UNIVERSIDADE D
COIMBRA

Sofia Ferreira Anastácio

Coxiella burnetii and Q Fever
An Emergent Zoonosis In Portugal?

Tese no âmbito do Doutoramento em Ciências Farmacêuticas, Ramo de Microbiologia e Parasitologia orientada pela Professora Doutora Gabriela Conceição Duarte Jorge da Silva e pelo Doutor Karim Sidi-Boumedine e apresentada à Faculdade de Farmácia da Universidade de Coimbra.

Junho de 2019

Faculdade de Farmácia
Universidade de Coimbra

COXIELLA BURNETII AND Q FEVER

AN EMERGENT ZOONOSIS IN PORTUGAL?

Sofia Ferreira Anastácio

VOLUME 1

Tese no âmbito do Doutoramento em Ciências Farmacêuticas, Ramo de Microbiologia e Parasitologia orientada pela Professora Doutora Gabriela Conceição Duarte Jorge da Silva e pelo Doutor Karim Sidi-Boumedine; e apresentada à Faculdade de Farmácia da Universidade de Coimbra.

Junho 2019

UNIVERSIDADE D
COIMBRA

Faculdade de Farmácia
Universidade de Coimbra

COXIELLA BURNETII AND Q FEVER

AN EMERGENT ZOONOSIS IN PORTUGAL?

SOFIA FERREIRA ANASTÁCIO

Doctoral dissertation

Dissertation presented to obtain a Ph.D. degree in Pharmaceutical Sciences, speciality Microbiology/Parasitology by University of Coimbra, Faculty of Pharmacy, University of Coimbra.

The work was carried out in the Microbiology Laboratory, at the Faculty of Pharmacy, and Center of Neurosciences and Cell Biology University of Coimbra, which provided the facilities and logistical supports.

Faculdade de Farmácia
Universidade de Coimbra

COXIELLA BURNETII AND Q FEVER

AN EMERGENT ZOONOSIS IN PORTUGAL?

Scientific supervisors

Professor Doctor Gabriela Jorge da Silva

Laboratory of Microbiology
Faculty of Pharmacy
Center of Neurosciences and Cell Biology
University of Coimbra, Portugal

Doctor Karim Sidi-Boumedine

Laboratory of Q Fever
ANSES - Sophia-Antipolis Laboratory
Sophia-Antipolis, France

Faculdade de Farmácia
Universidade de Coimbra

COXIELLA BURNETII AND Q FEVER

AN EMERGENT ZOONOSIS IN PORTUGAL?

Financial support

PhD Fellowship (SFRH/BD/77823/2011) provided by the Portuguese Foundation for Science and Technology (FCT) of the Portuguese Ministry of Science, Technology and Higher Education

Bolsa Individual de Doutoramento (SFRH/BD/77823/2011) da Fundação Portuguesa para a Ciência e a Tecnologia (FCT) do Ministério da Ciência, Tecnologia e Ensino Superior.

Acknowledgements

To the Faculty of Pharmacy, University of Coimbra, for accepting me and giving me the opportunity to get to know an excellent research team and a high quality educational training of the Pharmaceutical Sciences PhD programme.

To the Centre of Pharmaceutical Studies of University of Coimbra, for giving me the scientific and financial support needed for the development of this research.

To the Escola Universitária Vasco da Gama and Associação Cognitária Vasco da Gama, for helping me and for giving me the encouragement to conclude this work.

To the Portuguese Foundation for Science and Technology (FCT) of the Portuguese Ministry of Science, Technology and Higher Education, for providing me the PhD Fellowship (SFRH/BD/77823/2011).

To the Centre of Neurosciences and Cell Biology of University of Coimbra, for giving me the scientific and financial support needed for the development of this research.

Personal thanks

The development and conclusion of this dissertation and the herein described work was only possible with the assistance of many people, who I would like to remember and give my warm acknowledgements.

First, I would like to thank my supervisors, who had assisted me in acquiring additional skills and knowledge concerning my research, being an endless source of information and support.

I am very grateful to Professor Gabriela Jorge da Silva, who provided me all the help and experience. I deeply appreciate and recognize her scientific competence, accessibility, objectivity and constant encouragements. The enormous support in the most difficult times, that I will never forget, was very important to this successful overcoming.

To Doctor Karim Sidi-Boumedine, I am deeply grateful for the encouragement, for the persistent, helpful and meticulous scientific feedbacks in all the questions and doubts addressed. *Merci beaucoup pour votre gentillesse, votre orientation et pour les mots d'encouragement que vous m'avez donné.*

I am very thankful to Nuno Carolino and Ricardo Cabeças for their prompt assistance and help with the statistical analysis. Thank you for your extreme patience.

To Nuno Tavares and Carlos Cruz, I address my sincere thanks for their efforts and assistance in sampling procedures.

To João Simões and Luís Pimenta, I acknowledge the simplicity and honesty that allowed a great collaboration with the North of Portugal.

Heartfelt thanks to Madalena Vieira Pinto for a long friendship and for helping me with samples from wildlife. To Catarina Coelho and Andreia Gil, I thank all the efforts and assistance in sampling procedures.

In the Biochemistry lab, I thank the support and assistance of Professor Carla Pereira.

I also acknowledge the working group members of the Laboratory of Microbiology of the Faculty of Pharmacy, for the friendship and constant good mood: Sara, Vera, Nuno, Mrs. Sandra and last but not the least Rui, the researcher and the artist.

A special thanks to Mrs. Luísa and Mrs. Paula for their assistance in Escola Universitária Vasco da Gama. *Obrigada*!

To all my colleagues from Escola Universitária Vasco da Gama, I thank the support.

To my dear friends, I thank the support, for helping me and for just being there. I want to thank the insightful serenity of Anabela, the true and casual friendship of Carla and Teresa, the sweet tenderness of Liliana, the kindness and simplicity of Sofia Duarte and Eduarda, the complicity of Elsa, the quietness of Anália and the goodness of Francisco.

Last, but not least, I would like to thank my dear family for all their encouragement.

A special *OBRIGADO* goes to all my family and especially to my dear parents, José and Conceição. Your unconditional emotional support, encouragements and help are my strength. To my big sister, Isabel, thank you for being there when I most needed as a source of endless support.

To my little and sweet Dinis, I thank your never-ending happiness. You changed my life and every single day you teach me so many new things! You are my sunshine and an endless source of joy!

To my dear Duarte, you are my strong young man! I thank you for being what you are, focused and courageous. Never let go of your dreams and always believe in what your heart “tells” you. You will get there, just where your dreams guided you. I will always be proud of you!

And finally, to Sérgio, my big partner in life! I thank most of all your patience, your resilience and your never-ending support! Thank you for knowing me and understanding me so well! It was not easy, but we finally did it!

Abstract

Q fever is a zoonotic disease caused by *Coxiella burnetii* that occurs worldwide. It causes usually an asymptomatic infection or manifests as a flu-like syndrome with spontaneous recovery. Sometimes, acute Q fever can cause serious problems like pneumonia, abortions or meningitis, and long-term complications such as endocarditis and osteo-articular problems may occur in a lower percentage of patients. A wide range of hosts can be infected by *C. burnetii*. Domestic ruminants are considered as the main source of human infection; shedding bacteria principally through birth and abortion products, milk and faeces.

The risk that Q fever may represent to humans is recognized since its first description in 1937 in Australia; but after the largest ever reported Q fever outbreak in the Netherlands (2007-2011); public health authorities, namely the European Food and Safety Authority (EFSA), warned about the uncertainties that still exist in the understanding of Q fever epidemiology, including amongst the domestic ruminant populations throughout Europe. Some relevant facts pointed in a scientific report published by EFSA in 2010, mentioned that despite the knowledge about the wide distribution of *C. burnetii* among domestic ruminants, the true prevalence of infection is not known in many countries; and the lack of harmonized methodologies between the different existing studies hinder data comparison. Moreover, the role of other species, including domestic and wild animals, and even vectors; in the epidemiology of the disease is very limited. In Portugal, epidemiological data on Q fever is even scarcer. Only, a few studies reporting the infection in humans, in domestic ruminants and in zoo animals have been published and the prevalence of Q fever in Portuguese animal populations remains unclear. To gather information about *C. burnetii* epidemiology in Portugal, the present project aimed to provide information about Q fever prevalence among different animal species (domestic, companion and wild animals), but also to identify potential reservoirs in urban and rural environment and vectors (ticks), and to genetically characterize the circulating strains and compare them with those identified in other countries. To achieve our global objectives, a serologic survey was conducted in domestic and wild animals to screen for Q fever; then *C. burnetii* DNA was detected in biological samples collected from seropositive herds or animals, and from ticks; and finally, *C. burnetii* strains were genetically characterized. Harmonized methodologies

used in the French Reference Laboratory for Q fever were used, following the recommendations drawn by EFSA. The results obtained showed that in Portugal, independently of the type of production, herd seroprevalence is higher in small ruminants (32.6% meat herds and 51.6% dairy herds) than in cattle (23.5% meat herds and 37.8% dairy herds). Despite the non-evidence of exposure in domestic pigs, *C. burnetii* infection is not restricted to domestic ruminants as an exposure was demonstrated in companion animals, namely in dogs (12.6%) and in cats (17.2%); in feral pigs (5.6%); and in red deer (30.4%). However, *C. burnetii* DNA was only detected in domestic ruminants. The shedding of *C. burnetii* was demonstrated in cattle herds a higher percentage (20%) than in small ruminant herds (6.3%). Individually, the proportion of animal shedders was higher in goats (15%), followed by cattle (10.8%) and then sheep (3.6%). Milk appeared as the most important shedding route (10.9%) compared to vaginal swabs (2.1%). *C. burnetii* seems to be an important threat even in apparently healthy domestic ruminant populations, and milk appeared as an important shedding route. Despite the controversial data about the risk of infection by the consumption of unpasteurized milk, special attention should be given to occupational activities requiring contact with lactating domestic ruminants, and those requiring the manipulation of milk and milk products. The molecular characterization of *C. burnetii* obtained from clinical samples allowed the identification of six novel MLVA-6 profiles. These novel genotypes clustered with genotypes identified in cattle from other European countries and in Portuguese acute human infections.

Overall, our results demonstrate that in Portugal *C. burnetii* circulates among several domestic and sylvatic animals. Additionally, the close relation of the herein identified genotypes to those identified in cattle from other European countries suggests that a common pool of *C. burnetii* strains circulates in cattle in Europe and might be linked to human infection. Finally, this study highlights that harmonized methodologies are needed for epidemiological studies. It is central for comparing results from different studies and to cover different scenarios when controlling programs are needed.

Keywords

C. burnetii, Q fever, domestic ruminants, wild ungulates, companion animals, milk, PCR, ELISA, MLVA-6

Resumo

A Febre Q é uma doença zoonótica causada por *Coxiella burnetii* com uma ocorrência mundial. A Febre Q origina normalmente uma infeção assintomática, podendo manifestar-se como uma síndrome gripal com recuperação espontânea. Contudo, por vezes, formas agudas podem causar quadros mais graves como pneumonia, aborto ou meningite. Complicações a longo prazo, como endocardite e problemas osteoarticulares, podem também ocorrer com menor frequência. O espectro de hospedeiros recetivos a *C. burnetii* é largo. Porém, os ruminantes domésticos são considerados a principal fonte de infeção para humanos, excretando bactérias através de produtos do parto e aborto, do leite e das fezes.

O risco que a Febre Q representa para humanos é reconhecido desde a sua primeira descrição, em 1937 na Austrália. E, após um dos maiores surtos alguma vez reportados, ocorrido na Holanda (2007-2011), as autoridades de saúde, nomeadamente a *European Food and Safety Authority* (EFSA), alertaram para as incertezas existentes na compreensão da epidemiologia da Febre Q. Num relatório científico publicado pela EFSA em 2010, foi referido que, apesar da larga distribuição de *C. burnetii* entre ruminantes domésticos, a prevalência real de infeção não era conhecida em muitos países; e que a falta de metodologias harmonizadas entre os estudos existentes dificultava a comparação de dados. Além disso, foi realçada a falta de conhecimento sobre o papel de outras espécies, como animais domésticos e silvestres, ou mesmo vetores, na epidemiologia da doença. Em Portugal, a informação sobre a epidemiologia da Febre Q era ainda mais escassa. Apenas alguns estudos reportando a ocorrência de infeção em humanos, em ruminantes domésticos e em animais de zoo estavam publicados e a prevalência de infeção nas populações animais em Portugal permanecia pouco clara. Para reunir informação sobre a epidemiologia de *C. burnetii* em Portugal, o presente projeto teve como objetivo estimar a prevalência de infeção em diferentes espécies animais (animais domésticos de companhia e de produção, e animais silvestres), mas também identificar potenciais reservatórios de infeção em meio urbano e em meio rural e também em vetores (ixodídeos), e ainda caracterizar geneticamente as estirpes circulantes comparando com estirpes identificadas internacionalmente. Para atingir estes objetivos foi realizado um rastreio serológico em animais domésticos e silvestres; em seguida foi efetuada a pesquisa de DNA de *C. burnetii* em amostras

biológicas colhidas em explorações ou em animais seropositivos, e ainda em ixodídeos; e por fim as estirpes de *C. burnetii* detetadas foram geneticamente caracterizadas. As metodologias utilizadas foram harmonizadas com as metodologias utilizadas no Laboratório de Referência para a Febre Q sito em França e seguindo as recomendações da EFSA. Os resultados obtidos revelaram que em Portugal, independentemente do tipo de produção, a seroprevalência ao nível da exploração é mais elevada em pequenos ruminantes (32,6% em explorações de carne e 51,6% em explorações de leite) do que em bovinos (23,5% em explorações de carne e 37,8% em explorações de leite). Apesar de não ter sido evidenciada a exposição em suínos domésticos**,** demonstrou-se que a infeção por *C. burnetii* não está limitada aos ruminantes domésticos, tendo sido demostrada a exposição em animais de companhia, nomeadamente em cães (12,6%) e gatos (17,2%); em javalis (5,6%); e em veados (30,4%). No entanto, o DNA de *C. burnetii* foi apenas detetado em ruminantes domésticos. A evidência de excreção de *C. burnetii* foi superior (20%) em explorações de bovinos do que em pequenos ruminantes (6,3%). Individualmente, a proporção de animais excretores foi superior em caprinos (15%), seguindo-se os bovinos (10,8%) e por fim os ovinos (3,6%). O leite surgiu como a via de excreção mais importante (10,9%) comparativamente com zaragatoas vaginais (2,1%). *C. burnetii* parece ser um agente importante mesmo em ruminantes domésticos aparentemente saudáveis sendo o leite uma via de excreção importante. Apesar da informação controversa sobre o risco de infeção associado ao consumo de leite não pasteurizado, é necessário considerar as atividades ocupacionais que implicam o contacto com ruminantes domésticos em lactação e/ou que requerem a manipulação do leite e seus produtos derivados. A caracterização molecular de *C. burnetii* obtida a partir de amostras biológicas permitiu identificar seis novos perfis MLVA-6. Estes novos genótipos apresentam-se no mesmo *cluster* que genótipos identificados em bovinos de outros países Europeus e em infeções agudas de humanos em Portugal.

Globalmente, os resultados obtidos demonstram que em Portugal, *C. burnetii* circula entre animais domésticos e silvestres. Adicionalmente, a relação próxima entre os genótipos identificados neste estudo e os genótipos identificados em bovinos de outros países da Europa sugere que existe um *pool* comum de estirpes de *C. burnetii* que circulam entre bovinos na Europa e que podem estar associados com a infeção em humanos. Finalmente, este estudo realça a necessidade de uniformização de metodologias para a realização de estudos epidemiológicos. Tal é fundamental para a

comparação de resultados permitindo abranger diferentes cenários quando é necessário implementar planos de controlo.

Palavras-chave

C. burnetii, Febre Q, ruminantes domésticos, ungulados silvestres, animais de companhia, leite, PCR, ELISA, MLVA-6

Table of contents

List of figures

List of tables

List of abbreviations

ATP	Adenosine Triphosphate
BSL-3	Biosafety Level 3 Laboratories
BTM	Bulk Tank Milk
CCL	CC Chemokine Ligand
CCR	CC Chemokine Receptor
CCV	*Coxiella*-Containing Vacuole
CD	Cluster of Differentiation
CEVDI	*Centro de Estudos de Vectores e Doenças Infecciosas*
CFT	Complement Fixation Test
CI	Confidence Interval
CR	Complement Receptor
Ct	Cycle Threshold
CXCL	CXC Chemokines Ligand
DGS	*Direção Geral de Saúde*
DNA	Deoxyribonucleic Acid
EC	European Commission
ECDC	European Centre of Disease Prevention and Control
EEA	Early Endosome Antigen
EFSA	European Food and Safety Authority
EF-Ts	Elongation Factor Thermo Stable
EF-TU	Elongation Factor Thermo Unstable
ELISA	Enzyme Linked Immunosorbent Assay
GTP	Guanosine Triphosphate
HIV	Human Immunodeficiency Virus
Hq1	DNA binding protein specific to the SCV
HP	High Positive
IAP	Integrin Associated Protein
IFA	Indirect Immunofluorescent Assay
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
illD	Estimated Dose Causing Illness
InfD	Estimated Dose Causing Infection
iNOS	Isoform of Nitric Oxide Synthase
INSA	*Instituto Nacional de Saúde Dr. Ricardo Jorge*
IRS	Infrequent Restriction Site
IS	Insertion Sequence
LAMP	Lysosome-Associated Membrane Protein
LC-MS	Liquid Chromatography–Mass Spectrometry
LCV	Large Cell Variant
LP	Low Positive
LPS	Lipopolysaccharides

LRI	Leukocyte Response Integrin
MAPK	Mitogen Activated Protein Kinase
MAT	Microagglutination Test
MLVA	Multiple-Locus Variable Number Tandem-Repeat Analysis
MR	Mannose Receptor
MST	Multispacer Sequence Typing
Neg	Negative
ND	No Data Available
NM	Nine Mile
OD	Optical Density
OIE	World Organization for Animal Health
OMP	Outer Membrane Protein
PCR	Polymerase Chain Reaction
Pos	Positive
RFLP	Restriction Fragment Length Polymorphism Analysis
PFGE	Pulsed Field Gel Electrophoresis
PV	Parasitophorous Vacuole
RAB	Group of GTPases
RNA	Ribonucleic Acid
RpoS	RNA Polymerase Sigma S
rRNA	Ribosomal Ribonucleic Acid
SCV	Small Cell Variant
ScvA	DNA Binding Proteins Specific to the SCV
SD	Standard Deviation
SDC	Small Dense Cells
SE	Standard Error
SNP	Single Nucleotide Polymorphism
S/P	Ratio Sample/Positive
ST	Sequence Type
T1SS	Type 1 Secretion System
T4BSS	Type 4B Secretion System
TGF-b1	Transforming Growth Factor Beta 1
Th	T helper
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
UPGMA	Unweighted Pair Group Method with Arithmetic Mean Clustering
UV	Ultra-Violet
VHP	Very High Positive
WAHIS	World Animal Health Information System
WGA	Whole Genome Amplification
WHO	World Health Organization

1

General Introduction

1.1

A historical approach to Q fever

S. Anastacio, K. Sidi-Boumedine, G.J. da Silva. A historical approach to Q Fever. In: The Principles and Practice of Q Fever. Simões, J., Anastácio, S. and da Silva, G. J. Nova Science Publishers. New York. 2017. Pp 25-34.

The history of Q fever can be traced back to 1937, when it was described by Edward Holbrooke Derrick in Australia (Derrick, 1983). Q fever was discovered in the United States almost at the same time (Dyer, 1949).

1.1.1. Australia, one beginning

Since 1933, repeated outbreaks of an obscure febrile illness were detected in slaughterhouse workers and farmers in Brisbane-Queensland, Australia. In 1935, Edward Holbrooke Derrick, the Director of the Laboratory of Microbiology and Pathology, Queensland Health Department, Brisbane was assigned to investigate these outbreaks. He started by a detailed description of the disease and he recognized that it represented a previously non-described illness characterized predominantly by fever of unknown origin (MacKerras, 1998). His first step was to make a careful clinical study of all the cases available. He used his laboratory resources to determine whether it was an aberrant form of some febrile disease already known in Queensland, but the results were negative. A search among veterinary literature also failed to reveal any potential zoonosis from cattle origin. He used the guinea-pig, the standard experimental animal used in his laboratory. He found that guinea-pigs inoculated with blood from febrile patients developed a mild disease characterized by fever and enlargement of the spleen and ended by the recovery of the animals. He demonstrated that the disease could be transmitted among guinea-pigs by inoculation of spleen or liver emulsions. Guinea-pigs that had recovered from a previous infection were refractory to re-inoculation. He concluded that it was a specific infectious disease caused by an organism, which could not grow in any of the culture media available at the time (MacKerras, 1998), thus it was thought to be a virus. These findings led Derrick to name the disease by Q fever ("*Q*" for Query) in January 1939, by which it is still known (Burnet and Freeman, 1937; Cook, 2008).

Derrick suspected that the causative agent was probably a virus and sent infected spleens to Frank MacFarlane Burnet and Mavis Freeman, from the Walter and Eliza

Hall Institute of Medical Research in Melbourne, for further studies (MacKerras, 1998). Their tools in the laboratory were chorioallantoic membrane of developing chick and adult mice. The chorioallantoic membrane model proved to be very adequate for this research but the inoculated mice developed enlarged spleens, sometimes with exudate on the surface. Studying a section of an infected spleen, Burnet found large numbers of intracytoplasmic structures, which seemed to be a micro-colony of tiny, weakly stained rods. As the smears stained positive by Castenada method, they suspected the presence of a rickettsial organism. This suspicion was also supported by cross immunity tests which confirmed that the mouse "virus" was identical to the guinea-pig "virus" and that semi-purified rickettsial suspensions agglutinated with the sera of the infected mice and guinea-pigs. Additionally, they also found that the agent was filterable, but with difficulty, through relatively permeable (0.7 μm) gradocol membranes (MacKerras, 1998; Burnet and Freeman, 1937). Because the microorganism shared characteristic features with other *Rickettsiae* spp., Derrick suggested the name *Rickettsia burnetii* for the organism in honour to the researcher Burnet (MacKerras, 1998; Maurin and Raoult, 1999).

Burnet and Freeman also provided a valuable diagnostic tool. Miss Freeman was able to prepare a stable rickettsial suspension from infected mouse spleens, which was used to develop a micro-agglutination test for specific antibodies in human and animal sera. The test was later transferred to Brisbane, using Freeman mouse spleen antigen. In 1940, Wilbur Smith discovered the abundant multiplication of *Rickettsiae* in infected female *Rhipicephalus sanguineus* (dog ticks), which enabled him to prepare larger volumes of excellent suspensions. This tick antigen was employed until the complement-fixation test was introduced in 1950 (MacKerras, 1998).

1.1.2. United States of America, the other beginning

Meanwhile, in 1938 an unknown agent was isolated by Gordon Davis and Herald Cox from the tick *Dermacentor andersoni* in Montana. The ticks have been collected in the Spring of 1935, during an investigation of the ecology of Rocky Mountain spotted fever, in the Nine Mile Creek region, Montana, United States. In the laboratory they found that the agent, initially thought to be a virus, was transmitted to susceptible animals by *D. andersoni* in the process of feeding and that it survived in ticks through

successive moults and through the egg stage, being found in the gut and haemolymph. With these findings the investigators suggested ticks as natural vectors. Furthermore, they also found that the agent was infectious for guinea pigs, was Gram-negative, and presented an extracellular and intracellular pleomorphic, rickettsia-like, appearance (Dyer, 1949; Cox, 1938, Davis and Cox, 1938). The disease produced in animals was called "Nine Mile Fever" and the agent was identified as a rickettsial organism. In 1938, Cox developed a method of culture in chick embryos, which allowed the isolation of the strains for further investigation. Unlike other members of this group, it was filterable, and it was named *Rickettsia diaporica* (*diaporica* is derived from the Greek word for having the property or ability to pass through [a filter]) (Dyer, 1949; Lennette, 1948; Blut, 2014).

Rolla Eugene Dyer, at the time the director of the National Institutes of Health in the United States, joined Davis's and Cox's team at the Rocky Mountain Laboratory (Lennette, 1948; Dyer, 1949; Cooke, 2008). While on a visit to the Rocky Mountain Laboratory, a member of his staff suffered an accidental laboratory infection by the agent of the "Nine Mile Fever," at the time named "X strain." This occurrence led Dyer to consider a relationship between Australian Q Fever and the disease caused by the infectious agent isolated from ticks in Montana (Dyer, 1938).

1.1.3. Australian and American collaboration

The Australian and the American teams shared their findings and Burnet sent material to Dyer. To determine the origin of the "X strain" that caused human disease, cross-immunity and protective tests were developed. At the National Institutes of Health, Dyer found a negative cross-immunity between the agent isolated in guinea pigs from the human patient and Rocky Mountain Spotted Fever; whereas five guinea pigs recovered from Australian Q fever, previously sent by Dr. Burnet, were found to be immune to the "X strain." Tests with the "Q fever strain" (sent by Dr. Burnet in the form of two infected mouse spleens, maintained subsequently in mice and guinea pigs), the "X strain" (obtained by Dyer) and two strains of Rocky Mountain Spotted Fever were conducted. A complete cross-immunity between "Q fever strain" and the "X strain" was found (Bengston, 1941).

Burnet and Freeman also compared "Q fever strain" and "X strain." They observed that both strains were virulent in monkeys but "X strain" triggered a more acute infection in guinea pigs, being considered more virulent (Bengston, 1941).

The work of both teams suggested that Australian Q-fever and Montana Nine Mile fever were the same disease. Immunological research proved that *R. burnetii* and *R. diaporica* were identical (Lennette, 1948; Dyer, 1949; Cooke, 2008).

1.1.4. The global distribution

Until 1944, Q fever was considered confined to Australia and United States but during the Second World War, specifically between November 1944 and June 1945, eight outbreaks totalling 600 cases among allied troops were reported in Italy, Greece and Corsica, being described as pseudo-flu endemic syndromes (Dyer, 1949; Lennette, 1948). The disease was also reported in Panama in 1946 and in Switzerland in 1947 (Lennette, 1948). Prior to 1946, naturally acquired Q fever appeared to be rare in the United States but an outbreak reported in Texas in 1946 confirmed the suggestion of Cox that the disease could be widespread (Dyer, 1949).

Initially, a large diversity of names was attributed to this disease according to the circumstances of its occurrence (*e.g.* slaughterhouse fever, Queensland rickettsial fever, seven days fever, Balkan flu, Italy fever or Nine Mile Creek fever); however, Q fever was the name that persisted until our days (Dyer, 1949; Angelaskis and Raoult, 2010).

The potential danger of Q fever to public health and the large gaps in the knowledge of the disease were early recognized. In 1950, the World Health Organization (WHO) encouraged the epidemiological research and it was concluded that Q fever was present in 51 countries on five continents (Kaplan and Bertagna, 1955).

Nowadays, Q-fever is listed in the Terrestrial Animal Health Code of the World Organization for Animal Health (OIE) and all the Member Countries are required to report the occurrence of the disease.

In humans' Q fever is usually reported by the occurrence of outbreaks. Its worldwide distribution is globally accepted except in New Zealand where the disease has never been reported (WAHIS Interface, 2016).

1.1.5. Unravelling the characteristics of the etiologic agent

Although the etiological agent of Q fever was classified as a rickettsial organism, certain differences were early identified between this microorganism and other rickettsiae. The morphologic, tinctorial and cultural, characteristics supported its classification as a rickettsial organism. However, the relative easy passage through bacterial filters, the finding of a large number of organisms inside the infected cells, the great resistance to physical and chemical agents, the production of non-soluble antigen and the absence of the rash seen with other rickettsial diseases were characteristics that made it differ from *Rickettsia* (Lennette, 1948; Dyer, 1949; Maurin and Raoult, 1999).

These striking characteristics justified the proposal of placing the organism in a new genus to be known as *Coxiella*. The genus *Coxiella* was created in 1948 by Cornelius B. Philip and *Rickettsia burnetii* (Derrick) became *Coxiella burnetii* listed in the 6th edition of Bergey's Manual of Determinative Bacteriology (Lennette, 1948; MacKerras, 1998).

Coxiella burnetii supersedes *R. burnetii* and *R. diaporica* and honors the almost simultaneous isolation of the organism by American and Australian researchers, Harold Cox and MacFarlane Burnet (Maurin and Raoult, 1999).

C. burnetii was initially included in the order *Rickettsiales*, family *Ricketsiaceae* (Reimer, 1993). However, the sequence analysis of the 16S rRNA gene showed that *C. burnetii* falls in the gamma group of proteobacteria showing a closer relationship with the genus *Legionella* (Weisburg, 1989; Stein *et al*., 1993) than with the *Rickettsia* group, belonging to alpha group of proteobacteria. Other phylogenic neighbours of *C. burnetii* are *Francisella tularensis* and *Rickettsiella grylli* (Maurin and Raoult, 1999). These bacteria are able to multiply within cells (Seshadri *et al*., 2003). Thus, a phylogenically reclassification was performed and nowadays *C. burnetii* belongs to the order *Legionellales*, family *Coxiellaceae* and genus *Coxiella* (Drancourt *et al*. 2004). The genus *Coxiella* includes other members as *Coxiella cheraxi* (Tan and Owens, 2000) and *Coxiella*-like organisms recently identified in birds, in ticks (Psaroulaki *et al*., 2014; Duron *et al*., 2015) and in horses (Seo *et al*., 2016).

The development of molecular techniques also enabled the genetic characterisation of *C. burnetii* and allowed the study of its genotypic diversity among different geographical areas and dissemination of specific strains, the determination of relationships between variants of the bacterium, the investigation of Q fever outbreaks, particularly to clarify links regarding source of infection, the understanding of the

epidemiological emerging factors and the evaluation of control measures (Roest *et al*., 2011; Sulyok *et al*., 2014; OIE, 2015).

Nowadays, molecular epidemiology is a fundamental tool for the study of genetic surveillance of *C. burnetii* from different sources (Piñero *et al*., 2015). A systematic genotyping provides a descriptive database enabling to monitor the temporal and geographical evolution and dissemination of the strains. The possibility of a rapid surveillance of the dispersion of a strain in a specific host or between different hosts is an added value for an epidemiological study. Furthermore, the data may help to explain different scenarios of dissemination and contribute to find efficient control measures (Sidi-Boumedine and Rousset, 2011).

Eighty years after the first reports of Q fever, it is known that the disease is globally widespread. The scientific knowledge on Q fever increased over the last 40 years, especially after the largest human Q fever outbreak that occurred in the Netherlands between 2007 and 2010 (Dijkstra *et al*., 2012), which raised many questions, particularly on the control of the infection. Moreover, further research is needed to improve our understanding of the transmission of Q fever, and to clarify some of the characteristics of the microorganism responsible for it.

1.2

Coxiella burnetii, the pathogen

Coxiella burnetii is a highly infective small intracellular bacterium and it is the etiological agent of Q-fever (Mege *et al*., 1997; Raoult *et al*., 2005). Its high infectivity and stability in the environment explain why *C. burnetii* is listed as a Category B biothreat agent (Waag, 1997).

1.2.1. Phylogeny

Coxiella burnetii belongs to the genus *Coxiella* (Mege *et al*., 1997). Initially classified as a "Rickettsia-like" organism, it was included in the order Rickettsiales, family *Ricketsiaceae* (Reimer, 1993). However, the sequence analysis of the 16S rRNA gene showed that *C. burnetii* falls in the gamma group of proteobacteria presenting a closer relationship with the genus *Legionella* (Weisburg *et al*., 1989; Stein *et al*., 1993) than with the Rickettsia group, belonging to the alpha group of proteobacteria. Other phylogenetic neighbours of *C. burnetii* are *Francisella tularensis* and *Rickettsiella grylli* (Roux *et al*., 1997; Maurin and Raoult, 1999). All of these bacteria are able to multiply within cells (Seshadri *et al*., 2003). Thus, a phylogenetic reclassification was done and nowadays *C. burnetii* belongs to the order *Legionellales*, family *Coxiellaceae* and genus *Coxiella* (Drancourt *et al*. 2004). For years, *C. burnetii* was the only species of the genus *Coxiella*. In 2000, *Coxiella cheraxi* was considered a new member of the genus, showing 95.5% similarity with *C. burnetii* in 16S rRNA (Tan and Owens, 2000) and *Coxiella*-like organisms recently identified in ticks (Arthan *et al.*, 2015; Duron *et al*., 2015; Al-Deeb *et al*., 2016) and in horses (Seo *et al.*, 2016). The wide distribution of *Coxiella*-like endosymbionts among different tick species and the phylogenetic analysis of these *Coxiella*-like organisms suggest that *C. burnetii* has probably emerged from a tick-borne progenitor (Duron *et al*., 2015). Thus, it has been proposed that *C. burnetii* recently evolved to vertebrate pathogenicity from an inherited endosymbiont of ticks, due to spontaneous mutations or horizontal gene transfers from pathogens that co-infected the same tick or vertebrate (Duron *et al*., 2014; Duron *et al*., 2015).

1.2.2. The bacterial cell

C. burnetii is unique among intracellular bacteria as it replicates within a parasitophorous vacuole of eukaryotic host cells with an estimated doubling time of 20 to 45 hours (Heinzen *et al*., 1999; Angelaskis and Raoult, 2010).

1.2.2.1. Morphology

C. burnetii is a small pleomorphic rod, presenting 0.2–0.4 μm wide and 0.4–1.0 μm long, with a cellular membrane similar to that of Gram negative bacteria (Maurin and Raoult, 1999).

A characteristic of *C. burnetii* is the biphasic developmental cycle that generates biologically, ultra-structurally and compositionally distinct forms. The first observations of different forms of *C. burnetii* occurred through electron microscopy (McCaul and Williams, 1981). Two main different forms are described in regard to morphology, antigenic composition and physical and chemical resistance: large cell variants (LCVs) and small cell variants (SCVs) (McCaul and Williams, 1981; Seshadri *et al*., 1999).

LCVs are the exponentially replicating forms. They have a larger size (> 0.5 μm), they are more metabolically active and less electron dense forms. They have dispersed and filamentous chromatin and possess clearly distinguishable outer and cytoplasmic membranes with exposed lipopolysaccharides (LPS) on the surface, sharing features with Gram negative bacteria. These LCVs are sensitive to the decrease of osmotic pressure (McCaul and Williams, 1981; Heinzen *et al*., 1999; Seshadri *et al*., 1999).

SCVs are the stationary non-replicating forms, being observed after prolonged culture, such as 21 days in Vero cells. The size of these rod shape forms typically ranges from 0.2 and 0.5 μm, being filterable through 0.22 μm filters. They are very compact and present low metabolic activity (Heinzen *et al*., 1999, Coleman *et al*., 2004; Eldin *et al*., 2017). They are also stable in the environment, showing a high resistance to osmotic, mechanical, chemical, heat and desiccation stresses (Eldin *et al*., 2017). Some structural characteristics of SCVs are the electron-dense and condensed chromatin and the unusual cell envelope characterized by a high number of cross-links in peptidoglycans, which seems to enhance environmental stability (McCaul and Williams,

1981; Sandoz *et al.*, 2016). Additionally, SCVs harbour a complex system of internal membranes, arranged in whorls contiguous with the cytoplasmic membrane, favouring a compact organization of SCV (McCaul and Williams, 1981; Heinzen *et al*., 1999). The resistance properties of these SCVs strongly implicate this form as responsible for long-term extracellular survival and aerosol transmission of *C. burnetii* (McCaul and Williams, 1981; Coleman *et al*., 2004).

The environmental stability of *C. burnetii*, the possibility of its aerosolization and its high virulence led to the classification of this bacterium as a category B biological threat agent (Eldin *et al.*, 2017).

The different forms of *C. burnetii* are linked to its lifecycle as a strategy to survive in and out of the *Coxiella*-containing vacuole (CCV). Infection results in a CCV harbouring a mixture of cell types. Despite the infectious ability demonstrated by LCVs *in vitro*, it might have a little relevance in natural infections because the fragile LCVs do not persist in an infectious form in extracellular environment for long periods (Heinzen *et al*., 1999). SCVs are the environmentally stable form of *C. burnetii* and their extracellular stability facilitates dispersion and transmission (McCaul and Williams, 1981; Heinzen et al., 1999).

Since a few proteins are differentially expressed by SCV and LCV, the morphological differences between SCV and LCV are correlated with different protein composition. Elongation factors (EF-TU and EF-Ts), stationary phase sigma factors (RPOS) and a 29-kDa protein designated P1 with porin activity are expressed at high levels by LCV (Heinzen *et al*., 1999; Varghees *et al*., 2002). ScvA and Hq1 are DNA binding proteins specific to the SCV that likely play a role in chromatin condensation (Heinzen and Hackstadt, 1996; Coleman *et al*., 2004).

Another cell form, a ‘spore-like particle’ was described in the polar regions of some LCVs as an electron dense polar body. It was hypothesized that an endogenous spore could be a part of the developmental cycle of *C. burnetii* (McCaul and Williams, 1981). Despite its resistance to osmotic pressure, this form did not show an infectious ability (Heinzen *et al*., 1999; Raoult *et al*., 2005). Moreover, it could not be considered as a spore particle because it did not stain with spore stains, it was not detected by tests for dipicolinic acid, a traditional spore marker (McCaul *et al*., 1991) and sporulation genes have not been identified in *C. burnetii* genome (Seshadri *et al*., 2003). It was also hypothesized that the SDCs may develop into SCVs, but this was not proven yet (Oyston and Davis, 2011).

1.2.2.2. Staining

The structure of the cell wall of *C. burnetii* resembles those of Gram negative bacteria but it is not well stainable by the Gram technique. *C. burnetii* cells also exhibit properties of acid fast organisms similarly to tubercle bacilli, being stainable by the Ziehl-Nielsen method and the Gimenez method, usually used to stain *C. burnetii* in clinical specimens or laboratory cultures (Gimenez, 1964; McCaul and Williams, 1981; Maurin and Raoult, 1999).

1.2.3. Antigenic variation

All the LPS-encoding genes are in a 38 Kb region in the *C. burnetii* genome and it has been observed that mutational variations in this region result in antigenic and virulence shift, termed "phase variation" (Mege *et al*., 1997; Barry *et al*., 2012; Kuley *et al*., 2015). Thus, *C. burnetii* may exist in a virulent phase I and in an avirulent phase II (Van den Brom *et al*., 2015). Antigenic variation between these two phases is based on a mutation process in which there is an irreversible modification from smooth to rough-type LPS, presenting a dramatically reduced virulence (Mege *et al*., 1997; Van den Brom *et al*., 2015). The avirulent rough LPS is likely due to an additional point/frameshift mutation, small deletion or transposon insertion in a gene in the LPS biosynthetic pathway (Beare *et al*., 2006; Denison *et al*., 2007). Phase II variant (Nine Mile, phase II, clone 4, RSA439) of the virulent Nine Mile phase I reference strain (RSA493) contains a chromosomal deletion that eliminates genes involved in O-antigen biosynthesis (Moos and Hackstadt, 1987). The deleted region comprises genes responsible for the addition of sugars to the core. So, the sugar composition of each LPS is quite different, because phase I LPS contains sugars such as L-virenose dihydrohydroxystreptose and galactosamine uronyl-(1,6) glucosamine, which are lacking in phase II LPS (Mege *et al*., 1997; Hoover *et al*., 2002). The absence of L-virenose in the LPS of phase II *C. burnetii*, as well as in other bacteria, justifies the use of this sugar as a unique biomarker of phase I *C. burnetii* (Palkovicova *et al*., 2009). These findings suggested that the lack of virulence is associated to a shorter LPS and not to a defect in the synthesis of other virulence factors. However, it is interesting to note that avirulent forms of other strains besides Nine Mile show different patterns of

deletions/mutations suggesting that the biosynthesis of LPS in *C. burnetii* is not completely understood (Beare *et al*., 2006). Thus, the relevance of phase II *C. burnetii* in natural infections remains questionable (Van den Brom *et al*., 2015).

Phase I *C. burnetii* can be recovered from infected humans and animals being characterized by smooth-type LPS and high virulence (Mege *et al*., 1997). The smooth LPS in *C. burnetii* phase I disturbs an effective immune response, giving the phase I bacterium the opportunity to survive and multiply in the host cells. Therefore, phase I *C. burnetii* is highly infectious (Van den Brom *et al*., 2015). The avirulent form, phase II *C. burnetii*, has not yet been isolated from the host and can only be seen after culturing in non-immunocompetent cell cultures or hen eggs (Arricau-Bouvery and Rodolakis, 2005; Van den Brom *et al*., 2015). The shift from virulent phase I to avirulent phase II is likely due to repeated passages of the strains in cell cultures or embryonated eggs (Kuley *et al*., 2015).

1.2.4. Genomics

The first complete genome sequence of *C. burnetii* was published in 2003. It corresponded to the original strain (RSA 493 strain) isolated from ticks by Davis and Cox in 1935 also known as Nine Mile strain. This event led to significant advances in the knowledge of *C. burnetii*. RSA 493 strain revealed the presence of small, circular chromosome with 1.995.275 bp and a 37.393 bp plasmid (QpH1) (Seshadri *et al*., 2003; Eldin *et al.*, 2017). Since then, ten new isolates completely sequenced and 25 incomplete genomes have become available in Genbank (Beare *et al*., 2009; Rouli *et al*., 2012; D'Amato *et al*., 2014[a,b], Karlsson *et al*., 2014; Sidi-Boumedine *et al*., 2014; Walter *et al*., 2014, D'Amato et al., 2015; Hammerl *et al*., 2015; Kuley et al., 2016; Warrier *et al*., 2016; Beare et al., 2017[a,b,c]; Millar et al., 2017).

It is recognized that the genome size of *C. burnetii* varies from 1.5 to 2.4 Mb (Raoult *et al*., 2005) and four plasmid types may be present: QpH1, (36 kb), QpRS (39 kb), QpDG (42 kb), and QpDV (33 kb). Each bacterial cell has only one of the four plasmids, which contains about 2% of the genetic information (Angelakis and Raoult, 2010). However, some bacteria are plasmid-less, presenting a plasmid-homologous sequence with a length of 36–56 kb integrated in the chromosome (Voth *et al*., 2011). A correlation between plasmid types and virulence (*i.e.*, development of acute or chronic

Q fever) was initially proposed (Minnick *et al*., 1990; Minnick *et al*., 1991) but, a few years later, it was found to be inconsistent (Stein and Raoult, 1993; Thiele and Williams, 1994).

The chromosome of *C. burnetii* lacks bacteriophage or conjugation-mediated genes, and an important number of genes are of unknown function. Besides that, approximately 10% of genes in all strains are pseudogenes (Omsland and Heinzen., 2011) and many of them contain single frameshifts, point mutations, or truncations, which might be explained by a recent origin (Seshadri *et al*., 2003). Indeed, in opposition to the previous understanding of bacterial virulence, it is now accepted that gene loss, rather than acquisition of virulence factors, has been a driving force in the adaptation of parasites to eukaryotic cells (Merhej *et al*. 2009). Some non-virulent genes become inactivated because their functions are no longer required in highly specialized niches, and thus, highly pathogenic bacteria show reduced genome size (Cole *et al*., 2001; Sakharkar *et al*., 2004; D'Amato *et al*., 2015). For example, CB 175 strain, an hypervirulent strain specific from French Guiana, presents a deletion in the region of genes involved in T1SS (Eldin e*t al*., 2014; D'Amatto *et al*., 2015).

The genome of *C. burnetii* also contains genes that encode proteins with eukaryotic domains. These proteins mimic host cell proteins, aiding the pathogen's survival. Polymorphisms of these proteins have been observed and they contribute to strain specific differences in virulence potential (Carey *et al*., 2011). Z3055 strain that belongs to MST 33, just like NL-Limburg strain involved in the Netherlands outbreak, presents a high number of mutations involving genes coding ankyrin repeat domains proteins, membrane proteins and proteins participating in translation and transcription (D'Amatto *et al*., 2014[b]). These mutations could have been involved in the change of surface antigens which led to an absence of immune recognition in a naïve population allowing a rapid dissemination (Eldin *et al*., 2017).

Globally, the genome of *C. burnetii* contains conserved genomic regions as well as polymorphic regions (Sidi-Boumedine *et al*., 2015[a]). This genetic variability has been early recognized from the preliminary genotyping investigations. The analysis of the *adaA* genomic region revealed a polymorphic appearance allowing a good differentiation of *C. burnetii* strains that might be considered a useful tool to investigate the molecular epidemiology and the evolution of *C. burnetii* (Frangoulidis *et al*., 2013). Furthermore, the insertion sequence IS1111 plays an important role in the genomic plasticity of *C. burnetii*. The number of IS1111 elements is highly variable between

strains; many different locations are described, showing a direct impact in genotyping of *C. burnetii* (Sidi-Boumedine *et al*., 2015 [b]).

By the cross-genome comparison of strains it was suggested that *C. burnetii* isolates are at different stages of pathoadaptation (Beare *et al*., 2009). Indeed, isolates obtained from acute and chronic disease revealed to belong to distinct groups (van Schaik *et al*., 2013). Moreover, genetically different pathotypes have been described and displayed different virulence in animal models (Stein *et al*., 2005; Russel-Lodrigue *et al*., 2009).

1.2.5. Resistance

C. burnetii, an obligate intracellular organism, replicates in eukaryotic cells inside an acidic vacuole (CCV) as a strategy to avoid the microbicidal response of host cells (Mege *et al*., 1997). LCVs are the exclusive and pleomorphic intracellular forms of *C. burnetii,* which are metabolically active but very fragile in the extracellular environment (McCaul and Williams, 1981; Boarbi *et al.*, 2015). The impressive stability and resistance of this bacterium is attributed to SCVs (McCaul and Williams, 1981; Heinzen *et al*., 1999; Coleman *et al*., 2004; Roest *et al.* 2014; Boarbi *et al.*, 2015). These forms are highly resistant to environmental stress such as osmotic pressure, high temperature, desiccation, UV light and various chemical disinfectants (Coleman *et al*., 2004; Voth and Heinzen, 2007). Viable microorganisms can be recovered after several years in dust, two years at –20ºC, seven to ten months on wool at environment temperature, 150 days in soil, for more than one month on fresh meat and seven days in water or in milk at room temperature (Tissot-Dupont *et al*., 2004; van Woerden *et al*., 2004; Boarbi *et al*., 2015). These features allow the survival of this pathogen in the environment for long periods of time while keeping its infectivity (McCaul and Williams 1981; van Schaik *et al*., 2013).

1.3

Strategies of infection

As an obligate intra-cellular pathogen, successful intracellular replication of *Coxiella burnetii* is required. In fact, *C. burnetii* developed a range of mechanisms to invade, survive and replicate within large and acidic phagolysosome-like vacuoles known to fuse homo and heterotypically with other vesicles (Capo *et al*., 1999; Beron *et al*., 2002; van Schaik *et al*., 2013). This ability to prosper within such a hostile intracellular niche is central to its pathogenesis. However, little is known about genes that facilitate intracellular growth (Maurin *et al*., 1992; Voth and Heinzen, 2007; Larson *et al.*, 2016; Moses *et al*., 2017).

1.3.1. Life in the host cell

C. burnetii displays unique characteristics among intracellular bacteria since it can resist inside an acidic (pH≅5) parasitophorous vacuole (PV) with phagolysosomal characteristics, also called *Coxiella*-containing vacuole (CCV) (Heinzen *et al*., 1999; Howe and Mallavia., 2000; Voth and Heinzen, 2007).

Several genes encoding adhesion, invasion, detoxification and secretion system proteins seem to contribute to the formation of CCV (Beare *et al*., 2011[a]; Carey *et al*., 2011; Eldin *et al*., 2017). Adhesion genes encode proteins containing ankyrin repeats, which bind, for example, to αvβ3 integrin, one of the eukaryotic cell receptors for *C. burnetii*. Invasion genes encode proteins inducing cytoskeleton reorganization during cell infection (Capo *et al.*, 1999). Recently, OmpA was identified as a *Coxiella* invasin inducing cellular uptake, specifically by nonprofessional phagocytes (Martinez et al., 2014). Detoxification genes encode superoxide dismutase, catalase and acid phosphatase that allow *C. burnetii* to escape from microbicidal activity of macrophages by the detoxification of reactive oxygen produced in the host cells (Baca *et al.*, 1993; Mege *et al*., 1997). Furthermore, during host cell infection, *C. burnetii* expresses several *dot/icm* genes involved in secretion systems that seem to facilitate the formation of the CCV and *C. burnetii* replication (Zamboni *et al*., 2003; Weber et al., 2016). In fact, it was shown that *Dot/Icm* type 4B secretion system (T4BSS) delivers bacterial effectors

proteins into the host cell cytoplasm during the replication of *C. burnetii*; and prevents cells apoptosis. The lack of T4BSS-mediated secretion prevents the replication in eukaryotic cells and does not prevent intrinsic apoptosis (Voth *et al*., 2007; Carey *et al*., 2011; Beare *et al*., 2012; Winchell *et al*. 2014; Larson *et al*., 2015; Luedtke *et al*., 2017). Notwithstanding, recent findings showed that this cannot be extrapolated to all virulent strains. Regarding CB 175 strain, its virulence appears to be related to a genome reduction event by the loss of type I secretion system (D'Amatto *et al.*, 2015).

Despite the little knowledge about *C. burnetii* pathogenic mechanisms, it is globally accepted that inside the CCV the acidic pH activates *C. burnetii* metabolism, beginning its replication and allowing the penetration of nutrients needed for metabolism (Coleman *et al*., 2004; Voth and Heinzen, 2007).

1.3.1.1. Susceptible cells

C. burnetii survives in a wide range of cells, namely myeloid cells (*e.g.* monocytes, macrophages and dendritic cells) (Meconi *et al*., 1998; Capo *et al*., 1999). In human and in animal infection, the primary target cells are blood circulating monocytes, macrophages (*e.g.* lymph nodes, spleen, liver, lungs) (Mege *et al*., 1997; Heinzen *et al*., 1999; Shannon and Heinzen, 2009; Eldin *et al*., 2017) and trophoblasts (Amara *et al*., 2010).

The persistence of *C. burnetii* in free living amoeba (La Scola and Raoult, 2001) and murine adipose tissue (Bechah *et al*., 2014) has been described too, and macrophage-like cell lines can also be infected by this pathogen. Infrequently, *C. burnetii* is observed in epithelial cells, pneumocytes, fibroblasts and endothelial cells (Khavkin and Tabibzadeh, 1988; Heinzen *et al*., 1999).

1.3.1.2. Entry into the cells

The internalisation differs between phase I (virulent) and II (avirulent) LPS and involves the recognition of several receptors (Capo *et al*., 2003; Eldin *et al*., 2017).

The uptake of phase I *C. burnetii* by phagocytic cells is mediated by the Leukocyte Response Integrin (LRI) (αvβ3) and an Integrin Associated Protein (IAP) (Mege *et*

al., 1997; Dupuy and Caron, 2008). The entry occurs by passive microfilament-dependent endocytosis (Heinzen *et al*., 1999; Capo *et al*., 2003). Phase I LPS induces a rearrangement of F-actin cytoskeleton leading to pronounced membrane protrusions on the surface of monocytes at the site of bacterial adherence. This phenomenon, called membrane ruffling, requires contact between *C. burnetii* and host cells and depends on the expression of Toll Like Receptor type 4 (TLR4) on the host cell surface (Figure 1) (Meconi *et al*., 1998; 2001; Honstettre *et al*., 2004). The ability to use αvβ3 integrin for invasion might be exploited by *C. burnetii* as a mechanism to avoid the induction of an inflammatory response, since αvβ3 integrin is typically involved in the removal of apoptotic cells via phagocytosis being generally associated with an inhibition of inflammation (Dupuy and Caron, 2008). Thus, *C. burnetii* enters the cells without alerting the immune system (van Schaik *et al*., 2013).

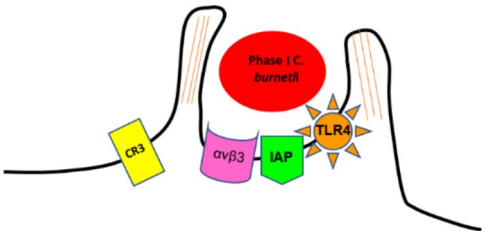


Figure 1: Scheme representing the internalization of phase I *C. burnetii* by monocyte-like cells.

While phase I *C. burnetii* is internalized by monocyte-like cells engaging the αvβ3 integrin complex, IAP and TLR-4, the uptake of phase II organisms is mediated by LRI (αvβ3 integrin), by IAP and by Complement Receptor 3 (CR3). TLR-4 is not involved in phase II *C. burnetii* internalization (Mege *et al*., 1997; Meconi *et al*., 1998; Capo *et al*., 1999) and the CR3 molecules remained outside the pseudopodal extensions induced by virulent *C. burnetii* (Capo *et al*., 2003).

In non-phagocytic cells the adherence and internalisation of phase II organisms occurs at higher rates than for phase I. Differential engagement of αvβ3 integrin and CR3 cannot account for this increased uptake as these cells lack such receptors. As phase II, *C. burnetii* produces a truncated LPS with lower content of carbohydrate than phase I, this avirulent *C. burnetii* is highly hydrophobic (Williams *et al*., 1981). Consequently, the increased uptake of phase II *C. burnetii* occurs by non-specific

hydrophobic interactions that facilitate contact of bacteria with host plasma membrane and receptors (Baca *et al*., 1993). The entry into these cells occurs after direct contact, through the zippering of bacterial ligands to host cell receptors (Beare *et al*., 2011[a]; Carey *et al*., 2011; van Schaik *et al.*, 2013).

Despite a lower internalisation of virulent *C. burnetii*, it survives successfully when compared to avirulent strains which are efficiently internalised but are rapidly eliminated (Meconi *et al*., 1998; Capo *et al*., 1999; Ghigo *et al*., 2009).

1.3.1.3. Phagosome maturation

Phase I and phase II *C. burnetii* behave differently depending on host cell type. In phagocytic cells, virulent bacteria survive and replicate while avirulent microorganisms are destroyed. In non-microbicidal cells, both virulent and avirulent microorganisms replicate (Barry *et al.*, 2011).

Briefly after entering the host cell, *C. burnetii* resides in a tight fitting nascent phagosome during the first 4–6 h post-infection (Howe and Mallavia, 2000). After this, the CCV matures through the endocytic pathway and, following a serial, ordered and regulated fusion and fission events, culminates in a phagolysosome with degradative lysosomal characteristics (Howe *et al*., 2010; Flannagan *et al*., 2012).

The subversion of microbicidal function of host cells by intracellular pathogens is critical for their survival and pathogenicity (Ghigo *et al*., 2002). Frequently, intracellular pathogens subvert endosomal cascade and arrest maturation of the phagosome at an early stage to avoid fusion with lysosomes (Flannagan *et al*., 2009). Regarding *C. burnetii*, after internalization in microbicidal cells, virulent and avirulent bacteria localize within the nascent CCV, which traffics through the endocytic cascade. It develops into an early phagosome acquiring the small GTPase RAB5. This GTPase stimulates the fusion with early endosomes, resulting in acidification of the lumen to approximately pH 5.4 and acquisition of the early-endosomal marker protein 1 (EEA1) (Heinzen and Hackstadt, 1996; Kinchen and Ravichandran, 2008). Early phagosome is converted into late phagosome acquiring acid hydrolases which are involved in pronounced degradative activity to which *C. burnetii* can resist (Howe *et al*., 2010). This late phagosome lacks RAB5 and EEA1 but acquires lysosome-associated membrane protein 1, 2 and 3 (LAMP1, LAMP2 and LAMP3) and vacuolar ATPase

which pumps protons into the maturing phagosome to further decrease the luminal pH to about 5.0 (Beron *et al*., 2002; Kinchen and Ravichandran, 2008; Schulze-Luehrmann *et al*., 2016; Eldin *et al*., 2017). *C. burnetii* persists in acidic vacuoles independently of virulence, the bacteria are able to replicate, in a slow rate, within the large CCV with an acidic environment (Mege *et al*., 1997; Ghigo *et al*., 2002; Howe *et al*., 2003). The process of phagosome maturation continues with its fusion with lysosomal compartments to acquire cathepsins and hydrolases. The vacuolar ATPase further reduces the pH to around 4.5 (Kinchen and Ravichandran, 2008; Flannagan *et al*., 2012). A complete maturation of CCV seems to occur only in phase II organisms since cathepsin D, a lysosomal hydrolase, does not seem to accumulate in phase I CCV. Virulent organisms seem to escape intracellular killing by inhibiting the final phagosome maturation step - cathepsin fusion (Ghigo *et al*., 2002, 2004). The mechanisms used by virulent bacteria are not completely known. However, several factors seem to be involved in hijacking conversion of phagosomes, such as the T4BSS and p38α-mitogen activated protein Kinase (p38α MAPK) (Barry *et al*., 2011, 2012). Phase II organisms stimulate p38α MAPK which is required for trafficking of CCV to phagolysosomes for their destruction. Contrarily, phase I *C. burnetii* avoids p38α MAPK activation through the disruption of TLR-4 and TLR-2 association via actin cytoskeleton reorganization induced by LPS (Conti *et al*., 2015) thus escaping from destruction in phagolysosomes (Barry *et al*., 2012). However, the above-mentioned events are not completely accepted, and discordant findings are found in the literature. A similar kinetics between both virulent and avirulent organisms has been described in mature CCVs harbouring phase I or phase II bacteria and containing proteolytically active cathepsins. It was proposed that despite phase I and phase II bacteria appear to engage different host cells receptors, it does not seem to result in different CCV maturation states (Howe *et al*., 2010).

In fact, other factors may interfere in CCV formation and maturation such as opsonisation and cytokine production (Desnues *et al*., 2009; Barry *et al*., 2011). Opsonisation of *C. burnetii* with specific antibodies in persistent infections prevent phagosome conversion since large CCV do not express cathepsin D (Desnues *et al*., 2009). Cytokines also modulate phagosome maturation. Phagosome maturation depends on the balance between pro-inflammatory (IFN-γ, IL-12 and IL-6) and anti-inflammatory (IL-10) cytokines (Barry *et al*., 2011).

It is globally accepted that *C. burnetii* modulates the genesis of CCV and has several strategies for adaptation to the stressful environment. It encodes an important number of basic proteins that are probably involved in buffering the acidic environment of the CCV. Also, four sodium-proton exchangers and transporters for osmoprotectants are found in its genome, allowing this bacterium to confront osmotic and oxidative stresses (Seshadri *et al.*, 2003; Eldin *et al*., 2017).

Considering non-microbicidal cells, phase II bacteria replicate within a compartment that displays all the characteristics of a phagolysosome but exhibits several markers of autophagosomes being considered a chimeric compartment that shares properties of phagolysosome and autophagosomes. It is likely that avirulent bacteria re-route phagolysosome through the autophagosome pathway to get a more suitable environment for replication such as a higher concentration of cholesterol (Barry *et al*., 2011; Pareja *et al*., 2017).

During its biogenesis process, CCV becomes large and contains numerous organisms (Ghigo *et al*., 2002). *C. burnetii* does not synthesize its own CCV membrane. Multiple fusion events with autophagosomes along with endolysosomal vacuoles are essential to provide sufficient membrane to enlarge the CCV (Voth and Heinzen, 2007; Pareja *et al*., 2017). *C. burnetii* continuously directs fusion with other host cell compartments and inhibits apoptotic cell death, allowing a prolonged infectious cycle (Howe *et al*., 2003; Lührmann and Roy, 2007; Voth *et al.*, 2007; Voth and Heinzen, 2009; Vázquez and Colombo, 2010).

1.3.1.4. Intracellular cycle

In the infected cell, *C. burnetii* exhibits a biphasic developmental cycle in which the bacterium transits between SCV and LCV forms under specific environmental conditions (Figure 2) (McCaul and Williams, 1981; Mege *et al*., 1997; Heinzen *et al*., 1999; Coleman *et al*., 2004).

The intracellular cycle of *C. burnetii* is typical of a closed bacterial system with defined lag, exponential and stationary phases (Coleman *et al*., 2004). After internalization by a eukaryotic host cell, the SCV is sequestered within the CCV. In the CCV, SCV differentiates into replicative and metabolically active LCV. The low intra-phagosomal pH and perhaps enzyme system and/or nutrient sources present in the

vacuole seem to trigger this differentiation. Lag phase extends to approximately two days post-infection and it is composed primarily of SCV to LCV morphogenesis (Howe and Mallavia, 2000; Coleman *et al*., 2004; Howe *et al*., 2010). Exponential phase occurs over the next four days with CCV harbouring replicating LCV almost exclusively. The LCV multiplies and persists within an expanding CCV that contains lysosomal elements including an acid pH (5.0) and degradative proteases (Heinzen *et al*. 1999; Howe and Mallavia, 2000; Coleman *et al*. 2004; Howe *et al*., 2010; van den Brom *et al*., 2015).

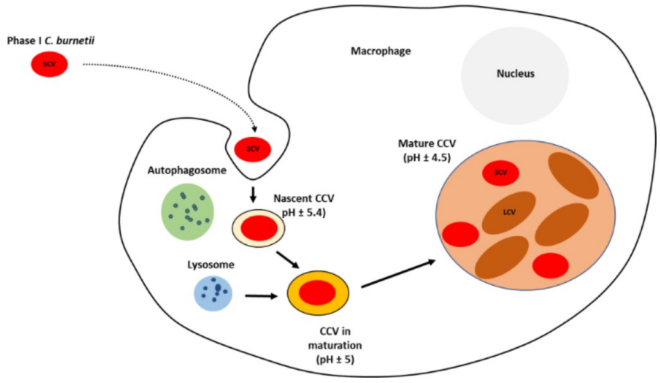


Figure 2: Diagram of the intracellular lifecycle of *C. burnetii* (Adapted from Boarbi *et al*., 2015). CCV - *Coxiella* containing vauole; SCV - Small cell variant; LCV - Large cell variant.

The generation time during this phase is estimated in 12.4 hours. A dramatic expansion of the CCV occurs concomitantly with the appearance of replicating LCV occupying nearly the entire cytoplasm (Coleman *et al*. 2004; Voth and Heinzen, 2007). These metabolically active LCV also play an important role in cell-to-cell spread during acute infection. This process is facilitated by the display of unique LCV antigens such as a porin protein termed P1. The stationary phase begins six days post-infection, concomitantly with the re-appearance of SCV. Following accumulation of large numbers of LCVs, *C. burnetii* converts back into SCVs, which are released from heavily infected cells by an undefined mechanism (Coleman *et al*., 2004).

C. burnetii metabolism is dependent on the moderately low pH inside CCV. Because the pH of the CCV is stable over many weeks it is unlikely that this condition regulates morphogenesis. Rather it is likely to be a response to a decline in the nutritional status

of the host, due in part to the parasitic burden imposed by *C. burnetii* growth (Coleman *et al*., 2004).

It is accepted that SCVs are not replicating forms, since they are scarce during exponential phase appearing in significant numbers during stationary phase. SCV apparently arise in stationary phase via condensation of LCV through a continuum of intermediate forms and increase in number as the phase progresses without further *C. burnetii* replication. Heavily infected host cells may reduce trafficking of nutrient-laden vesicles of the endocytic and/or the autophagic pathway to the CCV. Depletion of critical metabolites such as amino acids may drive to the development of a population dominated by SCV of *C. burnetii* (Coleman *et al*., 2004).

It was demonstrated that lightly and heavily infected cells are capable of division and in the process, they segregate the CCV into one of the emerging daughter cells and the companion daughter cell emerges parasite-free. This asymmetric division of infected cells, revealed via photomicrography of stained cells, accounts for the appearance of uninfected cells within persistently infected host cell populations (Roman *et al*., 1986).

1.3.1.5. Persistent cell infection

Since 1940s, it is recognized that *C. burnetii* can persist after a primary infection (Harris *et al*., 2000). Persistent infected cells harbour *C. burnetii* in the acidic CCV for as long as 153 days post infection (Maurin and Raoult, 1999). A recent study showed that *C. burnetii* DNA was found in the bone marrow of 88% of patients who had primary Q fever 12 years previously (Marmion *et al*., 2005). This explains why *C. burnetii* can be found in apparently cured people (Capo *et al*., 2003; Honstettre *et al*., 2004).

Persistent infection occurs due to the anti-apoptotic activity that *C. burnetii* exerts to maintain the host cell viability (Voth *et al*. 2007; Vázquez and Colombo, 2010). Distinct events have been described, relying on *Coxiella* protein synthesis and revealing that the organism secretes distinct effectors to modulate apoptotic pathways to inhibit host cell death (Voth *et al*., 2007; Luhrmann *et al*., 2010; Broederdorf and Voth, 2011). For example, caspase activation is prevented and a decrease of cytochrome c release from mitochondria is observed. These events affect intrinsic cell death pathway (Lührmann and Roy, 2007; Voth *et al*. 2007). Also, the Dot/Icm T4BSS plays an

essential role to protect infected cells from apoptosis (Beare *et al*., 2011[a]; Carey *et al*., 2011). Overall, the inhibition of apoptosis by *C. burnetii* represents an important virulence property that provides to this obligate intracellular pathogen a stable niche for the course of the pathogen's infectious cycle (Lührmann and Roy, 2007).

1.3.2. Pathogenesis

C. burnetii is a highly infective pathogen and even low doses (10 or fewer microorganisms) cause a high risk of illness (Jones *et al*., 2006; Brooke *et al*., 2015). A recent study estimated in 1.18 bacteria the dose required for fifty percent of the exposed population to become infected (InfD50%), revealing a high infectivity of *C. burnetii* via aerosol exposure (Brooke *et al*., 2013).

The virulence of the infective strain, the size of the inoculum, the health condition of the host and the route of infection influence the incubation period, the disease severity, the duration and the outcome of the infection. Some experimental studies showed an association between the inoculum size and disease severity. For example, high inoculum (10^5) were associated to myocarditis in guinea pigs (La Scola *et al*., 1997) and massive inoculum (10^7) resulted in the death of infected animals (Raoult *et al*., 2005). Considering the route of infection, it was shown that intra-nasal infection resulted in pneumonia and intra-peritoneal infection caused hepatitis (Marrie *et al*., 1996; La Scola *et al*., 1997; Million *et al*., 2009).

Recent work has been done to clarify *C. burnetii* infection mechanisms, but a full picture of the events does not exist yet (Graham *et al*., 2013). It is accepted that aerosol contact leads to entry through the lungs and systemic distribution (Maurin and Raoult, 1999); and the consumption of contaminated fluids such as raw milk leads to systemic distribution through the digestive tract (Tissot-Dupont *et al*., 1992).

After a primary infection, *C. burnetii* may persist covertly and later recrudescence may occur. The number of organisms, their viability and the underlying organ sites of latent infection remain obscure. However, the detection of *C. burnetii* DNA in bone marrow several years after a primary infection suggests bone marrow as a potential focus for recrudescent infection (Harris *et al*., 2000; Marmion *et al*., 2005).

Considering that the infection occurs usually by inhalation of contaminated aerosols, alveolar macrophages are the first line defence that confronts *C. burnetii* (Shannon and

Heinzen, 2009; Graham *et al*., 2013). In fact, the ability of these cells to rapidly respond recruiting additional immune cells is central for an effective antibacterial response in early stages of infection (Marriott and Dockrell, 2007; Barry *et al*., 2011).

In primary infections, after entry into the organism, a bacteraemia occurs leading to a systemic infection with the involvement of organs such as liver, spleen, lungs and bone marrow (Maurin and Raoult, 1999). The organism can subsequently disseminate to colonize and replicate in resident macrophages of different tissues and organs (Stein *et al.*, 2005). In early infections *C burnetii* DNA is detectable in the serum (Fournier and Raoult, 2003; Vincent *et al*., 2015) but a progressive decrease occurs as a specific immune response develops (Fournier and Raoult, 2003; Schneeberger *et al*., 2010). Usually, infection during pregnancy results in uncontrolled growth of the organism in the uteroplacental unit. This results in placentitis and necrosis of placental tissues (Baumgartner and Bachmann, 1992; Carcopino *et al*., 2007). In a recent study, it was shown that in pregnant goats the primary target cells were the trophoblasts in the allanthocorion. Although, the exact mechanism of infection in trophoblasts is not clear; it was observed that gradually, in two to four weeks, *C. burnetii* multiplied and became detectable in trophoblasts of the allanthocorion which developed signs of inflammation (Roest *et al*., 2012). The amount of *C. burnetii* DNA detected increased until parturition and decreased drastically after parturition. This probably occurred by the disappearing of trophoblasts, thus depriving *C. burnetii* of its replication niche (Sanchez *et al*. 2006; Roest *et al*., 2012). The strong tropism of *C. burnetii* towards placenta did not seem to occur for other tissues of goats and kids suggesting that only pregnant females are susceptible to *C. burnetii* infection. However, it is not clear if females should be pregnant to become infected; the possibility that following infection undetectable numbers of *C. burnetii* hide in the body to infect trophoblasts when they become available should not be excluded (Roest *et al*., 2012).

Circulating immune complexes because of specific host immune response may cause vasculitis and vascular thrombosis, which in turn may lead to the placental insufficiency and subsequent obstetric complications such as foetal death (Stein and Raoult, 1998; Sanchez *et al*., 2006). Obstetric complications are more common in patients who are infected during the first trimester of pregnancy than in those infected later (Carcopino *et al*., 2007).

It seems that *C. burnetii* can trigger blood coagulation through the extrinsic pathway, being responsible for the local deposition of fibrin on the surface of infected valves

leading to the development of large vegetations and endocarditis in chronic Q fever (Miragliotta *et al*., 1989). There is an increased risk of persistent infection and endocarditis in pregnancy probably due to a decline in cellular immunity (Stein *et al*., 2000).

1.3.2.1. Host immune response

The course of phase I *C. burnetii* infection depends on the effectiveness of the immune response (Cunha *et al*., 2015). Primary infection almost always resolves without antibiotic treatment, suggesting that host immune response is sufficient (Capo and Mege, 2012), despite the inability to eradicate *C. burnetii* from the organism (Honstettre *et al*., 2004).

Cell-mediated immunity probably plays a critical role in controlling *C. burnetii* infection (Honstettre *et al*., 2004; Shannon and Heinzen, 2009), and T cells are critical for clearance of *C. burnetii* (Andoh *et al*., 2007).

Cells belonging to monocyte-macrophage lineage express polarized functional properties (Gordon and Taylor, 2005). This polarization seems to be closely related with the ability to control *C. burnetii* infection, explaining the bacterial persistence in chronic infections (Benoit *et al*., 2008). M1 polarized macrophages secrete inflammatory cytokines (IL-1b, TNF, IL-12 and IL-6), chemokines (CCL2, CCL5 and CXCL8) and express the surface receptors CCR7, CD80, TLR-2 and TLR-4. They also secrete high levels of iNOS and reactive nitrogen intermediates such as nitrites (Honstettre *et al*., 2004; Benoit *et al*., 2008). M2 macrophages secrete high levels of inflammatory cytokines (IL-10, IL-1, receptor antagonist IL-1ra and TGF-b1), chemokines (CCL16, CCL17, CCL18 and CCL24) and express the surface receptors CXCR1, mannose receptor (MR), CD14 and arginase-1 which induces the blocking of the iNOS pathway. Classically, M1 polarized macrophages are induced by LPS, IFN-γ and TNF-α, participate in the resistance against intracellular pathogens and are involved in Th1 responses. In contrast, M2 polarized macrophages are induced by IL-4, IL-13 or IL-10 and promote Th2 responses. Considering this, it is thought that the course of infection differs according to the macrophage polarization in response to *C. burnetii* infection (Benoit *et al*., 2008). If M1 associated molecules are expressed by macrophages, the bacterial replication will be controlled (Ghigo *et al*., 2002; Benoit *et al*., 2008). While,

the stimulation of a M2 response will account for the persistence of *C. burnetii* in macrophages which become highly permissive to *C. burnetii* replication (Ghigo *et al*., 2001; Ghigo *et al*., 2003; Benoit *et al*., 2008).

Consequently, to the activation of myeloid cells induced by the binding of phase I *C. burnetii* to cell receptors, namely TLR4, a reorganization of the cytoskeleton occurs and early post-infection, infected cells respond to infection with a general up-regulation of Th1-related cytokines. IFN-γ and TNF-α, cytokines secreted by lymphocytes under mediation of TLR4 and TLR2, activate monocytes and macrophages (Honstettre *et al*., 2004; Zamboni *et al*., 2004; Shannon and Heinzen, 2009; Chen *et al*. 2011). This inflammatory response results in early control of infection by the production of reactive nitrogen and oxygen intermediates that lead to intracellular killing of the pathogen (Brennan *et al*., 2004; Andoh *et al*., 2007). However, *C. burnetii* has evolved some mechanisms to resist and to subvert the host immune response allowing its replication and propagation (Shannon *et al*., 2005; Graham *et al*., 2013). This immune evasion strategy, characterized by the ability of limiting pro-inflammatory response helps *C. burnetii* to protect itself from clearance by the host immune system (Capo *et al*., 1999; Barry *et al*., 2011; Capo and Mege, 2012; Sobotta *et al*., 2016) and it is related with the structure of LPS. Phase I LPS mask bacterial recognition by receptors TLR4 signalling of dendritic cells, and thus minimize detection by the host. This results in a less intense inflammatory immune response that allows the persistence of *C. burnetii* without significant maturation of dendritic cells and without inflammatory cytokine production (Zamboni *et al*., 2004; Shannon *et al*. 2005; Waag, 2007; van Schaik *et al.*, 2013). Because phase I *C. burnetii* does not induce maturation of primary dendritic cells, low levels of IL-12 and TNF-α are produced. In opposition, phase II LPS are promptly detected by TLR2, inducing the activation of dendritic cells and secretion of high levels of IL-6, IL-12 and TNF-α. Macrophages are then activated to mediate bacterial clearance (Shannon *et al*. 2005; van Schaik *et al*. 2013).

IFN-γ is synthesized by lymphocytes because of a Th1 response. Th1 response appears to be effective in the control of acute infections, inducing the formation of granulomas (Izzo and Marmion, 1993). Contrarily, chronic infections result from an ineffective cell-mediated response with poor lymphocyte proliferation and a defective synthesis of IFN-γ (Raoult *et al*., 2005).

So, one of the main features of the immune response is the formation of a granuloma under the control of gamma-interferon (γ-IFN). These granulomas are formed by the

migration of monocytes through the vascular endothelium. Granulomas are characterized by an accumulation of immune cells around a central open space, a lipid vacuole, and they are limited by a fibrinoid ring which leads to the definition of a "*doughnut granuloma*". Granulomas are rich in macrophages in different phases of maturation including epithelioid cells and multinucleated giant cells. Few isolated bacteria can be found in granulomas. TLR-4 seems to have a role in granuloma formation, since knock-out mice deficient for this receptor have decreased numbers of granulomas. This probably explains why phase II *C. burnetii* are less efficient than phase I in inducing granuloma formation (Honstettre *et al*., 2004; Argov *et al*., 2008; Eldin *et al*., 2017).

Beyond cell mediated response, an antibody-mediated immunity also seems to be important in *C. burnetii* infection (Shannon and Heinzen, 2009). Treatment of *C. burnetii* with immune sera makes the bacterium more susceptible to phagocytosis and destruction by macrophages (Zhang *et al*., 2007). Specific immunoglobulins are secreted following infection (Maurin and Raoult, 1999) and the infection of dendritic cells with antibody-opsonized bacteria results in increased expression of maturation markers and inflammatory cytokines in mice (Shannon and Heinzen, 2009). However, a large heterogeneity is described between hosts in regard to the time to onset serologic response as well as to the magnitude of the antibody peak titre and decay. In early stages of infection, especially during the increase of the antibody response, antibody concentration changes fast within a relatively short time period (Wielders *et al*., 2015). *C. burnetii* phase II specific antibodies, both IgM and IgG, can be detected within two or three weeks. Antibody titres remain increased for up to 13 weeks post-infection (Maurin and Raoult, 1999; Roest *et al*., 2013[a]) and its decline takes a long time with slowly decreasing concentrations (Wielders *et al*., 2015). Antibody anti-phase I *C. burnetii* increase about four weeks later when compared to phase II (Roest *et al*., 2013[a]). So, within three weeks after infection only low levels of anti-phase I IgM are detected (Dupuis *et al*., 1985). Specific phase I antibodies, namely IgG and IgA, are predominant in chronic forms of the disease (Fournier and Raoult, 1999). Globally, it might be considered that IgG phase II reaches the highest peak titre and that it is also the most persistent (Wielders *et al*., 2015). The duration of the antibody response is not exactly determined. However, it can be concluded from field studies that *C. burnetii* antibodies are highly persistent lasting for several months up to years (Roest *et*

al., 2013[a]; Teunis *et al*., 2013). Thus, both humoral and cellular immunity play a role in *C. burnetii* infection (Maurin and Raoult, 1999).

An effective response by the immune system leads to the formation of protective granulomatous lesions in the infected organs (Honstettre *et al*., 2004; Shannon and Heinzen, 2009). These granulomas are characteristic of the acute Q fever which is spontaneously resolutive (Raoult *et al*., 2005; Boucherit *et al*., 2012). Only, few isolated bacteria can be found in granulomas during the acute phase (Honstettre *et al*., 2004).

However, the immune control of *C. burnetii* might not lead to its eradication from the infected host (Honstettre *et al.*, 2004). *C. burnetii* DNA can be found in circulating monocytes or bone marrow of people infected months or years earlier (Capo *et al*., 2003). Furthermore, *C. burnetii* can persist in the liver, bone, heart valves and endocardium (Harris *et al*., 2000). It is also hypothesized that the uterus could be a site of latent infection hence reactivation during pregnancy can occur (Langley *et al*., 2003; Carcopino *et al*., 2007). For all these reasons persistence, recurrence or re-emergence of *C. burnetii* are a constant worry following an acute infection (Sabatier *et al*., 1997).

Chronic infections are characterized by defective cell-mediated immunity that is inadequate to eradicate the pathogen. Patients with chronic Q fever exhibit increased IL-1 and IL-10 secretion (Capo *et al*., 1996; Ghigo *et al*., 2001; Honstettre *et al.*, 2003; Raoult *et al.*, 2005) and suppression of Th1 mediated cellular response (Waag, 2007). *C. burnetii* continues to multiply despite high concentrations of all three classes of antibodies (IgG, M, and A) to phase I and II bacteria (Sabatier *et al*., 1997). Organ biopsies do not show granulomas (Boucherit *et al*., 2012) but large vacuoles containing *C. burnetii* can be detected in infected tissues such as heart valves and liver, and in aneurysms (Maurin and Raoult, 1999). Monocytes from these patients are not able to kill *C. burnetii* (Dellacasagrande *et al*., 2000) and do not migrate through the endothelium (Raoult *et al*., 2005).

1.4

Clinical patterns of *Coxiella burnetii* infection

The clinical manifestations of Q fever may be so variable that the diagnosis often occurs only if it has been systematically considered (Maurin and Raoult, 1999).

1.4.1. In humans

Following exposure to *C. burnetii* a primary infection develops. This primary infection can be asymptomatic (around 60% of cases) (Raoult *et al*., 2005; Anderson *et al*., 2013) or symptomatic (remaining 40%) also mentioned as acute Q fever. Most of the symptomatic patients recover spontaneously (Fournier *et al*., 2003, CSL Biotherapies 2009), but one to five per cent will develop long-term complications (Figure 3) (Million *et al*., 2015, 2017).

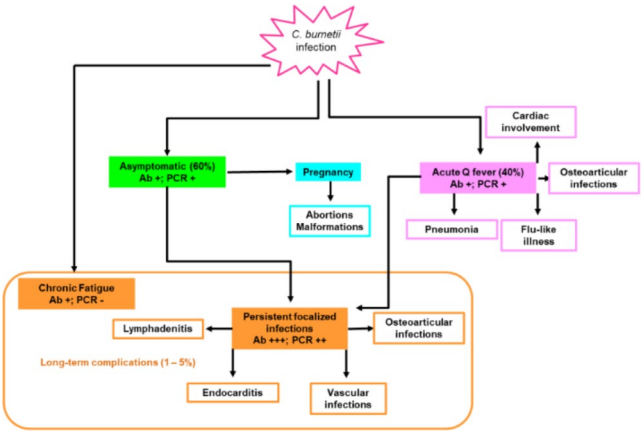


Figure 3: Natural history of *C. burnetii* infection (Adapted from Million *et al*., 2009 and from Eldin *et al*., 2017).

Previously, two main clinical patterns of Q fever were considered, the acute and the chronic Q fever. Nowadays the symptomatic primary infection is named as acute Q fever, but the term chronic Q fever was replaced by long-term complications which

includes two independent entities: persistent focalized infections and the chronic fatigue syndrome (Raoult *et al*., 2017).

1.4.1.1. Acute Q fever

The occurrence of a symptomatic primary infection depends on the strain (D'Amato *et al*., 2015) and on the patient's susceptibility (D'Amato *et al*., 2014[b]). Host determinants like age and sex are linked to clinical manifestations of infection. Older men are more frequently symptomatic than younger women and even pregnant women (Million *et al*., 2015); and children are less frequently symptomatic then adults (Maltezou *et al*., 2002, 2004). The role of immunosuppression in acute Q fever is not well defined. Some reports describe more severe cases in HIV patients (Raoult *et al*., 1993) while others do not find a relation (Montes *et al*., 1995). The virulence of the infective strain also plays an important role in symptomatic infections. MST 17 clone, the unique genotype recognized in human Q fever in French Guiana causes a high rate of symptomatic cases due to its high virulence (Edouard *et al*., 2014; D'Amato *et al*., 2015).

The clinical manifestation of acute Q fever may differ according to seasonal factors and geographic origin of the infection (Raoult *et al*., 2000; Raoult *et al*., 2005; Espejo *et al*., 2014). For example, a higher proportion of pneumonic forms seem to occur in colder months (Espejo *et al*., 2014). These pneumonic forms are more common than hepatitis in Eastern Canada, North of Spain and the Netherlands (Maurin and Raoult, 1999; Espejo *et al*., 2014; Wielders *et al*., 2014) while in Southern Spain and Southern France hepatitis appears more frequently than pneumonia (Tissot-Dupont *et al*., 1992; Raoult *et al*., 2000; Espejo *et al*., 2014). This geographical variation is likely due to differences in host factors, in *C. burnetii* strains (Raoult *et al*., 2005) and in infectious doses (Tiggertt *et al*., 1961; Oyston and Davies, 2011). Previously an association between the clinical pattern of disease and the route of the infection (aerosol or ingestion) was established (Tissot-Dupont *et al*., 1992; Marrie *et al*., 1996), but it is now recognized that this assumption is not true (Raoult *et al.*, 2000).

Nowadays, the symptomatic primary infection is characterized by a diversity of clinical manifestations. The incubation period ranges from two to three weeks depending on the size of the inoculum. In some cases, mild symptomatic forms

resembling a cold occur but often they are undiagnosed or are diagnosed retrospectively (Eldin *et al.*, 2017). The most common clinical presentations of acute Q fever are isolated febrile syndrome or flu-like illness, pneumonia and hepatitis. These manifestations may co-exist (Pebody *et al*., 1996; Maurin and Raoult, 1999; Espejo *et al*., 2014) and none is predictive of the development of long-term complications (Eldin *et al*., 2017).

Isolated febrile syndrome or flu-like illness

The predominant sign is high fever (39-40ºC) but it may be associated with myalgia and severe headaches (Raoult *et al*., 2005). The onset may be abrupt but typically it increases to a plateau within 2 to 4 days and then, after 5 to 14 days, the temperature returns to normal. A biphasic pattern of fever may be experienced by about 25% of patients. The first phase occurs as described above and the second phase corresponds to the reappearance of fever, usually to lower levels and intermittently, lasting from 1 to 19 days (Maurin and Raoult, 1999). Due its nonspecific presentation the diagnosis is misleading, and the screening for *C. burnetii* infection is suggested in the presence of an isolated fever of unknown origin (Eldin *et al*., 2017).

Pneumonia

The prevalence of pneumonia during *C. burnetii* primary infection is highly variable. It has been described as the main manifestation of acute Q fever in the Maritime Provinces of Canada (Marrie *et al.*, 2008), North of Spain (Espejo *et al*., 2014), Croatia (Luksic *et al*., 2006) and the Netherlands (Dijkstra *et al*., 2012). *C. burnetii* has also been identified as the causative agent of community-acquired pneumonia in Germany (Schack *et al.*, 2014), Cameroon (Koulla-Shiro *et al.*, 1997) and French Guiana (Epelboin *et al*., 2012).

Almost all patients suffering Q fever pneumonia present fever, cough, dyspnoea, chest pain and auscultation abnormalities. Pleural effusions may also occur and patients more likely complain of severe headaches. Extrapulmonary signs such as anorexia, fatigue, chills, myalgia, arthralgia, nausea, vomiting, abdominal pain diarrhoea or constipation and sweats, are regularly described. Less frequently skin rash or neurological signs are observed. The fatality rate is lower than 3% and death often occurs in patients with previous pulmonary or cardiac defects (Maurin and Raoult, 1999; Marrie *et al.*, 2003; Lai *et al.*, 2014; Wielders *et al.*, 2014).

Hepatitis

Q fever hepatitis occurs more frequently than pneumonia in France (Edouard *et al*., 2014), Spain (Espejo *et al*., 2014), Portugal (Palmela *et al*., 2012), Israel (Ergas *et al*., 2006) and Taiwan (Chang *et al.*, 2008).

The increased liver enzymes are usually accompanied clinically by fever, chills and headache (Chang *et al.*, 2008). Less frequently, it is observed by abdominal pain (especially in the right hypochondrium), anorexia, nausea, vomiting, and diarrhoea (Ergas *et al*., 2006; Palmela *et al*., 2012; Edouard *et al*., 2014, Espejo *et al*., 2014). Progressive jaundice and palpation of a mass in the right hypochondrium have also been referred. However, most of the times Q fever hepatitis is only revealed by an increase in hepatic enzyme levels and fatality due to hepatic injury is very rare (Maurin and Raoult, 1999).

Cardiac involvement

Clinical manifestations of cardiac involvement show a lower rate of reports, and include pericarditis, myocarditis and endocarditis (Maurin and Raoult, 1999).

Earlier, it was estimated that pericarditis and myocarditis occurred each one in about 1% of symptomatic primary infections (Pebody *et al*., 1996; Maurin and Raoult, 1999; Fournier *et al*., 2001). The systematic screening for *C. burnetii* in patients exhibiting signs of pericarditis revealed that Q fever was involved in 24% of cases and evidenced that *C. burnetii* pericarditis is not as rare as previously postulated (Levy *et al*., 2009). Signs of Q fever pericarditis are not specific and frequently correspond to fever and thoracic pain. Sometimes pericarditis occurs concomitantly with myocarditis or pleuritis (Levy *et al*., 1999; Maurin and Raoult, 1999). Myocarditis is a rare manifestation. It appears as the most severe manifestation of acute Q fever, with a worse prognosis comparing with other clinical forms (Fournier *et al*., 2001). In most patients, myocarditis is revealed only by abnormalities on the electrocardiogram (Maurin and Raoult, 1999; Vogiatzis *et al*., 2008) and considering Q fever in the diagnosis of acute myocarditis improves the prognosis of this severe condition by an early and appropriate treatment (Eldin *et al*., 2017).

Endocarditis was previously described as a manifestation of “chronic Q fever” (*i.e.*, Q fever long-term complications). The recognition of endocarditis in acute primary infections originated a new scenario, which might be associated with an

immunosuppressive condition, but it will need further characterization (Eldin *et al*., 2017).

Neurologic involvement

Neurological manifestations of acute Q fever might be observed alone or combined with another organ involvement. Headache is the most common neurological sign in symptomatic primary infection, but confusion, meningitis, meningoencephalitis and other peripheral neuropathies can also occur (Marrie, 1985; Pebody *et al*., 1996; Maurin and Raoult, 1999; Kofteridis *et al.*, 2004).

Rare clinical manifestations

Among the less common manifestations of acute Q fever are dermatological lesions. Cutaneous manifestations occurred in 4% and 1% of acute cases in France and in the Netherlands respectively (Raoult *et al*., 2000; Wielders *et al*., 2014), being likely more common than generally thought. These manifestations include transient maculopapular or vesicular exanthema and purpuric lesions (Raoult *et al*., 2000; Argov *et al*., 2008). Bone marrow involvement is usually reflected by the presence of characteristic doughnut granulomas in bone marrow (Okun *et al*., 1979; Bottieau *et al*., 2000). Acute lymphadenitis can affect several lymph nodes occurring usually in association with fever, headache, hepatitis or pneumonia (Ariga *et al*., 2000; Foucault *et al.*, 2004). Biological markers of autoimmunity have been detected in some acute Q fever cases (Ordi-Ros *et al*., 1994). Similarly, these have also been, sporadically, reported in acute renal failure cases (Morovic *et al*., 1993; Korkmaz *et al*., 2012).

1.4.1.2. *Coxiella burnetii* long-term complications

After the primary infection, one to five per cent of patients will suffer of long term complications (previously known as chronic Q fever). These may occur soon (within six weeks) after an acute infection, or years later. The duration of this form usually exceeds a period of six months and often evolves to death (Maurin and Raoult, 1999; Botelho-Nevers *et al*., 2007; Million *et al*., 2010).

Long-term complications include persistent focalized infections related to *C. burnetii* reactivation and chronic fatigue syndrome (Maurin and Raoult, 1999; Morroy *et*

al., 2016; Eldin *et al*., 2017) (Figure 3). Patients suffering Q fever long-term complications differ from those with acute disease in age, predisposing conditions and clinical and laboratory findings (Raoult *et al*., 2005; Million *et al*., 2013). Globally, these patients are older than those suffering from acute Q fever (Maurin and Raoult, 1999), and present some predisposing conditions such as immunosuppression, heart valve lesions, vascular abnormalities and pregnancy (Maurin and Raoult, 1999; Fennolar *et al*., 2000, 2001; Karakousis *et al*., 2006; Million *et al.*, 2013).

Persistent focalized infections

The most common manifestations of persistent focalized infections are endocarditis and infections of aneurysms or vascular prostheses, occurring nearly always in patients with underlying lesions (Dellacasagrande *et al*., 2000; Fennolar *et al*., 2000; Raoult *et al*., 2005; Botelho-Nevers *et al*., 2007; Million *et al*., 2009, 2010). They also are the most severe "chronic" forms (Boarbi *et al*., 2015). Q fever endocarditis can be spontaneously fatal if untreated, and mortality rate ranges from 5% to 65% without an appropriate antibiotic treatment (Million *et al*., 2013). For these reasons, systematic echocardiography to exclude underlying cardiac lesions is recommended in all people with acute Q fever, because the evolution to endocarditis can be prevented by antibiotic prophylaxis (Fennolar *et al*., 2000; Million *et al*., 2013; Million and Raoult, 2017). The clinical presentation of *C. burnetii* endocarditis is nonspecific. Patients may present an isolated relapsing fever, chills, night sweats, weight loss, and hepatosplenomegaly (Raoult *et al.*, 2005). Sudden cardiac insufficiency, stroke, or other embolic signs may also occur (Million *et al.*, 2010). Often *C. burnetii* vascular infections are latent and diagnosis is made when complications occur (Botelho-Nevers *et al*., 2007; Wegdam-Blans *et al*., 2011). To improve the early diagnosis of these infections a list of diagnostic procedures was proposed as major diagnostic criteria (Eldin *et al*., 2017).

Osteoarticular infections as an outcome of *C. burnetii* infection have been increasingly reported over the last years, with osteomyelitis occurring more frequently in children and presenting a multifocal pattern (Nourse *et al.*, 2004; Neth *et al*., 2011; Francis *et al*., 2016). Also, persistent lymphadenitis, chronic pulmonary infection and pulmonary fibrosis occurred sporadically (Tattevin *et al*., 2003; Silva *et al*., 2006[b]).

Q fever chronic fatigue syndrome

Another long-term complication is the post-Q fever chronic fatigue syndrome (Ayres *et al*., 1998; Maurin and Raoult, 1999; Ledina *et al*., 2007). Patients manifest fatigue, myalgia, arthralgia, night sweats, mood changes and sleep disturbance (Angelaskis and Raoult, 2010). This syndrome develops in approximately 15% of patients recovering from acute infection, can be disabling in severity and can persist for up to a decade (Marmion *et al*., 1996; Ayres *et al*., 1996, 1998; Strauss *et al*., 2012). It has been attributed to a dysregulation of cytokine production, induced by persistent antigens, including LPS and proteins, rather than persistent latent *C. burnetii* (Marmion *et al*., 2009).

1.4.1.3. Q fever in pregnancy

Despite the abortifacient potential of *C. burnetii* (Stein *et al*., 2000), most pregnant women infected with *C. burnetii* remain asymptomatic (Munster *et al*., 2012). It is suggested that untreated acute Q fever infection during pregnancy may result in adverse pregnancy outcome in up to 81% of cases (Stein and Raoult, 1998; Langley *et al*., 2003; Carcopino *et al*., 2007). These outcomes include oligoamnios, spontaneous abortion, intrauterine foetal death and premature delivery, or low birth weight (Raoult *et al*., 2002; Carcopino *et al*., 2007, 2009) and the risk of occurrence is similar between asymptomatic infections and symptomatic cases (Parker *et al*., 2006). It was also found that obstetric complications occur significantly more often in patients who are infected during the first trimester of pregnancy than in those infected later (Raoult *et al*., 2002; Carcopino *et al*. 2009). All these findings lead to recommendations which state that in outbreak situations all pregnant women should be serological screened for recent Q fever infection and, if found positive, long-term antibiotic treatment should be implemented (Raoult *et al*., 2002; Tissot-Dupont *et al*., 2007; Munster *et al*., 2012). Besides that, the risk of developing chronic Q fever infection is especially high for pregnant women being associated with recurrent miscarriages (Stein and Raoult, 1998; Raoult *et al*., 2002, 2005). Furthermore, not only acute infections associated with obstetric complications, but also previous infections seem to increase the risk (Langley *et al*., 2003). The best explanation for this is the hypothesis of intrauterine latency of the pathogen (Langley *et al*., 2003). During the large outbreak, ever recorded in The

Netherlands, no-evidence of adverse pregnancy outcome amongst women who had antibodies to *C. burnetii* during early pregnancy (van der Hoek et *al*., 2011). Thus, showing that the natural history of *C. burnetii* infection among pregnant women is not completely understood and several factors, including the infective strains, seem to have an important role in the pathogenesis (Munster et *al*., 2012).

1.4.2. In animals

The infection by *C. burnetii* is recognized worldwide by the presence of seropositive animals (Agerholm, 2013). The term Q fever has been adapted in veterinary medicine from the febrile illness originally observed in abattoir workers in Australia (Derrick, 1983). However, because the clinical pattern of Q fever in animals is pleomorphic (EFSA, 2010[a]) and different from humans, the term coxiellosis may be more appropriate to refer to *C. burnetii* infection in animals, especially in cases without fever (Agerholm, 2013).

The knowledge on acute infection in animals is scarce since only a few experimental studies have been carried out (Agerholm, 2013). Furthermore, ruminants do not seem to develop endocarditis because of chronic infection and the lesions occur mainly in the uterus and mammary gland (Maurin and Raoult, 1999). The infection of a pregnant uterus results in a range of conditions, including abortion, delivery of premature offspring, stillbirth and weak offspring in addition to clinically normal progeny that may or may not be congenitally infected. The intrauterine infection depends on, but it is not limited to, strain virulence, maternal and foetal immune responses, severity of placental lesions, possible spread to and dissemination in the foetus, gestation age, and number of infected foetuses (Agerholm, 2013). Infection of livestock with *C. burnetii* often goes unnoticed. Indeed, sporadic abortion of up to 5% does not prompt investigation (Cutler *et al*., 2007). So, in most cases, infection with *C. burnetii* remains non-apparent, with or without serological response (Maurin and Raoult, 1999).

1.4.2.1. Small ruminants

In small ruminants, in early stages of infection, *C. burnetii* can be demonstrated in blood, lungs, spleen and liver (Maurin and Raoult, 1999). However, it is not clear if its presence in organs other than placenta affects the functions of these organs, since only mild lesions have been described for sheep and goats (Lennette *et al*., 1952; Martinov *et al.*, 1989; Sanchez *et al*., 2006; Roest *et al*., 2012).

Some experimental infections conducted in pregnant ewes showed, in an acute phase of infection, the occurrence of fever in a biphasic pattern at the 5th and 12th days post-infection; depression, anorexia, thirst, conjunctivitis, rhinitis and tachypnoea in some cases (Martinov *et al*., 1989). In other cases, apart from irregular episodes of fever, which were usually most pronounced during the first 7 to 10 days post-infection, no apparent signs of illness were detected (Lenette *et al*., 1952) and the absence of clinical signs during the acute phase of infection has been also described (Berri *et al*., 2001, 2005). After the acute phase, which can also be asymptomatic, a latent infection develops reactivating late in pregnancy, several days before parturition (Martinov *et al.*, 1989; Arricau-Bouvery and Rodolakis, 2005). This reactivation manifests usually like abortion, stillbirth, delivery of weak offspring, premature delivery and metritis (Berri *et al*., 2002, 2005; Sánchez *et al*., 2006; Rousset *et al*., 2009; Astobiza *et al*., 2010; Roest *et al*., 2012; Van den Brom *et al*., 2015). Of these, one of the most important outcomes of the *C. burnetii* infection in domestic small ruminants is the abortion, which may occur without premonitory signs and associated with a deterioration of the general condition (Martinov *et al.*, 1989; Arricau-Bouvery and Rodolakis, 2005). In goats the abortion occurs uneventful although dystocia may develop due to foetal death or uterine inertia (Sanford *et al*., 1994).

Globally, when late-term abortions, stillbirths or birth of stunted animals are observed in sheep and goat flocks, Q fever should be suspected. Usually, up to 90% of the reproductive females within the flock are infected (Arricau-Bouvery and Rodolakis, 2005). That is why it is mentioned that *C. burnetii* may cause epidemic herd outbreaks with significant animal losses due to abortion and weak offspring during the parturition period (Zeman *et al.*, 1989; Arricau-Bouvery and Rodolakis, 2005; Eibach *et al.*, 2012). In the season that follows an abortion storm the multiplication of the organism may be reactivated during pregnancy leading to reproductive failures which can be less

prominent in ewes bearing in mind that shedding of the bacterium might still be important (Berri *et al*. 2005, 2007; Van den Brom and Vellema, 2009).

1.4.2.2. Cattle

The available data about the clinical findings in cattle are inconsistent (López-Gatius *et al*. 2012; Agerholm, 2013; García-Ispierto *et al*., 2013, 2014).

Before strict bio-safety measures, some experimental studies have been conducted in cattle. In one experiment, a transient fever was observed 2–3 days after subcutaneous (SC) inoculation of seronegative cows with *C. burnetii* Nile Mile strain. Additionally, two cows aborted late in pregnancy (Behymer *et al*., 1976). The occurrence of an acute self-cured pneumonia was reported in another experiment (Plommet *et al*., 1973).

Despite the frequent asymptomatic pattern of coxiellosis in cattle (Maurin and Raoult, 1999; Hansen *et al*., 2011), and the lack of scientific evidence showing a cause-effect relationship between *C. burnetii* and some reproductive disorders neither at individual level nor at herd level (Agerholm, 2013), a negative impact on animal health is recognized in *C. burnetii* infection. Clinical signs consist of reproductive disorders and occasionally pneumonia (Arricau-Bouvery and Rodolakis, 2005). The occurrence of late abortion (Woldehiwet, 2004; Agerholm, 2013), infertility (To *et al*., 1998; López-Gatius *et al*., 2012), placental retention, post-partum metritis (Bildfell *et al*., 2000; Martinov, 2008; Garcia-Ispierto *et al*., 2010; López-Gatius *et al*., 2012) and even mastitis (Barlow *et al*., 2008) have been described.

The abortion caused by *C. burnetii* is considered a rare event in cattle (Bildfell *et al*., 2000; Jensen *et al*., 2007; Rodolakis *et al*., 2007; Muskens *et al*., 2011). However, in naïve herds, the abortion rate may reach 5% (van den Brom and Vellema, 2009). It results from the placental inflammation induced by the infection of trophoblasts (Bildfell *et al*., 2000; Roest *et al*. 2012). The bacteria may remain confined to the placenta or it may spread to the foetus by the amniotic-oral route, if it penetrates the placenta, it contaminates the amniotic fluid and become aspirated/swallowed by the foetus. In such cases, bacteria become established in the intestinal tract or may invade the lungs by the trachea-bronchial route. This explains the cases of bronchopneumonia observed in aborted foetus (Bildfell *et al*., 2000; Cantas *et al*., 2011; Agerholm, 2013). *C. burnetii* also does not seem to be an important cause for metritis in dairy herds even

when apparently *C. burnetii* is present in Bulk Tank Milk (BTM) (Muskens *et al*., 2011).

Some studies could not find a consistent relationship between *C. burnetii* infection and reproductive disorders in dairy cows (Muskens *et al*., 2011; Agerholm, 2013). It was found that the pregnancy occurred sooner in seropositive shedders than in seronegative cows (López-Gatius *et al*., 2012; Garcia-Ispierto *et al*., 2013) and twin pregnancy appeared to be more likely in seropositive than in seronegative cows (Garcia-Ispierto *et al*., 2015). These somewhat unexpected events seem to be occurring after a previous exposure. The animals probably become protected against the detrimental effects of a recent infection or even against recrudescence of the bacterium during their fertile period (Garcia-Ispierto *et al*., 2014).

According to the European Food and Safety Authority (EFSA) recommendations, the major warning sign of Q fever, to be considered, is the number of abortions and calves with low birth weight in cattle herds (EFSA[a], 2010):

- three abortions in the year for herds with less than 100 cows and
- more than 4% of cows aborting during the year for herds of more than 100 cows.

1.4.2.3. Other species

C. burnetii can infect many animal species. It is though that the infection remains usually asymptomatic (Maurin and Raoult, 1999) as the role of *C. burnetii* in illness of animals other than ruminants is not very clear (Agerholm, 2013).

C. burnetii has been investigated as a potential causative agent of equine abortion in a few studies. No *C. burnetii* positive case of equine abortion was conclusive about the responsibility of this organism in equine pregnancy loss because of the concomitant infection by other abortifacient agents or by the absence of specific lesions (Agerholm, 2013; Marenzoni *et al*. 2013).

Also, in pigs the knowledge on porcine coxiellosis is almost absent (Agerholm, 2013). Some studies conducted in 1950's revealed that experimentally infected pigs develop specific antibodies. However, the bacterium was not detected in placentas from sows' cohabitants with dairy cattle, some of them known to be infected by *C. burnetii* (Marmion and Stocker, 1958). So, it remains to be proven if *C. burnetii* can cause clinical illness in domestic pigs.

Such as in pigs, there is no direct evidence that dogs may develop a clinical illness after exposure to *C. burnetii*. However, it is well known that dogs may become infected and develop a humoral immune response (Cooper *et al*., 2012; Shapiro *et al*., 2016). A human outbreak of Q fever has been linked to close contact to an infected parturient dog that gave birth to three pups that died shortly after birth while a fourth pup died within 24 h. However, as the pups were not tested some doubts remain about the involvement of *C. burnetii* in these events (Buhariwalla *et al*. 1996).

Besides the evidence of exposure in cats (Matthewman *et al.*, 1997; Komyia *et al.*, 2003; Shapiro *et al.*, 2015), *C. burnetii*'s DNA has been detected from uterine biopsies of healthy client-owned cats (Cairns *et al*., 2007) and isolated from the vagina of cats having abortion and fever (Nagaoka *et al.*, 1998). Furthermore, parturient cats delivering stillborn or healthy kittens have been implicated in several outbreaks of Q fever in man. (Kosatsky, 1984; Marrie *et al*., 1988; Pinsky *et al*., 1991). As none of the kittens have been examined and the association between the cat's parturition and the Q fever outbreak has been established in retrospective epidemiological investigations, the confirmation of the association between coxiellosis and the occurrence of reproductive disorders in cats is lacking. Some experimental investigations indicate that *C. burnetii* can cause fever, anorexia and lethargy in cats. However, in the field, infection usually remains subclinical (Egberink *et al*., 2013).

1.4.3. Pathological findings

The immune response during coxiellosis is associated with an inflammatory reaction that results in formation of granulomatous lesions most commonly involving the lungs, liver, and bone marrow (La Scola, 2002).

The hepatic lesions are different in acute and chronic coxiellosis. In acute cases, the characteristic findings are granulomatous lesions containing the so-called doughnut granuloma. In chronic cases, pathologic findings are nonspecific with lymphocytic infiltration and foci of spotty necrosis (La Scola, 2002).

The vegetations in coxiellosis endocarditis often are smooth and nodular. The valve often is infiltrated with foamy macrophages that are filled with *C. burnetii* cells (La Scola, 2002).

C. burnetii infection may cause placentitis in pregnant females which is characterized macroscopically by the presence of purulent yellow-brownish exudates, covering mainly the thickened inter-cotiledonary areas (Martinov *et al*., 1989; Van den Brom *et al*., 2012). Microscopically, in almost all cases, the trophoblasts of the inter-cotiledonary allantochorion and the chorionic epithelial cells, especially at the base of the cotyledonary villi are affected, showing a foamy vacuolated cytoplasm and contain basophilic intra-cytoplasmatic granulation. The severity of inflammation varies from mild mononuclear infiltration to severe necrosis and purulent exudation (Van den Brom *et al*., 2012).

When abortion occurs, foetuses show normal appearance although occasionally autolysis is described (Van den Brom *et al*., 2012). The liver of some foetus may show mild granulomatous hepatitis, usually with no abnormalities present in other organs (Roest *et al*., 2012; Van den Brom *et al*., 2012).

1.5

Diagnostic challenges

An accurate diagnosis of Q fever / coxiellosis requires laboratory testing due to the absence of specific clinical signs (McQuiston *et al.*, 2002; Arricau-Bouvery and Rodolakis, 2005). The lack of overt clinical signs exhibited among infected livestock make diagnosis of coxiellosis a challenge (Cutler *et al*., 2007).

1.5.1. Collection and handling of clinical specimens

Coxiella burnetii is a very infectious bacterium. The handling and cultivation of *C. burnetii* contaminated clinical samples should be performed in biosafety level 3 laboratories (BSL-3) by experienced personnel (La Scola, 2002; Hartzell *et al.*, 2008; De Bruin *et al*., 2009; Angelaskis and Raoult, 2011).

Several specimens are suitable for the detection of *C burnetii*, but their availability depends on the clinical presentation. Globally blood, cerebrospinal fluid, bone marrow, cardiac valves, vascular aneurysms or grafts, bone biopsy, liver biopsy, milk, placenta and foetal specimens after abortion can all be use in the diagnosis of Q fever (Angelaskis and Raoult, 2011).

It is recommended that specimens should be kept frozen at -80ºC and forwarded on dry ice to the diagnostic laboratory (Fournier *et al*., 1998). In acute Q fever cases, early serum samples are a valuable source of viable *C. burnetii* with no special handling of the specimens required to maintain the organism's viability. In these serum samples *C. burnetii* maintains its viability for 224 days or 371 days respectively in refrigerating or freezing condition (-20ºC) (Vincent *et al*., 2015). The demonstration of live organisms in the extracellular fraction of the blood during infection has been achieved by the successful isolation of *C. burnetii* by inoculation of serum from Q fever patients into immunosuppressed A/J mice (Nagaoka *et al*., 1998; Vincent *et al*., 2015).

1.5.2. Methods of laboratory diagnosis

Two main categories of diagnostic tests are available: i) detection of the agent and ii) serologic tests, including indirect immunofluorescent (IFA), enzyme immunoassay (ELISA), and complement fixation test (CFT) (McQuiston *et al.*, 2002; OIE, 2015).

1.5.2.1. Detection of the agent

The detection of the agent includes isolation of the organism; staining methods and specific detection methods such as immunohistochemistry, fluorescent in-situ hybridisation and polymerase chain reaction (PCR) assays. Nowadays, genotyping methods are a useful tool as a complement of isolation and PCR, allowing a detailed identification of strains (OIE, 2015).

Isolation of C. burnetii

Cultivation of *C. burnetii* may be obtained from the buffy coat of heparinised blood, whole blood, plasma, bone marrow, cerebrospinal fluid, cardiac valve biopsy, vascular aneurysm or graft, bone biopsy, liver biopsy, milk, faeces, vaginal swabs, placenta, and foetal specimens in case of an abortion (La Scola, 2002).

Isolation of *C. burnetii* can occur by inoculation of laboratory animals, embryonated eggs, cell cultures (Van den Brom *et al*., 2015) and insect larvae (Norville *et al*., 2014). Recently, the development of cell free culture media improved the methods of isolation of *C. burnetii* (Omsland *et al*., 2009, 2013; Omsland, 2012). However due to specific requirements this technique is not widely used.

Several rodent and non-human primate models have been developed and used for *C. burnetii* isolation. These animal models have the main advantage and the greater success of isolation from samples with low bacterial loads and/or contaminated with other bacteria (Van den Brom *et al*., 2015). However, the use of animals itself (ethically speaking), the high costs and the difficult manipulation are well recognised disadvantages (Norville *et al*., 2014).

In embryonated eggs, *C. burnetii* grows almost exclusively in the yolk sac endoderm cells. To reduce bacterial contamination, filtering the samples or adding antibiotics can be used however these procedures reduce the sensitivity of the test. Another

disadvantage of this culture system is that the growth of *C. burnetii* cannot be monitored by visual inspection (Van den Brom *et al*., 2015).

Compared with other techniques the cell culture system has several advantages; therefore, the cell culture system is currently the most widely used *in vitro* system to isolate and cultivate *C. burnetii*, and several cell lines can be used (Voth and Heinzen, 2007). In many cases, the Vero cell culture was more sensitive than the diagnostic qPCR assays for the detection of *C. burnetii* (Vincent *et al*., 2015). Cell-based cultivation methods closely mimics *in vivo* infection conditions (Kuley *et al*., 2015). Propagation of *C. burnetii* in cell-based cultures is useful for *in vitro* growth (Beare *et al*., 2011[b]; Omsland *et al*., 2011; Kuley *et al*., 2015).

However, over the recent years the concept of *C. burnetii* isolation has suffered some changes. On one hand, the use of insect models has been described for several pathogens (McMillan *et al*., 2015; Champion *et al*., 2016). The larvae of the greater wax moth, *Galleria mellonella*, were found to be susceptible to *C. burnetii* infection in a dose-dependent manner. Furthermore, *G. mellonella* survives at 37°C, allowing optimal expression of bacterial virulence factors that are active in a mammalian host; and *C. burnetii* resides and replicates within haemocytes which function as mammalian macrophages, phagocytosing bacteria and killing via an oxidative burst. These findings greatly support the use of this insect model for isolation and investigation of *C. burnetii* infection suppressing the disadvantages of the animal model (Norville *et al*., 2014). On the other hand, the development of an axenic (cell-free) growth medium for *C. burnetii* has greatly facilitated the isolation of single colonies on solid medium (Beare *et al*., 2011[b]; Omsland *et al*., 2011). This medium mimic the composition of the acidic environment in which *C. burnetii* replicates (Omsland *et al*., 2009, 2013; Omsland, 2012). An advantage of cell-free propagation of *C. burnetii* is the absence of host cell genetic material, which significantly accelerates the availability of the genome being particularly helpful in the genetic analysis of *C. burnetii* (Omsland *et al*., 2009; Kuley *et al.*, 2015; Van den Brom *et al*., 2015). Furthermore, the development in axenic culture system does not significantly influence the viability, phase variation and relative virulence of *C. burnetii* compared with cell-based culture (Kuley *et al*., 2015).

Since the isolation of *C. burnetii* is time consuming and can only be performed by specialized laboratories with BSL-3 facilities, it is not used routinely for the diagnosis of Q fever (Arricau-Bouvery and Rodolakis, 2005; Vincent *et al*., 2015).

Staining methods

Smears of placental cotyledons can be prepared on microscopic slides and stained according to several techniques such as Stamp, Gimenez, Macchiavello and Giemsa which are close to the modified Ziehl-Neelsen method (Rousset *et al*., 2009). A very large number of thin, pink-stained coccobacillary bacteria against a blue or green background characterizes *C. burnetii*. They may, sometimes, be difficult to detect because of their small size, but their large numbers compensate for this; often inclusions within the host cells appear as red masses against the blue or green background. However, attention must be taken in the interpretation of the results as, microscopically, *C. burnetii* can be confused with *Chlamydophila abortus* or *Brucella* spp. and all these agents are abortifacient. Diagnosis based on microscopy, coupled with positive serological results, is usually adequate for routine purposes. When biological staining is inconclusive, one of the above methods may be used as a confirmatory test (Rousset *et al*., 2009).

Specific detection methods

Detection of *C. burnetii* in samples can also be achieved by specific immunodetection or in-situ hybridisation (Jensen *et al*., 2007; Samuel and Hendrix, 2009). Immunodetection can be used in paraffin-embedded tissues or in acetone-fixed smears (Raoult *et al*., 1994). It is based on an indirect immunofluorescence or immunoperoxidase assay using polyclonal *C. burnetii* specific antibodies and a species-specific anti-IgG conjugate, labelled with fluorescein or peroxidase, which is used to visualise the bacteria. Control positive slides of *C. burnetii* antigen are needed for comparison (OIE, 2015). Fluorescent in-situ hybridisation using specific oligonucleotide probes targeting 16s rRNA may also be used on paraffin embedded tissues, especially placenta samples (Jensen *et al*., 2007).

Molecular methods

DNA based detection and quantification methods, like the Polymerase Chain Reaction (PCR), aim to detect *C. burnetii* directly by targeting one or more specific sequences in its genome. Several conventional and quantitative PCR assays have been developed for the detection and quantification of *C. burnetii* in clinical samples of different origins (Stein *et al*., 1992; Guatteo *et al*., 2005; Van den Brom *et al*., 2015).

The specific sequences targeted by PCR can occur in single or in multiple copies within the genome. Several PCR tests based on multiple copy genes like IS1111 have been developed. These are valuable for sensitive detection of the organism in each sample, especially when the organism is present in very low concentrations. IS1111 PCR tests showed a higher sensitivity than those based on single copy genes which are usually used for quantification purposes (Kim *et al*., 2005, Klee *et al*., 2006, Loftis *et al*., 2006, Panning *et al*., 2008, De Bruin *et al*., 2009; De Bruin, 2011).

However, the use of PCR based on IS1111 for the detection of *C. burnetii*, has recently been questioned by the demonstration of *Coxiella*-like bacteria in ticks (Duron, 2015; Jourdain *et al*., 2015). This finding raised the concern that PCR assays based on IS1111 detection may lead to misidentification with *Coxiella*-like bacteria (Jourdain *et al*., 2015). The addition of other targets than IS1111 has been proposed to overcome the false positive results, but it was found that other targets may also be contained in *Coxiella*-like bacteria (Duron, 2015, Duron *et al*., 2015, Jourdain *et al*., 2015). It has been suggested that coupling sensitive detection assays with single nucleotide polymorphism (SNP) genotyping methods provides an insurance mechanism for positive identification (and genotyping) of *C. burnetii* without the risk of false positives mainly when dealing with samples obtained from ticks (Pearson *et al*., 2016). Despite current evidence suggests that in most situations, the overall risk of false detection of *C. burnetii* is low and can be mitigated with current methods (Pearson *et al*., 2016); PCR-based surveys aiming to detect *C. burnetii* in ticks by the currently available methods must be interpreted with caution if the amplified products cannot be sequenced (Jourdain *et al*., 2015).

Another limitation of PCR methods can be the inability to differentiate viable and non-viable bacteria. The single detection of *C. burnetii* DNA by PCR is not convincing evidence of the presence of viable bacteria. However, repeated detection accompanied by a decrease in the cycle threshold (Ct) value in real-time PCR is indicative of an increase in the number of *C. burnetii* genome copies and therefore a good indication of bacterial growth (Vincent *et al*., 2015).

The availability of complete genome sequences of *C. burnetii* has increased the understanding of the genomic diversity of the agent (Massung *et al*., 2012). Molecular characterization of strains allows the comparison of genotypes and the discrimination between strains obtained from different animal species or geographic origin. This helps tracing back Q fever outbreaks to their source, assessing the relationships between

genotype and virulence, and the monitoring of different geographic areas (Sidi-Boumedine *et al*., 2009; Roest *et al*., 2013[b]). For instance, systematic genotyping of *C. burnetii* enhances the ability to identify the origin of an outbreak and, consequently, specific measures can be implemented to contain it, reducing the number of cases (Arricau-Bouvery *et al*., 2006; Roest *et al*., 2011). Over the years, various genotyping techniques have been described for the characterization of *C. burnetii* strains. Initially, the plasmid profile was assumed to be associated with acute or chronic presentations of Q fever (Samuel *et al*., 1988; Willems *et al*. 1993). Subsequent studies did not confirm this assumption (Stein *et al*., 1993; Thièle *et al*., 1994; Jagger *et al*., 2002). Analysis of the genome by techniques such as pulsed field gel electrophoresis (PFGE) and PCR restriction fragment length polymorphism analysis (PCR-RFLP) have been used to recognize different groups of *C. burnetii* isolates (Heinzen *et al*., 1990; Thièle *et al.*, 1993) as well as the differentiation between strains by sequence-based determination of *Com1* and *MucZ* encoding genes (Zhang *et al*., 1997; Sekeyova *et al*., 1999). Infrequent Restriction Site-PCR (IRS-PCR), IS1111 an insertion sequence PCR-based and tandem mass spectrometry coupled to nanoscale ultraperformance liquid chromatography (LC-MS/MS) have also been developed for the typing of *C. burnetii* isolates (Arricau-Bouvery *et al*., 2006; Denison *et al*., 2007; Hernychova *et al*., 2008). All these methods however, rely on the cultivation of the agent prior to analysis which is wearisome and may also create a potential for generation of genotypic variation due to multiple sequential cell divisions. Besides that, their discriminatory power is poor, and their reproducibility and transferability are not always straightforward. Some of these techniques are laborious and sophisticated and are not suitable for routine use (Arricau-Bouvery *et al*., 2006; Massung *et al*., 2012).

Several genotyping methods have been developed to be used directly on clinical samples, namely multispacer sequence typing (MST), single nucleotide polymorphism (SNP) based typing and multiple-locus variable number tandem-repeat analysis (MLVA) (Glazunova *et al*., 2005; Arricau-Bouvery *et al*., 2006; Svraka *et al*., 2006; Huijsmans *et al*. 2011; Honstra *et al*., 2012).

To date, MLVA and MST are considered as the most discriminating methods for *C. burnetii*, allowing the identification of up to 36 distinct genotypes (Chmielewski *et al*., 2009; Klaassen *et al*., 2009; Sulyok *et al*., 2014). As PCR-based methods, theoretically, these methods allow the characterization directly from clinical samples avoiding the culture step of this fastidious bacterium and can be performed in any

laboratory with basic equipment. They can easily determine relationships among *C. burnetii* from different origins by using PCR-positive samples, thus helping in the identification of the source of an outbreak in a rapid analysis (OIE, 2015; Sidi-Boumedine *et al*., 2015[b]). Moreover, they are reproducible, allowing inter-laboratory comparisons. The development of international databases for these two typing methods allow the comparison and understanding of the propagation of the *C. burnetii* isolates (Chmielewski *et al*., 2009; Klaassen *et al*., 2009; Sulyok *et al*., 2014).

The MLVA typing scheme is based on variation of number in tandemly repeated DNA elements, on multiple loci, in the genome of *C. burnetii* and shows a high discriminatory power (Svraka *et al*., 2006; Chmielewski *et al.*, 2009; De Bruin *et al*., 2009; Sidi-Boumedine *et al*., 2015[b]). Tandem repeats are sequences that contain a repetitive element (repeats), aligned next to each other. *C. burnetii* strains may differ in the number of repeats within a specific locus, and several different loci can be combined to obtain specific MLVA types (De Bruin *et al*., 2009). A total of 17 different minisatellite and microsatellite repeat markers have been described (Arricau-Bouvery *et al*., 2006; Sidi-Boumedine *et al.*, 2015[b]).

An MLVA type can be assigned to a specific strain (Svraka *et al.*, 2006). To obtain a robust result, DNA of high quality and yield is needed. Thus, some difficulties to obtain complete MLVA patterns from clinical samples have been observed. The presence of inhibiting substances in animal matrices and the background DNA from organisms other than *C. burnetii* originated in the matrix material can bind to the selected primers and cause problems (De Bruin *et al*., 2009; Sidi-Boumedine *et al*., 2015[b]). Furthermore, the low amounts of *C. burnetii* DNA in samples compromise the typeability (Ct > 31) (Piñero *et al*., 2015). Often this can be solved by prior cultivation of the organism (De Bruin *et al*., 2009). The Whole Genome Amplification (WGA) can also be used to amplify the total amount of DNA in a sample thus increasing the amount of *C. burnetii* DNA. This procedure can increase the robustness of molecular typing, like MLVA, if enough *C. burnetii* target DNA is amplified within a sample (De Bruin *et al*., 2009). Amplification failures leading to incomplete MLVA patterns and the presence of amplicons of unexpected sizes (>to 1.5 kbp) have also been described as difficulties encountered when using MLVA as a routine genotyping scheme (Sidi-Boumedine *et al*., 2015 [b]).

In MST, DNA sequence variations in non-coding intergenic spacer regions within the genome are used to compare *C. burnetii* strains. In this typing method, 10 short intergenic regions are used to investigate relationships between *C. burnetii* strains. An advantage is that sequence information is more robust than PCR fragment length analysis methods, like MLVA. However, MST can be hampered by the same factors as MLVA (Glazunova *et al*. 2005; Chmielewski *et al.*, 2009; De Bruin *et al*., 2009). Also, the costs related with sequencing hamper the use of this technique in routine diagnosis.

1.5.2.2. Serology

The diagnosis and the screening for Q fever often rely on serologic techniques, reflecting the host's humoral immune response (Schoffelen *et al.*, 2014). Demonstration of specific antibodies directed against *C. burnetii* is possible with several serological tests such as microagglutination (MAT), complement fixation test (CFT), indirect immunofluorescence (IFA) and Enzyme Linked Immunossorbent assay (ELISA) (Rousset *et al*., 2007; Herremans *et al.*, 2013; Bizzini *et al*., 2015). Among the serological techniques, the ELISA and the CFT are the most commonly used tests for detecting antibodies against *C. burnetii* in animals (Van den Brom *et al*., 2013), whereas IFA is considered the gold standard for human Q fever diagnosis. It is especially useful to differentiate IgM and IgG antibodies against phase II and I antigens, which occur in acute and chronic Q fever, respectively. Recently an automated epifluorescence immunoassay was developed and showed to be a valuable assay for chronic Q fever diagnosis (Bizzini *et al.*, 2015).

Among the disadvantages of using serology for diagnosis, there is the lag in antibody development of seven to fifteen days after the onset of symptoms (Maurin and Rault, 1999; Anderson *et al*., 2013) and the persistence of IgM phase II antibodies for diagnosis of acute cases in post-endemic and endemic situations (Wegdam-Blans *et al*., 2012). The presence of IgM phase II antibodies can be inaccurate for diagnosis because solitary IgM can be a false positive (Szymanska-Czerwinska *et al*., 2015).

Enzyme Linked Immunossorbent Assay

ELISA is a sensitive technique that is easy to perform, thus preferred for routine diagnosis in animals and for screening of large number of samples. However, it has

some limitations in monitoring activity (Jaspers *et al*., 1994; Natale *et al.*, 2012; Herremans *et al*., 2013; OIE, 2015).

The commercial ELISA assay for Q fever detect mainly IgG antibodies since IgM detecting ELISA are not currently available (Kittelberger *et al.*, 2009). Ready-to-use kits are commercially available and can detect anti-phase II antibodies or both anti-phase I and II antibodies (OIE, 2015). Most commercial tests detect both and the ratio between the two types differs, being influenced by the antigen extraction procedure (Van den Brom *et al*., 2015).

The sensitivity of the ELISA is high, comparable to IFA (Rousset *et al*., 2007) and it is higher than CFT. The specificity is good and similar between tests (Rousset *et al*., 2007, 2009; Kittelberger *et al*., 2009; Ruiz-Fons *et al*., 2010; Natale *et al.*, 2012). ELISA will detect most of the infected ruminants but may miss animals that have not developed an IgG response (Kittelberger et al., 2009).

Numerous reports described *C. burnetii* antigens and their influence on the results of serological testing (Rodolakis, 2006, 2009; Kittelberger *et al*. 2009). Discrepant results were described in complement fixation test (CFT) when using Nine Mile (NM) strain (ticks from Montana) and Henzerling strain (humans from Italy) as antigen (Stoker *et al*., 1955). An ELISA using an ovine aborted placenta antigen (Cb01) has been evaluated in Europe and an increased sensitivity was observed when compared to a similar ELISA using the NM strain antigen (Rodolakis *et al*. 2007; Horigan *et al*. 2011). In fact, the EFSA reported a higher diagnostic sensitivity of ELISA using ruminant *C. burnetii* antigens when compared to those using NM antigens (EFSA, 2010[b]). However, recently no differences in diagnostic sensitivity were found between two ELISA kits based on antigens isolated from ruminants and from ticks, when testing goat serum from a herd experiencing an abortion storm (Emery *et al*., 2014). These contradictory results need further investigation, expanding the target species, the clinical presentation and associating the characterization of the infective strains and the geographic origin.

Indirect Immunofluorescence (IFA)

IFA is highly sensitive, and it is the gold standard method for the serodiagnosis of Q fever in humans (Tissot-Dupont *et al*., 1994; Fournier *et al*., 1998; Maurin and Raoult, 1999). The assay uses both phases I and II *C. burnetii* antigens allowing the recognition of acute and chronic forms of infection, which have different serological

profiles. In acute Q fever, there is an increase of IgG antibodies against phase II, whereas in chronic Q fever, high levels of IgG antibodies against both phase I and II are observed. Therefore, IFA is an important tool in the follow-up of patients and in identifying patients at risk for developing chronic Q fever (Herremans *et al*., 2012).

Currently, IFA is not commercially available for ruminants and thus it is not often used for diagnosis of Q fever in animals (Rodolakis, 2006). However, antigen-spot slide wells coated by phase II or both I and II forms of *C. burnetii* may be purchased. Thus, IFA can be adapted by replacing the human conjugate by a conjugate adapted to the animal species (OIE, 2015).

Complement Fixation Test (CFT)

CFT can use a phase II antigen prepared from a mixture of two strains, Nine Mile and Henzerling or, alternatively, a mixture of phase I and II antigens prepared from Nine Mile strain can be used (Kittelberger *et al.*, 2009; OIE, 2015). In the past, the CFT was extensively used in serodiagnosis of Q fever. Nowadays, its use has decreased. It is specific but the lower sensitivity when compared to ELISA or IFA makes CFT not suitable for serological screening. CFT also is more time-consuming than IFA or ELISA (La Scola, 2002; Rousset *et al*., 2007; Kittelberger *et al*., 2009; Natale *et al*., 2012). Some technical difficulties are described in CFT namely the failure in detection of cases when anti-complementary substances are present in the tested sera, the non-evidencing of antibodies due to differences in the ability of IgG subclasses to activate complement and a pro-zone phenomenon may be present with serum specimens from patients with chronic Q fever that could result in a false-negative test. Moreover, seroconversion is detected later (ie. 2 to 3 weeks) compared to IFA or ELISA (ie. 10 to 15 days) (Maurin and Raoult, 1999; La Scola, 2002; Rousset *et al*., 2007).

1.6

Epidemiology of *Coxiella burnetii*

Current knowledge indicates that Q fever should be considered a public health problem in many countries, even where Q fever is unrecognized because of poor surveillance of the disease (EFSA, 2010[a,b]; OIE, 2015).

1.6.1. Descriptive epidemiology

Spatial and temporal distribution of *Coxiella burnetii* can be linked with agricultural and meteorological characteristics (de Rooij *et al*., 2016).

1.6.1.1. Spatial distribution

The potential danger of Q fever to public health and the large gaps in the knowledge of the disease were early recognized. In 1950, the World Health Organization (WHO) encouraged the epidemiological research and it was concluded that Q fever was present in 51 countries on five continents (Kaplan and Bertagna, 1955).

Nowadays, *C. burnetii* infection is usually reported by the occurrence of outbreaks in humans. It is globally accepted the worldwide distribution of *C. burnetii* except in New Zealand where the infection has never been reported (Maurin and Raoult, 1999; OIE WAHIS Interface, 2016). Q fever is listed in the Terrestrial Animal Health Code of the World Organization for Animal Health (OIE). All the Member Countries are obligated to report the occurrence of the disease. Based on those reports, Figure 4 shows the world distribution of Q fever cases reported in domestic and wild species from 2005 to 2015 (OIE WAHIS Interface, 2016).

Figure 4: World distribution of Q fever reports in domestic and wildlife animals from 2005 to 2015. (OIE WAHIS Interface, 2016)

Concerning the European countries, Tables 1 and 2 show the number of reports in humans and animals, respectively from 2005 to 2015. (OIE WAHIS Interface, 2016).

In most European countries, Q fever cases in humans and animals are reported regularly (ECDC, 2014; OIE WAHIS Interface, 2016). And after the largest ever recorded outbreak which involved more than 4000 human cases between 2007 and 2011 in The Netherlands, Q fever gained renewed attention (van Loenhout *et al.*, 2012). However, it is observed that since then the number of human cases has suffered in general a sustained decrease in the EU and, nowadays, small outbreaks still occur as evidenced on Figures 5 and 6, mainly in areas with infected livestock herds (ECDC, 2014; OIE WAHIS Interface, 2016).

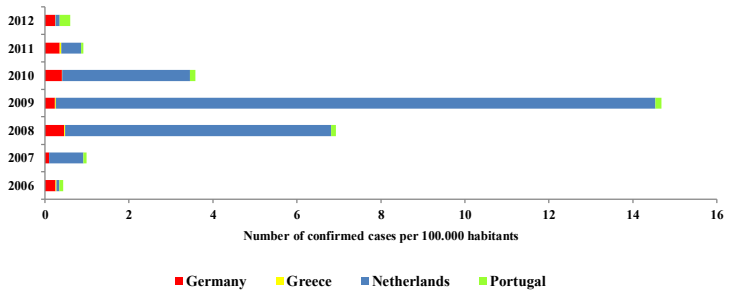


Figure 5: Rates of confirmed human cases of Q fever in four European Countries from 2006 to 2012 (ECDC, 2011, 2014).

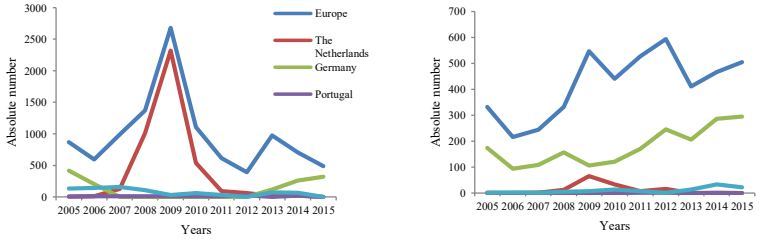
Table 1: Reported number of Q fever cases in humans in European countries from 2005 to 2015 (OIE WAHIS Interface, 2016)

European Countries	2005		2006		2007		2008		2009		2010		2011		2012		2013		2014		2015	
	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths
Albania	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Andorra	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Austria	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Azerbaijan	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Belarus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Belgium	-	-	23	-	107	-	27	-	33	-	29	-	38	-	-	-	29	-	-	-	-	-
Bosnia and Herzegovina	31	-	-	-	-	-	-	-	-	-	33	-	11	-	10	-	35	-	33	-	21	-
Bulgaria	49	-	27	1	36	-	17	-	24	-	-	-	-	-	24	-	23	1	17	-	18	-
Cyprus	---	---	---	---	8	-	31	-	3	-	4	-	5	-	-	-	-	-	-	-	4	-
Czech Republic	1	-	2	-	2	-	-	-	-	-	-	-	1	-	1	-	-	-	-	-	1	-
Denmark	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Estonia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Finland	3	-	3	-	2	-	2	-	1	-	5	-	4	-	-	-	5	-	-	-	3	-
France	-	-	-	-	244	-	-	-	-	-	-	-	138	-	-	-	100	-	-	-	-	-
Georgia	-	-	-	-	-	-	-	-	1	-	2	-	1	-	-	-	-	-	5	-	9	-
Germany	416	1	204	-	-	-	-	-	-	-	-	-	-	-	-	-	115	1	262	-	322	-
Greece	1	-	2	-	-	-	3	-	3	-	1	-	4	-	11	-	11	-	15	-	-	-
Greenland	---	---	---	---	---	---	---	---	---	---	1	-	-	-	-	-	-	-	21	-	-	-
Croatia	40	-	28	-	43	-	41	-	21	-	24	-	19	-	-	-	25	-	-	-	14	-
Hungary	-	-	-	-	-	-	11	-	19	-	-	-	37	-	36	-	175	-	59	1	35	2
Ireland	-	-	12	-	20	-	13	-	17	-	10	-	5	-	6	-	-	-	-	-	4	-
Italy	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Latvia	-	-	-	-	-	-	1	-	-	-	2	-	1	-	1	-	1	1	3	-	1	-
Liechtenstein	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	3	-
Lithuania	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Luxembourg	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rep. of Macedonia	2	-	4	-	8	-	-	-	-	-	5	-	5	-	4	-	8	-	9	-	-	-
Moldova	4	-	4	-	-	-	-	-	7	-	5	-	2	-	-	-	-	-	5	-	-	-
Netherlands	5	-	12	-	127	-	1014	1	2318	6	538	11	90	-	63	-	17	-	22	-	-	-
Norway	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4	-	1	-	1	-
Poland	59	-	-	-	-	-	24	-	5	-	-	-	-	-	-	-	-	-	-	-	-	-
Portugal	6	-	10	-	10	-	12	-	14	-	14	-	9	-	26	-	-	-	21	-	-	-
Romania	-	-	-	-	6	-	-	-	-	-	139	-	10	-	16	-	-	-	-	-	-	-
Russia	85	-	48	-	84	-	17	-	124	-	190	-	128	-	190	-	171	-	34	-	49	-
Serbia	3	-	46	-	12	-	15	-	17	-	24	-	8	-	-	-	102	-	18	-	-	-
Montenegro					1	-	6	-	-	-	-	-	2	-	-	-	2	-	-	-	-	-
Slovakia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Slovenia	3	-	3	-	64	-	-	-	-	-	1	-	-	-	-	-	1	-	2	-	1	-
Spain	134	-	140	-	159	-	109	-	34	-	61	-	33	-	-	-	74	-	64	-	-	-
Sweden	3	-	1	-	3	-	7	-	5	-	11	-	5	-	2	-	4	-	2	-	4	-
Switzerland	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	26	-	44	-	-	-
Turkey	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ukraine	-	-	-	-	-	-	7	-	8	-	6	-	15	-	4	-	-	-	7	-	1	-
United Kingdom	23	-	23	-	53	-	11	-	25	-	-	-	43	-	-	-	46	-	60	-	-	-

Table 2: Reported number of Q fever outbreaks in animals, in European countries from 2005 to 2015 (OIE WAHIS Interface, 2016)

European Countries	2005		2006		2007		2008		2009		2010		2011		2012		2013		2014		2015	
	Outbr	Species	Outbr	Species	Outbr	Species	Outbr	Species	Outbr	Species	Outbr	Species	Outbr	Species	Outbr	Species	Outbr	Species	Outbr	Species	Outbr	Species
Albania	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
Andorra	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
Austria	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Azerbaijan	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
Belarus	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
Belgium	0	-	0	-	0	-	0	-	1	C-	0	-	0	-	0	-	0	-	0	-	0	-
Bosnia and Herzegovina	46	CSG	4	CSG	3	CS	2	CG	2	C	30	CSG	48	CS	2	C	6	CSG	12	CSG	4	CS
Bulgaria	39	CS	19	CBSG	38	CSG	28	CSG	25	CSG	ND	ND	0	-	1	SG	0	-	1	C	ND	ND
Cyprus	0	-	0	-	0	-	0	-	0	-	2	G	3	CSG	2	SG	2	-	0	-	0	-
Czech Republic	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
Denmark	0	-	1	C	1	C	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
Estonia	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
Finland	0	-	0	-	0	-	2	C	3	C	0	-	0	-	0	-	0	-	0	-	0	-
France	0	-	0	-	0	-	0	-	15	WS	4	WS	0	-	0	-	0	-	0	-	0	-
Georgia	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
Germany	174	CSG	94	CSG	109	CS	157	CSG	106	CSGEq	121	CSG	171	CSG	246	CS	206	CSG	286	CSG	295	CSG
Greece	7	SG	9	SG	4	SG	2	SG	0	-	0	-	5	SG	0	-	0	-	0	-	0	-
Greenland	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
Croatia	11	CG	7	CSG	6	SG	31	CSG	180	CSGEq	97	C	176	CS	191	CS	63	CSG	16	CSG	15	CG
Hungary	2	SG	0	-	1	S	0	-	28	CSG	5	S	3	CS	6	C	34	ND	22	CS	19	CS
Ireland	ND	ND	ND	ND	ND	ND	ND	ND	1	C	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Italy	ND	ND	ND	ND	ND	ND	4	CSG	1	SG	3	WS	3	WS	14	SGWS	0	-	3	SG	4	SG
Latvia	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	35	C
Liechtenstein	0	-	0	-	0	-	5	C	0	-	0	-	0	-	0	-	0	-	0	-	0	-
Lithuania	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
Luxembourg	0	-	0	-	0	-	0	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Rep. of Macedonia	2	C	0	-	0	-	1	G	2	CG	8	SG	3	CSG	4	SG	2	SG	8	CSG	2	C
Moldova	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
Netherlands	1	S	ND	ND	1	C	12	CSG	65	SG	33	SG	8	G	16	GWS	ND	ND	2	GWS	ND	ND
Norway	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
Poland	7	C	4	C	2	C	4	C	2	C	38	CS	4	CG	3	C	0	-	23	CSWS	9	C
Portugal	0	-	0	-	1	C-	0	-	0	-	0	-	1	G	1	ND	0	-	0	-	ND	ND
Romania	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
Russia	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Serbia	3	S	5	CSSW	13	CSG	4	CS	3	CS	5	CS	1	C	5	CS	10	CSG	3	CG	17	CG
Montenegro					2	C	0	-	0	-	0	-	0	-	0	-	4	CSG	ND	ND	ND	ND
Slovakia	0	-	0	-	0	-	0	-	0	-	0	-	1	C	0	-	0	-	0	-	0	-
Slovenia	0	-	0	-	3	S	7	CSG	25	CSG	1	S	0	-	0	-	0	-	0	-	ND	ND
Spain	1	C	3	C	1	SG	5	C	7	CSG	13	CS	7	CG	2	GWS	13	CSGWS	33	CSGWS	22	CSGWS
Sweden	ND	ND	ND	ND	0	-	ND	ND	ND	ND	ND	ND	ND	ND	0	-	0	-	0	-	ND	ND
Switzerland	39	CSG	70	CSG	59	C	65	CSG	78	CSG	73	CSG	76	CG	87	CSG	67	CG	58	CS	83	CSG
Turkey	0	-	0	-	0	-	0	-	0	-	0	-	9	CS	8	SG	0	-	0	-	0	-
Ukraine	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-
United Kingdom	ND	ND	ND	ND	ND	ND	3	CS	3	CG	8	CSG	8	CS	6	CS	5	ND	ND	ND	ND	ND

Legend: Outb-Outbreaks; C-cattle; S-sheep; G-goat; B-Buffaloes; Eq-Equids; Sw-Swine; WS-Wild Species; ND-no data available



A **B**

Figure 6: Q fever human (A) and animal (B) cases reported in some European countries from 2005 to 2015 (OIE WAHIS Interface, 2016).

The figures show that Q fever is an endemic disease in Europe evidencing often an outbreak distribution in humans, which is not so evident in animals. The characteristic asymptomatic pattern of infection in animals probably contributes to a more regular distribution of the reported cases. Besides the worldwide distribution of *C. burnetii* infection, the number of reported cases varies geographically and over time.

Prevalence of infection

Prevalence studies of *C. burnetii* infection are very important to increase the knowledge of the epidemiology. Tables 3, 4 and 5 summarize prevalence studies conducted all over the world in humans and animals.

Despite their contribution for a better knowledge of *C. burnetii* epidemiology, often comparisons between studies are difficult due to the lack of harmonized serological techniques (*e.g.* different cut-offs or serologic tests) and to different study designs and methodologies (serologic *versus* molecular) (EFSA, 2010[a]; Georgiev *et al.*, 2013).

Table 3: Prevalence of *C. burnetii* infection in humans worldwide.

Country	Period	Sample	Group	Rural/Urban	Number	Test	Cut-off	Prevalence	Reference
Australia	2006-09	Serum	Residents	ND	2438	IFA	1/50	7%	Islam *et al.* 2011
Canada	1998	Serum	Shepherds	Rural	81	IFA	1/32	28%	Dolcé *et al.* 2003
Colombia	2012	Serum	Living on farms	Rural	61	IFA	1/64	61%	Contreras *et al.* 2015
Denmark	2008	Serum	Occupational	Rural	359	IFA	1/512	11%	Bosnak *et al.* 2010
	1996-2002	Serum	Pregnancy and occupation	Rural	397	IFA	1/128	14.8%	Nielsen *et al.* 2013
France	1988	Serum	Blood donors	ND	924	IFA	1/25	4.03%	Tissot-Dupont *et al.* 1992
	1996	Serum	Pregnancy	ND	12716	IFA	1/100	0,15%	Rey *et al.* 2000
Greece	1998	Serum	Residents	Rural	238	IFA	1/120	42%	Antoniou *et al.* 2002
	nd	Serum	residents	Both	1007	IFA	1/64	7.5%	Pape *et al.* 2009
	2005-06	Serum	Blood donors	Both	493	IFA	1/120	48.7%	Vranakis *et al.* 2012
Italy	nd	Serum	Dog owners	Urban	69	nd	nd	35%	Baldelli *et al.* 1992
	2002-04	Serum	Occupational	Rural	128	IFA	1/20	73.4%	Monno *et al.* 2009
	nd	Serum	Agricultural workers	Rural	126	IFA	1/16	23.8%	Verso *et al.* 2016
Kenya	2007-08	Serum	Patients	Rural	246	IFA	1/32	30.9%	Knobel *et al.* 2013
Nova Scotia	1988-91	Serum	Residents	Both	492	MIFA	1/8	14.6%	Marrie and Pollack 1995
Poland		Serum	Foresters	Rural	304	IFA	1/16	10.52%	Szymanska *et al.* 2013
Portugal	1989	Serum	Residents	Both	487	IFA	nd	2.2%	Bacellar *et al.* 1991
Senegal	2008	Serum	Residents	Rural	238	IFA	1/200	3.7%	Mediannikov *et al.* 2010
Spain	nd	Serum	Residents	Rural	130	IFA	1/80	15.4%	Tellez *et al.* 1989
				Urban	91			8.8%	
	1987	Serum	Residents	Both	400	IFA	1/40	50.2%	Ruiz-Beltran *et al.* 1990
	1994	Serum	Residents	Rural	406	IFA	1/80	40.6%	Suarez-Estrada *et al.* 1996
	1996	Serum	Residents	Both	662	IFA	1/80	21.5%	Bolanos *et al.* 2003
	nd	Serum	Residents	Both	216	IFA	1/80	8.8%	Cardenosa *et al.* 2006
Sweden	nd	Serum	Occupational	Both	454	ELISA	A_{405}>0.2	14.9%	Macellaro *et al.* 1993
The Netherlands	2007-09	Serum	Pregnancy	Both	2004	IFA	1/64	31%	Van der Hoek *et al.* 2011
	2009-10	Serum	Farmers and family	Rural	268	IFA	1/32	68.7%	Schimmer *et al.* 2012
	2008	Serum	Veterinarians	Rural	189	IFA	1/32	65.1%	Van den Brom *et al.* 2013
	2010-11	Serum	Blood donors	Both	1033	IFA	1/32	3%	Van Wijk *et al.* 2014
Turkey	1998	Serum	Occupational	Rural	102	IFA	1/80	7.8%	Cetinkaya *et al.* 2000
	2007	Serum	Blood donors	Both	601	ELISA/IFA	nd	32.3%	Kilic *et al.* 2008
United Kingdom	nd	Serum	Farm workers	Rural	385	IFA	1/32	27%	Thomas *et al.* 1995
United States	2003-04	Serum	Residents	Both	4437	IFA	1/16	3.1%	Anderson *et al.* 2009
United States	1989-2009	Serum	Military veterinarians	ND	500	IFA	1/16	17%	Vest and Clark 2014

Table 4: Herd prevalence of *C. burnetii* infection by country and animal species

Country (area)	Study Period	Type of sample	Sampling method	Number	Test	Cut-off value	Prevalence	Reference
Cattle								
Belgium	2006	Bulk Tank Milk	Random	206	ELISA	0.4	57.8%	Czaplicki *et al*. 2012
Denmark	2012	Bulk Tank Milk	Random	120	ELISA	0.4	79.2%	Agger and Paul 2014
Ecuador	2008-2010	Serum	Cluster sampling	386	ELISA	0.4	46.9%	Carbonero *et al*. 2015
Iran	2010	Bulk Tank Milk	Random	44	ELISA	0.4	45.4%	Khalili *et al*. 2011
Ireland (Republic of)	2009	Bulk Tank Milk	Stratified random	290	ELISA	0.4	37.9%	Ryan *et al*. 2011
	2005-2007	Serum		332			6.9%	
Ireland (Northern)	n.a.	Serum	Systematic stratified	273	ELISA	0.4	48.4%	McCaughey *et al*. 2010
Norway	2010	Bulk Tank Milk	Random	3289	ELISA	0.4	0%	Kampen *et al*. 2012
Portugal	2013	Bulk Tank Milk	Random	90	ELISA	0.4	61.1%	Pimenta *et al*. 2015
Spain (Bizkaia province)	2009-2010	Bulk Tank Milk	All herds	178	ELISA	0.4	66.9%	Astobiza *et al*. 2012
Spain	2009	Serum	Random	110	ELISA	0.40	30%	Alvarez *et al*. 2012
	2007-2008			42			43.00%	Ruiz-Fons *et al*. 2010
Sweden	2008-2009	Bulk Tank Milk	Random	1537	ELISA	0.4	8.2%	Ohlson *et al*. 2014
The Netherlands	2007	Bulk Tank Milk	Random stratified	341	ELISA	0.3	71.6%	Muskens *et al*. 2011
	2009-2011		Random	309			81.6%	Van Engelen *et al*. 2014
The Netherlands	2007	Bulk Tank Milk	Random	341	qPCR	Ct≤40	56.6%	Muskens *et al*. 2011
	2009-2011			309			18.8%	Van Engelen *et al*. 2014
Belgium	2006	Bulk Tank Milk	Random	50	qPCR	Ct≤40	30.0%	Czaplicki *et al*. 2012
Spain (Bizkaia province)	2009-2010	Bulk Tank Milk	All herds	178	qPCR	Ct≤40	51.7%	Astobiza *et al*. 2012
USA	2001-2003	Bulk Tank Milk	Convenience	316	PCR	n.a.	94.3%	Kim *et al*. 2005
	2011		Random	316	qPCR	Ct≤36.5	61.1%	Bauer *et al*. 2015
Goat								
Canada	2010-12	Serum	Multi-stage random	76	ELISA	0.4	63.2%	Meadows *et al*. 2015
Great Britain	2008	Serum	Random stratified	145	ELISA	0.4	3.0%	Lambton *et al*. 2016
Ireland (Republic of)	2005-2007	Serum	Random	66	ELISA	0.4	1.5%	Ryan *et al*. 2011
Norway	2009	Bulk Tank Milk	Random	348	ELISA	0.4	0%	Kampen *et al*. 2012
Spain	2007-2008	Serum	Random	11	ELISA	0.40	45.00%	Ruiz-Fons *et al*. 2010
Sweden	2010	Bulk Tank Milk	Random	58	ELISA	0.4	1.7%	Ohlson *et al*. 2014
Switzerland	2011	Serum	Random stratified	72	ELISA	0.4	11.1%	Magouras *et al*. 2017
The Netherlands	2008	Serum	Random	442	ELISA	0.4	17.9%	Van den Brom *et al*. 2013
USA	2012	Serum	Random	24	ELISA	0.4	4.2%	Baker and Pithua 2014
Sheep								
Canada	1998	Serum	Random	103	ELISA	0.3	21.3%	Lang *et al*. 1991
Germany	2009	Serum	Random	39	ELISA	0.4	28.0%	Hilbert *et al*. 2012
Great Britain	2008	Serum	Random stratified	384	ELISA	0.4	3.0%	Lambton *et al*. 2016
Ireland (Republic of)	2005-2007	Serum	Random	119	ELISA	0.4	8.4%	Ryan *et al*. 2011
Norway	2006-2008	Serum	Random	130	ELISA	0.4	0%	Kampen *et al*. 2012
Spain	2007-2008	Serum	Random	46	ELISA	0.40	74.00%	Ruiz-Fons *et al*. 2010
Sweden	2011	Bulk Tank Milk	Random	518	ELISA	0.4	0.6%	Ohlson *et al*. 2014
Switzerland	2011	Serum	Random stratified	100	ELISA	0.4	5.0%	Magouras *et al*. 2017
The Netherlands	2008	Serum	Random	1208	ELISA	0.4	14.5%	Van den Brom *et al*. 2013
Turkey	2001-2004	Serum	Random stratified	42	ELISA	0.4	81.00%	Kennerman *et al*. 2010

Table 5: Individual prevalence of *C. burnetii* infection by country and animal species

Country (area)	Study Period	Type of sample	Sampling method	Number of samples	Test	Cut-off value	Prevalence	Reference
Cattle								
Albania	1995-1997	Serum	n.a.	311	ELISA	0.4	10.9%	Cekani *et al*. 2008
Bangladesh	2009-2010	Serum	Convenience	620	ELISA	0.4	0.65%	Haider *et al*. 2015
Denmark	2012	Serum	Random	800	ELISA	0.4	5.5%	Paul *et al*. 2014
Ecuador	2008-2010	Serum	Cluster sampling	2668	ELISA	0.4	12.6%	Carbonero *et al*. 2015
Egypt	2012-2013	Serum	Convenience	158	ELISA	0.4	13.2%	Gwida *et al*. 2014
India	n.a.	Serum	Convenience	88	ELISA	0.4	11.36%	Vaidya *et al*. 2010
Ireland (Republic of)	2005-2007	Serum	Random stratified	1659	ELISA	0.4	1.8%	Ryan *et al*. 2011
Ireland (Northern)	n.a.	Serum	Systematic stratified	5182	ELISA	0.4	6.2%	McCaughey *et al*. 2010
Iran	n.a.	Serum	Cluster	246	ELISA	0.4	22.3%	Azizzzadeh *et al*. 2011
Ivory Coast	2012-2014	Serum	Cluster	633	ELISA	0.4	13.9%	Kanouté *et al*. 2017
Reunion Island	2011-2012	Serum	Random	245	ELISA	0.4	11.8%	Cardinale *et al*. 2014
Spain	2009	Serum	Random	1100	ELISA	0.4	6,76%	Alvarez *et al*. 2012
The Netherlands	2008	Serum	Random stratified	2871	ELISA	0.4	16.0%	Muskens *et al*. 2011
Cyprus	2008-2009	Aborted foetus	Convenience	51	PCR	n.a.	41.17%	Cantas *et al*. 2011
India	n.a.	Swabs	Convenience	88	PCR	n.a.	12.5%	Vaidya *et al*. 2010
Portugal	2006-2008	Organs	Convenience	29	PCR	n.a.	17.2%	Clemente *et al*. 2009
The Netherlands	2008	Milk and feces	Random stratified	2871	rtPCR	Ct≤40	8.7%%	Muskens *et al*. 2011
Goat								
Albania	1995-1997	Serum	n.a.	443	ELISA	0.4	8.8%	Cekani *et al*. 2008
Bangladesh	2009-2010	Serum	convenience	529	ELISA	0.4	0.76%	Haider *et al*. 2015
Canada	2010-12	Serum	Multi-stage random	2195	ELISA	0.4	32.5%	Meadows *et al*. 2015
Great Britain	2008	Serum	Random stratified	522	ELISA	0.4	0.8%	Lambton *et al*. 2016
India	n.a.	Serum	Convenience	53	ELISA	0.4	5.66%	Vaidya *et al*. 2010
Iran	n.a.	Serum	Multi-stage random	241	ELISA	0.4	22.4%	Ezatkhah *et al*. 2014
Ireland (Republic of)	2005-2007	Serum	Random	590	ELISA	0.4	0.3%	Ryan *et al*. 2011
Ivory Coast	2012-2014	Serum	Cluster	622	ELISA	0.4	12.4%	Kanouté *et al*. 2017
Reunion Island	2011-2012	Serum	Random	134	ELISA	0.4	13.4%	Cardinale *et al*. 2014
Spain	2007-2008	Serum	Random	115	ELISA	0.40	8.7%	Ruiz-Fons *et al*. 2010
Switzerland	2011	Serum	Random stratified	321	ELISA	0.4	3.4%	Magouras *et al*. 2017
The Gambia	2012	Serum	Multi-stage random	484	ELISA	0.4	24.2%	Klaasen *et al*. 2014
The Netherlands	2008	Serum	Random	3134	ELISA	0.4	7.8%	Van den Brom *et al*. 2013
USA	2012	Serum	Random	249	ELISA	0.4	1.2%	Baker and Pithua 2014
Iran	2010	Aborted feces	Convenience	744	PCR	Ct≤40	16.39%	Dehkordi 2011
Portugal	2006-2008	Organs	Convenience	37	PCR	n.a.	40.50%	Clemente *et al*. 2009
Sheep								
Albania	1995-1997	Serum	n.a.	350	ELISA	0.4	8.8%	Cekani *et al*. 2008
Canada	1998	Serum	n.a.	334	CFT	1/8	41.0%	Dolcé *et al*. 2003
	1991		Random	3765	ELISA	0.3	1.5%	Lang *et al*. 1991
Great Britain	2008	Serum	Random stratified	5791	ELISA	0.4	0.9%	Lambton *et al*. 2016
India	n.a.	Serum	Convenience	43	ELISA	0.4	9.3%	Vaidya *et al*. 2010
Iran	n.a.	Serum	Multi-stage random	127	ELISA	0.4	33.9%	Ezatkhah *et al*. 2014
Ireland (Republic of)	2005-2007	Serum	Random	2197	ELISA	0.4	0.7%	Ryan *et al*. 2011
Ivory Coast	2012-2014	Serum	Cluster	622	ELISA	0.4	9.4%	Kanouté *et al*. 2017
Reunion Island	2011-2012	Serum	Random	137	ELISA	0.4	1.4%	Cardinale *et al*. 2014
Switzerland	2011	Serum	Random stratified	500	ELISA	0.4	1.8%	Magouras *et al*. 2017
Spain	2007-2008	Serum	Random	1379	ELISA	0.40	11.8%	Ruiz-Fons *et al*. 2010
Turkey	2001-2004	Serum	Random stratified	743	ELISA	0.4	20.00%	Kennerman *et al*. 2010
The Gambia	2012	Serum	Multi-stage random	395	ELISA	0.4	18.5%	Klaasen *et al*. 2014

Sheep (cont.)								
The Netherlands	2008	Serum	Random	12052	ELISA	0.4	2.4%	Van den Brom *et al*. 2013
India	n.a.	Swabs	Convenience	43	PCR	n.a.	11.62%	Vaidya *et al*. 2010
Iran	2010	Aborted feces	Convenience	782	qPCR	Ct≤40	12.53%	Dehkordi 2011
Portugal	2006-2008	Organs	Convenience	25	PCR	n.a.	36.00%	Clemente *et al*. 2009
Dogs								
Australia	2006-2007	Serum	Random	101	ELISA	0.5	21.8%	Cooper *et al*. 2011
Canada	1983	Serum	Convenience	447	IFA	1:8	0.0%	Marrie *et al*. 1985
France	n.a.	Serum	Convenience	355	IFA	1/50	9.8%	Boni *et al*. 1998
Portugal	n.a.	Serum	Convenience	104	IFA	n.a.	4.8%	Bacellar *et al*. 1995
Wild Rodents								
Cape Verde	2009-2013	Serum	Random	38	ELISA	0.4	21.1%	Foronda *et al*. 2015
Great Britain	2007-2009	Serum	Random	793	ELISA	0.10	17.3%	Meredith *et al*. 2014
Spain (Canary)	2009-2013	Serum	Random	147	ELISA	0.4	10.2%	Foronda *et al*. 2015
The Netherlands	2008-2010	Serum	Random	202	ELISA	n.a.	11.4%	Reusken *et al*. 2011
	2008-2010	Organs	Random	330	PCR	n.a.	3.9%	
Cats								
Australia	unavailable	serum	Convenience	376	IFA	1/256	9.3	Shapiro *et al*. 2015
Canada	1983	Serum	Convenience	216	IFA	1:8	24.1%	Marrie *et al*. 1985
Great Britain	2007-2009	Serum	Random	26	ELISA	0.16	61.5%	Meredith *et al*. 2014
Southern Africa	n.a.	Serum	n.a.	117	IFA	1/40	13.0%	Matthewmann *et al*. 1997
Foxes								
Great Britain	2007-2009	Serum	Random	102	ELISA	0.16	41.2%	Meredith *et al*. 2014
Roe Deer								
France	1979	Serum	Random	695	CFT	n.a.	3.74%	Baradel *et al*. 1988
Czech Republic	1986-1991	Serum	Random	33	MAT	1:8	6.0%	Hubalek *et al*. 1993
Spain	2004-2005	Serum	Random	39	IFA	1:16	15.4%	Ruiz-Fons *et al*. 2008
	2001-2006	Organs	Convenience	78	PCR	n.a.	5.1%	Astobiza *et al*. 2011
The Netherlands	2008-2010	Organs	Convenience	79	PCR	Ct≤40	23%	Rijks *et al*. 2011
Red Deer								
Czech Republic	1986-1991	Serum	Random	24	MAT	1:8	25.0%	Hubalek *et al*. 1993
France	1979	Serum	Random	54	CFT	n.a.	1.85%	Baradel *et al*. 1988
Iberian Peninsula	n.a.	Serum	Random	1486	ELISA	0.4	14.1%	Gonzalez-Barrio *et al*. 2014
Spain	2004-2005	Serum	Random	34	IFA	1:16	2%	Ruiz-Fons *et al*. 2008
White Tail Deer								
USA	2009-2010	Serum	Random	1059	IFA	1/20	14.64%	Kirchgessner *et al*. 2012
Wild Boar								
Czech Republic	1986-1991	Serum	Random	32	MAT	1:8	6.0%	Hubalek *et al*. 1993
France	1979	Serum	Random	209	CFT	n.a.	0.0%	Baradel *et al*. 1988
Japan	n.a.	Serum	Random	30	ELISA	n.a.	0.0%	Ejercito *et al*. 1993
Spain	2001-2006	Organs	Convenience	93	PCR	n.a.	4.3%	Astobiza *et al*. 2011
European Hare								
Czech Republic	1986-1991	Serum	Random	23	MAT	1:8	0.0%	Hubalek *et al*. 1993
Spain	2001-2006	Organs	Convenience	22	PCR	n.a.	9.1%	Astobiza *et al*. 2011
Macropods								
Australia	n.a.	Serum	Random	500	ELISA	0.5	20.8%	Cooper *et al*. 2012

1.6.1.2. Temporal distribution

The number of Q fever cases varies geographically, and a seasonal variation is also described. In fact, in Europe, acute Q fever cases are more often reported in Spring and early Summer (Maurin and Raoult, 1999; Tissot-Dupont *et al*., 1999), showing a slow rise in reported cases in March and April, probably associated with the start of lambing/kidding, and the main peak occurs between May and July (ECDC, 2014). This occurs probably due to the "outside" lambing/kidding during Spring associated to heavy environmental contamination with *C. burnetii* (Arricau-Bouvery and Rodolakis, 2005). This is consistent with a recent study in which a significant prevalence of Q fever pneumonia in humans was observed in Summer season (Schack *et al*., 2014). However, besides the environmental contamination, climatic factors also may contribute to an increase of the incidence in a given region, such as the wind (Tissot-Dupont *et al*., 1999; O'Connor *et al*., 2015). It is known that the lambing season in October is not related to a higher incidence in humans which might be due to "indoor" lambing (Maurin and Raoult, 1999; Angelaskis and Raoult, 2011), which is also consistent with the study conducted in the south of France that concluded that Autumn is not a very windy season explaining the lower incidence of human Q fever in this time of the year (Tissot-Dupont *et al*., 1999). So, the wind plays a role in *C. burnetii* transmission and it might be related to unexpected outbreaks in each area since it can be monitored but not prevented (Tissot-Dupont *et al*., 2004).

Concerning to *C. burnetii* infections reported in animals, no seasonality has been observed from 2005 to 2015, as showed in Figure 7. However, a study conducted in Japan revealed that antibody positive cows and their antibody titters were significantly high in Winter and decreased in Summer (Yanase *et al*., 1997). Moreover, areas with high wind speed, open landscape and high temperature increase the risk of infection in animals, while precipitation seems to be a protective factor by decreasing the quantity of bacteria aerosolized (Nusinovici *et al*., 2015).

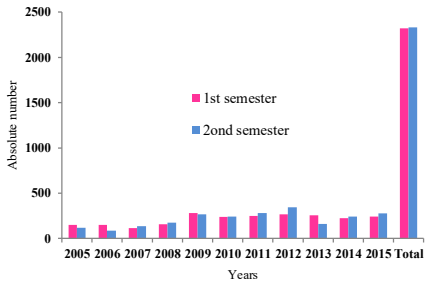


Figure 7: Number of *C. burnetii* infections in animals reported to OIE from 2005 to 2015. (WAHIS Interface – OIE, 2016)

1.6.2. Host determinants

1.6.2.1. Receptivity to *Coxiella burnetii*

C. burnetii has an impressively broad host range being present in virtually all animal kingdoms, including arthropods, but the disease affects mostly humans, cattle, sheep and goats (Maurin and Raoult 1999; OIE, 2015; Van den Brom *et al*., 2015).

Reservoirs

Domestic ruminants are considered the main reservoirs of *C. burnetii*, but cats, dogs, rabbits, birds and others, have also been reported to be implicated in human disease/infection (OIE, 2015).

For instance, *C. burnetii* has been isolated in more than 40 species of ticks and the risk of human transmission has been reported (Anderson *et al*., 2013). Ticks shed large concentrations of *C. burnetii* in their faeces contaminating the skin of their hosts or even being inhaled by them (Angelakis and Raoult, 2010; Boarbi *et al*., 2015). In Europe, the detection of *C. burnetii* in ticks is considered rare. In the Netherlands, the prevalence in questing *Ixodes ricinus* ticks was less than 0.2%, making the current risk of acquiring Q fever from a questing tick negligible (Sprong *et al*., 2012; Michelet *et al*., 2014). In Spain, there was no evidence of *C. burnetii* DNA in ticks suggesting that they do not

play an important role in the transmission of *C. burnetii* in the area (Astobiza *et al.*, 2011). In the South of Portugal, *C. burnetii* was identified in 19.4% of ticks identified as *Hyaloma lusitanicum*, *Dermacentor marginatus*, *Ixodes* spp. and *Rhipicephalus pusillus* collected from vertebrate hosts and vegetation, but their role in the transmission of *C. burnetii* in the current situation was not investigated (Silva *et al*., 2014).

It was also postulated that rats through their commensal nature, could be an important factor in the dissemination of *C. burnetii* to domestic animals, livestock and humans. *C. burnetii* DNA was detected in 4.9% of the brown rats (*Rattus norvegicus*) and 3.0% of the black rats (*Rattus rattus*). The presence of actively infected rats in multiple locations might suggest that rats might not be merely a spill-over host but might represent true reservoirs. In this case rats might be able to maintain *C. burnetii* and thereby contribute to spread and transmission of the pathogen (Reusken *et al*., 2011).

Human infections might also be associated with infected dogs and cats in rural or in urban areas (Kosatsky, 1984; Marrie *et al*., 1988; Buhariwalla *et al*., 1996; Komiya *et al*., 2013a, b). Several studies demonstrated that cats might be a significant source of *C. burnetii* for humans mainly by the contact with infected parturient cats (Cairns *et al*., 2007; Porter *et al*., 2011; Kopecny *et al*., 2013; Fujishiro *et al*., 2016). An epidemiological investigation conducted in the South of France suggested that faeces of pigeons were in the origin of a human outbreak (Stein and Raoult, 1999). Additionally, the finding of the bacterium in dogs and horses suggest their role as reservoirs of *C. burnetii* (Roest *et al*., 2013[b]).

The natural susceptibility to *C. burnetii* infection in pigs has been demonstrated by the presence of antibodies in serum after experimental infection (Marmion and Stocker, 1958) but there are no reports of an active infection in domestic swine so far.

The role of free living wild animals as sources of Q fever in humans has been questioned (EFSA, 2010[a]). The detection of *C. burnetii* DNA in wild animals has been investigated in several countries. In the Netherlands, the bacterium was found in free living roe deer and the highest bacterial load occurred in spring and summer (Rijks *et al.*, 2011). In Spain, it was found in roe deer (*Capreolus capreolus*), wild boar (*Sus scrofa*), European hare (*Lepus europeus*), and in wild birds such as vultures (*Gyps fulvus*) and black kites (*Milvus migrans*) (Astobiza *et al*., 2011). Moreover, in European rabbits (*Oryctolagus cuniculus*) a high prevalence of infection was found

together with the evidence of systemic infection, suggesting their role as *C. burnetii* reservoirs (Gonzalez-Barrio *et al.*, 2015[a]). Recently, the complexity of *C. burnetii* ecology was highlighted and it was proposed that red deer (*Cervus elaphus*) is able to maintain *C. burnetii* circulating in a region without third species; however, it is thought that most probably other wild and domestic host species are involved. These findings together with previous evidence of *C. burnetii* shedding by naturally infected red deer point at this wild ungulate as a true reservoir for *C. burnetii* in the Iberian Peninsula (Gonzalez-Barrio *et al*., 2015[b]).

1.6.3. Virulent materials and sources of infection

Infected animals shed the organism mostly in placental membranes and birth fluids (Arricau-Bouvery *et al*., 2003; Roest *et al*., 2012). High concentrations of *C. burnetii* are found in infected animal's birth products such as placenta and vaginal secretions (Sidi-Boumedine *et al*., 2015[a]). This is the most important excretion route which by far exceeds others. Bacteria are also excreted in milk (Roest *et al*., 2012, van den Brom *et al*., 2012), faeces and vaginal mucus (Guatteo *et al*., 2006, 2007). Some questions were raised about the possible contamination of faeces and vaginal mucus into the environment (Roest *et al*., 2012), but so far, their role as virulent materials is still considered.

After shedding, in the environment, bacteria can be easily aerosolized from desiccation of infected placenta and body fluids or from contaminated manure; and then can infect susceptible hosts (Maurin and Raoult 1999; Arricau-Bouvery and Rodolakis, 2005; Schimmer *et al*., 2010). However, during the Dutch human Q fever outbreak the role of goat manure as a source of infection was considered very limited. It has been suggested that the composting process within a dunghill result in a clear reduction in the number of viable *C. burnetii* (van den Brom *et al*. 2015).

1.6.4. Methods of transmission

Briefly, the transmission of *C. burnetii* may occur by direct, indirect or vectorial transmission as schematized on Figure 8. For humans and animals, the majority of

natural *C burnetii* infections occur by airborne transmission resulting from the inhalation of aerosolized bacteria (Arricau-Bouvery and Rodolakis, 2005; Berri *et al*., 2005; Cutler *et al*., 2007; Angelakis and Raoult, 2010; Roest *et al*., 2011a, 2012). *C. burnetii* is highly infectious through inhalation. It has been estimated that the probability of one single bacterium initiates an infection is 0.9 in guinea pigs (Jones *et al.*, 2006). The environmental survival of *C. burnetii* allows it to be dispersed by wind far away from its original source (Tissot-Dupont *et al*. 1999, 2004; de Rooij *et al*., 2016). This may cause a long-distance transmission of infection leading to inter-herd transmission of *C. burnetii* or even to dispersion of bacteria to residential locations (de Rooij *et al*., 2016; Pandi *et al*., 2016). This can account for the appearance of Q fever cases in urban areas, where an important percentage of patients fails to report direct contact with animals (Arricau-Bouvery and Rodolakis, 2005). Several epidemiological studies on human outbreaks report this long-distance transmission such as 400m in Germany (Gilsdorf *et al*., 2008), 5 km in the Netherlands (Schimmer *et al*., 2010), 18 Km in United Kingdom (Hawker *et al*., 1998) and 40 km in France (Tissot-Dupont *et al*., 2004).

Other less common, routes of transmission include ingestion or skin inoculation through the bite of an arthropod (Raoult *et al*., 2005). Q fever transmission to humans from tick bite or tick excreta has been recorded but is very rare; yet ticks may be essential in natural maintenance cycles (McQuiston *et al.*, 2002).

Milk was early recognized as a source of *C. burnetii* since it is one of the shedding routes (Bell *et al*., 1949). Thus, the likelihood of infection by the ingestion of contaminated milk cannot be excluded. There is much evidence that *C. burnetii* is viable in unpasteurized milk but the transmission of *C. burnetii* by ingestion is controversial (Eldin *et al.*, 2013; Gale *et al*., 2015). Early experiments conducted in 1940s and 1950s, recovered viable *C. burnetii* from raw milk raising some concerns due to the bacterium resistance to heat and the uncertainties about the efficacy of milk pasteurization in destroying *C. burnetii*. The experiments showed that pasteurization procedure by submitting milk to temperature of 72ºC for 15 seconds is adequate to eliminate viable *C. burnetii* from whole raw milk, as well as the temperature of 63ºC for 30 minutes (Huebner *et al*., 1949; Enright *et al*. 1957). More recently, some studies questioned the efficacy of these procedures on the inactivation of *C. burnetii* (Heinzen et al., 1999; Cerf and Condron, 2003). Also, in the United States, viable *C. burnetii* have been detected in commercial unpasteurized milk samples; but the viability of bacteria by oral

administration in mice was not confirmed which raised some doubts about the infection by ingestion of milk (Loftis *et al.*, 2010). A study conducted in France, showed that *C. burnetii* is commonly present in commercially available milk products but because its viability was not confirmed the transmission of Q fever by consumption of these products was not considered important (Eldin *et al*., 2013). Also, in a recent report from EFSA, concerning the public health risks related to raw drinking milk, *C. burnetii* is not mentioned as a biohazard to be transmitted via milk (EFSA, 2015). The pathogen biologic cycle might explain the low hazard attributed to this intracellular bacterium which will not grow outside the intracellular environment of the host cell. Thus, for risk assessment it is assumed that multiplication of the pathogen in milk and milk products does not occur (Gale *et al*., 2015). Furthermore, there are insufficient data for a dose-response model for the oral route in humans (Gale *et al*., 2015).

Globally, it seems more plausible that clinical Q fever results mainly from inhalation and sometimes from arthropods bites. The ingestion of *C. burnetii* contaminated milk or milk products may result in serological conversion but not necessarily disease. Furthermore, seroconversion may result from the ingestion of live or inactivated cells (Cerf and Condron, 2006).

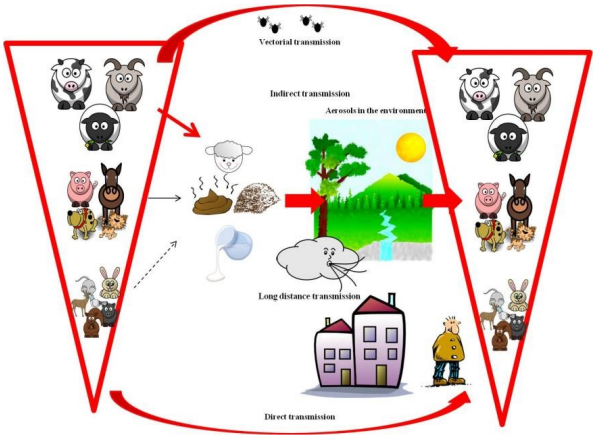


Figure 8: Diagram schematizing the transmission forms of *C. burnetii.*

Transmission among humans through sexual contact was reported rising the hypothesis of transmission by artificially insemination in animals which was confirmed

by the isolation of *C. burnetii* in bull semen (Chmielewski and Tylewska-Wierzbanowska, 2012). Similarly, the persistence of these bacteria after washing makes embryo a potential vehicle of transmission of the bacterium during embryo transfer from infected donor cows to healthy recipients and/or their offspring (Alsaleh *et al*., 2014).

Other than sexual transmission from man to man occur sporadically. Such infections have been described during autopsy from necropsy material, by blood transfusion and after contact with infected parturient woman (Maurin and Raoult, 1999). Recently, it was reported one case of *C. burnetii* contamination by the injection of ovine cells (foetus and placenta) in the context of cell therapy (Promed, 2014).

1.6.5. Risk factors

A risk factor is considered when its presence increases the likelihood of disease. Therefore, risk factors may be either casual or non-casual and their knowledge is useful in identifying populations at which attention should be directed (Thrusfield, 1995).

1.6.5.1. Human populations

C. burnetii infection in humans is mostly associated with infected ruminants (*e.g.* cattle, sheep and goats). Thus globally, the risk factors are included in the direct or indirect contact with virulent materials shed by infected animals (Angelakis and Raoult, 2010). Certainly, some occupations facilitate the contact with infectious materials and these called risk occupations will be analysed hereafter. Besides that, community outbreaks have been described very often. Thus, several investigations have been conducted, some of them in an outbreak context, to identify risk factors for the occurrence of Q fever in human population.

Environment

Concerning to the local of residence, adults living on farms (Karki *et al*., 2015), or living on rural or sub urban areas (van den Brom *et al*., 2013) and in the proximity of

positive farms (Schimmer *et al*., 2012), are at a substantially greater risk of contracting Q fever.

Regarding the animals, the exposure to farm animals, their breeding or their slaughter are also important risk factors (Valencia *et al*., 2000; Dorko *et al*., 2011; de Rooij *et al*., 2012; Njeru *et al*., 2016). Interestingly, despite the uncertainties about the role of pigs in the epidemiology of *C. burnetii*, the exposure to swine was also considered a risk factor for Q fever (van den Brom *et al*., 2013; Schimmer *et al*., 2014). Furthermore, the presence of cats is considered a risk factor (Schimmer *et al*., 2012), which agrees with some reports highlighting these animals in the epidemiology of *C. burnetii* and even describing them as sources of human outbreaks (Cairns *et al*., 2007). Risk factors were also considered the presence of birds, and the direct or indirect contact with rats or mice (Schimmer *et al*., 2014). Rats have been already described as a potential significant reservoir of *C. burnetii* (Reusken *et al*., 2011)

Considering the virulent materials previously described (see 6.3.), the contact with manure was considered a risk factor associated with serological positivity (Dal Pozzo *et al*., 2017). Also, the high frequency of refreshing stable bedding (de Lange *et al*., 2014) and the work in a dusty environment such as on fields, gardens, stables and construction sites were also considered risk factors (Dorko *et al*., 2011).

As previously discussed, (see 6.4. Methods of transmission) the consumption of unpasteurized cattle milk and fermented milk products were dietary factors associated with *C. burnetii* serological positivity (Njeru *et al*., 2016).

Individual Determinants

After the infection, in humans, the risk of acute illness appears to depend on age and gender (Brooke *et al*., 2015). In fact, age is considered a risk factor for Q fever (Raoult *et al*., 2005). The incidence increases with age and it is mostly reported between the 50 to 60-year age group (Schimmer *et al*., 2008; ECDC, 2014); and several studies suggested young age as a protective factor against *C. burnetii* (Angelakis and Raoult, 2010).

Gender is also considered a risk factor (Maurin and Raoult, 1999). In children the sex ratio of clinical cases as well as that of infections is 1:1 (Maltezou and Raoult, 2002; Leone *et al*., 2004). Several studies showed that infectivity is equal in both genders, with no differences in seropositivity rates. However, it is known that the occurrence of clinical symptoms depends upon gender (Maurin and Raoult, 1999); and the

male: female ratio ranges from 1.5 to 2.5 in adults (Dupuis *et al*., 1987; Raoult *et al*., 2000; Maltezou and Raoult, 2002; van der Hoek *et al*., 2010), but in 2012 achieved 2.91 (ECDC, 2014). Males tend to be slightly more susceptible than females which show a higher estimated dose causing 50% probability of illness (illD50) (Brooke *et al*., 2015). The predisposition for infection in males is explained by physiological events that occur at puberty (Leone et al., 2004). The gender difference is due to a protective effect of the female sex hormone 17βestradiol which control host responses to *C. burnetii* infection (Leone *et al*., 2004). This protective role of 17β estradiol was demonstrated in mice (Leone *et al*., 2004). These experimental data show the role of female hormones in Q fever. This finding could explain why the sex ratio is biased only after puberty (Maltezou *et al*., 2004).

Pre-existent cardiac valvular disease, aortic aneurysm, vascular grafts, immunocompromised state, and pregnancy are reported risk factors for the development of chronic Q fever (Fennolar *et al*., 2001; Landais *et al*., 2007).

Occupational

Human infections are mostly associated with infections in ruminants (*e.g.* sheep, goats and cows) and Q fever is often an occupational hazard. Various professional groups are occupationally exposed to infection with *C. burnetii*. The farming workforce constitutes an occupational risk group with an increased risk for *C. burnetii* infection presumably because of their contact with infected livestock, namely during breeding practices (Cutler *et al*., 2007; Szymanska-Czerwinska *et al*., 2015).

Furthermore, veterinarians, laboratory workers and abattoir workers are also at risk of being infected (Valencia *et al*., 2000; Dorko *et al*., 2011; de Rooij *et al*., 2012; Njeru *et al*., 2016; OIE, 2015), as well as workers in wool, tanneries, fur, meat, leather and timber industries (Szymanska-Czerwinska et al. 2015).

1.6.5.2. Animal populations

The risk of animal infection has been studied mainly for ruminants (dairy and beef) at the herd level and at the individual level.

At the herd level, and regarding the location of the farm, factors as the proximity (until 8Km) of an infected farm, in a municipality with high animal density and in areas

with high wind speed, open landscape, high temperature, increase the risk of infection (Schimmer *et al*., 2011; Cardenale *et al*., 2014; Meadows *et al*., 2015; Nusinovici *et al*., 2015). Considering the herd size, it was shown that the increased size of the herd is a risk factor for serological positivity (McCaughey *et al*., 2010; Ryan *et al*., 2011; Schimmer *et al*., 2011; Paul *et al*., 2012; van Engenlen *et al*., 2014; Meadows *et al*., 2015). Also, among the considered risk factors, at the herd level are: the poor hygiene and bio-security measures in the farm, the presence of ticks, the presence of dogs and cats in the farm, the artificial insemination by non-specialized technicians and the importation of straw of unknown (Cantas *et al*., 2011; Schimmer *et al*., 2011; Agger *et al*., 2013; Cardenale *et al*., 2014; van Engenlen *et al*. 2014). And finally, as reported in humans, the presence of swine on ruminant farm was considered a risk factor for *C. burnetii* infection (Meadows *et al*., 2015).

At an individual level, the risk factors identified were the age and number of animal movements. The risk of serological positivity increased with age and with the increasing number of movements between herds (Paul *et al*., 2014).

1.6.6. Synthetic epidemiology

Q fever epidemiology is complex as represented by its wide host range, its capacity to persist in the environment and its multifactorial air-borne transmission (OIE, 2015). When investigating outbreaks of Q fever, it is important to consider all possible sources of infection and to consider all the epidemiological cycles.

1.6.6.1. Infection cycles

The epidemiology of *C. burnetii* is characterized by the existence of two main cycles of transmission, one related with domestic animals, mainly ruminants, and the other related to wild animals. However, these cycles are not completely independent as shown in Figure 9.

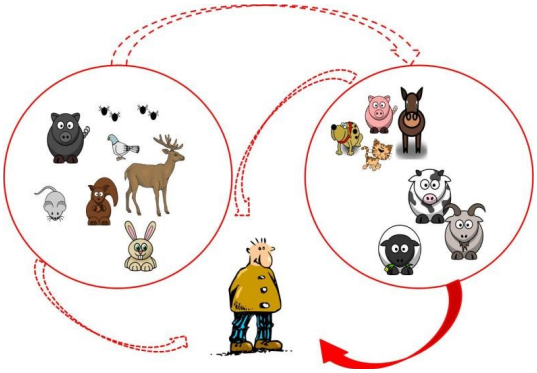


Figure 9: Diagram schematizing the epidemiological infection cycles of *C. burnetii*.

Wildlife cycle

Ticks seem to play an important role in the transmission of *C. burnetii* between wild vertebrates such as wild boars, cervids and other wild ruminants, rodents, lagomorphs, marsupials and birds. They are also able to infect domestic vertebrates, but this method of transmission is not major. The infected wild animals shed bacteria in the environment where it can survive for several weeks. The contaminated environment becomes a source of infection to wild species as well as to domestic animals.

The circulation of *C. burnetii* in wild species allows the persistence of the pathogen in the nature (Rousset *et al*., 2003).

Domestic cycle

Domestic animals can be infected eventually by tick bites but the most common is by the inhalation of infected aerosols. Generally, infected animals are asymptomatic, but they shed bacteria through the birth products, milk and faeces. This causes a very important contamination of the environment where bacteria will survive for several weeks. The transmission between domestic animals would form a cycle which would be the origin of most of the human infections (Rousset *et al.*, 2003).

1.6.7. Molecular epidemiology

The development of molecular techniques enabled the study of small genetic differences between microorganisms at a higher level of discrimination than it has been possible using conventional serological techniques (Thrusfield, 1995). The molecular characterisation of *C. burnetii* is a useful tool: i) to explore the genotypic diversity in an area and between geographical areas; ii) to determine relationships between variants of the bacterium; iii) to investigate Q fever outbreaks, particularly to clarify links regarding source of infection; and iv) to understand the epidemiological emerging factors and to evaluate control measures (Roest *et al*., 2011; Sulyok *et al*., 2014; OIE, 2015).

The genetic heterogeneity of *C. burnetii* can be assessed with several molecular techniques previously described (see 4.4). To date, among the typing methods that can be used directly on clinical samples, MLVA and MST are the most discriminating methods for *C. burnetii* (OIE, 2015). Moreover, databases have been established for MLVA and MST: http://mlva.u-psud.fr/MLVAnet/ and http://ifr48.timone.univ-mrs.fr, respectively enabling easy comparisons. The availability of such databases allows interlaboratory comparisons to be made easily and this will lead to a better understanding of the propagation of the *C. burnetii* isolates or to identify new emerging strains (OIE, 2015). Table 6 summarizes some of the identified genotypes in several countries.

Nowadays, molecular methods are a useful tool for genetic surveillance of *C. burnetii* from different sources. For instance, in a recent study in Northern Spain, 15 different MLVA genotypes were identified in 36 epidemiologically unrelated dairy cattle herds, indicating a high genetic diversity of *C. burnetii* in the region. Some genotypes worldwide distributed were identified, and only 11% of the overall detections corresponded to genotypes closely related to those identified in humans, suggesting that dairy cattle play a limited role in human Q fever infection in this region (Piñero *et al*., 2015).

Table 6: Genotypic diversity of *C. burnetii* from different countries

Country	Origin	Sample size	MST type	MLVA type	Reference
Ethiopia	*Amblyomma variegatum*	2	ST52	24/AH	Sulyok *et al*., 2014
	Amblyommacohaerens	1			
	Amblyomma cohaerens	2		26/AI	
France	Semi-skimmed milk	1	n.d.	I	Tilburg *et al*. 2012a
		2		J	
Germany	Low-fat and semi-skimmed milk	1		K	
Hungary	Cow / milk	1	ST20	I	Sulyok *et al*., 2014
	Cow / placenta	2			
		2		J	
		1		M	
	Sheep / placenta	4	ST37	AF	
		1	ST28	AG	
Poland	Human / blood	1	ST16	B	Chmielewski *et al*., 2009
	Human urine	1	ST18	E	
	Cattle placenta	2		C	
	Cattle placenta	1		D	
	Bull semen	1			
Portugal	Human / blood	1	ST8	T	Santos *et al*., 2012
		1	ST4	Y	
		4		V	
		1		W	
		3		U	
		1		X	
	Human/ mitral valve	1	ST13	S	
		1	ST8	T	
	Goat / abortion	2	ST13	S	
Slovak Republic	Semi-skimmed milk	2	n.d.	I	Tilburg *et al*. 2012a
	Semi-skimmed milk	1	n.d.	L	
		1		P	
Spain	Low-fat and semi-skimmed milk	2	n.d	I	
		2		J	
		1		M	
		1		N	
	Goat /placenta	2	n.d	S	Astobiza *et al*., 2012
		1		AE	
		1		T	
	Cattle / vaginal swabs	3		I	
	Cattle / individual milk	3		I	
		3		J	
		1		AD	
	Cattle / BTM	1		J	
		1		AC	
		1		S	
		1		AB	
		1		I	
		1		M	
	Sheep / Placenta	1		T	
		1		S	
	Sheep / vaginal swab	4		S	
		1		AA	
	Sheep / feces	2		AA	
		1		S	
	Sheep / individual milk	1		AA	
	Sheep / aerosol	2		S	
	Sheep / environmental samples	1		S	
Switzerland	Semi-skimmed milk	1	n.d.	I	Tilburg *et al*. 2012a
The Netherlands	Goats / Vaginal Swabs	43	n.d.	A	De Bruin *et al*., 2012
		6		B	
		2		C	
		9		D	
	Sheeps / Vaginal Swabs	14		A	
		5		B	
	Rats /Spleen	6		A	
		5		E	
	Low-fat and semi-skimmed milk	1	n.d	I	Tilburg *et al*. 2012a
		2		J	
	Human biological samples	7	n.d	A	Tilburg *et al*., 2012b
		1		B	
		1		C	
		1		D	
		3		E	
		1		F	
		18		G	
		1		H	
		13		P	
	Goat / Placenta	22		G	
United Kingdom	Semi-skimmed milk	1	nd	O	Tilburg *et al*. 2012a
USA	Individual milk (cattle)	1	ST20	n.d.	Pearson *et al*., 2014
	Bulk Tank (cattle)	3	ST20		
	Cattle / Bulk Tank Milk	71	ST20	n.d.	Bauer *et al*., 2015
		2	ST8		

n.d.- no data available

In another study, in Belgium, the molecular diversity of strains from goats was observed and an emerging CbNL01-like genotype was identified. This strain was isolated from half of field samples and it matches with MLVA and SNP genotyping of CbNL01 isolated from the Dutch outbreak. However, no impact on the number of human cases was observed (Boarbi *et al.*, 2014).

A systematic genotyping provides a descriptive database enabling to monitor the temporal and geographical evolution of strains. The possibility of rapid surveillance of the dispersion of a strain in a host or between different hosts is an added value for an epidemiological investigation. Furthermore, the genotypic data can help explaining different scenarios of dispersion as well as to find efficient control measures (Sidi-Boumedine and Rousset, 2011).

2

Objectives

Q fever is considered an emerging or re-emerging disease in many countries. Since the decade of 1990, the number of publications on Q fever outbreaks, or even on retrospective studies of isolated cases or on uncommon clinical manifestations, showed a remarkable increase (Arricau-Bouvery and Rodolakis, 2005). The increase in the number of human Q fever cases associated with small ruminant herds, in urban or residential areas, in Europe (Panaiotov *et al*., 2009, Medic *et al*., 2005, Porten *et al*., 2006, Gilsdorf *et al*., 2008) as well as the largest Q fever outbreak ever reported with 4108 acute human cases notified in the Netherlands between 2007 and 2011 (van Loenhout *et al*., 2012), called for special action of the human and animal health European Authorities.

Following a European Community (EC) demand, the European Food and Safety Authority (EFSA) in a close collaboration with the European Centre for Disease Prevention and Control (ECDC), prepared a scientific opinion to determine the distribution and impact of infection and disease in domestic ruminants and humans, the risk factors for the maintenance and spillover of *C. burnetii* and control options in domestic ruminant populations. In this document, the considerable uncertainty in the understanding of *C. burnetii* infection in domestic ruminant populations and the fair knowledge of its prevalence was highlighted. The harmonized field and laboratory data collection about *C. burnetii* infection in animals in European countries was recommended, to allow comparison of prevalence/incidence estimates over time and between countries (EFSA, 2010[a,b]).

In Portugal, Q fever was described for the first time in 1948. Although the occurrence of human outbreaks has never been reported in this country, its notification is mandatory since 1999 (Portaria nº1071/98 de 31 Dezembro). By analyzing the descriptive epidemiology of human Q fever notifications in Portugal, it is noted that from 1999 to 2014, the estimated incidence in humans ranged from 0.06 to 0.25 cases per 100 000 inhabitants (Figure 10), meaning a total of 217 cases notified in 16 years and an average of 13.6 cases/year (SD=5.9). An increase of the notification rate has been observed over the last years, mainly since 2011, which is probably explained by the greater awareness of the medical community after the large outbreak in The

Netherlands. However, these values may not correspond to the reality and the epidemiological situation of human Q fever in Portugal might be underestimated. In fact, the data on the number of diagnostic cases at the National Reference Laboratory, the "Centro de Estudos de Vectores e Doenças Infecciosas" of the "Instituto Nacional de Saúde Dr. Ricardo Jorge (CEVDI/INSA)", is significantly higher than the number of notifications in the same period (Santos *et al*., 2007). Additionally, the polymorphic characteristics of the disease may contribute to an under diagnosis of the disease in humans.

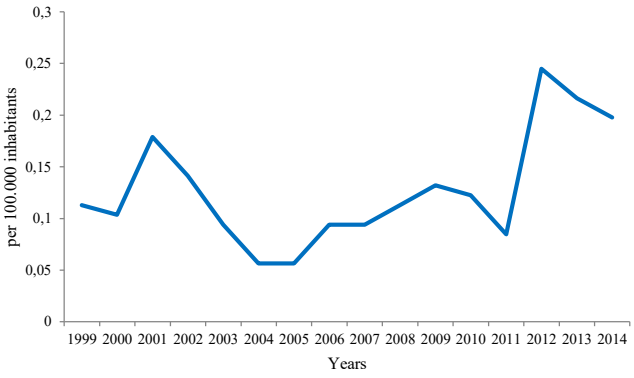


Figure 10: Incidence of notifications of human Q fever in Portugal from 1999 to 2014 (DGS, 2004, 2008, 2010, 2014, 2015).

The etiological agent and its main characteristics, the receptive hosts and the main transmission pathways are well established. However, and concerning animal population, the absence of mandatory notification to National Veterinary Authorities, the limited number of reports to the World Organization for Animal Health (OIE), the asymptomatic pattern in most cases of infection and the scarce information about the prevalence of infection in animals, namely in domestic ruminants, prompted us to develop this study with Portuguese samples.

To our knowledge, only a few reports on the epidemiology of *C. burnetii* were published. More than twenty years ago, a serosurvey was performed in dogs from Setubal (Lisboa region) and 4.8% of the animals evidenced an exposure to *C. burnetii* (Bacellar *et al*., 1995). Later, prevalence studies were conducted in sheep flocks in the region of Montemor-o-Novo (Alentejo) where a herd prevalence of 57% and a global

individual prevalence of 8.8% were observed (Fernandes, 2008). Also, the presence of *C. burnetii* DNA was evidenced in domestic ruminants and wildlife species (Clemente *et al*., 2008, 2009) and these data were supplemented with recent publications (Santos *et al*., 2012; Cumbassá *et al*., 2015).

The limited information concerning the epidemiological status of *C. burnetii* infection of Portuguese animals prompted us to develop the research herein presented. Our main goal was to provide available data in a national and international context on the epidemiology of *C. burnetii* in Portugal.

The global objective was to characterize the epidemiology of *C. burnetii* in animals from Portugal. First, our aim was to provide information about its prevalence among diverse animal species, identifying potential reservoirs in urban and rural environment and vectors (ticks). Second, we aimed to characterize at the molecular level the circulating strains and compare them with those identified in other countries.

This study was conducted in Center-North region of Portugal, which represents the second region with the largest number of human notifications (34.3%), following the Lisbon region and Tagus Valley (49.5%) (DGS, 2004, 2008, 2010, 2014, 2015). To our best knowledge, an epidemiological study on animal Q fever was never conducted in this region.

To achieve our global objective, serologic and molecular methods were used to unravel the potential sources of infection and determine the status of animal infection. Specific objectives are indicated:

1 –Serologic survey of *C. burnetii* in domestic and wild animals

- To optimize the ELISA testing to other species than ruminants
- To estimate the prevalence at individual level and at the herd level

2 –Detection of *C. burnetii* DNA in biological samples collected from domestic and wild animals

- To optimize a conventional PCR assay to screen DNA samples
- To screen for the presence of *C. burnetii* DNA in biological samples collected from domestic and wild animals
- To perform a quantitative assay using real time PCR to estimate the bacterial load in PCR positive samples

3 –Detection of *C. burnetii* DNA in ticks

- To perform the morphological identification of ticks

- To screen for *C. burnetii* DNA collected from animals

4 –Molecular characterization of *C. burnetii* strains

- To optimize the genotyping methods namely MLVA
- To provide a fine description of the biodiversity of *C. burnetii* in relation to host species, geographical areas and clinical impact.

2.1

Thesis outline

The present thesis has been divided into ten chapters and one appendix. Two initial chapters comprise a general introduction to the subject *C. burnetii* and Q fever, and an overview of this subject at the national level; raising some questions that support the study design and the objectives of this thesis. Following these two intial chapters, the original research of this thesis is organized in four chapters by animal groups to simplify the systematization of the results. The seventh chapter includes the genotyping of *C. burnetii* by MLVA. Finally, in the eighth and nineth chapters the results are discussed from an integrated point of view and conclusions are drawn, respectively. Chapter 10 refers to the references used in this thesis. An Appendix reporting the scientific work published that supports this thesis is included.

Chapter 1: General introduction

This chapter comprises the state of the art on the biology and epidemiology of *C. burnetii* and Q fever.

ABRIDGED CONTENTS: 1.1. A historical approach to Q fever; 1.2. *Coxiella burnetii*, the pathogen; 1.3. Strategies of infection; 1.4. Clinical patterns of *C. burnetii* infection; 1.5. Diagnostic challenges; 3.6. Epidemiolgy of *Coxiella burnetii*.

Chapter 2: Objectives

This chapter deals with some aspects of *C. burnetii* and Q fever in Portugal. Overall, it puts into context this topic at the national level and raises some questions that support the study design of this thesis. It also summarizes the motivation of the study of the epidemiology of *C. burnetii* in Portugal and the objectives of the thesis.

ABRIDGED CONTENTS: 2.1. Thesis outline

Chapter 3. Domestic ruminants

In this chapter the exposure to *C. burnetii* in small ruminants and cattle was evaluated by specific antibody testing using serum samples. This was followed by the molecular screening for *C. burnetii* DNA which was performed in vaginal swabs and individual milk samples. An exception occurred in dairy herds where Bulk Tank Milk samples were used for both antibody testing and molecular screening.

ABRIDGED CONTENTS: 3.1. Serological evidence of exposure to *Coxiella burnetii* in sheep and goats in central Portugal; 3.2. Serosurvey of Q fever in cattle in central Portugal; 3.3. Prevalence of *Coxiella burnetii* antibodies in Portuguese dairy cattle herds; 3.4. Q fever dairy herd status determination based on serological and molecular analysis of Bulk Tank Milk; 3.5. *Coxiella burnetii* is present in milk from dairy cattle herds in the Northwest Portugal; 3.6. Molecular screening for *Coxiella burnetii* in seropositive ruminant herds in Portugal.

Chapter 4. Domestic pigs

In this chapter the exposure to *C. burnetii* in domestic pigs was evaluated by specific antibody testing using serum samples.

ABRIDGED CONTENTS: 4.1. No evidence of specific anti-*C. burnetii* antibodies in domestic pigs.

Chapter 5. Companion animals and ticks

In this chapter the exposure to *C. burnetii* in companion animals was evaluated by specific antibody testing using serum samples. This was followed by the molecular screening for *C. burnetii* DNA in ticks collected from companion animals.

ABRIDGED CONTENTS: 5.1. *C. burnetii* in companion animals and ticks: serological and molecular screening.

Chapter 6. Wild ungulates

In this chapter the exposure to *C. burnetii* in wild boar and red deer was evaluated by specific antibody testing using serum samples. This was followed by the molecular screening for *C. burnetii* DNA in serum and feces.

ABRIDGED CONTENTS: 6.1. *C. burnetii* in wild boar (*Sus scrofa*): serological and molecular analysis. 6.2. *C. burnetii* in wild ungulates at central Portugal.

Chapter 7. Genotyping *C. burnetii*

In this chapter, molecular characterization of *C. burnetii* DNA from PCR positive samples obtained from previous chapters is conducted by MLVA-6 genotyping. Results are compared with national and international data.

ABRIDGED CONTENTS: 7.1. Genotyping *C. burnetii* from domestic ruminants.

Chapter 8. Integrated overview

This chapter comprises a general discussion of results from the studies conductive to the present dissertation. The independent results are combined to allow a contextualized and critical overview.

ABRIDGED CONTENTS: 8.1. Integrated overview of the obtained results

Chapter 9. Conclusions

This chapter systemathizes the general conclusions that can be taken from the studies conductive to the present dissertation. The independent results are combined to allow a conclusion.

ABRIDGED CONTENTS: 9.1. General conclusions. 9.2. Future perspectives.

Chapter 10. References

This closing part lists all cited references previously.

ABRIDGED CONTENTS: References.

Appendix 1

The following original publications were prepared in the scope of the works conductive to the present dissertation.

Edition of Books

1. João Carlos Caetano Simoes, Sofia Ferreira Anastácio and Gabriela Jorge da Silva. The Principles and Practice of Q Fever: The One Health Paradigm. New York: Nova Science Publishers, 2017. [427pp]

Book Chapters

1. Anastácio S, Sidi-Boumedine K, da Silva GJ. Chapter 2: A Historical Approach to Q Fever. *In: The Principles and Practice of Q Fever: The One Health Paradigm*. Ed. Simões J., Anastácio S., da Silva GJ. New York: NovaSciencePublishers, 2017. [25-34]

Articles in international peer-reviewed journals

1. Anastácio S, Carolino N, Sidi-Boumedine K, da Silva GJ. Q fever dairy herd status determination based on serological and molecular analysis of bulk tank milk. *Transboundary and Emerging Diseases*, 2016; 63: e293–e300.

2. Anastácio S, Pimenta L, Simões J, Alegria N, Rabiço A, Sidi-Boumedine K, da Silva GJ. *Coxiella burnetii* is present in milk from dairy cattle herds in the Northwest Portugal. *Experimental Pathology and Health Sciences*, 2016; 8 (1): 13-14.

3. Pimenta L, Alegria N, Anastácio S, Sidi-Boumedine K, da Silva GJ, Martins A, Simões J. Prevalence of *Coxiella burnetii* antibodies in Portuguese dairy cattle herds. *Journal of Tropical Animal Health and Production*, 2015; 47: 227-230.

4. Anastácio S, Tavares N, Carolino N, Sidi-Boumedine K, da Silva GJ. Serological evidence of exposure to Coxiella burnetii in small ruminants in central Portugal. *Veterinary Microbiology*, 2013; 167: 500-505.

Abstracts published in scientific journals with peer-review

1. Anastácio S, Cruz C, Pessoa D, Pegado J, Sidi-Boumedine K, da Silva G. Investigation of *Coxiella burnetii* infection in dairy ruminant herds with reproductive disorders in two different regions of Portugal. *Clinical Microbiology and Infection*, 2012; 18 (s3): 524.

Abstracts published in conference books, sticks or online (POSTERS)

1. Anastácio S, Tavares N, Cruz C, Sidi-Boumedine K, da Silva GJ. Molecular screening for *Coxiella burnetii* in seropositive ruminant herds in Portugal. *International Meeting on Emerging Diseases and Surveillance*, 4th to 7th November 2016, Vienna, Austria. Poster 19094.

2. Anastácio S, Sousa S, Almeida M, Vilhena H, Sidi-Boumedine K, da Silva GJ. *Coxiella burnetii* in companion animals and ticks: Serological and molecular screening. *International Meeting on Emerging Diseases and Surveillance*, 4th to 7th November 2016, Vienna, Austria. Poster 20180.

3. Anastácio S, Coelho C, Pereira M, Vieira-Pinto MM, Sidi-Boumedine K, da Silva GJ. *Coxiella burnetii* infection in wild boars (*Sus scrofa*). *International Meeting on Emerging Diseases and Surveillance*, 31st October to 3rdNovember 2014, Vienna, Austria. Poster nr 23064; pp 147.

4. Anastácio S, Carolino N, Sidi-Boumedine K, da Silva GJ. *Coxiella burnetii* in dairy ruminant herds: Informative value of BTM serological and molecular analysis. *International Meeting on Emerging Diseases and Surveillance*, 31st October to 3rd November 2014, Vienna, Austria. Poster nr 23100, pp 158.

5. Anastácio S, Tavares N, Carolino N, Sidi-Boumedine K, da Silva GJ. Evaluation of the Seroprevalence and Shedding of *Coxiella burnetii* in Portuguese Ruminant Herds. *54th Interscience Conference on Antimicrobial Agents and Chemotherapy*, Washington, DC; 05th to 09th September 2014. POP-007.

6. Anastácio S, Tavares N, Sidi-Boumedine K, da Silva GJ. Avaliação da eliminação de *Coxiella burnetii* em amostras biológicas e resposta serológica em pequenos ruminantes aparentemente saudáveis. XV Jornadas da Associação Portuguesa de Buiatria, Ilhavo; 24 a 26 de Maio de 2013.

7. Anastácio S, Tavares N, Sidi-Boumedine K, da Silva GJ. Evidence of *Coxiella burnetii* infection in small ruminants: a cross sectional study. *European Congress of Clinical Microbiology and Infectious Diseases*, 27 a 30 Abril 2013. Berlim (OC 332).

8. Anastácio S, Sidi-Boumedine K, da Silva GJ. Sero-epidemiology of *Coxiella burnetii* in companion animals. *International Meeting on Emerging Diseases and Surveillance*, 15th to 18th February 2013, Vienna, Austria. Poster nr 22075. Pp 153.

9. Anastácio S, Cruz C, Pessoa D, da Silva GJ. *Coxiella burnetii* in bulk tank milk samples, Portugal. Abstract book of *52nd ICAAC - International Conference of Antimicrobials Agents and Infectious Diaseases*, 9 to 12th September 2012, San Francisco, USA. Poster nr 1012.

10. Anastácio S, Tavares N, Martins L, Pereira A, Galvão G, Sidi-Boumedine K, da Silva G. Seroprevalence of Q fever in small ruminants at the center of Portugal. Abstract book of the *13th ISVEE International Symposium of Veterinary Epidemiology and Economics*, 20 to 24th August 2012, Maastrich, The Netherlands. Poster nr 33, pp 283.

11. Anastácio S, Pessoa D, Cruz C, Cannas da Silva J, da Silva GJ. Detection of *Coxiella burnetii* in milk samples of ruminant farms from the Center of Portugal. Abstract book of *World Buiatrics Congress*, 3rd to 8th June 2012. Lisbon, Portugal. Oral Communication nr 183, pp 38-39.

3

Domestic ruminants

3.1

Serological evidence of exposure to *Coxiella burnetii* in sheep and goats in central Portugal

S. Anastacio, N. Tavares, N. Carolino, K. Sidi-Boumedine, G.J. da Silva. Serological evidence of exposure to *Coxiella burnetii* in sheep and goats in central Portugal. *Veterinary Microbiology*, 2013; 167: 500–505.

Abstract

The recent outbreak of Q fever in The Netherlands warned European health authorities of the need of studying *Coxiella burnetii*. In Portugal, little is known about *C. burnetii* infection in animals. A cross-sectional study was designed to investigate the exposure to *C. burnetii* in sheep and goats in the Central region of Portugal, estimating the herd and individual prevalence. A serosurvey was conducted in a two levels random sampling of 89 herds and 460 animals. Individual blood samples were collected from animals older than 6 months, and specific antibodies anti-*C. burnetii* were detected by ELISA testing.

Results showed a global herd prevalence of 32.6% (95% CI: 23.1 to 42.1%). Herd prevalence was higher in mixed herds (38.5%; 95% CI: 12 to 65%) and in sheep herds (37.5%; 95% CI: 21 to 54%) than in goat herds (28.8%; 95% CI: 17 to 41%). Global individual prevalence was estimated at 9.6% (95% CI: 6.9 to 12.2%), and it was higher in goats (10.4%; 95% CI: 7.8 to 13%) than in sheep (8.6%; 95% CI: 5.8 to 11.4%). Sample positive percentages (S/P) ranged from 41.5 to 185.9%. S/P percent higher than 100 was found in 18.2% (8/44) of sera from distinct herds. Positive results were significantly associated with goats, older animals and larger herds. These results revealed the presence of *C. burnetii* in small ruminants evidencing their potential role in the infection cycle.

Keywords: *Coxiella burnetii*, epidemiology, seroprevalence, zoonosis.

Introduction

Q fever is a zoonotic infection caused by *Coxiella burnetii*, an obligate intracellular bacterium. It was described in Australia in 1937 for the first time (Maurin and Raoult, 1999). Currently, this disease presents a worldwide distribution, affecting a wide range of domestic and wildlife animals (Arricau-Bouvery and Rodolakis, 2005; Rousset *et al*., 2010).

The clinical signs of Q fever are not pathognomonic neither in humans nor in animals. This lack of specificity is the first major obstacle to its diagnosis (Arricau-Bouvery and Rodolakis, 2005; Angelakis and Raoult, 2010). In humans, acute Q fever can be asymptomatic, or it can manifest as a nonspecific flu-like illness. Complications associated with pneumonia or hepatitis requiring hospitalization may be observed in about 2% of patients. Chronic Q fever may appear as an endocarditis, an osteoarticular infection, a chronic hepatitis or as a chronic pneumonia in patients with predisposing factors and/or inappropriate antibiotherapy. The infection during pregnancy may lead to abortion (Maurin and Raoult, 1999; ECDC, 2010). Also, cases of chronic fatigue syndrome have been described infrequently following *C. burnetii* infection (Angelaskis and Raoult, 2010; van Asseldonk *et al.*, 2013). In animals, Q fever is mainly reported in livestock ruminants and occurs, usually, as an asymptomatic infection (Woldehiwet, 2004; Arricau-Bouvery and Rodolakis, 2005; Rousset *et al*., 2010). In small ruminants, abortions, premature delivery, delivery of weak offspring and stillbirth are reported (Rodolakis, 2006). In cattle, clinical signs of Q fever can be less obvious than in small ruminants. However, a very recent study demonstrated that abortion and irregular repeat breeding are important risk indicators in cattle dairy herds (Saegerman *et al*., 2013). Also, an association of Q fever with metritis and infertility has been suggested (To *et al*., 1998, Woldehiwet, 2004, EFSA, 2010[a]).

Epidemiological studies have demonstrated a relationship between the infection in humans and ruminants (Gilsdorf *et al*., 2008, Schimmer *et al*., 2010, van den Brom *et al*., 2012). However, the Q fever prevalence and incidence are not well known, and have been underestimated for many years (EFSA, 2010[a]). Shedding of bacteria occurs by secretions and excreta from infected animals, namely vaginal secretions, milk, faeces and urine. During birthing and/or abortion the bacterium is excreted massively in genital secretions, placenta and fetal fluids (Berri *et al*., 2001, Berri *et al*., 2002, Berri *et al*., 2005, Arricau-Bouvery *et al*., 2003, Guatteo *et al*., 2006). Because of the existence of fecal shedders and the high resistance of *C. burnetii*, bedding material must be considered as a source of infection (Rodolakis, 2006, Guatteo *et al*., 2007, Rousset *et al*., 2010).

Recently, the European Commission (EC) formulated concerns about the increase number of human Q fever cases associated with small ruminant herds, in urban or residential areas, in Europe (Panaiotov *et al*., 2009; Medic *et al*., 2005; Porten *et al*., 2006; Gilsdorf *et al*., 2008). In the Netherlands, 4108 acute human cases were

notified between 2007 and 2011 (van Loenhout *et al*., 2012). Typing of bacteria by multiple-locus variable number tandem repeat analysis (MLVA) showed a genetic similarity of isolates recovered from human and animal samples, indicating a relationship between human cases and the occurrence of infection in ruminant herds (Klaassen *et al*., 2009; van der Hoek *et al*., 2010; Roest *et al*., 2011[a,b]).

Following the EC's demand to assess the risk for humans and animals associated with Q fever, the European Food Safety Authority (EFSA), in a scientific opinion, highlighted the considerable uncertainty that still exists in the understanding of *C. burnetii* infection in domestic ruminant populations and the knowledge of its prevalence (EFSA, 2010[a]).

In Portugal, Q fever is a notifiable disease since 1999, and the average number of notifications is 0,10 cases per 10^5 inhabitants. However, these data might be underestimated. Between 2004 and 2005, 32 cases were diagnosed in the Centre for Vectors and Infectious Diseases at the National Health Institute but only 12 were notified, clearly suggesting an under-notification (Santos *et al*., 2007). Despite the zoonotic pattern of Q fever, the information about the occurrence of infection in animals is scarce. A few studies demonstrated the presence of bacteria in clinical samples from zoo animals and from ruminants (Clemente *et al*., 2008, 2009). Also, our previous results on screening bulk tank milk indicated the presence of *C. burnetii* in ruminant herds originated from different regions (Anastácio *et al*., 2012). A genotypic diversity among *C. burnetii* isolates from animals and human clinical samples was shown (Santos *et al*., 2012). These studies were based on a limited number of samples obtained from clinical cases. They highlighted the need of epidemiological study of *C. burnetii* in other geographical regions, increasing the number of samples randomly sampled. In this context, the present study aimed to understand the current status of small ruminants to the exposure of *C. burnetii* in the Center of Portugal. A cross-sectional study was designed to estimate the herd and the individual apparent prevalence of specific antibodies anti-*C. burnetii*.

Material and Methods

Study design and sampling approach

A cross-sectional survey was carried out during the 4th trimester of 2011 in small ruminant herds from the central region of Portugal.

The number of herds used in the study was calculated taken into account the regional census (N=1527 small ruminant's herds), obtained from the Official Regional Veterinary Services. The sample size calculation was performed using the program WinEpiscope version 2,0 based on the formula $n=[t^2 P_{esp} (1-P_{esp})]/d^2$, considering n the required size sample, t the student value for a 95% confidence level (1,96), P_{esp} the expected prevalence and d the desired absolute precision. Taking into account that the study population (N) was small (n/N > 5%), the required sample size was adjusted by the formula $n_{adj}=(N*n)/(N+n)$ (Thrusfield, 1995).

It was considered an expected herd prevalence of 57% (Fernandes, 2008) a desired absolute precision of 10%, and a 95% confidence interval, resulting in an estimated sample of 89 herds. The list of total herds was used for a simple random sampling, using the program Microsoft Excel®.

In each herd, the sample size was calculated to detect the presence of infection using the WinEpiscope version 2,0 based on the formula $n=[1-(1-p_1)^{1/d}][N-d/2]+1$ in which n is the required sample size, N is the population size, d is the minimal number of affected animals in the population and $p1$ is the probability of finding at least one case in the sample (Thrusfield, 1995). For this purpose, the herd size was considered, the expected proportion of seropositive animals was established in 15% (Guatteo *et al*., 2011) and a 95% confidence level was considered. On farms sized ≤ 10 animals, samples were taken from all the animals. The list of animals in each herd was used for a simple random sampling using the program Microsoft Excel®.

Blood samples were collected from selected animals simultaneously undergoing statutory routine brucellosis testing (animals aged > 6 months), by the veterinary practitioner group, in charge of the Official Sanitary Campaign. Individual apparent prevalence was calculated globally considering the total amount of samples. The serum obtained by centrifugation of blood samples was stored at -20ºC until serological testing. A questionnaire was filled up on the surveyed herds by interviewing farmers, during sample collection.

Serological analyses

Sera were tested for the presence of specific antibodies anti-*Coxiella burnetii* using an indirect commercial ELISA kit, LSIVET Ruminant Milk/Serum Q Fever® (LSI, France). Optical density (OD) values were measured at 450 nm. Sample/positive percentages (S/P percent) were calculated by the adjustment with the negative control,

using the formula ($OD_{sample} - OD_{negative}$) / ($OD_{positive} - OD_{negative}$) x 100. The resulting S/P percent were divided in different classes, according to manufacturer's instructions: negative (Neg; S/P per cent ≤ 40), low positive (LP; 40 < S/P ≤ 100), positive (Pos; 100 < S/P ≤ 200), high positive (HP; 200 < S/P ≤ 300) and very high positive (VHP; S/P > 300).

Statistical analysis

For statistical analysis purposes, it was considered the herd size (continuous), species in the herds (categorical nominal: sheep, goats or mixed herds) or species individually (categorical nominal: sheep/goats), productive system (categorical nominal: intensive, extensive, semi-extensive), age (continuous), geographic distribution (categorical nominal: counties), co-habitation with other species (categorical nominal: yes/no), and reports of reproductive disorders within the previous year (ie, at least one of the following disorders: abortion, premature delivery, infertility, metritis and/or placentary retention) (categorical nominal: presence/absence).

The response variables were the S/P percent (continuous) obtained in each individual serum by ELISA testing and its categorization in positive or negative (categorical nominal: positive/negative). So, herds were categorized as positive or negative, according to the results obtained for individual serum. A herd was considered positive when at least one serum showed a positive result to ELISA testing. The apparent prevalence of anti-*C. burnetii* antibodies was calculated at herd and at individual level. Statistical uncertainty was assessed by calculating the 95% confidence interval for each of the proportions according to the expression S.E. 95% C.I. = $1.96\ [p\ (1 - p)\ /n]^{1/2}$ (Thrusfield, 1995) and using WinEpiscope version 2.0.

Statistical analyses were performed using SAS® (version 9.1.2). Simple logistic regression test was performed to assess individually the main factors associated with *C. burnetii* seropositivity at herd and individual level. After evaluating these factors with significant influence ($p< 0.05$) on positive results, a multiple logistic regression analysis was conducted to assess the joint relationship between several independent factors and *C. burnetii* seropositivity. Also, a multiple logistic regression analysis was used to evaluate the combined effect of multiple variables in S/P percent (continuous) ($p< 0.05$).

Results

Descriptive analysis

Of all 1527 eligible herds, 89 (5.8%) were selected to this study. The mean herd size was 6.7 animals (SD=11.305, range 1-104) and 46 herds (51.7%) had less than 4 animals. Goat herds were predominant (n=52, 58.4%) followed by sheep herds (n=24, 27%) and mixed herds (n=13, 14.6%). It was also observed a predominance of meat producing herds (n=79, 88.8%), a semi-extensive grazing system (n=89, 100%) and herd localization at the county of Coimbra (n=58, 65.2%). In these herds, 460 animals were sampled (mean age 45.6 months) (SD=29.9, range 9-167).

Table 7 summarizes the descriptive characteristics and seroprevalence results of the ELISA test in herds.

Table 7: Descriptive characteristics and seroprevalence results in sheep and goat herds.

Variable	Frequency (n)	Seroprevalence (%)	[a]CI 95%
Selected	89	32.6	23.2-42.1
Herd size			
≤ 10	80	28.8	19.1-38.5
> 10	9	66.7	36-97.4
Herd species			
Goat	52	28.8	16.7-40.1
Sheep	24	37.5	18.3-56.7
Mixed	13	38.4	12.2-64.8
Type of production			
Meat	79	34.2	24.1-45.8
Milk	3	33.3	1.8-87.5
Mixed	7	14.3	1-58
Productive system			
Intensive	0	0	na[b]
Extensive	0	0	na[b]
Semi-extensive	89	32.6	23.2-42.1
County			
Coimbra	58	32.8	21.4-46.5
Miranda do Corvo	10	30	8.1-64.6
Lousã	1	0	na[b]
Pencova	4	0	na[b]
Vila Nova de Poiares	16	43.8	20.8-69.5
Cohabitation with other species			
Yes	61	34.4	23-47.8
No	28	28.5	14-48.9
Cohabitant species			
Pets	7	28.6	5.1-69.7
Farm animals	30	36.7	20.6-56.1
Pets and farm animals	20	40	20-63.6
Reproductive disorders			
Yes	6	33.3	6-75.9
No	83	32.5	22.9-43.8

[a]Confidence interval (range within which is reasonably confident to find the real prevalence)

[b] not aplicable

Global prevalence in herds was estimated on 32.6% (CI 95%: 23.1 to 42.1%). Herd prevalence was higher in mixed herds 38.5% (95% CI: 12 to 65%) and in sheep herds 37.5% (CI 95%: 21 to 54%) than in goat herds 28.8% (95% CI: 17 to 41%). Geographic distribution of positive herds showed a frequency of 32.8% (19/58) in Coimbra, 42.8% (7/16) in Vila Nova de Poiares, 30% (3/10) in Miranda do Corvo, 0% (0/1) in Lousã and 0% (0/4) in Penacova.

Co-habitation with other species was observed in 64% (57/89) of herds, and a positive result was obtained in 36.8% (21/57). Pets (dogs and/or cats), alone or together with farm animals, were reported in 47.4% (27/57) of herds, amongst which 37% (10/27) showed a positive result.

The occurrence of previous reproductive disorders was reported in 6.7% (6/89) of herds particularly abortion in 2.2% (2/89) and infertility in 4.5% (4/89).

In 27.6% (8/29) of positive herds at least one serum presented a high S/P per cent (>100), and in 31% (9/29) more than one serum was classified as positive (S/P per cent >40).

Table 8 shows the descriptive statistic of results at individual level. Global individual seroprevalence was estimated on 9.6% (CI 95%: 6.9 to 12.2%), but considering the ruminants species, seroprevalence was estimated on 10.4% (CI 95%: 7.8 to 13%) in goats and 8.6% (CI 95%: 5.8 to 11.4 %) in sheep. Mean age of positive animals was 50 months (SD 28.4, range 14-135), and 44 months for the negative animals (SD 30.2, range 9-167). S/P per cent ranged from 41.5 to 185.9 (mean 75.6, SD 34.07), and 18.2% (8/44) of samples were classified as positive ($100 < S/P \leq 200$), all of them from different herds.

Table 8: Descriptive statistics of *C. burnetii* antibodies in sheep and goats individually.

Test Category	Nr of animals	Mean age of animals (months)	Apparent prevalence	95% CI[a] (p)	Range of S/P[b]	Mean S/P value
Positive	44	50	0,096	[0,07;0,12]	41,5 - 185,9	75,6
Negative	416	44	0,904	[0,88;0,93]	0-38,82	4,2

[a] Confidence Interval (range within which is reasonably confident to find the real prevalence)

[b] S/P - Sample positive percentage

Univariable analysis

Individual factors were tested to find associations with positive results in herds and in animals individually. The variable production system was not included as the reference. Categories were inexistent as almost all the herds had a semi-extensive grazing system. Univariable analysis identified three factors with significant effect on *C. burnetii* seropositivity at herd or animal levels. At the herd level, only the herd size evidenced an association with seropositivity ($p< 0.01$), using the logistic regression test (Figure 11). Indeed, it was observed that all the herds with more than 14 (6.8%) animals were classified as positive. Individually, the logistic regression test evidenced an association between the increase of animal's age and seropositivity ($p< 0.01$). Also, it was observed that the probability of having a positive result is higher in goats than in sheep ($p< 0.05$), using the same statistic model.

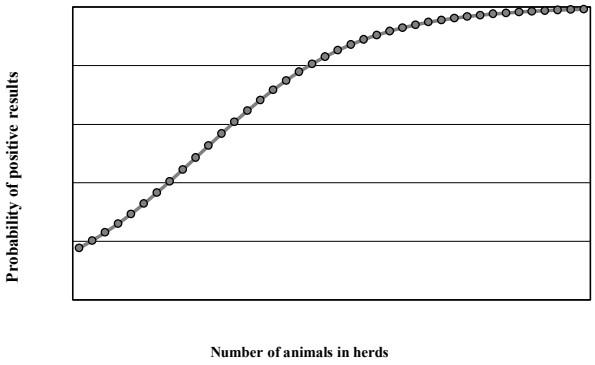


Figure 11: The probability of a positive result for *C. burnetii* antibodies increases with the number of animals in herds (Intercept= -1.7009±0.4459; β_1= 0.1644±0.0668).

Multivariable analysis

A multivariable model was performed to test simultaneously variables found to be associated in univariable analysis. A multiple logistic regression test confirmed that species and age were both associated with positive results ($p< 0.05$) (Figure 12).

Also, a linear regression model tested the effect of multiple variables in S/P per cent. The age of the animal was the only factor evidencing an influence with S/P per cent ($p<0.01$).

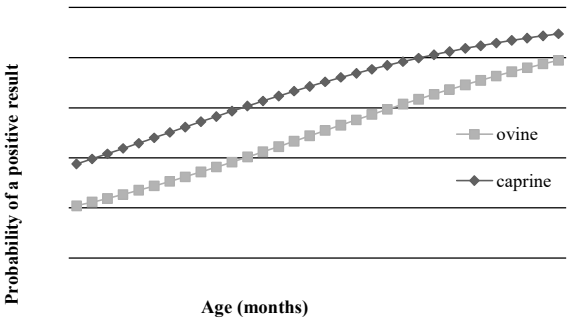


Figure 12: The probability of positive results for *C. burnetii* antibodies increases with age, in each month, by species using a logistic regression model (Ovine: Intercept= -1.5037±0.3204; Caprine: Intercept= -0.6783±0.3552; β_1= 0.0214±0.0084).

Discussion

Q fever is recognized as zoonotic disease worldwide with multiple animals acting as *C. burnetii* reservoirs. The present study was designed as an approach to evaluate the exposure of small ruminants to *C. burnetii* in the center of Portugal. A commercial ELISA test was used to detect IgG anti-*C. burnetii* (phase I and phase II).

Our results indicate a global herd prevalence of 32.6%, higher in mixed herds (38.4%) and in sheep herds (37.5%) than in goat herds (28.8%). These data are similar to those reported in sheep herds from Sardinia, Italy (38%) (Masala *et al*., 2004). However, higher values of seroprevalence (74%) were reported in sheep herds from Spain (Ruiz-Fons *et al*., 2010) and Turkey (83%) (Kennerman *et al*., 2010), while in Germany, sheep herd seroprevalence was shown to be lower (28%) (Hilbert *et al*., 2012). According the data from goat herds in other European countries, a higher seroprevalence was reported compared to this study, namely in The Netherlands (43.1%) (Schimmer *et al*., 2011), in Northern Ireland (42.9%) (McCaughey *et al*., 2010), in Spain (45%) (Ruiz-Fons *et al*., 2010) and in Sardinia, Italy (47%) (Masala

et al., 2004). Among these studies, only Ruis-Fonz *et al*. (2010) and Schimmer *et al*. (2011) performed the serologic test with the same commercial ELISA used in our study. Overall, it can be suggested that the herd prevalence in our study was lower than the range of herd prevalence described in other European countries.

The global individual seroprevalence was 9.6%. Goats were significantly related with seropositivity at animal level ($p< 0.05$). Indeed, individual seroprevalence was slightly higher (10.4%) in goats than in sheep (8.6%). These results are similar to those obtained in other European seroprevalence studies such as Spain, Ireland, Greece and Sardinia, Italy, in which values ranged from 6.5% and 13% (Ruiz-Fons *et al*., 2010; McCaughey *et al*., 2010; Pape *et al*., 2009; Masala *et al*., 2004). A higher individual seroprevalence (17.2%) was reported in The Netherlands, in 2008, during the Q fever epidemic outbreak (van den Brom *et al*., 2012).

The increase of the age of the animal was associated with seropositive results ($p< 0.01$). This is consistent with the report from Schimmer and collaborators, in The Netherlands, where they also found an increase of seroprevalence with age (Schimmer *et al*., 2011). This finding suggests the occurrence of horizontal transmission among animals and the maintenance of infection within adult populations (Garcia-Perez *et al*., 2009; Ruiz-Fons *et al*., 2010; Astobiza *et al*., 2012). It may be explained by the increase rate of contagion as a consequence of a higher probability of contact during lifetime (Ruiz-Fons *et al*., 2010). Furthermore, an IgG based antibody test was used, thus possibly evidencing past exposure to *C. burnetii* (McCaughey *et al*., 2010). The presence of such antibodies cannot be associated exclusively to a current infection, since animals can remain seropositive for years after the acute infection have been resolved (McQuiston *et al*., 2002). The high mean age of animals in our study (3.9 years) might be related to regional cultural habits and the traditional consumption of meat from older animals. Indeed, most of the sampled animals came from meat production herds in a semi-extensive grazing system.

The long-time contact with *C. burnetii* in the surveyed herds together with the random selection of sampled herds and animals can explain the lack of association found between reproductive disorders and seropositivity (Garcia-Perez *et al*., 2009; Ruiz-Fons *et al*., 2010; Astobiza *et al*., 2012). However, the presence of an asymptomatic infection in herds cannot be excluded. In fact, our previous results showed the presence of specific antibodies (Anastácio *et al*., 2012) and DNA of *C. burnetii*, detected by qPCR (unpublished data), in bulk milk tank from dairy

ruminant farms with reports of reproductive disorders. Indeed, an association between reproductive disorders and *C. burnetii* prevalence in ruminants has been reported in some studies (Cabassi *et al*., 2006; Garcia Perez *et al*., 2009).

Despite the significant association between goats and positive results ($p<0.05$), from an individual perspective, it was found a lower herd prevalence in goats than in sheep, which is in agreement with data from a study conducted in Northern Spain (Ruiz-Fons *et al*., 2010). The higher individual prevalence together with the lower herd prevalence may suggest that the within-herd prevalence is high in goats. Nevertheless, this could not be assessed in this study because sample size calculation in herds aimed the detection of infection, not the estimation of within-herd prevalence. Moreover, differences of prevalence between sheep and goats cannot be explained by different sampling periods in relation to the lambing season. Sample collection occurred in early pregnancy in both species and the reproductive cycle is similar among both species in this region.

The herd size was associated to seropositive results ($p< 0.01$), thus the probability of a positive result increases with the number of animals per herd. Other studies in goats (Schimmer *et al*., 2011; Schimmer *et al*., 2012) and in cattle (McCaughey *et al*., 2010) support our findings. The increased risk of introduction and/or transmission of pathogens in a large population is probably related with the increased number lambing females at lambing season (Woldehiwet, 2004) and by other management factors like larger amounts of feed, animal supply and a higher number of professionals working at or visiting the farm (Schimmer *et al*., 2011). Therefore, larger herds are more prone to acquire and develop Q fever, and the number of animals must be considered a risk factor to *C. burnetii* dissemination.

In conclusion, this study confirms the presence of specific anti-*C. burnetii* antibodies in goats and sheeps in Portugal. To our knowledge, this is the first seroprevalence survey performed in small ruminants in this country. To clarify the infection status in these herds, namely the presence of an active infection, the shedding of bacteria must be assessed. Also, a better elucidation of the epidemiology of Q fever in Portugal requires the inclusion of other animal species from a large geographical area.

Acknowledgements

The authors would like to thank the collaboration of farmers.

Financial support

Sofia Anastácio is supported by the grant SFRH/BD/77823/2011 given by Fundação para a Ciência e a Tecnologia (FCT), Lisbon, Portugal. This work was supported financially by the Center for Pharmaceutical Studies, University of Coimbra (CEF/UC), Portugal

Conflict of interest statement

The authors declare no conflict of interests.

3.2

Serosurvey of Q fever in cattle in central Portugal

S. Anastacio, N. Tavares, K. Sidi-Boumedine, G.J. da Silva. Serosurvey of Q fever in cattle in central Portugal. *4th Conference of the Centre of Pharmaceutical Studies,* 27th of November 2013. Faculty of Pharmacy, University of Coimbra. Oral Communication.

Abstract

Following the EC's demand, the European Food Safety Authority (EFSA) highlighted the need of studying *Coxiella burnetii* in European countries. Recently, we reported the exposure of *C. burnetii* in small ruminants evidencing their potential role in the infection cycle. But little is known about the exposure in cattle in Portugal. This study aimed to evaluate the status of cattle to the exposure of *C. burnetii* in central Portugal.

A serosurvey was developed in cattle herds (n=17) from November 2011 to December 2012. A total of 166 blood samples were collected and tested for the presence of specific antibodies anti-*C. burnetii* using a commercial ELISA. Laboratory results were expressed in S/P per cent and a positive herd was considered when at least one serum showed a positive result on ELISA testing.

The proportion of positive herds was estimated in 23.5% (CI 95%: 7.8 to 50.2%). and the proportion of positive animals was 15.1% (CI 95%: 10.2 to 21.6%). Also, it was observed that all the positive herds were dairy herds, in intensive production system and larger than 50 animals. Moreover, all the positive herds reported the occurrence of reproductive disorders in the previous year, and infertility, metritis and placentary retention were factors significantly associated with positive results ($p<0.05$). The results presented herein confirm the presence of specific antibodies anti-*C. burnetii* in cattle highlighting their potential role as reservoirs of *C. burnetii* in Portugal.

Keywords: *Coxiella burnetii*, ELISA, epidemiology, zoonosis.

Introduction

Q fever is a zoonotic infection caused by *Coxiella burnetii*, an obligate intracellular bacterium (Maurin and Raoult, 1999). Currently, this disease presents a worldwide distribution, affecting a wide range of domestic and wildlife animals (Arricau-Bouvery and Rodolakis, 2005; Rousset *et al*., 2010). Infected animals shed the organism mostly

in placental membranes and birth fluids (Arricau-Bouvery *et al*., 2003; Roest *et al*., 2012). Bacteria are also excreted in milk (Roest *et al*., 2012; van den Brom *et al*., 2012), feces and vaginal mucus (Guatteo *et al*., 2006, 2007). After shedding, in the environment, bacteria can be easily aerosolized and then can infect susceptible hosts (Maurin and Raoult 1999; Arricau-Bouvery and Rodolakis, 2005; Schimmer *et al*., 2010).

Because the clinical pattern of Q fever in animals is pleomorphic and mostly asymptomatic (EFSA, 2010[a]), the infection is usually recognized in livestock by the presence of seropositive animals (Agerholm, 2013). Some clinical signs decribed in cattle include reproductive disorders and occasionally pneumonia (Arricau-Bouvery and Rodolakis, 2005). The occurrence of late abortion (Agerholm, 2013), infertility (To *et al*., 1998; López-Gatius *et al*., 2012), placental retention, post-partum metritis (Martinov, 2008; Garcia-Ispierto *et al*., 2010; López-Gatius *et al*., 2012) and even mastitis (Barlow *et al*., 2008) have also been described. According to EFSA recommendations, the major warning sign of Q fever to be taken into account is the number of abortions and calves with low birth weight in cattle herds (e.g. three abortions in the year for herds with less than 100 cows and more than 4% of cows aborting during the course of the year for herds of more than 100 cows) (EFSA, 2010[b]). This lack of clinical specificity is a major obstacle to its diagnosis (Arricau-Bouvery and Rodolakis, 2005; Angelakis and Raoult, 2010).

Following the EC's demand to assess the risk for humans and animals associated with Q fever, the European Food Safety Authority (EFSA), in a scientific opinion, highlighted the considerable uncertainty that still exists in the understanding of *C. burnetii* infection in domestic ruminant populations and the knowledge of its prevalence (EFSA, 2010[a]).

In Portugal, the notification of Q fever in animals is not mandatory. Cases of Q fever in humans are regularly notified, but the information about the occurrence of infection in animals, namely in cattle, is very limited. A few studies demonstrated the presence of bacteria in clinical samples from zoo animals and from ruminants (Clemente *et al*., 2008, 2009). Also, our preliminary results on screening bulk tank milk indicated the presence of *C. burnetii* in ruminant herds originated from different regions (Anastácio *et al*., 2012). The present study aimed to extend the study and to screen for specific antibodies anti-*C. burnetii* in cattle, in the Centre of Portugal.

Material and Methods

Study design and sampling approach

A cross-sectional study was developed in cattle herds from November 2011 to December 2012, in the central region of Portugal (40º15'N 8º27'W). A serosurvey was performed (animals aged > 6 months) taking advance of undergoing statutory routine brucellosis testing. Blood samples were collected by the veterinary practitioner group, in charge of the Official Sanitary Campaign. In the study region (Coimbra), cattle population consists in 750 cattle in 65 herds, according to data obtained from the regional census of the Official Regional Veterinary Services. For sample size calculation purpose, the program WinEpiscope version 2,0 used. Briefly, it was based on the formula $n=[t^2 P_{esp} (1-P_{esp})]/d^2$, considering n the required size sample, t the student value for a 95% confidence level (1,96), P_{esp} the expected prevalence and d the desired absolute precision. Taking into account that the study population (N) was small (n/N > 5%), the required sample size was adjusted by the formula $n_{adj}=(N*n)/(N+n)$ (Thrusfield, 1995).

It was considered an expected herd prevalence of 30% (Alvarez *et al*., 2012) in cattle, a desired absolute precision of 10%, and a 95% confidence interval, resulting in an estimated sample of 39 cattle herd. The list of total herds was used for a simple random sampling, using the program Microsoft Excel®.

In each herd, the sample size was calculated to detect the presence of infection using the WinEpiscope version 2,0 based on the formula $n=[1-(1-p_1)^{1/d}][N-d/2]+1$ in which n is the required sample size, N is the population size, d is the minimal number of affected animals in the population and $p1$ is the probability of finding at least one case in the sample (Thrusfield, 1995). For this purpose, the herd size was considered, the expected proportion of seropositive animals was established in 6.7% (Alvarez *et al*., 2012) and a 95% confidence level was considered. On farms sized ≤ 10 animals, samples were taken from all the animals. The list of animals in each herd was used for a simple random sampling using the program Microsoft Excel®.

The serum obtained by centrifugation of blood samples was stored at -20ºC until serological testing. A questionnaire was filled up on the surveyed herds by interviewing farmers, during sample collection.

Serological analyses

Sera were tested for the presence of specific antibodies anti-*Coxiella burnetii* using an indirect commercial ELISA (LSIVET Ruminant Milk/Serum Q Fever®, Lifetechonogies). Optical density (OD) values were measured at 450 nm. Sample/positive percentages (S/P percent) were calculated by the adjustment with the negative control, using the formula $(OD_{sample} - OD_{negative}) / (OD_{positive} - OD_{negative}) \times 100$. The resulting S/P per cent were divided in different classes, according to manufacturer's instructions: negative (Neg; S/P per cent ≤ 40), low positive (LP; $40 < S/P \leq 100$), positive (Pos; $100 < S/P \leq 200$), high positive (HP; $200 < S/P \leq 300$) and very high positive (VHP; $S/P > 300$).

Statistical analysis

For statistical analysis purposes, the herd's explanatory variables were the herd size (continuous and categorical nominal: <50 or ≥50), production type (categorical nominal: dairy or beef), co-habitation with other species (categorical nominal: yes/no) and reports of reproductive disorders within the previous year (ie, at least one of the following disorders: abortion, premature delivery, infertility, metritis and/or placentary retention) (categorical nominal: presence/absence). The individual's explanatory variables were gender (categorical nominal: male/female) and age (continuous).

The response variables were the S/P percent (continuous) obtained in each individual serum by ELISA testing and its categorization in positive or negative (categorical nominal: positive/negative).

A herd was considered positive when at least one positive result was obtained in the ELISA testing. The antibody positivity rate (anti-*C. burnetii* antibodies) was calculated at herd and at individual level. Statistical uncertainty was assessed by calculating the 95% confidence interval for each of the proportions according to the expression S.E. 95% C.I. = $1.96 [p (1 - p) /n]^{1/2}$ (Thrusfield, 1995) and using WinEpiscope version 2.0.

Statistical analyses were performed using EpiInfo (version 3.5.4). Simple logistic regression test was performed to assess individually the main factors associated with *C. burnetii* seropositivity at herd and individual level. Also, a linear regression analysis was used to evaluate the combined effect of age (continuous) in S/P percent (continuous). Significant results were considered when $p<0.05$.

Results

Table 9 summarizes the descriptive characteristics and seropositive results in herds. A total of 166 animals from 17 herds were screened. The mean herd size was 75 animals (SD=125.5, range 1-400). The occurrence of previous reproductive disorders was reported in 47.0% (8/17) of herds particularly abortion in 41.2% (7/17). The global rate of positivity in herds was estimated on 23.5% (CI 95%: 7.8 to 50.2%).

Table 9: Descriptive characteristics and seroprevalence results in cattle herds.

Variable	Frequency (n)	Seropositivity (%)	[a]CI 95%
Selected	17	23.5	7.8-50.2
Herd size			
< 50	12	0	na[b]
≥ 50	5	80.0	29.9-98.9
Type of production			
Meat	6	0	na[b]
Milk	11	36.4	12.4-68.4
Productive system			
Intensive	8	50.0	17.5-82.5
Extensive	9	0	na[b]
Cohabitation with other species			
Yes	4	25.0	1.3-78.0
No	6	0	na[b]
Missing	7	na[b]	na[b]
Reproductive disorders			
Yes	8	50.0	17.5-82.5
No	7	0	na[b]
Missing	2	na[b]	na[b]

[a] Confidence Interval (range within which is reasonably confident to find the real prevalence)

[b] not aplicable

Table 10 shows the descriptive statistic of seropositive results at individual level. The global rate of positivity in animals was estimated on 15.1% (CI 95%: 10.2 to 21.6%). In seropositive animals, the mean age was 47 months (SD 17.5, range 29-85) against 58 months in seronegative animals (SD 24.6, range 31-120). S/P per cent in positive samples ranged from 44.9 to 231.4 (mean 125.5, SD 64.7).

Table 10: Descriptive statistics of *C. burnetii* antibodies individually.

Test Category	Nr of animals	Mean age of animals (months)	Frequency	95% CI[a] (p)	Range of S/P[b]	Mean S/P value
Positive	25	47	0,151	[0,102;0,216]	44,9 – 231.4	125.5
Negative	141	58	0,848	[0,783;0,898]	0-29.2	4.8

[a] Confidence interval (range within which is reasonably confident to find the real prevalence)

[b] S/P - Sample positive per cent

Individual factors were tested to find associations with positive results in herds and in animals individually. At the herd level, the report of infertility, metritis and placentary retention revealed an association with seropositivity ($p< 0.05$). Individually, a linear regression model tested the effect of age in S/P per cent showing that the increase of age might be a protective factor for the increased antibody titer ($p<0.05$).

Discussion

Q fever is a zoonotic disease and a multiple-host pathogen with worldwide distribution (Angelakis and Raoult, 2010). The present study was designed as an approach to evaluate the exposure of cattle to *C. burnetii* in the center of Portugal. The number of herds selected for the study was lower than the expected and exact error in the estimation of the herd prevalence was established in 18,72% (WinEpiscope version 2.0). Thus, the results are described in terms of positivity rate.

The global herd positivity rate was established in 23.5%. These data are lower to those reported in other countries using similar methodology such as Northern Ireland (48.4%) (McCaughey *et al*., 2010); Spain (30%) (Alvarez *et al*., 2012) and Ecuador (46.9%) (Carbonnero *et al*., 2015). Only one study conducted in the Republic of Ireland, reported a herd prevalence of 6.9% being suggested that it could be associated by the sampling procedure, as only 5 samples per herd were collected (Ryan *et al*., 2011).

The global individual antibody positivity was estimated in 15.1%. Despite an even higher individual seroprevalence was reported in Iran (22.3%) (Azizzzadeh *et al*., 2011), this result might reproduce a slightly imprecision caused by the small size of the sample. In fact, in other reports from other European countries the individual seroprevalence in cattle is lower, such as 1.8% in the Republic of Ireland (Ryan *et al*., 2011), 5.5% in Denmark (Paul *et al*., 2014), 6.2% in Northern Ireland (McCaughey

et al., 2010), 6.7% in Spain (Alvarez *et al*., 2012) and 16% in the Netherlands (Muskens *et al*., 2011).

The increase of the animal's age appeared to be related to a decrease of the S/P per cent ($p< 0.05$). This finding is somewhat unexpected because usually seropositivity is associated with aged animals (Schimmer *et al*., 2011). The rate of contagion increases as a consequence of a higher probability of contact during lifetime (Ruiz-Fons *et al*., 2010). In this study, all of the positive herds were dairy herds in an intensive production system which is considered a closed system. In these herds, the entry of *C. burnetii* occurs most likely by the introduction of new infected animals. On the other hand, in dairy cattle the average lifetime is high, which might explain these results if an exposure occurred for a long time.

A significant association was found between antibody positivity and reproductive disorders such as infertility ($p<0.05$), metritis ($p<0.05$) and placentary retention ($p<0.05$). Despite the frequent asymptomatic pattern of infection in cattle, these reproductive disorders have been reported (To *et al*., 1998; Arricau-Bouvery and Rodolakis, 2005; López-Gatius *et al*., 2012).

In conclusion, this study confirms the presence of specific anti-*C. burnetii* antibodies in cattle in Portugal. To clarify the infection status in these herds, namely the presence of an active infection, the shedding of bacteria must be assessed.

Acknowledgements

The authors would like to thank the collaboration of farmers.

Fianancial support

S. Anastácio is supported by the grant SFRH/BD/77823/2011 from Fundação para a Ciência e a Tecnologia, Lisbon, Portugal. This work was supported financially by the Center for Neuroscience and Cell Biology (CNC), University of Coimbra.

Conflict of interest statement

The authors declare no conflict of interests.

3.3

Prevalence of *Coxiella burnetii* antibodies in Portuguese dairy cattle herds

L. Pimenta, N. Alegria, S. Anastácio, K. Sidi-Boumedine, G. da Silva, A. Martins, J. Simões. Prevalence of *Coxiella burnetii* antibodies in Portuguese dairy cattle herds. *Tropical Animal Health and Production*, 2015; 47: 227-230.

Abstract

Q fever is an important zoonotic disease which has been recently diagnosed, mainly in sheep and goats, in Portugal. The aim of the present study was to determine the prevalence of bovine Coxiella burnetii antibodies in dairy farms from the northwest of Portugal. Bulk tank milk samples were randomly obtained, on November 2013, from 90 dairy farms and assayed using an ELISA kit. The apparent prevalence was 61.1 % (95 % C.I. from 50.8 to 70.5 %). The proportion of negative and intermediate (inconclusive) herds was 34.5 % (25.5 to 44.7 %) and 4.4 % (1.7 to 10.9 %), respectively. In conclusion, a high level of exposure to *Coxiella burnetii* was observed in Portuguese dairy cattle herds, highlighting the needs to better understand the epidemiology of Q fever in Portugal by the implementation of a monitoring program based on harmonized serologic and molecular methodologies and elucidation of the infection status of the herds.

Keywords: Cows, ELISA, Milk, Q fever, Survey

Introduction

The Q fever is a disease caused by Coxiella burnetii, an intracellular bacterium affecting a large range of domestic and wildlife animals. Livestock is the main vertebrate reservoir associated to human disease (Rousset *et al*., 2010).

The human Q fever outbreak in the Netherlands (2007–2011) raised the awareness of the European health authorities about the lack of scientific knowledge, concerning this worldwide spread zoonotic infection, in European countries (EFSA, 2010[a]).

The *C. burnetii* genotypes identified in the Dutch human outbreak were mostly associated with those recovered from sheep and goats (Klaassen *et al*., 2009; Roest *et al*., 2011[b]; Tilburg *et al*., 2012[a,c]). Nevertheless, cattle have also been described as an important reservoir (Rousset *et al*., 2010). Moreover, high prevalence of *C. burnetii* antibodies in bulk tank milk samples from bovine dairy herds were reported in several

countries such as Denmark (59.0 %) (Agger *et al*., 2010), Iran (45.4 %) (Khalili *et al*., 2011), and USA (>94 %) (Kim *et al*., 2005). More recently, Pearson *et al*. (2014) observed a high prevalence of *C. burnetii* associated with a lack in genotypic diversity (mostly two genotypes were identified) and a segregation of the genotypes between cows and goats, suggesting species-specific adaptations or interspecies dissemination barriers.

In Portugal, a recent serosurvey carried out in sheep and goats showed a herd seroprevalence of 32.6 % (Anastácio *et al*., 2013[a]). Furthermore, the shedding of *C. burnetii* in sheep and goat herds was confirmed using qPCR assays (Anastácio *et al*., 2013[b]). Concerning dairy cattle herds, the seroprevalence remains unreported in scientific literature and, unlike brucellosis, there is no national surveillance for Q fever in cattle or small ruminants. The availability of Q fever vacines in Portugal is very recent and under veterinary control. The aim of the present study was to assess the prevalence of Q fever in bovine dairy herds, and thus their exposure to *C. burnetii*, in the county of Barcelos, an important region of dairy industry in North Portugal.

Material and methods

Herd and milk sampling

A cross-sectional study was designed at herd level, in Barcelos, a county located in the north of Portugal (41°53N latitude and 08°61W longitude).

The sample size calculation considered the total number of herds (n=887) and adult dairy (n=23487) or beef (n=7641) cows registered in the Barcelos Agricultural Cooperative Society (http://www.agribar.pt/), on November 2013, according to the National Bovine Brucellosis Surveillance Plan of the Portuguese General Directorate for Food and Veterinary (http://www.dgv.min-agricultura.pt/).

The criteria for inclusion were the following: (1) only dairy herds were selected, (2) the existence of a bulk tank milk (BTM) sample delivered daily to dairy industry; and (3) the absence of vaccinated animals in each herd. The formula $n=Z^2pq/l^2$ (Thrusfield, 1995) was applied to calculate the sample size. A sample size n=90 was obtained considering an expected prevalence of 65 % (Agger *et al*., 2010), the desired precision of 0.10 at the 95 % confidence level, and Z=1.96 (95 % confidence interval for standardized normal distribution).

The random assignment for the determination of the 90 herds was performed in Microsoft Excel® 2013 using the "=RAND ()" function.

During the month of November 2013, BTM samples of each randomly selected herd were collected into a sterile 10-ml plastic tube and taken to the laboratory under refrigerated conditions. In each herd, the number of lactating cows was registered at the time of sampling. At the laboratory, the samples were centrifuged, and the non-fat fraction was frozen (−20 °C) until analysis.

Antibody ELISA analysis

All samples were tested in duplicate for the presence of specific antibodies to *C. burnetii* by using the commercial CHEKIT® Q-Fever Antibody ELISA Test (IDEXX, Liebefeld-Bern, Switzerland). This test is based on inactivated *C. burnetii* phase 1 and phase 2 antigens obtained from the reference Nine Mile strain (isolated from ticks).

The optical density (OD) of each sample was corrected by subtraction of the OD of the negative control included in the test kit. The results were estimated as the ratio of OD of the sample (S) *versus* OD of the positive control (P), also included in the test kit, and were expressed as S/P values.

A sample was considered positive for a S/P≥40 %, negative for a S/P≤30 %, and intermediate (suspect) for a 30%≤S/ P<40 %, according to the manufacturer's instructions.

Statistical analysis

Descriptive statistics of antibody S/P values and apparent prevalence of *C. burnetii* and their corresponding 95 % confidence intervals were estimated using the software JMP® version 7 (SAS Institute Inc. 2007). A Spearman's correlation between the number of lactating females in the herd and the S/P values of each herd was tested.

Results and discussion

The number of lactating cows per herd contributing to BTM ranged from 25 to 580, and the total number of lactating cows represented 30.9% (9610/31,128) of the total number of adult cows in the county.

The apparent prevalence of antibodies was 61.1 %. The proportion of negative and intermediate (inconclusive) herds was 34.5 and 4.4 %, respectively. The estimated 95 % confidence intervals are reported in Table 1.

Table 11: Descriptive statistic for S/P values of anti-*Coxiella burnetii* antibodies in positive, intermediate and negative farms.

Farm	Positive	Intermediate	Negative
Number	55	4	31
Apparent prevalence	61.11 %	4.44 %	34.45%
95 % confidence interval	50.78 to 70.53 %	1.74 to 10.88 %	25.45 to 44.72 %
Mean of S/P values	95.25	34.46	11.97
Range of S/P values	40.31 - 147.49	32.23 - 39.13	0.80 - 29.65

The S/P values ranged from 0.8 to 147.5 (Figure 13).

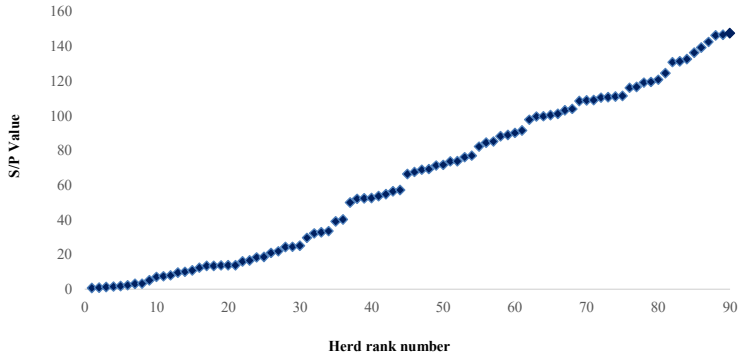


Figure 13. Array of antibody S/P values to *Coxiella burnetii* in bulk tank milk samples from 90 Portuguese dairy farms in November 2013.

The infection caused by *C. burnetii* has been reported in Portugal in recent years, especially in the south and center of the country. Few studies demonstrated the presence of the bacteria in clinical samples from zoo animals and ruminants (Clemente *et al*., 2008, 2009), and surveys were carried out on serum or BTM samples from ruminants in central Portugal using the LSIVet Ruminant Milk/Serum Q Fever® (LSI; Lissieu, France) (Anastácio *et al*., 2012, 2013[a]). However, some questions are still unclear, particularly whether the prevalence of infection differs among regions and in that case which factors may be involved. Milk production is an important economic activity in the coastal region of the northern and central Portugal, and in dairy herds,

BTM is a suitable sample to screen for *C. burnetii* exposure at the herd level. The present study was designed as an approach to estimate the apparent seroprevalence of *C. burnetii* in the dairy cattle from the northwest of Portugal.

Our study shows a relatively high apparent prevalence (61.1 %) of antibodies in cattle BTM samples. These results are similar to those obtained in other European countries such as Belgium (57.8 %) (Czaplicki *et al*., 2012), Denmark (59 %) (Agger *et al*., 2010), and Spain (66.9%) (Astobiza *et al*., 2012). Notwithstanding, they are slightly higher than those obtained in a survey performed at the center of Portugal (50 %) (Anastácio *et al*., 2012). However, different methodologies and study designs were used. Indeed, in the latter study, a convenience sampling was performed with the selection of herds with reports of reproductive disorders, and the lower seropositivity was somewhat unexpected. Furthermore, the antibody testing was performed using an ELISA based on antigens obtained from a European C. burnetii bovine strain. This ELISA is considered more sensitive than the ELISA using antigens prepared from the reference strain Nine Mile, isolated from ticks (EFSA, 2010[b]). In a very recent study, Paul *et al*. (2013) estimated a sensitivity of 86%and a specificity of 99% using milk antibody CHEKIT Q-Fever Antibody ELISA Test Kit (IDEXX®, Switzerland) in a Bayesian framework.

The correlation between the number of tested animals and antibody S/P values was rho=0.20 (P=0.055). The correlation between the number of tested animals and antibody S/P values in each herd did not reach, in limit, the statistical significance, suggesting the absence or a tendency for a low herd size effect.

However, an effect of the increase of the herd size on BTM antibody positivity to *C. burnetii* has been reported in some studies (Ryan *et al*., 2011; Agger *et al*., 2013). Thus, the infection status and specific risk factors on each herd, including the number of infected animals probably affect the rate of seropositivity (Agger *et al*., 2013; Paul *et al*., 2014).

It is concluded that there is a high level of exposure to *C. burnetii* in dairy cattle herds in the northwest of Portugal. Further studies are needed to investigate the status of the herds by assessing the shedding prevalence infection and to characterize the circulating genotypes in the herds. These data are crucial to characterize the epidemiology of infection and to decide the most appropriate control strategies to adopt.

These results, together with other published work in Portugal, highlight the need to implement a monitoring program for Q fever based on harmonized methodologies.

Acknowledgments: The authors thank the Barcelos Agricultural Cooperative Society (http://www.agribar.pt/) for the data availability of bovine and herd characterization for the present work.

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical standards: The manuscript does not contain clinical studies or patient data.

3.4

Q fever dairy herd status determination based on serological and molecular analysis of Bulk Tank Milk

S. Anastacio, N. Carolino, K. Sidi-Boumedine, G.J. da Silva. Q fever dairy herd status determination based on serological and molecular analysis of Bulk Tank Milk. *Transboundary and Emerging Diseases*, 2016; 63: e293–e300.

Abstract

Ruminants are recognized as the main reservoirs of *Coxiella burnetii*. EFSA highlighted the lack of knowledge about Q fever prevalence in many European countries. A cross-sectional study was carried out in randomly selected dairy herds (n=109) from central Portugal to screen for *C. burnetii* infection and to correlate it with herd factors. Bulk tank milk (BTM) samples from cattle (n=45) and small ruminant (n=64) herds were tested by ELISA and PCR. The apparent seroprevalence of Q fever was estimated in 45.9% (95%CI: 36.3-55.7) being higher in small ruminants (51.6; 95%CI: 39.6-63.4) than in cattle (37.8; 95%CI: 25.1-52.4). The shedding of *C. burnetii* in BTM was detected in 11.9% (95%CI: 7.1-19.4) of BTM and it was higher in cattle (20%; 95% CI: 10.9-33.8) than in sheep and mixed herds (6.3%; 95% CI: 2.5-15). A high bacterial load ($\geq 3x10^3$ bacteria/mL) was observed in 85% of PCR positive BTM. A significant correlation was found between the bacterial load and positive samples on ELISA ($p<0.001$). Antibody positivity was significantly associated with the increased herd size ($p<0.01$) and the occurrence of abortion ($p<0.05$) whereas the shedding of *C. burnetii* was significantly associated with the report of infertility ($p<0.05$). The results highlight that serological and molecular methods in combination are a useful tool to screen for Q fever and to clarify the herd infection status. The shedding of *C. burnetii* through milk is important, especially in dairy cattle, and thus the role of milk as a potential source of infection among dairy workers should not be neglected. To our knowledge, this is the first study reporting *C. burnetii* infection in dairy livestock in Portugal showing that Q fever is significant in dairy herds, leading to economic losses and being a risk for public health, which highlights the need of implementation of control measures.

Keywords: *Coxiella burnetii*, dairy ruminants, qPCR, ELISA, epidemiology

Introduction

Over the last years there has been an increased interest for Q fever and its causative agent *Coxiella burnetii* (Khalili *et al*., 2011; Muskens *et al*., 2011; Ryan *et al*., 2011; Astobiza *et al*., 2012; Czaplicki *et al*., 2012). The large-scale epidemic of human Q fever that occurred in The Netherlands between 2007 and 2011 (van Loenhout *et al*., 2012) has contributed to an increased awareness and case detection in diverse European countries (ECDC, 2011). Also, the scientific opinion issued by the European Food and Safety Agency (EFSA) encouraged the investigation in many countries since the control of the infection in animals is considered as the first step to prevent human exposure (EFSA, 2010[a]).

Some previous studies reported an association between human Q fever cases and infected ruminant herds (Porten *et al*., 2006; Gilsdorf *et al*., 2008). For this reason, domestic ruminants are generally considered the primary animal reservoir of *C. burnetii* (EFSA, 2010[a,b]).

Infected ruminants can be asymptomatic, but Q fever can cause abortion, stillbirth, delivery of weak offspring and premature delivery. These clinical manifestations are more often expressed in sheep and goats. In cattle clinical signs of Q fever can be less obvious (Rodolakis *et al*., 2007; EFSA, 2010[b]). The shedding of *C. burnetii* in infected ruminants occurs mainly during and after parturition or abortion, in birth products and vaginal mucus, but the shedding in urine, feces and milk are also documented (Guatteo *et al*., 2007[b]; Rousset *et al*., 2009; EFSA, 2010[b]). The impacts on public and animal health make Q fever a disease of interest for public policy makers and food industries, mainly dairy production (Guatteo *et al*., 2011).

In dairy herds, bulk tank milk (BTM) has been shown to be a suitable sample to screen for *C. burnetii* infection on lactating animals (Kim *et al*., 2005; Agger *et al*., 2010; Muskens *et al*., 2011; van den Brom *et al*., 2012). The milk antibody analysis by ELISA testing can provide information about the exposure to *C. burnetii* (Guatteo *et al*., 2007[a]) and the results are comparable to those obtained in serum samples because immunoglobulins are transferred from blood to milk in lactating females (Nielsen *et al*., 2011). However, to control the spread of bacteria among animals and from animals to environment and humans, detection of *C. burnetii* is a crucial step (Guatteo *et al*., 2007[a]). Nowadays, PCR is recognized as the most suitable tool for *C. burnetii* detection (Berri *et al*., 2000) and quantitative PCR (qPCR) allows quantification of the bacterial load (Guatteo *et al*., 2007[a], Czaplicki *et al*., 2012).

The aims of the present study were: i) to investigate the exposure to *C. burnetii* in dairy ruminant farms by testing the presence of specific anti-*C. burnetii* antibodies ii) to evaluate the infection status of dairy ruminant farms by assessing the prevalence of shedding in BTM samples iii) to evaluate the degree of bacterial excretion in shedding herds and iv) to identify associations between *C. burnetii* infection with herd factors and reproductive disorders.

Materials and methods

From February 2009 to July 2013, a cross-sectional survey was developed among dairy ruminant herds (n= 480502, Official Regional Veterinary Services) from the center region of Portugal. The number of herds to be studied was calculated using the program WinEpiscope version 2.0 and it was based on the formula $n = [t2\ P_{esp}\ (1\text{-}P_{esp})]/d2$, considering *n* the required size sample, *t* the student value for a 95% confidence level (1,96), P*esp* the expected prevalence and *d* the desired absolute precision (Thrusfield, 1995). For that purpose, an expected herd prevalence of 32.6% (Anastácio *et al*., 2013[a]), a desired absolute precision of 10% and a 95% confidence interval were considered, resulting in an estimated sample of 85 herds. The list of herds in databases of 8 collaborating livestock veterinarians from different locations of the region was used for a simple random sampling, using the program Microsoft Excel®. The registration of the official herd code ensured the non-duplication of herds. To each collaborator it was asked the collection of 50 mL of bulk tank milk (BTM) into sterile plastic tubes per herd, after the agreement of the farmer in a maximum of 15 herds. Also, it was asked to complete a short questionnaire containing some herd level variables (farm demographics, management practices and observed reproductive disorders during the 12 previous months) (Czaplicki *et al*., 2012).

A total of 109 samples were collected by the 8 collaborating veterinarians. These were tested for the presence of specific anti-*C. burnetii* antibodies using the commercial ELISA (LSIVET Ruminant Milk/Serum Q Fever, LSI) with a cut-off Sample/Positive percentage (S/P per cent) of 30% as recommended by the supplier. This test is based on antigens obtained from a European ovine strain of *C. burnetii* and gives a positive result on BTM samples when at least 10% of lactating cows in the herd are specific antibodies positive. The S/P per cent was categorized in 4 semi-quantitative classes: negative

(S/P ≤ 30), weak positive (+; 30<S/P≤100), positive (++; 100<S/P≤200) and strong positive (+++; S/P>200) and in 2 qualitative classes: negative (S/P≤30) and positive (S/P>30).

DNA was extracted from 200 µL of each BTM sample using the QIAmp DNA Mini Kit (Qiagen®, Izasa Portugal), following manufacturer's instructions. Firstly, a conventional PCR assay targeting IS1111, a transposon-like repetitive region of *C. burnetii*, was performed as described by Berri *et al*. (2000) with some modifications. Briefly, the amplicons, of 243 bp in size, were obtained using the primers described by Vaidya *et al*. (2008). DNA of *C. burnetii* Nine Mile strain was used as a positive control. The DNA amplification reaction was performed in a Biometra Thermocycler (Biometra®, Germany). The amplification products were analyzed by 2% agarose gel electrophoresis and visualized under UV light. Additionally, the bacterial load in PCR positive BTM samples was estimated using the commercial real-time qPCR assay (Taq-Vet^{TM} *Coxiella burnetii*–Absolute Quantification Kit®, LSI), according to the manufacturer's instructions. The PCR assays were performed using a CFX-96 thermocycler (Bio-Rad®, Portugal). Results, expressed in number of bacteria per milliliter, were categorized in weak positive (+; <300 bacteria/mL), positive (++; 300 bacteria/mL–3x10^3 bacteria/mL) and strong positive (+++; ≥3x10^3 bacteria/mL).

Confidence limits for the proportions were estimated with 95% confidence intervals (CI) assuming a binominal exact distribution (EpiInfo version 3.5.4). The agreement between ELISA and PCR results was investigated by the determination of the Kappa Coefficient using the WinEpiscope version 2.0. A univariable analysis was performed initially to explore associations between herd factors and reproductive disorders with the herd infection using the simple logistic regression test. Subsequently, a multivariable analysis included all the variables showing a p-value below 0.05. Moreover, associations between ELISA and PCR results were investigated by the Fisher exact test and by the determination of the Spearman Correlation coefficient. The analyses were performed using the statistical program SAS® (version 9.1.2).

Results

Descriptive characterization of herds

From the 109 dairy herds (9337 animals) included in this study, cattle herds were predominant (41.7%; 95% CI: 32.3 to 51.5%) followed by sheep herds (35.2%; 95% CI:

26.2 to 45.0%), goat herds (12%; 95%CI: 6.6 to 19.7%) and mixed herds (11.1%; 95% CI: 5.9 to 18.6%). An intensive production system was reported in 38% (95% CI: 28.8 to 47.8%) of the herds, most of them cattle herds (97.6%; 95% CI: 87.1 to 99.9%).

The herd mean size was 85.6 animals (SD=101.1, range 5-602) and 69.4% (95% CI: 59.8 to 77.9%) of the herds were under this mean size. Cohabitation with other animal species was reported in 49.5% (95% CI: 39.7 to 59.4%) of the herds and in 37% (95% CI: 27.9 to 46.9%) of herds it was reported the occurrence of tick's infestation at the time of sampling. Reproductive disorders were reported in 79.6% (95% CI: 70.8 to 86.8%) of the herds. These were significantly associated with cattle herds ($p<0.05$) and with an intensive production system ($p<0.05$).

BTM antibody testing

Fifty BTM samples (45.9%; 95% CI: 36.3 to 55.7%) were positive on ELISA testing; 20 from sheep (18.4%; 95% CI: 12.2 to 26.7%), 17 from cattle (15.6%; 95% CI: 10 to 23.6%), 7 (6.4%; 95% CI: 3.1 to 12.7%) from mixed herds and 6 (5.5%; 95% CI: 2.5 to 11.5%) from goat herds. In the whole positive results, 34 (31.2%; 95% CI: 23.5 to 41.7%) were weak positive (+), whereas 14 (12.8%; 95% CI: 7.2 to 20.6%) were positive (++) and 2 (1.8%; 95% CI: 0.2 to 6.5%) were classified as strong positive (+++).

BTM PCR testing

Thirteen BTM samples were PCR positive (11.9%; 95% CI: 7.1 to 19.4%). Cattle herds showed a high proportion of positive results (8.3%; 95% CI: 4.4 to 15%) followed by mixed and sheep herds with 2 (1.8%; 95% CI: 0.5 to 6.4%) positive BTM samples in each group. No PCR positive results were obtained in goat herds. Table 12 summarizes the results obtained by ELISA and PCR testing considering the species in herds.

In PCR positive BTM samples the bacterial load was estimated by qPCR and ranged from 195 bacteria/mL to 288001 bacteria/mL (mean = 37512.77 and SD=77865.23). Most of the BTM samples (11/13, 84.6%; 95% CI: 53.7 to 97.3%) evidenced a strong positive (+++) result corresponding to $\geq 3x10^3$ bacteria/mL.

Table 12: Distribution of positive and negative results on ELISA and PCR testing considering the species in herds.

Species	Number of herds	ELISA positive (%; 95% CI)	PCR positive (%; 95% CI)
Mixed	12	7 (58.3%; 32 to 80.7)	2 (16.7%; 4.7 to 44.8)
Goat	13	6 (46.2%; 23.2 to 70.9)	0
Sheep	39	20 (51.3%; 36.2 to 66.1)	2 (5.1%; 1.4 to 16.9)
Cattle	45	17 (37.8%; 25.1 to 52.4)	9 (20%; 10.9 to 33.8)
Total	109	50 (45.9; 36.8 to 55.2)	13 (11.9%; 7.1 to 19.4).

PCR versus ELISA results

Table 13 shows the results obtained by ELISA and qPCR results considering categories. A high proportion (38/96) of BTM PCR negative samples were antibodies positive by ELISA testing (39.6%; 95% CI: 29.9 to 50.1%), whereas only 7.7% (1/13; 95% CI: 0.4 to 37.9%) of BTM PCR positive samples were negative on BTM ELISA. Although this discrepancy, it was observed that 92.3% (12/13; 95% CI: 62.1 to 99.6%) of PCR positive results were associated with antibody positive results. Globally, the agreement between ELISA and PCR results was weak (Kappa=0.24, 95% CI: 10.7-36.6) but the Fisher exact test showed a significant association between PCR and ELISA categorical results ($p<0.001$). Moreover, a statistically significant correlation was observed between estimated bacterial load from qPCR and the S/P per cent by ELISA testing (Spearman's correlation coefficient =0.34, $p<0.001$).

Table 13: Relationship between ELISA and qPCR results on 109 BTM samples from dairy ruminant herds

ELISA	qPCR				
	Negative	Positive +	Positive ++	Positive +++	Total
	No (%; 95%CI)	No (%; 95%CI)	No (%; 95%CI)	No (%; 95%CI)	No (%; 95%CI)
Negative	58 (53.2; 43.5-62.8)	1 (0.9; 0.05-5.8)	0	0	59 (54.3; 63.6)
Positive +	27 (24.8; 17.2-34.2)	0	1 (0.9; 0.05-5.8)	6 (5.5; 2.3-12.1)	34 (31.2; 22.9-40.9)
Positive ++	9 (8.3; 4.1-15.5)	0	0	5 (4.6; 1.7-10.9)	14 (12.8; 7.5-20.9)
Positive +++	2 (1.8; 0.3-7.1)	0	0	0	2 (1.8; 0.3-7.1)
Total (%; 95 CI)	96 (88.1; 80.1-93.2)	1 (0.9; 0.05-5.8)	1 (0.9; 0.05-5.8)	11 (10.1; 5.4-17.7)	109 (100)

Effect of herd factors on C. burnetii infection

Individual factors were tested to find associations with positive results. Univariable analysis by logistic regression test identified only two risk factors significantly associated with BTM antibody positive results: the increasing of the herd size ($p<0.01$) and abortion ($p<0.05$). A multivariable model was performed to test simultaneously variables found to be associated in univariable analysis. The multiple logistic regression test confirmed that the increasing of the herd size and the occurrence of abortion were both associated with antibody positive results on BTM ($p<0.05$) as represented in Figure 14.

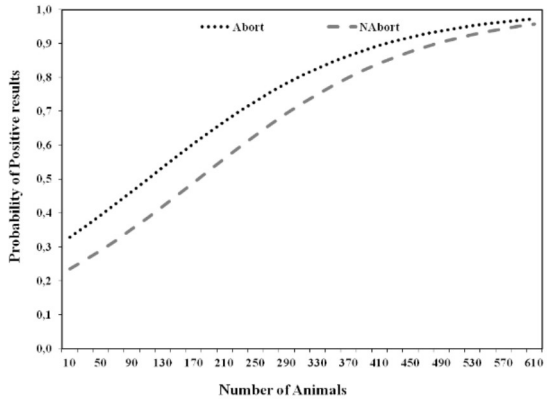


Figure 14: Association between the probability of positive results for C. burnetii antibodies with herd size and the occurrence of abortion or no abortion using a logistic regression model. [Abort (abortion): Intercept ±SE = -0.7889±0.3707, NAbort (no abortion): Intercept ±SE = -1.2528±0.4161, Logistic regression coeficient±SE = 0.00713±0.00274].

The detection of *C. burnetii* by PCR was higher in herds reporting reproductive disorders (14%) than in those that did not report reproductive disorders (0%; $p>0.05$) but only infertility evidenced a significant association with PCR positive results ($p<0.05$).

Discussion

C. burnetii is the etiologic agent of Q fever, an infection that affects both animals and humans (EFSA, 2010[a]). The prevalence of *C. burnetii* infection in ruminant herds in most of Portuguese regions is poorly known and the vaccination is very recent in Portugal, and not mandatory. The knowledge of the status of infection in herds contributes to a better understanding of the epidemiology of this pathogen. In this context, this study was designed as an approach to describe simultaneously at the herd level the exposure to *C. burnetii* and the milk shedding of this bacterium in dairy ruminant herds in central Portugal, which is unknown.

The results of antibody detection in BTM showed that almost half of the 109 dairy herds tested have been exposed to *C. burnetii* (global antibody apparent prevalence of 45.9%). At the time of the study, 11.9% of the herds showed an active infection illustrated by the detection of *C. burnetii* in BTM. These results confirm that this pathogen is endemic throughout the region. The antibody positivity was higher in sheep and goat herds (51.6%) than in cattle herds (37.8%) but the detection of *C. burnetii* in BTM was higher in cattle herds (20%) than in sheep and goat herds (6.3%). The ELISA results contrast with a recent report on *C. burnetii* infection in domestic ruminants that suggested a higher seroprevalence in cattle compared to goats and sheep (Guatteo *et al*., 2011). However, they are in accordance with the observations compiled in other countries evidencing that Q fever human outbreaks are more often related to small ruminants than to cattle (EFSA, 2010[a]). The results showing a higher detection of *C. burnetii* in cattle BTM than in small ruminants are identical to those obtained in the Netherlands, despite their higher global prevalence. In fact, in the Netherlands a higher prevalence of bacterial DNA was found in cattle BTM (56.6%) (Muskens *et al*., 2011), comparing to goat BTM (24.4%) (Schimmer *et al*., 2011).

Regarding dairy cattle herds, the antibody apparent prevalence in this study was very similar to that obtained in Ireland (37.9%) (Ryan *et al*., 2011), but it was lower than the obtained in other countries which ranged from 45.4 to 78.6% (Agger *et al*., 2010; Khalili *et al*., 2011; Muskens *et al*., 2011; Astobiza *et al*., 2012; Czaplicki *et al*., 2012). The antibody testing of BTM was performed using the same commercial ELISA test among the mentioned studies, with the exception of Agger *et al*. (2010) and Khalili *et al*. (2011). These results show that animals are exposed to *C. burnetii* in a significant percentage of dairy herds from several European countries, including Portugal.

Moreover, in our study 20% of dairy cattle herds shed *C. burnetii* through milk which is slightly lower than the results obtained in other countries, ranging from 29.6 to 94.3% (Kim *et al*., 2005; Fretz *et al*., 2007; Muskens *et al*., 2011; Astobiza *et al*., 2012; Czaplicki *et al*., 2012). All these results show that the shedding of *C. burnetii* through milk is widespread in dairy cattle herds in different countries. This might be explained by the fact that milk is the most frequent shedding route of *C. burnetii* in cows (Guatteo *et al*., 2012) and a longtime excretion through milk can extend for several months even in asymptomatic animals (Kim *et al*., 2005; Guatteo *et al*., 2007[b], 2011).

In sheep and goat dairy herds, the antibody apparent prevalence was estimated in 51.6%. Other studies assessing herd antibodies prevalence in sheep and goat herds found rates between 28% and 67.6% (Garcia-Perez *et al*., 2009; Hilbert *et al*., 2012; van den Brom *et al*., 2012). A discrepancy was observed between these BTM antibody results (51.6%) and the serum antibody results (32.6%) obtained in a recent serologic survey conducted in sheep and goats from the same Portuguese region (Anastácio *et al*., 2013[a]). This might be somewhat unexpected because the criterion for positivity in the serosurvey was set as at least one positive animal per herd and it is known that a positive result on BTM ELISA occurs when at least 10% of lactating females are seropositive. Notwithstanding, several factors may have influenced this difference. It is known that the herd size is correlated with antibody positivity (Ryan *et al*., 2011; Anastácio *et al*., 2013[a]). In this study the mean herd size for sheep and goat herds was 56 animals (data not shown), whereas in the serologic survey the mean herd size was 6.7 animals. So, the increased size of herds in this study might explain the increased antibody positivity. Moreover, an association between antibody positivity and dairy production has been described (Ryan *et al*., 2011; van den Brom *et al*., 2013). In the previous serologic survey there was a predominance of meet herds (88.8%). In this study only dairy herds were included. Also, the proportion of sheep and goat dairy herds reporting reproductive disorders was higher (67.2%) than in the previous serosurvey (6.7%). Higher antibody positivity has been described in herds reporting reproductive disorders (Bildfell *et al*., 2000; García-Perez *et al*., 2009; Khalili *et al*., 2011; Muskens *et al*., 2011).

Despite the higher percentage of antibody positive dairy sheep and goat herds, only 6.3% of them shed DNA of *C. burnetii* through milk. Other studies assessing the shedding of *C. burnetii* in BTM from small ruminant herds obtained results ranging

from 0% to 32.9% (Fretz *et al*., 2007; García-Perez *et al*., 2009; van den Brom *et al*., 2012). No positive PCR BTM samples were obtained in goat herds and similar results were obtained in Switzerland (Fretz *et al*., 2007) and in Iran (Abbasi *et al*., 2011). This might be due to the absence of the bacterium in goat farms at the time of sampling since, as in cattle, milk is considered the main route of bacterial shedding in the goat herds although for shorter periods. However, the presence of *C. burnetii* in other matrices than milk should not be excluded (Rodolakis *et al*., 2007). Moreover, it was described a discontinuous *C. burnetii* shedding in goats (Berri *et al*., 2007) and thus, false-negative results may be obtained in single samples (Rousset *et al*., 2009). Considering ewes, the low PCR positivity rate probably occurs because the shedding of *C. burnetii* in milk seems to be less important (Rodolakis *et al*., 2007) and thus the bacterium could be present in other matrices. In small ruminants *C. burnetii* is mainly shed after parturition or abortion in birth products (van den Brom *et al*., 2012). This discontinuous shedding might have contributed to the low shedding prevalence obtained in our study.

The estimation of the bacterial load allows the knowledge of the shedding pattern of infected animals. The qPCR targeted the repetitive sequence region (IS*1111*) present in different copy numbers in the genome of different *C. burnetii* strains (Klee *et al*., 2006). However, the low genetic diversity of *C. burnetii* strains found in Portuguese ruminants (Santos *et al*., 2012) was considered for quantification purposes. So, the estimated values presented herein were obtained assuming that the copy number of IS*1111* was the same in *C. burnetii* strains in all the herds. This methodology was also used in other studies (Guatteo *et al*., 2007[a]; Astobiza *et al*., 2012).

In 34.8% BTM specific antibodies were present but *C. burnetii* was not detected, suggesting a past infection by the absence of shedders among lactating females. A BTM sample from a cattle herd (0.9%) was antibodies negative but PCR positive with the estimated bacterial load being low (195 bacteria/mL). This finding suggests the presence of a small number of *C. burnetii* milk shedding cows with low antibody response (Guatteo *et al*., 2007[b]), or the presence of animals in an initial phase of infection which antibodies anti-*C. burnetii* were not yet detectable on ELISA.

Globally, a discrepancy between ELISA and PCR results was observed in 37.6% of BTM samples and similar findings were described in other studies (Schimmer *et al*., 2011; Czaplicki *et al*., 2012). Notwithstanding, most of PCR positive results were associated with antibody positive results. Actually, 84.6% of the PCR positive herds

were strong positive on qPCR and all of them were antibodies positive (mean S/P per cent = 97, SD = 29.4). Furthermore, it was found a positive and statistically significant correlation ($p<0.001$) between the estimated bacterial load on BTM qPCR and S/P per cent on BTM ELISA which is in accordance with the results obtained by Astobiza *et al*. (2012). Overall these findings support the suggestion that persistent shedder animals shed a high titer of bacteria and present a higher titer of antibodies (Guatteo *et al*., 2007[a]) and that in presence of negative antibody test, the probability to find a positive PCR test on BTM is considered low to negligible (Saegerman *et al*., 2013).

Herd size was significantly associated with antibody positive results, which is in agreement with previous serosurvey studies (Ryan *et al*., 2011; Schimmer *et al*., 2011; Agger *et al*., 2013). The increased risk of introduction and/or transmission of pathogens in a large population is probably related with the increased number of lambing females at lambing season (de Cremoux *et al*., 2012) and by other management factors like larger amounts of feed, animal supply and a higher number of professionals working at or visiting the farm (Schimmer *et al*., 2011). Therefore, larger herds are more prone to acquire and develop Q fever and the number of animals must be considered a risk factor to *C. burnetii* dissemination.

An association was found also between abortion and antibody positive results in BTM samples. However, a careful interpretation should be made because the cause of abortion in the herds was not clarified and many other infections can cause abortion in dairy ruminants (Agerholm, 2013) even if the bacterium is in the herd (Saegerman *et al*., 2013). So, it is uncertain whether *C. burnetii* is the sole cause of abortions or acts as a contributory factor (Cetinkaya *et al*., 2000). In fact, controversial data have been reported. An association has been described in some studies (Cetinkaya *et al*., 2000; García-Perez *et al*., 2009) while more recent ones did not find any (Anastácio *et al*., 2013[a]). Similarly, in this study an association between infertility and presence of *C. burnetii* in BTM samples was found. It is recognized that *C. burnetii* can cause infertility in cattle (Parisi *et al*., 2006) and interestingly 69% of PCR positive samples were from cattle herds.

Overall, *C. burnetii* is endemic in Central Portugal since a high percentage of herds evidenced an exposure by a positive serology. An active infection was confirmed in a lower percentage of herds by the shedding of *C. burnetii* through milk. Thus, milk should not be neglected as a source of infection to humans namely by the consumption

of unpasteurized milk or by the inhalation of aerosols in milking rooms (Loftis *et al*., 2010). To our knowledge, this is the first study in Portugal screening for *C. burnetii* in dairy ruminant herds and evidencing an active infection by the shedding of bacteria through milk, that was more significant in cattle herds. The cattle trade in Europe may contribute to the dissemination of *C. burnetii.* Further research is needed to characterize at molecular level the circulating strains and to compare them with those from other countries. Moreover, the economic impact of Q fever in ruminant herds should be investigated in more detail. This study reinforces the need of a global and harmonized control policy among European countries towards the prevention of Q fever in regard to public health and economic impact.

Acknowledgements

Authors are very grateful to farmers by their agreement and to veterinary practitioners that kindly collaborated on sample collection. Dr. Elodie Rousset is also acknowledged for the critical review of the manuscript.

Financial support

S. Anastácio is supported by the grant SFRH/BD/77823/2011 from Fundação para a Ciência e a Tecnologia, Lisbon, Portugal. This work was supported financially by the Center for Pharmaceutical Studies, University of Coimbra.

3.5

Coxiella burnetii is present in milk from dairy cattle herds in the Northwest Portugal

S. Anastacio, L. Pimenta, J. Simões, N. Alegria, A. Rabiço, K. Sidi-Boumedine, G.J. da Silva. *Coxiella burnetii* is present in milk from dairy cattle herds in the Northwest Portugal. *Experimental Pathology and Health Sciences* 2016, 8 (1), 13-14.

Abstract

Ruminants are recognized as the main reservoirs of *Coxiella burnetii*. EFSA highlighted the lack of knowledge about Q fever prevalence in many European countries. A cross-sectional study was carried out in randomly selected dairy herds from the Northwest of Portugal to screen for *C. burnetii* infection by PCR testing in antibody positive Bulk Tank Milk samples (BTM). The proportion of *C. burnetii* positive BTM samples was 30.9% of herds and the bacterial load ranged from 600 to 8513800 bacteria/mL. The results highlight that the shedding of *C. burnetii* through milk is important, especially in dairy cattle, and thus the role of milk as a potential source of infection among dairy workers should not be neglected.

Introduction

Q fever is a zoonotic disease whose etiological agent is *Coxiella burnetii* (Maurin and Raoult, 1999). In recent years, an increased incidence of human infection in Europe has been reported (Georgiev *et al*., 2013), mainly due to the 4173 cases associated to the Q fever outbreak that occurred in the Netherlands between 2007 and 2012 (Dijkstra *et al*., 2012). An association between human Q fever cases and infected ruminant herds has been referred (van den Brom *et al*., 2015). For this reason, domestic ruminants are generally considered the primary animal reservoir of *C. burnetii*. The shedding of *C. burnetii* in infected ruminants occurs mainly during and after parturition or abortion, in birth products and vaginal mucus, but the shedding in urine, feces and milk are also documented in countries other than Portugal (Rousset *et al*., 2009; EFSA, 2010[b]). The impact on public and animal health triggers Q fever a disease of interest for public policy makers and food industries, mainly dairy production (Guatteo *et al*., 2011). This study aimed i) to assess the *C. burnetii* shedding in antibody positive BTM samples and ii) to determine the bacterial load in *C. burnetii* PCR positive samples.

Materials and Methods

The present study was performed using BTM positive samples for anti-*C. burnetii* antibodies. These samples were previously detected in a survey conducted at the county of Barcelos, in the Northwest of Portugal. In this previous study, BTM samples (n=90) were tested for the presence of specific anti-*C. burnetii* antibodies by using a commercial ELISA based on inactivated *C. burnetii* phase I and phase II antigens, obtained from the reference Nine Mile strain, isolated from ticks (Pimenta *et al*., 2015). Considering that a positive and statistically significant correlation was established between the bacterial load on BTM qPCR and antibody positive results on BTM, and that in presence of negative antibody test, the probability to find a positive PCR test on BTM is considered low or negligible (Saegerman *et al*., 2015; Anastácio *et al*., 2016), the assessment of *C. burnetii* shedding was performed only in antibody positive BTM. DNA was extracted from 200 µL of each BTM sample using the QIAmp DNA Mini Kit (Qiagen®, Izasa Portugal), following manufacturer's instructions. The DNA samples were first screened by conventional PCR as previously described (Anastácio *et al*., 2016). PCR positive samples were additionally tested to estimate the bacterial load by a real-time qPCR assay (Taq-VetTM *C. burnetii*–Absolute Quantification kit; Lifetechonologies®, USA), according to manufacturer's instructions. The real-time PCR assays were performed on a CFX-96 thermocycler (Bio-Rad®, Amadora, Portugal). Results were expressed in number of bacteria per milliliter. Confidence limits for the proportions were estimated with 95% confidence intervals (CI) assuming a binominal exact distribution (EpiInfo version 3.5.4).

Results

Of the total of 55 BTM samples tested by PCR, 30.9% (17/55; 95% CI: 19.5-45.0%) were *C. burnetii* positive. Considering the total of herds, the proportion of PCR positive herds was estimated in at least 18.9% (17/90; 95% CI: 11.7-28.8%). In PCR positive BTM samples the bacterial load ranged from 600 to 8513800 bacteria/mL (mean = 2119694 and SD = 2944856). It was found that in 88.2% (15/17) of PCR positive samples the concentration of bacteria was higher than 10^4 and in 47.1% (8/17) the concentration was higher than 10^6.

Discussion and Conclusions

C. burnetii is the etiologic agent of Q fever, an infection that affects both animals and humans (EFSA, 2010[a,b]). The knowledge of the status of infection in herds contributes to a better understanding of the epidemiology of this pathogen. The shedding of *C. burnetii* through milk is widespread in dairy cattle herds in different countries. This might be explained by the fact that milk is the most frequent shedding route of *C. burnetii* in cows and a longtime excretion through milk can last for several months even in asymptomatic animals (Kim *et al*., 2005; Guatteo *et al*., 2011). In this context, this study was designed as an approach to investigate the milk shedding of *C. burnetii* in dairy ruminant herds in the Northwest of Portugal.

C. burnetii was detected in the BTM of 30.9% of tested herds. Overall, and considering the total of studied herds (n=90), an active infection occurred in at least 18.9% of herds which might be slightly underestimated as the antibody negative herds were not screened by PCR. However, recent findings suggest that in antibody negative BTM samples the probability of getting a positive PCR is low to negligible (Saegerman *et al*., 2015; Anastácio *et al*., 2016). Thereby the results were similar to that found in central Portugal (20%) (Anastácio *et al*., 2016) but were slightly lower than the results obtained in other countries, ranging from 29.6 to 94.3% (Kim *et al*., 2005; Fretz *et al*., 2007; Muskens *et al*., 2011; Astobiza *et al*., 2012; Czaplicki *et al*., 2012). The estimation of the bacterial load allows the knowledge of the shedding pattern of infected animals. The bacterial load of the PCR positive herds was found to be high in most of the PCR positive herds and these results are in agreement with the findings in central Portugal (Anastácio *et al*., 2016).

An active infection was confirmed in some herds where the exposure to *C. burnetii* has been showed previously by the presence of specific antibodies. Thus, milk should not be neglected as a source of infection to humans namely by the possible inhalation of aerosols in milking rooms (Loftis *et al*., 2010). Further research is needed to characterize, at the molecular level, the circulating strains and to compare them with those from other countries.

3.6

Molecular screening for *Coxiella burnetii* in seropositive ruminant herds in Portugal

S. Anastácio, N. Tavares, C. Cruz, K. Sidi-Boumedine, G.J. da Silva. Molecular screening for *Coxiella burnetii* in seropositive ruminant herds in Portugal. *Manuscript in submission.*

Abstract

Domestic ruminants are recognized as major sources of infection for *Coxiella burnetii,* the Q fever agent. In Portugal, recent surveys showed an individual seroprevalence of up to 15 % in ruminants. This study aimed to evaluate the shedding of *C. burnetii* in milk and vaginal mucus in ruminant females from seropositive non-vaccinated herds and to estimate their bacterial load. A cross-sectional study was conducted between February and July 2012. In 24 herds, 142 vaginal swabs and 92 milk samples were collected from females older than 6 months (n=142). The screening was performed by PCR, amplifying the IS1111. Bacterial load was estimated by qPCR in positive samples. *C. burnetii* shedding was confirmed in 20.8% of the herds and in 9.2% of females. The proportion of shedding was higher in cattle herds (66.6%) than in mixed (25.0%) and goat herds (22.2%). It was not found in sheep herds. Individually, excretion was higher in goats (15.0%), than in cows (10.9%) and ewes (3.6%). The quantification results categorized 7.7% of samples as strong positive ($\geq 3 \times 10^4$ bacteria/ml); the bacterial load ranged from 460 to 600000 bacteria/ml. A significant association was found between seropositive individuals and bacteria shedding ($p<0.05$); a lower risk of excretion ($p<0.005$) was observed in animals older than 36 months. To our knowledge, this is the first study confirming the shedding of *C. burnetii* in apparently healthy ruminants in Portugal. Overall, the study highlights that the identification of shedders is central in the assessment for the risk of human infection and in Q fever control or surveillance schemes.

Keywords: Q fever, domestic ruminants, serology, PCR, public health

Introduction

The intracellular bacterium *Coxiella burnetii* is the aetiological agent of Q fever, a zoonotic disease. It has a worldwide distribution, infecting mammals, birds and arthropods (Angelakis and Raoult, 2011).

The impact of Q fever on human health has been reported in many countries for several decades (Selvaggi *et al*., 1996; Lyytikäinen *et al*., 1998; Medic *et al*., 2005; Porten *et al*., 2006; Grilc *et al*., 2007; Gilsdorf *et al*., 2008; Panaiatov *et al*., 2009; Amitai *et al*., 2010; Wallesten *et al*., 2010; Medic *et al*., 2012; Bellini *et al*., 2014). But it was again perceived as a major public health problem when The Netherlands experienced the largest human Q fever epidemic ever described between 2007 and 2011 (van Loenhout *et al*., 2012; Georgiev *et al*. 2013).

Human Q fever has been associated to infected animals (Marrie *et al*., 1988; Stein and Raoult, 1999; Klaassen *et al*., 2009; van der Hoek *et al*., 2010) and domestic ruminants are recognized as the most important source of infection (Angelakis and Raoult, 2011). Infected ruminants are often asymptomatic. The most important clinical presentations of Q fever in animals, that are relevant to its zoonotic properties, are abortion and stillbirth that occur in a small percentage of animals within a flock, mainly in small ruminants (Rodolakis *et al*., 2007; EFSA, 2010[b]). Moreover, delivery of weak offspring and premature delivery have also been reported in small ruminants whereas metritis and infertility are mostly reported in cattle (Rodolakis, 2009). Pregnant ruminants are highly susceptible to infection and the abortions occur mainly at the first parturition after infection. The following gestations usually develop without reproductive failures (Berri *et al*., 2007). Infected animals shed bacteria in birth products and vaginal mucus during and after parturition or abortion; but the shedding through milk, urine and faeces are also reported (Guatteo *et al*., 2007[b]; Rousset *et al*., 2009). The shedding is normally very high at the first parturition after the infection but occasionally it occurs at subsequent pregnancies accompanied by a considerable number of bacteria excreted through placenta (Berri *et al*., 2007; Roest *et al*., 2012). So, normal deliveries in infected females contribute to the environmental contamination and should, therefore, be considered as a major zoonotic risk (Roest *et al*., 2012).

Transmission between animals and from animals to humans occurs mainly by the inhalation of contaminated aerosol particles generated from infected placenta, body fluids or dust after desiccation (Angelakis and Raoult, 2011; Nielsen *et al*., 2012). Given the high resistance of *C. burnetii* in the environment, windborne spread of infective particles occurs. The highest risk of infection was estimated within a radius of 5 Km from the source of infection (Tissot-Dupont *et al*., 1999; Schimmer *et al*., 2010). Although ingesting contaminated raw milk and derivatives is considered a minor source of contamination to humans (Fishbein and Raoult, 1992; Arricau-Bouvery and

Rodolakis, 2005; Angelakis and Raoult, 2010), the risks related to the consumption of unpasteurised milk and milk products (including fresh cheese) are not negligible, but they seem to be lower in comparison to transmission via inhalation of aerosols from parturient products (Gale *et al*., 2015; Signs *et al*., 2012).

The asymptomatic pattern in many cases of *C. burnetii* infection and the lack of specificity of the clinical signs of Q fever make its recognition difficult. Thus, the real distribution of animal Q fever is not well established. In some countries, epidemiological data have been obtained from investigations of defined outbreaks or from animal surveys (Angelakis and Raoult, 2011).

In Portugal, Q fever is a notifiable disease in humans and is characterized by a low incidence rate (Santos *et al*., 2007). In ruminants, our recent studies demonstrate an individual seroprevalence of up to 15 % (Anastácio *et al*., 2013[a]; Anastácio *et al*., 2013[b]). However, serological data does not allow to assess the risk of infection related to animal management practices. The objective of this study was to unravel the presence of an active infection in seropositive herds and to assess the risk of transmission of the bacteria, by evaluating the proportion of *C. burnetii* shedders in milk and vaginal mucus and estimating the bacterial load in positive samples.

Materials and Methods

Study design and sampling approach

A cross-sectional study was conducted between February and July 2012 in non-vaccinated ruminant farms located at the Centre of Portugal where a serosurvey was performed during the 4th trimester of 2011 (Anastácio *et al*., 2013[a,b]). All the flocks showing at least one seropositive animal for Q fever were revisited for individual sampling after farmer's agreement.

In participating farms, vaginal swabs (VS) and milk samples (MS) were collected from females older than 6 months. In small ruminants, sampling was conducted during the kidding season (February and March). Samples were taken from 15 females randomly selected using the list of animals in each herd and the "=RAND ()" function of the program Microsoft Excel® (Microsoft Corporation, Redmond, WA, USA). On farms sized ≤ 15 animals, samples were taken from all the females fulfilling the inclusion criterion.

All the samples were stored at – 20ºC until analysis. A short questionnaire, containing some herd level variables (farm demographics and observed reproductive disorders during the 12 previous months), was filled up during sample collection (Czaplicki *et al*., 2012) and the individual results of the previous serological testing were also recorded in the questionnaire.

PCR testing

DNA was extracted from 200 µl of milk sample or PBS suspension of vaginal swab using the QIAmp DNA Mini kit (Qiagen, Izasa, Portugal) and following the manufacturer's instructions.

All the DNA samples were first screened by conventional PCR as previously described (Anastácio *et al*., 2016). PCR positive samples were additionally tested to estimate the bacterial load by a real-time qPCR assay (Taq-VetTM *Coxiella burnetii* – Absolute Quantification kit; Lifetechonologies®, USA), according to manufacturer's instructions. The real-time PCR assays were performed on a CFX-96 thermocycler (Bio-Rad®, Amadora, Portugal). The results were expressed in number of bacteria per millilitre and categorized in weak positive (+; <300 bacteria/ml), positive (++; 300 bacteria/ml–3 x 10^4 bacteria/ml) and strong positive (+++; ≥3 x 10^4 bacteria/ml).

Statistical analysis

For statistical analysis purpose, it was considered the herd size (categorical nominal: <15 animals/≥15 animals), species in the herds (categorical nominal: sheep/goats/mixed/cattle herds) or species individually (categorical nominal: sheep/goats/cattle), productive system (categorical nominal: intensive/extensive/semi-extensive), type of production (categorical nominal: meat/milk), age (categorical nominal: 6-36 months/≥ 36 months), co-habitation with other species (categorical nominal: yes/no), individual results of the previous serological testing (categorical nominal: positive/negative) and reports of reproductive disorders (at least one of the following disorders: abortion, premature delivery, infertility, metritis and/or placental retention) within the previous year (categorical nominal: presence/absence). The response variables were the PCR result (categorical nominal: positive/negative) obtained in each sample and the categorization of the bacterial load (categorical nominal: negative/weak positive/positive/strong positive). An animal was considered positive when at least one of the tested samples was positive in PCR testing.

Herds were also categorized as positive or negative, according to the results obtained for individual testing. A herd was considered positive when at least one animal showed a positive result to PCR testing. Simple logistic regression test was performed to assess individually the main factors associated with *C. burnetii* PCR positive results at herd and individual level. After evaluating these factors with significant influence ($p<0.05$) on positive results, a multiple logistic regression analysis was conducted to assess the joint relationship between several independent factors and *C. burnetii* positivity. Statistical analyses were performed using EpiInfo (version 3.5.4; Center for Disease Control and Prevention, Atlanta, GA, USA). Confidence limits for the proportions were estimated with 95% confidence intervals (CI) assuming a binominal exact distribution (EpiInfo version 3.5.4; Center for Disease Control and Prevention, Atlanta, GA, USA).

Results

Descriptive analysis

From the total of 106 screened farms, 34 were eligible for the study and 24 farms agreed to participate. Goat herds were predominant (n=9, 37.5%) followed by sheep herds (n=8, 33.3%), mixed herds, i.e. sheep and goats (n = 4, 16.7%) and cattle herds (n=3, 12.5%). The estimated herd mean size was 33.1 animals (SD=59.4, ranging from 3 to 200) and 16 (66.7%) herds had less than 15 animals. A predominance of meat producing herds (n=20, 83.3%) in a semi-extensive grazing system (n=21, 87.5%) was observed. Table 14 summarizes the descriptive characteristics in herds and PCR test results. The shedding of *C. burnetii* was observed in five (20.8%; 95% CI: 7.1-42.2%) herds. The proportion of positive herds was higher in cattle herds (2/3; 66.6%; 95% CI: 20.8-93.9%) followed by mixed herds (1/4; 25.0%; 95% CI: 13.2-78.1%) and goat herds (2/9; 22.2%; 95% CI: 4.0-59.8%). No positive results were found in sheep herds. Reproductive disorders were reported in three (60.0%; 17.0-92.7) PCR positive herds. Moreover, all the PCR positive herds reported the cohabitation with other animal species.

Table 14: Descriptive characteristics and PCR results in domestic ruminant herds

Variable	Number of herds	PCR positive (%;[a]CI 95%)
Selected	24	5 (20.8; 7.1-42.2)
Herd size		
≤ 15	16	3 (18.8; 5.0-46.3)
> 15	8	2 (25.0; 4.5-64.4
Herd species		
Cattle	3	2 (66.7; 20.8-93.9)
Goat	9	2 (22.2; 4.0-59.8)
Sheep	8	0 (0.0; na[b])
Mixed	4	1 (25.0; 13.2-78.1)
Type of production		
Meat	20	3 (15.0; 4.0-38.9)
Milk	4	2 (50.0; 9.2-90.8)
Productive system		
Intensive	3	2 (66.7; 12.5-98.2)
Extensive	0	na[b]
Semi-extensive	21	3 (14.3; 3.8-37.4)
Cohabitation with other species		
Yes	15	5 (33.3; 13.0-61.3)
No	9	0 (0.0; na[b])
Cohabitant species		
Pets	5	2 (40.0; 7.3-83.0)
Farm animals	5	1 (20.0; 3.6-62.5)
Pets and farm animals	5	2 (40.0; 7.3-83.0)
Reproductive disorders		
Yes	5	3 (60.0; 17.0-92.7)
No	19	2 (10.5; 1.9-34.5)

[a] Confidence Interval (range within which is reasonably confident to find the real prevalence)

[b] not applicable

In the studied herds (794 animals), 142 females (ewes n=56, goats n=40, cows n=46) were sampled (142 VS and 92 MS) of which 132 (93%) have been previously submitted to serological testing. The mean age of sampled females was estimated to be 50.3 months (SD = 29.7, range 9–167). The mean age of positive females was 36 months (SD=26.4, range 11-105) and 51.8 months for negative females (SD=29.7, range 9-167). A PCR positive result was observed in 13 (9.2%; 95% CI: 5.2-15.5%) females. Considering species individually, a higher proportion of shedders was observed in goats (6/40; 15%; 95% CI: 6.3-30.5%) followed by cows (5/46; 10.9; 95% CI: 4.1-24.4) and ewes (2/56; 3.6%; 95% CI: 0.6-13.4). According to the type of sample, the proportion of positive results was higher in MS (10/92; 10.9%; 95% CI: 5.6-19.5%) than in VS (3/142; 2.1%; 95% CI: 0.5-6.5%). The milk shedding was observed in goats (6/23; 26.1; 95% CI: 11.1-48.7%) and in cows (4/46; 8.7%; 95%CI: 2.8-21.7%) whereas vaginal

shedding was observed in ewes (2/56; 3.6%; 95%CI: 0.6-13.4%) and in cows (1/46; 2.2%; 0.1-13.0).

The quantification results categorized 92.3% of the PCR positive samples as positive and 7.7% as strong positive. Overall, the bacterial load ranged from 460 bacteria/ml to 600000 bacteria/ml (mean=59.959, SD=163905).

Univariable analysis

Individual factors were tested to find associations with positive results in herds and in animals individually. Significant associations were found only at the individual level. Logistic regression test evidenced that animals older than 36 months present a lower risk of shedding *C. burnetii* (OR=0.09, 95% CI: 0.012-0.44; $p<0.005$). Also, a significant association was found between individual seropositive results and the shedding of bacteria (OR=3.4, 95% CI: 1.1-11.1; $p<0.05$).

Multivariable analysis

A multivariable model was performed to test simultaneously variables found to be associated in univariable analysis. The multiple logistic regression test confirmed that in animals older than 36 months the risk of shedding *C. burnetii* is lower ($p < 0.005$).

Discussion

Q fever outbreaks have a major public health impact when they occur, affecting not only rural but also urban populations. It has a worldwide significance (Angelakis and Raoult, 2010), being an OIE (Office International des Epizooties) notifiable disease (OIE, 2015). More than 30 different animal species are known to be susceptible to *C. burnetii*, but domestic ruminants (cattle, sheep and goats) are recognized as the main sources of infection (EFSA, 2010[a,b]) through their contaminated products (Georgiev *et al*., 2013). Q fever cases have been reported in many countries but numerous knowledge gaps in the understanding of *C. burnetii* epidemiology have been highlighted (Georgiev *et al*., 2013). Some human outbreaks were attributed to spill-over infection from goats and sheep, particularly during breeding season, nevertheless in most cases the sources of human infection remain unclear (EFSA, 2010[a,b], Georgiev *et al*., 2013).

The shedding of *C. burnetii* by ruminants is an important public health threat. The identification of shedders is crucial for monitoring the spread among animals and from animals to humans. Furthermore, the zoonotic risk also depends on the level of *C. burnetii* in the contaminated products of the infected animals (EFSA, 2010[a,b]; Cardinale *et al*., 2014).

In Portugal, a recent serosurvey for *C. burnetii* evidenced an apparent herd seroprevalence of 32.6 % and 23.5%, as well as an individual seroprevalence of 9.6% and 15% in small ruminants and cattle respectively (Anastácio *et al*., 2013[a,b]). However, in a flock with seropositive animals it is impossible to predict shedders based on immunologic results because serological tests only indicate exposure to *C. burnetii*, which may be due to a current clinical condition or to an earlier infection (Maurin and Raoult, 1999; Berri *et al*., 2002). Conventional PCR is a very specific and sensitive method for detection of *C. burnetii* DNA in biological samples (Willems *et al*., 1994; Berri *et al*., 2000). Moreover, the real-time PCR technique provides quantifiable information (Guatteo *et al*., 2005; Cardinale *et al*., 2014) which allows to scale the importance of the sources of bacterium with regards to the risk of transmission of *C. burnetii* among animals and from animals to humans (Guatteo *et al*., 2005).

In the present study, we aimed to evaluate the shedding of *C. burnetii* in seropositive ruminant females from non-vaccinated herds by assessing the excretion of *C. burnetii* in milk and vaginal mucus; and to estimate the bacterial load in positive samples. To our knowledge, this is the first screening study conducted in Portugal for the excretion of *C. burnetii* in domestic ruminants from seropositive ruminant herds, expanding the knowledge of *C. burnetii* epidemiology in this country.

The shedding of *C. burnetii* DNA was confirmed in 20.8% of the herds and in 9.2% of females. Recent PCR based studies identified *C. burnetii* in 9% of tested flocks in northern Spain (Oporto *et al*., 2006), in 15.9% of farms from the southern Italy (Parisi *et al*., 2006) and in 5% of sheep herds from Germany (Hilbert *et al*., 2012). Individual results showed that 8% of small ruminants were PCR positive in Sardinia (Masala *et al*., 2004), 11.1% of domestic ruminants in India (Vaidya et al., 2010), 18.9% of small ruminants in Italy (Parisi *et al*., 2006) and in 36% of domestic ruminants in the south of Portugal (Clemente *et al*., 2009). All these studies were performed in cases of reproductive disorders, namely abortions, except the study conducted in Germany (Hilbert *et al*., 2012), which render difficult the comparison of data due to differences in study design, sampling approaches and applied methods. In the current study, we

observed that 60% of positive herds reported the occurrence of reproductive disorders within the previous year. Despite reproductive disorders are considered the main clinical presentation of Q fever in ruminants, the aetiology of reproductive disorders in the studied herds was not determined; thus, causality was not established. Additionally, we did not find a significant association between the report of reproductive disorders and the shedding of *C. burnetii*, supporting that *C. burnetii* infection and consequently its shedding also occurs in asymptomatic herds (Rodolakis *et al*., 2007).

The randomly sampling of herds and females for serological and molecular purposes, demonstrates the role of domestic ruminants in the infection cycle of *C. burnetii* in Portugal. An underestimation of the herein presented results should not be excluded. It is generally accepted that the shedding of *C. burnetii* occurs mainly during parturition and lactation (Guatteo *et al*., 2007). The visit of the herds occurred during the kidding season of small ruminants and the lactation of cows. However, at the time of visiting small ruminant herds not all the females had given birth. This explains the smaller number of milk samples when compared with vaginal swabs and that some negative results in vaginal swabs can be due to the fact that females were not yet shedding *C. burnetii* as it is accepted that the shedding begins mainly after parturition (Roest *et al*., 2012).

The proportion of *C. burnetii* shedding was higher in cattle herds (66.6%) than in mixed herds (25.0%) and goat herds (22.2%), and it was not found in sheep herds (0.0%). Somewhat opposite results were obtained in Italy where *C. burnetii* DNA was detected in 21.5% of small ruminants and 10.8% of cattle herds (Parisi *et al*., 2006). Once again, the study design can affect the results between studies, since the Italian study was conducted in abortion cases and the abortion caused by *C. burnetii* is mainly reported in small ruminants rather than cattle (Rodolakis *et al*., 2007; EFSA, 2010[b]). At the individual level, the shedding of *C. burnetii* was higher in goats (15.0%), followed by cows (10.9%) and lastly ewes (3.6%). In India, *C. burnetii* DNA was detected in 12.5% of cattle, 11.6% of sheep and 5.6% of goats (Vaidya *et al*., 2010), whereas in Italy, the highest proportion of positive results was found in small ruminants (18.6%) followed by cattle (11.6%) (Parisi *et al*., 2006). Our results are somehow higher than the expected comparing to above mentioned studies, mainly due to the study design features that were previously pointed out. These findings suggest that probably different

epidemiologic scenarios occur among regions reinforcing the importance of harmonized monitoring schemes.

The recent Q fever outbreaks that occurred in Europe have been mainly associated with small ruminant herds (EFSA, 2010[a,b]). A higher proportion of shedding was observed in goats than in ewes whose mean age was estimated to be 49 months (data not shown), evidencing that they were not primiparous. These findings are supported by the fact that after a Q fever outbreak in a goat flock more than one-half of goats may shed bacteria through milk and vaginal mucus at subsequent pregnancies without manifestation of clinical signs (Berri *et al*., 2007; Rousset *et al*., 2009). Although the contact with sheep is described as a major zoonotic risk factor for Q fever (Marrie and Raoult, 1997), infected ewes are highly infective in the first parturition which is usually accompanied with reproductive failures. Whereas, the shedding of *C. burnetii* at later parturitions, usually carried to term with normal deliveries, may not occur (Berri *et al*., 2002; Berri *et al*., 2005). Overall, considering goats as long-term shedders and the environmental resistance of *C. burnetii* (Rousset *et al*., 2009), goats constitute an important risk of direct or indirect exposure to *C. burnetii*; explaining why living close to goat farms has been identified as an important risk factor for Q fever (Schimmer *et al*., 2010).

Considering the results obtained by sample type, a higher proportion of excretion was observed in milk samples (10.9%) compared with vaginal swabs (2.1%) but differences were observed among animal species. In milk samples a higher positivity rate was observed in goats and cows, 26.1% and 8.7% respectively, being null in ewes (0.0%); while in vaginal swabs the opposite was observed, and the positivity rate was higher in ewes (3.6%) and in cows (2.2%) but null in goats (0.0%). These differences may occur because the shedding routes differ among animal species (Rodolakis *et al*., 2007). The higher proportion of excretion in milk is in accordance with the reports that milk is the main shedding route of *C. burnetii* in dairy cattle (Beaudau *et al*., 2006; Rodolakis, 2006; Guatteo *et al*., 2007; Rodolakis *et al*., 2007; Mohammed *et al*., 2014). As well as in goats that, even in the post-partum, excrete *C. burnetii* DNA for long-periods (Berri *et al*., 2007; Rodolakis, 2009; Roest *et al*., 2012) and perhaps during successive lactating periods (Arricau-Bouvery *et al*., 2003). In our study, and considering the quantification results, one strong positive result (7.7%) was obtained, corresponding to a milk sample from one cow, which was highly-seropositive on the ELISA testing. Persistent shedder cows are usually persistently highly seropositive

probably due to a strong immune stimulation (Guatteo *et al*., 2007; Courcoul *et al*., 2010). However, to confirm that this cow is a heavy-shedder female further milk samples should have been tested.

The lower proportion of cows shedding through vaginal mucus confirms that this shedding route is not very important in cattle (Rodolakis, 2006; Rodolakis *et al*., 2007). Although it is globally accepted that goats shed *C. burnetii* via vaginal mucus and milk (de Cremoux *et al*., 2012; Rodolakis *et al*., 2007), even at successive parturitions and normal deliveries (Berri *et al*., 2007), in this study there was no evidence of vaginal shedding in goats. Despite the suggestion that the detection of *C. burnetii* in vaginal mucus after parturition possibly results from environmental contamination (Roest *et al*., 2012), it is possible that our findings result from the intermittent shedding of bacteria described in goats leading to false negative results in cross-sectional studies as previously referred (Rousset *et al*., 2009). The evidence of vaginal shedding observed in ewes in opposition to the absence of milk shedding is in agreement to the reports that the vaginal mucus is considered the primary shedding route in ewes (Rodolakis *et al*., 2007; Rodolakis, 2009) while milk is not the preferred route of *C. burnetii* shedding in sheep (Rodolakis, 2006; Mohammed *et al*., 2014). However, an intermittent shedding in milk has been reported in ewes (Rodolakis, 2006).

We also observed that all the animals shed bacteria by only one route which is in accordance with previous reports (Beaudau *et al*., 2006; Guatteo *et al*., 2006; Rodolakis, 2009).

The age seems to be an important factor to limit the environment contamination. The shedding was significantly lower in females over 36 months, suggesting that antibody persist for long periods after the bacteria have been cleared from the organism (Berri *et al*., 2002). Herds with a high number of younger females are more prone to develop Q fever and to be a source of infection for humans.

A significant association was found between individual seropositive results and the shedding of bacteria. Notwithstanding, five shedder females (two ewes and three goats) were antibody negative, a result also described in a previous study (de Cremoux *et al*., 2012). These seronegative results can be explained by the localisation of bacteria only in the udder, placenta, uterus or vagina of animals without inducing systemic antibodies (Berri *et al*., 2002) or by the fact that *C. burnetii* antigens used in ELISA kits

may not be suitable for post-infection antibody response detection (Berri *et al*., 2002; Rodolakis, 2009).

Conclusions

To the best of our knowledge, this is the first screening confirming the shedding of *C. burnetii* in vaginal swabs and milk samples of apparently healthy ruminants in Portugal. In opposition to the serological testing observations where seropositivity was significantly associated to the increased age, we noticed that the shedding of *C. burnetii* is significantly associated with younger females, which represent a higher risk for environmental contamination. Moreover, it was observed that reproductive disorders maybe predictive of *C. burnetii* infection notwithstanding other methods should be used to confirm the suspicion. Some of our results are not consistent with those obtained in other countries. This might be explained by different epidemiological scenarios among regions or by the different methodologies applied. This reinforces the need of harmonized screening methods and study designs to ease the sharing and comparison of data leading to a better understanding of the impact of Q fever in animal and public health. The molecular methods such as qPCR should be considered as a standard method, for identifying animal shedders, due to their reliability and sensitivity. Therefore, strategies combining the interests of public and veterinary health should include the identification of shedders, which is central to any control or surveillance scheme.

Acknowledgements

Authors are very grateful not only to farmers by their agreement in participating in the study but also to the animals included in this study.

Financial support

S. Anastácio is supported by the grant SFRH/BD/77823/2011 from Fundação para a Ciência e a Tecnologia, Lisbon, Portugal. This work was supported financially by the Center for Neuroscience and Cell Biology (CNC), University of Coimbra.

4

Domestic pigs

4.1

No evidence of specific anti-*Coxiella burnetii* antibodies in domestic pigs

S. Anastácio, J. Andrade, T. Silva, A. Almeida, S. Duarte, K. Sidi-Boumedine, G.J da Silva. No evidence of specific anti-*Coxiella burnetii* antibodies in domestic pigs.

Abstract

Coxiella burnetii is a zoonotic and multiple-host pathogen transmitted mainly by the inhalation of contaminated aerosols. Among hosts, feral pigs have been described as reservoirs contributing to the wildlife cycle of infection. However, the role of domestic pigs in the epidemiology of *C. burnetii* is unknown however the feral pigs have been described as reservoirs belonging to the wildlife cycle of infection. This study aimed to investigate the exposure of domestic pigs to *C. burnetii*. Blood samples (n=50) were obtained following a systematic random sampling in a slaughterhouse at the Centre of Portugal. Sera were tested in duplicate using a commercial ELISA adapted with an anti-multi-species conjugate (protein AG). The results were negative for antibody anti-*C. burnetii*. No evidence of exposure was found in pigs. To our knowledge, this is the first serosurvey of *C. burnetii* in domestic pigs in Portugal and it seems that the exposure to *C. burnetii* in pigs in intensive production system is very limited or even inexistent. Considering that the age of the animals is important in the epidemiology of *C. burnetii*; further studies including adult animals and different types of production systems (intensive and extensive) are needed for a better elucidation about the role of suine on the epidemiology of *C. burnetii*.

Keywords: Q fever, zoonosis, swine, ELISA

Coxiella burnetii, the etiological agent of Q fever presents a worldwide occurrence. It is a zoonotic and multiple-host pathogen that infects wild and domestic vertebrates and ticks. Transmission occurs mainly by inhalation of infected aerosols or dust (Maurin and Raoult, 1999). So far, domestic ruminants have been considered the main reservoirs of the pathogen (Angelakis and Raoult, 2010). Pigs have been described has reservoirs of several zoonotic pathogens as *Brucella suis*, *Trichinella spiralis* or *Toxoplasma gondii* (Khan et al, 2013). Also, the presence of antibodies in serum after experimental infection (Marmion and Stocker, 1958) indicates their susceptibility to *C. burnetii* but, at the moment there are no reports of an active infection in these domestic animals.

Therefore, the aim of this study was to investigate the exposure to *C. burnetii* infection among domestic pigs.

A cross-sectional study was designed and developed during February 2013. The sampling procedure included the collection of blood samples by systematic random sampling in a slaughterhouse located at the Centre of Portugal (39º 42' N 8º 17' W). Using the software Microsoft Excell® the number 8 was randomly selected from 0 to 100; and thus, it was considered the unit of sampling. A total of 50 blood samples were collected one day per week and for four weeks. Blood (5 ml) was kept refrigerated at 4°C until centrifugation and sera were stored at - 20°C until analysis. At the time of collecting blood samples a small questionnaire containing information about the animals was filled.

Each serum was tested in duplicate using a commercial indirect ELISA, which is based on the use of an antigen (phase I and II) isolated from domestic ruminants (LSIVET Ruminant Milk/Serum Q Fever®, Laboratoire Service International, Lissieu, France). The ELISA was adapted for multi-species detection, using an anti-multi-species conjugate (protein AG) provided by the manufacturer. Before testing, an assay was performed to find the best working dilution for protein AG, using the ruminant positive and negative control sera provided in the kit. Briefly, serial dilutions of the protein AG were performed (from 1 to 1/100.000) and ruminant positive and negative control sera were tested in duplicate, using the ruminant conjugate and the protein AG dilutions. The mean optical densities (OD) of the positive and negative controls of each protein AG dilutions were compared with the mean OD of the ruminant conjugate and the dilution with an OD closer to the ruminant conjugate was used (Laboratoire Service International, personal communication, 2011). For the identification of *C. burnetii* positive and negative samples to use as control sera, a prior test is needed according to Cooper *et al*. (2011, 2012). Briefly, a screening of samples is carried out using the ruminant positive and negative control sera provided by the kit. The mean OD of duplicates is calculated, and samples are not considered if the coefficient of variation (CV) is > 10%. The pool of the three high-reacting (HR) and the three low-reacting (LR) sera (mean OD HR / mean OD LR $\geq$ 4) are then used as positive and negative control sera, respectively.

The ELISA was performed according manufacturer's instructions. The ruminant conjugate was replaced by the protein AG. OD values were measured at 450 nm. Sample/positive percentages (S/P per cent) were calculated by the adjustment with the negative control [S/P per cent=(OD_{sample} $OD_{negative}$)/($OD_{positive}$-$OD_{negative}$)x100]. Sera with an S/P per cent <50% were considered negative. Samples with an S/P per cent between

50% and 75% were considered positive and those >75% were considered strongly positive (Cooper *et al.*, 2011, 2012).

At the preliminary screening of the 50 sera, no significant differences were observed in the mean OD of the duplicates. This means that it was not possible to identify a positive serum with a mean OD four-fold higher than the others.

Considering this, it is not possible to assess the exposure to *C. burnetii* in domestic pigs.

At the present, the methods described for serological testing in animals are the ELISA, the Indirect Immunofluorescence (IF) test and the Complement Fixation (CF) test. ELISA tests are commercially available for domestic ruminants and often used (Rousset *et al*., 2010). However, the lack of commercially available and validated ELISA tests for other species raises some difficulties in their use, namely by the absence of species-specific antibody and by the lack of reference control sera. The use of the protein AG has been described as a strategy to solve the problem of the absence of a species-specific antibody. Since it has a strong ability to bind to IgG of mammalian species, it has been used in the adaptation of ELISA tests to multiple species (Zhang *et al*., 2010; Al-Adhami and Gajadhar, 2014). This strategy was also used in other surveys for *C. burnetii* in other species than ruminants (Meredith *et al*., 2014).

To the best of our knowledge this is the first serosurvey of *C. burnetii* in pigs in Portugal. However, it seems that the exposure of domestic pigs to *C. burnetii* is very limited or even inexistent. The intensive production system and the high turnover of the pig population in the farm might explain the obtained results. Further studies are needed to clarify about the exposure in pigs, namely by the sampling of adult animals used in reproduction in intensive production systems and by the study of pigs in extensive production systems.

5

Companion animals and ticks

5.1

Coxiella burnetii in companion animals and ticks: serological and molecular screening

S. Anastácio, S. Sousa, M. Almeida, H. Vilhena, M. Portugal, F. Ramalho, K. Sidi-Boumedine, G.J. da Silva. *Coxiella burnetii* in companion animals and ticks: serological and molecular screening.

Abstract

Dogs and cats are potential sources of infection for some zoonotic diseases such as Q fever, caused by *Coxiella burnetii*, a multiple host pathogen. Ticks are considered *C. burnetii* vectors in wild and domestic cycles. This study aimed to screen for *C. burnetii* in pets and in ticks collected from infested animals. A cross-sectional study was conducted from 2011 to 2014. Sera obtained from pets (n=180) were tested for *C. burnetii* antibodies using a commercial ELISA adapted for multi-species detection. *C. burnetii* in ticks was screened by PCR assay targeting IS1111. An exposure to *C. burnetii* was observed in 17.2% (95%CI:5.8-35.8%) of cats and in 12.6% (95%CI:7.7-19.0%) of dogs. The exposure occurred in cats living in rural habitat, but there were no differences in the exposure between dogs living in urban or rural habitats. Ticks (n=91) were identified as *Rhipicephalus sanguineus*, *Ixodes ricinus* and *Dermacentor reticulatus,* being the former being the most common. None was positive for *C. burnetii* DNA. The higher exposure in cats might be explained by their hunting activities and contact with wildlife. An increase of antibody positivity in dogs was observed over the last 20 years in Portugal, but we could not associate wild, domestic animals and ticks as a source of infection. This study revealed that ticks do not seem to be involved in the transmission of *C. burnetii*. This highlights the need of more research to clarify the potential sources of infection in dogs and the role of ticks in *C. burnetii* transmission.

Keywords: Q fever, ELISA, PCR, dogs, cats, ticks

Introduction

Coxiella burnetii, a small Gram negative intracellular bacterium, is the causative agent of Q fever, a zoonotic infection worldwide distributed. The host range of *C. burnetii* includes mammals, birds, reptiles and arthropods (Angelaskis and Raoult, 2010). Domestic ruminants are recognized as the main sources of human

infection and the shedding occurs mostly at the time of parturition, when the heavily infected placenta results in aerosolization of *C. burnetii* (Rodolakis, 2009).

However, human Q fever outbreaks have also been documented from the contact with dogs and cats (Buhariwalla *et al*., 1996; Kosaksy, 1984; Marrie *et al*., 1988[a], 1988[b]; Kopecny *et al*., 2013). Pets can potentially be infected by inhalation, tick bites, consumption of placentas or milk from infected ruminants (Porter *et al*., 2011). The finding of *C. burnetii* in vaginal and uterine samples of healthy cats (Nagaoka *et al*., 1998; Cairns *et al*., 2007; Tozer *et al*., 2014) and dogs (Roest *et al*., 2013[b]; Mares-Guia *et al*., 2014; Tozer *et al*., 2014) suggest a potential zoonotic risk for humans. Experimentally, in cat *C. burnetii* can cause fever, anorexia and lethargy but in the field, the infection remains asymptomatic and frequently undiagnosed (Egberink *et al*., 2013). In infected parturient dogs, early death of the pups has been reported (Buhariwalla *et al*., 1996).

Few reports indicate that ticks, ectoparasites of mammals including pets, can act as vectors of *C. burnetii* (Spitalska *et al*., 2003). Ticks acquire *C. burnetii* during a blood meal on infected animals and the infection is maintained in the ticks by vertical transmission, meaning that transtadically and transovarially transmission may occur. Transmission from ticks to vertebrate might occur during the next blood meal or by aerogenic spread of dried tick faecal excretions (Szymanska-Czerwinska *et al*., 2013). Over than 40 species of ticks have been found to carry *C. burnetii*, and eventually they may serve as indicators of infection in nature (Spitalska *et al*., 2003).

In Portugal, only one serosurvey of *C. burnetii* was performed in dogs, using an indirect fluorescent antibody test (Bacellar *et al*., 1995). Moreover, this study was performed many years before the public concern raised by the *European Food and Safety Authority* (EFSA) triggered by the large human Q fever outbreak in The Netherlands in 2007-2011 (EFSA 2010). Likewise, there is a lack of information about studies in cats.

This study aimed to determine the seroprevalence of *C. burnetii* infection in dogs and cats from central Portugal and to detect the presence of *C. burnetii* DNA in ticks collected from the animals.

Methods

Study design and sampling approach

A cross-sectional study was conducted in central Portugal from 2011 to 2014, in a region of 1937 Km^2 in a latitude 40.0º to 40.7º N and a longitude 8.2º to 8.7º W. For sample size calculation, the size of the domestic dog and cat population in the study region was estimated according to Alves *et al*. (2005) and Cooper *et al*. (2011). Furthermore, an expected prevalence of 4.8% was considered (Bacellar *et al*. 1995) following the method described by Thrusfield (1995), considering a desired absolute precision of 10% and a 95% confidence interval. The calculation was performed using the software WinEpiscope version 2.0, resulting in a minimum sample size of 19 dogs and 19 cats. A convenience sampling was performed in dogs and cats attending on veterinary clinics and housed at the municipal kennel of Coimbra. Only animals older than six months were included in the study. At veterinary clinics, only animals subjected to a blood sampling during the routine procedures of veterinary examination, were included in the study, after the consent of the owners. In that case, the surplus blood was centrifuged, and the serum or plasma was stored at -20ºC until analysis. At the municipal kennel, blood samples were collected only from animals that would be euthanized according to the national legislation (Portugal: DL315/2003). This procedure was carried out under approval of Portuguese National Authority for Animal Health (no.C.12.014.UDER).

Additionally, ticks were collected from infested cats and dogs and preserved in 70% alcohol. Ticks were identified to species level using a standard morphological key (Zajac and Conboy, 2006) and the data was recorded, including the developmental stage and the gender.

A small questionnaire was filled-up, containing some individual factors as gender, breed, age, habitat, and exposure to wildlife or to other domestic animals.

Serological testing

Sera (n=180) were tested using a commercial indirect ELISA, which is based on the use of an antigen (phase I and II) isolated from domestic ruminants (LSIVET Ruminant Milk/Serum Q Fever®, Laboratoire Service International, Lissieu, France). The sensibility and specificity of this Kit were estimated to be 87% and 99.1%, respectively, by comparison with complement fixation (Laboratoire Service International, Lissieu, France, personal communication, 2012). This ELISA was adapted for multi-species

detection, using an anti-multi-species conjugate (protein A/G) provided by the manufacturer. Before testing, an assay was performed to find the best working dilution for protein AG, using the ruminant positive and negative control sera provided in the kit. Briefly, serial dilutions of the protein AG were performed (from 1 to 1/100.000) and ruminant positive and negative control sera were tested in duplicate, using the ruminant conjugate and the protein AG dilutions. The mean optical densities (OD) of the positive and negative controls of each protein AG dilutions were compared with the mean OD of the ruminant conjugate, and the dilution with an OD closer to the ruminant conjugate was used (Laboratoire Service International, Lissieu, France, personal communication, 2011). The identification of *C. burnetii* positive and negative samples, for use as control sera, was performed prior to testing as recently described (Cooper *et al*., 2011, 2012). A previous screening of 92 serum samples from dogs (67) and cats (n=25) was carried out using the ruminant positive and negative as control sera. The mean OD of duplicates was calculated, and samples were not considered if the coefficient of variation (CV) was > 10%. The three high-reacting (HR) and the three low-reacting (LR) sera (mean OD HR / mean OD LR ≥ 4) were pooled and used as positive and negative control sera, respectively. These selected samples were re-tested and a CV<15% was required between assays.

The ELISA was performed according to the manufacturer's instructions. Each serum sample was tested in duplicate and the ruminant conjugate was replaced by the protein AG. OD values were measured at 450 nm. Sample/positive percentages (S/P per cent) were calculated by the adjustment with the negative control [S/P per cent=(OD_{sample}-$OD_{negative}$)/($OD_{positive}$–$OD_{negative}$)x100]. Sera with an S/P per cent <50% were considered negative. Samples with an S/P per cent between 50% and 75% were considered positive and those >75% were considered strongly positive (Cooper *et al*., 2011, 2012).

Molecular analysis

The PCR testing for detection of *C. burnetii* DNA was performed on ticks after DNA isolation. Ticks (n=91) were washed three times in 1x phosphate buffered saline, rinsed with distilled water and dried on sterile filter paper prior to DNA extraction (Reye *et al*., 2012). Ticks were crushed individually and aseptically, using surgical scalpel blades in sterile Petri dishes and 25 mg of homogenate was used for lysis using Qiamp DNA Mini Kit (Qiagen®, Isaza, Portugal) according to manufacturer's instructions.

A conventional PCR assay targeting IS1111, a transposon-like repetitive region of *C. burnetii*, was performed as described previously (Anastácio *et al*., 2016). To discard the presence of *Taq* Polymerase inhibitors, DNA samples were tested in duplicate, adding 2.5 μL of positive control (*C. burnetii* RSA 493) to each DNA sample duplicate (2.5 μL). The DNA amplification was performed in a Biometra Thermocycler (Biometra®, Germany). The expected amplicons (243 bp) were separated by electrophoresis on 2% agarose gel and observed under UV light. In case of positive results, a real time PCR testing, conducted on a CFX-96 thermocycler (Bio-Rad®, Portugal) and using the commercial Taq-Vet™ *Coxiella burnetii* kit (LifeTechnologies®, USA) enabled the confirmation of the positive results and the determination of Ct values for comparisons.

Statistical analyses

For statistical analyses purposes, simple logistic regression analysis was performed to explore associations between individual factors and response variables. Confidence limits for the proportions were estimated with 95% confidence intervals (CI) assuming a binominal exact distribution (EpiInfo version 3.5.4; Center for Disease Control and Prevention, Atlanta, GA, USA).

Results

ELISA testing

A total of 180 surveyed serum samples were obtained from 151 dogs and 29 cats. Table 15 shows the descriptive characteristics and serological results in both species.

The calculated mean age was 63.6 months (range: 6 to 192 months, SD=60.8) for cats and 71 months (range: 6 to 168 months, SD=51) for dogs. Male gender was slightly predominant in both species: 55.2% in cats (95% CI: 36-73%) and 62.9% in dogs (95% CI: 53.7-71.3%); as well as the rural habitat: 58.6% of cats (95% CI: 39.1-75.9%) and 54.9% of dogs (95% CI: 46.0-63.6%) lived in a rural area.

An exposure to *C. burnetii* was evidenced in five cats (17.2%; 95% CI: 5.8 to 35.8%). The proportion of positive results was slightly higher in female cats (10.3%, 95% CI: 2.7-28.5%) and in cats younger than 24 months (10.3%, 95% CI: 2.7-28.5%). All the positive cats lived in rural area. Among them, 80% (95% CI:

29.9-98.9%) referred the exposure to wildlife and 20% (95% CI: 1.1-7.0%) cohabited with other domestic animal species.

In dogs the exposure to *C. burnetii* was evidenced in 19 animals (12.6%; 95% CI: 7.7-19.0%). Despite the lack of data in some animals, the proportion of positive results was equal in both genders as well as in rural or urban habitat. A higher proportion of positive results was found in animals older than 24 months (5.3%; 95% CI: 2.7-10.1%), in animals with owner (8%; 95% CI: 4.4-13.8%), without cohabiting with domestic animals (4%; 95% CI: 1.6-8.8%) but exposed to wildlife (4%; 95% CI: 1.6-8.8%).

Table 15: Descriptive characteristics and seropositive results (cats and dogs).

Variables	Cats			Dogs		
	Frequency (n)	Positivity (%)	[a]CI 95%	Frequency (n)	Positivity (%)	[a]CI 95%
Gender						
Male	16	6.9	1.2-24.2	78	4.0	1.6 – 8.8
Female	13	10.3	2.7-28.5	49	4.0	1.6 – 8.8
Missing	0	0	na[b]	24	4.6	2.1 – 9.7
Age						
< 24 months	10	10.3	2.7-28.5	7	1.3	0.3-4.7
≥ 24 months	16	6.9	1.2-24.2	100	5.3	2.7-10.1
Missing	3	0	na[b]	44	6.0	3.2-11.0
Habitat						
Rural	17	17.2	5.8-35.8	72	5.3	2.5-10.5
Urban	12	0	na[b]	59	5.3	2.5-10.5
Missing	0	0	na[b]	20	2.0	0.5-6.1
Origin						
Owner	28	17.2	5.8-35.8	71	8.0	4.4-13.8
Kennel	1	0	na[b]	80	4.6	2.1-9.7
Cohabitation with other species						
Yes	10	3.5	0.2-19.6	11	0	na[b]
No	13	10.3	2.7-28.5	40	4.0	1.6-8.8
Missing	6	3.5	0.2-19.6	100	9.9	5.9-16.1
Exposure to wildlife						
Yes	13	13.8	4.5-32.6	63	4.0	1.6-8.8
No	16	3.5	0.2-19.6	37	2.7	0.9-7.1
Missing	0	0	na[b]	51	6.0	2.9-11.4
Total	**29**	**17.2**	**5.8-35.8**	**151**	**12.6**	**7.7-19.0**

[a] Confidence Interval (range within which is reasonably confident to find the real prevalence)

[b] not applicable

Table 16 describes the categories of ELISA results in relation with the origin of animals.

Table 16: Categories of ELISA results in relation with the origin of the animals.

	Negative n (%; 95% CI)	**Positive n (%; 95% CI)**	**Strong positive n (%; 95% CI)**
Dogs			
With owner	59 (39.1%; 31.3-47.45%)	7 (4.6%; 2.1-9.7%)	5 (3.3%; 1.2-8.0%)
Kennel	73 (48.3%; 40.2-56.6%)	2(1.3; 0.2-5.2%)	5 (3.3%; 1.2-8.0%)
Cats			
With owner	23 (79.3%; 59.7-91.3%)	2 (6.9%; 1.2-24.2)	3 (10.3%; 2.7-28.5%)
Kennel	1 (3.5; 0.2-19.6%)	0	0

Ticks identification

Table 17 summarizes the data about tick identification and PCR results. A total of 91 hard ticks comprising 88 adults (96.7%) and 3 nymphs (3.3%) were collected. Among those, 77 (84.6%) were collected from dogs and 14 (15.4%) from cats. In the adult forms, females were predominant (61; 69.3%) and most of them (40/61; 65.6%) were engorged. The most common species in both dogs and cats was *Rhipicephalus sanguineus*, followed by *Ixodes ricinus*. *Dermacentor reticulatus* was only identified in one dog.

Table 17. Characterisation of the ticks and PCR results in each animal species.

Tick species	Stage / Sex					Nº ticks	Nº hosts	PCR result
	Engorged female	Non-engorged female	Male	Nymph	Larva			
Dogs								
R. sanguineus	34	16	23	1	0	74	14	Negative
I. ricinus	0	0	1	1	0	2	2	Negative
D. reticulatus	0	0	1	0	0	1	1	Negative
Cats								
R. sanguineus	6	3	1	0	0	10	6	Negative
I. ricinus	0	2	1	1	0	4	3	Negative

Molecular testing

No positive results were obtained in ticks by molecular analysis (Table 17). Furthermore, no inhibitions of the *Taq* Polymerase were observed on PCR.

Discussion

Dogs and cats are very common as companion animals over the world. However, these animals can serve as sources of infection for human bacterial diseases, even when they are asymptomatic (Skerget *et al*., 2003). Q fever is a zoonotic disease characterized

by an acute febrile illness with nonspecific clinical signs in humans, whose non-specific manifestations include hepatitis and atypical pneumonia in severe cases, and in a small proportion of infected people a chronic infection with life-threatening valvular endocarditis may occur (Angelakis and Raoult, 2010). Both cats and dogs have been referred as sources of infection (Pinsky *et al*., 1991; Marrie *et al*., 1988[a,b]; Buhariwalla *et al*., 1996) and the role of ticks in bacterial transmission in wild and peridomestic cycles is described (Mancini *et al*., 2014). Establishing the seroprevalence of *C. burnetii* in dogs and cats is essential to obtain an overview of the exposure to this bacterium, to gauge the likelihood of *C. burnetii* being an agent of disease in these species and to determine the risk of infection to veterinary personnel, professional breeders and pet owners (Shapiro *et al*., 2015). Moreover, the detection of *C. burnetii* DNA in ticks provides information about their role in the peridomestic cycle and whether this screening is needed in surveillance studies.

In this study the proportion of positive results was 17.2% and 12.6% in cats and dogs, respectively. Previous studies conducted in cats reported positivity rates of 14.2% in Japan (Komiya *et al*., 2003), 9% in California (Willeberg *et al*., 1980), 13% in Zimbabwe and 2% in South Africa (Matthewman *et al*., 1997). A serosurvey conducted in Canada - Nova Scotia, where an outbreak of human Q fever was reported (Marrie *et al*., 1988[a]), revealed a seroprevalence of 6% in cats (Marrie *et al*., 1985). Interestingly, in a similar study performed in the region of Ontario, no seropositive results were found in cats (Lang *et al*., 1992) revealing that the patterns of infection may differ geographically. A higher seroprevalence (61.5%) was obtained in cats from United Kingdom but the sampling strategy included cats with owner living outdoor with hunting habits which might have biased results by the exposure to wildlife (Meredith *et al*., 2014). Overall, this agrees with our findings in which all the positive cats lived in rural area and among them, 80% (95% CI: 29.9-98.9) were exposed to wildlife (data not shown). This can be explained by the predatory activity of cats in wildlife, living close to prey animals or even to livestock (Marrie *et al*., 1988[a]; Meredith *et al*., 2014). This suggestion is also supported by the results obtained in Japan, where a seropositive rate of 41.7% in stray cats was reported, being higher than the seropositivity in domestic cats (14.2%) (Komiya *et al*., 2003). These findings demonstrate that the feline environment influences the exposure to *C. burnetii.*

The increased seropositivity in dogs (12.6%) compared to the last serosurvey in Portugal (4.8%) (Bacellar *et al*., 1995) suggests a change in the scenario of *C. burnetii*

infection in dogs over the last 20 years, but the different sensibility and specificity of methods applied should not be neglected. Previous surveys of *C. burnetii* in dogs have reported a seropositivity of 21.8% in Australia (Cooper *et al*., 2011), 66% in California (Willeberg *et al*., 1980), 59.3% in Bulgaria (Martinov *et al*., 1989[b]), 12% in Croatia (Punda-Polic *et al*., 1995), 9.8% in France, 11.6% in Senegal (Boni *et al*., 1998), 1% in Italy (Baldelli *et al*., 1992) and in Canada no antibodies were detected in dogs (Marrie *et al*., 1985). Differences on seroprevalence may occur during time and between geographic locations. Nevertheless, the comparison of data of published studies must be done carefully due to differences in study design, sampling approach and methods applied.

Interestingly, we found higher antibody positivity (8.0%) in dogs with owner than in dogs from the municipal kennel (4.6%), which is opposite to that found in California, where a higher positivity was observed in stray dogs than in dogs with owner (Willeberg *et al*., 1980). Moreover, we obtained similar antibody positivity in dogs from rural (5.3%) and from urban (5.3%) areas. These findings are somewhat unexpected. Most of the animals kept at the municipal kennel were captured from the streets suggesting a higher chance of exposure to *C. burnetii* by the contact with wildlife, and then a higher positivity would be expected in stray dogs than in dogs with owner. Besides, in rural areas a higher positivity would be expected, due to contact with livestock and wildlife that might favour the exposure to *C. burnetii* (Maurin and Raoult, 1999; Anastácio *et al*., 2014). Q fever outbreaks in urban areas might be related to windborne spreading of *C. burnetii* (Popescu *et al*., 2014) but questions about the potential sources of infection in urban areas remain unclear.

Ticks are important vectors of infectious agents, playing an essential role in the eco-epidemiology of diseases, such as Lyme borreliosis, rickettsiosis, babesiosis, ehrlichiosis or tularaemia (Parola and Raoult, 2001). The transmission of *C. burnetii* by vector route has also been referred (Psatouraki *et al*., 2014). These arthropods are ubiquitous both in rural and urban areas and the possibility of ticks being a source of infection in dogs should not be excluded.

Among the 850 species of ticks identified all over the world (Silva *et al*., 2006[a]), more than 40 species of ticks have been found to carry *C. burnetii* (Angelaskis and Raoult, 2010). Ticks show some geographical and host specificity (Duron *et al*., 2014), and pathogen diversity differs among tick species (Reye *et al*., 2013). Moreover, a host-specific adaptation of *C. burnetii* has been hypothesized (Sulyok *et al*., 2014). In

this context, the identification of ticks was a substantial objective in this study, so it enables us to relate tick species to their hosts as well as an association with *C. burnetii* infection

In Portugal, the climatic and ecological conditions are favourable for the development of several species of ticks and 24 species have been identified so far (Silva *et al*., 2006[a]). We found *Rhipicephalus sanguineus* as the predominant tick species which agrees with other studies in Portugal (Maia *et al*., 2014). Dogs are recognized as major hosts of *R. sanguineus* however a wide range of domestic and sylvatic animals can be infected by this tick. *Ixodes ricinus,* the second most frequent tick in our study, is mainly found in regions rich in vegetation and presents an exceptional ability to infect several hosts, like birds, mammals and even humans. Only one specimen of Genus *Dermacentor* was found in a dog, the less frequent genus found in Portugal, but also presenting a large broad of hosts, including humans (Silva *et al*., 2006[a]).

In our screening for *C. burnetii* in ticks collected from dogs and cats, all the DNA samples were negative. Therefore, it was not possible to associate a tick species with the transmission of *C. burnetii*. Similar results were obtained recently in The Netherlands (Sprong *et al*., 2012) and in Japan (Andoh *et al*., 2013). However, positive results were obtained in Belarus (1%) (Reye *et al*., 2013), in Slovakia (3%) (Rehacek *et al*., 1991), in Poland (15.9%) (Szymanska-Czerwinska *et al*., 2013), in Italy (22%) (Mancini *et al*., 2014) and in pools of ticks from Kenya (50%) (Knobell *et al*., 2013). In these studies *C. burnetii* was found in the genus *Rhipicephalus*, *Ixodes*, *Dermacentor* and *Haemaphisalis* collected in dogs or in the environment (Rehacek *et al*., 1991; Knobell *et al*., 2013; Reye *et al*., 2013; Szymanska-Czerwinska *et al*., 2013; Mancini *et al*., 2014). Based on the results obtained from this study, currently the risk of acquiring *C. burnetii* from ticks is negligible. However, the recent identification of a hotspot of *C. burnetii* in ticks at a regional level in Belarus (Reye *et al*., 2013) and the high prevalence of *C. burnetii* in ticks collected in a public park in Rome, suggest their potential role in the maintenance of infection in some regions (Mancini *et al*., 2014). In this context, monitoring of vectors should be performed on a regular basis.

The exposure to *C. burnetii* was confirmed in both cats and dogs. Overall, cats seem to be more prone to be infected by *C. burnetii,* which might be related to their living habits since indoor cats having frequently free access to the backyard where they express their hunting instinct, and possibly being exposed to potential infected wildlife prey. In dogs, the potential sources of infection need to be clarified. Ticks cannot be

discarded as a source of transmission, but in this study, we could not establish an association between pets and ticks taken from infested animals. This reinforces the need of more investigation to really clarify the role of ticks in transmission of *C. burnetii*, namely at species level and their habitat.

Acknowledgements

Authors are very grateful not only to owners by their agreement in participating in the study but also to the animals included in this study and the veterinary clinics by their kind collaboration.

Financial support

S. Anastácio is supported by the grant SFRH/BD/77823/2011 from Fundação para a Ciência e a Tecnologia, Lisbon, Portugal. This work was supported financially by the Center for Neuroscience and Cell Biology (CNC), University of Coimbra.

Conflict of Interest

The authors declare that they have no conflict of interest.

6

Wild ungulates

6.1

Coxiella burnetii in wild boars (*Sus scrofa*): serological and molecular analysis

S. Anastácio, R. Cabeças, C. Coelho, M. Pereira, M. Almeida, M.M. Vieira-Pinto, K. Sidi-Boumedine, G.J. da Silva. (manuscript in submission)

Abstract

The role of wild boars in the epidemiology of Q fever is poorly understood. This study aimed to investigate their exposure to *C. burnetii* and to determine the presence of *C. burnetii* DNA in sera and faeces. In a cross-sectional study, the screening for specific antibodies was performed by a modified ELISA and *C. burnetii* DNA was detected by PCR. Results showed an exposure in five animals (5.6%; 95%CI: 1.8-12.6%) but *C. burnetii* DNA was not detected. The risk of wild boar as sources of infection for both animals and humans, especially during hunting activities, must not be neglected.

Keywords: Q fever, feral swine, epidemiology, serosurvey, PCR

Coxiella burnetii is a multiple-host pathogen and the causative agent of Q fever, a worldwide zoonosis. Transmission to humans occurs by inhalation of infected aerosols or dust when working with or near infected animals, animal tissues, secretions and excreta, mostly associated with birth (Maurin and Raoult, 1999).

In Europe, Q fever has been reported mainly in domestic ruminants and the role of other animal species in Q fever epidemiology is not well understood (EFSA, 2010[a]). *C. burnetii* antibodies were found in serum of pig after experimental infection (Marmion and Stocker, 1958) but there are no reports of an active infection in domestic swine so far. Only one study reports an active infection in wild boars by the detection of *C. burnetii* in tissues (Astobiza *et al*., 2011).

Wild species can be infected by livestock pathogens and, simultaneously, be a risk for the re-infection of livestock (Conner *et al*., 2008; Meng *et al*., 2009). They are known (*e.g.* wild boar) to be the source of infection of zoonotic pathogens such as *Brucella suis* (Giovannini *et al*., 1988; Pilo *et al*., 2015), *Leptospira interrogans*, *Mycobacterium bovis* (Meng *et al*., 2009), *Trichinella spiralis* (Baradel *et al*., 1988; Moskwa *et al*., 2015) or even *Toxoplasma gondii* (Clark *et al*., 1983; Racka *et al*., 2015).

Wild boar is the main large game species hunted in Portugal. Hunting activities might favour the exposure to *C. burnetii* by handling fresh meat or body fluids from infected animals and contact with contaminated environment (Maurin and Raoult, 1999). Moreover, the need of studying and monitoring *C. burnetii* infection in wildlife to ensure a broader evaluation of their epidemiological role as a source of infection for domestic animals and humans has recently been highlighted (Rijks *et al*., 2011; Billinis, 2013; Ciliberti *et al*., 2015). The aims of this study were to investigate the exposure to *C. burnetii* infection among wild boars and to determine the presence of *C. burnetii* DNA in their sera and faeces.

A cross-sectional study was conducted in the Northeast of Portugal during the hunting season 2011/2012 (October 2011 to February 2012). Samples were collected from 89 hunted wild boars in two districts (Bragança and Vila Real). Blood (5 ml) was collected directly from the thoracic cavity upon evisceration and kept at 4°C until centrifugation. Faeces were collected into sterile containers. Sera and faeces were stored at -20°C until analysis. The specific region where animals were hunted (Nordeste Transmontano, Douro, Tâmega and Barroso-Padrela), gender and age (determined on the tooth eruption patterns) were registered. Animals were categorized as juveniles (6-14 months) and sub-adult/adult (≥15 months) (Saenz de Buraga *et al*., 1991).

Sera (n=89) were tested using a commercial indirect ELISA which is based on the use of an antigen (phase I and II) isolated from domestic ruminants (LSIVET Ruminant Milk/Serum Q Fever®, Laboratoire Service International, Lissieu, France). This ELISA was adapted for multi-species detection, using an anti-multi-species conjugate (protein AG) provided by the manufacturer. Before testing, an assay was performed to find the best working dilution for protein AG. The optical densities (OD) of the positive and negative control sera of each protein AG dilutions were compared to those obtained with the ruminant conjugate. The dilution with an OD closer to the ruminant conjugate was used (Laboratoire Service International, personal communication, 2011). Also, prior testing was performed to identify *C. burnetii* positive and negative samples to use as control sera, as described by Cooper *et al*. (2012). Each serum sample was tested in duplicate following the manufacturer's instructions and Sample/Positive per cent (S/P%) was calculated. As the ELISA used in this study has only been validated for ruminants which may develop different immune responses to *C. burnetii* compared to wild boar, the results were analyzed following two methods. A cut-off of 50% was considered to differentiate positive and negative samples (Cooper *et al*., 2011, 2012).

Additionally, a statistical approach of the S/P% results was performed. Assuming that the sampled population includes a mixture of seropositive and seronegative animals, the frequency distribution of S/P% should show two components. A bi-modal normal distribution was fitted to the observed S/P%, using Bayesian Markov chain Monte Carlo model implemented using "rjags" package from R (R Core Team, 2015) and the results were analyzed according Meredith *et al*. (2014). The agreement between the two methods was investigated by the determination of the kappa coefficient using the WinEpiscope version 2.0 (University of Edinburgh, Roslin, UK).

C. burnetii DNA screening was performed in serum (n=28) and faeces (n=57) by PCR. A randomly sampling of 25% of the 89 tested sera (n=23) was performed. Then, a convenience sampling was performed to add five seropositive sera. All the collected faeces samples were tested, and they consisted of 53 seronegative and 4 seropositive animals. DNA was isolated from 200 µL of serum and 220 mg of faeces using the QIAamp DNA Mini Kit and QIAamp DNA Stool Kit (Qiagen®, Izasa, Portugal), respectively. A PCR assay targeting IS1111was performed (Anastácio *et al*., 2016). PCR positive samples were confirmed by real time PCR, using the commercial Taq-Vet^{TM} *Coxiella burnetii* kit (LifeTechnologies®, USA). PCR inhibitors were controlled by testing each DNA sample (2.5 µL) added with 2.5 µL of positive control (*C. burnetii* RSA 493).

A simple logistic regression analysis was performed to explore associations between individual factors and response variables. Confidence limits for the proportions were estimated with 95% confidence intervals (CI) assuming a binominal exact distribution (EpiInfo version 3.5.4; Center for Disease Control and Prevention, Atlanta, GA, USA).

The lack of validated tests for wild species raises some difficulties, namely by the absence of species-specific antibody and reference control sera (Rousset *et al*., 2010). The protein AG has a strong ability to bind IgG of mammalian species and has been used in ELISA tests to multiple species (Zhang *et al*., 2010, Al-Adhami and Gajadhar, 2014, Meredith *et al*., 2014). This strategy was used in this study. To overcome the lack of species-specific reference control and to set the cut-off value for the ELISA, we followed the method of Cooper *et al*. (2011, 2012).

The descriptive characteristics and the seroprevalence results are summarized on Table 18. From the 89 tested animals, five (5.6%, 95% CI: 1.8 to 12.6%) evidenced an exposure to *C. burnetii.* Data from other countries are variable. In Japan (Ejercito *et*

al., 1993), Italy and France (Giovanni *et al*., 1988; Baradel *et al*., 1988) there was no evidence of exposure. In Czech Republic and in United States (California), 6% and 50% of the animals were positive, respectively (Hubalek *et al*., 1993; Clark *et al*., 1983).

Table 18: Descriptive characteristics and seroprevalence results in hunted wild boars in the North region of Portugal.

	Number of animals	Seropositivity n (%)	CI 95%[a]
Selected	89	5 (5.6)	1.8 – 12.6
A. Distribution according to the geographic area			
Nordeste Transmontano	42	2 (4.8)	0.8 – 17.4
Douro	37	2 (5.4)	0.9 – 19.5
Tâmega	4	1 (25)	1.3 – 78
Barroso – Padrela	6	0	na[b]
B. Distribution according to the animal's category			
Gender			
Male	28	0	na[b]
Female	61	5 (8.2)	3.1 – 18.8
Age			
6-14 months	24	0	na[b]
≥15 months	65	5 (7.7)	2.9 – 17.8

[a] CI – Confidence interval
[b] – Not applicable

However, comparison of data may not be feasible. The above-mentioned studies were conducted in various geographical areas, using different methods, and changes in the epidemiology throughout the years might have occurred. The harmonization of serological tools is critical to compare data (Rousset *et al*., 2010; EFSA, 2010[b]). So, we additionally performed a statistical approach of S/P% values as referred by Meredith *et al*. (2014). The proportional frequency of S/P% showed a bi-modal normal distribution (Figure 15) obtained by the MCMC model, representing two subpopulations. The mean S/P% was estimated in 6.0 (SD=7.9) and 40.4 (SD=8.2) for the negative and positive sub-population, respectively, and a statistically significant difference was observed in the estimated means of the two groups ($p<0,001$). Two S/P% values (92 and 146) were excluded to improve convergence of distributions since it would not interfere in threshold determination. The threshold was estimated in 35% resulting in a slight increase of the apparent prevalence (12.3%; 95% CI: 6.6 to 21.5%) comparing with the method of Cooper *et al*. (2012) and the sensitivity and specificity were calculated in 100% and 92.8%, respectively.

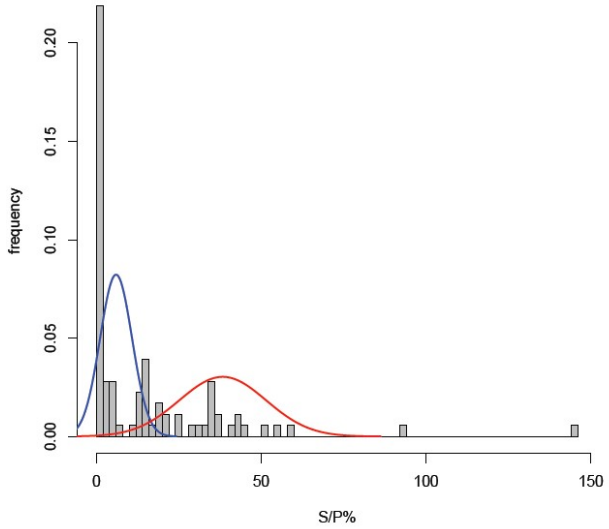


Figure 15. Frequency distribution of observed S/P% values (grey bars) in the modified indirect ELISA for wild boar (n=89). Solid curves correspond to the fitted distributions using the mean and variance of the two distributions from the MCMC analysis.

The agreement between the two methods was calculated and classified in fair to good (kappa=0.59; 95% IC: 0.40-0.78), leading us to conclude that the cut-off of 50% decreases the sensitivity of the test.

It was found that the seropositivity occurred only in females older than 15 months, but none of the explanatory variables was identified as a risk factor for *C. burnetii* exposure. This finding is similar to that described in Spain, where only female wild boar older than 15 months presented *C. burnetii* DNA in tissues (Astobiza *et al*., 2011). This suggests that postpubescent wild boar females are more prone to be infected, as reported for ruminants (Arricau-Bouvery and Rodolakis, 2005; Anastácio *et al*., 2013[a]). *C. burnetii* replicates strongly in the reproductive tissues and birth products (Rousset *et al*., 2010). The gregarious habits of wild boar living close in small groups of females may favour the infection. Beyond the intra-species transmission, the occurrence of indirect or vector-borne interspecies transmission might happen. The proximity between domestic ruminants and wild species in areas of pasture where livestock were being

grazed enables the occurrence of a spillover event (Rijks *et al*., 2011; Meredith *et al*., 2014), which can be confirmed by genotyping the strains circulating among wild and domestic animals (Rijks *et al*., 2011).

No positive results were obtained in serum and faeces by PCR, suggesting that no active infection was present at the time of sample collection. No inhibition of *Taq* Polymerase was observed. Serum has been described as a suitable sample for detection of bacterial DNA. In humans, *C. burnetii* is found in the blood during the acute phase of infection (Angelakis and Raoult, 2010) but it is expected that DNA becomes undetectable as the immunological response develops (Zhang *et al*., 1998; Schneeberger *et al*., 2010). In domestic ruminants, the shedding of *C. burnetii* occurs mainly in birth products but also in milk, faeces and urine. The contaminated dust resulting from contaminated excrements can lead to the inhalation of infected aerosols by other animals or humans as *C. burnetii* is a very resistant bacterium (Arricau-Bouvery and Rodolakis, 2005). Among the studied animals, there was no evidence of faecal shedding. Faeces might not be a privileged shedding route in wild boars as occurs in cattle. The evidence of specific antibodies indicates a previous exposure which is partially supported by the PCR negative results in sera. To clarify the potential sources of infection among wild boar population further studies with a large diversity of biological samples are surely needed.

To our knowledge this is the first report of the exposure of *C. burnetii* in wild boar in Portugal. Wild boar may be a potential source of infection, highlighting the importance of monitoring this population. Furthermore, and considering the low infectious dose for humans (Maurin and Raoult, 1999) and the finding of the same *C. burnetii* genotype in a wild boar and humans, suggesting transmission (Jado *et al*., 2012), the zoonotic risk associated with hunting activities especially when handling hunted wild boars should not be neglected.

Financial support

S. Anastácio is supported by the grant SFRH/BD/77823/2011 from Fundação para a Ciência e a Tecnologia, Lisbon, Portugal. This work was supported financially by the Center for Pharmaceutical Studies, University of Coimbra and by the strategic research Project PEst-OE/AGR/UI0772/2014 financed by the Foundation for Science and Technology (FCT).

6.2

Coxiella burnetii in wild ungulates at central Portugal

S. Anastácio, A. Gil, M. Almeida, M. M. Vieira-Pinto M.M., K. Sidi-Boumedine, G.J. da Silva. *C. burnetii* in wild ungulates at central Portugal.

Presented as a poster at the 6th Meeting of Wild Iberian Ungulates, 4th and 5th September 2015, São Pedro do Sul – Viseu, Portugal.

Abstract

Q fever, caused by *Coxiella burnetii*, is a zoonotic infection worldwide distributed. The role of wild animals in the transmission of the infection is recognized but little known. This study aimed to investigate the exposure to *C. burnetii* infection among wild boars and red deers.

A cross-sectional study was conducted during the hunting season 2014/2015 in the center of Portugal. Blood samples were collected from wild boar (n=47) and red deer (n=46) and tested, using a commercial ELISA, for specific antibodies anti-*C. burnetii* using a multi-antispecies conjugate.

Of the tested animals, an exposure to *C. burnetii* was shown in 6.4% (95% CI: 1.7 to 18.6%) of wild boars and 30.4% (95% CI: 18.2 to 45.9%) of red deers.

This study revealed a rate of exposure to *C. burnetii* in wild boars similar to that found in a previous study (5.6%) in the North of Portugal. The exposure in red deers was higher than in wild boars. Ruminants seem to play a major role in the infection cycle of *C. burnetii*. The large resistance of this bacterium in the environment enables the infection to other species as wild boars. Notwithstanding, and considering that only 1-10 bacteria are needed to infect humans, the zoonotic risk when handling hunted animals should not be neglected.

Keywords: Q fever, serosurvey, wild boar, red deer

C. burnetii has an impressively broad host range being present in virtually all animal kingdoms, including arthropods. Domestic ruminants are considered the main reservoirs of *C. burnetii*, but cats, dogs, rabbits, birds and others, have also been reported to be implicated in human disease/infection (OIE, 2015).

After the large Q fever outbreak occurred in the Netherlands (2007-2011) the role of wild animals as sources of Q fever in humans has been questioned (EFSA, 2010[a]). Some studies have been conducted and the detection of *C. burnetii* DNA in wild animals has been investigated in several countries. In the Netherlands, the bacterium

was found in free living roe deer (Rijks *et al.*, 2011). In Spain, it was found in roe deer (*Capreolus capreolus*), wild boar (*Sus scrofa*), European hare (*Lepus europeus*) (Astobiza *et al*., 2011). Very recently, the complexity of *C. burnetii* ecology was highlighted. It was proposed that red deer (*Cervus elaphus*) can maintain *C. burnetii* circulating in a region without third species. However, it is thought that most probably other wild and domestic host species are involved (Gonzalez-Barrio *et al*., 2015b). This study aimed to investigate the exposure to *C. burnetii* infection among wild boars and red deers from the Centre of Portugal.

A cross-sectional study was conducted during the hunting season 2014/2015 in the region of Idanha-a-Nova (39º55'N; 7º14'W) of Portugal. Blood samples were collected from wild boars (n=47) and red deers (n=46) using a convenience sampling strategy.

Each serum was tested in duplicate using a commercial indirect ELISA which is based on the use of an antigen (phase I and II) isolated from domestic ruminants (LSIVET Ruminant Milk/Serum Q Fever®, Laboratoire Service International, Lissieu, France). The ELISA was adapted for multi-species detection, using an anti-multi-species conjugate (protein AG) provided by the manufacturer. Before testing, an assay was performed to find the best working dilution for protein AG, using the ruminant positive and negative control sera provided in the kit. Briefly, serial dilutions of the protein AG were performed (from 1 to 1/100.000) and ruminant positive and negative control sera were tested in duplicate, using the ruminant conjugate and the protein AG dilutions. The mean optical densities (OD) of the positive and negative controls of each protein AG dilutions were compared with the mean OD of the ruminant conjugate and the dilution with an OD closer to the ruminant conjugate was used (Laboratoire Service International, personal communication, 2011). For the identification of *C. burnetii* positive and negative samples to use as control sera, a prior test is needed according to Cooper *et al*. (2011, 2012). Briefly, a screening of samples is carried out using the ruminant positive and negative control sera provided by the kit. The mean OD of duplicates is calculated, and samples are not considered if the coefficient of variation (CV) was > 10%. The pool of the three high-reacting (HR) and the three low-reacting (LR) sera (mean OD HR / mean OD LR ≥ 4) are then used as positive and negative control sera, respectively.

The ELISA was performed according to manufacturer's instructions. The ruminant conjugate was replaced by the protein AG. OD values were measured at 450 nm. Sample/positive percentages (S/P per cent) were calculated by the adjustment with the

negative control [S/P per cent=(OD_{sample}–$OD_{negative}$)/($OD_{positive}$–$OD_{negative}$) x100]. Sera with an S/P per cent <50% were considered negative. Samples with an S/P per cent between 50% and 75% were considered positive and those >75% were considered strongly positive (Cooper *et al.*, 2011, 2012).

In the total of 47 wild boars, three (6.4%; 95% CI: 1.7 to 18.6%) evidenced an exposure to *C. burnetii* and only one serum evidenced a strong positive result (S/P per cent = 138.6). There are only a few studies referring to the infection by *C. burnetii* in wild boars. In Japan, there was no evidence of exposure (Ejercito *et al*., 1993) while in Czech Republic, six percent (6%) of animals showed a seropositive result (Hubalek *et al*., 1993). But the herein obtained results are similar to that found in a previous study in the Northeast of Portugal (5.6%) (see 6.1).

Concerning red deers, the estimated rate of exposure was 30.4% (95% CI: 18.2 to 45.9%) of red deers and 8 sera (57.1%) evidenced a strong positive result (S/P per cent > 75%). The exposure in red deers appear to be higher than in wild boars and even higher to that found in Spain (14.1%) (González-Barrio *et al*., 2014). Similar results were obtained in other studies, such as in Czech Republic (25%) (Hubalek *et al*., 1993) but the use of different methodology (MAT) compromises comparison. Results similar to those here presented were obtained in farmed red deer in Spain (32%) but once again the methodology (IFA) differs (Ruiz-Fons *et al*., 2008). Globally, ruminants seem to play a major role in the infection cycle of *C. burnetii* which is supported by the evidence of *C. burnetii* shedding by naturally infected red deer pointing this wild ungulate as a true reservoir for *C. burnetii* (Gonzalez-Barrio *et al*., 2015[b]).

In the present study, it should be noted that the convenience sampling might compromise the representativity as well as the low sample size that increases the imprecision of the determination. Notwithstanding, the circulation and the exposure to *C. burnetii* was evidenced among wild ungulates in the centre region of Portugal. Further research should be done, by increasing the number of samples and by the detection of *C. burnetii* in organs such as spleen, feces, and vaginal swabs of the animals.

Financial support

S. Anastácio is supported by the grant SFRH/BD/77823/2011 from Fundação para a Ciência e a Tecnologia, Lisbon, Portugal. This work was supported financially by the Center for Pharmaceutical Studies, University of Coimbra.

7

Genotyping
Coxiella burnetii

7.1

Genotyping *Coxiella burnetii* from domestic ruminants in Portugal

S. Anastácio, N. Tavares, C. Cruz, K. Sidi-Boumedine, G.J. da Silva, G.J. Molecular screening for *Coxiella burnetii* in seropositive ruminant herds in Portugal. *Manuscript in submission.*

Abstract

Molecular epidemiology is essential in surveillance of *C. burnetii.* Multiple-Locus Variable Number Tandem-Repeat Analysis (MLVA) is one of the most discriminating methods for *C. burnetii* and it can be used directly from clinical samples. A cross-sectional study was conducted, between 2010 and 2013, in *Coxiella burnetii* seropositive ruminant herds, from the Northwest and Center of Portugal, to genotype *C. burnetii* DNA recovered from cattle, sheep and goats, to analyze the host and geographic distribution of the identified genotypes and to compare results with genotypes previously reported in Portugal and abroad. After a *C. burnetii* PCR testing in milk samples and vaginal swabs from dairy and meat herds, only PCR positive samples showing a Ct value ≤34 were selected for genotyping (n=31). A panel of six MLVA markers was used for genotyping using two different sets of primers. The results showed a strong consistency between the two sets of primers on the calcultion of the number of repeats. Seven MLVA-6 complete genotypes were obtained in this study and six were identified for the first time. All the profiles were obtained from milk samples originated from dairy ruminant farms. One genotype was identified in one cattle herd from the Northwest Portugal, showing a close relation with the other ones identified in the Center Portugal. These genotypes clustered with genotypes recently described in cattle from Spain and from several European countries, and also showed a close relation with genotypes previously identified in acute human infections in Portugal.

Keywords: MLVA-6, Q fever, ruminants, milk

Introduction

Coxiella burnetii, an intracellular bacterium with worldwide occurrence, is the causative agent of Q fever. The high resistance of *C. burnetii* makes Q fever as an important public health issue that should be considered worldwide, even in regions where it is unrecognized (EFSA, 2010[a]; OIE, 2015). A large range of domestic and wild animal species are receptive hosts of this bacterium. Infected animals usually develop a specific

serological response but often the infection remains asymptomatic (Maurin and Raoult, 1999). In humans, besides the existence of non-aparent infections, an acute illness may occur, and in a low proportion of cases long-term complications can develop (Million and Raoult, 2017). Q fever in humans is usually reported by the occurrence of outbreaks mainly in areas with infected livestock herds (ECDC, 2014; OIE, 2016).

Nowadays, molecular epidemiology is essential in surveillance of *C. burnetii* and in the investigation of Q fever outbreaks. A systematic genotyping provides a descriptive database enabling to monitor the temporal and geographical evolution of strains, thus helping to trace the origins of the outbreaks and to identify interspecies transmission. These data can help to explain different scenarios of dissemination and contribute to find efficient control measures (Roest *et al*., 2011[a]; Sidi-Boumedine and Rousset, 2011; Massung *et al*., 2012; Sulyok *et al*., 2014; OIE, 2015; Piñero *et al*., 2015).

The genetic heterogeneity of *C. burnetii* can be assessed by several molecular techniques. To date, among the typing methods that can be used directly on clinical samples, Multiple-Locus Variable Number Tandem-Repeat Analysis (MLVA) is one of the most discriminating methods for *C. burnetii* (Arricau-Bouvery *et al*., 2006; Svraka *et al*., 2006) (also see 1.5.2.1. Identification of the agent – Molecular methods). So far, two *C. burnetii* MLVA genotyping schemes have been described, one uses two panels of markers comprising a totality of 17 *loci* (Arricau-Bouvery *et al*., 2006) and the other one uses seven *loci* (Svraka *et al*., 2006). Overtime, some modifications have been proposed to the previously described schemes. For example, during the Q fever outbreak in The Netherlands, alternative strategies to the scheme described by Arricau-Bouvery *et al*. (2006) were developed, using a lower number of markers to simplify the analysis, and selecting different primers focusing short repeat units to increase the chance of successful amplification (Klaassen *et al*., 2009; de Bruin 2009, Tilburg *et al*., 2012[c]). Globally, MLVA genotyping proved to be discriminatory, useful to be used directly from clinical samples and thus a valuable tool in epidemiological studies. Furthermore, the availability of a free-access database at the internet, increased the interest in this method to characterize *C. burnetii* strains circulating in a given region in a normal context or in case of outbreak (Tilburg *et al*., 2012[c]; Boarbi *et al*., 2014; Sulyok *et al*., 2014[b]). However, a lack of standardization and harmonization occurs because few studies used the same MLVA typing scheme or marker panels, hampering the comparison of results (Sidi-Boumedine *et al*., 2015).

In Portugal, evidence of *C. burnetii* infection based on detection of DNA has been reported in domestic (Clemente *et al*., 2009; Santos *et al*., 2012; Cumbassá *et al*., 2015; Anastácio *et al*., 2016), in wild and in captive animals (Clemente *et al*., 2008; Cumbassá *et al*., 2015). Nonetheless, the data on the genotypes that circulate in the country are limited regarding the host and geographical origin (Santos *et al*., 2012; Cumbassá *et al*., 2015).

The present study aimed: i) to genotype *C. burnetii* DNA recovered from cattle, sheep and goats, ii) to compare the MLVA typing results using two different sets of primers described in the literature, iii) to analyze the host distribution of the identified genotypes, iii) to evaluate the geographic distribution of genotypes from the Northwest and Center Portugal and iv) to compare results with genotypes previously reported in Portugal and abroad.

Materials and Methods

Samples collection

A cross-sectional study was conducted in 140 *Coxiella burnetii* seropositive ruminant herds tested between 2010 and 2013, located in the Northwest (n=62) and in the Center (n=78) regions of Portugal. Sampling was performed under the agreement of farmers. The sample collection strategy was different in dairy (n=108) and in meat herds (n=32). In dairy herds, one bulk tank milk sample was collected per herd, while in meat herds, random individual samples (i.e. vaginal mucus and milk) were collected from a maximum of 15 females per herd.

The DNA isolation was performed using the QIAmp DNA Mini kit (Qiagen, Izasa, Portugal), according to manufacturer's instructions. All the DNA samples were screened firstly by conventional PCR. Then, PCR positive samples were further tested by quantitative real-time qPCR assay to confirm positive results and to obtain the Ct value as previously described (Anastácio et al., 2016).

The bacterial load and the quality of the DNA influence genotyping results (de Bruin, 2009). It has been observed that samples showing PCR cycle threshold (Ct) values <32 yield complete genotypes, Ct values of 32-34 give only partial genotypes are obtained and Ct values >34 are poorly typable (Roest et al., 2011[a]). So, a Ct value ≤34 was the criterion to select PCR positive samples for genotyping.

From the 32 antibody positive meat herds, PCR positive results were obtained in five herds: milk samples from cattle (n=4) and goats (n=6) and vaginal swabs from cattle (n=1)

and sheep (n=2). Among these, only ten samples accomplished the inclusion criterion: cattle milk samples (n=2), goat milk samples (n=6) and sheep vaginal swabs (n=2). Regarding the 108-antibody positive dairy herds, 39 showed a PCR positive result and 21 accomplished the inclusion criterion: cattle (n=18), sheep (n=2) and sheep-goat (n=1).

Multiple-Locus Variable number tandem repeats Analysis (MLVA)

MLVA genotyping was performed by single PCRs targeting six microsatellite markers (MLVA-6) belonging to the panel two described by Arricau-Bouvery *et al*. (2006), as performed in other studies (Astobiza *et al*., 2012; Santos *et al*., 2012; Ceglie *et al*., 2015): Ms27, Ms28 and Ms34 contain repeat units of six base pairs and Ms23, Ms24 and Ms33 contain repeat units of seven base pairs (Arricau-Bourvery *et al*., 2006). Each one of the above-mentioned microsatellite was tested twice, using two different sets of primers: set 1 described by Arricau-Bourvery *et al*. (2006) and set 2 indicated by Klaassen *et al*. (2009) and Tilburg *et al*. (2012[c]) (Table 19).

Table 19: Sets of primers targeting six microsatellite markers in MLVA

Set	Microsatellite	Primer (Forward)	Primer (Reverse)	Repeat Unit Size (pb)	Reference
1	Ms27	TTTTGAGTAAAGGCAACCCAAT	CAAACGTCGCACTAACTCTACG	6	Arricau Bouvery *et al*., 2006
	Ms28	TAGCAAAGAAATGTGAGGATCG	ATTGAGCGAGAGAATCCGAATA	6	
	Ms34	TGACTATCAGCGACTCGAAGAA	TCGTGCGTTAGTGTGCTTATCT	6	
	Ms23	GGACAAAAATCAATAGCCCGTA	GAAAACAGAGTTGTGTGGCTTC	7	
	Ms24	ATGAAGAAAGGATGGAGGGACT	GATAGCCTGGACAGAGGACAGT	7	
	Ms33	TAGGCAGAGGACAGAGGACAGT	ATGGATTTAGCCAGCGATAAAA	7	
2	Ms27	TCTTTATTTCAGGCCGGAGT	GAACGACTCATTGAACACACG	6	Klaasen *et al*., 2009
	Ms28	AGCAAAGAAATGTGAGGATCG	GCCAAAGGGATATTTTTGTCCTTC	6	
	Ms34	TTCTTCGGTGAGTTGCTGTG	GCAATGACTATCAGCGACTCGAA	6	
	MS23	CGCMTAGCGACACAACCAC	GACGGGCTAAATTACACCTGCT	7	Tilburg *et al*., 2012[c]
	Ms24	TGGAGGGACTCCGATTAAAA	GCCACACAACTCTGTTTTCAG	7	
	Ms33	TCGCGTAGCGACACAACC	GTAGCCCGTATGACGCGAAC	7	

PCR amplifications occurred in a total volume of 25 µl containing 1 ng of DNA, 1× PCR reaction buffer (Invitrogen®), 1 U of *Taq* DNA polymerase (Platinum *Taq* DNA Polymerase, Invitrogen®), 200 µM of each deoxynucleotide triphosphate (ThermoScientific®), 3 mM MgCl2 (Invitrogen®), 0.1 mg/ mL BSA (ThermoScientific®) and 0.3 µM of each flanking primer. The DNA amplification reaction was performed in a Biometra Thermocycler (Biometra®, Göttingen, Germany). Initial denaturation at 94°C for 5 minutes was followed by 40 cycles consisting of denaturation at 94°C for 30 s, primer annealing at 60ºC (Set1: Ms27, Ms28; Set2: Ms24,

Ms27) or 62º (Set1: Ms23, Ms24, Ms33, Ms34; Set 2: Ms23, Ms28, Ms33, Ms34) for 30 s, and elongation at 70°C for 1 min. The final extension step was at 72°C for 10 min. Five microliters of amplification product were loaded on a 4% standard agarose gel (MetaPhor, Lonza®) stained with ethidium bromide and submitted to 3,5V/cm for three hours. Gels were visualized under UV light, and photographed (Vilber Lourmat®, Marnes-la-Vallée, France).

DNA from the Nine Mile phase II strain (RSA 493 isolate) was used as reference in all the experiments to normalize the number of repetitions in each assay and to compare the MLVA-6 profiles. For each MLVA marker, the number of repeats was determinated by extrapolation by using the fragment length of the sample and the fragment length of the reference strain. According to *in silico* analysis, the genotype of the Nine Mile strain is 9-27-4-6-9-5 for markers Ms23-Ms24-Ms27-Ms28-Ms33-Ms34, respectively (Available online: http://mlva.u-psud.fr/MLVAnet/spip.php?rubrique50 accessed on 01/2015). The MLVA codification of the results was based on the methodology used by Arricau-Bouvery and colleagues (Arricau-Bouvery *et al*., 2006) and the new MLVA recommendations issued by Université de Paris-Sud (Available online: http://mlva.u-psud.fr/MLVAnet/spip.php?rubrique50). Distinct genotypes were considered when the number of repeats of at least one of the 6 markers differed, as indicated by Svraka *et al*. (2006) and recommended on the above-mentioned website.

Data analysis

Data obtained from MLVA-6 typing was imported into Multi-Variate Statistic Package v3.22 (Kovach Computing Services, Anglesey, Wales, United Kingdom), together with published *C. burnetii* genotypes from different countries that used the same MLVA-6 *C. burnetii* typing. The latest data were obtained by accessing the International *C. burnetii* Cooperative Database (http://mlva.u-psud.fr/mlvav4/genotyping/). A cluster analysis was performed by Unweighted Pair Group Method with Arithmetic mean clustering (UPGMA) to determine genetic similarity among DNA (Multi-Variate Statistic Package v3.22). Also, the discriminatory ability of the MLVA method of each marker individually was calculated using Simpson's index of diversity.

Results

Among the 31 DNA samples used for MLVA-6 typing, a complete genotype was achieved in seven samples (22.6%; CI 95%: 10.3-41.5%); a partial genotype was obtained

in 15 samples (48.4%; CI 95%: 30.6-66.6%) and in nine samples (29.0%; 95% CI: 14.9-48.2%) none of the markers was amplified. The mean of Ct values was lower in samples showing a complete genotype (30.3; range from 26.1 to 31.9) comparing with those showing a partial genotype (31.9; range from 30.7 to 33.5) or those with no amplifications (32.36; range from 30,89 to 33.78) (Table 20).

Table 20: Positive *C. burnetii* DNA samples used for MLVA typing. Geographical origin, year of collection, host, Ct value and typing result are indicated.

Sample ID	Origin	Year	Host	Sample type	Ct	Genotype status
09-05	Portugal – Center	2009	Goat/Sheep	Bulk Tank Milk	32,64	ND[a]
09-10	Portugal – Center	2009	Sheep	Bulk Tank Milk	33,78	ND[a]
10-04	Portugal – Northwest	2010	Cattle	Bulk Tank Milk	31,9	Complete
10-09	Portugal – Northwest	2010	Cattle	Bulk Tank Milk	31,13	Partial
11-26	Portugal – Center	2011	Cattle	Bulk Tank Milk	31,16	Complete
11-29	Portugal – Center	2011	Cattle	Bulk Tank Milk	32,69	Partial
11-31	Portugal – Center	2011	Cattle	Bulk Tank Milk	27,7	Complete
11-34	Portugal – Center	2011	Cattle	Bulk Tank Milk	32,76	Partial
11-36	Portugal – Center	2011	Cattle	Bulk Tank Milk	32,08	Partial
11-40	Portugal – Center	2011	Cattle	Bulk Tank Milk	32,88	Partial
12-1035	Portugal – Center	2012	Sheep	Vaginal swab	32,5	ND[a]
12-1036	Portugal – Center	2012	Sheep	Vaginal swab	31,4	ND[a]
12-1038	Portugal – Center	2012	Goat	Milk	30,89	Partial
12-1039	Portugal – Center	2012	Goat	Milk	31,37	Partial
12-1041	Portugal – Center	2012	Goat	Milk	31,37	Partial
12-1043	Portugal – Center	2012	Goat	Milk	32,64	Partial
12-1092	Portugal – Center	2012	Cattle	Bulk Tank Milk	31,59	Complete
12-1107	Portugal – Center	2012	Cattle	Milk	31,75	ND[a]
12-1243	Portugal – Center	2012	Cattle	Milk	26,1	Complete
12-1040	Portugal – Center	2012	Goat	Milk	30,89	ND[a]
12-1042	Portugal – Center	2012	Goat	Milk	33,78	ND[a]
13-1215	Portugal – Center	2013	Cattle	Bulk Tank Milk	31,87	Complete
13-JT	Portugal – Center	2013	Sheep	Bulk Tank Milk	31,88	Complete
13-01	Portugal – Northwest	2013	Cattle	Bulk Tank Milk	33,5	Partial
13-19	Portugal – Northwest	2013	Cattle	Bulk Tank Milk	31,2	Partial
13-52	Portugal – Northwest	2013	Cattle	Bulk Tank Milk	30,7	Partial
13-59	Portugal – Northwest	2013	Cattle	Bulk Tank Milk	33,07	Partial
13-72	Portugal – Northwest	2013	Cattle	Bulk Tank Milk	30,75	Partial
13-79	Portugal – Northwest	2013	Cattle	Bulk Tank Milk	32,51	ND[a]
13-82	Portugal – Northwest	2013	Cattle	Bulk Tank Milk	31,61	Partial
13-90	Portugal – Northwest	2013	Cattle	Bulk Tank Milk	33,46	ND[a]

[a] ND – Not determined due to no amplification

PCR amplification with the Set 1 primers was not successful for Ms23 *locus*, while for the other *loci* it was possible to determine the number of repeats for Ms24 (51.6%), Ms27 (54.8%), Ms28 (45,2%), Ms33 (29.0%) and Ms34 (51.6%). By using the Set 2, it was not possible to determine the number of repeats for Ms27 *locus.* However, the number of

repeats was achieved for Ms23 (45.2%), Ms24 (61.3%), Ms28 (58.1%), Ms33 (35.5%) and Ms34 (19.3%) (Table 21).

Table 21: Results on MLVA-6 gentotyping using the two different sets of primers.

Set 1							Set 2					
Ms23	**Ms24**	**Ms27**	**Ms28**	**Ms33**	**Ms34**	**Sample ID**	**Ms23**	**Ms24**	**Ms27**	**Ms28**	**Ms33**	**Ms34**
9	27	4	6	9	5	**NM RSA 493**	9	27	4	6	9	5
ND [a]	ND [a]	2	6	ND [a]	9	**10-04**	8	15	ND [a]	6	9	ND [a]
ND [a]	15	ND [a]	6	ND [a]	9	**10-09**	7	15	ND [a]	6	ND [a]	9
ND [a]	15	2	6	9	9	**11-26**	7	15	ND [a]	6	9	9
ND [a]	ND [a]	ND [a]	8	ND [a]	8	**11-29**	ND [a]	14	ND [a]	8	ND [a]	8
ND [a]	15	2	8	9	8	**11-31**	8	15	ND [a]	8	9	8
ND [a]	ND [a]	2	8	ND [a]	8	**11-34**	7	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]
ND [a]	14	ND [a]	8	9	ND [a]	**11-36**	7	14	ND [a]	8	9	ND [a]
ND [a]	15	2	ND [a]	ND [a]	8	**11-40**	8	ND [a]	ND [a]	8	ND [a]	ND [a]
ND [a]	19	4	ND [a]	ND [a]	2	**12-1038**	ND [a]	ND [a]	ND [a]	4	ND [a]	ND [a]
ND [a]	15	2	6	9	8	**12-1039**	ND [a]	15	ND [a]	6	ND [a]	8
ND [a]	ND [a]	2	ND [a]	ND [a]	8	**12-1041**	ND [a]	15	ND [a]	8	ND [a]	ND [a]
ND [a]	ND [a]	2	ND [a]	9	8	**12-1043**	ND [a]	14	ND [a]	ND [a]	ND [a]	ND [a]
ND [a]	15	2	8	9	8	**12-1092**	7	15	ND [a]	8	ND [a]	ND [a]
ND [a]	15	2	6	9	8	**12-1243**	7	15	ND [a]	6	9	8
ND [a]	14	2	ND [a]	9	9	**13-1215**	7	14	ND [a]	8	9	ND [a]
ND [a]	15	2	ND [a]	9	8	**13-JT**	7	15	ND [a]	8	9	ND [a]
ND [a]	14	ND [a]	ND [a]	ND [a]	ND [a]	**13-01**	ND [a]	14	ND [a]	ND [a]	ND [a]	ND [a]
ND [a]	14	2	ND [a]	ND [a]	ND [a]	**13-19**	7	14	ND [a]	8	9	ND [a]
ND [a]	14	2	8	ND [a]	ND [a]	**13-52**	7	14	ND [a]	8	9	ND [a]
ND [a]	14	2	8	ND [a]	7	**13-59**	ND [a]	14	ND [a]	ND [a]	ND [a]	ND [a]
ND [a]	15	2	8	ND [a]	ND [a]	**13-72**	7	15	ND [a]	8	9	ND [a]
ND [a]	ND [a]	ND [a]	8	ND [a]	ND [a]	**13-82**	ND [a]	14	ND [a]	8	11	ND [a]

[a] ND – Not determined due to no amplification

Globally, a higher percentage of amplifications occurred using Set 2 except for *locus* Ms34. Despite the differences regarding the success of PCR amplification, the calcultion of the number of repeats revealed a strong consistency between the two sets of primers. This consistency allowed a global interpretation, considering the missing results from the two sets (Ms23 and Ms27) (Table 22).

On this basis, considering the six markers used to characterize genotypically *C. burnetii*, it was observed that Ms24, Ms27, Ms28 and Ms34 allowed the characterization of a higher percentage of samples (67.7%, 54.8%, 64.5% and 51.6% respectively) than Ms23 and Ms33 (45.16% in each one). The Simpson's index of diversity calculated for each marker ranged from 0.928 for Ms23 and Ms33, to 0.952 for Ms24, as displayed in

Table 22. Seven complete genotypes were found in cattle (n=6) and sheep (n=1) and 15 partial genotypes were obtained in cattle (n=11) and goats (n=4) (Table 19 and 21).

Table 22: Global partial and complete profiles on MLVA-6 typing.

	Ms23	**Ms24**	**Ms27**	**Ms28**	**Ms33**	**Ms34**
Reference strain						
NM RSA 493	9	27	4	6	9	5
Samples						
10-04	8	15	2	6	9	9
10-09	7	15	-	6	-	9
11-26	7	15	2	8	9	9
11-29	-	14	-	8	-	8
11-31	8	15	2	8	9	8
11-34	7	-	2	8	-	8
11-36	7	14	-	8	9	-
11-40	8	15	2	8	-	8
12-1038	-	19	4	4	-	2
12-1039	-	15	2	6	9	8
12-1041	-	15	2	8	-	8
12-1043	-	14	2	-	9	8
12-1092	7	15	2	8	9	8
12-1243	7	15	2	6	9	8
13-1215	7	14	2	8	9	9
13-JT	7	15	2	8	9	8
13-01	-	14	-	-	-	-
13-19	7	14	2	8	9	-
13-52	7	14	2	8	9	-
13-59	-	14	2	8	-	7
13-72	7	15	2	8	9	-
13-82	-	14	-	8	11	-
Number of alleles	2	3	2	3	2	4
Number of samples with results	14	21	17	20	14	16
Simpson's index of diversity	0,928	0,952	0,938	0,949	0,928	0,935

Of the complete genotypes, only one profile (7-15-2-8-9-8) occurred simultaneously in two dairy herds (cattle and sheep), both from the center of Portugal. Among the remaining, a discrepancy was observed in one or two *loci*. Considering all the profiles, an exception was observed in one goat milk sample, regardless the partial profile obtained. This sample revealed a different number of repeats in the four *loci* that it was possible to characterize: Ms24, Ms27, Ms28 and Ms34 showed 19, 4, 4 and 2 tandem repeat units, respectively (Table 22).

Regarding the geographical distribution of genotypes, DNA samples were selected from the Northwest (n=10) and from the Center (n=21) of Portugal. In both regions, complete (one and six, respectively) and partial (seven and eight, respectively) genotypes were observed as well as an absence of amplification in all the *loci* (two and seven,

respectively). One complete genotype identified in the Northwest showed a discrepancy from the others in two or three *loci* (Figure 16). Figure 1 displays an UPGMA cluster analysis that includes all the complete and partial profiles lacking only one marker.

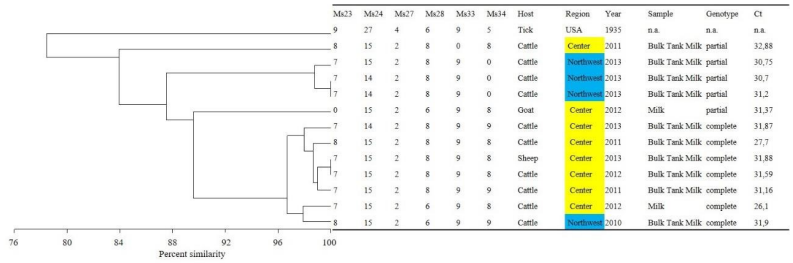


Figure 16: UPGMA cluster analysis of *C. burnetii* genotypes obtained in this study using multiple-locus variable-number tandem-repeat analysis. Only partial genotypes lacking one marker were considered (scheduled with 0). Number of repeats per locus, hosts, geographical origin, year of collection, type of genotype, and Ct values are indicated. (Yellow: Portugal-Center; Blue: Portugal-Northwest).

The complete profiles herein identified were compared with those from other studies conducted in Portugal, and it was found that none have been previously described (Table 23).

Table 23: *C. burnetii* genotypes described in Portugal.

Host	Origin	Ms23	Ms24	Ms27	Ms28	Ms33	Ms34	Source
Cattle	Northwest	8	15	2	6	9	9	this study
Cattle	Center	8	15	2	8	9	8	this study
Cattle	Center	7	15	2	6	9	8	this study
Cattle	Center	7	15	2	8	9	9	this study
Cattle	Center	7	15	2	8	9	8	this study
Sheep	Center	7	15	2	8	9	8	this study
Cattle	Center	7	14	2	8	9	9	this study
Sheep	Center	3	9	4	5	7	2	Cumbassá et al. 2015
Goat	Azores	6	13	2	7	9	9	Cumbassá et al. 2015
Goat	Center	8	9	4	5	7	2	Cumbassá et al. 2015
Goat	Center	1	11	2	3	7	3	Santos et al. 2012
Mongoose	Northeast	0	16	3	7	7	2	Cumbassá et al. 2015
Mongoose	South	0	24	3	7	8	2	Cumbassá et al. 2015
Mongoose	Center	0	15	3	7	7	2	Cumbassá et al. 2015
Human	Center	3	9	4	5	7	2	Santos et al. 2012
Human and goat	Center	1	11	2	3	7	3	Santos et al. 2012
Human	Center	3	14	3	7	7	2	Santos et al. 2012
Human	South	3	16	3	7	7	2	Santos et al. 2012
Human	South	3	16	2	7	7	2	Santos et al. 2012
Human	Center-South	2	14	3	7	6	2	Santos et al. 2012
Human	Center	3	18	3	7	7	2	Santos et al. 2012
Number of alleles		8	8	3	5	4	5	n.a.

n.a. – Not applicable

Furthermore, the consultation of the International *Coxiella burnetii* Cooperative Database (http://mlva.u-psud.fr/mlvav4/genotyping/ - accessed on the 24th February 2018) did not show a complete compatibility between the genotypes found in this study and those published. For comparison purposes, an analysis was conducted accepting a maximum difference of three markers between our profiles and those published in the international database. Results are displayed in Table 24.

Table 24: Complete profiles of this study are compared with published genotypes (Source: International *Coxiella burnetii* Cooperative Database, accessed on 24th February 2018)

Sample	Profile	Database profile	Different *loci*	Number of reports	Geographical origin	Host	Year
10-04	**8-15-2-6-9-9**	6-**15**-**2**-7-**9**-12	3	1	Spain	Cattle	2011
		5-13-**2**-7-**9**-**9**	3	1	Spain	Cattle	2010
		6-13-**2**-**6**-**9**-10	3	1	Germany	Cattle	2010
		6-13-**2**-7-**9**-**9**	3	31	Hungary, Netherlands, Italy, Switzerland, France, Spain, and **Portugal (mainland and Azores)**	Cattle, sheep, goat and human	1994-2012
		6-12-**2**-7-**9**-**9**	3	7	France	Cattle Goat	n.a.
11-26	**7-15-2-8-9-9**	5-13-**2**-7-**9**-**9**	3	1	Spain	Cattle	2010
		6-**15**-**2**-7-**9**-12	3	1	Spain	Cattle	2011
		6-13-**2**-7-**9**-**9**	3	31	Hungary, Netherlands, Italy, Switzerland, France, Spain, and **Portugal (mainland and Azores)**	Cattle, sheep, goat and human	1994-2012
		6-12-**2**-**8**-**9**-8	3	2	France	Cattle	n.a.
		6-12-**2**-7-**9**-**9**	3	7	France	Cattle Goat	n.a.
11-31	**8-15-2-8-9-8**	6-12-**2**-**8**-**9**-**8**	2	2	France	Cattle	n.a.
		6-13-**2**-7-**9**-**8**	3	1	Netherlands	Cattle	2011
		6-**15**-**2**-7-**9**-12	3	1	Spain	Cattle	2011
12-1243	**7-15-2-6-9-8**	6-13-**2**-7-**9**-**8**	3	1	Netherlands	Cattle	2011
		6-**15**-**2**-7-**9**-12	3	1	Spain	Cattle	2011
		6-13-**2**-**6**-**9**-10	3	1	Germany	Cattle	2010
		9-7-4-**6**-**9**-**8**	3	1	Sweden	Sheep	n.a.
		6-12-**2**-**8**-**9**-8	3	2	France	Cattle	n.a.
12-1092 and 13-JT	**7-15-2-8-9-8**	6-12-**2**-**8**-**9**-**8**	2	2	France	Cattle	n.a.
		6-13-**2**-7-**9**-**8**	3	1	Netherlands	Cattle	2011
		6-**15**-**2**-7-**9**-12	3	1	Spain	Cattle	2011
13-1215	**7-14-2-8-9-9**	5-13-**2**-7-**9**-**9**	3	1	Spain	Cattle	2010
		6-14-2-7-9-11	3	2	Italy	Cattle	2011
		6-13-**2**-7-**9**-**9**	3	31	Hungary, Netherlands, Italy, Switzerland, France, Spain, and **Portugal (mainland and Azores)**	Cattle, sheep, goat and human	1994-2012
		6-12-**2**-**8**-**9**-8	3	2	France	Cattle	n.a.

n.a. – not available

Overall, the profiles herein identified differed in three or more markers from those published at the international *Coxiella burnetii* cooperative database. Profiles differing in

three markers have been reported in Portugal (mainland and Azores) and in other European countries such as Hungary, the Netherlands, Sweden, Switzerland, Italy, France and Spain. An exception was observed in two genotypes (7-15-2-8-9-8 and 8-15-2-8-9-8) from three samples (BTM-12-1092, BTM-13-JT and BTM-11-31) which differed in only two markers from the genotype 6-12-2-8-9-8 previously identified in France (Arricau-Bouvery et al., 2006).

Figure 17 shows an UPGMA cluster analysis comparing MLVA-6 *C. burnetii* profiles identified in Portugal so far and others available on the online International *Coxiella burnetii* Cooperative Database.

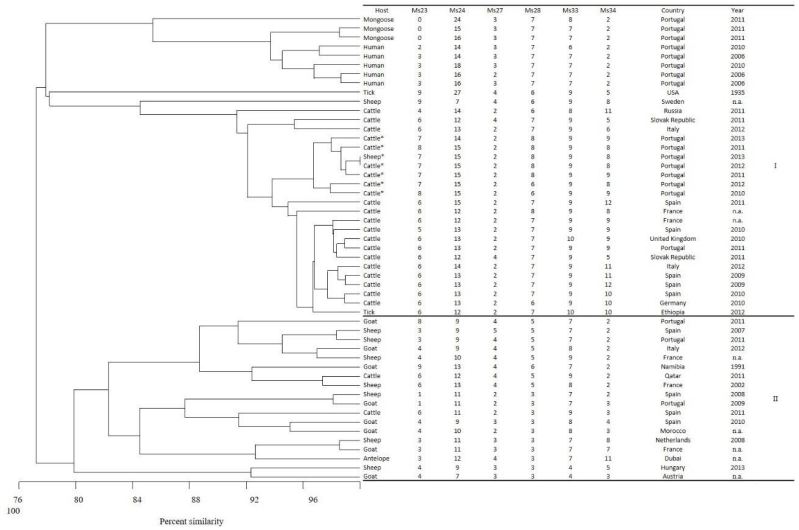


Figure 17: UPGMA cluster analysis of *C. burnetii* genotypes using multiple-locus variable-number tandem-repeat analysis. Hosts, number of repeats per locus, geographical origin and year of collection are indicated. *Genotypes obtained in this study.

This analysis shows *C. burnetii* MLVA profiles clustering in two main groups (I and II) (Figure 17). Group I appear genetically more homogeneous than group II, with the majority of the samples showing >90% of genetic similarity and including most of the Portuguese *C. burnetii* DNA. Within group I, two subgroups can be recognized: one subgroup comprises some of the MLVA profiles previously described in Portugal, in

human acute infections and in mongoose. The other includes all the profiles identified in this study, the profiles identified in cattle from several European countries such as Spain, France, Italy, United Kingdom, Russia and Slovak Republic, one profile identified in sheep from Sweden, and two profiles identified in ticks from Ethiopia. Group II shows a higher genetic heterogeneity and have been associated mainly with sheep and goats in several countries (Europe and other continents).

Discussion

Molecular epidemiology plays a crucial role on the study of genetic surveillance of *C. burnetii.* In Portugal, information about the genetic diversity of *C. burnetii* is scarce. To our best knowledge, only two studies describe *C. burnetii* genotypes from a few clinically affected cases in humans and domestic ruminants, and from wild species at the Center-South Regions (Santos *et al*., 2012; Cumbassá *et al*., 2015).

The present study was part of a whole project developed in apparently healthy herds to screen for *C. burnetii*. Genotyping was performed in *C. burnetii* DNA positive samples collected from antibody positive ruminant farms. A complete genotype was achieved in a low percentage of samples (22.6%), which is probably due to a poor quality of *C. burnetii* DNA in samples and/or a low bacterial load leading to failures in PCR amplification (de Bruin, 2009). In fact, the non-typeable samples showed Ct values above 30.7 and it has been recognized that typeability might be compromised in samples showing a Ct>31 (Roest *et al*., 2011[a], de Bruin *et al*., 2012). The misidentification of complete genotypes has been commonly reported regardless the markers or the primers used for genotyping purpose (Roest *et al*., 2011[a], Astobiza *et al*., 2012; Santos *et al*., 2012; Tilburg *et al*., 2012[c]; Sulyok *et al*., 2014[b]; Ceglie *et al*., 2015).

Since the first description of MLVA schemes for *C. burnetii* genotyping (Arricau-Bouvery *et al*., 2006; Svraka *et al*., 2006), alternative approaches have been developed, to simplify analysis or to overcome technical difficulties subsequently identified (Klaassen *et al*., 2009; Roest *et al*., 2011[a]). Nowadays, a panel of six *loci* (MLVA-6) is frequently used (Astobiza *et al*., 2012; Santos *et al*., 2012; Sulyok *et al*. 2014[b]; Ceglie *et al*., 2015) but different primers for the same *loci* have been described (Klaassen *et al*., 2009; de Bruin, 2009; Roest *et al*., 2011[a]; de Bruin *et al*., 2012; Vincent *et al*., 2016). This study was performed with the MLVA-6 panel using two sets of primers: one set (Set 2) used in previous national studies (Santos *et al*., 2012; Cumbassá *et al*., 2015) described by Klaassen *et al*. (2009) and Tilburg *et al*. (2012[c]), which would be

useful for comparison of our data; and a second set of primers (Set 1) described by (Arricau-Bouvery *et al*., 2006) used to overcome the non-amplification of the *locus* Ms27 with the former set.

Indeed, we observed that PCR amplification was not successful for *locus* Ms23 with Set 1 and for Ms27 with Set 2. For the remaining markers, the results were consistent and an identical profile was obtained. The consistency of the results obtained with both sets indicated that results of the alternative set could be accepted. A higher percentage of amplification was obtained in Set 2 than in Set 1, except for Ms34, that might be due to the use of primers targeting short repeat units, which may increase the chance of successful amplification (Klaassen *et al*., 2009; de Bruin 2009, Tilburg *et al*., 2012[c]). Furthermore, the lower percentage of amplification observed in markers Ms23 and Ms33, was also reported in other studies (Sidi-Boumedine *et al.*, 2015 [b]; Aurélien *et al*., 2017).

Our data showed that the two primer sets used in parallel can complement the profiles obtained; neverteless, some samples did not amplify and complete/partial profiles were only achieved with a Ct<32, sustaining previous suggestion that the amount and quality of DNA is a key component on successful MLVA genotyping (Roest *et al*., 2011[a], de Bruin *et al*., 2012). Seven MLVA-6 complete genotypes were obtained in this study and six were identified for the first time. The limited data on *C. burnetii* genotypes in Portugal turns the report of novel genotypes predictable (Frangoulidis *et al*., 2014; Sulyok *et al*., 2014). All the profiles were originated from dairy ruminant farms, due to the non-detection or non-typeability of *C. burnetii* DNA in meat farms or in other animal species (see chapters 3, 4, 5 and 6). All the genotypes herein identified were obtained from milk samples. Milk is considered an important shedding route in ruminants, but small ruminants show an intermittent shedding beginning mostly after parturition (Guatteo *et al*., 2007; Roest *et al*., 2012), which might explain the identification of genotypes mainly in dairy cattle (one common genotype in sheep and in cattle).

Regarding the geographical distribution, one genotype was identified in one cattle herd from the Northwest Portugal. This genotype seems to be closely related with the other ones, but the low number of genotypes hinders the regional comparison and a regional geographical nidality of genotypes.

MLVA is considered a high discriminatory method (Arricau-Bouvery *et al*., 2006). The genetic diversity of *C. burnetii* circulating in domestic ruminants was demonstrated by the finding of six novel genotypes, despite their close genetic relationship. Also, they were closely related with genotypes recently described in cattle from Spain and from

several European countries (Arricau-Bouvery *et al*., 2006; Astobiza *et al*., 2012; Tilburg *et al*., 2012[b]; Ceglie *et al*., 2015; Piñero *et al*., 2015; International *C. burnetii* Cooperative Database), suggesting a host specificity and circulation of closely genetic related *C. burnetii* in cattle from European countries. This finding is supported by other studies (Piñero *et al*., 2015; Aurélien *et al*., 2017). Additionally, the concept of geographical nidality also might occur in a wider perspective since European countries seem to act as one single region with no borders. This might be explained by cattle circulation between countries, and a clone might disseminate by the introduction in the herds of asymptomatic *C. burnetii* infected animals (Tilburg *et al*., 2012[b]). The hypothesis that *C. burnetii* may undergo species-specific adaptations showing an association with a specific host has been recently proposed since *C. burnetii* genotypes seem to cluster by host species (Frangoulidis *et al*., 2014; Aurélien *et al*, 2017). In this context, a common pool of *C. burnetii* strains infecting cattle seems to exist in Europe (Piñero *et al*., 2015).

The genotypes herein identified showed a close relation with genotypes previously identified in acute human infections in different regions of Portugal (Santos *et al*., 2012). This finding suggests that a link might occur between human and cattle infection, but also reinforces the hypothesis that *C. burnetii* may evolve genetically and adapt to different hosts reinforcing the hypothesis of host specificity of *C. burnetii* genotypes circulating in a large geographic region.

Finally, special attention should be given to occupational activities requiring contact with animals, and manipulation of milk and milk products in inefected herds, or even the consumption of unpasteurized milk that should not be neglected as a source of infection to humans (Loftis *et al*., 2010).

Conclusions

In conclusion, a close genetic relationship was observed among *C. burnetii* from unrelated dairy ruminant farms in North and Center of Portugal. Yet, six novel genotypes were identified mainly in cattle herds. These genotypes are closely related to those identified in cattle from other European countries and in acute human infections from Portugal, suggesting that a common pool of *C. burnetii* strains that infect cattle exists in Europe and may play a role in human infection. As one of these genotypes was also found in one sheep dairy farm in the same region, further studies on *C. burnetii* genotyping in

Portugal are required, to better elucidate the host specificity and the geographical nidality of particular *C. burnetii* genotypes.

Financial support

S. Anastácio is supported by the grant SFRH/BD/77823/2011 from Fundação para a Ciência e a Tecnologia, Lisbon, Portugal. This work was supported financially by the Center for Neuroscience and Cell Biology (CNC), University of Coimbra.

8

Integrated overview

Q fever is a disease shared by human and animal population. In humans the infection could be appointed as an accidental occurrence, as humans are not described as reservoirs of infection and the transmission between humans has been reported as occasional (Maurin and Raoult, 1999). So, it is globally accepted that Q fever is centred in animal population. The main objective of this research was to characterize the epidemiology of *C. burnetii* in animals in Portugal, which is barely known, and consequently, to provide useful data in the context of One Health approach. Serologic and molecular methods were used to unravel the potential sources of infection and determine the status of infection in animals. The study was conducted with the concern of using standardized methodologies to enable comparison with other international studies.

Domestic ruminants have been recognized as important reservoirs of *C. burnetii* (OIE, 2015). Therefore, initially a serologic survey was conducted in these species to screen for specific antibodies anti-*C. burnetii*, namely in cattle and small ruminants from the Centre region, and cattle from the Northwest region of Portugal.

At the Centre region, the herd prevalence was higher in small ruminants (32.6%) than in cattle (23.5%). Results obtained for antibodies screening in BTM are comparable to those obtained in serum samples (Guatteo *et al*., 2007[a]; Nielsen *et al*., 2011; van den Brom *et al*., 2012), so BTM samples were also used in dairy herds. Antibody positivity in BTM was higher in small ruminant herds (51.6%) than in cattle herds (37.8%), which was consistent with the serosurvey results obtained in both herds type.

Notwithstanding, a discrepancy was observed: as antibody positivity appeared to be higher in the BTM survey than in the serosurvey. This might be explained by the mean herd size, which was higher in the BTM survey than in the serologic survey. Our results (Chapter 3) showed that in domestic ruminants the exposure to *C. burnetii* increases with the herd size, as reported in other studies (McCaughey et al., 2010; Schimmer *et*

al., 2011; Schimmer *et al*., 2012). Therefore, larger herds are more prone to acquire and develop *C. burnetii* infection, which might be related with the larger number of parturient females in birth season (Woldehiwet, 2004). Furthermore, an association between antibody positivity and dairy production has been reported (Ryan *et al*., 2011; van den Brom *et al*., 2013). In the serosurvey, a predominance of meet herds was observed and in the BTM survey exclusively dairy herds were studied. Finally, the proportion of reports of reproductive disorders, namely abortion, was higher in the BTM survey than in the serosurvey; and higher antibody positivity has been described in herds reporting reproductive disorders (Bildfell *et al*., 2000; García-Perez *et al*., 2009; Khalili *et al*., 2011; Muskens *et al*., 2011). However, a careful interpretation should be done regarding the occurrence of reproductive disorders, because many other infections can cause reproductive disorders in dairy ruminants (Agerholm, 2013) and the cause should be clarified, even if *C. burnetii* is present in the herd (Saegerman *et al*., 2013). In fact, controversial data have been reported. Although an association has been reported in some studies (Bildfell *et al*., 2000), others did not find any (Cetinkaya *et al*., 2000; García-Perez *et al*., 2009).

In the BTM survey conducted in dairy cattle herds from the Northwest region, a higher antibody positivity (61.1%) was observed comparing within the Centre region. So, the exposure to *C. burnetii* seems to be higher in dairy cattle herds from the Northwest region.

Comparing our results with other studies, the herd seroprevalence in small ruminants fits in the wide range reported in small ruminants (3% to 83%) but is lower in cattle (30% to 48.4%) (Kennerman *et al*., 2010; McCaughey *et al*., 2010; Ruiz-Fons *et al*., 2010; Schimmer *et al*., 2011, Alvarez *et al*., 2012; Lambton *et al*., 2016).

In the BTM survey in the Centre region, the exposure in dairy small ruminant herds and in dairy cattle herds fits in the range reported in other countries (0.6% to 67.6% and 8.2% to 78.6%, respectively) (Garcia-Perez *et al*., 2009; Hilbert *et al*., 2012; van den Brom *et al*., 2012; Agger *et al*., 2010; Khalili *et al*., 2011; Muskens *et al*., 2011; Ryan *et al*., 2011; Astobiza *et al*., 2012; Czaplicki *et al*., 2012; Ohlson *et al*., 2014). Despite the higher exposure rate observed in dairy cattle herds from the North Region comparing with the Centre Region, it fits in the range reported in other countries.

The results of the serosurvey at the individual level showed that individual seroprevalence in small ruminants (9.6%) fits in the range of results (1.8% to 13%) described in other European countries (Masala *et al*., 2004; Ruiz-Fons *et al*., 2010; McCaughey *et al*., 2010; Pape *et al*., 2009; Magouras *et al*. 2017). Goats (10.4%) appeared to be more prone to evidence an exposure to *C. burnetii* ($p<0.05$) compared to sheep (8.6%).

The antibody positivity in cattle (15.1%) was higher than reported in other European countries (1.8% to 6.7%) (McCaughey *et al*., 2010; Ryan *et al*., 2011; Alvarez *et al*., 2012; Paul *et al*., 2014) but the small size of the sample should be considered a possible cause of imprecision of the results.

All together, these results evidenced that the exposure to *C. burnetii* in domestic ruminants is important in Portugal. Although regional variations may occur, this pathogen is circulating amongst Portuguese domestic ruminants.

It is not possible to predict shedders in seropositive flocks by their antibody response because animals can remain seropositive for years after the acute infection has been resolved (Berri *et al*., 2002; McQuiston *et al*., 2002). Thus, the shedding of C. *burnetii* was investigated in seropositive herds to assess the risk of transmission of the bacteria. This was achieved individually by testing individual milk samples and vaginal swabs collected from females in identified seropositive herds from the centre region. Moreover, BTM obtained from dairy herds from the Centre and Northwest regions were also tested to assess the milk shedding in a herd level. The detection of *C. burnetii* DNA was performed by molecular methods such as conventional PCR, a sensitive method for detection of *C. burnetii* DNA (Berri *et al*., 2000) and real-time PCR, which enabled the confirmation of previous results and, especially the quantification of the bacterial load (Guatteo *et al*., 2005). This approach allowed scaling the importance of the sources of bacterium with regards to the risk of transmission of *C. burnetii* among animals and from animals to humans through direct contact or environment contamination.

Considering the results from individual testing of the animals, the shedding was confirmed in 20.8% of the seropositive herds and 9.2% of the females. The main shedding route was milk (10.9%) followed by vaginal swabs (2.1%). However, differences were observed among animal species as already reported (Rodolakis *et*

al., 2007). A higher proportion of shedders were observed in goats (15%) followed by cattle (10.8%) and then sheep (3.6%).

In goats, after a Q fever outbreak, most of the females shed bacteria at subsequent pregnancies without manifestation of clinical signs (Berri *et al*., 2007; Rousset *et al*., 2009). The only shedding route observed in goats was milk. Milk is the main *C. burnetii* shedding route described in goats that, even in the post-partum, excrete *C. burnetii* DNA for long-periods intermittently (Berri *et al*., 2007; Rodolakis, 2009; Roest *et al*., 2012) and perhaps during successive lactating periods (Arricau-Bouvery *et al*., 2003). Vaginal mucus is also considered an important shedding route in goats, although our results did not confirm it, probably due to the intermittent shedding that might lead to false negative results (Rousset *et al*., 2009).

In cattle, after infection the shedding occurs at any stage of lactation and not only focused on periparturient and aborted cows (Guatteo *et al*., 2007). We observed the shedding in milk and in vaginal swabs, but proportionally it was higher in milk. Milk is considered the main shedding route in dairy cattle (Beaudau *et al*., 2006; Rodolakis *et al*., 2006; Guatteo *et al*., 2007; Rodolakis *et al*., 2007; Mohammed *et al*., 2014), and vaginal mucus is not so important (Rodolakis *et al*., 2006; Rodolakis *et al*., 2007).

In sheep, after an outbreak, infected ewes are highly infective in the first parturition and the shedding of *C. burnetii* at later parturitions, usually carried to term with normal deliveries, may not occur (Berri *et al*., 2002, 2005). In this study, the shedding in ewes was lower than in cattle and in goats, and it was only observed in vaginal swabs. In fact, vaginal mucus is considered the primary shedding route in ewes (Rodolakis *et al*., 2007; Rodolakis, 2009) and milk it is not the preferred one, but an intermittent shedding has been reported (Rodolakis *et al*., 2007; Mohammed *et al*., 2014). We observed that most of the studied females were multiparous which might have contributed to the lower proportion of shedders observed in ewes.

With regards to the PCR screening of BTM in the Centre region, the shedding was evidenced in 11.9% of the herds being higher in cattle herds (20%) than in small ruminant herds (6.3%). This higher detection of *C. burnetii* in cattle BTM comparing to small ruminants as also been reported in other countries (Muskens *et al*., 2011; Schimmer *et al*., 2011).

Despite the higher exposure observed dairy small ruminant herds, the shedding in BTM was observed only in 6.3% of herds being null in goat herds. Other studies

assessing the shedding of *C. burnetii* in BTM from small ruminant herds obtained results ranging from 0% to 32.9% (Fretz *et al*., 2007; García-Perez *et al*., 2009; van den Brom *et al*., 2012). The reduced shedding in sheep BTM probably occurred because the shedding of *C. burnetii* in milk seems to be less important in ewes (Rodolakis *et al*., 2007). The absence of shedding in BTM from goat herds was also reported in Switzerland (Fretz *et al*., 2007) and in Iran (Abbasi *et al*., 2011). This might be due to the absence of the bacterium in goat farms at the time of sampling but considering that milk is an important route of shedding in goats, an intermittent shedding might cause false-negative results (Berri *et al*., 2007; Rousset *et al*., 2009, van den Brom *et al*., 2012).

In the Northwest region, the shedding was confirmed in 18.9% of the herds which is identical to that found in the Centre. Together these results were slightly lower than the results obtained in other countries, ranging from 29.6 to 94.3% (Kim *et al*., 2005, Fretz *et al*., 2007, Muskens *et al*., 2011, Astobiza *et al*., 2012, Czaplicki *et al*., 2012).

All these results show that the shedding of *C. burnetii* through milk is widespread in dairy cattle herds in different countries. This might be explained by the fact that milk is the most frequent shedding route of *C. burnetii* in cows (Guatteo *et al*., 2012) and a long-time excretion through milk can extend for several months even in asymptomatic animals (Kim *et al*., 2005; Guatteo *et al*., 2007[b]; Guatteo *et al*., 2011).

The bacterial load was moderate in 92.3% of the individual positive samples, with no differences between types of samples or species. Regarding BTM, the bacterial load was considered high in more than 85% of the herds and no differences were observed between regions.

Another interesting finding was that concerning the exposure, a positive association was found between the increase of the age and seropositivity ($p< 0.01$). This was already reported in other studies (Schimmer *et al*., 2011). Particularly, in this study most of the animals were old (3.9 years) and from small meat production herds. These characteristics are probably related with regional cultural habits involving the traditional consumption of goat meat from old animals. But regarding the shedding, the increase of the age appears as an important factor to limit the environment contamination. The shedding was significantly lower in females over 36 months. This suggests that herds

with a high number of younger females (primiparous) are more prone to develop Q fever and to be a source of infection for humans. Furthermore, it can be concluded that antibodies persist for long periods after the bacteria have been cleared from the organism (Berri *et al*., 2002).

It was also observed that most of BTM PCR positive results were associated with antibody positive results. A positive and statistically significant correlation ($p<0.001$) between the bacterial load on BTM qPCR and S/P per cent on BTM ELISA was obtained, which is in accordance with the results reported by Astobiza *et al*. (2012). Overall, these findings suggest that persistent shedders shed a high titre of bacteria and present a higher titre of antibodies (Guatteo *et al*., 2007[a]) and in the presence of BTM negative antibody test, the probability to find a positive PCR test on BTM is considered low or negligible (Saegerman *et al*., 2013). This information might be useful when monitoring programs to be implemented on dairy herds.

Globally, considering the serosurvey and the screening for *C. burnetii* in recognised shedding routes, it was possible to observe that the shedding of *C. burnetii* is an important threat even in apparently healthy domestic ruminant populations. The three-investigated species demonstrated to have an active role in the domestic cycle of *C. burnetii.* Small ruminants showed a higher exposure compared to cattle, but the shedding seemed to be more important in cows and goats. The proportion of shedding at the herd level and at the individual level should be retained not only by the risk of direct transmission to other animals or even to humans but also by environmental contamination, which is important given the high resistance and the low infectious dose reported for this pathogen (Maurin and Raoult, 1999).

The milk as an individual sample or as a herd sample appeared as the most important shedding route. The controversial data about the risk of infection by the consumption of unpasteurized milk needs clarification. However, besides the risk of transmission by ingestion, it should not be forgotten the risk of infection by the inhalation of aerosols in milking rooms (Loftis *et al*., 2010). Thus, special attention should be given not only to occupational activities requiring contact with lactating domestic ruminants but also to those requiring the manipulation of milk and milk products as aerosol transmission may occur from infected milk.

Furthermore, it is important to note that this screening was conducted in apparently healthy herds thus reflecting the "normal" circulation of *C. burnetii* among animals and herds, which would be probably different in an outbreak context.

Domestic ruminants are considered the main reservoirs of *C. burnetii*. However, human outbreaks have been related with other animal species such as cats, dogs, rabbits, birds and others (OIE, 2015). With the objective to evaluate the involvement of other animal species, serological surveys were developed in other animal species, namely companion animals (*i.e.* dogs and cats), domestic pigs, and wild animals (*i.e.* wild boar and red deer).

However, the lack of a validated ELISA test for other species than ruminants raised some difficulties, namely by the absence of species-specific antibody and reference control sera (Rousset *et al*., 2010). The use of protein AG, presenting a strong ability to bind IgG of mammalian species, was used such as described in other ELISA tests to multiple species (Zhang *et al*., 2010; Al-Adhami and Gajadhar, 2014; Meredith *et al*., 2014). To overcome the lack of species-specific reference control and to set the cut-off value for the ELISA, the method of Cooper *et al*. (2011, 2012) was followed. But, because the harmonization of serological tools is critical to compare data (Rousset *et al*., 2010; Sidi-Boumedine *et al*., 2010), we additionally performed a statistical approach of S/P% values using a MCMC model, which allowed to conclude that the cut-off set according to Cooper *et al*. (2011, 2012) decreases the sensitivity of the test but the specificity is 100%. So, and because no other tests would be used for screening, we followed the method described by Cooper *et al*. (2011, 2012) to estimate the serological positivity, assuming that the likelihood of false positive results would be very low and accepting that the final results could be underestimated.

The serosurvey in dogs was conducted in the Centre region. Samples from two origins were analysed, dogs with owners and stray dogs from the municipal kennel. Globally, an increase of seropositivity was observed (12.6%) comparing to the last serosurvey in Portugal (4.8%) (Bacellar *et al*., 1995). This finding suggests a change in the scenario of *C. burnetii* infection in dogs over the last 20 years. However, the different methodology used for screening should not be neglected. Comparing our results with those obtained in other countries, the range is wide (0% to 66%) (Willeberg *et al*., 1980; Marrie *et al*., 1985; Martinov *et al*., 1989; Baldelli *et al*., 1992; Punda-Polic

et al., 1995; Boni *et al*., 1998; Cooper *et al*., 2011). Differences on seroprevalence might have occurred throughout the time and between geographic locations, but these comparisons must be done carefully because different methodologies for screening antibodies were used.

A higher exposure to *C. burnetii* was found in dogs with owner compared to stray dogs from the municipal kennel. This finding was somewhat unexpected, and an opposite result was found in California, USA (Willeberg *et al*., 1980). Stray dogs seem to have a higher chance of exposure to *C. burnetii* by the contact with wildlife (Cooper *et al*., 2011). Additionally, the exposure in dogs was similar in rural and urban areas, and this was an interesting finding too. In rural areas the contact with livestock and wildlife seems to favour the exposure to *C. burnetii* (Boni *et al*., 1998; Cooper *et al*., 2011). It is known that Q fever outbreaks in urban areas might be related to windborne spreading of *C. burnetii* (Popescu *et al*., 2014) but questions about the potential sources of infection in urban areas remain uncleared.

Considering the results of the serosurvey in cats from the Centre region, the proportion of positive results was estimated in 17.2%. The range of positivity among different countries along several years vary from 2% to 61.5% (Willeberg *et al*., 1980; Marrie *et al*., 1985; Matthewman *et al*., 1997; Komiya *et al*., 2003; Meredith *et al*., 2014). All the seropositive cats lived in rural area and, in most of them, an exposure to wildlife was reported. This might be explained by the predatory activity of cats in wildlife, living close to prey animals or even to livestock (Marrie *et al*., 1988; Meredith *et al*., 2014). These findings are supported by the results obtained in Japan, where the serological positivity in stray cats was higher than in domestic cats (Komiya *et al*., 2003). These findings demonstrate that the feline environment might influence the exposure to *C. burnetii.*

Overall, cats seem to be more prone to be infected by *C. burnetii,* which might be related to their living habits. Indoor cats having frequently free access to the backyard where they express their hunting instinct, are possibly exposed to potential infected wildlife prey. In dogs, the potential sources of infection need to be elucidated and vector borne transmission should also be considered. More investigation is required, namely on molecular epidemiology, to explain relations concerning the sources of infection (OIE, 2015).

In domestic pigs the susceptibility to *C. burnetii* was confirmed experimentally but the evidence of a natural infection was never reported, to our knowledge. We carried out a serosurvey in domestic fattening pigs in intensive production system, using the same methodology described in other species; but, it was not possible to demonstrate the exposure in domestic pigs. Probably, the high turnover of animals in finishing farms occurred in the intensive production system explain our results. Further studies, including reproduction animals in intensive production systems and animals from extensive production systems, should be conducted. These suggestions are supported by the results obtained in the serological survey of in feral pigs (*e.g.* wild boar), as mentioned below.

The epidemiology of *C. burnetii* involves a wildlife cycle where several wild species are included, such as rodents, birds, rabbits, ruminants and wild boars among others. So, we intended to evaluate the role of some wild species in the epidemiology of *C. burnetii* in Portugal. A serological survey was conducted in wild boars from the Northeast region and in wild boar and red deer from the centre region.

The serological positivity in wild boars was similar between the Northeast (5.6%) and the Centre region (6.4%). Similar results were obtained in Czech Republic (6%) (Hubalek *et al*., 1993). Data from other countries range from 0% to 50% (Giovanni *et al*., 1988; Baradel *et al*., 1988; Ejercito *et al*., 1993; Clark *et al*. 1983). Although the comparison of data should be careful analysed because these studies were conducted for a long-time period, in different geographical areas, using different methodologies, and also changes in the epidemiology throughout the years might have occurred. Our results suggested that wild boar females of reproducing age are more prone to be infected, as reported for ruminants (Arricau-Bouvery and Rodolakis, 2005; Anastácio *et al*., 2013[a]). These findings are supported by one study in Spain, where only wild boar females older than 15 months presented *C. burnetii* DNA in tissues (Astobiza *et al*., 2011).

The results of the serological survey in red deer showed that the estimated rate of exposure was higher than in wild boar (30.4%) and even higher than that found in Spain (14.1%) (González-Barrio *et al*., 2014), using ELISA testing. Some other studies showed similar results, but the use of different methodologies difficult comparison (Hubalek *et al*., 1993; Ruiz-Fons *et al*., 2008).

Overall, wild ruminants also seem to play a role in the infection cycle of *C. burnetii,* which is supported by the evidence of *C. burnetii* shedding by naturally infected red deer, pointing this wild ungulate as a true reservoir for *C. burnetii* also in Portugal (Gonzalez-Barrio *et al*., 2015[b]).

Besides the evaluation of the exposure to the pathogen, another purpose of this study was to identify sources of infection. As described for domestic ruminants, a molecular screening was conducted in biological samples from wild boars (serum and faeces). The presence of *C. burnetii* DNA was not evidenced in none of the tested samples. Faeces might not be a privileged shedding route in wild boars as described in ruminants (Arricau-Bouvery and Rodolakis, 2005). The presence of specific antibodies indicated a previous exposure, which is partially supported by the PCR negative results in sera. Further studies with a large diversity of biological samples are surely needed to clarify the potential sources of infection among wild ungulates.

Ticks are considered reservoirs of *C. burnetii* and potential environmental sources of infection. In Portugal, the climatic and ecological conditions are favourable for the development of several species of ticks and 24 species of ticks have been identified so far (Silva *et al*., 2006[a]). *Rhipicephalus sanguineus*, *Ixodes ricinus* and Genus *Dermacentor*, which present a large broad of hosts (Silva *et al*., 2006[a]), were the three-main species/genus identified in this study. In the present study, no positive results were obtained by molecular analysis of ticks collected from dogs and cats, as reported in some other studies (Sprong *et al*., 2011; Andoh *et al*., 2013).

In Europe, the detection of *C. burnetii* in ticks is considered rare (Astobiza *et al.*, 2011; Sprong *et al*., 2012; Michelet *et al*., 2014); however, *C. burnetii* was identified in 19.4% of ticks in the South of Portugal belonging to species *Hyaloma lusitanicum*, *Dermacentor marginatus*, *Ixodes* spp. and *Rhipicephalus pusillus* and collected from vertebrate hosts and vegetation (Silva *et al*., 2014). Positive results were also obtained by others elsewhere (Rehacek *et al*., 1991; Knobell *et al*., 2013; Reye *et al*., 2013; Szymanska-Czerwinska *et al*., 2013; Mancini *et al*., 2014). However, PCR-based surveys aiming to detect *C. burnetii* in ticks by the currently available methods must be interpreted with caution (Jourdain *et al*., 2015). The detection of *C. burnetii* in ticks has recently been questioned by the demonstration of *Coxiella*-like bacteria in ticks using PCR methods based on the detection of IS1111 (Duron, 2015;

Jourdain *et al*., 2015). Notwithstanding, it has been purposed that coupling sensitive detection assays with genotyping methods provides an insurance mechanism for positive identification (and genotyping) of *C. burnetii* without the risk of false positives mainly when dealing with samples obtained from ticks (Pearson *et al*., 2016).

The study of molecular epidemiology is essential to monitor *C. burnetii* dissemination. In the present study we aimed to characterize genetic profiles of *C. burnetii* DNA detected in ruminant samples. It was possible to achieve a complete genotype in only seven DNA samples. Despite the reduced number of complete genotypes, a close genetic relationship was observed among *C. burnetii* from epidemiologically unrelated dairy cattle farms in north and center of Portugal. Yet, six novel genotypes were identified. These genotypes are closely related, but not identical, to those identified in cattle from other European countries and in acute human infections, suggesting that a common pool of *C. burnetii* strains that infect cattle exists in Europe and may play a role in human infection. One of these genotypes was also found in one sheep dairy farm in the same region. Further studies are required to elucidate about the host specificity and the geographical nidality of *C. burnetii*.

For an accurate understanding on the epidemiology of *C. burnetii* in Portugal, a larger collection of isolates representing a greater geographical and host range is mandatory.

9

Conclusions

The results obtained and discussed in this study allowed to reach the following conclusions:

i) The serologic survey conducted in domestic ruminants in Portugal showed that **in Portugal small ruminants and cattle are exposed to *C. burnetii*,** although some differences in the prevalence rate were observed between species and regions. *C. burnetii* is an important biological threat even in **apparently healthy** domestic ruminant populations.

ii) The exposure to *C. burnetii* infection was not restricted to domestic ruminants in Portugal. Despite the **non-evidence of exposure in domestic fattening pigs,** it was **demonstrated in companion animals (*i.e.* dogs and cats); in feral pigs and in red deer.**

iii) Among the studied animal species, ***C. burnetii* DNA was only detected in domestic ruminants**.

- At the herd level, the shedding of *C. burnetii* was demonstrated in a higher percentage in cattle herds than in small ruminant herds. Individually**, shedding of *C. burnetii* was higher in goats followed by cattle and then ewes**.

- Considering the studied shedding routes in domestic ruminants, the **milk appeared as the most important shedding route**, as an **individual** sample or as a **herd** sample.

- **Age seemed to be significantly associated to *C. burnetii* infection**. The increase of the age was associated with an increased serological positivity (*i.e.* exposure) to *C. burnetii*. Contrarily, the increase of the age was related with a decreased shedding.

- A significant positive correlation occurred between antibody titer and the bacterial load in BTM. Thus, **in presence of negative antibody test, the probability to find a positive PCR test on BTM is considered very low**.

iv) ***C. burnetii* DNA was not detected in ticks** collected from companion animals.

v) **Six new MLVA-6 profiles were identified** in Portugal. These novel genotypes clustered with genotypes identified in cattle from other European countries.

Considering the global objective of this PhD project, we believe that we have successfully addressed most of the questions we have wished to answer. The results herein presented highlighted that in Portugal, *C. burnetii* circulates among several domestic and sylvatic animals. Additionally, genotyping of *C. burnetii* DNA detected in domestic ruminants showed that genotypes are closely related to those identified in cattle from other European countries and in acute human infections, suggesting that a common pool of *C. burnetii* strains that infect cattle exists in Europe and may play a role in human infection.

It is globally recognized that epidemiological studies are crucial to monitor infection in different regions. Regarding *C. burnetii* epidemiology, the use of standardized methodologies is crucial to compare results from different studies and to cover different scenarios when controlling programs are needed**.**

9.1

Future Perspectives

The results of this study clearly increased the scientific knowledge on *C. burnetii* epidemiology in Portugal. However, data obtained from this work raised some questions and challenges that need to be elucidated, namely:

- To clarify the role of domestic pigs from extensive production systems. This will be achieved by the serological screening of this subpopulation. Results will help to understand the role of domestic pigs in the epidemiology of *C. burnetii*.

- To characterize genetically circulating strains in companion animals and in wild ungulates. This will be achieved by increasing the number and the variety of biological samples on molecular screening. In companion animals, sampling of reproductive tissues/secretions will be conducted in bitches and queens attending on veterinary clinics specifically for surgical purposes, particularly hysterectomy. In wild ungulates, sampling of reproductive and reticuloendothelial organs (*e.g.* spleen, liver, uterus) will take place during the hunting season. Firstly, a molecular screening will be carried out to detect *C. burnetii* DNA. Secondly, genetic characterisation will allow the comparison with national and international data and the analysis of the genetic diversity of strains circulating among animal species.

- To determine if the environment or even other animal species not included in this study (*e.g.* pigeons) play an important role in the epidemiology of *C. burnetii* in urban areas. This will include a molecular screening for *C. burnetii* in the environment (*i.e.* soil and ticks) and in wild bird excrements. *C. burnetii* DNA will be further genotyped to investigate the genetic variability and to clarify links regarding the source of infection.

- Strain viability is an important issue to assess the infectious risk. Additionally, strain virulence is related to its potential pathogenicity. To assess strain viability and virulence, qPCR *Coxiella burnetii* positive samples will be inoculated on a mouse model to isolate

strains and to assess their virulence, namely the novel genotypes found. These data will be useful in the design of control programs.

10

References

Abbasi S, Farzan R, Momtaz H. Molecular detection of *Coxiella burnetii* in goat bulk milk samples in some provinces of Iran. *Afr J Biotechnol*, 2011; 10: 18513-18515.

Agerholm JS. *Coxiella burnetii* associated reproductive disorders in domestic animals-a critical review. *Acta Vet. Scand*., 2013; 55:13.

Agger JF, Christoffersen AB, Rattenborg E, Nielsen J, Agerholm JS. Prevalence of *Coxiella burnetii* antibodies in Danish dairy herds. *Acta Vet Scand*, 2010; 52: 5.

Agger JF, Paul S, Christoffersen AB, Agerholm JS. Risk factors for *Coxiella burnetii* antibodies in bulk tank milk from Danish dairy herds. *Acta Vet Scand*, 2013; 55: 80.

Agger JF, Paul S. Increasing prevalence of *Coxiella burnetii* seropositive Danish dairy cattle herds. *Acta Vet Scand*, 2014; 56:46.

Al-Adhami BH, Gajadhar AA. A new multi-host species indirect ELISA using protein A/G conjugate for detection of anti-*Toxoplasma gondii* IgG antibodies with comparison to ELISA-IgG, agglutination assay and Western blot. *Vet Parasitol*, 2014; 200: 66-73.

Al-Deeb M. A, Frangoulidis D, Walter MC, Kömpf D, Fischer SF, Petney T, Muzaffara SB. *Coxiella*-like endosymbiont in argasid ticks (*Ornithodoros muesebecki*) from a Socotra Cormorant colony in Umm Al Quwain, United Arab Emirates. *Ticks and Tick-borne Dis*, 2016; 7: 166–171.

Alsaleh A, Fieni F, Moreno D, Rousset E, Tainturier D, Bruyas JF, Pellerin JL. Risk of *Coxiella burnetii* transmission via embryo transfer using in vitro early bovine embryos. *Theriogenology*, 2014; 81(6): 849-853.

Alvarez J, Perez A, Mardones FO, Pérez-Sancho M, García-Seco T, Pagés E, Mirat F, Díaz R, Carpintero J, Domínguez L. Epidemiological factors associated with the exposure of cattle to Coxiella burnetii in the Madrid region of Spain. *Vet J*, 2012; 194: 102–107.

Alves MCGP, de Matos MR, Reichmann ML, Dominguez MH. Estimation of the dog and cat population in the state of São Paulo. *Rev Saúde Pública*, 2005; 39(6): 1-7.

Amara AB, Ghigo E, Le Priol Y, Lépolard C, Salcedo SP, Lemichez E, Bretelle F, Capo C, Mege JL. *Coxiella burnetii*, the agent of Q fever, replicates within trophoblasts and induces a unique transcriptional response. *PloS One*, 2010; 5(12): e15315.

Amitai Z, Bromberg M, Bernstein M, Raveh D, Keysary A, David D, Pitlik S, Swerdlow D, Massung R, Rzotkiewicz S, Halutz O, Shohat T. A Large Q Fever Outbreak in an Urban School in Central Israel. *Clin Infect Dis*, 2010; 50: 1433–1438.

Anastácio S, Carolino N, Sidi-Boumedine K, da Silva GJ. Q fever dairy herd status determination based on serological and molecular analysis of bulk tank milk. *Transbound Emerg Dis*, 2016; 63: e293–e300.

Anastácio S, Pessoa D, Pegado J, Cruz C, Sidi-Boumedine K, da Silva GJ. Investigation of Coxiella burnetii infection in dairy ruminant herds with reproductive disorders in two different regions of Portugal. *Clin Microbiol Infect*, 2012; 18 (S3): 524.

Anastacio S, Tavares N, Carolino N, Sidi-Boumedine K, da Silva GJ. [a] Serological evidence of exposure to *Coxiella burnetii* in sheep and goats in central Portugal. *Vet Microbiol*, 2013; 167: 500-505.

Anastácio S, Tavares N, Sidi-Boumedine K, da Silva GJ. [b] Evidence of Coxiella burnetii infection in small ruminants: a cross sectional study. Abstract OC 332. *23rd European Congress of Clinical Microbiology and Infectious Diseases*. Berlin, Germany. 2013.

Anderson A, Bijlmer H, Fournier PE, Graves S, Hartzell J, Kersh GJ, Limonard G, Marrie TJ, Massung RF, McQuiston JH, Nicholson WL, Paddock CD, Sexton DJ. Diagnosis and management of Q fever - United States, 2013: recommendations from CDC and the Q Fever Working Group. *MMWR Recomm Rep*, 2013; 62:1-30.

Anderson AD, Kruszon-Moran D, Loftis AD, McQuillan G, Nicholson WL, Priestley RA, Candee AJ, Patterson NE, Massung RF. Seroprevalence of Q Fever in the United States, 2003–2004. *Am J Trop Med Hyg*, 2009; 81(4): 691–694.

Andoh M, Andoh R, Teramoto K, Komiya T, Kaneshima T, Takano A, Hayashidani H, Ando S. Survey of *Coxiella burnetii* in ticks collected from dogs in Japan. *J Vet Med Sci*, 2013; 75(8): 1115-1117.

Andoh M, Zhang G, Russell-Lodrigue KE, Shive HR, Weeks BR, Samuel JE. T cells are essential for bacterial clearance, and gamma interferon, tumor necrosis factor alpha, and B cells are crucial for disease development in Coxiella burnetii infection in mice. *Infect Immun*, 2007;75(7): 3245–3255.

Angelaskis E, Raoult D. Emergence of Q fever. *Iran J Public Health*, 2011; 40:1-18.

Angelaskis E, Raoult D. Q fever. *Vet Microbiol*, 2010; 140:297-309.

Antoniou M, Economou I, Wang X, Psaroulaki A, Spyridaki I, Papadopoulos B, Christidou A, Tsafantakis E, Tselentis Y. Fourteen-year seroepidemiological study of zoonoses in a Greek village. *Am J Trop Med Hyg*, 2002; 66(1): 80-85.

Argov O, Weintraub M, Charach G. "Doughnut" granulomas from erythema nodosum in acute Q fever. *Isr Med Assoc J*, 2008; 10(3): 241–242.

Ariga T, Nagaoka H, Miyanoshita A, Kusunoki Y, Watanabe T, Shinohara T, Sakiyama Y. *Coxiella burnetii* lymphadenitis: a possible fever focus in acute Q fever. *Pediatr Int*, 2000; 42(6): 711-714.

Arricau-Bouvery N, Souriau A, Lechopier P, Rodolakis A. Experimental *Coxiella burnetii* infection in pregnant goats: excretion routes. *Vet Res*, 2003; 34(4): 423-433.

Arricau-Bouvery N, Hauck Y, Bejaoui A, Frangoulidis D, Bodier CC, Souriau A, Meyer H, Neubauer H, Rodolakis A, Vergnaud G. Molecular characterization of *Coxiella burnetii* isolates by infrequent restriction site-PCR and MLVA typing. *BMC Microbiol*, 2006; 6:38.

Arricau-Bouvery N, Rodolakis A. Is Q fever an emerging or re-emerging Zoonosis? *Vet Res*, 2005; 36:327-49.

Arthana W, Sumrandee C, Hirunkanokpun S, Kitthawee S, Baimai V, Trinachartvanit W, Ahantarig A. Detection of *Coxiella*-like endosymbiont in *Haemaphysalis* tick in Thailand. *Ticks and Tick-borne Dis*, 2015; 6, 63–68.

Astobiza I, Barandika JF, Hurtado A, Juste RA, García-Pérez AL. Kinetics of *Coxiella burnetii* excretion in a commercial dairy sheep flock after treatment with oxytetracycline. *Vet J*, 2010; 184: 172–175.

Astobiza I, Barral M, Ruiz-Fons F, Barandika JF, Gerrikagoitia X, Hurtado A, García-Pérez AL. Molecular investigation of the occurrence of *Coxiella burnetii* in wildlife and ticks in an endemic area. *Vet Microbiol*, 2011; 147:190-194.

Astobiza I, Ruiz-Fons F, Piñero A, Barandika JF, Hurtado A, Garcia-Perez AL. Estimation of *Coxiella burnetii* prevalence in dairy cattle in intensive systems by serological and molecular analyses of bulk-tank milk samples. *J Dairy Sci*, 2012; 95: 1632-1638.

Astobiza I, Tilburg JJ, Piñero A, Hurtado A, García-Pérez AL, Nabuurs-Franssen MH, Klaassen CH. Genotyping of *Coxiella burnetii* from domestic ruminants in northern Spain. *BMC Vet Res*, 2012; 8: 241.

Ayres JG, Flint N, Smith EG, Tunnicliffe WS, Fletcher TJ, Hammond K, Ward D, Marmion BP. Post-infection fatigue syndrome following Q fever. *Q J Med*, 1998; 91:105–123.

Ayres JG, Smith EG, Flint N. Protracted fatigue and debility after acute Q fever. *Lancet*, 1996; 347(9006): 978-979.

Azizzadeh M, Mohammadi GM, Haghparast AR, Heidarpour-Bami M. Seroepidemiology of *Coxiella burnetii* in commercial dairy herds in northeast of Iran. *Iran J Vet Sci Technol*, 2011; 3(2):33-40.

Baca OG, Klassen DA, Aragon AS. Entry of *Coxiella burnetii* into host cells. *Acta Virol*, 1993; 37(2-3): 143-155.

Bacellar F, Dawson JE, Silveira CA, Filipe AR. Antibodies against Rickettsiaceae in dogs of Setubal, Portugal. *Cent Eur J Public Health*, 1995;3(2): 100-102.

Bacellar F, Núncio MS, Lehacek J, Filipe AR. Rickettsiae and Rickettsioses in Portugal. *Eur J Epidemiol*, 1991; 7(3): 291-293.

Baker MD, Pithua PO. Low seroprevalence of *Coxiella burnetii* in Boer goats in Missouri. *BMC Res Notes*, 2014;7:421.

Baldelli R, Cimmino C, Pasquinelli M. Dog-transmitted zoonoses: a serological survey in the province of Bologna. *Ann Ist Super Sanita*, 1992;28(4):493-496.

Baradel JM, Barrat J, Blancou J, Boutin JM, Chastel C, Dannacher G, Delorme D, Gerard Y, Gourreau JM, Kihm U, Larenaudie B, Le Goff C, Pastoret PP, Perreau P, Schwers A, Thiry E, Trap D, Uilenberg G, Vannier P. Results of a serological survey of wild mammals in France. *Rev Sci Tech Off Int Epiz*, 1988; 7 (4):873-883.

Barlow J, Rauch B, Welcome F, Kim SG, Dubovi E, Schukken Y. Association between *Coxiella burnetii* shedding in milk and subclinical mastitis in dairy cattle. *Vet Res*, 2008; 39:23.

Barry A, Mege J, Ghigo E. Hijacked phagosomes and leukocyte activation: an intimate relationship. *J Leukoc Biol*, 2011; 89(3), 373–382.

Barry AO, Boucherit N, Mottola G, Vadovic P, Trouplin V, Soubeyran P, Capo C, Bonatti S, Nebreda A, Toman R, Lemichez E, Mege JL, GhigoE. Impaired Stimulation of p38a-MAPK/Vps41-HOPS by LPS from Pathogenic *Coxiella burnetii* Prevents Trafficking to Microbicidal Phagolysosomes. *Cell Host Microbe*, 2012; 12: 751-763.

Bauer AE, Olivas S, Cooper M, Hornstra H, Keim P, Pearson T, Johnson AJ. Estimated herd prevalence and sequence types of *Coxiella burnetii* in bulk tank milk samples from commercial dairies in Indiana. *BMC Vet Res*, 2015;11:186.

Baumgartner W, Bachmann S. Histological and immunocytochemical characterization of *Coxiella burnetii* associated lesions in the murine uterus and placenta. *Infect Immun*, 1992; 60(12), 5232–5241.

Beare PA, Gilk SD, Larson CL, Hill J, Stead CM, Omsland A, Cockrell DC, Howe D, Voth DE, Heinzen RA. [a] Dot/Icm Type IVB Secretion System Requirements for *Coxiella burnetii* Growth in Human Macrophages. *mBio*, 2011; 2(4): e00175-11.

Beare PA, Jeffrey BM, Martens CA, Heinzen RA [a]. Draft Genome Sequences of Three *Coxiella burnetii* Strains Isolated from Q Fever Patients. *Genome Announc*, 2017; 5(38), e00986-17.

Beare PA, Jeffrey BM, Martens CA, Heinzen RA [b]. Draft Genome Sequences of the Avirulent *Coxiella burnetii* Dugway 7D77-80 and Dugway 7E65-68 Strains Isolated from Rodents in Dugway, Utah. *Genome Announc*, 2017; 5(39), e00984-17.

Beare PA, Jeffrey BM, Martens CA, Pearson T, Heinzen RA [c]. Draft Genome Sequences of Historical Strains of *Coxiella burnetii* Isolated from Cow's Milk and a Goat Placenta. *Genome Announc*, 2017; 5(39), e00985-17.

Beare PA, Larson CL, Gilk SD, Heinzen RA. Two systems for targeted gene deletion in *Coxiella burnetii*. *Appl Environ Microbiol*, 2012;78(13):4580-4589.

Beare PA, Samuel JE, Howe D, Virtaneva K, Porcella SF, Heinzen RA. Genetic Diversity of the Q Fever Agent, *Coxiella burnetii*, Assessed by Microarray-Based Whole-Genome Comparisons. *J Bacteriol*, 2006; 188(7):2309-2324.

Beare PA, Sandoz KM, Omsland A, Rockey DD, Heinzen RA. [b] Advances in genetic manipulation of obligate intracellular bacterial pathogens. *Front Microbiol*, 2011; 2: 97.

Beare PA, Unsworth N, Andoh M, Voth DE, Omsland A, Gilk SD, Williams KP, Sobral BW, Kupko JJ, Porcella SF, Samuel JE, Heinzen RA. Comparative genomics reveal extensive transposon-mediated genomic plasticity and diversity among potential effector proteins within the genus *Coxiella*. *Infect Immun*, 2009; 77, 642-656.

Beaudau F, Guatteo R, Seegers H. Voies d'excretion de *Coxiella burnetii* par la vache laitière: implication pour le dépistage et la maitrison de l'infection en élevage. *Rev Epid San Anim*, 2006; 49:1-4.

Bechah Y, Verneau J, Amara AB, Barry AO, Lépolard C, Achard V, Panicot-Dubois L, Textoris J, Capo C, Ghigo E, Mege JL. Persistence of *Coxiella burnetii*, the agent of Q fever, in murine adipose tissue. *PLoS One*, (2014); 9(5): e97503.

Behymer DE, Biberstein EL, Riemann HP, Franti CE, Sawyer M, Ruppanner R, Crenshaw GL. Q fever (*Coxiella burnetii*) investigations in dairy cattle: challenge of immunity after vaccination. *Am J Vet Res*, 1976; 37:631–634.

Bell EJ, Parker RR, Stoenner HG. Experimental Q fever in cattle. *Am J Public Health*, 1949;39(4):478-484.

Bellini C, Magouras I, Chapuis-Taillard C, Clerc O, Masserey E, Peduto G, Péter O, Schaerrer S, Schuepbach G, Greub G. Q fever outbreak in the terraced vineyards of Lavaux, Switzerland. *New Microbes New Infect*, 2014; 2: 93–99.

Bengston IA. Complement Fixation in Q fever. *Exp Biol Med*, 1941; 46:665-8.

Benoit M, Barbarat B, Bernard A, Olive D, Mege JL. *Coxiella burnetii*, the agent of Q fever, stimulates an atypical M2 activation program in human macrophages. *Eur J Immunol*, 2008; 38(4): 1065–1070.

Berón W, Gutierrez MG, Rabinovitch M, Colombo MI. *Coxiella burnetii* Localizes in a Rab7-Labeled Compartment with Autophagic Characteristics. *Infect Immun*, 2002; 70(10): 5816–5821.

Berri M, Laroucau K, Rodolaskis A. The detection of *Coxiella burnetii* from ovine genital swabs, milk and fecal samples by the use of a single touchdown polymerase chain reaction. *Vet Microbiol*, 2000; 72: 285-293.

Berri M, Rousset E, Champion JL, Russo P, Rodolaskis A. Goats may experience reproductive failures and shed *Coxiella burnetii* at two successive parturitions after a Q fever infection. *Res Vet Sci*, 2007; 83:47-52.

Berri M, Rousset E, Hechard C, Champion JL, Dufour P, Russo P, Rodolaskis A. Progression of Q Fever and *Coxiella burnetii* shedding in milk after an outbreak of enzootic abortion in a goat herd. *Vet Rec*, 2005; 156:548-9.

Berri M, Souriau A, Crosby M, Crochet D, Lechopier P, Rodolaskis A. Relationships between the shedding of *Coxiella burnetii*, clinical signs and serological responses of 34 sheep. *Vet Rec*, 2001; 148: 502-505.

Berri M, Souriau A, Crosby M, Rodolaskis A. Shedding of *Coxiella burnetii* in ewes in two pregnancies following an episode of Coxiella abortion in a sheep flock. *Vet Microbiol*, 2002; 85:55-60.

Bildfell RJ, Thomson GW, Haines DM, McEwen BJ, Smart N. *Coxiella burnetii* infection is associated with placentitis in cases of bovine abortion. *J Vet Diagn Invest*, 2000; 12: 419–425.

Billinis C. Wildlife diseases that pose a risk to small ruminants and their farmers. *Small Rum Res*, 2013; 110: 67-70.

Bizzini A, Péter O, Baud D, Edouard S, Meylan P, Greub G. Evaluation of a new serological test for the detection of anti-*Coxiella* and anti-*Rickettsia* antibodies. *Microbes Infect*, 2015; 17(11-12):811-816.

Blut A. *Coxiella burnetii* − pathogenic agent of Q fever. *Transfus Med Hemother* 2014; 41:60-72.

Boarbi S, Fretin D, Mori M. *Coxiella burnetii*, agent de la fièvre Q. *Can J Microbiol*, 2015; 62: 1-21.

Boarbi S, Mori M, Rousset E, Sidi-Boumedine K, Van Esbroeck M, Fretin D. Prevalence and molecular typing of *Coxiella burnetii* in bulk tank milk in Belgian dairy goats, 2009-2013. *Vet Microbiol*, 2014;170(1-2):117-124.

Bolaños M, Santana OE, Angel-Moreno A, Pérez-Arellano JL, Limiñana JM, Serra-Majem L, Martín-Sánchez AM. Seroprevalence of infection by *Coxiella burnetii* in Canary Islands (Spain). *Eur J Epidemiol*, 2003; 18: 259–262.

Boni M, Davoust B, Tissot-Dupont H, Raoult D. Survey of seroprevalence of Q fever in dogs in the southeast of France, French Guyana, Martinique, Senegal and the Ivory Coast. *Vet Microbiol*, 1998;64:1-5.

Bosnjak E, Hvass AMSW, Villumsen S, Nielsen H. Emerging evidence for Q fever in humans in Denmark: role of contact with dairy cattle. *Clin Microbiol Infect*, 2010; 16: 1285–1288.

Botelho-Nevers E, Fournier PE, Richet H, Fenollar F, Lepidi H, Foucault C, Branchereau A, Piquet P, Maurin M, Raoult D. *Coxiella burnetii* infection of aortic aneurysms or vascular grafts: report of 30 new cases and evaluation of outcome. *Eur J Clin Microbiol Infect Dis*, 2007; 26(9): 635-640.

Bottieau E, De Raeve H, Colebunders R, Van den Ende J, Vervoort T, Van Marck E. Q fever after a journey in Syria: a diagnosis suggested by boné marrow biopsy. *Acta Clin Belg*, 2000; 55(1): 30-33.

Boucherit N, Barry AO, Mottola G, Trouplin V, Capo C, Mege JL, Ghigo E. Effects of *Coxiella burnetii* on MAPKinases phosphorylation. *FEMS Immunol Med Microbiol*, 2012; 64(1): 101–103.

Brennan RE, Russell K, Zhang G, Samuel JE. Both inducible nitric oxide synthase and NADPH oxidase contribute to the control of virulent phase I *Coxiella burnetii* infections. *Infect Immun*, 2004; 72(11), 6666–6675.

Broederdorf LJ, Voth DE. Cheating death: A *Coxiella* effector prevents apoptosis. *Front Microbiol*, 2011; 2: 1–2.

Brooke RJ, Kretzschmar ME, Mutters NT, Teunis PF. Human dose response relation for airborne exposure to *Coxiella burnetii*. *BMC Infect Dis*, 2013; 13(1): 488.

Brooke RJ, Mutters NT, Péter O, Kretzschmar MEE, Teunis PFM. Exposure to low doses of *Coxiella burnetii* caused high illness attack rates: Insights from combining human challenge and outbreak data. *Epidemics*, 2015; 11: 1–6.

Buhariwalla F, Cann B, Marrie TJ. A dog-related outbreak of Q fever. *Clin Infec Dis*, 1996; 23: 753-755.

Burnet FM, Freeman M. Experimental studies on the virus of "Q" fever. *Med J Aus*, 1937; 2:299-305.

Cabassi CS, Taddei S, Donofrio G, Ghidini F, Piancastelli C, Flammini CF, Cavirani S. Association between *Coxiella burnetii* seropositivity andabortion in dairy cattle of Northern Italy. *New Microbiol*, 2006; 29: 211-214.

Cairns K, Brewer M, Lappin MR. Prevalence of *Coxiella burnetii* DNA in vaginal and uterine samples from healthy cats of north-central Colorado. *J Feline Med Surg,* 2007;9(3):196-201.

Cantas H, Muwonge A, Sareyyoupoglu B, Yardimci H, Skjerve E. Q fever abortions in ruminants and associated on-farm risk factors in Northern Cyprus. *BMC Vet Res*, 2011; 7: 13.

Capo C, Lindberg FP, Meconi S, Zaffran Y, Tardei G, Brown EJ, Raoult D, Mege JL. Subversion of Monocyte Functions by *Coxiella burnetii*: Impairment of the Cross-Talk Between avb3 Integrin and CR31. *J Immunol*, 1999; 163:6078-6085.

Capo C, Mege JL. Role of innate and adaptive immunity in the control of Q Fever. *Adv Exp Med Biol*, 2012;984:273-286.

Capo C, Moynault A, Collette Y, Olive D, Brown EJ, Raoult D, Mege JL. *Coxiella burnetii* avoids macrophage phagocytosis by interfering with spatial distribution of complement receptor 3. *J Immunol*, 2003; 170: 4217–4225.

Capo C, Zugun F, Stein A, Tardei G, Lepidi H, Raoult D, Mege JL. Upregulation of Tumor Necrosis Factor Alpha and Interleukin-1β in Q Fever Endocarditis. *Infect Immun*, 1996; 64(5): 1638-1642.

Carbonero A, Guzmán LT, Montaños K, Torralbo A, Arenas-Montes A, Saa LR. *Coxiella burnetii* seroprevalence and associated risk factors indairy and mixed cattle farms from Ecuador. *Prev Vet Med*, 2015; 118: 427–435.

Carcopino X, Raoult D, Bretelle F, Boubli L, Stein A. Q Fever during pregnancy: a cause of poor fetal and maternal outcome. *Ann N Y Acad Sci*, 2009;1166:79-89.

Carcopino X, Raoult D, Bretelle F, On Boubli L, Stein A. Managing Q Fever during Pregnancy: The Benefits of Long-Term Cotrimoxazole Therapy. *Clin Infect Dis*, 2007; 45: 548–555.

Cardeñosa N, Sanfeliu I, Font B, Muñoz T, Nogueras MM, Segura F. Seroprevalence of human infection by *Coxiella burnetii* in Barcelona (Northeast of Spain). *Am J Trop Med Hyg*, 2006; 75(1): 33–35.

Cardinale E, Esnault O, Beral M, Naze F, Michault A. Emergence of *Coxiella burnetii* in Ruminants on Reunion Island? Prevalence and Risk Factors. *PLoS Neglect Trop Dis*, 2014; 8: e3055.

Carey KL, Newton HJ, Lührmann A, Roy CR. The *Coxiella burnetii* Dot/Icm System Delivers a Unique Repertoire of Type IV Effectors into Host Cells and Is Required for Intracellular Replication. *PLoS Pathog*, 2011; 7(5), e1002056.

Ceglie L, Guerrini E, Rampazzo E, Barberio A, Tilburg JJ, Hagen F, Lucchese L, Zuliani F, Marangon S, Natale A. Molecular characterization by MLVA of *Coxiella burnetii* strains infecting dairy cows and goats of north-eastern Italy. *Microbes Infect*, 2015; 17(11-12): 776-81.

Cekani M, Papa A, Kota M, Velo E, Berxholi K. Report of a serological study of *Coxiella burnetii* in domestic animals in Albania. *Vet J,* 2008;175(2):276-278.

Cerf O, Condron R. Coxiella burnetii and milk pasteurization: an early application of the precautionary principle? *Epidemiol Infect*, 2006; 134:946-951.

Cetinkaya B, Kalender H, Ertas HB, Muz A, Arslan N, Ongor H, Gurfay M. Seroprevalence of coxiellosis in cattle, sheep and people in the east of Turkey. *Vet Rec*, 2000; 146: 131-136.

Champion OL, Wagley S, Titball RW. *Galleria mellonella* as a model host for microbiological and toxin research. *Virulence*, 2016;7(7):840-845.

Chang K, Lee NY, Chen YH, Lee HC, Lu PL, Chang CM, Wu CJ, Chen TC, Hsieh HC, Ko WC. Acute Q fever in southern Taiwan: atypical manifestations of hyperbilirubinemia and prolonged fever. *Diagn Microbiol Infect Dis*, 2008;60(2):211-216.

Chen C, Dow C, Wang P, Sidney J, Read A, Harmsen A, Samuel JE, Peters B. Identification of CD4+ T cell epitopes in *C. burnetii* antigens targeted by antibody responses. *PloS One*, 2011; 6(3): e17712.

Chmielewski T, Sidi-Boumedine K, DuquesneV, Podsiadly E, Thiéry R, Tykewska-Wierzbanowska S. Molecular Epidemiology of Q fever in Poland. *Pol J Microbiol*, 2009; 58(1):9-13.

Chmielewski T, Tylewska-Wierzbanowska S. Q fever at the turn of the century. *Pol J Microbiol*, 2012; 61 (2), 81-93.

Ciliberti A, Gavier-Widén D, Yon L, Hutchings M R, Artois M. Prioritisation of wildlife pathogens to be targeted in European surveillance programmes: Expert-based riskanalysis focus on ruminants. *Prev Vet Med*, 2015; 118(4): 271-284.

Clark RK, Jessup DA, Hird DW, Ruppanner R, Meyer ME. Serologic survey of California wild hogs for antibodies against selected zoonotic disease agents. *J Am Vet Med Assoc*, 1983; 183(11): 1248-1251.

Clemente L, Fernandes TL, Barahona MJ, Bernardino R, Botelho A. Confirmation by PCR of *Coxiella burnetii* infection in animals at a zoo in Lisbon, Portugal. *Vet Rec*, 2008; 163: 221-222.

Clemente ML, Barahona MJ, Andrade MF, Botelho A. Diagnosis of *Coxiella burnetii* by PCR in aborted foetuses of domestic ruminants in Portugal. *Vet Rec*, 2009; 164, 373-374.

Cole ST, Eiglmeier K, Parkhill J, James KD, Thomson NR, Wheeler PR, Honoré N, Garnier T, Churcher C, Harris D, Mungall K, Basham D, Brown D, Chillingworth T, Connor R, Davies RM, Devlin K, Duthoy S, Feltwell T, Fraser A, Hamlin N, Holroyd S, Hornsby T, Jagels K, Lacroix C, Maclean J, Moule S, Murphy L, Oliver K, Quail MA, Rajandream MA, Rutherford KM, Rutter S, Seeger K, Simon S, Simmonds M, Skelton J, Squares R, Squares S, Stevens K, Taylor K, Whitehead S, Woodward JR, Barrell BG. Massive gene decay in the leprosy bacillus. *Nature*, 2001; 409: 1007-1011.

Coleman SA, Fischer ER, Howe D, Mead DJ, Heinzen RA, 2004. Temporal Analysis of *Coxiella burnetii* Morphological Differentiation. *J Bacteriol*, 186(21), 7344-7352.

Conner MM, Ebinger MR, Blanchong JA, Cross PC. Infectious disease in cervids of North America: data, models, and management challenges. *Ann N Y Acad Sci*, 2008; 1134: 146-172.

Conti F, Boucherit N, Baldassarre V, Trouplin V, Toman R, Mottola G, Mege JL, Ghigo E. *Coxiella burnetii* lipopolysaccharide blocks p38α-MAPK activation through the disruption of TLR-2 and TLR-4 association. *Front Cell Inf Microbiol*, 2015; 4: 182.

Contreras V, Máttar S, González M, Álvarez J, Oteo JA. *Coxiella burnetii* in bulk tank milk and antibodies in farm workers at Montería, Colombia. *Rev Colomb Cienc Pec*, 2015; 28:181-187.

Cooke RA. Q Fever. Was Edward Derrick's contribution undervaluated? *Med J Aust*, 2008; 189: 660-662.

Cooper A, Barnes T, Potter A, Ketheesan N, Govan B. Determination of *Coxiella burnetii* seroprevalence in macropods in Australia. *Vet Microbiol*, 2012;155:317-323.

Cooper A, Hedlefs R, Ketheesan N, Govan B. Serological evidence of *Coxiella burnetii* infection in dogs in a regional centre. *Aust Vet J*, 2011;89(10):385-387.

Courcoul A, Vergu E, Denis JB, Beaudeau F. Spread of Q fever within dairy cattle herds: key parameters inferred using a *Bayesian* approach. *P R Soc B*, 2010; 277: 2857–2865.

Cox HR. A filter-passing Infectious Agent isolated from Ticks. III. Description of Organism and Cultivation Experiments. *Public Health Rep*, 1938; 53: 2270-6.

Cumbassá A, Barahona MJ, Cunha MV, Azórin B, Fonseca C, Rosalino LM, Tilburg J, Hagen F, Santos AS, Botelho A. *Coxiella burnetii* DNA detected in domestic ruminants and wildlife from Portugal. *Vet Microbiol*, 2015; 180(1-2): 136-141.

Cunha LD, Ribeiro JM, Fernandes TD, Massis LM, Khoo CA, Moffatt JH, Newton HJ, Roy CR, Zamboni DS. Inhibition of inflammasome activation by *Coxiella burnetii* type IV secretion system effector IcaA. *Nature Comm*, 2015; 6: 10205.

Cutler SJ, Bouzid M, Cutler RR. Q fever. *J Infect*, 2007; 54: 313-318.

Czaplicki G, Houtain JY, Mullender C, Porter SR, Humblet MF, Manteca C, Saegerman C. Apparent prevalence of antibodies to *Coxiella burnetii* (Q fever) in bulk tank milk from dairy herds in southern Belgium. *Vet J*, 2012;192: 529–531.

D'Amato F, Eldin C, Georgiades K, Edouard S, Delerce J, Labas N, Raoult D. Loss of TSS1 in hypervirulent *Coxiella burnetii* 175, the causative agentof Q fever in French Guiana. *Comp Immunol, Microbiol Infect Dis*, 2015; 41: 35-41.

D'Amato F, Million M, Edouard S, Delerce J, Robert C, Marrie T, Raoult D. [a] Draft genome sequence of *Coxiella burnetii* Dog Utad, a strain isolated from a dog-related outbreak of Q fever. *New Microb New Infect*, 2014; 2: 136-137.

D'Amato F, Rouli L, Edouard S, Tyczka J, Million M, Robert C, Nguyen TT, Raoult D. [b] The genome of *Coxiella burnetii* Z3055, a clone linked to the Netherlands Q fever outbreaks, provides evidence for the role of drift in the emergence of epidemic clones. *Comp Immunol, Microbiol Infect Dis*, 2014; 37(5-6): 281-288.

Dal Pozzo F, Martinelle L, Léonard P, Renaville B, Renaville R, Thys C, Smeets F, Czaplicki G, Van Esbroeck M, Saegerman C. Q Fever Serological Survey and Associated Risk Factors in Veterinarians, Southern Belgium, 2013. *Transbound Emerg Dis*, 2017;64(3):959-966.

Davis GE, Cox HR. A filtering-passing infectious agent isolated from ticks. I: isolation from *Dermacentor andersoni*, reactions in humans and filtration experiments. *Public Health Rep*, 1938; 53:2259-67.

De Bruin A, Janse I, van Rotterdam. Molecular detection and typing of *Coxiella burnetii*. *RIVM*, 2009; Letter report 330291002.

de Bruin A, van Alphen PT, van der Plaats RQ, de Heer LN, Reusken CB, van Rotterdam BJ, Janse I. Molecular typing of *Coxiella burnetii* from animal and environmental matrices during Q fever epidemics in the Netherlands. *BMC Vet Res*, 2012;8:165.

De Bruin A. Detection of *Coxiella burnetii* by (q)PCR: a comparison of available assays. *RIVM*, 2011; Letter report 330071002.

de Cremoux R, Rousset E, Touratier A, Audusseau G, Nicollet P, Ribaud D, David V, Le Pape M. *Coxiella burnetii* vaginal shedding and antibody responses in dairy goat herds in a context of clinical Q fever outbreaks. *FEMS Immunol Med Microbiol*, 2012; 64 (1): 120-122.

De Lange MM, Schimmer B, Vellema P, Hautvast JL, Schneeberger PM, Van Duijnhoven YT. *Coxiella burnetii* seroprevalence and risk factors in sheep farmers and farm residents in The Netherlands. *Epidemiol Infect*, 2014;142(6):1231-1244.

de Rooij MM, Schimmer B, Versteeg B, Schneeberger P, Berends BR, Heederik D, van der Hoek W, Wouters IM. Risk factors of *Coxiella burnetii* (Q fever) seropositivity in veterinary medicine students. *PLoS One*, 2012;7(2): e32108.

de Rooij MMT, Borlée F, Smit LAM, de Bruin A, Janse I, Heederik DJJ, Wouters IM. Detection of *Coxiella burnetii* in Ambient Air after a Large Q Fever Outbreak. *PLoS One*, 2016; 11(3): e0151281.

Dehkordi FS. Prevalence Study of *Coxiella burnetii* in Aborted Ovine and Caprine Fetuses by Evaluation of Nested and Real-Time PCR Assays. *Am J Anim Vet Sci*, 2011; 6 (4): 180-186.

Dellacasagrande J, Ghigo E, Capo C, Raoult D, Mege JL. Coxiella burnetii Survives in Monocytes from Patients with Q Fever Endocarditis: Involvement of Tumor Necrosis Factor. *Infect Immun*, 2000; 68(1): 160-164.

Dellacasagrande J, Ghigo E., Capo C., Raoult, D, Mege JL. Coxiella burnetii Survives in Monocytes from Patients with Q Fever Endocarditis: Involvement of Tumor Necrosis Factor. *Infect Immun*, 2000; 68(1): 160-164.

Denison AM, Massung RF, Thompson HE. Analysis of theO-antigen biosynthesis regions of phase II Isolates of Coxiella burnetii. *FEMS Microbiol Lettters*, 2007; 267: 102–107.

Derrick EH. "Q" fever, a new fever entity: clinical features, diagnosis and laboratory investigation". *Rev Infect Dis*, 1983; 5:790-800.

Derrick, EH. Epidemiology of Q fever. *J Hyg (Lond)*, 1944; 43:357-361.

Desnues B, Imbert G, Raoult D, Mege JL, Ghigo E. Role of specific antibodies in Coxiella burnetii infection of macrophages. *Clin Microbiol Infect*, 2009; 15 (Suppl. 2): 161–162.

DGS - Direcção-Geral da Saúde. Doenças de Declaração Obrigatória, 1999-2003. Direcção de Serviços de Informação e Análise. Divisão de Epidemiologia. - Lisboa: DGS. 2004. [38 pp].

DGS - Direcção-Geral da Saúde. Doenças de Declaração Obrigatória, 2003-2007. Direcção-Geral da Saúde. Direcção de Serviços de Informação e Análise. Divisão de Epidemiologia. - Lisboa: DGS. 2008. [49 pp].

DGS - Direcção-Geral da Saúde. Doenças de Declaração Obrigatória, 2004-2008. Direcção-Geral da Saúde. Direcção de Serviços de Informação e Análise. Divisão de Epidemiologia. - Lisboa: DGS. 2010. [88 pp].

DGS - Direcção-Geral da Saúde. Doenças de Declaração Obrigatória, 2009-2012. Direcção-Geral da Saúde. Direcção de Serviços de Informação e Análise. Divisão de Epidemiologia. - Lisboa: DGS. 2014. [67 pp].

DGS - Direcção-Geral da Saúde. Doenças de Declaração Obrigatória, 2012-2015. Direcção-Geral da Saúde. Direcção de Serviços de Informação e Análise. Divisão de Epidemiologia. - Lisboa: DGS. 2016. [82 pp].

Dijkstra F, van der Hoek W, Wijers N, Schimmer B, Rietveld A, Wijkmans CJ, Vellema P, Schneeberger PM. The 2007–2010 Q fever epidemic in The Netherlands: characteristics of notified acute Q fever patients and the association with dairy goat farming. *FEMS Immunol Med Microbiol*, 2012; 64(1): 3-12.

Dolcé P, Bélanger MJ, Tumanowicz K, Gautier CP, Jutras P, Massé R, Montpetit C, Bernartchez H, McColl D, Artsob H. *Coxiella burnetii* seroprevalence of shepherds and their flocks in the lower Saint-Lawrence River region of Quebec, Canada. *Can J Infect Dis*, 2003; 14 (2): 97-102.

Dorko E, Rimárová K, Kecerová A, Pilipčinec E, Dudríková E, Lovayová V, Petrovičová J, Boroš E. Potential association between *Coxiella burnetii* seroprevalence and selected risk factors among veterinary students in Slovakia. *Ann Agric Environ Med*, 2011;18(1):47-53.

Drancourt M, Roux V, Dang LV et al. Genotyping, Orientalis-like *Yersinia pestis*, and Plague Pandemics. *Emerg Infect Dis*, 2004; 10:1585-92.

Dupuis G, Peacock M, Burgdorfer W, Haller E. Immunoglobulin Responses in Acute Q Fever, *J Clin Microbiol*, 1985; 22(4):484.

Dupuis G, Petite J, Péter O, Vouilloz M. An important outbreak of human Q fever in a Swiss Alpine valley. *Int J Epidemiol*, 1987;16(2):282-287.

Dupuy AG, Caron E. Integrin-dependent phagocytosis: spreading from microadhesion to new concepts. *J Cell Sci*, 2008; 121(11): 1773–1783.

Duron O, Jourdain E, McCoy KD. Diversity and global distribution of the *Coxiella* intracellular bacterium in seabird ticks. *Ticks Tick Borne Dis*, 2014;5(5):557-63.

Duron O, Noël V, McCoy KD, Bonazzi M, Sidi-Boumedine K, Morel O, Vavre F, Zenner L, Jourdain E, Durand P, Arnathau C, Renaud F, Trape1 JF, Biguezoton AS, Cremaschi J, Dietrich M, Léger E, Appelgren A, Dupraz M, Gómez-Díaz E, Diatta G, Dayo GK, Adakal H, Zoungrana S, Vial L, Chevillon C. The Recent Evolution of a Maternally-Inherited Endosymbiont of Ticks Led to the Emergence of the Q Fever Pathogen, *Coxiella burnetii*. *PLoS Pathog*, 2015; 11(5): e1004892.

Duron O. The IS1111 insertion sequence used for detection of *Coxiella burnetii* is widespread in Coxiella-like endosymbionts of ticks. *FEMS Microbiol Lett*, 2015; 362(17): fnv132.

Dyer RE. A filter-passing Infectious Agent isolated from Ticks. IV. Human Infection. *Public Health Rep*, 1938; 53:2277-82.

Dyer RE. Q fever – history and presente status. *Am J Public Health*, 1949; 39:471-7.

ECDC (European Centre for Diasease Prevention and Control). Annual Epidemiological Report of Communicable Diseases in Europe. ECDC, Stockholm. 2010. 181 pp.

Edouard S, Mahamat A, Demar M, Abboud P, Djossou F, Raoult D. Comparison between Emerging Q Fever in French Guiana and Endemic Q fever in Marseille, France. *Am J Trop Med Hyg*, 2014; 90(5): 915–919.

EFSA (European Food Safety Authority). Panel on Animal Health and Welfare (AHAW); Scientific Opinion on Q Fever. *EFSA Journal*, 2010: 8(5):1595. [114 pp]. Available online: www.efsa.europa.eu

EFSA (European Food Saftey Authority)[b]. Development of harmonized schemes for the monitoring and reporting of Q-fever in animals in the European Union. Scientific Report submitted to EFSA, Question No EFSAQ-2009-00511; 2010. [48 pp.]

EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards). Scientific Opinion on the public health risks related to the consumption of raw drinking milk. *EFSA Journal* 2015; 13:3940.

Egberink H, Addie D, Belák S, Boucraut-Baralon C, Frymus T, Gruffydd-Jones T, Hartmann K, Hosie MJ, Lloret A, Lutz H, Marsilio F, Möstl K, Pennisi MG, Radford AD, Thiry E, Truyen U, Horzinek MC. Coxiellosis/Q fever in cats: ABCD guidelines on prevention and management. *J Feline Med Surg*, 2013;15(7):573-575.

Eibach R, Bothe F, Runge M, Fischer SF, Philipp W, Ganter M. Q fever: baseline monitoring of a sheep and a goat flock associated with human infections. *Epidemiol Infect*, 2012; 140:1939-1949.

Ejercito CLA, Cai L, Htwe KK, Taki M, Inoshima Y, Kondo T, Kano C, Abe S, Shirota K, Sugimoto T, Yamaguchi T, Fukushi H, Minamoto N, Kinjo T, Isogai E, Hirai K. Serological evidence of *Coxiella burnetii* infection in wild animals in Japan. *J Wildl Dis*, 1993; 29:481-484.

Eldin C, Angelakis E, Renvoisé A, Raoult D. *Coxiella burnetii* DNA, but not viable bacteria, in dairy products in France. *Am J Trop Med Hyg*, 2013;88(4):765-769.

Eldin C, Mahamat A, Demar M, Abboud P, Djossou F, Raoult D. Q Fever in French Guiana. *Am J Trop Med Hyg*, 2014, 91(4): 771–776.

Eldin C, Mélenotte C, Mediannikov O, Ghigo E, Million M, Edouard S, Mege JL, Maurin M, Raoult D. 2017. From Q Fever to *Coxiella burnetii* Infection: a Paradigm Change. *Clin Microbiol Rev*, 2017; 30(1): 115-190.

Emery MP, Ostlund EN, Ichou MA, Ballin JD, McFarling D, McGonigle L. *Coxiella burnetii* serology assays in goat abortion storm. *J Vet Diagn Inv*, 2014; 26(1): 141-145.

Enright JB, Sadler WW, Thomas RC. Pasteurization of milk containing the organism of Q Fever. *Am J Public Health*, 1957; 47, 695-700.

Epelboin L, Chesnais C, Boullé C, Drogoul AS, Raoult D, Djossou F, Mahamat A. Q Fever Pneumonia in French Guiana: Prevalence, Risk Factors, and Prognostic Score. *Clin Infect Dis*, 2012; 55(1):67–74.

Ergas D, Keysari A, Edelstein V, Sthoeger ZM. Acute Q Fever in Israel: Clinical and Laboratory Study of 100 Hospitalized Patients. *IMAJ*, 2006; 8: 337-341.

Espejo E, Gil-Díaz A, Oteo JA, Castillo-Rueda R, García-Alvarez L, Santana-Báez S, Bella F. Clinical presentation of acute Q fever in Spain: seasonal and geographical diferences. *Int J Infect Dis*, 2014; 26: 162–164.

European Centre for Disease Prevention and Control. Annual epidemiological report 2014: emerging and vector-borne diseases. Stockholm: ECDC; 2014. [52 pp]. Available online:www.ecdc.europa.eu (accessed on August 08, 2016).

European Centre for Disease Prevention and Control. Annual Epidemiological Report 2011: Reporting on 2009 surveillance data and 2010 epidemic intelligence data. Stockholm: ECDC; 2011. [224 pp]. Available online:www.ecdc.europa.eu (accessed on August 02, 2016).

Ezatkhah M, Alimolaei M, Khalili M, Sharifi H. Seroepidemiological study of Q fever in small ruminants from Southeast *Iran. J Infect Public Health*, 2015;8(2):170-6.

Fenollar F, Fournier PE, Carrieri MP, Habib G, Messana T, Raoult D. Risks Factors and Prevention of Q Fever Endocarditis. *Clin Infect Dis*, 2001; 33:312–316.

Fernandes ACB. Rastreio serológico de Febre Q em ovinos no concelho de Montemor-o-Novo. [Serosurvey on Q fever in sheep at the county of Montemor-o-Novo]. Tese de Dissertação de Mestrado em Saúde Pública Veterinária. Faculdade de Medicina Veterinária. Universidade Técnica de Lisboa. Lisboa. 2008. [108 pp]. (in portuguese).

Fishbein DB, Raoult D. A cluster of *Coxiella burnetii* infections associated with exposure to vaccinated goats and their unpasteurized dairy products. *Am J Trop Med Hyg*, 1992; 47(1): 35-40.

Flannagan RS, Cosio G, Grinstein S. Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. *Nat Rev Microbiol*, 2009; 7: 355–366.

Flannagan RS, Jaumouillé V, Grinstein S. The cell biology of phagocytosis. *Ann Rev Pathol*, 2012; 7: 61–98.

Foronda P, Plata-Luis J, del Castillo-Figueruelo B, Fernández-Álvarez Á, Martín-Alonso A, Feliu C, Cabral MD, Valladares B. Serological survey of antibodies to Toxoplasma gondii and Coxiella burnetii in rodents in north-western African islands (Canary Islands and Cape Verde). *Onderstepoort J Vet Res*, 2015;82(1):899.

Foucault C, Lepidi H, Poujet-Abadie JF, Granel B, Roblot F, Ariga T, Raoult D. Q fever and lymphadenopathy: report of four new cases and review. *Eur J Clin Microbiol Infect Dis*, 2004;23(10):759-64.

Fournier P, Marrie TJ, Raoult D. Diagnosis of Q Fever. *J Clin Microbiol*, 1998;36 (7), 1823-1834.

Fournier PE, Etienne J, Harle JR, Habib G, Raoult D. Myocarditis, a Rare but Severe Manifestation of Q Fever: Report of 8 Cases and Review of the Literature. *Clin Infect Dis*, 2001; 32:1440–7.

Fournier PE, Raoult D. Comparison of PCR and Serology Assays for Early Diagnosis of Acute Q Fever. *J Clin Microbiol*, 2003;41(11):5094–5098.

Fournier PE, Raoult D. Predominant Immunoglobulin A Response to Phase II Antigen of *Coxiella burnetii* in Acute Q Fever. *Clin Diagn Lab Immunol*, 1999; 6(2): 173-177.

Francis JR, Robson J, Wong D, Walsh M, Astori I, Gill D, Nourse C. Chronic Recurrent Multifocal Q Fever Osteomyelitis in Children: An Emerging Clinical Challenge. *Pediatr Infect Dis J*, 2016; 35(9): 972-976.

Frangoulidis D, Splettstoesser WD, Landt O, Dehnhardt J, Henning K, Hilbert A, Bauer T, Antwerpen M, Meyer H, Walter MC, Knobloch JKM. Microevolution of the Chromosomal Region of Acute Disease Antigen A (adaA) in the Query (Q) Fever Agent *Coxiella burnetii*. *PLoS One*, 2013; 8(1), e53440.

Frangoulidis D, Walter MC, Antwerpen M, Zimmermann P, Janowetz B, Alex M, Böttcher J, Henning K, Hilbert A, Ganter M, Runge M, Münsterkötter M, Splettstoesser WD, Hanczaruk M. Molecular analysis of *Coxiella burnetii* in Germany reveals evolution of unique clonal clusters. *Int J Med Microbiol*, 2014; 304(7): 868-876.

Fretz R, Schaeren W, Tanner M, Baumgartner A. Screening of various foodstuffs for occurrence of *Coxiella burnetii* in Switzerland. *Int J Food Microbiol*, 2007; 116: 414–418.

Fujishiro MA, Scorza AV, Gookin JL, Lappin MR. Evaluation of associations among *Coxiella burnetii* and reproductive abnormalities in cats. *J Feline Med Surg*, 2016; 18(4): 344-347.

Gale P, Kelly L, Mearns R, Duggan J, Snary EL. Q fever through consumption of unpasteurised milk and milk products – a risk profile and exposure assessment. *J Appl Microbiol*, 2015; 118:1083–1095.

García-Ispierto I, López-Helguera I, Tutusaus J, Mur-Novales R, López-Gatius F. Effects of long-term vaccination against *Coxiella burnetii* on the fertility of high-producing dairy cows. *Acta Vet Hung*, 2015; 63 (2): 223–233.

García-Ispierto I, López-Helguera I, Tutusaus J, Serrano B, Monleón E, Badiola JJ, López-Gatius F. *Coxiella burnetii* Shedding During the Peripartum Period and Subsequent Fertility in Dairy Cattle. *Reprod Dom Anim*, 2013; 48: 441–446.

García-Ispierto I, Nogareda C, Yániz JL, Almería S, Martínez-Bello D, de Sousa NM, Beckers JF, López-Gatius F. *Neospora caninum* and *Coxiella burnetii* seropositivity are related to endocrine pattern changes during gestation in lactating dairy cows. *Theriogenology*, 2010; 74(2):212-220.

Garcia-Ispierto I, Tutusaus J, López-Gatius F. Does *Coxiella burnetii* affect reproduction in cattle? A clinical update. *Reprod Domest Anim*, 2014;49(4):529-535.

García-Pérez AL, Astobiza I, Barandika JF, Atxaerandio R, Hurtado A, Juste RA. Investigation of *Coxiella burnetii* occurrence in dairy sheep flocks by bulk-tank milk analysis and antibody level determination. *J Dairy Sci*, 2009; 92: 1581-1584.

Georgiev M, Afonso A, Neubauer H, Needham H, Thiéry R, Rodolakis A, Roest J, Stärk KD, Stegeman JA, Vellema P, van der Hoek W, More SJ. Q fever in humans and farm animals in four European countries, 1982 to 2010. *Euro Surveill*, 2013;18(8): pii=20407.

Ghigo E, Capo C, Raoult D, Mege JL. Interleukin-10 stimulates coxiella burnetii replication in human monocytes through tumor necrosis factor down-modulation: Role in microbicidal defect of Q fever. *Infect Immun*, 2001; 69(4): 2345–2352.

Ghigo E, Capo C, Tung CH, Raoult D, Gorvel JP, Mege JL. *Coxiella burnetii* survival in THP-1 monocytes involves the impairment of phagosome maturation: IFN-g mediates its restoration and bacterial killing. *J Immunol*, 2002; 169(8), 4488–4495.

Ghigo E, Honstettre A, Capo C, Gorvel J, Raoult D, Mege J. Link between impaired maturation of phagosomes and defective *Coxiella burnetii* killing in patients with chronic Q fever. *J Infect Dis*, 2004; 190(10), 1767–1772.

Ghigo E, Imbert G, Capo C, Raoult D, Mege JL. Interleukin-4 induces *Coxiella burnetii* replication in human monocytes but not in macrophages. *Ann N Y Acad Sci*, 2003;990:450-459.

Ghigo E, Pretat L, Desnues B, Capo C, Raoult D, Mege JL. Intracellular Life of *Coxiella burnetii* in Macrophages. *Ann N Y Acad Sci*, 2009; 1166: 55–66.

Gilsdorf A, Kroh C, Grimm S, Jensen E, Wagner-Wiening C, Alpers K. Large Q fever outbreak due to sheep farming near residential areas, Germany, 2005. *Epidemiol Infect*, 2008; 136:1084-1087.

Gimenez DF. Staining Rickettsiae in Yolk-Sac Cultures. *J Stain Technol*, 1964; 39(3): 135-140.

Giovannini A, Cancellotti FM, Turilli C, Randi E. Serological investigations for some bacterial and viral pathogens in fallow deer (*Cervus dama*) and wild boar (*Sus scrofa*) of the San Rossore Preserve, Tuscany, Italy. *J Wildl Dis*, 1988; 24(1): 127-132.

Glazunova O, Roux V, Freylikman O, Sekeyova Z, Fournous G, Tyczka J, Tokarevich N, Kovacova E, Marrie TJ, Raoult D. *Coxiella burnetii* Genotyping. *Emerg Infect Dis*, 2005; 11(8): 1211-1217.

González-Barrio D, Fernández-de-Mera IG, Ortiz JA, Queirós J, Ruiz-Fons F.[a] Long-Term Dynamics of *Coxiella burnetii* in Farmed Red Deer (*Cervus elaphus*). *Front Vet Sci*, 2015; 2: 74.

González-Barrio D, Santos JPV, Queiros J, Boadella M, Barasona JA, Beltran-Beck B, Ruiz-Fons F. Are wild ruminants a relevant node in *Coxiella burnetii* maintenance and transmission? The role of red deer (*Cervus elaphus*) in the Iberian Peninsula. Procedures of the 12th Biennial Conference of the Society for Tropical Veterinary Medicine (STVM) and the VIII International Conference on Ticks and Tick-borne Pathogens (TTP-8): Abstract number 0024. Cape Town, South Africa. 2014.

González-Barrio D, Velasco Ávila AL, Boadella M, Beltrán-Beck B, Barasona JÁ, Santos JP, Queirós J, García-Pérez AL, Barral M, Ruiz-Fons F.[b] Host and Environmental Factors Modulate the Exposure of Free-Ranging and Farmed Red Deer (*Cervus elaphus*) to *Coxiella burnetii*. *Appl Environ Microbiol*, 2015; 81(18): 6223-6231.

Gordon S, Taylor PR. Monocyte and macrophage heterongeneity. *Nat Rev Immunol*, 2005; 5(12): 953-964.

Graham JG, MacDonald LJ, Hussain SK, Sharma UM, Kurten RC, Voth DE. Virulent *Coxiella burnetii* Pathotypes Productively Infect Primary Human Alveolar Macrophages. *Cell Microbiol*, 2013; 15(6): 1012–1025.

Grilc E, Sočan M, Koren N, Učakar V, Avšič T, Pogačnik M, Kraigher A. Outbreak of Q fever among a group of high school students in Slovenia, March-April 2007. *Euro Surveill*, 2007; 12(29): pii=3237.

Guatteo R, Beaudau F, Berri M, Rodolaskis A, Joly A, Seegers H. Shedding routes of *Coxiella burnetii* in dairy cows: implications for detection and control. *Vet Res*, 2006; 37: 827-833.

Guatteo R, Beaudau F, Joly A, Seegers H.[a] Assessing the Within-herd Prevalence of *Coxiella burnetii* Milk-shedder Cows using a Real-time PCR Applied to Bulk Tank Milk. *Zoonoses Public Health*, 2007; 54: 191-194.

Guatteo R, Beaudau F, Joly A, Seegers H.[b] *Coxiella burnetii* shedding by dairy cows. *Vet Res*, 2007; 8: 849-860.

Guatteo R, Beaudeau F, Descarsin V, Sellal E, Rodolakis A, Joly A. Intérêt de la technique PCR dite temps reel pour la détection de *Coxiella burnetii*, agent d'une zoonose: la Fièvre Q. *Renc Rech Rum*, 2005; 12:281.

Guatteo R, Beaudeau F, Joly A, Seegers H. *Coxiella burnetii* shedding by dairy cows. *Vet Res*, 2007; 38: 849-860.

Guatteo R, Seegers H, Taurel AF, Joly A, Beaudeau F. Prevalence of *Coxiella burnetii* in domestic ruminants: a critical review. *Vet Microbiol*, 2011; 149: 1-16.

Guatteo R., Joly A, Beaudeau F. Shedding and serological patterns of dairy cows following abortions associated with *Coxiella burnetii* DNA detection. *Vet Microbiol*, 2012; 155: 430-433.

Gwida M, El-Ashker M1, El-Diasty M, Engelhardt C, Khan I, Neubauer H. Q fever in cattle in some Egyptian Governorates: a preliminary study. *BMC Res Notes*, 2014;7:881.

Haider N, Rahman MS, Khan SU, Mikolon A, Osmani MG, Gurley ES, Shanta IS, Paul SK, Macfarlane-Berry L, Islam A, Islam A, Desmond J, Epstein JH, Priestley RA, Kersh GJ, Rahman MZ, Daszak P, Luby SP, Massung RF, Zeidner N. Serological Evidence of *Coxiella burnetii* Infection in Cattle and Goats in Bangladesh. *Ecohealth*, 2015;12(2):354-348.

Hammerl JA, Mertens K, Sprague LD, Hackert VH, Buijs J, Hoebe CJ, Henning K, Neubauer H, Al Dahouka S. First Draft Genome Sequence of a Human *Coxiella*

burnetii Isolate, originating from the Largest Q Fever Outbreak Ever Reported, the Netherlands, 2007 to 2010. *Genome Announc*, 2015; 3(3): e00445-15.

Hansen MS, Rodolakis A, Cochonneau D, Agger JF, Christoffersen AB, Jensen TK, Agerholm JS. *Coxiella burnetii* associated placental lesions and infection level in parturient cows. *Vet J*, 2011;190(2): e135-9.

Harris RJ, Storm PA, Lloyd A, Arens M, Marmion BP. Long-term persistence of *Coxiella burnetii* in the host after primary Q fever. *Epidemiol Infect*, 2000 ;124: 543-549.

Hartzell JD, Wood-Morris RN, Martinez LJ, Trotta RF. Q Fever: Epidemiology, Diagnosis, and Treatment. *Mayo Clin Proc*, 2008;83(5):574-579.

Hawker JI1, Ayres JG, Blair I, Evans MR, Smith DL, Smith EG, Burge PS, Carpenter MJ, Caul EO, Coupland B, Desselberger U, Farrell ID, Saunders PJ, Wood MJ. A large outbreak of Q fever in the West Midlands: windborne spread into a metropolitan area? *Commun Dis Public Health*, 1998;1(3):180-187.

Heinzen R, Stiegler GL, Whiting LL, Schmitt SA, Mallavia LP, Frazier ME. Use of pulsed field gel electrophoresis to differentiate *Coxiella burnetii* strains. *Ann N Y Acad Sci*, 1990; 590:504-513.

Heinzen RA, Hackstadt T, Samuel JE. Developmental biology of *Coxiella burnetii. Trends Microbiol*, 1999; 7 (4), 149-154.

Heinzen RA, Hackstadt T. A developmental stage-specific histone H1 homolog of *Coxiella burnetii*. *J Bacteriol*, 1996; 78(16): 5049-5052.

Hernychova L, Toman R, Ciampor F, Hubalek M, Vackova J, Macela A, Skultety L. Detection and identification of *Coxiella burnetii* based on the mass spectrometric analyses of the extracted proteins. *Anal Chem*, 2008; 80 (18): 7097–7104.

Herremans T, Hogema BM, Nabuurs M, Peeters M, Wegdam-Blans M, Schneeberger P, Nijhuis C, Notermans DW, Galama J, Horrevorts A, van Loo IHM, Vlaminckx B, Zaaijer HL, Koopmans MP, Berkhout H, Socolovschi C, Raoult D, Stenos J, Nicholson W, Bijlmer H. Comparison of the performance of IFA, CFA, and ELISA assays for the serodiagnosis of acute Q fever by quality assessment. *Diagn Microbiol Infect Dis*, 2013; 75: 16–21.

Hilbert A, Schmoock G, Lenzko H, Moog U, Diller R, Fröhlich A, Hoffmann L, Horner S, Elschner M, Tomaso H, Henning K, Neubauer H, Sprague LD. Prevalence of *Coxiella burnetii* in clinically healthy German sheep flocks. *BMC Res Notes*, 2012; 5: 152.

Honstettre A, Ghigo E, Moynault A, Capo C, Toman R, Akira S, Takeuchi O, Lepidi H, Raoult D, Mege JL. Lipopolysaccharide from *Coxiella burnetii* is involved in bacterial phagocytosis, filamentous actin reorganization, and inflammatory responses through Toll-like receptor 4. *J Immunol*, 2004; 172(6): 3695–3703.

Honstettre A, Imbert G, Ghigo E, Gouriet F, Capo C, Raoult D, Mege JL. Dysregulation of cytokines in acute Q fever: role of interleukin-10 and tumor necrosis factor in chronic evolution of Q fever. *J Infect Dis*, 2003; 187(6), 956–962.

Hoover TA, Culp DW, Vodkin MH, Williams JC, Thompson HA. Chromosomal DNA Deletions Explain Phenotypic Characteristics of Two Antigenic Variants, Phase II and RSA 514 (Crazy), of the *Coxiella burnetii* Nine Mile Strain. *Infect Immun*, 2002; 70(12), 6726-6733.

Horigan MW, Bell MM, Pollard TR, Sayers AR, Pritchard GC. Q fever diagnosis in domestic ruminants: comparison between complement fixation and commercial enzyme-linked immunosorbent assays. *J Vet Diagn Invest*, 2011; 23(5): 924-931.

Hornstra HM, Priestley RA, Georgia SM, Kachur S, Birdsell DN, Hilsabeck R, Gates LT, Samuel JE, Heinzen RA, Kersh GJ, Keim P, Massung RF, Pearson T. Rapid Typing of *Coxiella burnetii. PLoS One*, 2011; 6(11): e26201.

Howe D, Mallavia LP. Coxiella Burnetii Exhibits Morphological Change and Delays Phagolysosomal Fusion after Internalization by J774A. 1 Cells. *Infect Immun*, 2000; 68 (7): 3815–21.

Howe D, Melnicáková J, Barák I, Heinzen RA. Maturation of the *Coxiella burnetii* parasitophorous vacuole requires bacterial protein synthesis but not replication. *Cell Microbiol*, 2003; 5, 469–480.

Howe D, Shannon JG, Winfree S, Dorward DW, Heinzen RA. *Coxiella burnetii* phase I and II variants replicate with similar kinetics in degradative phagolysosome-like compartments of human macrophages. *Infect Immun*, 2010; 78(8), 3465–74.

Hubalek Z, Juricová Z, Svobodová S Halouzka J. A serological survey for some bacterial and viral zoonoses in game animals in Czech Republic. *J Wildl Dis*, 1993; 29:604-607.

Huebner RJ, Jellison WL, Beck MD, Wilcox FP. Q fever studies in Southern California; effects of pasteurization on survival of *C. burnetii* in naturally infected milk. *Public Health Rep*, 1949;64(16):499-511.

Huijsmans CJJ, Schellekens JJA, Wever PC, Toman R, Savelkoul PHM, Janse I, Hermans MHA. Single-Nucleotide-Polymorphism Genotyping of *Coxiella burnetii*

during a Q Fever Outbreak in The Netherlands. *Appl Environ Microbiol*, 2011; 77(6): 2051–2057.

Islam A, Ferguson J, Givney R, Graves S. Seroprevalence to *Coxiella burnetii* Among Residents of the Hunter New England Region of New South Wales, Australia. *Am J Trop Med Hyg*, 2011; 84(2): 318–320.

Izzo AA, Marmion BP. Variation in interferon-gamma responses to *Coxiella burnetii* antigens with lymphocytes from vaccinated or naturally infected subjects. *Clin Exp Immunol*, 1993; 94:507-515.

Jado I, Carranza-Rodríguez C, Barandika JF, Toledo A, García-Amil C, Serrano B, Bolaños M, Gil H, Escudero R, García-Pérez AL, Olmeda AS, Astobiza I, Lobo B, Rodríguez-Vargas M, Pérez-Arellano JL, López-Gatius F, Pascual-Velasco F, Cilla G, Rodríguez NF, Anda P. Molecular method for the characterization of *Coxiella burnetii* from clinical and environmental samples: variability of genotypes in Spain. *BMC Microbiol*, 2012; 12:91.

Jäger C, Lautenschläger S, Willems H, Baljer G. *Coxiella burnetii* plasmid types QpDG and QpH1are closely related and likely identical. *Vet Microbiol*, 2002; 89:161-166.

Jaspers U, Thiele D, Krauss H. Monoclonal antibody based competitive ELISA for the detection of specific antibodies against *Coxiella burnetii* in sera from different animal species. *Zentralbl Bakteriol*, 1994; 281(1): 61-66.

Jensen TK, Montgomery DL, Jaeger PT, Lindhardt T, Agerholm JS, Bille-Hansen V, Boye M. Application of fluorescent in situ hybridisation for demonstration of *Coxiella burnetii* in placentas from ruminant abortions. *APMIS*, 2007; 115: 347–353.

Jones RM, Nicas M, Hubbard AE, Reingold AL. The Infectious Dose of *Coxiella burnetii* (Q Fever). *Appl Biosaf*, 2006; 11(1): 32–41.

Jourdain E, Duron O, Séverine B, González-Acuña D, Sidi-Boumedine K. Molecular methods routinely used to detect *Coxiella burnetii* in ticks, cross-react with *Coxiella*-like bacteria. *Infect Ecol Epidemiol*, 2015; 5: 29230.

Kampen AH, Hopp P, Groneng GM, Melkild I, Urdahl AM, Karlsson AC, Tharaldsen J. No indication of *Coxiella burnetii* infection in Norwegian farmed ruminants. *BMC Vet Res*, 2012; 8:59.

Kanouté YB, Gragnon BG, Schindler C, Bonfoh B, Schelling E. Epidemiology of brucellosis, Q Fever and Rift Valley Fever at the human and livestock interface in northern Côte d'Ivoire. *Acta Trop*, 2017;165:66-75.

Kaplan MM, Bertagna P. The geographical distribution of Q fever. *Bull Wld Hlth Org*, 1955; 13:829-60.

Karakousis PC, Trucksis M, Dumler JS. Chronic Q Fever in the United States. *J Clin Microbiol*, 2006; 44(6): 2283–2287.

Karki S, Gidding HF, Newall AT, McIntyre PB, Liu BC. Risk factors and burden of acute Q fever in older adults in New South Wales: a prospective cohort study. *Med J Aust*, 2015; 203(11):438.

Karlsson E, Macellaro A, Byström M, Forsman M, Frangoulidis D, Janse I, Larsson P, Lindgren P, Öhrman C, van Rotterdam B, Sjödin A, Myrtennäs K. Eight New Genomes and Synthetic Controls Increase the Accessibility of Rapid Melt-MAMA SNP Typing of *Coxiella burnetii*. *PLoS One*, 2014; 9(1): e85417.

Kennerman E, Rousset E, Golcu E, Dufour P. Seroprevalence of Q fever (coxiellosis) in sheep from the southern Marmara region, Turkey. *Comp Immunol Microb*, 2010; 33: 37-45.

Khalili M, Sakhaee E, Aflatoonian MR, Shahabi-Nejad N. Herd-prevalence of *Coxiella burnetii* (Q fever) antibodies in dairy cattle farms based on bulk tank milk analysis. *Asian Pacific J Trop Med*, 2011; 54: 58-60.

Khan SU, Atanasova KR, Krueger WS, Ramirez A, Gray GC. Epidemiology, geographical distribution, and economic consequences of swine zoonoses: a narrative review. *Emerg Microbes Infect*, 2013; 2(12): e92.

Khavkin T, Tabibzadeh SS. Histologic, immunofluorescence, and electron microscopic study of infectious process in mouse lung after intranasal challenge with *Coxiella burnetii*. *Infect Immun*, 1988; 56(7): 1792–1799.

Kilic S, Yilmaz GR, Komiya T, Kurtoglu Y, Karakoc EA. Prevalence of *Coxiella burnetii* antibodies in blood donors in Ankara, Central Anatolia, Turkey. *New Microbiol*, 2008; 31, 527-534.

Kim SG, Kim EH, Lafferty CJ, Dubovi E. *Coxiella burnetii* in Bulk Tank Milk Samples, United States. *Emerg Infect Dis*, 2005;11: 619-621.

Kinchen JM, Ravichandran KS. Phagosome maturation: going through the acid test. *Nat Rev Mol Cell Biol*, 2008; 9(10), 781–795.

Kirchgessner MS, Dubovi EJ, Whipps CM. Seroepidemiology of *Coxiella burnetii* in wild white-tailed deer (Odocoileus virginianus) in New York, United States. *Vector Borne Zoonotic Dis*, 2012;12(11):942-947.

Kittelberger R, Mars J, Wibberley G, Sting R, Henning K, Horner GW, Garnett KM, Hannah MJ, Jenner JA, Pigott CJ, O'Keefe JS. Comparison of the Q-fever complement fixation test and two commercial enzyme-linked immunosorbent assays for the detection of serum antibodies against *Coxiella burnetii* (Q-fever) in ruminants: Recommendations for use of serological tests on imported animals in New Zealand. *N Z Vet J*, 2009; 57(5):262-268.

Klaassen CHW, Nabuurs-Franssen MH, Tilburg JJHC, Hamans MAWM, Horrevorts AM. Multigenotype Q Fever outbreak, The Netherlands. *Emerg Infect Dis*, 2009; 15: 613-614.

Klee SR, Tyczka J, Ellerbrok H, Franz T, Linke S. Highly sensitive real-time PCR for specific detection and quantification of *Coxiella burnetii. BMC Microbiol*, 2006; 6:2.

Knobel DL, Maina AN, Cutler SJ, Ogola E, Feikin DR, Junghae M, Halliday JEB, Richards AL, Breiman RF, Cleaveland S, Njenga MK. *Coxiella burnetii* in Humans, Domestic Ruminants, and Ticks in Rural Western Kenya. *Am J Trop Med Hyg*, 2013;88(3):513–518.

Knobel DL, Maina AN, Cutler SJ, Ogola E, Feikin DR, Junghae M, Halliday JEB, Richards AL, Breiman RF, Cleaveland S, Njenga MK. *Coxiella burnetii* in Humans, Domestic Ruminants, and Ticks in Rural Western Kenya. *Am J Trop Med Hyg*, 2013; 88(3): 513–518.

Kofteridis DP, Mazokopakis EE, Tselentis Y, Gikas A. Neurological complications of acute Q fever infection. *Eur J Epidemiol*, 2004; 19: 1051–1054.

Komiya T, Sadamasu K, Kang MI, Tsuboshima S, Fukushi H, Hirai K.[a] Seroprevalence of *Coxiella burnetii* infections among cats in different living environments. *J Vet Med Sci*, 2003; 65(9): 1047-1048.

Komiya T, Sadamasu K, Toriniwa H, Kato K, Arashima Y, Fukushi H, Hirai K, Arakawa Y.[b] Epidemiological survey on the route of *Coxiella burnetii* infection in an animal hospital. *J Infect Chemother*, 2003; 9(2): 151-155.

Kopecny L, Bosward KL, Shapiro A, Norris JM. Investigating *Coxiella burnetii* infection in a breeding cattery at the centre of a Q fever outbreak. *J Feline Med Surg*, 2013; 15(12): 1037-1045.

Korkmaz S, Elaldi N, Kayatas M, Sencan M, Yildiz E. Unusual manifestations of acute Q fever: autoimmune hemolytic anemia and tubulointerstitial nephritis. *Ann Clin Microbiol Antimicrob*, 2012; 11:14.

Kosatski T. Household outbreak of Q fever pneumonia related to a parturient cat. *Lancet*, 1984; 2(8417-8418):1447-1449.

Koulla-Shiro S, Kuaban C, Belee L. Microbial etiology of acute community-acquired pneumonia in adult hospitalized patients in Yaounde-Cameroon. *Clin Microbiol Infect*, 1997; 3 (2): 180-186.

Kuley R, Smith HE, Frangoulidis D, Smits MA, Roest HIJ, Bossers A. Cell-Free Propagation of *Coxiella burnetii* Does Not Affect Its Relative Virulence. *PLoS One*, 2015; 10(3): e0121661.

Kuley R, Smith HE, Janse I, Harders FL, Baas F, Schijlen E, Nabuurs-Franssen MH, Smits MA, Roest HIJ, Bossersa A. First Complete Genome Sequence of the Dutch Veterinary *Coxiella burnetii* Strain NL3262, originating from the Largest Global Q Fever Outbreak, and Draft Genome Sequence of Its Epidemiologically Linked Chronic Human Isolate NLhu3345937. *Genome Announc*, 2016; 4(2): e00245-16.

La Scola B, Lepidi H, Raoult D. Pathologic Changes during Acute Q Fever: Influence of the Route of Infection and Inoculum Size in Infected Guinea Pigs. *Infect Immun*, 1997; 65(6): 2443–2447.

La Scola B, Raoult D. Survival of *Coxiella burnetii* within free-living amoeba Acanthamoeba castellanii. *Clin Microbiol Infect*, 2001; 7(2): 75–9.

La Scola B. Current Laboratory Diagnosis of Q Fever. *Semin Pediatr Infect Dis*, 2002; 13(4): 257-262.

Lai CH, Chang LL, Lin JN, Chen WF, Wei YF, Chiu CT, Wu JT, Hsu CK, Chen JY, Lee HS, Lin HH, Chen YH. Clinical Characteristics of Q Fever and Etiology of Community-Acquired Pneumonia in a Tropical Region of Southern Taiwan: A Prospective Observational Study. *PLoS One*, 2014; 9(7): e102808.

Lambton SL, Smith RP, Gillard K, Horigan M, Farren C, Pritchard GC. Serological survey using ELISA to determine the prevalence of *Coxiella burnetii* infection (Q fever) in sheep and goats in Great Britain. *Epidemiol Infect*, 2016;144(1):19-24.

Landais C, Fenollar F, Thuny F, Raoult D. From acute Q fever to endocarditis: serological follow-up strategy. *Clin Infect Dis*, 2007;44(10):1337-1340.

Lang G, Waltner-Toews D, Menzies P. The seroprevalence of coxiellosis (Q fever) in Ontario sheep flocks. *Can J Vet Res*, 1991;55(2):139-142.

Lang G. Prevalence of antibodies to *Coxiella* and *Chlamydia* spp. in cats in Ontario. *Can J Vet Res*, 1992; 33: 134.

Langley JM, Marrie TJ, Leblanc JC, Almudevar A, Resch L, Raoult D. *Coxiella burnetii* seropositivity in parturient women is associated with adverse pregnancy outcomes. *Am J Obstet Gynecol*, 2003; 189(1), 228-232.

Larson CL, Beare PA, Voth DE, Howe D, Cockrell DC, Bastidas RJ, Valdivia RH, Heinzen RA. *Coxiella burnetii* effector proteins that localize to the parasitophorous vacuole membrane promote intracellular replication. *Infect Immun*, 2015; 83(2), 661–670.

Larson CL, Martinez E, Beare PA, Jeffrey B, Heinzen RA, Bonazzi M. Right on Q: genetics begin to unravel *Coxiella burnetii* host cell interactions. *Future Microbiol*, 2016; 11(7): 919–939.

Ledina D, Bradarić N, Milas I, Ivić I, Brnčić N, Kuzmičić N. Chronic Fatigue Syndrome after Q fever. *Med Sci Monit*, 2007; 13(7): CS88-92.

Lennette EH, Holmes MA, Abinanti FR. Remove from marked Records Q. Fever Studies. XIV. Observations on the Pathogenesis of the Experimental Infection induced in Sheep by the Intravenous Route. *Am J Hyg*, 1952; 55(2): 254-267.

Lennette EH. Q fever in California. *Calif. Med.* 1948; 69:91-5.

Leone M, Honstettre A, Lepidi H, Capo C, Bayard F, Raoult D, Mege JL. Effect of sex on *Coxiella burnetii* infection: protective role of 17 beta-estradiol. *J Infect Dis*, 2004; 189(2):339-345.

Levy PY, Gouriet F, Habib G, Bonnet JL, Raoult D. Diagnosis of *Coxiella burnetii* pericarditis by using a systematic prescription kit in cases of pericardial effusion: an 8-year experience. *Clin Microbiol Infect*, 2009; 15 (Suppl. 2): 173–175.

Loftis AD, Priestley RA, Massung RF. Detection of *Coxiella burnetii* in Commercially Available Raw Milk from the United States. *Foodborne Pathog Dis*, 2010; 7: 1453-1456.

López-Gatius F, Almeria S, Garcia-Ispierto I. Serological screening for *Coxiella burnetii* infection and related reproductive performance in high producing dairy cows. *Res Vet Sci*, 2012; 93: 67–73.

Luedtke BE, Mahapatra S, Lutter EI, Shaw EI. The *Coxiella burnetii* Type IVB Secretion System (T4BSS) Component DotA is Released/Secreted During Infection of Host Cells and During in vitro Growth in a T4BSS Dependent Manner. *Pathog Dis*, 2017; 75(4): doi.org/10.1093/femspd/ftx047.

Lührmann A, Nogueira CV, Carey KL, Roy CR. Inhibition of pathogen-induced apoptosis by a *Coxiella burnetii* type IV effector protein. *PNAS*, 2010; 107(44): 18997–19001.

Lührmann A, Roy CR. *Coxiella burnetii* inhibits activation of host cell apoptosis through a mechanism that involves preventing cytochrome c release from mitochondria. *Infect Immun*, 2007; 75(11): 5282–5289.

Lukšić B, Punda-Polić V, Ivić I, Bradarić I, Bradarić N. Clinical and epidemiological features of hospitalized acute Q fever cases from Split-Dalmatia County (Croatia), 1985–2002. *Med Sci Monit*, 2006; 12(3): 126-131.

Lyytikäinen O, Ziese T, Schwartländer B, Matzdorff P, Kuhnhen C, Jäger C, Petersen L. An outbreak of sheep-associated Q fever in a rural community in Germany. *Eur J Epidemiol*, 1998; 14: 193–199.

Macellaro A, Akesson A, Norlander L. A survey of Q fever in Sweden. *Eur J Epidemiol*, 1993; 9(2): 213–216.

MacKerras IM. *Edward Holbrook Derrick* 1898-1976. 1998. Available from http:// www.asap.unimelb.edu.au/bsparcs/aasmemoirs/derrick.htm#mac; Accessed on July 10, 2016.

Magouras I, Hunninghaus J, Scherrer S, Wittenbrink MM, Hamburger A, Stärk KD, Schüpbach-Regula G. *Coxiella burnetii* Infections in Small Ruminants and Humans in Switzerland. *Transbound Emerg Dis*, 2017;64(1):204-212.

Maia C, Ferreira A, Nunes M, Vieira ML, Campino L, Cardoso L. Molecular detection of bacterial and parasitic pathogens in hard ticks from Portugal. *Ticks Tick Borne Dis*, 2014; 5(4): 409-414.

Maltezou HC, Constantopoulou I, Kallergi C, Vlahou V, Georgakopoulos D, Kafetzis DA, Raoult D. Q Fever in children in Greece. *Am J Trop Med Hyg*, 2004; 70(5): 540–544.

Maltezou HC, Raoult D. Q fever in children. *Lancet Infect Dis*, 2002; 2: 686–91.

Mancini F, Di Luca M, Toma L, Vescio F, Bianchi R, Khoury C, Marini L, Rezza G, Ciervo A. Prevalence of tick-borne pathogens in an urban park in Rome, Italy. *Ann Agric Environ Med*, 2014; 21(4): 723-727.

Marenzoni ML, Stefanetti V, Papa P, Proietti PC, Bietta A, Coletti M, Passamonti F, Henning K. Is the horse a reservoir or an indicator of *Coxiella burnetii* infection? Systematic review and biomolecular investigation. *Vet Microbiol*, 2013;167(3-4):662-669.

Mares-Guia MAMM, Rozental T, Guterres A, Gomes R, de Almeida DN, Moreira NS, Barreira JD, Favacho AR, Santana AL, de Lemos ERS. Molecular identification of the agent of Q fever – *C. burnetii* – in domestic animals in State of Rio de Janeiro, Brazil. *Rev Soc Bras Med Trop*, 2014; 47(2): 231-234.

Marmion BP, Shannon M, Maddocks I, Storm P, Penttila I. Protracted debility and fatigue after acute Q fever. *Lancet*, 1996;347(9006):977-8.

Marmion BP, Stocker MGP. The epidemiology of Q fever in Great Britain. *Br Med J*, 1958; 2:809-816.

Marmion BP, Storm PA, Ayres JG, Semendric L, Mathews L, Winslow W, Turra M, Harris RJ. Long-term persistence of *Coxiella burnetii* after acute primary Q fever. *QJM*, 2005; 98: 7-20.

Marmion BP, Sukocheva O, Storm PA, Lockhart M, Turra M, Kok T, Ayres J, Routledge H, Graves S. Q fever: persistence of antigenic non-viable cell residues of *Coxiella burnetii* in the host—implications for post Q fever infection fatigue syndrome and other chronic sequelae. *QJM*, 2009; 102:673–684.

Marrie TJ, Buren JV, Fraser J, Haldane EV, Faulkner RS, Williams JC, Kwan C. Seroepidemiology of Q Fever among Domestic Animals in Nova Scotia. *Am J Public Health*, 1985;75(7):763-766.

Marrie TJ, Campbell N, McNeil SA, Webster D, Hatchette TF. Q Fever Update, Maritime Canada. *Emerg Infect Dis*, 2008; 14(1): 67-69.

Marrie TJ, Durant H, Williams JC, Mintz E, Waag DM. Exposure to Parturient Cats: A Risk Factor for Acquisition of Q Fever in Maritime Canada. *J Infect Dis*, 1988; 158:101-108.

Marrie TJ, MacDonald A, Durant H, Yates L, McCormick L.[b] An outbreak of Q fever probably due to contact with a parturient cat. *Chest,* 1988; 93(1): 98-103.

Marrie TJ, Pollack PT. Seroepidemiology of Q fever in Nova Scotia: evidence for age dependent cohorts and geographical distribution. *Eur J Epidemiol*, 1995; 11: 47-54.

Marrie TJ, Raoult D. Q Fever – a review and issues for the next century. *Int J Antimicrob Agents*, 1997; 8: 145-161.

Marrie TJ, Stein A, Janigan D, Raoult D. Route of infection determines the clinical manifestation of acute Q Fever. *J Infect Dis*, 1996; 173: 484-487.

Marrie TJ. Coxiella burnetii pneumonia. *Eur Respir J*, 2003; 21: 713–719.

Marrie TJ. Pneumonia and meningo-encephalitis due to *C. burnetii. J Infect*, 1985, II: 59-61.

Marriott HM, Dockrell DH. The role of the macrophage in lung disease mediated by bacteria. *Exp Lung Res*., 2007;33(10):493-505.

Martinez E, Cantet F, Fava L, Norville I, Bonazzi M. Identification of OmpA, a *Coxiella burnetii* Protein Involved in Host Cell Invasion, by Multi-Phenotypic High-Content Screening. *PLoS Pathog*., 2014; 10(3): e1004013.

Martinov SP, Neikov P, Popov GV.[a] Experimental Q fever in sheep. *Eur J Epidemiol*, 1989;5(4):428-431.

Martinov SP, Pandarov S, Popov GV.[b] Seroepizootology of Q fever in Bulgaria during the last five years. *Eur J Epidemiol*, 1989;5(4):425-427.

Martinov SP. *Coxiella burnetii* Endometritis and Metritis in Cows. Abstract book of the 13th International Congress on Infectious Diseases, 2008; Poster number 18.006: e126.

Masala G, Porcu R, Sanna G, Chessa G, Cillara G, Chisu V, Tola S. Occurrence, distribution, and role in abortion of *Coxiella burnetii* in sheep and goats in Sardinia, Italy. *Vet Microbiol*, 2004; 99: 301-305.

Massung RF, Cutler SJ, Frangoulidis D. Molecular Typing of *Coxiella burnetii* (Q Fever*). Adv Exp Med Biol*, 2012;984:381-396.

Matthewman L, Kelly P, Hayter D, Downie S, Wray S, Bryson N, Rycroft A, Raoult D. Exposure of cats in southern Africa to *Coxiella burnetii*, the agent of Q fever. *Eur J Epidemiol*, 1997;13: 477–479.

Maurin M, Benolieil AM, Bongrand P, Raoult D. "Phagolysosomal Alkalinization and the Bactericidal Effect of Antibiotics: The *Coxiella burnetii* Paradigm." *J Infect Dis*, 1992; 166 (5): 1097–1102.

Maurin M, Raoult D. Q Fever. *Clin Microbiol Rev*, 1999; 12:518-53.

McCaughey C, Murray LJ, McKenna JP, Menzies FD, McCullough SJ, O'Neill HJ, Wyatt DE, Cardwell CR, Coyle PV. *Coxiella burnetii* (Q fever) seroprevalence in cattle. *Epidemiol Infect*, 2010; 138: 21-27.

McCaul TF, Banerjee-Bhatnagar N., Williams JC. Antigenic Differences between *Coxiella burnetii* Cells Revealed by Postembedding Immunoelectron Microscopy and Immunoblotting. *Infect Immun*,1991 ;59(9):3243-3253.

McCaul TF, Williams JC. Developmental Cycle of *Coxiella burnetii*: Structure and Morphogenesis of Vegetative and Sporogenic Differentiations. *J Bacteriol*, 1981; 147(3), 1063-1076.

McMillan S, Verner-Jeffreys D, Weeks J, Austin B, Desbois AP. Larva of the greater wax moth, *Galleria mellonella*, is a suitable alternative host for studying virulence of fish pathogenic Vibrio anguillarum. *BMC Microbiol*, 2015; 15:127.

McQuiston JH, Childs JE, Thompson HA. Q fever. *JAVMA*, 2002; 221(6):796-799.

Meadows S, Jones-Bitton A, McEwen S, Jansen J, Menzies P. *Coxiella burnetii* seropositivity and associated risk factors in goats in Ontario, Canada. *Prev Vet Med*, 2015;121(3-4):199-205.

Meconi S, Jacomo V, Boquet P, Raoult D, Mege JL, Capo C. *Coxiella burnetii* Induces Reorganization of the Actin Cytoskeleton in Human Monocytes. *Infect Immun*, 1998; 66(11):5527-5533.

Mediannikov O, Fenollar F, Socolovschi C, Diatta G, Bassene H, Molez JF, Sokhna C, Trape JF, Raoult D. *Coxiella burnetii* in Humans and Ticks in Rural Senegal. *PLoS Negl Trop Dis*, 2010; 4(4): e654.

Medic A, Dzelalija B, Punda Polic V, Gjenero Marjan I, Turkovic I, Gilic V. Q fever epidemic among employees in a factory in the suburb of Zadar. Croatia. *Croat Med J*, 2005; 46: 315-319.

Medic S, Kaluski DN, Seguljev Z, Obrenovic J, Rudan P, Lazarevic M, Kocic JJ, Sajenkovic D, Pusic I, Bugarski D, Vidanovic D, Sekler M. Q fever outbreak in the village of Nocaj, Srem County, Vojvodina Province, Serbia, January to February 2012. *Euro Surveill*, 2012; 17(15): pii=20143.

Mege JL, Maurin M, Capo C, Raoult D. *Coxiella burnetii*: the "query" fever bacterium – A model of immune subversion by a strictly intracellular microorganism. *FEMS Microbiol Rev,* 1997; 19, 209-217.

Meng XJ, Lindsay DS, Sriranganathan N. Wild boars as sources for infectious diseases in livestock and humans. *Phil Trans R Soc B*, 2009; 364: 2697-2707.

Meredith AL, Cleaveland SC, Denwood MJ, Brown JK, Shaw DJ. *Coxiella burnetii* (Q Fever) Seroprevalence in Prey and Predators in the United Kingdom: Evaluation of Infection in Wild Rodents, Foxes and Domestic Cats Using a Modified ELISA. *Transbound Emerg Dis*, 2015;62(6):639-49.

Merhej V, Royer-Carenzi M, Pontarotti P, Raoult D. Massive comparative genomic analysis reveals convergent evolution of specialized bacteria. *Biol Direct*, 2009; 4, 13.

Michelet L, Delannoy S, Devillers E, Umhang G, Aspan A, Juremalm M, Chirico J, van der Wal FJ, Sprong H, Boye Pihl TP, Klitgaard K, Bødker R, Fach P, Moutailler S.

High-throughput screening of tick-borne pathogens in Europe. *Front Cell Infect Microbiol*, 2014; 4: 103.

Millar JA, Beare PA, Moses AS, Martens CA, Heinzen RA, Raghavana R. Whole-Genome Sequence of *Coxiella burnetii* Nine Mile RSA439 (Phase II, Clone 4), a Laboratory Workhorse Strain. *Genome Announc*, 2017; 5(23), e00471-17.

Million M, Lepidi H, Raoult D. [Q fever: current diagnosis and treatment options]. *Med Mal Infect*, 2009; 39(2): 82–94.

Million M, Raoult D. No Such Thing as Chronic Q Fever. *Emerg Infect Dis*, 2017; 23(5):856-857.

Million M, Raoult D. Recent advances in the study of Q fever epidemiology, diagnosis and management. *J Infect*, 2015;71(1): S2-9. doi: 10.1016/j.jinf.2015.04.024.

Million M, Walter G, Thuny F, Habib G, Raoult D. Evolution From Acute Q Fever to Endocarditis Is AssociatedWith Underlying Valvulopathy and Age and Can Be Prevented by Prolonged Antibiotic Treatment. *Clin Infect Dis*, 2013;57(6):836–844.

Million M1, Thuny F, Richet H, Raoult D. Long-term outcome of Q fever endocarditis: a 26-year personal survey. *Lancet Infect Dis*, 2010;10(8):527-535.

Minnick MF, Heinzen RA, Frazier ME, Mallavia LP. Characterization and expression of the cbbE gene *Coxiella burnetii*. *J Gen Microbiol*, 1990; 136: 1099-1107.

Minnick MF, Heinzen RA, Reschke DK, Frazier ME, Mallavia LP. A Plasmid-Encoded Surface Protein Found in Chronic-Disease Isolates of *Coxiella burnetii*. *Infect Immun*, 1991; 59(12), 4735-4739.

Miragliotta G, Fumarola D, Brezina R. The ability of mononuclear cells to coagulate blood in response to *Coxiella burnetii*. *J Med Microbiol*, 1989; 29: 9-12.

Mohammed OB, Jarelnabi AA, Aljumaah RS, Alshaikh MS, Bakhiet AO, Omer SA, Alagaili AN, Hussein MF. *Coxiella burnetii*, the causative agent of Q fever in Saudi Arabia: molecular detection from camel and other domestic livestock. *Asian Pac J Trop Med*, 2014: 715-719.

Monno R, Fumarola L, Trerotoli P, Cavone D, Giannelli G, Rizzo C, Ciceroni L, Musti M. Seroprevalence of Q fever, brucellosis and leptospirosis in farmers and agricultural workers in Bari, Southern Italy. *Ann Agric Environ Med*, 2009; 16: 205–209.

Montes M, Cilla G, Marimon JM, Diaz de Tuesta JL, Perez-Trallero E. *Coxiella burnetii* infection in subjects with HIV infection and HIV infection in patients with Q fever. *Scand J Infect Dis*, 1995; 27(4):344-6.

Moos A, Hackstadt T. Comparative Virulence of Intra- and Interstrain Lipopolysaccharide Variants of *Coxiella burnetii* in the Guinea Pig Model. *Infect Immun*, 1987; 55(5):1144-1150.

Morovic M, Dzelalija B, Novakovic S, Stankovic S, Dujella J. Acute Renal Failure as the main complication of acute infection with *Coxiella burnetii*. *Nephron*, 1993; 64: 335.

Morroy G, Keijmel SP, Delsing CE, Bleijenberg G, Langendam M, Timen A, Bleeker-Rovers CP. Fatigue following Acute Q-Fever: A Systematic Literature Review. *PLoS One*, 2016; 11(5): e0155884.

Moses AS, Millar JA, Bonazzi M, Beare PA, Raghavan R. Horizontally Acquired Biosynthesis Genes Boost *Coxiella burnetii*'s Physiology. *Front Cell Infect Microbiol*, 2017; 7:174.

Moskwa B, Cybulska A, Kornacka A, Cabaj W, Bień J. Wild boars meat as a potential source of human trichinellosis in Poland: current data. *Acta Parasitol*, 2015; 60(3): 530-535.

Munster JM, Steggerda LM, Leenders AC, Aarnoudse JG, Hak E. Screening for *Coxiella burnetii* infection during pregnancy: pros and cons according to the Wilson and Jungner criteria. *Euro Surveill*, 2012;17(3):pii=20061.

Muskens J, van Engelen E, van Maanen C, Bartels C, Lam TJGM. Prevalence of *Coxiella burnetii* infection in Dutch dairy herds based on testing bulk tank milk and individual samples by PCR and ELISA. *Vet Rec*, 2011; 168: 79.

Nagaoka H, Sugieda M, Akiyama M, Nishina T, Akahane S, Fujiwara K. Isolation of *Coxiella burnetii* from the vagina of feline clients at veterinary clinics. *J Vet Med Sci*, 1998;60(2):251-252.

Natalea A, Buccia G, Capello K, Barberio A, Tavella A, Nardelli S, Marangon S, Ceglie L. Old and new diagnostic approaches for Q fever diagnosis: Correlation among serological (CFT, ELISA) and molecular analyses. *Comp Immunol Microbiol Infect Dis*, 2012; 35: 375– 379.

Neth OW, Falcon D, Peromingo E, Soledad Camacho M, Rodríguez-Gallego C, Obando I. Successful management of chronic multifocal Q fever Osteomyelitis with adjuvant interferon-gamma therapy. *Pediatr Infect Dis J*, 2011; 30(9):810-812.

Nielsen KT, Nielsen SS, Agger JF, Christoffersen AB, Agerholm JS. Association between antibodies to *Coxiella burnetii* in bulk tank milk and perinatal mortality of Danish dairy calves. *Acta Vet Scand*, 2011; 53: 64.

Nielsen SY, Hjøllund NH, Andersen A-MN, Henriksen TB, Kantsø B, Krogfelt KA, Mølbak K. Presence of Antibodies Against *Coxiella burnetii* and Risk of Spontaneous Abortion: A Nested Case-Control Study. *PLoS One*, 2012; 7(2): e31909.

Nielsen SY, Mølbak K, Andersen AMN, Henriksen TB, Kantsø B, Krogfelt KA, Hjøllund NH. Prevalence of *Coxiella burnetii* in women exposed to livestock animals, Denmark, 1996 to 2002. *Euro Surveill*, 2013;18(28): pii=20528.

Njeru J, Henning K, Pletz MW, Heller R, Forstner C, Kariuki S, Fèvre EM, Neubauer H. Febrile patients admitted to remote hospitals in Northeastern Kenya: seroprevalence, risk factors and a clinical prediction tool for Q-Fever. *BMC Infect Dis*, 2016;16:244.

Norville IH, Hartley MG, Martinez E, Cantet F, Bonazzi M, Atkins TP. *Galleria mellonella* as an alternative model of Coxiella burnetii infection. *Microbiol*, 2014;160(6):1175-1181.

Nourse C, Allworth A, Jones A, Horvath R, McCormack J, Bartlett J, Hayes D, Robson JM. Three Cases of Q Fever Osteomyelitis in Children and a Review of the Literature. *Clin Infect Dis*, 2004; 39:e61–e66.

Nusinovici S, Frössling J, Widgren S, Beaudeau F, Lindberg A. Q fever infection in dairy cattle herds: increased risk with high wind speed and low precipitation. *Epidemiol Infect*, 2015;143(15):3316-3326.

O'Connor BA, Tribe IG, Givney R. A windy day in a sheep saleyard: an outbreak of Q fever in rural South Australia. *Epidemiol Infect*, 2015;143(2):391-398.

Ohlson A, Malmsten J, Frössling J, Bölske G, Aspán A, Dalin AM, Lindberg A. Surveys on *Coxiella burnetii* infections in Swedish cattle, sheep, goats and moose. *Acta Vet Scand*, 2014; 56:39.

OIE (Office International des Épizooties) – World Organisation for Animal Health. Chapter 2.1.16. Q Fever. In: Manual of Diagnostic Tests and Vaccines for Terrestrial animals. 2015. Available from: http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.16_Q_FEVER.pdf; accessed on August 2nd 2016.

OIE (Office International des Épizooties) – World Organisation for Animal Health. WAHIS Interface. Available from: http://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home; acessed on August 5th 2016.

Okun DB, Sun NC, Tanaka KR. Bone marrow granulomas in Q fever. *Am J Clin Pathol*, 1979; 71(1):117-121.

Omsland A, Beare PA, Hill J, Cockrell DC, Howe D, Hansen B, Samuel JE, Heinzen RA. Isolation from Animal Tissue and Genetic Transformation of *Coxiella burnetii* Are Facilitated by an Improved Axenic Growth Medium. *Appl Environ Microbiol*, 2011; 77(11):3720-5.

Omsland A, Cockrell DC, Howe D, Fischer ER, Virtaneva K, Sturdevant DE, Porcella SF, Heinzen RA. Host cell-free growth of the Q fever bacterium *Coxiella burnetii*. *PNAS*, 2009; 106(11): 4430-4434.

Omsland A, Hackstadt T, Heinzen RA. Bringing Culture to the Uncultured: *Coxiella burnetii* and Lessons for Obligate Intracellular Bacterial Pathogens. *PLoS Pathog*, 2013; 9(9): e1003540.

Omsland A, Heinzen RA. Life on the outside: the rescue of *Coxiella burnetii* from its host cell. *Ann Rev Microbiol*, 2011; 65, 111-128.

Omsland A. Axenic growth of *Coxiella burnetii*. *Adv Exp Med Biol*, 2012; 984:215-229.

Oporto B, Barandika JF, Hurtado A, Aduriz G, Moreno B, Garcia-Perez AL. Incidence of ovine abortion by *Coxiella burnetii* in northern Spain. *Ann N Y Acad Sci*, 2006; 1078: 498-501.

Ordi-Ros J, Selva-O'Callaghan A, Monegal-Ferran F, Monasterio-Aspiri Y, Juste-Sanchez C, Vilardell-Tarres M. Prevalence, Significance, and Specificity of Antibodies to Phospholipids in Q Fever. *Clin Infect Dis*, 1994;18:213-218.

Oyston PCF, Davies C. Q fever: the neglected biothreat agent. *J Med Microbiol*, 2011; 60: 9–21.

Palkovicova K, Ihnatko R, Vadovic P, Betinova E, Skultety L, Frangoulidis D, Toman R. A monoclonal antibody specific for a unique biomarker, virenose, in a lipopolysaccharide of *Coxiella burnetii*. *Clin Microbiol Infect*, 2009; 15(suppl 2), 183-184.

Palmela C, Badura R, Valadas E. Acute Q fever in Portugal. Epidemiological and clinical features of 32 hospitalized patients. *Germs*, 2012; 2(2):43-59.

Panaiotov S, Ciccozzi M, Brankova N, Levterova V, Mitova-Tiholova M, Amicosante M, Rezza G, Kantardjiev T. An outbreak of Q fever in Bulgaria. *Ann Ist Super Sanita*, 2009; 45: 83-86.

Pandit P, Hoch T, Ezanno P, Beaudeau F, Vergu E. Spread of *Coxiella burnetii* between dairy cattle herds in an enzootic region: modelling contributions of airborne transmission and trade. *Vet Res*, 2016;47:48.

Panning M, Kilwinski J, Greiner-Fischer S, Peters M, Kramme S, Frangoulidis D, Meyer H, Henning K, Drosten C. High throughput detection of *Coxiella burnetii* by real-time PCR with internal control system and automated DNA preparation. *BMC Microbiol*, 2008, 8:77.

Pape M, Bouzalas EG, Koptopoulos GS, Mandraveli K, Arvanitidou-Vagiona M, Nikolaidis P, Alexiou-Daniel S. The serological prevalence of *Coxiella burnetii* antibodies in sheep and goats in northern Greece. *Clin Microbiol Infect*, 2009; 15: 146-147.

Pareja MEM, Bongiovanni A, Lafont F, Colombo MI. Alterations of the *Coxiella burnetii* Replicative Vacuole Membrane Integrity and Interplay with the Autophagy Pathway. *Front Cell Infect Microbiol*, 2017; 7: 112.

Parisi A, Fraccalvieri R, Cafiero M, Miccolupo A, Padalino I, Montagna C, Capuano F, Sottili R. Diagnosis of *Coxiella burnetii*-related abortion in Italian domestic ruminants using single-tube nested PCR. *Vet Microbiol*, 2006; 118: 101–106.

Parker NR, Barralet JH, Bell AM. Q fever. *Lancet*, 2006; 367: 679-688.

Parola P, Raoult D. Ticks and Tickborne Bacterial Diseases in Humans: An Emerging Infectious Threat. *Clin Infect Dis*, 2001; 32: 897-928.

Paul S, Agger JF, Agerholm JS, Markussen B. Prevalence and risk factors of Coxiella burnetii seropositivity in Danish beef and dairy cattle at slaughter adjusted for test uncertainty. *Prev Vet Med*, 2014; 113: 504-511.

Paul S, Toft N, Agerholm JS, Christoffersen AB, Agger JF. Bayesian estimation of sensitivity and specificity of *Coxiella burnetii* antibody ELISA tests in bovine blood and milk. *Prev Vet Med*, 2013; 109: 258-263.

Pearson T, Cocking JH, Hornstra HM, Keim P. False detection of *Coxiella burnetii*—what is the risk? *FEMS Microbiol Lett*, 2016; 363: fnw088.

Pearson T, Hornstra HM, Hilsabeck R, Gates LT, Olivas SM, Birdsell DM, Hall CM, German S, Cook JM, Seymour ML, Priestley RA, Kondas AV, Friedman CLC, Price EP, Schupp JM, Liu CM, Price LB, Massung RF, Kersh GJ, Keim P. High prevalence and two dominant host-specific genotypes of *Coxiella burnetii* in U.S. milk. *BMC Microbiol*, 2014; 14: 41.

Pebody RG, Wall PG, Ryan MJ, Fairley C. Epidemiological features of *Coxiella burnetii* infection in England and Wales: 1984 to 1994. *Commun Dis Rep CDR Rev*, 1996; 6(9): 128-132.

Pilo C, Addis G, Deidda M, Tedde MT, Liciardi M. A serosurvey for Brucellosis in wild boar (*Sus Scrofa*) in Sardinia, Italy. *J Wildl Dis*, 2015; ;51(4): 885-888.

Pimenta L, Alegria N, Anastácio S, Sidi-Boumedine K, da Silva G, Rabiço A, Simões J. Prevalence of *Coxiella burnetii* antibodies in Portuguese dairy cattle herds. *Trop Anim Health Prod*, 2015;47(1): 227-230.

Piñero A, Barandika JF, García-Pérez AL et al. Genetic diversity and variation over time of *Coxiella burnetii* genotypes in dairy cattle and the farm environment. *Infect Genet Evol*, 2015; 31:231-5.

Pinsky RL, Fishbein DB, Greene CR, Gensheimer KF. An outbreak of cat-associated Q fever in the United States. *J Infect Dis*., 1991;164(1):202-204.

Plommet M, Capponi M, Gestin J, Renoux G: Experimental Q fever in cattle [in French]. *Ann Rech Vétér*, 1973, 4:325–346.

Popescu C, Lobodan A, Dulama R, Negru AR, Radulesku M, Tiliscan C, Popescu GA, Poghirc V, Popescu R, Juganaru G, Arama V. Q fever in urban area – an emerging zoonosis. *BMC Infect Dis*, 2014; 14(Suppl 7): P86.

Porten K, Rissland J, Tigges A, Broll S, Hopp W, Lunemann M, Van Treeck U, Kimmig P, Brockmann SO, Wagner-Wiening C, Hellenbrand W, Buchholz U. A superspreading ewe infects hundreds with Q fever at a farmers' market in Germany. *BMC Infect Dis*, 2006; 6: 147.

Porter SR, Czaplicki G, Mainil J, Guatteo R, Saegerman C. Q fever: current state of knowledge and perspectives of research of a neglected zoonosis. *Int J Microbiol*, 2011; 2011: 248418.

Portugal: Decreto de Lei 315/2003 (17 de Dezembro de 2003).

Portugal: Portaria nº 1071/98 (31 de Dezembro de 1998).

Psaroulaki A, Chochlakis D, Angelakis E, Ioannou I, Tselentis Y. *Coxiella burnetii* in wildlife and ticks in an endemic area. *Trans R Soc Trop Med Hyg*, 2014; 108(10):625-31.

Punda-Polic V, Poljak S, Bubic A, Bradaric N, Klismanic-Nuber Z. Antibodies to spotted fever group rickettsiae and *Coxiella burnetii* among domestic animals in southern Croatia. *Acta Microbiol Immunol Hung*, 1995; 42(4): 339-344.

Racka K, Bartová E, Budikova M, Vodrazka P. Survey of *Toxoplasma gondii* antibodies in meat juice of wild boar (*Sus scrofa*) in several districts of the Czech Republic. *Ann Agric Environ Med*, 2015; 22(2): 231-235.

Raoult D, Dupont HT, Foucault C, Gouvernet J, Fournier PE, Bernit E, Stein A, Nesri M, Harle JR, Weiler PJ. Q fever 1985-1998 – Clinical and Epidemiologic Features of 1383 infections. *Medicine*, 2000; 79(2): 109-123.

Raoult D, Fenollar F, Stein A. Q fever during pregnancy: diagnosis, treatment, and follow-up. *Arch Intern Med*., 2002;162(6):701-704.

Raoult D, Laurent JC, Mutillod M. Monoclonal Antibodies to *Coxiella burnetii* for Antigenic Detection in Cell Cultures and in Paraffin-embedded Tissues. *Am J Clin Pathol*, 1994;101:318-320.

Raoult D, Levy PY, Dupont HT, Chicheportiche C, Tamalet C, Gastaut JA, Salducci J. Q fever and HIV infection. *AIDS*, 1993;7(1):81-6.

Raoult D, Marrie TJ, Mege JL. Natural history and pathophysiology of Q fever. *Lancet Infect Dis*, 2005; 5:219-226.

Raoult D. Q fever: Confusion between chronic infection and chronic fatigue. *Clin Infect Dis*, 2017; 65(6):1054-1055.

Rehacek J, Urvolgyi J, Kocianová E, Sekeyová Z, Vavreková M, Kovacová E. Extensive examination of different tick species for infestation with *Coxiella burnetii* in Slovakia. *Eur J Epidemiol*, 1991; 7(3): 299-303.

Reimer LG. Q Fever. *Clin Microbiol Rev*, 1993; 6:193-8.

Reusken C, van der Plaats R, Opsteegh M, de Bruin A, Swart A. *Coxiella burnetii* (Q fever) in Rattus norvegicus and Rattus rattus at livestock farms and urban locations in the Netherlands; could *Rattus* spp. represent reservoirs for (re)introduction? *Prev Vet Med*, 2011;101(1-2):124-30.

Rey D, Obadia Y, Tissot-Dupont H, Raoult D. Seroprevalence of *Coxiella burnetii* among pregnant women in South Eastern France. *Eur J Obstet Gynecol Reprod Biol*, 2000; 93: 151-156.

Reye AL, Arinola OG, Hübschen JM, Muller CP. Pathogen prevalence in ticks collected from the vegetation and livestock in Nigeria. *Appl Environ Microbiol*, 2012; 78(8): 2562-2568.

Reye AL, Stegniy V, Mishaeva NP, Velhin S, Hubschen JM, Ignatyev G, Muller CP. Prevalence of tick-borne pathogens in *Ixodes ricinus* and *Dermacentor reticulatus* ticks from different geographical locations in Belarus. *PLoS One*, 2013; 8(1): e54476.

Rijks JM, Roest HIJ, van Tulden PW, Kik MJL, IJzer J, Gröne A. *Coxiella burnetii* infection in roe deer during Q fever epidemic, the Netherlands. *Emerg Infect Dis*, 2011; 17:2369-2371.

Rodolakis A, Berri M, Héchard C, Caudron C, Souriau A, Bodier CC, Blanchard B, Camuset P, Devillechaise P, Natorp JC, Vadet JP, Arricau-Bouvery N. Comparison of *Coxiella burnetii* shedding in milk of dairy bovine, caprine and ovine herds. *J Dairy Sci*, 2007; 90(12):5352-5360.

Rodolakis A. Q Fever in Dairy Animals. *Ann N Y Acad Sci*, 2009; 1166:90–93.

Rodolakis A. Q fever, state of art: Epidemiology, diagnosis and prophylaxis. *Small Rumin Res*, 2006; 62:121-124.

Roest HIJ, Bossers A, Rebel JMJ.[a] Q Fever Diagnosis and Control in Domestic Ruminants. *Dev Biol (Basel)*, 2013;135:183-189.

Roest HIJ, Bossers A, van Zijderveld FR, Rebel JML. Clinical microbiology of *Coxiella burnetii* and relevant aspects for the diagnosis and control of the zoonotic disease Q fever. *Vet Q*, 2014; 33(3), 148-160.

Roest HIJ, Ruuls RC, Tilburg JJHC, Nabuurs-Franssen MH, Klaassen CHW, Vellema P, van den Brom R, Dercksen D, Wouda W, Spierenburg MAH, van der Spek AN, Buijs R, de Boer AG, Willemsen PTJ, van Zijderveld FG.[a] Molecular Epidemiology of *Coxiella burnetii* from Ruminants in Q Fever Outbreak, the Netherlands. *Emerg Infect Dis*, 2011; 17(4):668-675.

Roest HIJ, Tilburg JJHC, van der Hoek W, Vellema P, van Zijderveld FG, Klaassen CHW, Raoult D.[b] The Q fever epidemic in The Netherlands: history, onset, response and reflection. *Epidemiol Infect*, 2011; 139:1-12.

Roest HIJ, van Gelderen B, Dinkla A, Frangoulidis D, van Zijderveld F, Rebel J, van Keulen L. Q fever in pregnant goats: pathogenesis and excretion of *C. burnetii*. *PLoS One*, 2012; 7(11): e48949.

Roest HIJ, van Solt CB, Tilburg JJHC, Klaassen CHW, Hovius EK, Roest FTF, Vellema P, van den Brom R, van Zijderveld FG.[b] Search for Possible Additional Reservoirs for Human Q Fever, the Netherlands. *Emerg Infect Dis*, 2013;19(5): 834–835.

Roman MJ, Coriz PD, Baca OG. A Proposed Model to Explain Persistent Infection of Host Cells with *Coxiella burnetii*. *J Gen Microbiol*, 1986; 132: 1415-1422.

Rouli L, Rolain JM, Filali AE, Robert C, Raoult D. Genome Sequence of *Coxiella burnetii* 109, a Doxycycline-Resistant Clinical Isolate. *J Bacteriol*, 2012; 194(24): 6939.

Rousset E, Arricau-Bouvery N, Souriau A, Huard C, Rodolakis A, Pepin M, Aubert M. Les modalités de transmission de la fièvre Q à l'homme. *Bull Epidemiol*, 2003; 7: 1-2.

Rousset E, Berri M, Durand B, Dufour P, Prigent M, Delcroix T, Touratier A, Rodolaskis A. Coxiella burnetii Shedding Routes and Antibody Response after Outbreaks of Q Fever-Induced Abortion in Dairy Goat Herd. *Appl Environ Microbiol*, 2009; 75(2):428-433.

Rousset E, Durand B, Berri M, Dufour P, Pringent M, Russo P, Delcroix T, Touratier A, Rodolakis A, Aubert M. Comparative diagnostic potential of three serological tests for abortive Q fever in goat herds. *Vet Microbiol*, 2007; 124: 286–297.

Rousset E, Sidi-Boumedine K, Thiery R. Chapter 2.1.1.2 – Q fever. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Mammals, Birds and Bees). 7th ed. OIE. 2010. Available at http://www.oie.int/en/internationalstandard-setting/terrestrial-manual/access-online/ (accessed 20-08-2011)

Roux, V, Bergoin M, Lamaze N, Raoult D. Reassessment of the taxonomic position of *Rickettsia grylli*. *Int J Syst Bacteriol*, 1997; 47(4), 1255-1257.

Ruiz-Beltran R, Herrero-Herrero JI, Martin-Sanchez AM, Martin-González JA. Prevalence of antibodies to *Rickettsia connorii*, *Coxiella burnetii* and *Rickettsia typhi* in Salamanca Province (Spain). *Eur J Epidemiol*, 1990; 6(3): 293-299.

Ruiz-Fons F, Astobiza I, Barandika JF, Hurtado A, Atxaerandio R, Juste RA, Garcia-Perez AL. Seroepidemiological study of Q fever in domestic ruminants in semi-extensive grazing systems. *BMC Vet Res*, 2010; 6: 3.

Ruiz-Fons F, Rodríguez O, Torina A, Naranjo V, Gortázar C, de la Fuente J. Prevalence of *Coxiella burnetii* infection in wild and farmed ungulates. *Vet Microbiol*, 2008; 126 (1-3):282-286.

Russell-Lodrigue KE, Andoh M, Poels MHJ, Shive HR, Weeks BR, Zhang CQ, Tersteeg C, Masegi T, Hotta A, Yamaguchi T, Fukushi H, Hirai K, McMurray DN, Samuel JE. *Coxiella burnetii* Isolates Cause Genogroup-Specific Virulence in Mouse and Guinea Pig Models of Acute Q Fever. *Infect Immun*, 2009; 77(12), 5640-5650.

Ryan ED, Kirby M, Collins DM, Sayers R, Mee JF, Clegg T. Prevalence of *Coxiella burnetii* (Q fever) antibodies in bovine serum and bulk-milk samples. *Epidemiol Infect*, 2011;139: 1413-1417.

Sabatier F, Dignat-George F, Mège JL, Brunet C, Raoult D, Sampol J. CD41 T-Cell Lymphopenia in Q Fever Endocarditis. *Clin Diagn Lab Immunol*, 1997; 4(1), 89-92.

Saegerman C, Speybroeck N, Dal Pozzo F, Czaplicki G. Clinical Indicators of Exposure to *Coxiella burnetii* in Dairy Herds. *Transbound Emerg Dis*, 2013; 62(1): 46-54.

Saenz de Buraga M, Calero AL, Iraizoz FJP. Reconocimiento de sexo y edad en especies cinegéticas. Edilesa, Léon. 1991.

Sakharkar KR,1 Dhar PK, Chow VTK. Genome reduction in prokaryotic obligatory intracellular parasites of humans: a comparative analysis. *Int J Syst Evol Microbiol*, 2004; 54: 1937–1941.

Samuel JE, Frazier ME, Mallavia LP. Stability of Plasmid Sequences in an Acute Q-Fever Strain of *Coxiella burnetii*. *J Gen Microbiol*, 1988; 134:1795-1 805.

Samuel JE, Hendrix LR. Laboratory maintenance of *Coxiella burnetii*. *Curr Protoc Microbiol*, 2009; Chapter 6: Unit 6C.1.

Sánchez J, Souriauy A, Buendía AJ, Arricau-Bouvery N, Matínez CM, Salinas J, Rodolakis A, Navarro JA. Experimental *Coxiella burnetii* Infection in Pregnant Goats: a Histopathological and Immunohistochemical Study. *J Comp Path*, 2006;135:108-115.

Sandoz KM, Popham DL, Beare PA, Sturdevant DE, Hansen B, Nair V, Heinzen RA. Transcriptional Profiling of *Coxiella burnetii* Reveals Extensive Cell Wall Remodeling in the Small Cell Variant Developmental Form. *PLoS One*, 2016; DOI:10.1371/journal.pone.0149957.

Sanford SE, Josephson GKA, MacDonald A. *Coxiella burnetii* (Q fever) abortion storms in goat herds after attendance at an annual fair. *Can Vet J*, 1994; 35:376-378.

Santos AS, Bacellar F, França A. Q fever: a revision of concepts. [in portuguese]. *Med Int*, 2007; 14: 90-99.

Santos AS, Tilburg JJHC, Botelho A, Barahona MJ, Núncio MS, Nabuurs-Franssen MH, Klaassen CHW. Genotypic diversity of clinical *Coxiella burnetii* isolates from Portugal based on MST and MLVA typing. *Int J Med Microbiol*, 2012; 302: 253-256.

Schack M, Sachse S, Rödel J, Frangoulidis D, Pletz MW, Rohde GU, Straube E, Boden K. *Coxiella burnetii* (Q fever) as a cause of community-acquired pneumonia during the warm season in Germany. *Epidemiol Infect*, 2014;142(9):1905-1910.

Schimmer B, Lenferink A, Schneeberger P, Aangenend H, Vellema P, Hautvast J, van Duynhoven Y. Seroprevalence and Risk Factors for Coxiella burnetii (Q Fever) Seropositivity in Dairy Goat Farmers' Households in The Netherlands, 2009–2010. *PLoS One*, 2012; 7(7): e42364.

Schimmer B, Luttikholt S, Hautvast JLA, Graat EAM, Vellema P, van Duynhoven YTHP. Seroprevalence and risk factors of Q fever in goats on commercial dairy goat farms in the Netherlands, 2009-2010. *BMC Vet Res*, 2011; 7: 81.

Schimmer B, Morroy G, Dijkstra F, Schneeberger P M, Weers-Pothoff G, Timen A, Wijkmans C, van der Hoek W. Large ongoing Q fever Outbreak in the south of The Netherlands, 2008. *EuroSurveill*, 2008; 13(31):pii=18939.

Schimmer B, Schegget R, Wegdam M et al. The use of a geographic information system to identify a dairy goat farm as the most likely source of an urban Q-fever outbreak. *BMC Infect. Dis*, 2010; 10:69.

Schimmer B, Schotten N, van Engelen E, Hautvast JL, Schneeberger PM, van Duijnhoven YT. *Coxiella burnetii* seroprevalence and risk for humans on dairy cattle farms, the Netherlands, 2010-2011. *Emerg Infect Dis*, 2014;20(3):417-425.

Schneeberger PM, Hermans MH, van Hannen EJ, Schellekens JJ, Leenders ACP, Wever PC. Real-time PCR with serum samples is indispensable for early diagnosis of acute Q fever. *Clin Vaccine Immunol*, 2010; 17(2): 286–290.

Schoffelen T, Limonard GJM, Bleeker-Rovers CP, Bouwman JJM, van der Meer JWM, Nabuurs-Franssen M, Sprong T, van Deuren M. Diagnosis of Coxiella burnetii Infection: Comparison of a Whole Blood Interferon-Gamma Production Assay and a Coxiella ELISPOT. *PLoS One,* 2014; 9(8): e103749.

Schulze-Luehrmann J, Eckart RA, Ölke M, Saftig P, Liebler-Tenorio E, Lührmann A. LAMP proteins account for the maturation delay during the establishment of the *Coxiella burnetii*-containing vacuole. *Cell Microbiol*, 2016;18(2):181-194.

Sekeyová S, Roux V, Raoult D. Intraspecies diversity of *Coxiella burnetii* as revealed by com1 and mucZ sequence comparison. *FEMS Microbiol Lett*, 1999; 180: 61-67.

Selvaggi TM, Rezza G, Scagnelli M, Rigoli R, Rassu M, De Lalla F, Pellizzer GP, Tramarin A, Bettini C, Zampieri L, Belloni M, Dalla Pozza E, Marangon S,

Marchioretto N, Togni G, Giacobbo M, Todescato A, Binkin N. Investigation of a Q-fever outbreak in Northern Italy. *Eur J Epidemiol*, 1996; 12: 403-408.

Seo M-G, Lee S-H, VanBik D et al. Detection and Genotyping of *Coxiella burnetii* and *Coxiella*-Like Bacteria in Horses in South Korea. *PLoS One* 2016; 11: e0156710.

Seshadri R, Hendrix LR, Samuel JE. Differential Expression of Translational Elements by Life Cycle Variants of *Coxiella burnetii*. *Infect Immun*, 1999; 67(11), 6026-6033.

Seshadri R, Paulsen IT, Eisen JA, Read TD, Nelson KE, Nelson WC, Ward NL, Tettelin H, Davidsen TM, Beanan MJ, Deboy RT, Daugherty SC, Brinkac LM, Madupu R, Dodson RJ, Khouri HM, Lee KH, Carty HA, Scanlan D, Heinzen RA, Thompson HA, Samuel JE, Fraser CM, Heidelberg JF. Complete genome sequence of the Q-fever pathogen *Coxiella burnetii*. *Proc Natl Acad Sci U S A*, 2003; 100(9), 5455-5460.

Shack M, Sachse S, Rodel J, Frangoulidis D, Pletz MW, Rhodes GU, Straube E, Boden K. *Coxiella burnetii* (Q fever) as a cause of community-acquired pneumonia during the warm season in Germany. *Epidemiol Infect*, 2013; doi:10.1017/S0950268813002914.

Shannon JG, Heinzen RA. Adaptive immunity to the obligate intracellular pathogen *Coxiella burnetii*. *Immunol Res*, 2009; 43(1-3):138–148.

Shannon JG, Howe D, Heinzen RA. Virulent *Coxiella burnetii* does not activate human dendritic cells: Role of lipopolysaccharide as a shielding molecule, (Track II). *PNAS*, 2005; 102(24): 8722-8727.

Shapiro AJ, Bpsward KL, Heller J, Norris JM. Seroprevalence of *Coxiella burnetii* in domesticated and feral cats in eastern Australia. *Vet Microbiol*, 2015;177:154-161.

Shapiro AJ, Norris JM, Heller J, Brown G, Malik R, Bosward KL. Seroprevalence of *Coxiella burnetii* in Australian dogs. *Zoonoses Public Health*, 2016;63(6):458-466.

Sidi-Boumedine K, Adam G, Angen O, Aspan A, Bossers A, Roest HJ, Prigent M, Thiéry R, Rousset E. [a] Whole genome PCR scanning (WGPS) of *Coxiella burnetii* strains from ruminants. *Microbes Infect*, 2015; 17(11-12):772-775.

Sidi-Boumedine K, Duquesne V, Fernandes I, Marro S, Thiéry R. Evaluation of randomly amplified polymorphic DNA (RAPD) for discrimination of *Coxiella burnetii* ruminant strains isolated in France. *Clin Microbiol Infect*, 2009; 15 (Suppl. 2): 194–195.

Sidi-Boumedine K, Duquesne V, Prigent M, Yang E, Joulié A, Thiéry R, Rousset E.[b] Impact of IS1111 insertion on the MLVA genotyping of *Coxiella burnetii*. *Microbes Infect*, 2015;17(11-12):789-794.

Sidi-Boumedine K, Ellis RJ, Adam G, Prigent M, Angen Ø, Aspán A, Thiéry R, Rousset E. Draft genome sequences of six ruminant *Coxiella burnetii* isolates of European origin. *Genome Announc*, 2014; 2(3): e00285-14.

Sidi-Boumedine K, Rousset E. Epidémiologie moléculaire de la fièvre Q: une revue des méthodes de génotypage de *Coxiella burnetii* et des principales réalisations. *Euroreference*, 2011; 5:30-38.

Signs KA, Stobierski MG, Gandhi TN. Q fever cluster among raw milk drinkers in Michigan, 2011. *Clin Infect Dis*, 2012; 55: 1387–1389.

Silva MM, Santos AS, Formosinho P, Bacellar F.[a] Carraças associadas a patologias infecciosas em Portugal. *Acta Med Port*, 2006; 19:39-48.

Silva MMS, Antunes S, Domingues N, Ferrolho J, Duarte LR, Melo P, Santos N, Santos PT, Domingos A, Santos AS. A pesquisa de agentes zoonóticos em ixodídeos (Ixodida, Ixodidae) no restabelecimento de populações de lince ibérico no sul de Portugal. *Acta Parasitol Port*, 2014; 20 (1/2): 234.

Silva T, Chapmana A, Kudesiab G, McKendricka M.[b] Ongoing queries: Interpretation of serology in asymptomatic or atypical chronic Q fever. *J Infect*, 2006; 52: e113–e116.

Skerget M, Wenisch C, Daxboek F, Krause R, Haberl R, Stuenzner D. Cat or Dog Ownership and Seroprevalence of Ehrlichiosis, Q Fever, and Cat-Scratch Disease. *Emerg Inf Dis*, 2003; 9(10): 1337-1340.

Sobotta K, Hillarius K, Mager M, Kerner K, Heydel C, Menge C. *Coxiella burnetii* Infects Primary Bovine Macrophages and Limits Their Host Cell Response. *Infect Immun*, 2016;84(6):1722-1734.

Spitalska E, Kocianova E. Detection of *C. burnetii* in ticks collected in Slovakia and Hungary. *Eur J Epidemiol*, 2003; 18: 263-266.

Sprong H, Tijsse-Klasen E, Langelaar M, De Bruin A, Fonville M, Gassner F, Takken W, Van Wieren S, Nijhof A, Jongejan F, Maassen CBM, Scholte EJ, Hovius JW, Hovius KE, Spitalska E, Van Duynhoven YT. Prevalence of *Coxiella burnetii* in ticks after a large outbreak of Q fever. *Zoonoses Public Health*, 2012; 59: 69-75.

Stein A, Lepidi H, Mege JL, Marrie TJ, Raoult D. Repeated Pregnancies in BALB/c Mice Infected with *Coxiella burnetii* Cause Disseminated Infection, Resulting in Stillbirth and Endocarditis. *J Infect Dis*, 2000;181:188–194.

Stein A, Louveau C, Lepidi H, Ricci F, Baylac P, Davoust B, Raoult D. Q fever pneumonia: virulence of *C. burnetii* pathovars in a murine model of aerosol infection. *Infect Immun*, 2005; 73: 2469-2477.

Stein A, Raoult D. Detection of *Coxiella burnetii* by DNA Amplification Using Polymerase Chain Reaction. *J Clin Microbiol*, 1992; 30(9):2462-2466.

Stein A, Raoult D. Lack of pathotype specific gene in human *Coxiella burnetii* isolates. *Microb Pathog*, 1993;15(3):177-185.

Stein A, Raoult D. Pigeon pneumonia in Provence: a bird-borne Q Fever outbreak. *Clin Infect Dis*, 1999; 29: 617-620.

Stein A, Raoult D. Q Fever During Pregnancy: A Public Health Problem in Southern France. *Clin Infect Dis*, 1998; 27:592–596.

Stein A, Saunders NA, Taylor AG et al. Phylogenic homogeneity of *Coxiella burnetii* strains as determinated by 16S ribosomal RNA sequencing. *FEMS Microbiol Lett*, 1993; 113:339-44.

Stoker MGP, Page Z, Marmion BP. Problems in the diagnosis of Q fever by Complement Fixation Test. *Bull Wld Hlth Org*, 1955; 13: 807-827.

Strauss B, Löschau M, Seidel T, Stallmach A, Thomas A. Are fatigue symptoms and chronic fatigue syndrome following Q fever infection related to psychosocial variables? *J Psychosom Res*, 2012; 72(4):300-304.

Suarez-Estrada J, Rodriguez-Barbosa JI, Gutierrez-Martin CB, Castañeda-López MR, Fernandez-Marcos JM, González-Llamazares OR, Rodriguez-Ferri EF. Seroepidemiological survey of Q fever in León Province, Spain. *Eur J Epidemiol*, 1996; 12: 245-250.

Sulyok KM, Hornok S, Abichu G, Erdélyi K, Gyuranecz M.[a] Identification of Novel *Coxiella burnetii* Genotypes from Ethiopian Ticks. *PLoS One*, 2014;9(11):e113213.

Sulyok KM, Kreizinger Z, Hornstra HM, Pearson T, Szigeti A, Dán Á, Balla E, Keim PS, Gyuranecz M.[b] Genotyping of *Coxiella burnetii* from domestic ruminants and human in Hungary: indication of various genotypes. *BMC Vet Res*, 2014;10:107.

Svraka S, Toman R, Skultety L, Slaba K, Homan WL. Establishment of a genotyping scheme for *Coxiella burnetii*. *FEMS Microbiol Lett*, 2006; 254: 268–274.

Szymanska-Czerwinska M, Galinska EM, Niemczuk K, Knap JP. Prevalence of *Coxiella burnetii* Infection in Humans Occupationally Exposed to Animals in Poland. *Vector Borne Zoonotic Dis*, 2015; 15(4):261-267.

Szymanska-Czerwinska M, Galinska EM, Niemczuk K. Prevalence of *C. burnetii* infection in foresters and ticks in south-eastern Poland and comparison of diagnostic methods. *Ann Agric Environ Med*, 2013;20(4):699-704.

Tan CK, Owens L. Infectivity, transmission and 16S rRNA sequencing of a rickettsia, *Coxiella cheraxi* sp. nov., from the freshwater crayfish *Cherax quadricarinatus*. *Dis Aquat Org*, 2000, 41:115-22.

Tattevin P, Arvieux C, Dupont M, Guggenbuhl P, Lemeur A, Michelet C. Granulomatous Lymphadenitis as a Manifestation of Q Fever. *Emerg Infect Dis*, 2003; 9(1): 137-138.

Tellez A, Martin A, Anda P, de la Fuente L, Benitez P, Garcia C, Leon P. Study of *C. burnetii* in human and animal seroprevalence in a rural population in Madrid community. *Eur J Epidemiol*, 1989; 5(4): 444-446.

Teunis PFM, Schimmer B, Notermans DW, Leenders ACAP, Wever PC, Kretzschmar MEE, Schneeberger PM. Time-course of antibody responses against *Coxiella burnetii* following acute Q fever. *Epidemiol Infect*, 2013; 141: 62-73.

Thièle D, Willems H, Kopf G, Krauss H. Polymorphisms in DNA Restriction Patterns of *Coxiella burnetii* isolates investigated by Pulsed Field Gel Electrophoresis and Image Analysis. *Eur J Epidemiol*, 1993; 9(4): 419-425.

Thièle D, Willems H. Is plasmid-based differentiation of *Coxiella burnetii* in 'acute' and 'chronic' isolates still valid? *Eur J Epidemiol*, 1994; 10(4), 427-434.

Thomas DR, Treweek L, Salmon RL, Kench SM, Coleman TJ, Meadows D, Morgan-Capner P, Caul EO. The risk of acquiring Q fever on farms: a seroepidemiological study. *Occup Environ Med*, 1995;52:644-647.

Thrusfield M. Veterinary Epidemiology. 2ond ed. Oxford: Blackwell Science, 1995. [483 pp]

Tigertt WD, Benenson AS, Gochenour WS. Airborne Q fever. *Bacteriol Rev*, 1961; 25(3): 285–293.

Tilburg JJHC, Roest HJ, Buffet S, Nabuurs-Franssen MH, Horrevorts AM, Raoult D, Klaassen CH.[a] Epidemic genotype of *Coxiella burnetii* among goats, sheep, and humans in the Netherlands. *Emerg Infect Dis*, 2012; 18: 887-889.

Tilburg JJHC, Roest HJIJ, Nabuurs-Franssen MH, Horrevorts AM, Klaassen CHW.[b] Genotyping Reveals the Presence of a Predominant Genotype of *Coxiella burnetii* in Consumer Milk Products. *J Clin Microbiol*, 2012; 50(6): 2156.

Tilburg JJHC, Rossen JW, van Hannen EJ, Melchers WJ, Hermans MH, van de Bovenkamp J, Roest HJ, de Bruin A, Nabuurs-Franssen MH, Horrevorts AM, Klaassen CH.[c] Genotypic diversity of *Coxiella burnetii* in the 2007-2010 Q fever outbreak episodes in The Netherlands. *J Clin Microbiol*, 2012; 50(3): 1076-1078.

Tissot-Dupont H, Amadei MA, Nezri M, Raoult D. Wind in November, Q fever in December. *Emerg Infect Dis*, 2004; 10:1264-1269.

Tissot-Dupont H, Raoult D, Brouqui P, Janbon F, Peyramond D, Weiller PJ, Chicheportiche C, Nezri M, Poirier R. Epidemiologic features and clinical presentation of acute Q fever in hospitalized patients: 323 French cases. *Am J Med*, 1992; 93(4):427-434.

Tissot-Dupont H, Torres S, Nezri M, Raoult D. Hyperendemic Focus of Q Fever Related to Sheep and Wind. *Am J Epidemiol*, 1999; 150 (1): 67-74.

Tissot-Dupont H, Vaillant V, Rey S, Raoult D. Role of Sex, Age, Previous Valve Lesion, and Pregnancy in the Clinical Expression and Outcome of Q Fever after a Large Outbreak. *Clin Infect Dis*, 2007; 44:232–237.

To H, Htwe KK, Kako N, Kim HJ, Yamaguchi T, Fukushi H, Hirai K. Prevalence of *Coxiella burnetii* infection in dairy cattle with reproductive disorders. *J Vet Med Sci*, 1998; 60(7): 859-861.

Tozer SJ, Lambert SB, Strong CL, Field HE, Sloots TP, Nissen MD. Potential animal and environmental sources of Q fever infection for humans in Queensland. *Zoonoses Public Health*, 2014; 61(2): 105-112.

Vaidya VM, Malik SV, Kaur S, Kumar S, Barbuddhe SB. Comparison of PCR, immunofluorescence assay, and pathogen isolation for diagnosis of q fever in humans with spontaneous abortions. *J Clin Microbiol*, 2008; 46(6): 2038-2044.

Vaidya VM, Malik SVS, Bhilegaonkar KN, Rathore RS, Kaur S, Barbuddhe SB. Prevalence of Q fever in domestic animals with reproductive disorders. *Comp Immunol Microbiol Infect Dis*, 2010; 33:307-321.

Valencia MCS, Rodriguez CO, Puñet OG, Giral IB. Q fever seroprevalence and associated risk factors among students from the Veterinary School of Zaragoza, Spain. *Eur J Epidemiol*, 2000; 16: 469-476.

van Asseldonk MA, Prins J, Bergevoet RH. Economic assessment of Q fever in the Netherlands. *Prev Vet Med*, 2013; 112(1-2): 27-34.

van den Brom R, Moll L, van Schaik G, Vellema P. Demography of Q fever seroprevalence in sheep and goats in The Netherlands in 2008. *Prev Vet Med*, 2013; 109: 76-82.

van den Brom R, van Engelen E, Luttikholt S, Moll L, van Maanen K, Vellema P. *Coxiella burnetii* in bulk tank milk samples from dairy goat and sheep farms in The Netherlands. *Vet Rec*, 2012; 170:310.

van den Brom R, van Engelen E, Roest HI et al. Coxiella burnetii infections in sheep or goats: an opinionated review. *Vet Microbiol*, 2015; 181:119-29.

Van den Brom R, Vellema P. Q fever outbreaks in small ruminants and people in the Netherlands. *Small Rum Res*, 2009; 86: 74–79.

van den Brom, R., L. Moll, G. van Schaik, Vellema P. Demography of Q fever seroprevalence in sheep and goats in The Netherlands in 2008. *Prev Vet Med*, 2013; 109, 76-82.

van der Hoek W, Dijkstra F, Schimmer B, Schneeberger PM, Vellema P, Wijkmans C, ter Schegget R, Hackert V, van Duynhoven Y. Q fever in the Netherlands: an update on the epidemiology and control measures. *Euro Surveill*, 2010; 15(12), pii 19520.

van der Hoek W, Versteeg B, Meekelenkamp JCE, Renders NHM, Leenders ACAP, Weers-Pothoff I, Hermans MHA, Zaaijer HL, Wever PC, Schneeberger PM. Follow-up of 686 Patients With Acute Q Fever and Detection of Chronic Infection. *Clin Infect Dis*, 2011;52(12):1431–1436.

van Engelen E, Schotten N, Schimmer B, Hautvast JL, van Schaik G, van Duijnhoven YT. Prevalence and risk factors for Coxiella burnetii (Q fever) in Dutch dairy cattle herds based on bulk tank milk testing. *Prev Vet Med*, 2014;117(1):103-109.

van Loenhout JAF, Paget WJ, Vercoulen JH, Wijkmans CJ, Hautvast JLA, van der Velden K. Assessing the long-term health impact of Q-fever in the Netherlands: a prospective cohort study started in 2007 on the largest documented Q-fever outbreak to date. *BMC Infect Dis*, 2012; 12: 280.

van Schaik EJ, Chen C, Mertens K, Weber MM, Samuel JE. Molecular pathogenesis of the obligate intracellular bacterium *Coxiella burnetii*. *Nature Rev Microbiol*, 2013; 11(8), 561-573.

van Wijk MJ, Maas DW, Renders NHM, Hermans MHA, Zaaijer HL, Hogema BM. Screening of post-mortem tissue donors for *Coxiella burnetii* infection after large outbreaks of Q fever in The Netherlands. *BMC Infect Dis*, 2014; 14:6.

van Woerden HC, Mason BW, Nehaul LK, Smith R, Salmon RL, Healy B, Valappil M, Westmoreland D, de Martin S, Evans MR, Lloyd G, Hamilton-Kirkwood M, Williams NS. Q Fever Outbreak in Industrial Setting. *Emerg Infect Dis*, 2004; 10(7):1282-1289.

Varghees S, Kiss K, Frans G, Braha O, Samuel JE. Cloning and Porin Activity of the Major Outer Membrane Protein P1 from *Coxiella burnetii*. *Infect Immun*, 2002; 70(12):6741-6750.

Vázques CL, Colombo MI. *Coxiella burnetii* modulates Beclin 1 and Bcl-2, preventing host cell apoptosis to generate a persistent bacterial infection. *Cell Death Differ*, 2010; 17: 421–438.

Verso MG, Vesco G, Villari S, Galluzzo P, Gargano V, Matranga D, De Marchis P, Picciotto D. Analysis of seroprevalence against *Coxiella burnetii* in a sample of farm workers in Western Sicily. *Ann Agric Environ Med*, 2016; 23(1): 71–74.

Vest KG, Clark LL. Serosurvey and Observational Study of US Army Veterinary Corps Officers for Q Fever Antibodies from 1989 to 2008. *Zoonoses Public Health*, 2014;61(4):271-282.

Vincent G, Stenos J, Latham J, Fenwick S, Graves S. Novel genotypes of *Coxiella burnetii* identified in isolates from Australian Q fever patients. *Int J Med Microbiol*, 2016; 306(6): 463-470.

Vincent GA, Graves SR, Robson JM, Nguyen C, Hussain-Yusuf H, Islam A, Fenwick SG, Stenos J. Isolation of *Coxiella burnetii* from serum of patients with acute Q fever. *J Microbiol Methods*, 2015; 119: 74–78.

Vogiatzis I, Dimoglou G, Sachpekidis V. Q fever myocarditis. *Hippokratia*, 2008; 12(1): 46-49.

Voth DE, Beare PA, Howe D, Sharma UM, Samoilis G, Cockrell DC, Omsland A, Heinzen RA. The *Coxiella burnetii* Cryptic Plasmid Is Enriched in Genes Encoding Type IV Secretion System Substrates. *J Bacteriol*, 2011; 193(7): 1493–1503.

Voth DE, Heinzen RA. *Coxiella* Type IV Secretion and Cellular Microbiology. *Curr Opin Microbiol*, 2009; 12(1): 74-80.

Voth DE, Heinzen RA. Lounging in a lysosome: the intracellular lifestyle of *Coxiella burnetii*. *Cell Microbiol*, 2007; 9(4), 829–840.

Voth DE, Howe D, Heinzen RA. *Coxiella burnetii* inhibits apoptosis in human THP-1 cells and monkey primary alveolar macrophages. *Infect Immun*, 2007; 75(9): 4263–4271.

Vranakis I, Kokkini S, Chochlakis D, Sandalakis V, Pasparaki E, Minadakis G, Gikas A, Tselentis Y, Psaroulaki A. Serological survey of Q fever in Crete, southern Greece. *Comp Immunol Microbiol Infect Dis*, 2012; 35: 123–127.

Waag DM. Q fever. *In*: Medical Aspects of Biological Warfare. Lenhart MK, Washington D.C. 1997. pp 199-213.

Waag DM. *Coxiella burnetii*: host and bacterial responses to infection. *Vaccine*, 2007; 25(42), 7288–7295.

Wallensten A, Moore P, Webster H, Johnson C, van der Burgt G, Pritchard G, Ellis-Iversen J, Oliver I. Q fever outbreak in Cheltenham, United Kingdom, in 2007 and the use of dispersion modelling to investigate the possibility of airborne spread. *Euro Surveill*, 2010; 15(12): pii=19521.

Walter MC, Vincent GA, Stenos J, Graves S, Frangoulidis D. Genome sequence of *Coxiella burnetii* strain AuQ01 (Arandale) from an Australian patient with acute Q fever. *Genome Announc*, 2014; 2(5): e00964-14. doi:10.1128/genomeA.00964-14.

Warrier I, Walter MC, Frangoulidis D, Raghavan R, Hicks LD, Minnick MF. The Intervening Sequence of *Coxiella burnetii*: Characterization and Evolution. *Front Cell Infect Microbiol*, 2016; 6:83.

Weber MM, Faris R, van Schaik EJ, McLachlan J, Wright WU, Tellez A, Roman VA, Rowin K, Di Russo Case E, Luo ZQ, Samuel JE. The type IV secreted effector protein CirA stimulates the GTPase activity of RhoA and is required for virulence in a mouse model of *Coxiella burnetii* infection. *Infect Immun*, 2016; 84(9): 2524–2533.

Wegdam-Blans MCA, Vainas T, van Sambeek MR, Cuypers PW, Tjhie HTJ, van Straten AHM, Teijink JA. Vascular Complications of Q-fever Infections. *Eur J Vasc Endovasc Surg*, 2011; 42: 384-392.

Wegdam-Blans MCA, Wielders CCH, Meekelenkamp J, Korbeeck JM, Herremans T, Tjhie HT, Bijlmer HA, Koopmans MPG, Schneebergerb PM. Evaluation of Commonly Used Serological Tests for Detection of *Coxiella burnetii* Antibodies in Well-Defined Acute and Follow-Up Sera. *Clin Vaccine Immunol*, 2012; 19(7): 1110–1115.

Weisburg WG, Dobson ME, Samuel JE, Dasch GA, Mallavia LP, Baca O, Mandelco L, Sechrest JE, Weiss E, Woese CR. Phylogenetic diversity of the Rickettsiae. *J Bacteriol*, 1989;171(8), 4202-4206.

Wielders CC, Teunis PF, Hermans MH, van der Hoek W, Schneeberger PM. Kinetics of antibody response to *Coxiella burnetii* infection (Q fever): Estimation of the seroresponse onset from antibody levels. *Epidemics*, 2015; 13: 37–43.

Wielders CCH, Wuister AMH, Visser VL, Jager-Leclercq MG, Groot CAR, Dijkstra F, van Gageldonk-Lafeber AB, van Leuken JPG, Wever PC, van der Hoek W, Schneeberger PM. Characteristics of hospitalized acute Q fever patients during a large epidemic, The Netherland. *Plos One*, 2014; 9(3): e91764.

Willeberg T, Ruppanner R, Behymer DE, Haghighi S, Kaneko JJ, Franti CE. Environmental exposure to *Coxiella burnetii*: a sero-epidemiologic survey among domestic animals. *Am J Epidemiol*, 1980; 111(4): 437-443.

Willems H, Thiele D, Krauss H. Plasmid based differentiation and detection of *Coxiella burnetii* in clinical samples. *Eur J Epidemiol*, 1993; 9(4):411-418.

Williams JC, Peacock MG, McCaul TF. Immunological and biological characterization of *Coxiella burnetii*, phases I and II, separated from host components. *Infect Immun*, 1981; 32(2): 840–851.

Winchell CG, Graham JG, Kurten RC, Voth DE. *Coxiella burnetii* type IV secretion-dependent recruitment of macrophage autophagosomes. *Infect Immun*, (2014); 82(6), 2229–2238.

Woldehiwet, Z. Q fever (Coxiellosis): epidemiology and pathogenesis. *Res Vet Sci*, 2004; 77: 93-100.

Yanase T1, Muramatsu Y, Ueno H, Morita C. Seasonal variations in the presence of antibodies against *Coxiella burnetii* in dairy cattle in Hokkaido, Japan. *Microbiol Immunol*, 1997; 41(2): 73-75.

Zajac AM, Conboy GA. Veterinary Clinical Parasitology. 7th edition. Ames, Iowa: Blackwell Publications. 2006. [305pp]

Zamboni DS, Campos MA, Torrecilhas ACT, Kiss K, Samuel JE, Golenbock DT, Lauw FN, Roy CR, Almeida IC, Gazzinelli RT. Stimulation of Toll-like receptor 2 by *Coxiella burnetii* is required for macrophage production of pro-inflammatory cytokines and resistance to infection. *J Biol Chem*, 2004; 279(52): 54405–54415.

Zamboni DS, McGrath S, Rabinovitch M, Roy CR. Coxiella burnetii express type IV secretion system proteins that function similarly to components of the *Legionella pneumophila* Dot/Icm system. *Mol Microbiol*, (2003); 49(4): 965–976.

Zeman DH, Kirkbride CA, Leslie-Steen P, Duimstra JR. Ovine abortion due to *Coxiella burnetii* infection. *J Vet Diagn Invest*, 1989; 1:178-180.

Zhang D, Wang Z, Fang R, Nie H, Feng H, Zhou Y, Zhao J. Use of Protein AG in an Enzyme-Linked Immunosorbent Assay for Serodiagnosis of *Toxoplasma gondii* Infection in Four Species of Animals. *Clin Vaccine Immunol*, 2010; 17: 485–486.

Zhang G, Russell-Lodrigue KE, Andoh M, Zhang Y, Hendrix LR, Samuel JE. Mechanisms of vaccine-induced protective immunity against *Coxiella burnetii* infection in BALB/c mice. *J Immunol*, 2007;179(12):8372-8380.

Zhang GQ, Hotta A, Mizutani M, Ho T, Yamaguchi T, Fukushi H, Hirai K. Direct Identification of *Coxiella burnetii* Plasmids in Human Sera by Nested PCR. *J Clin Microbiol*, 1998; 36: 2210–2213.

Zhang GQ, To H, Yamaguchi T, Fukushi H, Hirai K. Differentiation of *Coxiella burnetii* by Sequence analysis of the gene (com 1) encoding a 27-kDa Outer Membrane Protein. *Microbiol Immunol*, 1997; 41(11):871-877.

11

Appendix

Transboundary and Emerging Diseases

SHORT COMMUNICATION

Q Fever Dairy Herd Status Determination Based on Serological and Molecular Analysis of Bulk Tank Milk

S. Anastácio[1,2], N. Carolino[2,3], K. Sidi-Boumedine[4] and G. J. da Silva[1]

[1] Faculty of Pharmacy and Center of Neurosciences and Cell Biology, University of Coimbra, Coimbra, Portugal
[2] University School of Vasco da Gama, Coimbra, Portugal
[3] INIAV, Vale de Santarém, Portugal
[4] Sophia-Antipolis Laboratory, ANSES, Sophia-Antipolis, France

Keywords:
Coxiella burnetii; dairy ruminants; qPCR; ELISA; Epidemiology

Correspondence:
G. J. da Silva. Faculty of Pharmacy, University of Coimbra, Health Sciences Campus, Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal. Tel.: +351 239488460; Fax: +351 239488503; E-mails: gjsilva@ci.uc.pt; silva.gj@gmail.com

Received for publication May 5, 2014

doi:10.1111/tbed.12275

Summary

Ruminants are recognized as the main reservoirs of *Coxiella burnetii*. EFSA highlighted the lack of knowledge about Q fever prevalence in many European countries. A cross-sectional study was carried out in randomly selected dairy herds ($n = 109$) from central Portugal to screen for *C. burnetii* infection and to correlate it with herd factors. Bulk tank milk (BTM) samples from cattle ($n = 45$) and small ruminant ($n = 64$) herds were tested by ELISA and PCR. The apparent seroprevalence of Q fever was estimated in 45.9% (95% CI: 36.3–55.7) being higher in small ruminants (51.6; 95% CI: 39.6–63.4) than in cattle (37.8; 95% CI: 25.1–52.4). The shedding of *C. burnetii* in BTM was detected in 11.9% (95% CI: 7.1–19.4) of BTM, and it was higher in cattle (20%; 95% CI: 10.9–33.8) than in sheep and mixed herds (6.3%; 95% CI: 2.5–15). A high bacterial load ($\geq 3 \times 10^3$ bacteria/ml) was observed in 85% of PCR-positive BTM. A significant correlation was found between the bacterial load and positive samples on ELISA ($P < 0.001$). Antibody positivity was significantly associated with the increased herd size ($P < 0.01$) and the occurrence of abortion ($P < 0.05$), whereas the shedding of *C. burnetii* was significantly associated with the report of infertility ($P < 0.05$). The results highlight that serological and molecular methods in combination are a useful tool to screen for Q fever and to clarify the herd infection status. The shedding of *C. burnetii* through milk is important, especially in dairy cattle, and thus, the role of milk as a potential source of infection among dairy workers should not be neglected. To our knowledge, this is the first study reporting *C. burnetii* infection in dairy livestock in Portugal showing that Q fever is significant in dairy herds, leading to economic losses and being a risk for public health, which highlights the need of implementation of control measures.

Introduction

Over the last years, there has been an increased interest for Q fever and its causative agent *Coxiella burnetii* (Khalili et al., 2011; Muskens et al., 2011; Ryan et al., 2011; Astobiza et al., 2012; Czaplicki et al., 2012). The large-scale epidemic of human Q fever that occurred in the Netherlands between 2007 and 2011 (van Loenhout et al., 2012) has contributed to an increased awareness and case detection in diverse European countries (ECDC, 2011). Also, the scientific opinion issued by the European Food and Safety Agency (EFSA) encouraged the investigation in many countries because the control of the infection in animals is considered as the first step to prevent human exposure (EFSA, 2010).

Some previous studies reported an association between human Q fever cases and infected ruminant herds (Porten et al., 2006; Gilsdorf et al., 2008). For this reason, domestic

ruminants are generally considered the primary animal reservoir of *C. burnetii* (EFSA, 2010; Sidi-Boumedine et al., 2010).

Infected ruminants can be asymptomatic, but Q fever can cause abortion, stillbirth, delivery of weak offspring and premature delivery. These clinical manifestations are more often expressed in sheep and goats. In cattle clinical signs of Q fever can be less obvious (Rodolaskis et al., 2007; Sidi-Boumedine et al., 2010). The shedding of *C. burnetii* in infected ruminants occurs mainly during and after parturition or abortion, in birth products and vaginal mucus, but the shedding in urine, faeces and milk is also documented (Guatteo et al., 2007b; Rousset et al., 2009; Sidi-Boumedine et al., 2010). The impacts on public and animal health make Q fever a disease of interest for public policy makers and food industries, mainly dairy production (Guatteo et al., 2011).

In dairy herds, bulk tank milk (BTM) has been shown to be a suitable sample to screen for *C. burnetii* infection on lactating animals (Kim et al., 2005; Agger et al., 2010; Muskens et al., 2011; van den Brom et al., 2012). The milk antibody analysis by ELISA testing can provide information about the exposure to *C. burnetii* (Guatteo et al., 2007a), and the results are comparable to those obtained in serum samples because immunoglobulins are transferred from blood to milk in lactating females (Nielsen et al., 2011). However, in order to control the bacterium spread among animals and from animals to environment and humans, detection of *C. burnetii* is a crucial step (Guatteo et al., 2007a). Nowadays, PCR is recognized as the most suitable tool for *C. burnetii* detection (Berri et al., 2000), and quantitative PCR (qPCR) allows quantification of the bacterial load (Guatteo et al., 2007a; Czaplicki et al., 2012).

The aims of this study were as follows: (i) to investigate the exposure to *C. burnetii* in dairy ruminant farms by testing the presence of specific anti-*C. burnetii* antibodies (ii) to evaluate the infection status of dairy ruminant farms by assessing the prevalence of shedding in BTM samples (iii) to evaluate the degree of bacterial excretion in shedding herds and (iv) to identify associations between *C. burnetii* infection with herd factors and reproductive disorders.

Materials and Methods

From February 2009 to July 2013, a cross-sectional survey was developed among dairy ruminant herds (n = 480 502, Official Regional Veterinary Services) from the centre region of Portugal. The number of herds to be studied was calculated using the program WinEpiscope version 2.0, and it was based on the formula $n = [t2\ Pesp\ (1\text{-}Pesp)]/d2$, considering n the required size sample, t the student value for a 95% confidence level (1.96), $Pesp$ the expected prevalence and d the desired absolute precision (Thrusfield, 1995). For that purpose, an expected herd prevalence of 32.6% (Anastacio et al., 2013), a desired absolute precision of 10% and a 95% confidence interval were considered, resulting in an estimated sample of 85 herds. The list of herds in databases of 8 collaborating livestock veterinarians from different locations of the region was used for a simple random sampling, using the program Microsoft Excel® (Microsoft Corporation, Redmond, WA, USA). The registration of the official herd code ensured the non-duplication of herds. To each collaborator, it was asked the collection of 50 ml of bulk tank milk (BTM) into sterile plastic tubes per herd, after the agreement of the farmer in a maximum of 15 herds. Also, it was asked to complete a short questionnaire containing some herd level variables (farm demographics, management practices and observed reproductive disorders during the 12 previous months) (Czaplicki et al., 2012).

A total of 109 samples were collected by the 8 collaborating veterinarians. These were tested for the presence of specific anti-*C. burnetii* antibodies using the commercial ELISA (LSIVET Ruminant Milk/Serum Q Fever; Laboratoire Service International, Lissieu, France) with a cut-off sample/positive percentages (S/P per cent) of 30% as recommended by the supplier. This test is based on antigens obtained from a European ovine strain of *C. burnetii* and gives a positive result on BTM samples when at least 10% of lactating cows in the herd are specific antibodies positive. The S/P per cent was categorized in four semi-quantitative classes: negative ($S/P \leq 30$), weak positive (+; $30 < S/P \leq 100$), positive (++; $100 < S/P \leq 200$) and strong positive (+++; $S/P > 200$) and in two qualitative classes: negative ($S/P \leq 30$) and positive ($S/P > 30$).

DNA was extracted from 200 μl of each BTM sample using the QIAmp DNA Mini kit (Qiagen, Izasa, Portugal), following manufacturer's instructions. Firstly, a conventional PCR assay targeting IS1111, a transposon-like repetitive region of *C. burnetii*, was performed as described by Berri et al. (2000) with some modifications. Briefly, the amplicons, of 243 bp in size, were obtained using the primers described by Vaidya et al. (2008). DNA of *C. burnetii* Nine Mile strain was used as a positive control. The DNA amplification reaction was performed in a Biometra Thermocycler (Biometra, Göttingen, Germany). The amplification products were analyzed by 2% agarose gel electrophoresis and visualized under UV light. Additionally, the bacterial load in PCR-positive BTM samples was estimated using the commercial real-time qPCR assay (Taq-Vet™ *Coxiella burnetii*–Absolute Quantification kit; LSI), according to the manufacturer's instructions. The PCR assays were performed using a CFX-96 thermocycler (Bio-Rad, Amadora, Portugal). Results, expressed in number of bacteria per millilitre, were categorized in weak positive (+; <300

bacteria/ml), positive (++; 300 bacteria/ml–3 × 10^3 bacteria/ml) and strong positive (+++; ≥3 × 10^3 bacteria/ml).

Confidence limits for the proportions were estimated with 95% confidence intervals (CI) assuming a binominal exact distribution (EpiInfo version 3.5.4; Center for Disease Control and Prevention, Atlanta, GA, USA). The agreement between ELISA and PCR results was investigated by the determination of the kappa coefficient using the WinEpiscope version 2.0 (University of Edinburgh, Roslin, UK). A univariable analysis was performed initially to explore associations between herd factors and reproductive disorders with the herd infection using the simple logistic regression test. Subsequently, a multivariable analysis included all the variables showing a *P*-value below 0.05. Moreover, associations between ELISA and PCR results were investigated by the Fisher's exact test and by the determination of the Spearman's Correlation coefficient. The analysis was performed using the statistical program SAS (version 9.1.2; SAS Institute Inc., Cary, NC, USA).

Results

Descriptive characterization of herds

From the 109 dairy herds (9337 animals) included in this study, cattle herds were predominant (41.7%; 95% CI: 32.3–51.5%) followed by sheep herds (35.2%; 95% CI: 26.2–45.0%), goat herds (12%; 95% CI: 6.6–19.7%) and mixed herds (11.1%; 95% CI: 5.9–18.6%). An intensive production system was reported in 38% (95% CI: 28.8–47.8%) of the herds, most of them cattle herds (97.6%; 95% CI: 87.1–99.9%).

The herd mean size was 85.6 animals (SD = 101.1, range 5–602) and 69.4% (95% CI: 59.8–77.9%) of the herds were under this mean size. Cohabitation with other animal species was reported in 49.5% (95% CI: 39.7–59.4%) of the herds, and in 37% (95% CI: 27.9–46.9%) of herds, it was reported the occurrence of ticks infestation at the time of sampling. Reproductive disorders were reported in 79.6% (95% CI: 70.8–86.8%) of the herds. These were significantly associated with cattle herds ($P < 0.05$) and with an intensive production system ($P < 0.05$).

BTM antibody testing

Fifty BTM samples (45.9%; 95% CI: 36.3–55.7%) were positive on ELISA testing; 20 from sheep (18.4%; 95% CI: 12.2–26.7%), 17 from cattle (15.6%; 95% CI: 10–23.6%), 7 (6.4%; 95% CI: 3.1–12.7%) from mixed herds and 6 (5.5%; 95% CI: 2.5–11.5%) from goat herds. In the whole positive results, 34 (31.2%; 95% CI: 23.5–41.7%) were weak positive (+), whereas 14 (12.8%; 95% CI: 7.2–20.6%) were positive (++) and 2 (1.8%; 95% CI: 0.2–6.5%) were classified as strong positive (+++).

BTM PCR testing

Thirteen BTM samples were PCR positive (11.9%; 95% CI: 7.1–19.4%). Cattle herds showed a high proportion of positive results (8.3%; 95% CI: 4.4–15%) followed by mixed and sheep herds with 2 (1.8%; 95% CI: 0.5–6.4%) positive BTM samples in each group. No PCR-positive results were obtained in goat herds. Table 1 summarizes the results obtained by ELISA and PCR testing considering the species in herds.

In PCR-positive BTM samples, the bacterial load was estimated by qPCR and ranged from 195 bacteria/ml to 288 001 bacteria/ml (mean = 37 512.77 and SD = 77 865.23). Most of the BTM samples (11/13, 84.6%; 95% CI: 53.7–97.3%) evidenced a strong positive (+++) result corresponding to ≥ 3 × 10^3 bacteria/ml.

PCR versus ELISA results

Table 2 shows the results obtained by ELISA and qPCR results considering categories. A high proportion (38/96) of BTM PCR-negative samples were antibodies positive by ELISA testing (39.6%; 95% CI: 29.9–50.1%), whereas only 7.7% (1/13; 95% CI: 0.4–37.9%) of BTM PCR-positive samples were negative on BTM ELISA. Although this discrepancy, it was observed that 92.3% (12/13; 95% CI: 62.1–99.6%) of PCR-positive results were associated with antibody-positive results. Globally, the agreement between ELISA and PCR results was weak ($\kappa = 0.24$, 95% CI: 10.7–36.6) but the Fisher's exact test showed a significant association between PCR and ELISA categorical results ($P < 0.001$). Moreover, a statistically significant correlation was observed between estimated bacterial load from qPCR and the S/P per cent by ELISA testing (Spearman's correlation coefficient = 0.34, $P < 0.001$).

Effect of herd factors on *C. burnetii* infection

Individual factors were tested to find associations with positive results. Univariable analysis by logistic regression test identified only two risk factors significantly associated

Table 1. Distribution of positive and negative results on ELISA and PCR testing considering the species in herds

Species	Number of herds	ELISA positive (%; 95% CI)	PCR positive (%; 95% CI)
Mixed	12	7 (58.3%; 32–80.7)	2 (16.7%; 4.7–44.8)
Goat	13	6 (46.2%; 23.2–70.9)	0
Sheep	39	20 (51.3%; 36.2–66.1)	2 (5.1%; 1.4–16.9)
Cattle	45	17 (37.8%; 25.1–52.4)	9 (20%; 10.9–33.8)
Total	109	50 (45.9; 36.8–55.2)	13 (11.9%; 7.1–19.4)

Table 2. Relationship between ELISA and qPCR results on 109 BTM samples from dairy ruminant herds

	qPCR				Total
ELISA	Negative No (%; 95% CI)	Positive + No (%; 95% CI)	Positive ++ No (%; 95% CI)	Positive +++ No (%; 95% CI)	Total No (%; 95% CI)
Negative	58 (53.2; 43.5–62.8)	1 (0.9; 0.05–5.8)	0	0	59 (54.3; 63.6)
Positive +	27 (24.8; 17.2–34.2)	0	1 (0.9; 0.05–5.8)	6 (5.5; 2.3–12.1)	34 (31.2; 22.9–40.9)
Positive ++	9 (8.3; 4.1–15.5)	0	0	5 (4.6; 1.7–10.9)	14 (12.8; 7.5–20.9)
Positive +++	2 (1.8; 0.3–7.1)	0	0	0	2 (1.8; 0.3–7.1)
Total (%; 95 CI)	96 (88.1; 80.1–93.2)	1 (0.9; 0.05–5.8)	1 (0.9; 0.05–5.8)	11 (10.1; 5.4–17.7)	109 (100)

with BTM antibody-positive results: the increasing of the herd size ($P < 0.01$) and abortion ($P < 0.05$). A multivariable model was performed to test simultaneously variables found to be associated in univariable analysis. The multiple logistic regression test confirmed that the increasing of the herd size and the occurrence of abortion were both associated with antibody-positive results on BTM ($P < 0.05$) as represented in Fig. 1.

The detection of *C. burnetii* by PCR was higher in herds reporting reproductive disorders (14%) than in those that did not report reproductive disorders (0%; $P > 0.05$) but only infertility, evidenced a significant association with PCR-positive results ($P < 0.05$).

Discussion

Coxiella burnetii is the aetiologic agent of Q fever, an infection that affects both animals and humans (EFSA, 2010). The prevalence of *C. burnetii* infection in ruminant herds in most of Portuguese regions is poorly known and the vaccination is very recent in Portugal, and not mandatory. The knowledge of the status of infection in herds contributes to a better understanding of the epidemiology of this pathogen. In this context, this study was designed as an approach to describe simultaneously at the herd level the exposure to *C. burnetii* and the milk shedding of this bacterium in dairy ruminant herds in central Portugal, which is unknown.

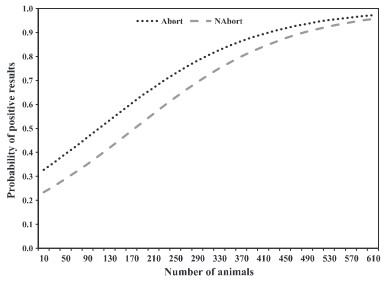


Fig. 1. Association between the probability of positive results for *Coxiella burnetii* antibodies with herd size and the occurrence of abortion or no abortion using a logistic regression model. [Abort (abortion): Intercept ± SE = −0.7889 ± 0.3707, NAbort (no abortion): Intercept ± SE = −1.2528 ± 0.4161, Logistic regression coefficient ± SE = 0.00713 ± 0.00274].

The results of antibody detection in BTM showed that almost half of the 109 dairy herds tested have been exposed to *C. burnetii* (global antibody apparent prevalence of 45.9%). At the time of the study, 11.9% of the herds showed an active infection illustrated by the detection of *C. burnetii* in BTM. These results confirm that this pathogen is endemic throughout the region. The antibody positivity was higher in sheep and goat herds (51.6%) than in cattle herds (37.8%), but the detection of *C. burnetii* in BTM was higher in cattle herds (20%) than in sheep and goat herds (6.3%). The ELISA results contrast with a recent report on *C. burnetii* infection in domestic ruminants that suggested a higher seroprevalence in cattle compared to goats and sheep (Guatteo et al., 2011). However, they are in accordance with the observations compiled in other countries evidencing that Q fever human outbreaks are more often related to small ruminants than to cattle (EFSA, 2010). The results showing a higher detection of *C. burnetii* in cattle BTM than in small ruminants are identical to those obtained in the Netherlands, despite their higher global prevalence. In fact, in the Netherlands, a higher prevalence of bacterial DNA was found in cattle BTM (56.6%) (Muskens et al., 2011), comparing to goat BTM (24.4%) (Schimmer et al., 2011).

Regarding dairy cattle herds, the antibody apparent prevalence in this study was very similar to that obtained in Ireland (37.9%) (Ryan et al., 2011), but it was lower than the obtained in other countries which ranged from 45.4 to 78.6% (Agger et al., 2010; Khalili et al., 2011; Muskens et al., 2011; Astobiza et al., 2012; Czaplicki et al., 2012). The antibody testing of BTM was performed using the same commercial ELISA test among the mentioned studies, with the exception of Agger et al. (2010) and Khalili et al.

(2011). These results show that animals are exposed to *C. burnetii* in a significant percentage of dairy herds from several European countries, including Portugal.

Moreover, in our study, 20% of dairy cattle herds shed *C. burnetii* through milk which is slightly lower than the results obtained in other countries, ranging from 29.6 to 94.3% (Kim et al., 2005; Fretz et al., 2007; Muskens et al., 2011; Astobiza et al., 2012; Czaplicki et al., 2012). All these results show that the shedding of *C. burnetii* through milk is widespread in dairy cattle herds in different countries. This might be explained by the fact that milk is the most frequent shedding route of *C. burnetii* in cows (Guatteo et al., 2012) and a long-time excretion through milk can extend for several months even in asymptomatic animals (Kim et al., 2005; Guatteo et al., 2007b, 2011).

In sheep and goat dairy herds, the antibody apparent prevalence was estimated in 51.6%. Other studies assessing herd antibodies prevalence in sheep and goat herds found rates between 28% and 67.6% (García-Pérez et al., 2009; van den Brom et al., 2012; Hilbert et al., 2012). A discrepancy was observed between these BTM antibody results (51.6%) and the serum antibody results (32.6%) obtained in a recent serologic survey conducted in sheep and goats from the same Portuguese region (Anastacio et al., 2013). This might be somewhat unexpected because the criterion for positivity in the serosurvey was set as at least one positive animal per herd, and it is known that a positive result on BTM ELISA occurs when at least 10% of lactating females are seropositive. Notwithstanding, several factors may have influenced this difference. It is known that the herd size is correlated with antibody positivity (Ryan et al., 2011; Anastacio et al., 2013). In this study, the mean herd size for sheep and goat herds was 56 animals (data not shown), whereas in the serologic survey the mean herd size was 6.7 animals. So, the increased size of herds in this study might explain the increased antibody positivity. Moreover, an association between antibody positivity and dairy production has been described (Ryan et al., 2011; van den Brom et al., 2013). In the previous serologic survey, there was a predominance of meet herds (88.8%). In this study, only dairy herds were included. Also, the proportion of sheep and goat dairy herds reporting reproductive disorders was higher (67.2%) than in the previous serosurvey (6.7%). Higher antibody positivity has been described in herds reporting reproductive disorders (Bildfell et al., 2000; García-Pérez et al., 2009; Khalili et al., 2011; Muskens et al., 2011).

Despite the higher percentage of antibody-positive dairy sheep and goat herds, only 6.3% of them shed DNA of *C. burnetii* through milk. Other studies assessing the shedding of *C. burnetii* in BTM from small ruminant herds obtained results ranging from 0% to 32.9% (Fretz et al., 2007; García-Pérez et al., 2009; van den Brom et al., 2012). No positive PCR BTM samples were obtained in goat herds, and similar results were obtained in Switzerland (Fretz et al., 2007) and in Iran (Abbasi et al., 2011). This might be due to the absence of the bacterium in goat farms at the time of sampling because, as in cattle, milk is considered the main route of bacterial shedding in the goat herds although for shorter periods. However, the presence of *C. burnetii* in other matrices than milk should not be excluded (Rodolaskis et al., 2007). Moreover, it was described a discontinuous *C. burnetii* shedding in goats (Berri et al., 2007) and thus, false-negative results may be obtained in single samples (Rousset et al., 2009). Considering ewes, the low PCR positivity rate probably occurs because the shedding of *C. burnetii* in milk seems to be less important (Rodolaskis et al., 2007), and thus, the bacterium could be present in other matrices. In small ruminants, *C. burnetii* is mainly shed after parturition or abortion in birth products (van den Brom et al., 2012). This discontinuous shedding might have contributed to the low shedding prevalence obtained in our study.

The estimation of the bacterial load allows the knowledge of the shedding pattern of infected animals. The qPCR targeted the repetitive sequence region (IS*1111*) present in different copy numbers in the genome of different *C. burnetii* strains (Klee et al., 2006). However, the low genetic diversity of *C. burnetii* strains found in Portuguese ruminants (Santos et al., 2012) was considered for quantification purposes. So, the estimated values presented herein were obtained assuming that the copy number of IS*1111* was the same in *C. burnetii* strains in all the herds. This methodology was also used in other studies (Guatteo et al., 2007a; Astobiza et al., 2012).

In 34.8% BTM-specific antibodies were present but *C. burnetii* was not detected, suggesting a past infection by the absence of shedders among lactating females. A BTM sample from a cattle herd (0.9%) was antibodies negative but PCR positive with the estimated bacterial load being low (195 bacteria/ml). This finding suggests the presence of a small number of *C. burnetii* milk shedding cows with low antibody response (Guatteo et al., 2007b), or the presence of animals in an initial phase of infection which antibodies anti-*C. burnetii* were not yet detectable on ELISA.

Globally, a discrepancy between ELISA and PCR results was observed in 37.6% of BTM samples, and similar findings were described in other studies (Schimmer et al., 2011; Czaplicki et al., 2012). Notwithstanding, most of PCR-positive results were associated with antibody-positive results. Actually, 84.6% of the PCR-positive herds were strong positive on qPCR, and all of them were antibodies positive (mean S/P per cent = 97, SD = 29.4). Furthermore, it was found a positive and statistically significant correlation ($P < 0.001$) between the estimated bacterial load on BTM qPCR and S/P per cent on BTM

ELISA which is in accordance with the results obtained by Astobiza et al. (2012). Overall, these findings support the suggestion that persistent shedder animals shed a high titre of bacteria and present a higher titre of antibodies (Guatteo et al., 2007a) and that in the presence of negative antibody test, the probability to find a positive PCR test on BTM is considered low to negligible (Saegerman et al., 2013).

Herd size was significantly associated with antibody-positive results, which is in agreement with previous serosurvey studies (Ryan et al., 2011; Schimmer et al., 2011; Agger et al., 2013). The increased risk of introduction and/or transmission of pathogens in a large population is probably related with the increased number of lambing females at lambing season (de Cremoux et al., 2012) and by other management factors such as larger amounts of feed, animal supply and a higher number of professionals working at or visiting the farm (Schimmer et al., 2011). Therefore, larger herds are more prone to acquire and develop Q fever and the number of animals must be considered a risk factor to *C. burnetii* dissemination.

An association was found also between abortion and antibody-positive results in BTM samples. However, a careful interpretation should be made because the cause of abortion in the herds was not clarified, and many other infections can cause abortion in dairy ruminants (Agerholm, 2013) even if the bacterium is in the herd (Saegerman et al., 2013). So, it is uncertain whether *C. burnetii* is the sole cause of abortions or acts as a contributory factor (Cetinkaya et al., 2000). In fact, controversial data have been reported. An association has been described in some studies (Cetinkaya et al., 2000; García-Pérez et al., 2009) while more recent ones did not found any (Anastacio et al., 2013). Similarly, in this study, an association between infertility and presence of *C. burnetii* in BTM samples was found. It is recognized that *C. burnetii* can cause infertility in cattle (Parisi et al., 2006), and interestingly, 69% of PCR-positive samples were from cattle herds.

Overall, *C. burnetii* is endemic in Central Portugal because a high percentage of herds evidenced an exposure by a positive serology. An active infection was confirmed in a lower percentage of herds by the shedding of *C. burnetii* through milk. Thus, milk should not be neglected as a source of infection to humans, namely by the consumption of unpasteurized milk or by the inhalation of aerosols in milking rooms (Loftis et al., 2010). To our knowledge, this is the first study in Portugal screening for *C. burnetii* in dairy ruminant herds and evidencing an active infection by the shedding of bacteria through milk, that was more significant in cattle herds. The cattle trade in Europe may contribute to the dissemination of *C. burnetii.* Further research is needed to characterize at molecular level of the circulating strains and to compare them with those from other countries. Moreover, the economic impact of Q fever in ruminant herds should be investigated in more detail. This study reinforces the need of a global and harmonized control policy among European countries towards the prevention of Q fever in regard to public health and economic impact.

Acknowledgements

Authors are very grateful to farmers by their agreement and to veterinary practitioners that kindly collaborated on sample collection. Dr. Elodie Rousset is also acknowledged for the critical review of the manuscript.

Financial Support

S. Anastácio is supported by the grant SFRH/BD/77823/2011 from Fundação para a Ciência e a Tecnologia, Lisbon, Portugal. This work was supported financially by the Center for Pharmaceutical Studies, University of Coimbra.

References

Abbasi, S., R. Farzan, and H. Momtaz, 2011: Molecular detection of *Coxiella burnetii* in goat bulk milk samples in some provinces of Iran. *Afr. J. Biotechnol.* 10, 18513–18515.

Agerholm, J. S., 2013: *Coxiella burnetii* associated reproductive disorders in domestic animals-a critical review. *Acta Vet. Scand.* 55, 13.

Agger, J. F., A. B. Christoffersen, E. Rattenborg, J. Nielsen, and J. S. Agerholm, 2010: Prevalence of *Coxiella burnetii* antibodies in Danish dairy herds. *Acta Vet. Scand.* 52, 5.

Agger, J.F., S. Paul, A.B. Christoffersen, and J.S. Agerholm, 2013: Risk factors for *Coxiella burnetii* antibodies in bulk tank milk from Danish dairy herds. *Acta Vet. Scand.* 55, 80.

Anastacio, S., N. Tavares, N. Carolino, K. Sidi-Boumedine, and G. J. da Silva, 2013: Serological evidence of exposure to *Coxiella burnetii* in sheep and goats in central Portugal. *Vet. Microbiol.* 167, 500–505.

Astobiza, I., F. Ruiz-Fons, A. Piñero, J. F. Barandika, A. Hurtado, and A. L. García-Pérez, 2012: Estimation of *Coxiella burnetii* prevalence in dairy cattle in intensive systems by serological and molecular analyses of bulk-tank milk samples. *J. Dairy Sci.* 95, 1632–1638.

Berri, M., K. Laroucau, and A. Rodolaskis, 2000: The detection of *Coxiella burnetii* from ovine genital swabs, milk and fecal samples by the use of a single touchdown polymerase chain reaction. *Vet. Microbiol.* 72, 285–293.

Berri, M., E. Rousset, J. L. Champion, P. Russo, and A. Rodolaskis, 2007: Goats may experience reproductive failures and shed *Coxiella burnetii* at two successive parturitions after a Q fever infection. *Res. Vet. Sci.* 83, 47–52.

Bildfell, R. J., G. W. Thomson, D. M. Haines, B. J. McEwen, and N. Smart, 2000: *Coxiella burnetii* infection is associated with

placentitis in cases of bovine abortion. *J. Vet. Diagn. Invest.* 12, 419–425.

van den Brom, R., E. van Engelen, S. Luttikholt, L. Moll, K. van Maanen, and P. Vellema, 2012: *Coxiella burnetii* in bulk tank milk samples from dairy goat and dairy sheep farms in The Netherlands in 2008. *Vet. Rec.* 170, 310.

van den Brom, R., L. Moll, G. van Schaik, and P. Vellema, 2013: Demography of Q fever seroprevalence in sheep and goats in The Netherlands in 2008. *Prev. Vet. Med.* 109, 76–82.

Cetinkaya, B., H. Kalender, H. B. Ertas, A. Muz, N. Arslan, H. Ongor, and M. Gurfay, 2000: Seroprevalence of coxiellosis in cattle, sheep and people in the east of Turkey. *Vet. Rec.* 146, 131–136.

de Cremoux, R., E. Rousset, A. Touratier, G. Audusseau, P. Nicollet, D. Ribaud, V. David, and M. Le Pape, 2012: *Coxiella burnetii* vaginal shedding and antibody responses in dairy goat herds in a context of clinical Q fever outbreaks. *FEMS Immunol. Med. Microbiol.* 64, 120–122.

Czaplicki, G., J. Y. Houtain, C. Mullender, S. R. Porter, M. F. Humblet, C. Manteca, and C. Saegerman, 2012: Apparent prevalence of antibodies to *Coxiella burnetii* (Q fever) in bulk tank milk from dairy herds in southern Belgium. *Vet. J.* 192, 529–531.

European Centre for Disease Prevention and Control (ECDC) 2011: Annual Epidemiological Report. ECDC, Stockholm. Available at http://www.ecdc.europa.eu (accessed January 25, 2012).

European Food Safety Authority (EFSA) 2010: Panel on Animal Health and Welfare (AHAW); scientific opinion on Q fever. *EFSA J.* 8, 1595. Available at http://www.efsa.europa (accessed March 20, 2012).

Fretz, R., W. Schaeren, M. Tanner, and A. Baumgartner, 2007: Screening of various foodstuffs for occurrence of *Coxiella burnetii* in Switzerland. *Int. J. Food Microbiol.* 116, 414–418.

García-Pérez, A. L., I. Astobiza, J. F. Barandika, R. Atxaerandio, A. Hurtado, and R. A. Juste, 2009: Investigation of *Coxiella burnetii* occurrence in dairy sheep flocks by bulk-tank milk analysis and antibody level determination. *J. Dairy Sci.* 92, 1581–1584.

Gilsdorf, A., C. Kroh, S. Grimm, E. Jensen, C. Wagner-Wiening, and K. Alpers, 2008: Large Q fever outbreak due to sheep farming near residential areas, Germany, 2005. *Epidemiol. Infect.* 136, 1084–1087.

Guatteo, R., F. Beaudau, A. Joly, and H. Seegers, 2007a: Assessing the within-herd prevalence of *Coxiella burnetii* milk-shedder cows using a real-time PCR applied to bulk tank milk. *Zoonoses Public Health* 54, 191–194.

Guatteo, R., F. Beaudeau, A. Joly, and H. Seegers, 2007b: *Coxiella burnetii* shedding by dairy cows. *Vet. Res.* 8, 849–860.

Guatteo, R., H. Seegers, A. F. Taurel, A. Joly, and F. Beaudeau, 2011: Prevalence of *Coxiella burnetii* in domestic ruminants: a critical review. *Vet. Microbiol.* 149, 1–16.

Guatteo, R., A. Joly, and F. Beaudeau, 2012: Shedding and serological patterns of dairy cows following abortions associated with *Coxiella burnetii* DNA detection. *Vet. Microbiol.* 155, 430–433.

Hilbert, A., G. Schmoock, H. Lenzko, U. Moog, R. Diller, A. Fröhlich, L. Hoffmann, S. Horner, M. Elschner, H. Tomaso, K. Henning, H. Neubauer, and L. D. Sprague, 2012: Prevalence of *Coxiella burnetii* in clinically healthy German sheep flocks. *BMC Res. Notes* 5, 152.

Khalili, M., E. Sakhaee, M. R. Aflatoonian, and N. Shahabi-Nejad, 2011: Herd-prevalence of *Coxiella burnetii* (Q fever) antibodies in dairy cattle farms based on bulk tank milk analysis. *Asian Pac. J. Trop. Med.* 54, 8–60.

Kim, S. G., E. H. Kim, C. J. Lafferty, and E. Dubovi, 2005: *Coxiella burnetii* in bulk tank milk samples, United States. *Emerg. Infect. Dis.* 11, 619–621.

Klee, S. R., J. Tyczka, H. Ellerbrok, T. Franz, and S. Linke, 2006: Highly sensitive real-time PCR for specific detection and quantification of *Coxiella burnetii*. *BMC Microbiol.* 6, 2.

van Loenhout, J. A. F., W. J. Paget, J. H. Vercoulen, C. J. Wijkmans, J. L. A. Hautvast, and K. van der Velden, 2012: Assessing the long-term health impact of Q-fever in the Netherlands: a prospective cohort study started in 2007 on the largest documented Q-fever outbreak to date. *BMC Infect. Dis.* 12, 280.

Loftis, A. D., R. A. Priestley, and R. F. Massung, 2010: Detection of *Coxiella burnetii* in commercially available raw milk from the United States. *Foodborne Pathog. Dis.* 7, 1453–1456.

Muskens, J., E. van Engelen, C. van Maanen, C. Bartels, and T. J. G. M. Lam, 2011: Prevalence of *Coxiella burnetii* infection in Dutch dairy herds based on testing bulk tank milk and individual samples by PCR and ELISA. *Vet. Rec.* 168, 79.

Nielsen, K. T., S. S. Nielsen, J. F. Agger, A. B. Christoffersen, and J. S. Agerholm, 2011: Association between antibodies to *Coxiella burnetii* in bulk tank milk and perinatal mortality of Danish dairy calves. *Acta Vet. Scand.* 53, 64.

Parisi, A., R. Fraccalvieri, M. Cafiero, A. Miccolupo, I. Padalino, C. Montagna, F. Capuano, and R. Sottili, 2006: Diagnosis of *Coxiella burnetii*-related abortion in Italian domestic ruminants using single-tube nested PCR. *Vet. Microbiol.* 118, 101–106.

Porten, K., J. Rissland, A. Tigges, S. Broll, W. Hopp, M. Lunemann, U. van Treeck, P. Kimmig, S. O. Brockmann, C. Wagner-Wiening, W. Hellenbrand, and U. Buchholz, 2006: A superspreading ewe infects hundreds with Q fever at a farmers' market in Germany. *BMC Infect. Dis.* 6, 147.

Rodolaskis, A., M. Berri, C. Héchard, C. Caudron, A. Souriau, C. C. Bodier, B. Blanchard, P. Camuset, P. Devillechaise, J. C. Natorp, J. P. Vadet, and N. Arricau-Bouvery, 2007: Comparison of *Coxiella burnetii* shedding in milk of dairy bovine, caprine and ovine herds. *J. Dairy Sci.* 90, 5352–5360.

Rousset, E., M. Berri, B. Durand, P. Dufour, M. Prigent, T. Delcroix, A. Touratier, and A. Rodolaskis, 2009: *Coxiella burnetii* shedding routes and antibody response after outbreaks of Q fever-induced abortion in dairy goat herd. *Appl. Environ. Microbiol.* 75, 428–433.

Ryan, E. D., M. Kirby, D. M. Collins, R. Sayers, J. F. Mee, and T. Clegg, 2011: Prevalence of *Coxiella burnetii* (Q fever) antibodies in bovine serum and bulk-milk samples. *Epidemiol. Infect.* 139, 1413–1417.

Saegerman, C., N. Speybroeck, F. Dal Pozzo, and G. Czaplicki, 2013: Clinical indicators of exposure to *Coxiella burnetii* in dairy herds. *Transbound Emerg. Dis.* doi: 10.1111/tbed.12070

Santos, A. S., J. J. H. C. Tilburg, A. Botelho, M. J. Barahona, M. S. Núncio, M. H. Nabuurs-Franssen, and C. H. W. Klaassen, 2012: Genotypic diversity of clinical *Coxiella burnetii* isolates from Portugal based on MST and MLVA typing. *Int. J. Med. Microbiol.* 302, 253–256.

Schimmer, B., S. Luttikholt, J. L. A. Hautvast, E. A. M. Graat, P. Vellema, and Y. T. H. P. van Duynhoven, 2011: Seroprevalence and risk factors of Q fever in goats on commercial dairy goat farms in the Netherlands, 2009–2010. *BMC Vet. Res.* 7, 81.

Sidi-Boumedine, K., E. Rousset, K. Henning, M. Ziller, K. Niemczuck, H. I. J. Roest, and R. Thiery, 2010: Development of harmonized schemes for the monitoring and reporting of Q-fever in animals in the European Union. Scientific Report submitted to EFSA, Question No EFSAQ-2009-00511; 2010.

Thrusfield, M., 1995: Veterinary Epidemiology, 2nd edn. Blackwell Science, Oxford.

Vaidya, V. M., S. V. S. Malik, K. N. Bhilegaonkar, R. S. Rathore, S. Kaur, and S. B. Barbuddhe, 2008: Prevalence of Q fever in domestic animals with reproductive disorders. *Comp. Immunol. Microbiol. Infect. Dis.* 33, 307–321.

Short Communication

Experimental Pathology and Health Sciences
2016;8 (1): 13-14

Coxiella burnetii is present in milk from dairy cattle herds in the Northwest Portugal

Anastácio Sa,b, Pimenta Lc, Simões Jd, Alegria Nd, Rabiço Ac, Sidi-Boumedine Ke, da Silva GJ.a

a. Faculty of Pharmacy and Center of Neurosciences and Cell Biology, Health Science Campus, University of Coimbra, Coimbra, Portugal.
b. University School of Vasco da Gama, Coimbra, Portugal.
c. Barcelos Agricultural Cooperative Society, Barcelos, Portugal.
d. CECAV, Department of Veterinary Science, Agricultural and Veterinary Sciences School. University of Trás-os-Montes e Alto Douro, Vila Real, Portugal
e. ANSES, Sophia-Antipolis Laboratory, Sophia-Antipolis, France.

Abstract

Ruminants are recognized as the main reservoirs of Coxiella burnetii. EFSA highlighted the lack of knowledge about Q fever prevalence in many European countries. A cross-sectional study was carried out in randomly selected dairy herds from the Northwest of Portugal to screen for C. burnetii infection by PCR testing in antibody positive Bulk Tank Milk samples (BTM). The proportion of C. burnetii positive BTM samples was 30.9% of herds and the bacterial load ranged from 600 to 8513800 bacteria/mL. The results highlight that the shedding of C. burnetii through milk is important, especially in dairy cattle, and thus the role of milk as a potential source of infection among dairy workers should not be neglected.

INTRODUCTION

Q fever is a zoonotic disease whose etiological agent is Coxiella burnetii (Maurin and Raoult 1999). In recent years, an increased incidence of human infection in Europe has been reported (Georgiev et al., 2013), mainly due to the 4173 cases associated to the Q fever outbreak occurred in the Netherlands between 2007 and 2012 (Dijkstra et al., 2012). An association between human Q fever cases and infected ruminant herds has been referred (van den Brom et al. 2015). For this reason, domestic ruminants are generally considered the primary animal reservoir of *C. burnetii*. The shedding of *C. burnetii* in infected ruminants occurs mainly during and after parturition or abortion, in birth products and vaginal mucus, but the shedding in urine, feces and milk are also documented in countries other than Portugal (Rousset et al. 2009, Sidi-Boumedine et al. 2010). The impact on public and animal health triggers Q fever a disease of interest for public policy makers and food industries, mainly dairy production (Guatteo et al. 2011). This study aimed i) to assess the *C. burnetii* shedding in antibodies positive BTM samples and ii) to determine the bacterial load in *C. burnetii* positive samples.

MATERIALS AND METHODS

The present study was performed using the BTM positive samples for specific anti-*C. burnetii* antibodies detected previously in survey conducted at the county of Barcelos, in the Northwest of Portugal . In the previous study, BTM samples (n=90) were tested for the presence of specific anti-*C. burnetii* antibodies by using a commercial ELISA based on inactivated *C. burnetii* phase I and phase II antigens, obtained from the reference Nine Mile strain, isolated from ticks (Pimenta et al. 2015). Considering that a positive and statistically significant correlation was established between the bacterial load on BTM qPCR and antibody positive results on BTM, and that in presence of negative antibody test, the probability to find a positive PCR test on BTM is considered low or negligible (Anastácio et al. 2014, Saegerman et al. 2015), the assessment of *C. burnetii* shedding was performed only in antibody positive BTM. DNA was extracted from 200 μL of each BTM sample using the QIAmp DNA Mini Kit (Qiagen, Izasa Portugal), following manufacturer's instructions. The DNA samples were first screened by conventional PCR as previously described (Anastácio et al. 2014). PCR positive samples were additionally tested to estimate the bacterial load by a real-time qPCR assay (Taq-VetTM C. burnetii–Absolute Quantification kit; Lifetechonologies, USA), according to manufacturer's instructions. The real-time PCR assays were performed on a CFX-96 thermocycler (Bio-Rad, Amadora, Portugal). Results were expressed in number of bacteria per milliliter. Confidence limits for the proportions were estimated with 95% confidence intervals (CI) assuming a binominal exact distribution (EpiInfo version 3.5.4).

RESULTS

Of the total of 55 BTM samples tested by PCR, 30.9% (17/55; 95% CI: 19.5-45.0%) were C. burnetii positive. Considering the total of herds, the proportion of PCR positive herds was estimated in at least 18.9% (17/90; 95% CI: 11.7-28.8%). In PCR positive BTM samples the bacterial load ranged from 600 to 8513800 bacteria/mL (mean = 2119694 and SD=2944856). It was found that in 88.2% (15/17) of PCR positive samples the concentration

of bacteria was higher than 10^4 and in 47.1% (8/17) the concentration was higher than 10^6.

DISCUSSION AND CONCLUSIONS

C. burnetii is the etiologic agent of Q fever, an infection that affects both animals and humans (Sidi-Boumedine et al. 2010). The knowledge of the status of infection in herds contributes to a better understanding of the epidemiology of this pathogen. The shedding of *C. burnetii* through milk is widespread in dairy cattle herds in different countries. This might be explained by the fact that milk is the most frequent shedding route of *C. burnetii* in cows and a longtime excretion through milk can be extended for several months even in asymptomatic animals (Kim et al. 2005, Guatteo et al. 2011). In this context, this study was designed as an approach to investigate the milk shedding of C. burnetii in dairy ruminant herds in the Northwest of Portugal.

C. burnetii was detected in the BTM of 30.9% of tested herds. Overall, and considering the total of studied herds, an active infection occurred in at least 18.9% of herds which might be slightly underestimated as the antibody negative herds were not screened by PCR. However, recent findings suggest that in antibody negative BTM samples the probability of getting a positive PCR is low to negligible (Anastácio et al. 2014, Saegerman et al. 2015). Thereby the results were similar to that found in central Portugal (20%) (Anastácio et al. 2014) but is was slightly lower than the results obtained in other countries, ranging from 29.6 to 94.3% (Kim et al. 2005, Fretz et al. 2007, Muskens et al. 2011, Astobiza et al. 2012, Czaplicki et al. 2012). The estimation of the bacterial load allows the knowledge of the shedding pattern of infected animals. The bacterial load of the PCR positive herds was found to be high in most of the PCR positive herds and these results are in agreement to that found in central Portugal (Anastácio et al. 2014).

An active infection was confirmed in some herds where the exposure to *C. burnetii* has been showed previously by the presence of specific antibodies. Thus, milk should not be neglected as a source of infection to humans namely by the consumption of unpasteurized milk or by the inhalation of aerosols in milking rooms (Loftis et al. 2010). Further research is needed to characterize at molecular level the circulating strains and to compare them with those from other countries.

REFERENCES

Anastácio S, Carolino N, Sidi-Boumedine K, da Silva GJ. Q fever dairy herd status determination based on serological and molecular analysis of bulk tank milk. Transbound Emerg Dis. 2014. doi:10.1111/tbed.12275.

Astobiza I, Ruiz-Fons F, Piñero A, Barandika J F, Hurtado A, Garcia-Perez AL. Estimation of Coxiella burnetii prevalence in dairy cattle in intensive systems by serological and molecular analyses of bulk-tank milk samples. J. Dairy Sci. 2012:95 :1632–1638.

Brom RV, Engelen EV, Roest HI, Hoek WV, Vellema P. Coxiella burnetii infections in sheep or goats: an opinionated review. Vet Microbiol. 2015. doi: 10.1016/j.vetmic.2015.07.011.

Czaplicki G, Houtain JY, Mullender C, Porter SR, Humblet MF, Manteca C, Saegerman C. Apparent prevalence of antibodies to *Coxiella burnetii* (Q fever) in bulk tank milk from dairy herds in southern Belgium. Vet. J. 2012:192, 529–531.

Dijkstra F, van der Hoek W, Wijers N, Schimmer B, Rietveld A, Wijkmans CJ, Vellema P, Schneeberger PM. The 2007–2010 Q fever epidemic in The Netherlands: characteristics of notified acute Q fever patients and the association with dairy goat farming. FEMS Immunol Med Microbiol. 2012:64(1),3-12.

Fretz R, Schaeren W, Tanner M, Baumgartner A. Screening of various foodstuffs for occurrence of Coxiella burnetii in Switzerland. Int. J. Food Microbiol. 2007:116, 414–418.

Georgiev M, Afonso A, Neubauer H, Needham H, Thiéry R, Rodolakis A, Roest J, Stärk KD, Stegeman JA, Vellema P, van der Hoek W, More SJ. Q fever in humans and farm animals in four European countries, 1982 to 2010. Euro Surveill. 2013:18(8):pii=20407.

Guatteo R, Seegers H, Taurel AF, Joly A, Beaudeau F. Prevalence of Coxiella burnetii in domestic ruminants: a critical review. Vet Microbiol. . 2011:149, 1-16.

Kim SG, Kim EH, Lafferty CJ, Dubovi E. Coxiella burnetii in Bulk Tank Milk Samples, United States. Emerging Infect. Dis. 2005:11(4), 619-621.

Loftis AD, Priestley RA, Massung RF. Detection of Coxiella burnetii in Commercially Available Raw Milk from the United States. Foodborne Pathog. Dis. 2010:7(12), 1453-6.

Maurin, M., Raoult, D. Q Fever. Clin. Microbiol. Rev. 1999:12(4), 518-553.

Muskens J, van Engelen E, van Maanen C, Bartels C, Lam TJGM. Prevalence of C*oxiella burnetii* infection in Dutch dairy herds based on testing bulk tank milk and individual samples by PCR and ELISA. *Vet. Rec.* 2011:168, 79.

Pimenta L, Alegria N, Anastácio S, Sidi-Boumedine K, da Silva G, Rabiço Â, Simões J. Prevalence of Coxiella burnetii antibodies in Portuguese dairy cattle herds. Trop Anim Health Prod. 2015:47(1), 227-230.

Rousset E, Berri M, Durand B, Dufour P, Prigent M, Delcroix T, Touratier A, Rodolaskis A. *Coxiella burnetii* Shedding Routes and Antibody Response after Outbreaks of Q Fever-Induced Abortion in Dairy Goat Herd. Appl. Environ. Microbiol. 2009:*75* (**2**), 428-433.

Saegerman C, Speybroeck N, Dal Pozzo F, Czaplicki G. Clinical Indicators of Exposure to Coxiella burnetii in Dairy Herds. Transbound Emerg Dis. 2013:62(1):46-54.

Sidi-Boumedine K, Rousset E, Henning K, Ziller M, Niemczuck K, Roest HIJ, Thiery R. Development of harmonized schemes for the monitoring and reporting of Q-fever in animals in the European Union. Scientific Report submitted to EFSA, Question No EFSAQ-2009-00511; 2010.

Trop Anim Health Prod (2015) 47:227–230
DOI 10.1007/s11250-014-0679-1

SHORT COMMUNICATIONS

Prevalence of *Coxiella burnetii* antibodies in Portuguese dairy cattle herds

Luís Pimenta · Nuno Alegria · Sofia Anastácio · Karim Sidi-Boumedine · Gabriela da Silva · Ângela Rabiço · João Simões

Received: 27 May 2014 / Accepted: 16 September 2014 / Published online: 23 October 2014

Abstract Q fever is an important zoonotic disease which has been recently diagnosed, mainly in sheep and goats, in Portugal. The aim of the present study was to determine the prevalence of bovine *Coxiella burnetii* antibodies in dairy farms from the northwest of Portugal. Bulk tank milk samples were randomly obtained, on November 2013, from 90 dairy farms and assayed using an ELISA kit. The apparent prevalence was 61.1 % (95 % C.I. from 50.8 to 70.5 %). The proportion of negative and intermediate (inconclusive) herds was 34.5 % (25.5 to 44.7 %) and 4.4 % (1.7 to 10.9 %), respectively. In conclusion, a high level of exposure to *Coxiella burnetii* was observed in Portuguese dairy cattle herds, highlighting the needs to better understand the epidemiology of Q fever in Portugal by the implementation of a monitoring program based on harmonized serologic and molecular methodologies and elucidation of the infection status of the herds.

L. Pimenta · N. Alegria · J. Simões (✉)
CECAV, Department of Veterinary Science, Agricultural and Veterinary Sciences School, University of Trás-os-Montes e Alto Douro, Quinta de Prados, 5000-811 Vila Real, Portugal
e-mail: jsimoes@utad.pt

S. Anastácio · G. da Silva
Faculty of Pharmacy, Center for Neurosciences and Cell Biology, Health Science Campus, University of Coimbra, Azinhaga de Sta Comba, 3000-548 Coimbra, Portugal

S. Anastácio
University School of Vasco da Gama, Rua José R. Sousa Fernandes, 197, Bloco B, 3020-210 Coimbra, Portugal

K. Sidi-Boumedine
Sophia-Antipolis Laboratory, ANSES, Les Templiers 105 route des Chappes, BP 111, 06902 Sophia Antipolis, France

Â. Rabiço
Barcelos Agricultural Cooperative Society, Rua Fernando de Magalhães 206, 4750-290 Barcelos, Portugal

Keywords Cows · ELISA · Milk · Q fever · Survey

Introduction

The Q fever is a disease caused by *Coxiella burnetii*, an intracellular bacterium affecting a large range of domestic and wildlife animals. Livestock is the main vertebrate reservoir associated to human disease (Rousset et al. 2010).

The human Q fever outbreak in the Netherlands (2007–2011) raised the awareness of the European health authorities about the lack of scientific knowledge, concerning this worldwide spread zoonotic infection, in European countries (EFSA 2010).

The *C. burnetii* genotypes identified in the Dutch human outbreak were mostly associated with those recovered from sheep and goats (Klaassen et al. 2009; Roest et al. 2011; Tilburg et al. 2012). Nevertheless, cattle have also been described as an important reservoir (Rousset et al. 2010). Moreover, high prevalence of *C. burnetii* antibodies in bulk tank milk samples from bovine dairy herds were reported in several countries such as Denmark (59.0 %) (Agger et al. 2010), Iran (45.4 %) (Khalili et al. 2011), and USA (>94 %) (Kim et al. 2005). More recently, Pearson et al. (2014) observed a high prevalence of *C. burnetii* associated with a lack in genotypic diversity (mostly two genotypes were identified) and a segregation of the genotypes between cows and goats, suggesting species-specific adaptations or interspecies dissemination barriers.

In Portugal, a recent serosurvey carried out in sheep and goats showed a herd seroprevalence of 32.6 % (Anastácio et al. 2013a). Furthermore, the shedding of *C. burnetii* in sheep and goat herds was confirmed using qPCR assays (Anastácio et al. 2013b). Concerning dairy cattle herds, the seroprevalence remains unreported in scientific literature and, unlike brucellosis, there is no national surveillance for Q fever

in cattle or small ruminants. The availability of Q fever vaccines in Portugal is very recent and under veterinary control.

The aim of the present study was to assess the prevalence of Q fever in bovine dairy herds, and thus their exposure to *C. burnetii*, in the county of Barcelos, an important region of dairy industry in North Portugal.

Material and methods

Herd and milk sampling

A cross-sectional study was designed at herd level, in Barcelos, a county located in the north of Portugal (41° 53 N latitude and 08° 61 W longitude).

The sample size calculation considered the total number of herds (n=887) and adult dairy (n=23487) or beef (n=7641) cows registered in the Barcelos Agricultural Cooperative Society (http://www.agribar.pt/), on November 2013, according to the National Bovine Brucellosis Surveillance Plan of the Portuguese General Directorate for Food and Veterinary (http://www.dgv.min-agricultura.pt/).

The criteria for inclusion were the following: (1) only dairy herds were selected, (2) the existence of a bulk tank milk (BTM) sample delivered daily to dairy industry, and (3) the absence of vaccinated animals in each herd. The formula n= Z2pq/l2 (Dohoo et al. 2003, p. 41) was applied to calculate the sample size. A sample size n=90 was obtained considering an expected prevalence of 65 % (Agger et al. 2010), the desired precision of 0.10 at the 95 % confidence level, and Z=1.96 (95 % confidence interval for standardized normal distribution).

The random assignment for the determination of the 90 herds was performed in Microsoft Excel® 2013 using the "=RAND ()" function.

During the month of November 2013, BTM samples of each randomly selected herd were collected into a sterile 10-ml plastic tube and taken to the laboratory under refrigerated conditions. In each herd, the number of lactating cows was registered at the time of sampling. At the laboratory, the samples were centrifuged and the non-fat fraction was frozen (−20 °C) until analysis.

Antibody ELISA analysis

All samples were tested in duplicate for the presence of specific antibodies to *C. burnetii* by using the commercial CHEKIT® Q-Fever Antibody ELISA Test (IDEXX, Liebefeld-Bern, Switzerland). This test is based on inactivated *C. burnetii* phase 1 and phase 2 antigens obtained from the reference Nine Mile strain (isolated from ticks).

The optical density (OD) of each sample was corrected by subtraction of the OD of the negative control included in the test kit. The results were estimated as the ratio of OD of the sample (S) versus OD of the positive control (P), also included in the test kit, and were expressed as S/P values.

A sample was considered positive for a $S/P \geq 40$ %, negative for a $S/P \leq 30$ %, and intermediate (suspect) for a $30\ \% \leq S/P < 40$ %, according to the manufacturer's instructions.

Statistical analysis

Descriptive statistics of antibody S/P values and apparent prevalence of *C. burnetii* and their corresponding 95 % confidence intervals were estimated using the JMP® version 7 (SAS Institute Inc. 2007) software. A Spearman's correlation between the number of lactating females in the herd and the S/P values of each herd was tested.

Results and discussion

The number of lactating cows per herd contributing to BTM ranged from 25 to 580, and the total number of lactating cows represented 30.9 % (9610/31,128) of the total number of adult cows in the county.

The apparent prevalence of antibodies was 61.1 %. The proportion of negative and intermediate (inconclusive) herds was 34.5 and 4.4 %, respectively. The estimated 95 % confidence intervals are reported in Table 1. The S/P values ranged from 0.8 to 147.5 (Fig. 1).

The infection caused by *C. burnetii* has been reported in Portugal in recent years, especially in the south and center of the country. Few studies demonstrated the presence of the

Table 1 Descriptive statistics for S/P values of anti-*Coxiella burnetii* antibodies in positive, intermediate, and negative farms

Farm	Positive	Intermediate	Negative
Number	55	4	31
Apparent prevalence/proportion	61.11 %	4.44 %	34.45 %
95 % confidence interval	50.78–70.53 %	1.74–10.88 %	25.45–44.72 %
Mean of S/P values	95.25	34.46	11.97
Range of S/P values	40.31–147.49	32.23–39.13	0.80–29.65

S/P value is the ratio between the optical density of the sample (S) and the optical density of positive control (P) delivered in the test kit

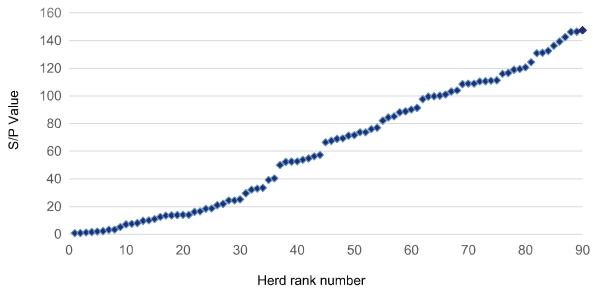


Fig. 1 Array of antibody *S/P* values to *Coxiella burnetii* in bulk tank milk samples from 90 Portuguese dairy farms in November 2013

bacteria in clinical samples from zoo animals and ruminants (Clemente et al. 2008, 2009), and surveys were carried out on serum or BTM samples from ruminants in central Portugal using the LSIVet Ruminant Milk/Serum Q Fever® (LSI; Lissieu, France) (Anastácio et al. 2012, 2013a). However, some questions are still unclear, particularly whether the prevalence of infection differs among regions and in that case which factors may be involved. Milk production is an important economic activity in the coastal region of the northern and central Portugal, and in dairy herds, BTM is a suitable sample to screen for *C. burnetii* exposure at the herd level. The present study was designed as an approach to estimate the apparent seroprevalence of *C. burnetii* in the dairy cattle from the northwest of Portugal.

Our study shows a relatively high apparent prevalence (61.1 %) of antibodies in cattle BTM samples. These results are similar to those obtained in other European countries such as Belgium (57.8 %) (Czaplicki et al. 2012), Denmark (59 %) (Agger et al. 2010), and Spain (66.9 %) (Astobiza et al. 2012). Notwithstanding, they are slightly higher than those obtained in a survey performed at the center of Portugal (50 %) (Anastácio et al. 2012). However, different methodologies and study designs were used. Indeed, in the latter study, a convenience sampling was performed with the selection of herds with reports of reproductive disorders, and the lower seropositivity was somewhat unexpected. Furthermore, the antibody testing was performed using an ELISA based on antigens obtained from a European *C. burnetii* bovine strain. This ELISA is considered more sensitive than the ELISA using antigens prepared from the reference strain Nine Mile, isolated from ticks (EFSA 2010). In a very recent study, Paul et al. (2013) estimated a sensitivity of 86 % and a specificity of 99 % using milk antibody CHEKIT Q-Fever Antibody ELISA Test Kit (IDEXX, Switzerland) in a Bayesian framework.

The correlation between the number of tested animals and antibody *S/P* values was rho=0.20 (*P*=0.055). The correlation between the number of tested animals and antibody *S/P* values in each herd did not reach, in limit, the statistical significance, suggesting the absence or a tendency for a low herd size effect. However, an effect of the increase of the herd size on BTM antibody positivity to *C. burnetii* has been reported in some studies (Ryan et al. 2011; Agger et al. 2013). Thus, the infection status and specific risk factors on each herd, including the number of infected animals probably affect the rate of seropositivity (Agger et al. 2013; Paul et al. 2014).

It is concluded that there is a high level of exposure to *C. burnetii* in dairy cattle herds in the northwest of Portugal. Further studies are needed to investigate the status of the herds by assessing the shedding prevalence infection and to characterize the circulating genotypes in the herds. These data are crucial to characterize the epidemiology of infection and to decide the most appropriate control strategies to adopt. These results, together with other published work in Portugal, highlight the need to implement a monitoring program for Q fever based on harmonized methodologies.

Acknowledgments The authors thank the Barcelos Agricultural Cooperative Society (http://www.agribar.pt/) for the data availability of bovine and herd characterization for the present work.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The manuscript does not contain clinical studies or patient data.

References

Agger, J.F., Christoffersen, A.B.., Rattenborg, E., Nielsen, J., and Agerholm, J.S., 2010. Prevalence of *Coxiella burnetii* antibodies in Danish dairy herds. Acta Veterinaria Scandinavica, 52, 5.

Agger, J.F., Paul, S., Christoffersen, A.B.. and Agerholm, J.S., 2013. Risk factors for *Coxiella burnetii* antibodies in bulk tank milk from Danish dairy herds. Acta Veterinaria Scandinavica, 55, 80.

Anastacio, S., Tavares, N., Carolino, N., Sidi-Boumedine, K. and da Silva, G.J., 2013a. Serological evidence of exposure to *Coxiella burnetii* in sheep and goats in central Portugal. Veterinary Microbiology 167, 500–505.

Anastácio S., Tavares N., Sidi-Boumedine K., da Silva G.J., 2013b. Evidence of *Coxiella burnetii* infection in small ruminants: a cross

sectional study. Abstract OC 332. 23rd European Congress of Clinical Microbiology and Infectious Diseases.

Anastácio, S., Pessoa, D., Pegado, J., Cruz, C, Sidi-Boumedine, K. and da Silva, G.J., 2012. Investigation of *Coxiella burnetii* infection in dairy ruminant herds with reproductive disorders in two different regions of Portugal. Clinical Microbiology and Infection,18 (S3), 524.

Astobiza, I., Ruiz-Fons, F., Piñero, A., Barandika, J.F., Hurtado, A. and García-Pérez, A.L., 2012. Estimation of *Coxiella burnetii* prevalence in dairy cattle in intensive systems by serological and molecular analyses of bulk-tank milk samples. Journal of Dairy Sciences, 95, 1632–1638.

Clemente, M. L., Barahona, M. J., Andrade, M. F., Botelho, A., 2009. Diagnosis of *Coxiella burnetii* by PCR in aborted foetuses of domestic ruminants in Portugal. Veterinary Record 164, 373–374.

Clemente, L., Fernandes, T.L., Barahona, M.J., Bernardino, R., Botelho, A., 2008. Confirmation by PCR of *Coxiella burnetii* infection in animals at a zoo in Lisbon, Portugal. Veterinary Record 163, 221–222.

Czaplicki, G., Houtain, J.Y., Mullender, C., Porter, S.R., Humblet, M.F., Manteca, C. and Saegerman, C., 2012. Apparent prevalence of antibodies to *Coxiella burnetii* (Q fever) in bulk tank milk from dairy herds in southern Belgium. Veterinary Journal, 192, 529–531.

Dohoo, I., Martin, W. and Stryhn, H., 2003. Veterinary Epidemiologic Research, (AVC Inc., Charlottetown, Prince Edward Island, Canada).

EFSA (EUROPEAN FOOD SAFETY AUTHORITY), 2010: Panel on Animal Health and Welfare (AHAW); Scientific Opinion on Q Fever. EFSA Journal 8, 1595. Available online: http://www.efsa.europa.eu/.

Khalili, M., Sakhaee, E., Aflatoonian, M.R. and Shahabi-Nejad, N., 2011: Herd-prevalence of *Coxiella burnetii* (Q fever) antibodies in dairy cattle farms based on bulk tank milk analysis. Asian Pacific Journal Tropical Medicine, 54, 8–60.

Kim, S.G., Kim, E.H., Lafferty, C.J. and Dubovi, E., 2005: *Coxiella burnetii* in Bulk Tank Milk Samples, United States. Emerging Infectious Diseases, 11, 619–621.

Klaassen, C.H.W., Nabuurs-Franssen, M.H., Tilburg, J.J.H.C., Hamans, M.A.W.M. and Horrevorts, A.M., 2009. Multigenotype Q Fever Outbreak, the Netherlands. Emerging and Infectious Diseases, 15, 613–614.

Paul, S., Agger, J.F., Agerholm, J.S. and Markussen, B., 2014. Prevalence and risk factors of *Coxiella burnetii* seropositivity in Danish beef and dairy cattle at slaughter adjusted for test uncertainty. Preventive Veterinary Medicine, 113, 504–511.

Paul, S., Toft, N., Agerholm, J.S., Christoffersen, A.B.. and Agger, J.F., 2013. Bayesian estimation of sensitivity and specificity of *Coxiella burnetii* antibody ELISA tests in bovine blood and milk. Preventive Veterinary Medicine, 109, 258–263.

Pearson, T., Hornstra, H.M., Hilsabeck, R., Gates, L.T., Olivas, S.M., Birdsell, D.M., Hall, C.M., German, S., Cook, J.M., Seymour, M.L., Priestley, R.A., Kondas, A.V., Friedman, C.L.C., Price, E.P., Schupp, J.M., Liu, C.M., Price, L.B., Massung, R.F., Kersh, G.J. and Keim, P., 2014. High prevalence and two dominant host-specific genotypes of *Coxiella burnetii* in U.S. milk. BMC Microbiology, 14, 41.

Roest, H.I., Ruuls, R.C., Tilburg, J.J., Nabuurs-Franssen, M.H., Klaassen, C.H., Vellema, P., van den Brom, R., Dercksen, D., Wouda, W., Spierenburg, M.A., van der Spek, A.N., Buijs, R., de Boer, A.G., Willemsen, P.T. and van Zijderveld, F.G., 2011. Molecular epidemiology of *Coxiella burnetii* from ruminants in Q fever outbreak, the Netherlands. Emerging Infectious Diseases, 17, 668–675.

Rousset, E., Sidi-Boumedine, K., Thiery, R., 2010. Chapter 2.1.1.2 - Q fever. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (mammals, birds and bees). OIE. 7th Ed. Available online: http://www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/.

Ryan, E.D., M. Kirby, D.M. Collins, R. Sayers, J.F. Mee and T. Clegg, 2011: Prevalence of *Coxiella burnetii* (Q fever) antibodies in bovine serum and bulk-milk samples. Epidemiology and Infection, 139, 1413–1417.

SAS Institute Inc. 2007. JMP User's Guide. Version 7.0. Cary, NC, USA: SAS Institute Inc

Tilburg, J.J., Roest, H.J., Buffet, S., Nabuurs-Franssen, M.H., Horrevorts, A.M., Raoult, D. and Klaassen, C.H., 2012. Epidemic genotype of *Coxiella burnetii* among goats, sheep, and humans in the Netherlands. Emerging and Infectious Diseases, 18, 887–889.

Veterinary Microbiology xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Serological evidence of exposure to *Coxiella burnetii* in sheep and goats in central Portugal

S. Anastácio [a,b], N. Tavares [b,c], N. Carolino [b], K. Sidi-Boumedine [d], G.J. da Silva [a,*]

[a] Center of Pharmaceutical Studies, Faculty of Pharmacy, University of Coimbra, Coimbra, Portugal
[b] University School of Vasco da Gama, Coimbra, Portugal
[c] OPP Coimbra, Coimbra, Portugal
[d] ANSES, Sophia-Antipolis Laboratory, Sophia-Antipolis, France

ARTICLE INFO

Article history:
Received 16 January 2013
Received in revised form 2 August 2013
Accepted 4 August 2013

Keywords:
Coxiella burnetii
Epidemiology
Seroprevalence
Zoonosis

ABSTRACT

The recent outbreak of Q fever in The Netherlands warned European health authorities of the need of studying *Coxiella burnetii*. In Portugal, little is known about *C. burnetii* infection in animals. A cross-sectional study was designed to investigate the exposure to *C. burnetii* in sheep and goats in the Central region of Portugal, estimating the herd and individual prevalence. A serosurvey was conducted in a two levels random sampling of 89 herds and 460 animals. Individual blood samples were collected from animals older than 6 months, and specific antibodies anti-*C. burnetii* were detected by ELISA testing.

Results showed a global herd prevalence of 32.6% (95% CI: 23.1–42.1%). Herd prevalence was higher in mixed herds (38.5%; 95% CI: 12–65%) and in sheep herds (37.5%; 95% CI: 21–54%) than in goat herds (28.8%; 95% CI: 17–41%). Global individual prevalence was estimated at 9.6% (95% CI: 6.9–12.2%), and it was higher in goats (10.4%; 95% CI: 7.8–13%) than in sheep (8.6%; 95% CI: 5.8–11.4%). Sample positive percentages (S/P) ranged from 41.5% to 185.9%. S/P percent higher than 100 was found in 18.2% (8/44) of sera from distinct herds. Positive results were significantly associated with goats, older animals and larger herds. These results revealed the presence of *C. burnetii* in small ruminants evidencing their potential role in the infection cycle.

1. Introduction

Q fever is a zoonotic infection caused by *Coxiella burnetii*, an obligate intracellular bacterium. It was described in Australia in 1937 for the first time (Maurin and Raoult, 1999). Currently, this disease presents a worldwide distribution, affecting a wide range of domestic and wildlife animals (Arricau-Bouvery and Rodolakis, 2005; Rousset et al., 2010).

The clinical signs of Q fever are not pathognomonic neither in humans nor in animals. This lack of specificity is the first major obstacle to its diagnosis (Arricau-Bouvery and Rodolakis, 2005; Angelaskis and Raoult, 2010). In humans, acute Q fever can be asymptomatic or it can manifest as a nonspecific flu-like illness. Complications associated with pneumonia or hepatitis requiring hospitalization may be observed in about 2% of patients. Chronic Q fever may appear as an endocarditis, an osteoarticular infection, a chronic hepatitis or as a chronic pneumonia in patients with predisposing factors and/or inappropriate antibiotherapy. The infection during pregnancy may lead to abortion (Maurin and Raoult, 1999; ECDC, 2010). Also, cases of chronic fatigue syndrome have been described infrequently following *C. burnetii* infection (Angelaskis and Raoult, 2010; van Asseldonk et al., 2013). In animals, Q fever is mainly reported in livestock ruminants and occurs,

* Corresponding author at: Laboratory of Microbiology, Center of Pharmaceutical Studies, Faculty of Pharmacy, University of Coimbra, Health Sciences Campus, Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal. Tel.: +351 239488460.
E-mail addresses: gjsilva@ci.uc.pt, silva.gj@gmail.com, gjsilva@gmail.com (G.J. da Silva).

0378-1135/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.vetmic.2013.08.004

usually, as an asymptomatic infection (Woldehiwet, 2004; Arricau-Bouvery and Rodolakis, 2005; Rousset et al., 2010). In small ruminants, abortions, premature delivery, delivery of weak offspring and stillbirth are reported (Rodolaskis, 2006). In cattle, clinical signs of Q fever can be less obvious than in small ruminants. However, a very recent study demonstrated that abortion and irregular repeat breeding are important risk indicators in cattle dairy herds (Saegerman et al., 2013). Also, an association of Q fever with metritis and infertility has been suggested (To et al., 1998; Woldehiwet, 2004; EFSA, 2010).

Epidemiological studies have demonstrated a relationship between the infection in humans and ruminants (Gilsdorf et al., 2008; Schimmer et al., 2010; van den Brom et al., 2012). However, the Q fever prevalence and incidence are not well known, and have been underestimated for many years (EFSA, 2010). Shedding of bacteria occurs by secretions and excreta from infected animals, namely vaginal secretions, milk, feces and urine. During birthing and/or abortion the bacterium is excreted massively in genital secretions, placenta and fetal fluids (Berri et al., 2001, 2002, 2005; Arricau-Bouvery et al., 2003; Guatteo et al., 2006). Because of the existence of fecal shedders and the high resistance of *C. burnetii*, bedding material must be considered as a source of infection (Rodolaskis, 2006; Guatteo et al., 2007; Rousset et al., 2010).

Recently, the European Commission (EC) formulated concerns about the increase number of human Q fever cases associated with small ruminant herds, in urban or residential areas, in Europe (Panaiotov et al., 2009; Medic et al., 2005; Porten et al., 2006; Gilsdorf et al., 2008). In the Netherlands, 4108 acute human cases were notified between 2007 and 2011 (van Loenhout et al., 2012). Typing of bacteria by multiple-locus variable number tandem repeat analysis (MLVA) showed a genetic similarity of isolates recovered from human and animal samples, indicating a relationship between human cases and the occurrence of infection in ruminant herds (Klaassen et al., 2009; van der Hoek et al., 2010; Roest et al., 2011a,b).

Following the EC's demand to assess the risk for humans and animals associated with Q fever, the European Food Safety Authority (EFSA), in a scientific opinion, highlighted the considerable uncertainty that still exists in the understanding of *C. burnetii* infection in domestic ruminant populations and the knowledge of its prevalence (EFSA, 2010).

In Portugal, Q fever is a notifiable disease since 1999, and the average number of notifications is 0.10 cases per 10^5 inhabitants. However, these data might be underestimated. Between 2004 and 2005, 32 cases were diagnosed in the Centre for Vectors and Infectious Diseases at the National Health Institute but only 12 were notified, clearly suggesting an under-notification (Santos et al., 2007). Despite the zoonotic pattern of Q fever, the information about the occurrence of infection in animals is scarce. A few studies demonstrated the presence of bacteria in clinical samples from zoo animals and from ruminants (Clemente et al., 2008, 2009). Also, our previous results on screening bulk tank milk indicated the presence of *C. burnetii* in ruminant herds originated from different regions (Anastácio et al., 2012). A genotypic diversity among *C. burnetii* isolates from animals and human clinical samples was shown (Santos et al., 2012). These studies were based on a limited number of samples obtained from clinical cases. They highlighted the need of epidemiological study of *C. burnetii* in other geographical regions, increasing the number of samples randomly sampled. In this context, the present study aimed to understand the current status of small ruminants to the exposure of *C. burnetii* in the Center of Portugal. A cross-sectional study was designed to estimate the herd and the individual apparent prevalence of specific antibodies anti-*C. burnetii*.

2. Materials and methods

2.1. Study design and sampling approach

A cross-sectional survey was carried out during the 4th trimester of 2011 in small ruminant herds from the central region of Portugal.

The number of herds used in the study was calculated taken into account the regional census ($N = 1527$ small ruminant's herds), obtained from the Official Regional Veterinary Services. The sample size calculation was performed using the program WinEpiscope version 2.0 based on the formula $n = [t^2 P_{esp}(1 - P_{esp})]/d^2$, considering n the required size sample, t the student value for a 95% confidence level (1.96), P_{esp} the expected prevalence and d the desired absolute precision. Taking into account that the study population (N) was small ($n/N > 5\%$), the required sample size was adjusted by the formula $n_{adj} = (N*n)/(N + n)$ (Thrusfield, 1995).

It was considered an expected herd prevalence of 57% (Fernandes, 2008) a desired absolute precision of 10%, and a 95% confidence interval, resulting in an estimated sample of 89 herds. The list of total herds was used for a simple random sampling, using the program Microsoft Excel®.

In each herd, the sample size was calculated to detect the presence of infection using the WinEpiscope version 2.0 based on the formula $n = [1 - (1 - p_1)^{1/d}][N - d/2] + 1$ in which n is the required sample size, N is the population size, d is the minimal number of affected animals in the population and p_1 is the probability of finding at least one case in the sample (Thrusfield, 1995). For this purpose, the herd size was considered, the expected proportion of seropositive animals was established in 15% (Guatteo et al., 2011) and a 95% confidence level was considered. On farms sized $\leq$10 animals, samples were taken from all the animals. The list of animals in each herd was used for a simple random sampling using the program Microsoft Excel®.

Blood samples were collected from selected animals simultaneously undergoing statutory routine brucellosis testing (animals aged >6 months), by the veterinary practitioner group, in charge of the Official Sanitary Campaign. Individual apparent prevalence was calculated globally considering the total amount of samples. The serum obtained by centrifugation of blood samples was stored at $-20\,^{\circ}C$ until serological testing. A questionnaire was filled up on the surveyed herds by interviewing farmers, during sample collection.

Please cite this article in press as: Anastácio, S., et al., Serological evidence of exposure to *Coxiella burnetii* in sheep and goats in central Portugal. Vet. Microbiol. (2013), http://dx.doi.org/10.1016/j.vetmic.2013.08.004

2.2. Serological analyses

Sera were tested for the presence of specific antibodies anti-*C. burnetii* using an indirect commercial ELISA kit, LSIVET Ruminant Milk/Serum Q Fever® (LSI, France). Optical density (OD) values were measured at 450 nm. Sample/positive percentages (S/P percent) were calculated by the adjustment with the negative control, using the formula (OD sample − OD negative)/(OD positive − OD negative) × 100. The resulting S/P percent were divided in different classes, according to manufacturer's instructions: negative (Neg; S/P per cent ≤ 40), low positive (LP; 40 < S/P ≤ 100), positive (Pos; 100 < S/P ≤ 200), high positive (HP; 200 < S/P ≤ 300) and very high positive (VHP; S/P > 300).

2.3. Statistical analysis

For statistical analysis purposes, it was considered the herd size (continuous), species in the herds (categorical nominal: sheep, goats or mixed herds) or species individually (categorical nominal: sheep/goats), productive system (categorical nominal: intensive, extensive, semi-extensive), age (continuous), geographic distribution (categorical nominal: counties), co-habitation with other species (categorical nominal: yes/no), and reports of reproductive disorders within the previous year (i.e., at least one of the following disorders: abortion, premature delivery, infertility, metritis and/or placentary retention) (categorical nominal: presence/absence).

The response variables were the S/P percent (continuous) obtained in each individual serum by ELISA testing and its categorization in positive or negative (categorical nominal: positive/negative). So, herds were categorized as positive or negative, according to the results obtained for individual serum. A herd was considered positive when at least one serum showed a positive result to ELISA testing. The apparent prevalence of anti-*C. burnetii* antibodies was calculated at herd and at individual level. Statistical uncertainty was assessed by calculating the 95% confidence interval for each of the proportions according to the expression S.E. 95% C.I. = 1.96 $[p(1-p)/n]^{1/2}$ (Thrusfield, 1995) and using WinEpiscope version 2.0.

Statistical analyses were performed using SAS (version 9.1.2). Simple logistic regression test was performed to assess individually the main factors associated with *C. burnetii* seropositivity at herd and individual level. After evaluating these factors with significant influence ($p < 0.05$) on positive results, a multiple logistic regression analysis was conducted to assess the joint relationship between several independent factors and *C. burnetii* seropositivity. Also, a multiple logistic regression analysis was used to evaluate the combined effect of multiple variables in S/P percent (continuous) ($p < 0.05$).

3. Results

3.1. Descriptive analysis

Of all 1527 eligible herds, 89 (5.8%) were selected to this study. The mean herd size was 6.7 animals (SD = 11.305, range 1–104) and 46 herds (51.7%) had less than 4 animals.

Table 1
Descriptive characteristics and seroprevalence results in sheep and goat herds.

Variable	Frequency (*n*)	Seroprevalence (%)	CI 95%[a]
Selected	89	32.6	23.2–42.1
Herd size			
≤10	80	28.8	19.1–38.5
>10	9	66.7	36–97.4
Herd species			
Goat	52	28.8	16.7–40.1
Sheep	24	37.5	18.3–56.7
Mixed	13	38.4	12.2–64.8
Type of production			
Meat	79	34.2	24.1–45.8
Milk	3	33.3	1.8–87.5
Mixed	7	14.3	1–58
Productive system			
Intensive	0	0	na[b]
Extensive	0	0	na[b]
Semi-extensive	89	32.6	23.2–42.1
County			
Coimbra	58	32.8	21.4–46.5
Miranda do Corvo	10	30	8.1–64.6
Lousã	1	0	na[b]
Pencova	4	0	na[b]
Vila Nova de Poiares	16	43.8	20.8–69.5
Cohabitation with other species			
Yes	61	34.4	23–47.8
No	28	28.5	14–48.9
Cohabitant species			
Pets	7	28.6	5.1–69.7
Farm animals	30	36.7	20.6–56.1
Pets and farm animals	20	40	20–63.6
Reproductive disorders			
Yes	6	33.3	6–75.9
No	83	32.5	22.9–43.8

[a] Confidence interval (range within which is reasonably confident to find the real prevalence).
[b] Not applicable.

Goat herds were predominant ($n = 52$, 58.4%) followed by sheep herds ($n = 24$, 27%) and mixed herds ($n = 13$, 14.6%). It was also observed a predominance of meat producing herds ($n = 79$, 88.8%), a semi-extensive grazing system ($n = 89$, 100%) and herd localization at the county of Coimbra ($n = 58$, 65.2%). In these herds, 460 animals were sampled (mean age 45.6 months) (SD = 29.9, range 9–167).

Table 1 summarizes the descriptive characteristics and seroprevalence results of the ELISA test in herds. Global prevalence in herds was estimated on 32.6% (CI 95%: 23.1–42.1%). Herd prevalence was higher in mixed herds 38.5% (95% CI: 12–65%) and in sheep herds 37.5% (CI 95%: 21–54%) than in goat herds 28.8% (95% CI: 17–41%). Geographic distribution of positive herds showed a frequency of 32.8% (19/58) in Coimbra, 42.8% (7/16) in Vila Nova de Poiares, 30% (3/10) in Miranda do Corvo, 0% (0/1) in Lousã and 0% (0/4) in Penacova.

Co-habitation with other species was observed in 64% (57/89) of herds, and a positive result was obtained in 36.8% (21/57). Pets (dogs and/or cats), alone or together with farm animals, were reported in 47.4% (27/57) of

Please cite this article in press as: Anastácio, S., et al., Serological evidence of exposure to *Coxiella burnetii* in sheep and goats in central Portugal. Vet. Microbiol. (2013), http://dx.doi.org/10.1016/j.vetmic.2013.08.004

Table 2
Descriptive statistics of *C. burnetii* antibodies in sheep and goats individually.

Test Category	No. of animals	Mean age of animals (months)	Apparent prevalence	95% CI[a] (*p*)	Range of S/P[b]	Mean S/P value
Positive	44	50	0.096	[0.07;0.12]	41.5–185.9	75.6
Negative	416	44	0.904	[0.88;0.93]	0–38.82	4.2

[a] Confidence interval (range within which is reasonably confident to find the real prevalence).
[b] S/P – sample positive percentage.

herds, amongst which 37% (10/27) showed a positive result.

The occurrence of previous reproductive disorders was reported in 6.7% (6/89) of herds particularly abortion in 2.2% (2/89) and infertility in 4.5% (4/89).

In 27.6% (8/29) of positive herds at least one serum presented a high S/P per cent (>100), and in 31% (9/29) more than one serum was classified as positive (S/P per cent > 40).

Table 2 shows the descriptive statistic of results at individual level. Global individual seroprevalence was estimated on 9.6% (CI 95%: 6.9–12.2%), but considering the ruminants species, seroprevalence was estimated on 10.4% (CI 95%: 7.8–13%) in goats and 8.6% (CI 95%: 5.8–11.4%) in sheep. Mean age of positive animals was 50 months (SD 28.4, range 14–135), and 44 months for the negative animals (SD 30.2, range 9–167). S/P per cent ranged from 41.5 to 185.9 (mean 75.6, SD 34.07), and 18.2% (8/44) of samples were classified as positive ($100 < S/P \leq 200$), all of them from different herds.

3.2. Univariable analysis

Individual factors were tested to find associations with positive results in herds and in animals individually. The variable production system was not included as the reference. Categories were inexistent as almost all the herds had a semi-extensive grazing system. Univariable analysis identified three factors with significant effect on *C. burnetii* seropositivity at herd or animal levels. At the herd level, only the herd size evidenced an association with seropositivity ($p < 0.01$), using the logistic regression test (Fig. 1). Indeed, it was observed that all the herds with more than 14 (6.8%) animals were classified as positive. Individually, the logistic regression test evidenced an association between the increase of animal's age and seropositivity ($p < 0.01$). Also, it was observed that the probability of having a positive result is higher in goats than in sheep ($p < 0.05$), using the same statistic model.

3.3. Multivariable analysis

A multivariable model was performed to test simultaneously variables found to be associated in univariable analysis. A multiple logistic regression test confirmed that species and age were both associated with positive results ($p < 0.05$) (Fig. 2).

Also, a linear regression model tested the effect of multiple variables in S/P per cent. The age of the animal was the only factor evidencing an influence with S/P per cent ($p < 0.01$).

4. Discussion

Q fever is recognized as zoonotic disease worldwide with multiple animals acting as *C. burnetii* reservoirs. The present study was designed as an approach to evaluate the exposure of small ruminants to *C. burnetii* in the center of Portugal. A commercial ELISA test was used to detect IgG anti-*C. burnetii* (phase I and phase II).

Our results indicate a global herd prevalence of 32.6%, higher in mixed herds (38.4%) and in sheep herds (37.5%) than in goat herds (28.8%). These data are similar to those reported in sheep herds from Sardinia, Italy (38%) (Masala et al., 2004). However, higher values of seroprevalence (74%) were reported in sheep herds from Spain (Ruiz-Fons et al., 2010) and Turkey (83%) (Kennerman et al., 2010), while in Germany, sheep herd seroprevalence was shown to be lower (28%) (Hilbert et al., 2012). According the data

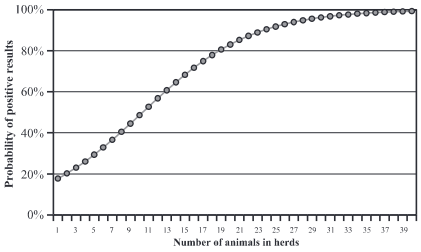


Fig. 1. The probability of a positive result for *C. burnetii* antibodies increases with the number of animals in herds (Intercept = −1.7009 ± 0.4459; β_1 = 0.1644 ± 0.0668).

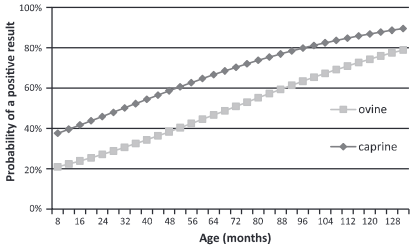


Fig. 2. The probability of positive results for *C. burnetii* antibodies increases with age, in each month, by species using a logistic regression model (Ovine: Intercept = −1.5037 ± 0.3204; Caprine: Intercept = −0.6783 ± 0.3552; β_1 = 0.0214 ± 0.0084).

Please cite this article in press as: Anastácio, S., et al., Serological evidence of exposure to *Coxiella burnetii* in sheep and goats in central Portugal. Vet. Microbiol. (2013), http://dx.doi.org/10.1016/j.vetmic.2013.08.004

from goat herds in other European countries, a higher seroprevalence was reported compared to this study, namely in The Netherlands (43.1%) (Schimmer et al., 2011), in Northern Ireland (42.9%) (McCaughey et al., 2010), in Spain (45%) (Ruiz-Fons et al., 2010) and in Sardinia, Italy (47%) (Masala et al., 2004). Among these studies, only Ruiz-Fons et al. (2010) and Schimmer et al. (2011) performed the serologic test with the same commercial ELISA used in our study. Overall, it can be suggested that the herd prevalence in our study was lower than the range of herd prevalence described in other European countries.

The global individual seroprevalence was 9.6%. Goats were significantly related with seropositivity at animal level ($p < 0.05$). Indeed, individual seroprevalence was slightly higher (10.4%) in goats than in sheep (8.6%). These results are similar to those obtained in other European seroprevalence studies such as Spain, Ireland, Greece and Sardinia, Italy, in which values ranged from 6.5% and 13% (Ruiz-Fons et al., 2010; McCaughey et al., 2010; Pape et al., 2009; Masala et al., 2004). A higher individual seroprevalence (17.2%) was reported in The Netherlands, in 2008, during the Q fever epidemic outbreak (van den Brom et al., 2012).

The increase of the age of the animal was associated with seropositive results ($p < 0.01$). This is consistent with the report from Schimmer and collaborators, in The Netherlands, where they also found an increase of seroprevalence with age (Schimmer et al., 2011). This finding suggests the occurrence of horizontal transmission among animals and the maintenance of infection within adult populations (García-Pérez et al., 2009; Ruiz-Fons et al., 2010; Astobiza et al., 2012). It may be explained by the increase rate of contagion as a consequence of a higher probability of contact during lifetime (Ruiz-Fons et al., 2010). Furthermore, an IgG based antibody test was used, thus possibly evidencing past exposure to *C. burnetii* (McCaughey et al., 2010). The presence of such antibodies cannot be associated exclusively to a current infection, since animals can remain seropositive for years after the acute infection have been resolved (McQuiston et al., 2002). The high mean age of animals in our study (3.9 years) might be related to regional cultural habits and the traditional consumption of meat from older animals. Indeed, most of the sampled animals came from meat production herds in a semi-extensive grazing system.

The long-time contact with *C. burnetii* in the surveyed herds together with the random selection of sampled herds and animals can explain the lack of association found between reproductive disorders and seropositivity (García-Pérez et al., 2009; Ruiz-Fons et al., 2010; Astobiza et al., 2012). However, the presence of an asymptomatic infection in herds cannot be excluded. In fact, our previous results showed the presence of specific antibodies (Anastácio et al., 2012) and DNA of *C. burnetii*, detected by qPCR (unpublished data), in bulk milk tank from dairy ruminant farms with reports of reproductive disorders. Indeed, an association between reproductive disorders and *C. burnetii* prevalence in ruminants has been reported in some studies (Cabassi et al., 2006; García-Pérez et al., 2009).

Despite the significant association between goats and positive results ($p < 0.05$), from an individual perspective, it was found a lower herd prevalence in goats than in sheep, which is in agreement with data from a study conducted in Northern Spain (Ruiz-Fons et al., 2010). The higher individual prevalence together with the lower herd prevalence may suggest that the within-herd prevalence is high in goats. Nevertheless, this could not be assessed in this study because sample size calculation in herds aimed the detection of infection, not the estimation of within-herd prevalence. Moreover, differences of prevalence between sheep and goats cannot be explained by different sampling periods in relation to the lambing season. Sample collection occurred in early pregnancy in both species and the reproductive cycle is similar among both species in this region.

The herd size was associated to seropositive results ($p < 0.01$), thus the probability of a positive result increases with the number of animals per herd. Other studies in goats (Schimmer et al., 2011, 2012) and in cattle (McCaughey et al., 2010) support our findings. The increased risk of introduction and/or transmission of pathogens in a large population is probably related with the increased number lambing females at lambing season (Woldehiwet, 2004) and by other management factors like larger amounts of feed, animal supply and a higher number of professionals working at or visiting the farm (Schimmer et al., 2011). Therefore, larger herds are more prone to acquire and develop Q fever, and the number of animals must be considered a risk factor to *C. burnetii* dissemination.

In conclusion, this study confirms the presence of specific anti-*C. burnetii* antibodies in goats and sheeps in Portugal. To the best of our knowledge, this is the first seroprevalence survey performed in small ruminants in this country. To clarify the infection status in these herds, namely the presence of an active infection, the shedding of bacteria must be assessed. Also, a better elucidation of the epidemiology of Q fever in Portugal requires the inclusion of other animal species from a large geographical area.

Conflict of interest statement

The authors declare no conflict of interests.

Acknowledgements

The authors would like to thank the collaboration of farmers.

Sofia Anastácio is supported by the grant SFRH/BD/77823/2011 given by Fundação para a Ciência e a Tecnologia (FCT), Lisbon, Portugal. This work was supported financially by the Center for Pharmaceutical Studies, University of Coimbra (CEF/UC), Portugal.

References

Anastácio, S., Pessoa, D., Pegado, J., Cruz, C., Sidi-Boumedine, K., da Silva, G., 2012. Investigation of *Coxiella burnetii* infection in dairy ruminant herds with reproductive disorders in two different regions of Portugal. Clin. Microbiol. Infect. 18, S524.

Angelaskis, E., Raoult, D., 2010. Q fever. Vet. Microbiol. 140, 297–309.

Please cite this article in press as: Anastácio, S., et al., Serological evidence of exposure to *Coxiella burnetii* in sheep and goats in central Portugal. Vet. Microbiol. (2013), http://dx.doi.org/10.1016/j.vetmic.2013.08.004

Arricau-Bouvery, N., Rodolakis, A., 2005. Is Q fever an emerging or re-emerging zoonosis? Vet. Res. 36, 327–349.

Arricau-Bouvery, N., Souriau, A., Lechopier, P., Rodolakis, A., 2003. Experimental *Coxiella burnetii* infection in pregnant goats: excretion routes. Vet. Res. 34, 423–433.

Astobiza, I., Ruiz-Fons, F., Piñero, A., Barandika, J.F., Hurtado, A., Garcia-Perez, A.L., 2012. Estimation of *Coxiella burnetii* prevalence in dairy cattle in intensive systems by serological and molecular analyses of bulk-tank milk samples. J. Dairy Sci. 95, 1632–1638.

Berri, M., Souriau, A., Crosby, M., Crochet, D., Lechopier, P., Rodolaskis, A., 2001. Relationships between the shedding of *Coxiella burnetii*, clinical signs and serological responses of 34 sheep. Vet. Rec. 148, 502–505.

Berri, M., Souriau, A., Crosby, M., Rodolaskis, A., 2002. Shedding of *Coxiella burnetii* in ewes in two pregnancies following an episode of *Coxiella* abortion in a sheep flock. Vet. Microbiol. 85, 55–60.

Berri, M., Rousset, E., Hechard, C., Champion, J.L., Dufour, P., Russo, P., Rodolaskis, A., 2005. Progression of Q Fever and *Coxiella burnetii* shedding in milk after an outbreak of enzootic abortion in a goat herd. Vet. Rec. 156, 548–549.

Cabassi, C.S., Taddei, S., Donofrio, G., Ghidini, F., Piancastelli, C., Flammini, C.F., Cavirani, S., 2006. Association between *Coxiella burnetii* seropositivity andabortion in dairy cattle of Northern Italy. New Microbiol. 29, 211–214.

Clemente, L., Fernandes, T.L., Barahona, M.J., Bernardino, R., Botelho, A., 2008. Confirmation by PCR of *Coxiella burnetii* infection in animals at a zoo in Lisbon, Portugal. Vet. Rec. 163, 221–222.

Clemente, M.L., Barahona, M.J., Andrade, M.F., Botelho, A., 2009. Diagnosis of *Coxiella burnetii* by PCR in aborted foetuses of domestic ruminants in Portugal. Vet. Rec. 164, 373–374.

ECDC (European Centre for Diasease Prevention and Control), 2010. Annual Epidemiological Report of Communicable Diseases in Europe. ECDC, Stockholm, pp. 181.

European Food Saftey Authority (EFSA) Panel on Animal Health and Welfare (AHAW), 2010. Scientific opinion on Q Fever. EFSA J. 8, 1595 (114 pp).

Fernandes, A.C.B., 2008. Rastreio serológico de Febre Q em ovinos no concelho de Montemor-o-Novo. (Serosurvey on Q fever in sheep at the county of Montemor-o-Novo).Faculdade de Medicina Veterinária. Universidade Técnica de Lisboa, Lisboa, pp. 108 (in Portuguese) (Tese de Dissertação de Mestrado em Saúde Pública Veterinária).

García-Pérez, A.L., Astobiza, I., Barandika, J.F., Atxaerandio, R., Hurtado, A., Juste, R.A., 2009. Investigation of *Coxiella burnetii* occurrence in dairy sheep flocks by bulk-tank milk analysis and antibody level determination. J. Dairy Sci. 92, 1581–1584.

Gilsdorf, A., Kroh, C., Grimm, S., Jensen, E., Wagner-Wiening, C., Alpers, K., 2008. Large Q fever outbreak due to sheep farming near residential areas. Germany, 2005. Epidemiol. Infect 136, 1084–1087.

Guatteo, R., Beaudau, F., Berri, M., Rodolaskis, A., Joly, A., Seegers, H., 2006. Shedding routes of *Coxiella burnetii* in dairy cows: implications for detection and control. Vet. Res. 37, 827–833.

Guatteo, R., Beaudeau, F., Joly, A., Seegers, H., 2007. *Coxiella burnetii* shedding by dairy cows. Vet. Res. 38, 849–860.

Guatteo, R., Seegers, H., Taurel, A.F., Joly, A., Beaudeau, F., 2011. Prevalence of *Coxiella burnetii* in domestic ruminants: a critical review. Vet. Microbiol. 149, 1–16.

Hilbert, A., Schmoock, G., Lenzko, H., Moog, U., Diller, R., Fröhlich, A., Hoffmann, L., Horner, S., Elschner, M., Tomaso, H., Henning, K., Neubauer, H., Sprague, L.D., 2012. Prevalence of *Coxiella burnetii* in clinically healthy German sheep flocks. BMC Res. Notes 5, 152.

Kennerman, E., Rousset, E., Golcu, E., Dufour, P., 2010. Seroprevalence of Q fever (coxiellosis) in sheep from the southern Marmara region, Turkey. Comp. Immunol. Microb. 33, 37–45.

Klaassen, C.H.W., Nabuurs-Franssen, M.H., Tilburg, J.J.H.C., Hamans, M.A.W.M., Horrevorts, A.M., 2009. Multigenotype Q Fever outbreak, The Netherlands. Emerg. Infect. Dis. 15, 613–614.

Masala, G., Porcu, R., Sanna, G., Chessa, G., Cillara, G., Chisu, V., Tola, S., 2004. Occurrence, distribution, and role in abortion of *Coxiella burnetii* in sheep and goats in Sardinia, Italy. Vet. Microbiol. 99, 301–305.

Maurin, M., Raoult, D., 1999. Q Fever. Clin. Microbiol. Rev. 12, 518–553.

McCaughey, C., Murray, L.J., McKenna, J.P., Menzies, F.D., McCullough, S.J., O'Neill, H.J., Wyatt, D.E., Cardwell, C.R., Coyle, P.V., 2010. *Coxiella burnetii* (Q fever) seroprevalence in cattle. Epidemiol. Infect. 138, 21–27.

McQuiston, J.H., Childs, J.E., Thompson, H.A., 2002. Q fever. JAVMA 221 (6) 796–799.

Medic, A., Dzelalija, B., Polic, V.P., Marjan, I.G., Turkovic, I., Gilic, V., 2005. Q fever epidemic among employees in a factory in the suburb of Zadar. Croatia. Croat. Med. J. 46, 315–319.

Panaiotov, S., Ciccozzi, M., Brankova, N., Levterova, V., Mitova-Tiholova, M., Amicosante, M., Rezza, G., Kantardjiev, T., 2009. An outbreak of Q fever in Bulgaria. Ann. Ist. Sup. Sanità 45, 83–86.

Pape, M., Bouzalas, E.G., Koptopoulos, G.S., Mandraveli, K., Arvanitidou-Vagiona, M., Nikolaidis, P., Alexiou-Daniel, S., 2009. The serological prevalence of *Coxiella burnetii* antibodies in sheep and goats in northern Greece. Clin. Microbiol. Infect. 15, 146–147.

Porten, K., Rissland, J., Tigges, A., Broll, S., Hopp, W., Lunemann, M., Van Treeck, U., Kimmig, P., Brockmann, S.O., Wagner-Wiening, C., Hellenbrand, W., Buchholz, U., 2006. A superspreading ewe infects hundreds with Q fever at a farmers' market in Germany. BMC Infect. Dis. 6, 147.

Rodolaskis, A., 2006. Q fever, state of art: Epidemiology, diagnosis and prophylaxis. Small Ruminant Res. 62, 121–124.

Roest, H.I.J., Ruuls, R.C., Tilburg, J.J.H.C., Nabuurs-Franssen, M.H., Klaassen, C.H.W., Vellema, P., van den Brom, R., Dercksen, D., Wouda, W., Spierenburg, M.A.H., van der Spek, A.N., Buijs, R., de Boer, A.G., Willemsen, P.T.J., van Zijderveld, F.G., 2011a. Molecular Epidemiology of *Coxiella burnetii* from ruminants in Q fever outbreak, the Netherlands. Emerg. Infect. Dis. 17, 668–675.

Roest, H.I.J., Tilburg, J.J.H.C., van der Hoek, W., Vellema, P., van Zijderveld, F.G., Klaassen, C.H.W., Raoult, D., 2011b. The Q fever epidemic in The Netherlands: history, onset, response and reflection. Epidemiol. Infect. 139, 1–12.

Rousset, E., Sidi-Boumedine, K., Thiery, R., 2010. Chapter 2.1.1.2 – Q fever. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Mammals, Birds and Bees). 7th ed. OIE. http://www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/

Ruiz-Fons, F., Astobiza, I., Barandika, J.F., Hurtado, A., Atxaerandio, R., Juste, R.A., Garcia-Perez, A.L., 2010. Seroepidemiological study of Q fever in domestic ruminants in semi-extensive grazing systems. BMC Vet. Res. 6, 3.

Saegerman, C., Speybroeck, N., Dal Pozzo, F., Czaplicki, G., 2013. Clinical indicators of exposure to *Coxiella burnetii* in dairy herds. Transbound. Emerg. Dis., http://dx.doi.org/10.1111/tbed.12070.

Santos, A.S., Bacellar, F., França, A., 2007. Q fever: a revision of concepts. Med. Int. 14, 90–99 (in Portuguese).

Santos, A.S., Tilburg, J.J.H.C., Botelho, A., Barahona, M.J., Núncio, M.S., Nabuurs-Franssen, M.H., Klaassen, C.H.W., 2012. Genotypic diversity of clinical *Coxiella burnetii* isolates from Portugal based on MST and MLVA typing. Int. J. Med. Microbiol. 302, 253–256.

Schimmer, B., Schegget, R., Wegdam, M., Züchner, L., Bruin, A., Schneeberger, P.M., Veenstra, T., Vellema, P., van der Hoek, W., 2010. The use of a geographic information system to identify a dairy goat farm as the most likely source of an urban Q-fever outbreak. BMC Infect. Dis. 10, 69.

Schimmer, B., Luttikholt, S., Hautvast, J.L.A., Graat, E.A.M., Vellema, P., van Duynhoven, Y.T.H.P., 2011. Seroprevalence and risk factors of Q fever in goats on commercial dairy goat farms in the Netherlands, 2009–2010. BMC Vet. Res. 7, 81.

Schimmer, B., Lenferink, A., Schneeberger, P., Aangenend, H., Vellema, P., Hautvast, J., van Duynhoven, Y., 2012. Seroprevalence and risk factors for *Coxiella burnetii* (Q fever) seropositivity in dairy goat farmers' households in The Netherlands, 2009–2010. PLoS ONE 7 (7) e42364, http://dx.doi.org/10.1371/journal.pone.0042364.

Thrusfield, M., 1995. Veterinary Epidemiology, 2nd ed. Blackwell Science Ltd., Oxford, pp. 178–198.

To, H., Htwe, K.K., Kako, N., Kim, H.J., Yamaguchi, T., Fukushi, H., Hirai, K., 1998. Prevalence of *Coxiella burnetii* infection in dairy cattle with reproductive disorders. J. Vet. Med. Sci. 60 (7) 859–861.

van Asseldonk, M.A., Prins, J., Bergevoet, R.H., 2013. Economic assessment of Q fever in the Netherlands. Prev. Vet. Med.. (in press).

van den Brom, R., van Engelen, E., Luttikholt, S., Moll, L., van Maanen, K., Vellema, P., 2012. *Coxiella burnetii* in bulk tank milk samples from dairy goat and sheep farms in The Netherlands. Vet. Rec. 170, 310.

van der Hoek, W., Dijkstra, F., Schimmer, B., Schneeberger, P.M., Vellema, P., Wijkmans, C., ter Schegget, R., Hackert, V., van Duynhoven, Y., 2010. Q fever in the Netherlands: an update on the epidemiology and control measures. Euro Surveill. 15 (12) . http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19520.

van Loenhout, J.A.F., Paget, W.J., Vercoulen, J.H., Wijkmans, C.J., Hautvast, J.L.A., van der Velden, K., 2012. Assessing the long-term health impact of Q-fever in the Netherlands: a prospective cohort study started in 2007 on the largest documented Q-fever outbreak to date. BMC Infect. Dis. 12, 280.

Woldehiwet, Z., 2004. Q fever (Coxiellosis): epidemiology and pathogenesis. Res. Vet. Sci. 77, 93–100.

Please cite this article in press as: Anastácio, S., et al., Serological evidence of exposure to *Coxiella burnetii* in sheep and goats in central Portugal. Vet. Microbiol. (2013), http://dx.doi.org/10.1016/j.vetmic.2013.08.004

Table: Clinical and laboratory features, treatment modalities and outcomes of infective endocarditis cases

Patient no.	Age	Sex	Site of infection	ECHO findings	Isolate on BC	DA MIC (mg/l)(E-test)	Initial a.b.	Reason for switch from initial a.b. to DA	Surgery	Result
1	38	M	AV	1.2*0.4 cm veg	S. mutans	0.5	VA+gen(7d.)	VA-renal insufficiency	-	↑
2	64	M	MV	1.1*1.3 cm veg	S. mutans	0.5	VA+gen (4d)	VA-susceptibility	-	↑
3	62	F	MV	1*1.3 cm veg	MRSA	0.75	DA (4d)	-	-	↑
4	72	M	Lead tip	0.6 cm veg	MRSA	0.75	S (4d)	MRSA	-	↑
5	68	F	MV	Torn anter.SEK 0.4cm (ventricle)	MRSA	0.25	VA+gen+CRO (7d)	Renal failure	-	↑
6	60	M	MV	1.6*0.8 cm vegetation	MRSA	0.5	VA+gen (7d)	Renal failure	-	↑
7	71	M	Left ventricle mass	0.9*0.5 cm mass	MRSA	0.5	DA (42d)	-	-	↑
8	54	M	TV	1.5*0.8 cm veg	MRSA	0.25	VA+gen+CRO (7d)	DAT	-	↑
9	67	M	MV	0.9*0.5 cm mass	S.mutans	1.0	Pen-G (10d)	Pen-R	-	↑
10	77	F	AV	Iron (struc. MR) Dense calcification - on AV	MRSA	0.5	TEC (7d)	Clinical failure	-	↑
11	70	F	MV	0.6*0.3 cm veg	MRSA	0.5	TEC (6d)	Clinical failure	-	↑
12	68	F	MV	1*0.5cm	MRSA	0.5	DA (42d)	-	-	↑
13	65	M	AV	1*0.7cm	MRSA	0.5	DA (42d)	-	-	↑
14	75	F	MV	0.6*0.3 cm	MRSA	0.5	S (3d)	MRSA	-	↑
15	73	M	MV	1.5*0.6 cm	MRSA	0.5	DA (7d)	-	-	↑

VA: vancomycin, DA: daptomycin, CRO: ceftriaxon, S: cefazolin
Pen-G: penicillin-G, gen: gentamicin, a.b: antibiotic, R: resistance, DAT: outpatient antibiotic treatment
AV: aortic valve, MV: mitral valve, TV: tricuspic valve, ECHO: echocardiogram, SEK:postoperative ECHO centrer
s.mutans: streptococcus mutans, mrsa+: mrsa+ eucaryone staphylococcus aureus
MRSA: Meticillin resistant Staphylococcus aureus, MIC : minimum inhibitory concentration
E: infective endocarditis, d: day, veg: vegetation, BC : blood culture
M: male, F: female, ↑: Success, ↓Exitus

minimal rises in serum CPK levels during treatment, these values returning to normal after treatment. Clinical and laboratory features, treatment modalites and outcomes of infective endocarditis cases.

Conclusion: Daptomycin can be used successfully in both complicated left heart endocarditis and right heart endocarditis, with no significant side-effects. Studies involving wider patient series are now needed to support the use of daptomycin in left heart endocarditis.

P1849 Investigation of *Coxiella burnetii* infection in dairy ruminant herds with reproductive disorders in two different regions of Portugal

S. Anastácio, D. Pessoa, J. Pegado, C. Cruz, K. Sidi-Boumedine, G. Da Silva (Coimbra, PT; Sophia Antipolis, FR)*

Objectives: Recently, an increased number of Q fever outbreaks in some European countries warned the health authorities for the need of knowing its real prevalence in Europe. In Portugal, little is known about the epidemiology of Q fever. The aim of this study was to determine the infection rate and the geographical distribution of *Coxiella burnetii* antibody positive ruminant herds in two different regions from Portugal.

Methods: A cross-sectional study was performed on bulk tank milk samples (BTMs) from dairy herds with clinical reports of abortion or other reproductive disorders within 3 months before sample collection. In the first semester of 2010, BTMs were collected from dairy cattle herds in the Northwest region (n = 27) and, between November 2010 and May 2011, BTMs were collected in the Center region from dairy cattle herds (n = 20) and from small ruminant dairy herds (n = 27). All the samples were tested for the presence of anti-*C. burnetii* antibodies by the Enzyme-Linked Immunosorbent Assay, using the LSIVET Ruminant Milk/Serum Q Fever® (LSI; Lissieu, France). Laboratory results were used to determine the infection rate at regional level and by species.

Results: Test positive herds were detected in both regions. Considering the Northwest region, a positive result was obtained in 15 (55.5%) cattle herds. In the center region, a positive result was obtained in 10 (50%) cattle herds and in 11 (40.7%) small ruminant herds. Positive titers (S/P) ranged from 34 to 115. In the Northwest region only one (3.7%) herd showed an antibody titer higher than 100. In the center region five (25%) cattle herds and two (7.4%) small ruminant herds showed an antibody titer higher than 100.

Conclusions: To our knowledge, this is the first report of the occurrence of *C. burnetii* infection in ruminants in these regions of Portugal. This study demonstrates that infection by *C. burnetii* is important in Portugal. It is crucial to know the real prevalence in ruminants and to investigate the involvement of other species, in order to develop a control program for *C. burnetii*, which does not exist in Portugal, to reduce the economic impact at herd level and the risk for public health.

P1850 Empirical antibiotic treatment and 3–30 day mortality in hospitalised patients with monomicrobial bacteraemia. A prospective population-based cohort study

U.S. Jensen, J.D. Knudsen, H.C. Schønheyder, C. Østergaard, M. Arpi, M. Søgaard, K.O. Gradel (Copenhagen, Aalborg, Odense, DK)*

Objectives: In few countries bacteraemia (Ba) is a reportable infection unless the causative agent is subject to national surveillance. Appropriate empirical antibiotic treatment (EAT) is an important clinical goal in order to limit mortality in Ba patients. However, population-based data on appropriateness of EAT and mortality are rare. Therefore, we investigated EAT and 3–30 day mortality in a prospective semi-national Danish cohort.

Methods: We included all hospitalized patients with incident Ba 2007–2008 from a collaborative network in Denmark with prospective registration of Ba in a population of ~1.7 mill. inhabitants. Incident Ba was defined as a clinical episode with ≥1 positive blood cultures deemed clinically significant without a prior episode in the preceding 365 days. EAT was defined as the antibiotic treatment given at 1st notification. EAT was recorded as appropriate if given intravenously (except fluoroquinolones, fluconazole and metronidazole) and if the blood isolate/s were susceptible to ≥1 of the antibiotics given. The primary outcome was mortality from day 3 to day 30 after the blood draw; the 2-day interval accounted for the delay of culture reports and precipitous death from sepsis unrelated to antibiotic therapy.

Results: We found 6834 patients with Ba. Of these, 4653 had monomicrobial Ba and a record af EAT and were alive at day 3. *E. coli*

Table. 3-30 day mortality of patients with monomicrobial incident bacteraemia by empirical antibiotic treatment status

Department of Clinical Microbiology	Empirical antibiotic treatment (EAT)		3–30 day mortality	
	Appropriate	P value	Appropriate EAT	P value
Escherichia coli	**N (%)**		**N (%)**	
Aalborg	382 (79.1)		28 (7.3)	
Herlev	482 (86.8)	0.001	61 (12.7)	0.02
Hvidovre	512 (86.2)		65 (12.7)	
Klebsiella pneumoniae	**N (%)**		**N (%)**	
Aalborg	68 (80.0)		9 (13.2)	
Herlev	75 (90.4)	0.14	11 (14.7)	0.43
Hvidovre	88 (87.1)		18 (20.5)	
Pseudomonas aeruginosa	**N (%)**		**N (%)**	
Aalborg	25 (51.0)		3 (12.0)	
Region	26 (66.7)	0.25	4 (14.5)	0.90
Hvidovre	26 (65.0)		3 (11.6)	
Anaerobe bacteria	**N (%)**		**N (%)**	
Aalborg	32(66.7)		4 (12.5)	
Herlev	38 (67.9)	0.77	4 (10.5)	0.37
Hvidovre	32 (61.5)		7 (21.9)	
Staphylococcus aureus	**N (%)**		**N (%)**	
Aalborg	145 (76.3)		28 (19.3)	
Herlev	206 (88.0)	<0.001	35 (17.0)	0.17
Hvidovre	161 (89.9)		40 (24.8)	
Streptococcus pneumoniae	**N (%)**		**N (%)**	
Aalborg	138 (90.8)		16 (11.6)	
Herlev	160 (92.0)	0.30	16 (10.0)	0.83
Hvidovre	157 (95.2)		19 (12.1)	
Enterococccus faecalis	**N (%)**		**N (%)**	
Aalborg	15 (37.5)		4 (26.7)	
Herlev	17 (23.3)	0.008	5 (29.4)	0.98
Hvidovre	4 (9.1)		1 (25.0)	
Enterococcus faecium	**N (%)**		**N (%)**	
Aalborg	3 (25.0)		0	
Herlev	4 (16.0)	0.57	2 (50.0)	
Hvidovre	2 (10.5)		0	

Chi2 was calculated to determine statistical significance (p<0.05).

Specific recommendations were formulated for each risk factor in order to prevent transmission.

19.093 Brucellosis in Cameroon: Seroprevalence and risk factors in beef-type cattle

B. Ojong[1], E. Macleod[2], M. L. Ndip[3], **A. Zuliani**[4], E. Piasentier[4]
[1]Société de Développement et d'Exploitation des Productions Animales (SODEPA), Yaoundé, Cameroon, [2]University of Edinburgh, Edinburgh, United Kingdom, [3]University of Buea, Buea, Cameroon, [4]University of Udine, Udine, Italy

Purpose: Bovine brucellosis is a well-known zoonosis which also has profound negative impacts on cattle productivity and on international trade. In this study we aimed at investigating seroprevalence and possible risk factors associated to seropositivity of bovine brucellosis in two agro-pastoral ecologic zones of Cameroon: the Western Highland Plateau Savannah (WHPS) and the Guinea Highland Savannah (GHS) as they encompass the most productive regions in the country in terms of cattle production.
Methods & Materials: The subdivision and village herds were considered as clusters for a two-stage cluster sampling procedure. To enable us to sample animals by probability proportional to size, cattle population of each village (cluster) was noted against the corresponding village. Each beef-type cattle in Cameroon was assigned to a single cluster (village). Using C-survey 2.0 a final list of villages to be visited was established. Blood samples were collected and screened using the Rose Bengal Plate Test. A field questionnaire was designed and distributed to herd managers during sampling to collect information on intrinsic risk factors (age, sex and breed) and extrinsic risk factors (ecological zone, herd size, herd management system, third trimester abortion, interaction with wildlife, and interaction with sheep and goats during grazing) that can affect Brucella sero-status of the target population. Data generated from the field questionnaire were tested for significance against seropositivity using ANOVA and Chi-Square in R-Software®.
Results: The results of this work confirm that bovine brucellosis is endemic in Cameroon. With an uneven but wide distribution the study reve