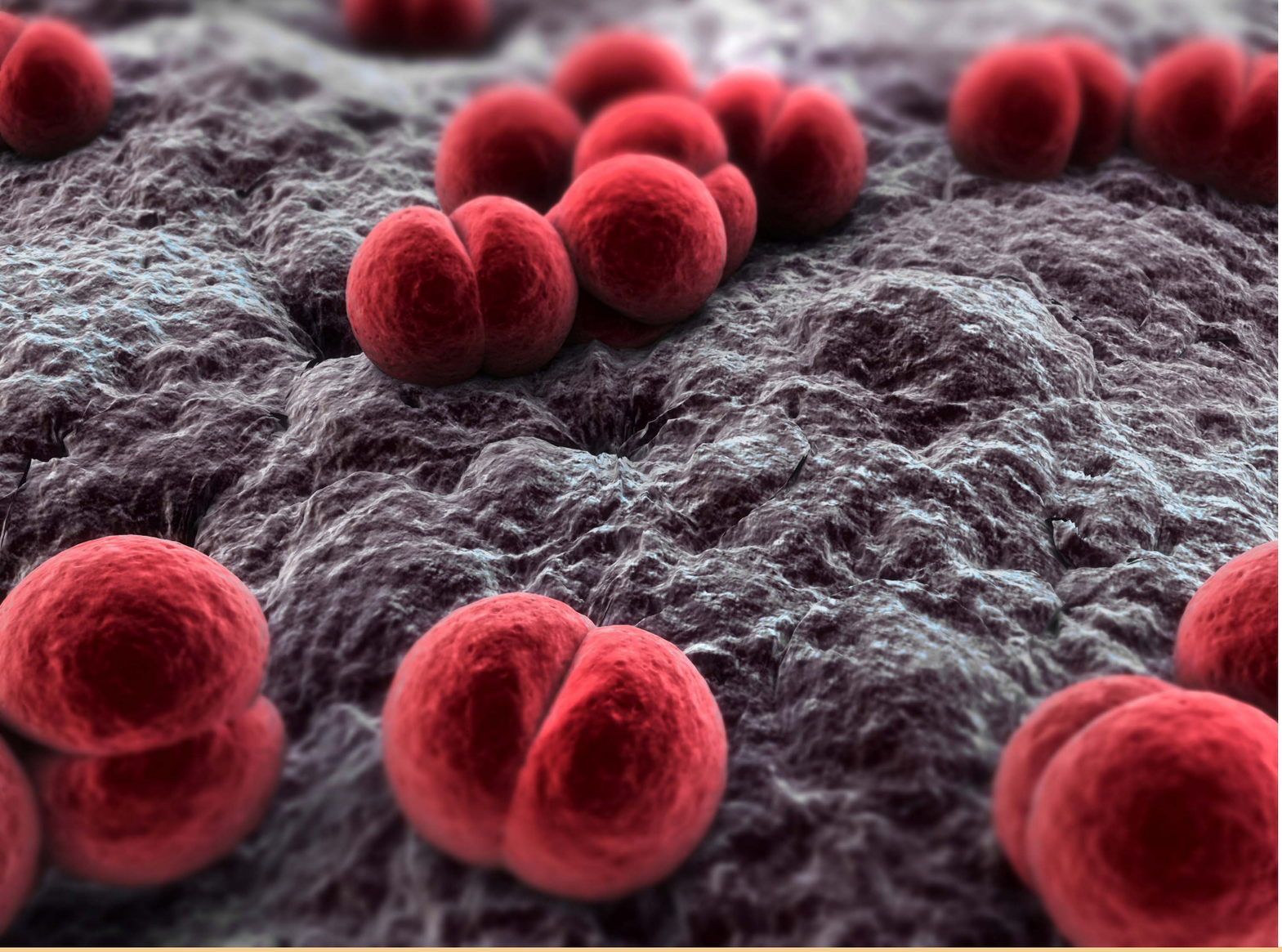

Jessica Aguiar Neves

Establishment of a PCR based method for detection of Neisseria meningitidis DNA DUS as a marker for meningococcal carriage

Monografia realizada no âmbito da unidade de Estágio Curricular do Mestrado Integrado em Ciências Farmacêuticas, orientada pela Professora Doutora Sara Margarida dos Santos Domingues e apresentada à Faculdade de Farmácia da Universidade de Coimbra

Setembro 2016

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Coimbra, 16 de setembro de 2016.

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O tutor da Monografia

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(Professora Doutora Sara Margarida dos Santos Domingues)

A aluna

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(Jessica Aguiar Neves)

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Ever tried. Ever failed. No matter. Try again. Fail again. Fail better.

(Samuel Beckett)

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Abstract

Neisseria meningitidis is the main causative agent of meningitis and septicaemia. This feared pathogen has been the focus of intense studies since the end of the 19th century. Although meningococcal disease is a life-threatening illness, *N. meningitidis* is a strict human commensal that commonly colonizes the throat's epithelial lining without causing any detectable symptoms. This phenomenon, commonly referred to as carriage, represents a successful commensal relationship between the host and the bacterium, with the host experiencing no detectable pathology. During periods of endemic disease, about 10% of the general population harbour *N. meningitidis* in the nasopharynx.

In all sexual organisms, adaptations exist that secure the safe re-assortment of homologous alleles and prevent the intrusion of hazardous alien DNA. In the human pathogen *N. meningitidis*, transformation by exogenous DNA is regulated by the presence of a specific DNA Uptake Sequence (DUS), which is present in thousands of copies in the respective genomes. DUS affects transformation by limiting DNA uptake and recombination in favour of homologous DNA.

Based on our comprehensive characterization of DUS in *N. meningitidis,* this work has been developed with the aim of establish a rapid and cost-effective procedure to detect *N. meningitids* DNA, using the DUS as the target. Several protocols of polymerase chain reaction (PCR) have been performed in order to achieve this goal. After a few attempts we found the ideal conditions to use DUS as a marker of the presence of *N. meningitidis* genome. This tool was designed with the future aim of further characterization of the carriage rates of *N. meningitidis* and other *Neisseria sp.* in Norwegian military recruits.

Key-words: *Neisseria meningitidis,* meningococcal carriage, meningitis, DNA Uptake Sequence.

Resumo

Neisseria meningitidis é o principal agente causador de meningite e septicémia. Este temido agente patogénico tem sido alvo de um intenso estudo desde o fim do século XIX. Apesar da meningite ser uma doença que põe em risco a vida, *N. meningitidis* é uma bactéria que pertence à flora comensal humana e que normalmente coloniza o epitélio nasofaríngeo, sem causar qualquer tipo de sintomas. Este fenômeno, normalmente referido como transporte, representa uma relação comensal de sucesso entre o hospedeiro e a bactéria, sendo que o hospedeiro não apresenta qualquer tipo de patologia. Durante períodos de doença endémica, estima-se que cerca de 10% da população esteja colonizada com *N. meningitidis*.

Em todos os organismos sexuais existem adaptações que promovem o rearranjo de alelos homólogos de forma segura, evitando a intrusão de ADN alienígeno e potencialmente perigoso. Em particular na bactéria *N. meningitidis*, a transformação por ADN exógeno é regulada pela presença de uma sequência específica (DNA *Uptake sequence* - DUS), que está presente em milhares de cópias nos respectivos genomas. Esta sequência afeta a transformação, limitando o *uptake* e recombinação de ADN.

Tendo em conta o nosso conhecimento acerca da sequência DUS em *N. meningitidis*, este trabalho foi desenvolvido com o objetivo de estabelecer um protocolo rápido, eficaz e de baixo custo, que permitisse detetar ADN de *N. meningitidis*, usando a sequência DUS como alvo. Diversos protocolos utilizando a técnica de reação em cadeia da polimerase (PCR) foram testados de forma a atingir esse objetivo. Após várias tentativas, conseguimos encontrar as condições reacionais ideais para a utilização da sequência DUS como um marcador da presença de genoma de *N. meningitidis*. Esta ferramenta foi desenvolvida com o objetivo de no futuro ser possível a caracterização das taxas de transporte de *N. meningitidis* e de outras bactérias do género *Neisseria* em militares Noruegueses.

Palavras-chave: *Neisseria meningitidis*, transporte de meningococos, meningite, *DNA Uptake Sequence.*

Abbreviations

bp – base pairs

CPS – Capsular polysaccharides

CSF – Cerebrospinal fluid

CTAB – Hexadecyltrimethylammonium bromide

dNTPS – Deoxyribonucleotides

DUS – DNA Uptake Sequence

fHbp – Factor H binding protein

HGT – Horizontal gene transfer

LOS – Lipooligosaccharides

Mb – Mega base pairs

MC 58 – *Neisseria meningitidis* strain 58

MC Z1099 – *Neisseria meningitidis* strain Z1099

NadA – Neisserial adhesin A

NHBA – Neisserial heparin binding antigen

TFP – Type IV pili

USS – Uptake Signal Sequence

1. Introduction

The *Neisseriaceae* family is constituted by Gram-negative cocci, harbouring many unique species that are part of the human flora. Among those species is *Neisseria meningitidis,* a feared pathogen which is frequently carried in healthy individuals, residing benignly in the respiratory tract (Davies and Kahler, 2014). Although it usually inhabits as part of the commensal flora, it can traverse from this nonpathogenic carrier state and disseminate into the blood stream (Davidsen *et al.*, 2007).

Despite advances in preventive and therapeutic medicine, meningococcal disease (meningitis and/or septicaemia) is the cause of significant morbidity and mortality in industrialized and developing countries worldwide. In this way, the application of insights gained from studies of meningococcal population biology and evolution is important in understanding the spread of disease, as well as in vaccine development and implementation (Caugant and Maiden, 2009).

Thus, this work represents a contribution to the continuous study of this microorganism.

2. Background

2.1 *Neisseria meningitidis*

2.1.1 Morphology and general features

Neisseria meningitidis, the meningococcus, is a Gram-negative bacterium with a coccoid shape. In particular they can be coffee bean shaped (Kayser *et al.*, 2005) or kidney shaped (Nester *et al.*, 2009). These diplococci are pathogenic members of the class β-proteobacteria, belonging to the *Neisseriaceae* family and to the genus *Neisseria*. Regarding the mobility they are classified as nonmotile since they don't have flagella, which primary role is locomotion. In addition these bacteria have long hair-like structures that extend from bacterial surface: type IV pili (TFP) (Yazdankhah and Caugant, 2004). Beyond their role in adherence, TFP also have an important function in twitching motility (Benam *et al.*, 2011). The sole ecological niche of *N. meningitidis* is the nasopharyngeal mucosa of humans (Yazdankhah and Caugant, 2004), so they are typically aerobes. However, they can grow anaerobically if a suitable terminal electron acceptor such as nitrite is present (Nester *et al.*, 2009). Metabolically, these bacteria are classified as oxidase and catalase positive. They can also produce acid from fermentation of glucose and maltose (Kayser *et al.*, 2005). *N. meningitidis* grows on media containing blood such as blood agar plate or chocolate agar plate. *N. meningitidis* is a nutritionally fastidious microorganism, with optimal growth at 35 °C - 37 °C. A concentration of 5-10% Carbon Dioxide (CO_2) encourages proliferation (Kayser *et al.*, 2005).

2.1.2 Meningococcal carriage

Although meningococcal disease is a life-threatening illness, *N. meningitidis* is a strict human commensal that commonly colonizes the throat's epithelial lining without causing any detectable symptoms (Caugant, 1998). This phenomenon, commonly referred to as carriage, represents a successful commensal relationship between the host and the bacterium, with the host experiencing no detectable pathology. On the other hand, disease represents a failed or dysfunctional relationship between the two species (Caugant and Maiden, 2009). Since *N. meningitidis* is an obligatory human pathogen and most patients have not been in contact with other cases, asymptomatic carriers are presumably the major source of the pathogenic strains. Despite this fact, carriage can be an immunizing process resulting in

systemic protective antibody responses (Yazdankhah and Caugant, 2004). Mucosal immunity in carriers can be detected by increased concentration of IgA in saliva. Even though mucosal immunity is not sufficient to prevent further colonization of the nasopharynx, it may play an important role in prevision of the invasion of epithelial cells (Caugant *et al*., 2007).

During periods of endemic disease, about 10% of the general population harbour *N. meningitidis* in the nasopharynx (Yazdankhah and Caugant, 2004). It's believed that microorganisms that share the same ecological niche as *N. meningitidis* form biofilms – matrix-enclosed populations of bacteria adherent to each other and/or surfaces. These biofilms can confer a number of advantages to the bacteria, including increased resistance to antimicrobial agents and host immune responses (O'Dwyer *et al.*, 2009). Co-colonization with other pathogenic and non-pathogenic bacteria may lead to genetic exchange, which may result in the emergence of new meningococcal clones (Yazdankhah and Caugant, 2004).

The bacterium is transmitted from person to person by contact with upper respiratory secretions of nasopharyngeal carriers (Yazdankhah and Caugant, 2004) or when respiratory droplets from an infected, but asymptomatic, person are spread to susceptible individuals (Caugant *et al*., 2007). Although often protected by a polysaccharide capsule, meningococci are particularly susceptible to drying. Thus, spread requires close contact. In this way, it is easy to understand why in closed or semi-closed populations, such as military recruit camps, kindergartens or university dormitories, transmission increases and carriage rate may approach 100% (Caugant *et al.*, 2007; Caugant and Maiden, 2009). In other words, the highest carriage prevalence is among young people occupying a confined space. Physical exhaustion may also influence the immune defence unfavourably.

Asymptomatic carriage is an age-dependent phenomenon, with point prevalence carriage rates ranging from 10 to 35% in young adults. Studies performed in Europe and North America have shown that carriage rates are very low in the first years of life, and then suddenly increase in teenagers, reaching a maximum in those aged between 20 and 24 years. Carriage rates in older ages are lower than 10% (Caugant and Maiden, 2009). However, there are other factors that increase the risk of being a meningococcal carrier such as: male gender (there are slightly more carriers in males than females); coincident respiratory tract infections of viral or bacterial origin; and smoking, passive as well as active. In addition, people with low socio-economic status are more likely to be carriers. Finally, the number and closeness of social contacts is one of the most important factors (Caugant *et al.*, 2007; Caugant and Maiden, 2009). Some studies have provided information on factors which are assumed to be related to carriage rates (Yazdankhah and Caugant, 2004). The role of these

factors was confirmed by a study in a randomly sampled population in Norway. The aim of this study was to estimate the extend of meningococcal carriage (Caugant *et al.*, 1994). It was found that age between 15 and 24, male sex and active and passive smoking were associated with meningococcal carriage (Yazdankhah and Caugant, 2004).

Knowledge regarding the duration of carriage is limited because most longitudinal studies of carriage have used only phenotypic techniques for strain characterization and many carried isolates are both no-serogroupable and non-serotypable (Caugant *et al.*, 2007). Studies in military recruits have demonstrated that the commensal association of particular clones with a host is a long term relationship, with 90% of the carriers keeping the same clone for at least 5-6 months (Caugant *et al.*, 1992).

2.1.3 Meningococci virulence factors

The ability of meningococci to cause disease in its exclusive human host is correlated with adherence to mucosal epithelial cells in the nasopharynx (Alfsnes, 2012), utilization of the local available nutrients and evasion of the human immune system (Yazdankhah and Caugant, 2004). After invading subepithelial tissues and the blood stream, it may cross the blood-brain barrier and enter the meninges, causing septicemia and meningitis, respectively.

The primary meningococci virulence factors are the capsular polysaccharides (CPS) and TFP. The CPS are the outermost antigens on the meningococcal surface (Yazdankhah and Caugant, 2004) and they can prevent activation of the alternate complement pathway (Alfsnes, 2012). On the other hand TFP facilitate primary adherence to the epithelial cell surfaces (Yazdankhah and Caugant, 2004). These structures are crucial to the niche specialization of the meningococcus because they mediate tropism specially for human respiratory tract epithelia (Tettelin *et al.*, 2000). The lipooligosaccharides (LOS) are a major virulence factor, which induce the production of pro-inflammatory mediators leading to septic shock (Yazdankhah and Caugant, 2004) and the opacity proteins are involved in the adhesion to the host cells (Alfsnes, 2012). Two outer membrane proteins, the porins PorA and PorB are also linked to virulence. PorA negatively regulates the complement response while PorB facilitates attachment and invasion of host cells (Alfsnes, 2012). Finally, IgA protease and factor H binding protein (fHbp) are factors promoting survival and pathogenicity, where the former has the capability to cleave human IgA and the latter is capable of binding to human transferrin and lactoferrin. These are assumed to be sources of

iron, a basic component essential for growth of meningococci (Yazdankhah and Caugant, 2004).

2.1.4 Meningococcal disease: Meningitis

As mentioned before, *N. meningitidis* is one of the main causative agents of meningitis and severe sepsis with an often fatal outcome. This bacteria may also cause, more rarely, other diseases such as septic arthritis, pneumonia, purulent pericarditis, conjutivitis, otitis, sinusitis and urethritis (Yazdankhah and Caugant, 2004). The majority of individuals will, at one time or another throughout life, harbour the bacterium asymptomaticaly in the throat (Caugant *et al.*, 2007). The mechanisms that allow some meningococcal strains to disseminate from their local niche and cause systemic disease are not very clear yet. Most cases of meningococcal disease are caused by hyperinvasive lineages. These lineages are underrepresented in healthy carriers, and significant numbers of individuals are colonized with strains that rarely cause disease (Benam *et al.*, 2011). The high diversity of meningococcal carrier strains, compared with hypervirulent strains, supports the idea that transmissibility, rather than invasion, is essential in the life cycle of *N. meningitidis* (Yazdankhah and Caugant, 2004).

Occasionally, shortly after the colonization, and usually less than 10 days from first exposure, meningococcus can pass through the epithelial cells and enter the blood stream (Caugant and Maiden, 2009). The blood carries the microorganisms to the meninges and to CSF. There they multiply faster than they can be engulfed and destroyed by the polymorphonuclear neutrophils that enter the fluid in response to the infection. The inflammatory response leads to obstruction of the normal outflow of CSF, causing the brain to be squeezed against the skull by the build-up of internal pressure. *N. meningitidis* circulating in the bloodstream releases endotoxin, causing a drop in blood pressure that can lead to shock. The initial symptoms of meningitis are not especially alarming: mild cold followed by headache, fever, pain, stiff neck and back. Usually these symptoms are followed by nausea, vomiting and photophobia (eye sensitivity to light). It can progress to convulsions and coma. Usually, people who survive a meningococcal invasion suffer some degree of neurological damage (Nester *et al.*, 2009). Lack of circulating protective bactericidal antibodies and defects in the complement system are the most important factors predisposing individuals to invasive meningococcal disease (Caugant and Maiden, 2009).

2.1.5 Epidemiology

Meningococcal disease in Europe and North America usually occurs as sporadic cases and the highest age-specific incidence rates are seen in children less than 5 years old, which is in contrast to the low prevalence of asymptomatic carriage in this age group. However, the disease may present different epidemiological features. In some areas hyperendemic disease occurs, with incidence rates 5-10 cases per 100,000. Sometimes, disease can also occur in clusters and localized outbreaks. Nonetheless, the most dramatic epidemiological manifestations are the periodic large countrywide epidemics or pandemics that occur in some parts of the world. Nowadays, epidemic and pandemic disease appears restricted to countries of sub-Saharan Africa, in the so-called "meningitis belt", which goes from Ethiopia in the East to Senegal in the West (Caugant and Maiden, 2009).

The incidence of the disease caused by *N. meningitidis* reached an epidemic level in the northern part of Norway in 1975. In the following years, the epidemic spread to the whole country, and not until the late 1980s did the incidence of disease start to decrease (Caugant *et al.*, 1994). Currently, around 10% of Norwegians are asymptomatic carriers of *N. meningitidis.* Consequently, Norway has always been at the forefront in meningococcal research, and some discoveries in this field have been performed in Norwegian institutions.

Meningococcal strains are classified by the antigenic properties of the CPS, which defines their serogroup (Alfsnes, 2012). Of the 12 serogroups identified, 6 (A, B, C, X, Y, W_{135}) are responsible for 90% of the invasive disease worldwide (Caugant, 2008). Serogroup A meningococci, which have essentially disappeared from Europe and North America since World War II, have been associated to the large epidemics in Africa. On the other hand, serogroup B meningococci, which are generally absent in sub-Saharan Africa, are the primary concern in industrialized countries. Outbreaks of serogroup C meningitis occur worldwide, while serogroup W_{135} and X have been responsible for epidemics in sub-Saharan Africa since 2002 (Caugant and Maiden, 2009).

Development of vaccines based on high molecular weight cell-surface CPS can prevent infections of serogroup A, C, Y and W_{135} (quadrivalent menigococcal conjugate vaccine – MenACWY). Although effective in adults, vaccines based on CPS are poor immunogens in infants and young children below the age of 2-5 years (who do not have fully developed immune systems) and in individuals that do not induce immunological memory (Alfsnes, 2012). With the use of a serogroup C vaccine in the United Kingdom, disease caused by these microorganisms has been almost eliminated (Gorringe and Pajón, 2016). The phased introduction in meningitis belt counties of MenAfriVac, a novel serogroup A

meningococcal conjugate vaccine, holds great promise to end epidemic meningitis as a public health concern (Centers for Disease Control and Prevention, 2015). It was administered to more than 217 million people in 2010-14, resulting in an estimated 142 000 lives saved and more than a million cases of meningitis prevented (Glassman and Miriam, 2016).

Vaccines base on the serogroup B capsule have been poorly immunogenic. The low immunogenicity is likely to be due to the structural homology between B polysaccharide and carbohydrate residues present in human tissue leading to immune tolerance (Gorringe and Pajon, 2012). Recently, Novartis Vaccines has developed a vaccine for the prevention of MenB disease that contains four antigenic components: factor H binding protein fHbp, neisserial adhesin A (NadA), neisserial heparin binding antigen (NHBA) and outer membrane vesicles from a New Zealand epidemic strain (which provides PorA). fHbp is a surface-exposed lipoprotein which induces high levels of bactericidal antibodies that are also protective following passive immunization. This protein was named fHbp because it binds to the human factor H, a negative regulator of the alternative complement pathway. The adhesin NadA is involved in cell adhesion, invasion and induction of pro-inflammatory cytokines. Finally, NHBA is a lipoprotein that appears to increase survival of *N. meningitidis* by binding glycosaminoglycans. The outer membrane vesicles from the New Zealand epidemic strain was added with the aim of increasing cross-protection. This vaccine, previously known as 4CMenB, has been given the registered name Bexsero® (Gorringe and Pajon, 2012).

2.2 Meningococcus genome

2.2.1 Genome characteristics

The genomes of meningococcus strains Z2491 and MC 58, representing serogroup A and B, respectively, were the first to be sequenced and annotated (Alfsnes, 2012). The complete genome sequence was obtained by the random shotgun sequencing strategy. *N. meningitidis* strain MC 58 has a genome size of approximately 2.2-2.3 mega base pairs (Mb) (Tettelin *et al.*, 2000), and harbour approximately 2000 coding regions making up between 75-80% of the genome (Alfsnes, 2012). The meningococcus genomes have an average G+C content of 51.5% (Tettelin *et al.*, 2000) and are characterized by an abundance of repeat sequences and phase variable genes (Alfsnes, 2012).

2.2.2 Transformation in *N. meningitidis*

Horizontal gene transfer (HGT) mediates the exchange of the genetic information between bacterial cells belonging to the same generation (Lång *et al.*, 2009). It allows genes and genetic information to flow between microorganisms, to cross species barriers and beyond, and for the reshuffling of genes or the acquisition of novel genes (Alfsnes, 2012). Three different means of HGT between bacteria are known. These are plasmid-mediated conjugation, phage-mediated transduction and natural transformation (Ambur, 2014), which is defined by binding, uptake and genomic integration of naked DNA through recombination (Fig. 1) (Alfsnes, 2012). The mechanisms, genetic consequences and evolutionary rationales are fundamentally distinct among these processes (Ambur, 2014). Transformation is comparable to sexual reproduction in eukaryotes; they both involve active translocation of DNA from one cell to another, as well as homologous recombination (Alfsnes, 2012). On the other hand, conjugation and transduction primarily transfer novel genetic sequences (Ambur, 2014).

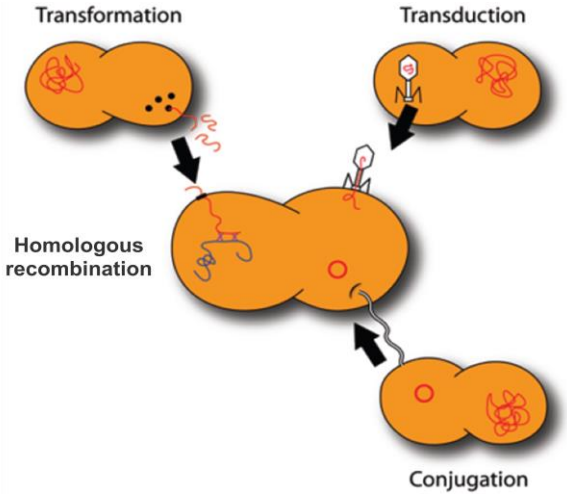

Figure 1 – The three modes of HGT in bacteria (Alfsnes, 2012).

The evolution of the pathogenic neisseriae, particularly *N. meningitidis*, is closely linked to their ability to be transformed with extracellular DNA (Ambur, 2014). Natural transformation is the predominant route for exchange of chromosomal DNA between neisserial strains. Meningococcus is naturally competent for transformation throughout its growth cycle (Benam *et al.*, 2011). A strong tradition of studying these microorganisms has documented that they exchange alleles with commensal *Neisseria*, and they also repeatedly utilize DNA from their own separate clonal lineages for this purpose (Ambur, 2014). In 1953, Alexander and Redman had described for the first time transformation of type specificity by DNA in *N. meningitidis*. Some years later, in 1961, Catlin and Cunningham, have performed studies with species of *Neisseria* in order to understand interspecies genetic transfer among this group (Ambur, 2014). Despite substantial variation between the transformability of individual strains and species, intraspecies transformation frequencies (transformation of one species with DNA from the same species) were repeatedly reported to be higher than interspecies transformation (transformation of one species with DNA

from another) frequencies. This work alluded to the presence of a barrier or barriers of unknown mechanism(s) that opposes the free mobility of DNA between different species (Ambur, 2014).

2.2.3 DNA Uptake Sequence – DUS

HGT is associated with the risk of allowing entry of alien and potentially harmful DNA (Ambur, *et al.*, 2009). Different reproductive barriers have evolved in diverse transformation-competent bacteria, which distinguish in favour of acquisition and recombination of homologous DNA sequences and discriminate against heterologous and potentially hazardous DNA (Frye *et al.*, 2013). This can occur at an early stage in the transformation process by recognizing and engaging specific uptake sequences present in the extracellular DNA (Ambur , 2014).

This phenomenon is a well-studied characteristic and seems a unique property of two very phylogenetically distinct bacterial families, *Pasteurellaceae* and the *Neisseriaceae*. The presence and identity of the Uptake Signal Sequence (USS), 5'-AAGTGCGGTCA-3', responsible for selective DNA uptake in *Pasteurellaceae* family members was first described in *Haemophilus influenzae* and *Haemophilus parainfluenzae.* Soon thereafter, Graves *et al.* have shown that *N. gonorrhoeae* also displayed sequence-specific DNA uptake in transformation. They have also determined experimentally that the gonococcal DUS was different from that of *Haemophilus* spp. (Ambur, 2014; Graves *et al*., 1982). Even though the uptake pathways in *Neisseriaceae* and *Pasteurellaceae* families are closely related, the species have evolved their own specific DUS and USS sequences, respectively, for increased transformation efficiency (Alfsnes, 2012).

The correct DUS was first identified as a 10-mer 5'-GCCGTCTGAA-3', present in DNA fragments able to competitively inhibit acquisition of foreign DNA in *N. gonorrhoeae.* The functional identity of DUS was later expanded to encompass the 12-mer 5'-atGCCGTCTGAA-3', where the lowercase letters denote two semiconserved nucleotides of DUS (Ambur, 2014). Ambur and his co-workers have shown that the two semiconserved residues 5' of the 10-mer DUS are both present in 76% of all DUS occurrences in every neisserial genome sequence available. They have also demonstrated a positive functional effect on transformation by the stepwise addition of these two nucleotides. Their data suggested that the 10-mer DUS compared to the 12-mer would interact more weakly in DUS affinity studies (Ambur *et al*., 2007). In this way, the 12-mer was found to outperform

the 10-mer DUS in transformation efficiency (Ambur, 2014). Furthermore a longer recognition signal would increase specificity and would, as a consequence, facilitate the discrimination of foreign DNA entering the neisserial cell. Thus, Ambur *et al.* have suggested that the established and commonly applied 10-mer DUS should be replaced by the 12-mer DUS when possible for synthetically produced fragments for use in transformation, at least if optimal transformation rates are desirable (Ambur *et al.*, 2007).

2.2.4 DUS-dependent transformation mechanism

Uptake of DNA and neisserial transformation have been associated with the presence of TFP, filamentous appendages emanating from the bacterial surface (Frye *et al.*, 2015). Considering this, the expression of TFP, the presence of DUS in the incoming DNA and RecA-mediated homologous recombination are prerequisites for transformation in *Neisseria* (Alfsnes, 2012). The TFP structures required for transformation include the pilin structural subunit PilE and the minor pilin ComP, as well as the outer membrane secretin PilQ which forms a pore through which pili are extruded and retracted. Inner membrane proteins PilG, PilP and PilT are also involved in this process for pilus assembly, secretin stabilization and pilus retraction respectively (Alfsnes, 2012). In the current model for transformation extracellular double-stranded DNA binds to ComP in the protruding pilus, which is constituted primarily of PilE monomers. After this, double-stranded DNA is pulled into the periplasm by the retracting pilus through the secretin pore. The energy required for pilus retraction is provided by the intracellular ATPase PilT (Ambur, 2014). Once DNA is inside of the outer membrane of the meningococcus cell, non-pilus associated proteins ensures transport through periplasm, the peptidoglycan layer supported by ComL, and through the cytoplasmic membrane facilitated by ComE binding. Finally, the DNA is assisted through the cytoplasmic membrane by transmembrane channel ComA. Restriction modification enzymes and the helicase activity of the RecBCD pathway transformed double-stranded DNA to single-stranded DNA in the cytoplasm. In the terminal steps of the transformation process, the cytoplasmic protein SSB protects the DNA from degradation, followed by recruitment of DprA and RecA enabling homologous recombination (Alfsnes, 2012).

2.2.5 Distribution and genomics of DUS

DUS are extremely abundant in the genomes of *Neisseria*, by far the most frequent small repeats (Fig. 2). The 2.2 Mb genomes of *N. gonorrhoeae* and *N. meningitidis* harbour nearly 2000 DUS, occupying as much as 1% of the chromosome. The abundance of DUS across the chromosome allows for transformation of the most parts of the genome (Ambur, 2014).

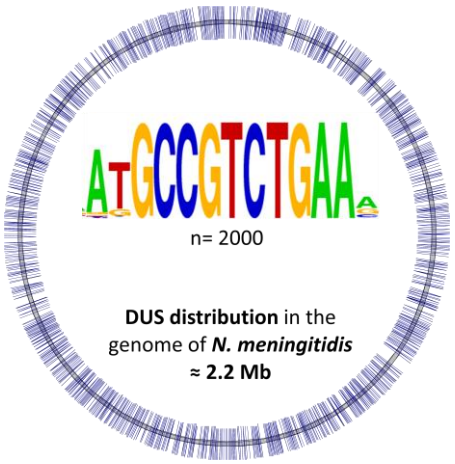

Figure 2 – The genomic distribution of DUS (represented as blue lines) in a representative meningococcal genome (Ambur, 2014).

DUS are an integrated part of their host genomes and are located both inside coding regions (25%) and in intergenic locations (75%). The former is suggestive of long term adaptive evolution due to the integration into the reading frame. On the other hand, the latter are often found as inverted repeat and suggestive of a transcriptional termination function (Alfsnes, 2012). Goodman and Scocca noted in their pre-genomic analysis that DUS in three different loci was arranged as an inverted repeats able to form stem-loop structures on RNA that potentially could attenuate or terminate transcription (Goodman e Scocca, 1988). It therefore seems that DUS was involved in two important processes: sequence-specific transformation and regulation of transcription (Ambur, 2014). In fact, intergenic DUS often occur in pairs arranged as inverted repeats and are likely to function as rho-independent transcriptional terminators or attenuators by forming loop structures in mRNA (Ambur *et al.*, 2007). Generally, DUS and the reverse complement DUS constitute the stem of the transcriptional terminator and are separated by five random nucleotides on average that constitute the loop. Of the two possible configurations of the inverted DUS-repeat, one is most common (5'-atGCCGTCTGAA (N) TTCAGACGGCat-3'). The abundance of inverted repeat DUS in intergenic locations may reflect the evolutionary costs of harbouring DUS inside coding regions (Ambur, 2014). Notably, there is a stop codon (UGA) in one reading frame, which imposes an obvious limitation on the liberty of positioning DUS in intragenic positions (Frye *et al.*, 2013; Ambur, 2014).

A biased distribution of the intragenic DUS towards genome maintenance genes, and a preserving function of the genome by DUS-mediated transformation was proposed as a driving force behind the over-representation of DUS in coding sequences (Davidsen *et al.*, 2004). To a considerable extent, it seems that homologous genes in *Neisseria* and *Pasteurellaceae* have accumulated DUS/USS inside their coding regions during evolution. DUS

and USS are non-homologous sequences and their bias towards genome maintenance genes is suggestive of convergent evolution and that sequence specific transformation has exerted similar influences on the evolution of these organisms (Ambur, 2014).

In 2013, analysis of the genome of *Neisseriaceae* family members has revealed that many, but not all, contain DUS or completely new dialects of DUS, outlining an evolving "sexual language". In total, eight DUS dialects have been detected that display considerable variation between species but that are remarkably well conserved and over-represented within each genome (Fig. 3) (Frye *et al.*, 2013). Different variants of DUS were termed DUS dialects alluding to their role as nucleotide "words" in genetic "communication" (Frye *et al.*, 2013). Importantly, an experimentally defined 5'-CTG-3' DUS core (5'-atGCCGT**CTG**AA-3') was found to be conserved in all eight dialects of DUS. Transformation of four phylogenetically separate *Neisseriaceae* species – *Kingella denitrificans*, *Eikenella corrodens*, *Neisseria elongata* and *N. meningitidis* – using DNA with different DUS-dialects can function as efficient barriers to inter-species transformation/sex although their corresponding DUS share the same core. Finding that DUS have evolved into different dialects, each carefully maintained and integrated in respective genomes in the *Neisseriaceae* family, supports the notion of the adaptive value of DUS (Ambur, 2014; Frye *et al.*, 2013).

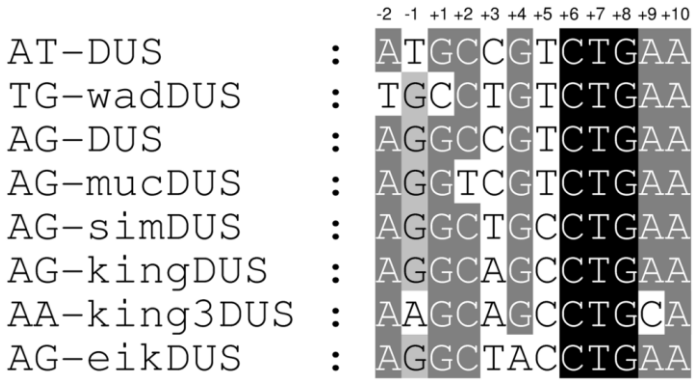

Figure 3 – Alignment of eight distinct DUS dialects identified in the genomes of *Neisseriaceae* family members. Relative shading indicate the level of sequence conservation. Shading black represents the 5'-CTG-3' DUS core (Frye *et al.*, 2013).

3. Aim of the study

Kingella kingae is a fastidious Gram-negative coccobacillus that belongs to the family *Neisseriaceae.* This bacterium is a normal component of the oropharyngeal microbiota in young children. Although *K. kingae* is the most common etiology of osteoarticular infections in young children, is a frequent cause of bacteremia in those younger than 4 years (Basmaci *et al.*, 2014). Frye *et al.* have identified in *K. kingae* a new DUS dialect, AA-king3DUS (Fig. 3) that is present on either the positive or negative DNA strand and is repeated in 2787 copies in the *K. kingae* genome (Frye *et al.*, 2013; Basmaci *et al.*, 2014). Given that the genome size of *K. kingae* is approximately 2 Mb, the DUS should be randomly repeated, on average, every 500 to 1000 bp. Therefore, Basmaci *et at.* have hypothesized that DUS may serve as a potential polymerase chain reaction (PCR) target for studying the genomic polymorphism of the strains using a one-primer amplification method. In this way, they have developed a rapid molecular typing tool for *K. kingae* (Basmaci *et al.*, 2014).

Based on our comprehensive characterization of DUS in *N. meningitidis* and the work performed by Basmaci and co-workers, the aim of this study was using the DUS dialect AT-DUS (Fig. 3) that is found in *N. meningitidis* genomes, to establish a rapid and cost-effective procedure for detecting *N. meningitidis* DNA. This tool was designed with the aim of further characterization of the carriage rates of *N. meningitidis* and other *Neisseria sp.* in Norwegian military recruits.

4. Materials, Methods and Results

4.1 Polymerase chain reaction (PCR)

PCR is a technique by which small samples of DNA can be quickly amplified, that is, increased to quantities that are large enough for analysis. Starting with just one gene-sized of DNA, PCR can be used to make billions of copies in only a few hours (Nester *et al.*, 2009).
The method consists of 20-40 cycles and each cycle is comprised of three steps called denaturation, annealing and elongation. During the first step, the double-stranded DNA containing the region to be amplified is denatured by heat, which creates single-stranded DNA. In the next step, the temperature is lowered so oligonucleotide primers can anneal to their complementary sequences in the single-stranded DNA. Two primers are required to amplify the DNA, and each must be complementary to a sequence on the appropriated

strand. In the third step, a DNA polymerase will extend the primers in a 5'-3' direction, elongating the DNA chain so that the DNA between those primers is copied. This happens in the presence of deoxyribonucleotides (dNTPS), and the temperature is set accordingly to the optimal temperature for the polymerase used (Evensen, 2014; Nester *et al.*, 2009). At the end of each cycle, the result is the synthesis of two new strands of DNA, each complementary to the other. In other words, the three-step cycle results in the duplication of the original target DNA. After each cycle of synthesis, the DNA is heated to convert all the new DNA into single strands. Each newly synthesized DNA strand serves in turn as a template for more new DNA (Nester *et al.*, 2009). The result of 20-40 cycles of these three steps is an exponential amplification of the DNA sequence of interest (Evensen, 2014). All of the necessary reagents are added to a tube, which is placed in a thermal cycler. The thermal cycler can be set for the desired temperatures, times and number of cycles (Nester *et al.*, 2009).

All the enzymes tested were used according to the manufacturer's instructions.

4.1.1 One-primer amplification method

4.1.1.1 Primer design

As mentioned previously, the 2.2 Mb genomes of *N. meningitidis* harbour nearly 2000 DUS, occupying as much as 1% of the chromosome (Ambur, 2014). Regarding the genome size, the DUS should be randomly repeated, on average every 500 to 1000 bp. It is also known that *N. meningitidis* harbour the AT-DUS (Fig. 3) and it is present on either the positive and negative strand. In this way, this sequence was used as a PCR target for detecting meningococcal DNA. However, instead of a regular PCR, this technique is a one-primer amplification method because only one primer is required to amplification, since the target sequence is present in both positive and negative strands. Regarding that AT-DUS was the PCR target, the AT-DUS sequence (5'-ATGCCGTCTGAA-3') was used to design the primer DUS Forward (5'-TTCAGACGG*C*A-3') and DUS Reverse (5'-TGCCGTCTG*A*A-3') (Fig. 4). The * represents a thio sulphate binding that blocks the 3'-5' exonuclease activity of some polymerases. This activity allows enzymes to cleave nucleotides one at a time from the end of a polynucleotide chain. The length of the primers is 11 bp and the melting temperature is 34 °C. The annealing temperature is approximately 30 °C, and it has been calculated with the help of the OligoCalc (Kibbe, 2007). Different polymerases have been used in this work and the different protocols will be presented in the next section.

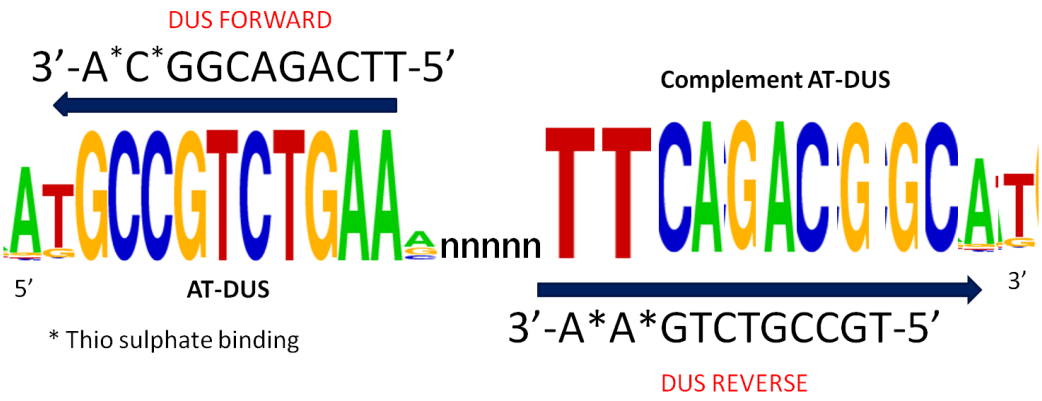

Figure 4 – Schematic representation of the primers DUS Forward and DUS Reverse, as well as the AT-DUS sequences.

4.1.1.2 Agarose gel electrophoresis

Agarose gel electrophoresis uses an electrical field to separate DNA fragments according to their size and charge. The negatively charged DNA fragments will migrate through a matrix of agarose towards a positive electrode**.** A low percentage of agarose will separate bigger fragments and a higher concentration will separate smaller fragments. Short fragments will migrate faster and further than the bigger fragments due to the pores in the agarose gel. This method can also be used to isolate and purify a DNA fragment of interest (Evensen, 2014). All the gels used in this study were 1% agarose gel. The amplification products were stained with GelRed™ Nucleic Acid Gel Stain (Biotium). This is a sensitive, stable and environmentally safe fluorescent nucleic acid dye designed to replace the highly toxic ethidium bromide (Biotium, 2013). All the gels were visualized using High Performance UV Transilluminator UVP after a 60-min migration at 100 V.

Regarding that there are 2000 places in the *N. meningitidis* genome where the primer can bind, there is no single predictable length of the PCR product. In this way we are expecting amplicons of different sizes, which means different bands in the agarose gel. Note that the DUS typing method amplifies sequences located between two DUS and not the DUS by itself. Therefore, each sequence between two DUS is unique (Basmaci *et al.*, 2014).

4.1.1.3 DNA PCR amplification using Taq DNA Polymerase

Taq DNA Polymerase is a highly thermostable DNA polymerase of the thermophilic bacterium *Thermus aquaticus*. The enzyme catalyzes 5'-3' synthesis of DNA and has no detectable 3'-5' exonuclease (proofreading) activity which would increase the fidelity

(Thermo Scientific, 2015). This was the first polymerase that we have used for the amplification method. Before the DNA PCR amplification we have done a dilution series of *N. meningitidis* strain 58 (MC 58) DNA, previously extracted by the hexadecyltrimethylammonium bromide (CTAB) method by co-workers of our lab. The first DNA concentration of the dilution series was 100 ng/µl (10^{-1}) and the last was 0,00001 ng/µl (10^{-8}). In this way we have used two strips of eight wells each, using the dilution series. This means that in each well the concentration of DNA was different (from 100 ng/µl to 0,00001 ng/µl). DNA PCR amplification was performed in a 25 µl reaction mixture that contained 24 µl of a master mixture (10X *Taq* Buffer, dNTPS, primer, magnesium, *Taq* DNA Polymerase (Thermo Scientific, United States of America) and 1µl of MC 58 DNA. Two different master mixtures were used, one with the DUS Forward and the other with the DUS Reverse. The amplification was performed in an ESCO Swift MaxPro PCR thermal cycler with an initial step of 5 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 40 °C, 1 min 30 s at 72 °C, and a final extension step of 10 min at 72 °C. The amplification products were stained and visualized as described before.

Result: The result was negative, or in other words, no bands were observed in the gel. This means that we did not get amplification with this PCR set up.

4.1.1.4 DNA PCR amplification using Phusion High-Fidelity DNA Polymerase

Once we did not get results with *Taq* DNA Polymerase, we tried Phusion High-Fidelity DNA Polymerase (Thermo Scientific, United States of America). Phusion DNA Polymerase brings together a novel *Pyrococcus*-like enzyme with a processivity-enhancing domain. The Phusion DNA Polymerase generates long templates with an accuracy and speed previously unattainable with a single enzyme. The error rate of Phusion DNA Polymerase in Phusion High-Fidelity Buffer is determined to be 4.4×10^{-7}, which is approximately 50-fold lower than that of *Taq* DNA polymerase. Phusion DNA Polymerase possesses 5'-3' DNA polymerase activity and 3'-5' exonuclease (proofreading) activity, which enables the polymerase to correct nucleotide incorporation errors (Thermo Scientific, 2013). With this enzyme we had troubleshooting. We tested PCR amplification protocols using: different annealing temperatures (31 °C, 25 °C and 23 °C); fresh dNTPS; Phusion high-fidelity buffer and Phusion GC buffer (this buffer can improve the performance of Phusion DNA Polymerase on some difficult or long templates, such as GC-rich templates); increment of magnesium concentration from 1,75 mM to 3,5 mM (the concentration of magnesium is

critical since Phusion DNA Polymerase is a magnesium dependent enzyme); increment of primers from 1 µl to 8 µl and gradient PCR. This specific PCR allows a variation of annealing temperature at the two extremes of the sample block in the thermal cycler, with the left column (column 1) being the coolest and the right column (column 12 in a 96-well block) the hottest. Our gradient PCR was performed using a range of 30 °C – 40 °C annealing temperature. In some protocols we have used as positive template control 10 ng/µl of MC 58 DNA with primer oh1 and oh22 (primers used before in our lab and that are known to promote amplification of a single target in MC 58 DNA) and as negative template control 10 ng/µl of *Helicobacter pylori* DNA. This negative control was chosen since *H. Pylori* does not have the DUS sequence in its genome. As referred before all the amplification products were stained and visualized as described in section 3.1.1.2.

Result: All the results were negative. However, in the positive control reaction we got amplification. This means that all the PCR conditions were working (dNTPS, buffer, polymerase, template, thermal cycler) but not with our primers DUS Forward and DUS Reverse.

4.1.1.5 DNA PCR amplification using Pfu DNA Polymerase

Pfu DNA Polymerase (Thermo Scientific, United States of America) is a highly thermostable DNA polymerase from the hyperthermophilic archaeum *Pyrococcus furious.* The enzyme catalyzes the template-dependent polymerization of nucleotides into duplex DNA in the 5'-3' direction. Pfu DNA Polymerase also exhibits 3'-5' exonuclease (proofreading) activity. This time the PCR was performed with an increment of primer from 1 µl to 8 µl, using 10 ng/µl of MC 58 DNA, and the positive and negative controls previously mentioned.

Result: Once again we only got amplification with the positive control.

4.1.1.6 DNA PCR amplification using phi29 DNA Polymerase

Phi29 DNA Polymerase (New England Biolabs, United Kingdom) is the replicative polymerase from the *Bacillus subtilis* phage phi29. This polymerase has exceptional strand displacement and processive synthesis properties. The polymerase has an inherent 3'-5' proofreading exonuclease activity. Strand displacement activity is a non-PCR based DNA amplification technique. This method amplifies rapidly small amounts of DNA samples to a reasonable quantity for genomic analysis. The reaction starts by annealing random hexamer

primers. When DNA synthesis proceeds to the next starting site, the polymerase displaces the newly produced DNA strand and continues its strand elongation. The strand displacement generates newly synthesized single stranded DNA template for more primers to anneal (Spits *et al.*, 2006).

Result: This reaction has demonstrated no specificity since we got amplification with the negative control.

4.1.1.7 DNA PCR amplification using QIAGEN® Multiplex PCR Plus Kit

The QIAGEN® Multiplex PCR *Plus* Kit (QIAGEN, Germany) is available in a convenient ready-to-use master mix format. This master mix contains pre-optimized concentrations of HotStarTaq *Plus* DNA Polymerase, a modified form of *Taq* DNA Polymerase that has no polymerase activity at ambient temperatures. This prevents extension of nonspecifically annealed primers and primer dimers formed at low temperatures during PCR setup and the initial PCR cycle. HotStarTaq *Plus* DNA Polymerase, is activated by a 5-minute incubation at 95 °C. The master mix also contains magnesium cloride, dNTPS and a unique PCR buffer containing the novel synthetic Factor MP. Together with optimized salt concentrations (K^+ and NH_4^+), this additive stabilizes specifically bound primers and enables efficient extension of the primers in the reaction. The Factor MP increases the local concentration of primers at the template promoting specific primer annealing (Fig. 5) (QIAGEN).

Using this kit we performed a gradient PCR using a range of 30 °C – 40 °C annealing temperature. DNA PCR amplification was performed in a 25 µl reaction mixture that contained 12,5 µl of 2x Multiplex PCR Master Mix, 2 µl of Dus Forward (10 µM) and 1 µl of DNA (100 ng/µl MC 58 DNA). The cycling protocol used was initial PCR activation step of 5 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 90 s at 30 °C – 40 °C, 90 s at 72 °C, and a final extension step of 10 min at 68 °C. All the amplification products were stained and visualized as described in section

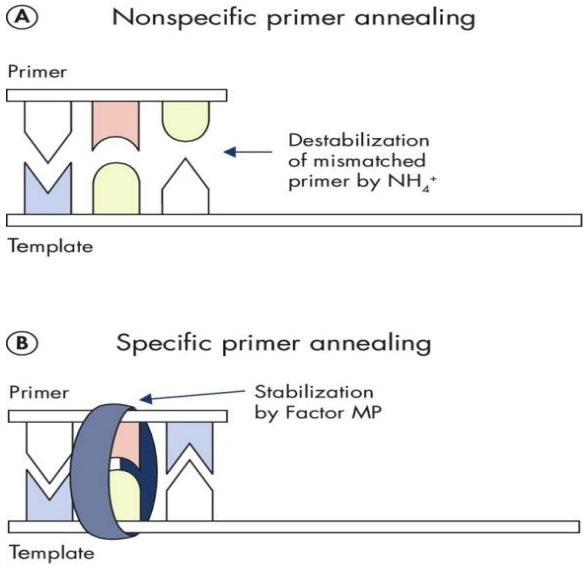

Figure 5 – Schematic representation of the activity of Factor MP. **[A]** NH_4^+ ions prevent nonspecific primers from annealing to the template.**[B]** Synthetic factor MP, increases the local concentration of primers at the template (QIAGEN).

3.1.1.2.

Results: Finally we got amplification with this kit. The gels electrophoresis of the PCRs are shown above (Fig.6). Lanes with the 100 bp designation are DNA ladders, lane 1 is a negative control (100 ng/µl of *H. pylori* DNA) at the lowest temperature (30 °C) and lane 14 is the same negative control at the highest temperature (40 °C). Lanes 2 to 13 are the PCR products of the gradient PCR with 100 ng/µl MC58 DNA and 2 µl of DUS Forward (10 µM). The optimal annealing temperature is 34,5 °C, lane 7. This PCR was performed in an Agilent Technologies Sure Cycler 8800.

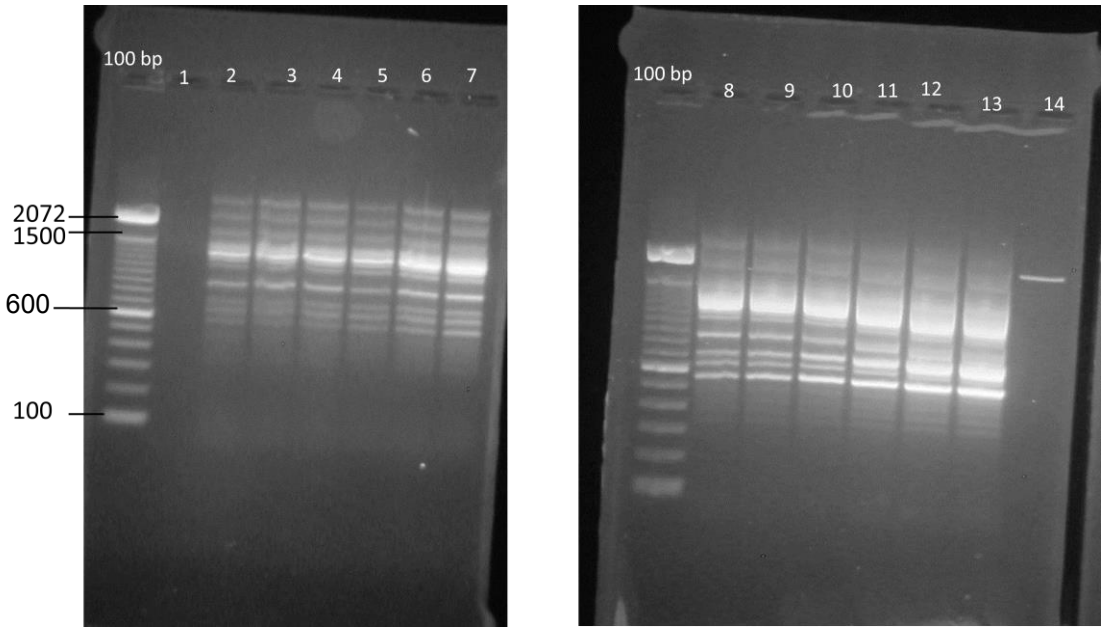

Figure 6 – Gel electrophoresis of the gradient PCR using the QIAGEN® Multiplex PCR *Plus* Kit.

Taking these results in consideration, we have performed the PCR protocol mencioned aboved but using dilution series (from 100 ng/µl to 0,00001 ng/µl) with DNA of two different *N. meningitidis* strains (MC 58 and MC Z1099).

Results: We got amplification with both MC 58 and MC Z1099. For MC 58 the optimal concentration of template was 10 ng/µl and for concentrations below we could not detect amplification product. For MC Z1099 the optimal concentration of template was 1 ng/µl and for concentrations below 0,1 ng/µl of template we could not detect amplfication products. Comparing the two gels it is evident that bands between 500 bp and 1400 bp are present in both. This fact supports the specificity of this method, once the primer DUS-Forward was able to target the AT-DUS sequence in different strains of *N. meningitidis*.

One last PCR with the aim of testing the specificity of the reaction was performed. In this way we have tested if, in the presence of alien DNA, the DUS primer could target the AT-DUS. The *H. pylori*, *H. influenzae*, and *Eikenella corrodens* DNA were used as alien DNA.

The first was chosen as a negative control regarding that this specie does not have the DUS sequence. On the other hand *H. influenzae* was chosen because it has the USS sequence and *E. corrodens* because it has the AG-eikDUS (Fig. 3). We were interested in understand if the presence of alien DNA could disturb the binding between the primer DUS and the AT-DUS sequence present in the *N. meningitidis* genome. With this purpose we have performed a PCR using the protocol described in page 18, using 34,5 °C as annealing temperature. Increments of alien DNA concentration were performed in order to test 1ng/µl of MC Z1099 DNA against 1, 10, 100 and 1000 ng/µl of alien DNA. The gel electrophoresis are shown below. For all gels (Fig. 7) **lanes** 100 bp are DNA ladders; **lanes 1** are 0 ng/µl of MC Z1099 DNA + 1 ng/µl of alien DNA's (negative controls); **lanes 2** are 0 ng/µl of MC Z1099 DNA + 0 ng/µl of alien DNA's (no template controls); **lanes 3** are 1 ng/µl of MC Z1099 DNA + 0 ng/µl of alien DNA's (positive controls); **lanes 4** are 1 ng/µl of MC Z1099 DNA + 1 ng/µl of alien DNA's; **lanes 5** are 1 ng/µl of MC Z1099 DNA + 10 ng/µl of alien DNA's; **lanes 6** are 1 ng/µl of MC Z1099 DNA + 100 ng/µl of alien DNA's and **lanes 7** are 1 ng/µl of MC Z1099 DNA + 1000 ng/µl of alien DNA's.

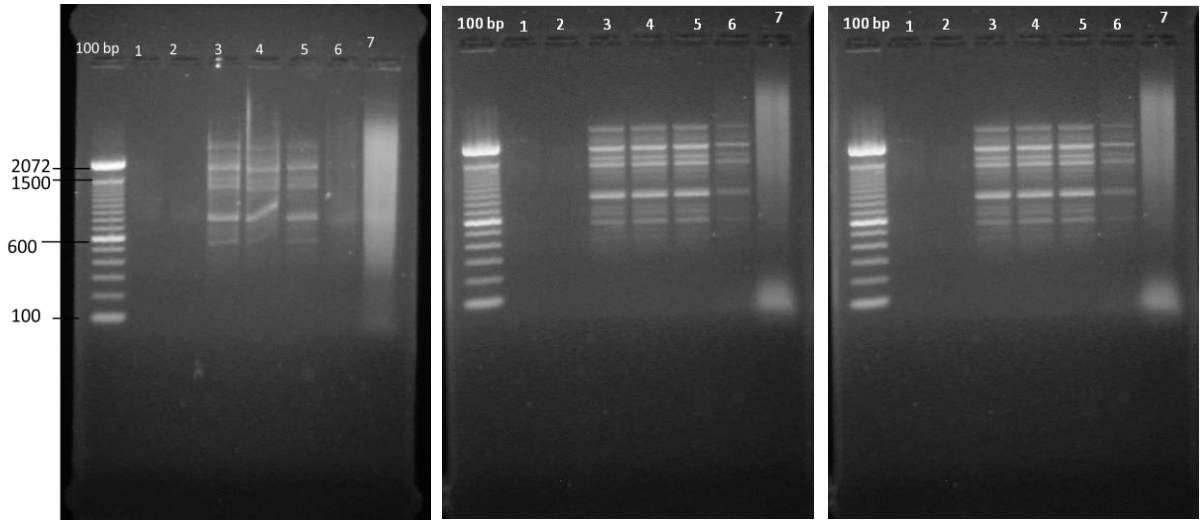

Figure 7 – Gel electrophoresis of the PCR. The first picture is the competition with *H. pylori*, the second with *H. influenzae*, and the third with *Eikenella corrodens.*

Results: With these results we can conclude that the reaction between the primer DUS Forward and the AT-DUS sequence is specific since we got the same bands even with 100 ng/µl of alien DNA present in the PCR reaction. We conclude that 1000 ng/µl was a too high template concentration.

5. Conclusions and Future Trends

We have established a single primer PCR protocol specific for the amplification of DUS-containing DNA such as in *N. meningitidis*. This protocol can be used in further studies due to the specificity of the protocol. We can conclude that DUSs are attractive targets for diagnostic purposes, and they can be used in epidemiological carriage studies.

The development of this DUS-dependent genome amplification technique opened doors to future projects. Suitable throat samples for *N. meningitidis* carriage designation have already been collected from Norwegian military recruits in January 2013 and December 2013 using ESwabs (Copan Diagnostics). These were cultivated within 24 h of collection and *N. meningitidis* and commensal *Neisseria sp.* strains were cloned and preserved. Consequently the application of this technique in these samples will allow the characterization of the carriage rates of *N. meningitidis* and *Neisseria sp*. in the Norwegian military recruits; the establishment of procedures for whole genome sequencing of *N. meningitidis* and other *Neisseria*; the investigation of genetic diversity in *N. meningitidis* strains; the study of potential spread and stability of individual lineages amongst cohabiting recruits at two time points one year apart (enrollment and end of service) and the establishment of a protocol using primers with individual DUS-dialects in real time PCR (qPCR) to resolve the *Neisseriaceae* family into individual genera/species. Since samples were collected twice from the same individual one year apart, this characterization will illuminate about strain evolution (how one strain may have evolved over one year), dissemination (how one clone may have spread from one individual to others) and potentially the dynamics of inter- and intraspecies genetic transformation.

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