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# ASSESSMENT OF JC VIRUS IN PORTUGUESE WASTEWATERS: IMPACT ON PUBLIC HEALTH

Tese de Mestrado em Análises Clínicas, orientada pela Professora Doutora Ana Miguel Duarte Matos Silva e apresentado à Faculdade de Farmácia da Universidade de Coimbra

Setembro 2015

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*A special thanks to Prof. Dr. Ana Miguel Matos*

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## ABBREVIATURES

| | |
|---|---|
| **Ct** | Cycle Threshold |
| **GC** | Genome Copies |
| **JCV** | JC Virus |
| **NCCR** | Noncoding Control Region |
| **ori** | Replication origin region |
| **PML** | Progressive Multifocal Leukoencephalopathy |
| **PP2A** | Protein Phosphatase 2A |
| **pRB** | Retinoblastoma Protein |
| **qPCR** | Real-Time Polymerase Chain Reaction |
| **TAg** | Large Tumor Antigen |
| **tAg** | Small Tumor Antigen |
| **WWE** | Effluent Wastewater |
| **WWI** | Influent Wastewater |
| **WWTP** | Wastewater Treatment Plant |

## ABSTRACT

**Background** Despite the increasing number of global population with access to an improved drinking-water source and to a better-quality sanitation, waterborne and water-related diseases still represent a major human health risk with massive socio-economic repercussions. The frequent detection of viruses in effluent sewage treatment facilities has been reported in different countries, which has led to the hypothesis that additional indicators of human contamination, apart from the commonly used bacteriological indicators may be needed to assure the microbiological quality of water. In this order, we aimed to create the first national profile of the ubiquitous human JC virus (JCV) in Portuguese wastewaters and to evaluate its removal efficiency by Wastewater Treatment Plants (WWTPs).

**Methods** Influent (WWI) and effluent (WWE) wastewater samples from fifteen different Portuguese WWTPs were collected during winter time. After ultracentrifugation and nucleic acid extraction, they were evaluated by qPCR for the presence and quantification of JCV.

**Results** Two (13.3%) of the 15 evaluated WWTP did not present JCV detectable in both influent and effluent samples. JCV DNA was detected in 13 WWI samples, and quantified in 12, being present in a concentration that ranged from $6.79 \times 10^3$ to $1.70 \times 10^6$ genome copies (GC) per liter (average $5.22 \times 10^5$ GC/L). After treatment, JCV was detected in 9 (69.2%) of the 13 WWI initial positive assessed samples and quantified in 8 WWE of them, revealing a concentration that ranged from $6.58 \times 10^3$ to $1.26 \times 10^6$ GC/L (average of $1.96 \times 10^5$ GC/L). The mean removal ratio of JCV in the treatment process was 2.88 log GC/L, with 4 WWTP accomplishing a complete (100%) removal of JCV to an undetectable viral load, with a reduction of more than 4 JCV logs.

**Conclusions** This was the first epidemiologic study regarding the presence of JCV in waters made in Portugal. The results demonstrate that JCV is frequent in Portuguese wastewaters, and 69.2% of WWTP were not able to efficiently eliminate this virus. For that reason, viral surveillance of water quality proves itself crucial with regards to identifying possible sources of contamination and prevent the widespread dispersion of certain potentially pathogenic agents.


**Keywords** JC Virus, Wastewater, Real-Time Polymerase Chain Reaction, Surveillance

## RESUMO

**Introdução** Apesar do número crescente da população mundial com acesso a uma fonte de água segura e a um saneamento de melhor qualidade, as doenças transmitidas pela água e com ela relacionadas ainda representam um grande risco para a saúde humana com massivas repercussões socioeconómicas. A frequente deteção de vírus em efluentes de estações de tratamento de esgotos foi reportada em diferentes países, o que levou à hipótese de que indicadores adicionais de contaminação humana, para além dos indicadores bacteriológicos frequentemente utilizados, possam ser necessários para assegurar a qualidade microbiológica da água. Por isso, pretendemos criar o primeiro perfil nacional do vírus ubiquitário humano JC (JCV) em águas residuais portuguesas e avaliar a sua eficiência de remoção pelas estações de tratamento de águas residuais (ETARs).

**Métodos** Amostras de águas residuais de influentes (WWI) e efluentes (WWE) de quinze ETARs portuguesas diferentes foram colhidas durante o inverno. Após ultracentrifugação e extração do ácido nucleico, foi utilizado um protocolo de qPCR para detetar e quantificar o genoma do JCV.

**Resultados** Em 2 (13,3%) das 15 ETARs avaliadas não foi detetada a presença de JCV, tanto nas amostras de influentes como de efluentes. A concentração do DNA do JCV em 12 das 13 outras amostras positivas de WWI variou de $6{,}79 \times 10^3$ a $1{,}70 \times 10^6$ cópias de genoma (CG) por litro (média $5{,}22 \times 10^5$ CG/L). Após o tratamento, o JCV foi detetado em 9 (69,2%) das 13 ETARs com amostras WWI positivas, revelando uma concentração que ia de $6{,}58 \times 10^3$ a $1{,}26 \times 10^6$ CG/L (média $1{,}96 \times 10^5$ CG/L) em 8 delas. A taxa de remoção média do JCV pelo processo de tratamento foi de 2,88 log CG/L, tendo 4 ETARs alcançado uma remoção completa (100%) do JCV a uma carga viral indetetável (100%), com a redução de mais de 4 logs de JCV.

**Conclusões** Este foi o primeiro estudo epidemiológico realizado em Portugal referente à presença de JCV nas águas. Os resultados demonstram que o JCV é frequente nas águas residuais portuguesas, e que 69,2% das ETARs não foram capazes de eliminar este vírus de forma eficiente. Por esta razão, a vigilância viral da qualidade da água revela-se crucial com o objetivo de identificar possíveis fontes de contaminação e prevenir a dispersão de determinados agentes potencialmente patogénicos.



**Palavras-Chave** Vírus JC, Águas Residuais, Reação de Polimerização em Cadeia em Tempo Real, Vigilância

# 1. INTRODUCTION

Despite the increasing number of global population with access to an improved drinking-water source and to a better-quality sanitation, waterborne and water-related diseases still represent a major human health risk with massive socio-economic repercussions. Throughout the world several waterborne outbreaks of acute gastroenteritis, respiratory diseases, meningitis and conjunctivitis have been described associated with potentially pathogenic organisms present in water such as enteric viruses, bacteria and protozoa. The majority of these microorganisms are eliminated in the urine and feces of both clinically ill and asymptomatic individuals. As a consequence, raw sewage may present a high number of such pathogenic agents and constitute an important source of water contamination[1].

## 1.1. Current Water Quality Surveillance

It is crucial to monitor and survey the water quality with the intention of identifying possible sources of contamination and prevent the widespread dispersion of certain potentially pathogenic agents. If there is no appropriate treatment of domestic wastewaters, microorganisms will not be completely eliminated and will become vastly distributed in the environment, some of them able to remain stable for long periods of time[2]. These circumstances facilitate the fecal-oral transmission which can happen through domestic water supplies, recreational waters, along with surface and ground waters contaminated with untreated sewage water[3].

Unfortunately, this is a very common situation regarding some viruses, which can be due to its higher resistance to inactivation by most disinfectants such as chlorination and ultraviolet light[4].

The human fecal contamination indicators frequently used in the microbiological water quality assessment are mainly bacterial indicators such as heterotrophic bacteria, total coliform bacteria, thermotolerant coliform *E. coli*, *Enterococci* and coliphages, which may not correlate well with the presence of human viruses[5].

Recent molecular techniques like end-point PCR and real-time qPCR, have significantly improved the detection and quantification of viruses in different types of water samples in a rapid, specific and sensitive approach[6]. These techniques have revealed an high frequency of viral genomes in effluent sewage treatment facilities, especially from noroviruses[7], adenoviruses[8], enteroviruses[8], rotaviruses[9], astroviruses[9], hepatitis A viruses[10], hepatitis E viruses[10], and polyomaviruses[11], which has led to the hypothesis that alternative indicators of fecal contamination may be needed to assess the presence of enteric viruses in different types of water.

The analysis of different wastewater matrices by several authors, led them to propose the JC virus (JCV), a ubiquitous human polyomavirus, as a useful tool for assessing human water contamination as well as the WWTPs viral removal efficiency[12–14]. Due to its high stability in the aquatic environment and resistance to treatments, additionally to its non-seasonal excretion profile, this polyomavirus may represent a valuable human contamination indicator[13].

## 1.2. JC Virus

JCV, a member of the *Polyomaviridae* family, is a small, nonenveloped virus with a closed circular, double-stranded DNA genome[15].

### 1.2.1. JCV structure and genome

Three viral structural proteins compose its 40 nm diameter icosahedral capsid: VP1, VP2 and VP3. Organized into 72 pentameric capsomers, VP1 is the major and most abundant capsid protein, responsible for the virus-cell interaction and the receptor specificity. Each VP1 pentamer is associated with one single molecule of the other two minor proteins, VP2 and VP3[15].

The viral genome, with an approximate length of 5.13 kb, is divided into three functional sections: an early and a late coding regions, separated by a noncoding control region (NCCR)[16] as represented in ***Figure 1***.

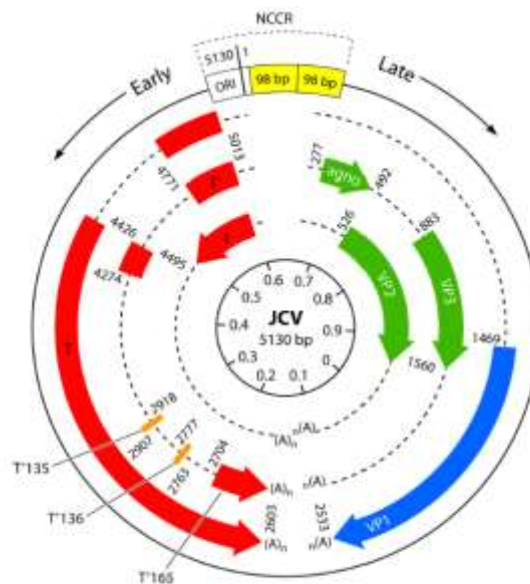


***Figure 1.*** *Illustrative scheme of JCV genome with a rearranged NCCR[15].*

The early region is the first to be transcribed, before the genome replication process takes place. It encodes the large tumor antigen (TAg) and a small tumor antigen (tAg). The TAg has the main purpose of driving the host cell into the S phase by interacting with both viral and host cell genome and proteins and further binding to the viral replication origin region (*ori*). It also regulates the transcription of the late region proteins[15]. Essential to viral replication and transcribed from a conserved genetic region, the TAg coding region is a target normally used to detect JCV in molecular techniques. The tAg function is currently not very clearly understood but it seems to interact with TAg via PP2A and pRB proteins, determining cycle cell progression[17].

After the beginning of viral DNA replication, the late region is transcribed originating the capsid structural proteins, VP1, VP2 and VP3. A fourth element transcribed from this region is the regulatory agnoprotein, which appears to be multifunctional, participating in viral replication and transcription regulation and inhibiting the host cell DNA repairing process to, consequently, successfully release the infectious virions[18].

The NCCR not only has the DNA replication origin but also the viral promotors and enhancers essential to control the transcription process of both early and late genes, bidirectionally transcribed from initiations sites close to the *ori*. Although early and late regions are genetically conserved, hypervariability of this regulatory region has been observed. Based on the differences noticed in the nucleotide sequence, the NCCR can adopt two main configurations: archetype and rearranged[19].

The archetype, or non-rearranged NCCR, is considered the main strain circulating in the human population[20] and contains, apart from the *ori,* a single copy of a 98bp sequence. If this genome region displays mutated sequences, such as deletions and/or duplications, the JCV variant is called rearranged or PML-type.

These alterations may be the main contributing factor for the infected host cell type and the kind of infection that will be developed. As a matter of fact, the rearranged type has frequently been found in cerebrospinal fluid, lymphocytes and brain tissue of patients with Progressive Multifocal Leukoencephalopathy (PML), a neurological disease due to JCV infection, suggesting its contribution for an active infection while the archetype has been found in urine and kidney tissue of both non-immunocompromised healthy individuals and PML patients, probably associated with a latent infection[21].

### 1.2.2. JCV replication cycle and pathogenesis

JCV enters the host cell by endocytosis in a clathrin-coated vesicle. Once within the nucleus, replication takes places with the cell's machinery and virions are assembled and released to the exterior by cell lysis[22].

With a human restricted host and a specificity to infect cells with $\alpha(2,6)$-sialic acid receptors, it was hypothesized that the tonsillar and lymph nodes could act as a primary replication site for the virus, and the oropharyngeal and respiratory routes are possible JCV transmission mechanisms[23]. After an asymptomatic or a mild upper respiratory infection, the virus possibly disseminates via B-lymphocytes to the kidneys and bone marrow where it establishes its latency or a low-level persistent infection[24].

### 1.2.3. JCV seroprevalence and viruria

As primary infection normally occurs in childhood or early adulthood, JCV seroprevalence can reach 50% after the first two decades of life, increasing in older individuals. By the age of 70, 80% of the population have specific JCV antibodies, demonstrating the ubiquitous dispersion of this virus in the human population[2526].

Furthermore, JCV viruria has been detected in different geographical areas, with worldwide values ranging from 19 up to 54%[26], rising in selected groups such as immunocompromised individuals, and elderly people[27].

### 1.2.4. Progressive Multifocal Leukoencephalopathy

Despite its high prevalence worldwide, JCV is not associated with disease in healthy individuals. Nevertheless, in immunocompromised individuals it causes a demyelinating disease of the CNS with a debilitating evolution and deadly outcome.

Upon severe cellular immunodeficiency, JCV can reactivate and disseminate to the CNS causing a productive infection in the brain's oligodendrocytes designated as Progressive Multifocal Leukoencephalopathy (PML). Occurring in approximately 5% of all AIDS patients worldwide, it has a mortality rate of 6.1 deaths per 10 million individuals[28].

Overall, the presence of the aforementioned characteristics, such as being a non-enveloped virus with an human specificity host, along with an elevated seroprevalence and a high, constant viruria, suggests JCV as a valuable marker of human waste contamination in waters[29]. However, concerning the specific case of Portugal, until the present time, no study has yet been reported in the literature evaluating the occurrence of JCV in Portuguese wastewaters.

The aim of this study was to create the first national profile of the JCV present in Portuguese domestic and industrial wastewaters and to evaluate the viral removal efficiency by wastewater treatment plants (WWTPs). Therefore, we aimed to (i) investigate the presence of human polyomavirus JCV in wastewaters of WWTP in different locations throughout Portugal in the winter time, (ii) to quantify these viruses before and after the water treatment by means of molecular techniques, and finally (iii) to determine the efficiency of the viral removal process and its impact on Public Health.

# 2. METHODS

**2.1. Wastewater Sample Collection.** Influent (WWI) and effluent (WWE) wastewater samples from fifteen different Portuguese Wastewater Treatment Plants were collected during 2013's winter time. Two hundred fifty mililiters of each water sample was collected in a sterile container, kept at 4ºC for less than 8h until it was stored at -20ºC.

**2.2. Wastewater Treatment Plants.** The location, population equivalent, average load capacity, type of wastewater treated and the process of the wastewater treatment of the analyzed WWTP is summarized in ***Table 1***.

***Table 1.*** *Characteristics of the WWTPs analyzed*

| WWTP Code | Region | Population equivalent | Type of wastewater treated | Discharging points | Average loads ($m^3$/d) | Type of treatment | Process |
|---|---|---|---|---|---|---|---|
| **A** | North | 41955 | Domestic. industrial (residual) | Fervença River | 5685 | Secondary | Activated Sludge with conventional aeration |
| **B** | North | 10000 | Domestic, industrial (mainly) | Tua River | 349 | Tertiary with UV | Activated Sludge with extended aeration |
| **C** | North | 57748 | Domestic, industrial (residual) | Tâmega River | 8069 | Tertiary with UV | Activated Sludge with extended aeration |
| **D** | North | 45257 | Domestic, hospital and industrial | Atlantic Ocean | 8580 | Tertiary with UV | Activated Sludge with medium load aeration |
| **E** | North | 255557 | Domestic and industrial | Ave River | 15000 | Tertiary with UV | Activated Sludge with conventional and extended aeration |
| **F** | North | 300000 | Domestic | Atlantic Ocean | 66718 | Tertiary with UV | Activated Sludge with extended aeration |
| **G** | Center | 213000 | Domestic and industrial | Mondego River | 36000 | Secondary | Trickling Filters |
| **H** | Center | 6850 | Domestic, hospital and industrial | Mondego River | 600 | Secondary | Activated Sludge |
| **I** | Lisbon and Tagus Valley | 700000 | Domestic and industrial | Trancão River | 60000 | Secondary with biofiltration | Activated Sludge |
| **J** | Lisbon and Tagus Valley | 215000 | Domestic and industrial | Tagus River | 50000 | Tertiary with UV | Activated Sludge |
| **K** | Lisbon and Tagus Valley | 756000 | Domestic | Tagus River | 140000 | Tertiary with UV | Biofiltration |
| **L** | Alentejo | 60000 | Domestic, hospital and industrial | Xarrama River | 13720 | Tertiary with UV | Activated Sludge with medium load aeration |
| **M** | Alentejo | 8700 | Domestic | Álamo Brook | 1239 | Tertiary with UV | Activated Sludge with extended aeration |
| **N** | Algarve | 49547 | Domestic | Atlantic Ocean | 9239 | Tertiary with UV | Activated Sludge with extended aeration |
| **O** | Algarve | 30766 | Domestic | Guadiana River | 6141 | Tertiary with UV | Lagoons with extended aeration |

**2.3. Concentration of viral particles.** Seventy five mililiters of WWI and WWE samples were ultracentrifuged at 41000rpm for 90 minutes at room temperature. The pellets were resuspended in 500μL of supernatant and 500μL of chloroform were added. After homogenization and centrifugation at 1500rpm for 10 minutes, the aqueous phase was collected and stored at -20°C until nucleic acid extraction.

**2.4. Nucleic Acid Extraction.** Nucleic acid extraction was performed in 140μL of the sediment suspension obtained in the concentration step, using the commercial kit QIAmp® Viral RNA Mini Kit (QIAGEN®, Izasa, Carnaxide, Portugal) according to the manufacturer's instructions. Viral genome was eluted in 60μL of elution buffer and stored at -20°C until amplification.

**2.5. JCV Nucleic Acid Amplification.** Detection and quantification of JCV DNA in wastewater samples was obtained by a quantitative real-time PCR protocol with a TaqMan probe chemistry using CFX96 Biorad® equipment. For this purpose, a set of four amplification primers and two internal TaqMan probes, previously described were used[21]. Two amplification primers target a highly-conserved region from the JCV genome that codes for T-antigen protein. The amplification of this region provides a measure of JCV copy number regardless of variant origin. The remaining two primers target a specific sequence of the NCCR region of JCV genome from the archetype variant.

Amplification reactions were performed in a final volume of 25μL containing 7.5μL of extracted DNA, 17.5μL of Maxima Probe qPCR Master Mix (2X) (Thermo Fisher Scientific, Lisbon, Portugal), 300 nM of each primer, and 200 nM of TaqMan probe. For the thermal cycling protocol an initial 2-min incubation at 50°C, was followed by 10 min at 95°C, and 45 cycles of 95°C for 15 sec, and 60°C sec for 60 sec.

**2.6. JCV Quantification.** A standard curve for the JCV viral load quantification was constructed using serial decimal dilutions of a plasmid containing the full JCV genome (Advanced Biotechnologies®, Inc.), ranging from 1 to $10^5$ genome copies of JCV per PCR reaction. Quantification of the JCV was determined by comparing the cycle threshold (Ct) value against the appropriate standard curve and the titers were expressed as genome copies/L of wastewater.

**2.7. Quality Control of the extraction and amplification methods.** For each series of nucleic acid extraction, a negative control was made corresponding to the final product obtained from the elution when replacing the wastewater sample by sterile water. For each set of amplification reactions, a positive control and a negative control were included in addition to the samples. Nucleic acid extract obtained from the tissue culture supernatant of JCV Mad-4 infected SVG-cells was used as positive control. The negative amplification control corresponded to the PCR mixture with water instead of the DNA template. Wastewater samples and controls underwent the same conditions as the amplification.

**2.8. Data Analysis.** Following the quantification of viruses, the log removal of JCV achieved for each WWTP and the associated percentage of the removed viruses was calculated using the following equations:

$$Log\ removal = log\left(\frac{WWI\ concentration}{WWE\ concentration}\right)$$

$$\%\ virus\ removed = \frac{(WWI\ concentration - WWE\ concentration)}{WWI\ concentration}$$

# 3. RESULTS

## 3.1. Prevalence and Quantification of JCV in Portuguese wastewaters samples

A total of 30 samples corresponding to both influent (WWI) and effluent (WWE) wastewater were collected during winter time from fifteen different WWTPs distributed throughout Portugal. They were analyzed for the presence of JCV and each positive result was quantified and the JCV concentration expressed as genome copies (GC) per liter. The detection limit of the qPCR was found to be 1 to 10 GC per reaction (corresponding to 3,81 x $10^2$ to 3,81 x $10^3$ CG per liter).

JCV genome was detected in 13 (86.7%) WWI of the 15 evaluated WWTPs but its quantification was only possible in 12 of the positive WWI samples. JCV was present in a concentration that ranged from 6.79 x $10^3$ to 1.70 x $10^6$ GC/L with a median titer of 5.22 x $10^5$ GC/L.

After the treatment process, JCV remained present in 9 (69.2%) WWE of the 13 initial positive assessed WWI. However, we were only able to quantify JCV in 8 out of the 9 WWE positive samples. The effluent concentration of JCV ranged from 6.58 x $10^3$ to 1.26 x $10^6$ GC/L with an average of 1.96 x $10^5$ GC/L. The 4 (30.8%) effluent samples where JCV was not detected in corresponded to WWTPs B, C, I and N. These data are summarized in ***Tables 2*** and ***3***. As it would be expected, the 2 (13.3%) WWTPs (F and O, respectively) without JCV genome detectable at the influent, remained without detectable JCV genome at its effluent wastewater.

***Table 2.*** *Summary of results obtained by analysis of WWI and WWE from 15 Portuguese WWTPs for the detection and quantification of JCV*

| | Prevalence | | Quantification |
|---|---|---|---|
| ***WWI*** | No. Positive/total samples | No. Negative/total samples | Min – Max (median value) for the 12 WWI quantified samples[a] |
| | 13/15 (86.7%) | 2/15 (13.3%) | 6.79 x $10^3$ – 1.70 x $10^6$ (5.22 x $10^5$) GC/L |
| ***WWE*** | No. Positive/Positive WWI | No. Negative/Positive WWI | Min - Max (median value) for the 8 WWE quantified samples[a] |
| | 9/13 (69.2%) | 4/13 (30.8%) | 6.58 x $10^3$ – 1.26 x $10^6$ (1.96 x $10^5$) GC/L |

[a] One influent and one effluent positive samples were not quantified.

## 3.2. Efficiency of the WWTP viral removal process

The efficiency of the viral removal process by the WWTPs was expressed by the log genome copies of JCV removed per liter and was calculated each time a WWI sample was positive in the qPCR. The results obtained were included in ***Table 3***.

***Table 3.*** *Efficiency of JCV removal by different WWTPs*

| WWTP | JCV Detection | | Log GC removed/L | Removed viral load (%) |
|---|---|---|---|---|
| | WWI | WWE | | |
| A | + | + | 1.402 | 96 |
| B | + | - | 5.790 | 100 |
| C | + | - | 6.058 | 100 |
| D | + | + | 0.939 | 88.5 |
| E | + | + | ND[a] | ND[a] |
| F | - | - | -------- | -------- |
| G | + | + | 0.131 | 26.1 |
| H | + | + | 1.867 | 98.6 |
| I | + | - | 4.836 | 100 |
| J | + | + | 0.826 | 85.1 |
| K | + | + | ND[a] | ND[a] |
| L | + | + | -1.372 | -22.5 |
| M | + | + | 1.499 | 96.8 |
| N | + | - | 5.484 | 100 |
| O | - | - | -------- | -------- |
| | **13/15 (86.7%)** | **9/13 (69.2%)** | **2.88**[b] | **89.1**[b] |

+ Detected; - Undetected;
[a] ND – Not Determined; [b] Data from the *WWTP L* not included.

The mean removal ratio of JCV in the treatment process was 2.88 log genome copies/L, with the lowest value being achieved by WWTP G, with 0.131 (26.1%) log GC removed/L. In contrast, most WWTPs attained a JCV removal superior to 0.826 (85.1%) log GC/L being the total removal (100%) of JCV to an undetectable viral load accomplished by 4 WWTPs - WWTP B, C, I and N -, where the removal efficiency reached more than 4 logs.

Furthermore, one WWTPs - WWTP P - exhibited an increase of 1.372 (22.5%) log GC/L in the WWE when compared to the WWI. Therefore, it was not included in the calculations of the average values regarding the WWTP efficiency for viral removal, such as the median log GC removed/L and the median percentage of viral load removed by the totality of the WWTPs.

# 4. DISCUSSION

The current Portuguese legislation that establishes the safety and quality of water for human use and that protects the waters against the pollution caused by the discharge of residual waters does not currently address the control of some highly stable pathogens, like viruses[30]. Regardless of the lack of data regarding the viral profile in Portuguese waters, some viruses like Hepatitis A virus and Norovirus genotype I have been detected in Tagus recreational waters[31]. No reference has yet been made to JCV, a ubiquitous human virus that has been found in several sewage and environmental waters from around the world and suggested, by some authors, as a possible alternative marker of human waste contamination in the aquatic environment[14].

In this study, we intended to evaluate the presence of JCV in Portuguese influent and effluent wastewaters as well as the removal efficiency of this virus by fifteen different WWTPs distributed across the country.

Previous studies have demonstrated that in Africa, more precisely in Egypt and South Africa, in several countries across Europe such as Spain, France and Sweden, in New Zealand and also in USA, rarely does an influent wastewater sample not present JCV[13,32]. The prevalence of JCV in WWI in our study was 86.7% with a median concentration titer of $5.22 \times 10^5$ GC/L. This is comparable to the values reported in the influent wastewaters of our neighbor Spain, where it was found with an average concentration of $2.6 \times 10^6$ GC/L in a WWTP in Barcelona[33], but it is also similar to the results described in Addis Ababa, Ethiopia, where its concentration was in the order of $10^5$ GC/L[34], and from across the ocean, in Rio de Janeiro, Brazil, where JCV had the mean concentration of $5.98 \times 10^5$ GC/L in influent samples[35]. Due to the high JCV seroprevalence and viruria observed worldwide, and in the specific case of Portugal[26], and since the totality of the analyzed WWTPs handled domestic residues, urine may represent the main source of JCV virions, which will end up incorporating sewage waters.

However, JCV was not detected in both WWI and WWE of 2 WWTPs despite its domestic origin. One of these WWTPs - WWTP F - was located in the north, and the other - WWTP O - in the south, in Algarve. Since both WWTPs were in different regions of Portugal and had a difference in the average load and in the population equivalent served of almost 10-fold, we may postulate that the people living in those areas may not excrete JCV

viral particles into sewage in a concentration sufficiently high to be detected in the wastewaters by the molecular techniques used. In order to confirm this hypothesis, it would be advisable to investigate JCV viruria and seroprevalence in the inhabitants of these regions.

Regarding the 13 WWTPs where JCV was detected at entrance, its concentration was also evaluated at the exit, after the treatment process. The wastewater prevalence of JCV showed a reduction from 86.7% to 69.2%, corresponding to a mean removal efficiency of 2.88 logs of JCV, which is in agreement with previous studies where it was superior to 2 logs[33,36]. The lowest viral load removed from the wastewater samples was achieved by WWTP G in the Center region of Portugal, which might be explained by this WWI sample presenting the highest JCV viral load of all the analyzed wastewaters, as well as the use of secondary treatment with trickling filters, which does not seem a very efficient treatment process.

In contrast, the effluent samples of 4 WWTPs did not present detectable JCV, achieving a JCV removal superior to 4 log GC/L. They all used an activated sludge process to clear the water of organic material, suspended solids and other components such as viruses by adsorption to the sludge flocs in the aeration tank. One WWTP employed a secondary treatment with biofiltration, whereas the other 3 used a tertiary treatment with UV.

Since an activated sludge process followed by a tertiary treatment with UV has shown a reduction in effluent viral loads from 85.1% to 100%, this appears to be the most efficient type of wastewater treatment. This situation does not appear to be related with the WWTP location or with the population equivalent served, because they were located in different regions throughout the country such as the north, Lisbon, Tagus Valley and Algarve, and handled a range of average loads varying from 349 to 50000 $m^3$/day. As JCV was detected in effluent waters of some WWTPs using an activated sludge process coupled with an UV disinfection but not in others WWTPs with the same type of treatment, it is critical to define the procedures when addressing viral removal from wastewaters.

However, none of the calculations made regarding the WWTP efficiency of JCV removal included WWTP L. This south-located WWTP registered an increase of 1.372 log GC/L in the WWE, which we hypothesize to be related to the lack of efficiency of the filtering system which may release the previously retained viruses and/or with the adsorption phenomenon that occurs during the activated sludge process which might after affect the disinfection process. As a matter of fact, during the activated sludge process occasionally

sludge flocs with low settling properties may be formed, which can adsorb and concentrate the viruses present in the reactor. As a consequence, these aggregates remain in the treated water and can protect JCV from disinfection with UV light, which then will not be properly eliminated from the wastewater[37].

Regardless of the wide range of average loads, there is a high discrepancy in the population equivalents covered by the WWTPs. Although a previous study reported that the virus presence and concentration were generally independent of the size of population served by the WWTP[8], a study of the viruria and antibodies throughout the country is suggested in order to detect the main focuses of this virus.

Although qPCR represents a sensitive method to detect JCV viral genome in urban wastewaters, it cannot distinguish between infectious and noninfectious virions[38]. Since cell culture lines for JCV replication have limited availability and robustness, nor there is an animal model to study this virus viability, we cannot estimate the number of infectious particles present in the analyzed samples. Therefore, the principal limitation of this highly specific and sensitive technique remains the inexistence of direct correlation between the viral genome copies quantified by qPCR and the viral infectivity[6].

## 5. CONCLUSIONS

To our knowledge, this was the first epidemiologic study performed in Portugal's wastewaters regarding JCV. The results demonstrate that JCV is frequent in Portuguese wastewaters, and 69.2% of WWTP were not able to efficiently eliminate this virus. These findings may suggest JCV as an indicator of human waste contamination and as a model for the presence of other potentially pathogenic viruses. If these viruses are not properly eliminated before water introduction into the environment, human exposure to such viruses through contact or accidental ingestion of contaminated surface waters could result in waterborne infections with harmful consequences.

For that reason, viral surveillance of water quality proves itself crucial with regards to identifying possible sources of contamination and preventing the widespread dispersion of certain potentially pathogenic agents which could cause a public health water-related hazard.

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