Effect of disinfection agents and quantification of potentially viable Leptospira in fresh water samples using a highly sensitive integrity-qPCR assay
Elise Richard, Pascale Bourhy, Mathieu Picardeau, Laurent Moulin, Sébastien Wurtzer

To cite this version:
Elise Richard, Pascale Bourhy, Mathieu Picardeau, Laurent Moulin, Sébastien Wurtzer. Effect of disinfection agents and quantification of potentially viable Leptospira in fresh water samples using a highly sensitive integrity-qPCR assay. PLoS ONE, Public Library of Science, 2021, 16 (5), pp.e0251901. 10.1371/journal.pone.0251901. pasteur-03249529

HAL Id: pasteur-03249529
https://hal-pasteur.archives-ouvertes.fr/pasteur-03249529
Submitted on 4 Jun 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution 4.0 International License
Effect of disinfection agents and quantification of potentially viable Leptospira in fresh water samples using a highly sensitive integrity-qPCR assay

Elise Richard1,2, Pascale Bourhy2, Mathieu Picardeau2,*, Laurent Moulin1,*, Sébastien Wurzer1

1 Eau de Paris, DRDQE, Ivry-sur-Seine, France, 2 Institut Pasteur, Unité Biologie des Spirochètes, Paris, France

* mathieu.picardeau@pasteur.fr (MP); laurent.moulin@eaudeparis.fr (LM)

Abstract

Leptospirosis is an emerging worldwide zoonotic disease, but the general biology of the causative agents is still poorly understood. Humans are an occasional host. The main risk factors are water-associated exposure during professional or recreational activities or during outbreaks in endemic areas. Detecting the presence of pathogenic bacteria in aquatic environments and their capacity to resist various inactivation processes are research fields that need to be further developed. In addition, the methods used for detecting and enumerating Leptospira still need to be improved. We aimed to describe a new quantitative polymerase chain reaction coupled to propidium monoazide treatment (PMAqPCR) that targets not only total Leptospira but also discriminates pathogenic from non-pathogenic Leptospira while also addressing PCR inhibitors, a frequently encountered problem when studying environmental water. In a second step, the killing efficiency of Leptospira to different treatments was tested and PMAqPCR compared to culture-based enumeration. This provided information about the effects of temperature, as well as ultraviolet and chlorine disinfection, that are both related to water treatment processes, in particular for the production of drinking water, on the persistence of both saprophytic and pathogenic Leptospira. Finally, PMAqPCR was used for the detection of Leptospira in freshwater samples for a proof-of-concept. In conclusion, our method could be used for routine freshwater monitoring and allows better evaluation of the presence of Leptospira, allowing evaluation of the bacterial dynamics in a designated area or assessment of the efficacy of water disinfection processes.

Introduction

Pathogenic Leptospira are responsible for a global zoonosis, leptospirosis, in which humans are found to be occasional hosts in a cycle involving wild and domestic animals. One million severe cases are reported every year worldwide [1]. Leptospirosis can take various forms, from a flu-like syndrome (fever, myalgia, or headache) to a multisystem disorder, with icteric and hemorrhagic syndrome accounting for 20% of cases, causing at least 60,000 deaths a year.
This disease occurs worldwide but the incidence is the highest in tropical regions [1–3]. However, developed countries, including those in Europe [4, 5], have also observed an increase in the number of reported cases.

Animal reservoirs, mainly rodents, excrete Leptospira through urine and contaminate the environment and, potentially, water resources. The dissemination of these bacteria into the environment can allow other animals or humans to be newly infected. Humans can be infected through direct or indirect contact with urine or water contaminated by Leptospira. In this context, leptospirosis can be both an occupational disease (affecting veterinarians, farmers, sewer workers, etc.) and a recreational disease associated with water-related activities (bathing, kayaking, canyoning, etc.) [6, 7]. Leptospirosis is considered to be an emerging zoonotic disease partially due to global warming [8] and more frequent and severe flooding events [9]. Water exposure appears to be the major risk factor [10]. Floods increase the risk by exposing humans and animals to Leptospira that are flushed out of their environment. In France, a study conducted from 1995 to 2005 showed that 42% of patients became infected after practicing water sports, 19% after contact with backwater (ponds, swamps, wells, water holes), and 19% during professional activities [11]. Recreational water activities are becoming increasingly popular and, for example, a recent study described a cluster of 14 kayakers that exhibited leptospirois symptoms after contact with water in Brittany, France [12]. Leptospirosis cases have also been reported after the consumption of drinking water. Contamination may be caused by failure during water treatment processes or the lack of any water treatment [13, 14].

To date, sixty-four species and more than three hundred serovars of Leptospira have been described and classified into four phylogenetic subclades: pathogenic P1 and P2 and saprophytic S1 and S2 [15]. Saprophytes are non-infectious species that can multiply in the environment, whereas pathogens are mostly isolated from both humans and animals and, occasionally, the environment, in which they can survive for a few weeks [16]. Leptospira are slow-growing bacteria (generation time of 5 and 20 hours for saprophytes and pathogens, respectively), requiring a specific and rich medium [17, 18]. They are fastidious to isolate because of possible contamination with fast-growing interfering flora [19–21]. The recent development of a cocktail of antibacterial and antifungal antibiotics to which Leptospira are resistant (sulfamethoxazole, trimethoprim, Fosfomycin, 5-fluorouracil, amphotericin B), thus limiting the development of interfering microorganisms [22], should facilitate their culture.

Little information is available on Leptospira contamination of surface water and its seasonal evolution. Several authors have highlighted the importance of assessing the bacterial concentration in water resources [19]. Such evaluation is essential for monitoring population exposure to Leptospira to ensure public health. Currently, scarce data are available about the persistence of Leptospira in the environment and their resistance to physico-chemical parameters or disinfection. According to Huang et al. [23], Leptospira genomes were still detected in tap water after treatment, whereas most other pathogens disappeared.

The effects of temperature and pH on Leptospira were studied by Chang et al. [24]. In this study, the authors showed that optimal survival conditions for Leptospira are neutral to slightly alkaline pH and a temperature of approximately 25 to 27°C. Other studies [25, 26] showed that pathogenic strains of Leptospira could survive for > 20 months, despite deleterious storage conditions (cold, nutrient-poor acidic water, etc.). To date, no study has described the antimicrobial effect of ultraviolet (UV) radiation exposure, a process commonly used in tap water production, on the viability of Leptospira.

Molecular diagnostic methods appear to be more sensitive, specific, and rapid than culture [27, 28]. qPCR appears to be applicable for Leptospira detection, although there is no consensus concerning molecular methods. In most recent studies, real-time PCR assays have been based on Taqman technology [29, 30] instead of the SYBR Green approach [27, 31] due to its
higher specificity [29]. Molecular methods for *Leptospira* detection have been mainly based on the detection of housekeeping genes, such as *rrs* 16S [32], *gyrB* [33], or *secY* [27]. Pathogen-specific *Leptospira* can be detected using genes such as *LipL32* [29], *ligA*, or *ligB* [34]. However, most analyses solely allow quantification of the *Leptospira* genome, irrespective of the viability of the bacteria. These methods tend to overestimate the true risk, which is solely linked to viable bacteria. The use of qPCR coupled with a DNA intercalating agent pretreatment was already described to evaluate the integrity of *Leptospira* [35]. This approach avoids the amplification of permeable bacteria (i.e. damaged or dead bacteria) and, thus, partially limits their overestimation while maintaining the advantages of molecular methods [36].

Our objective was to create a rapid and sensitive method for the quantification of potentially viable *Leptospira* in water samples, discriminating pathogens from saprophytes. This PMAqPCR detection method was also used to evaluate the resistance of *Leptospira* to different treatments such as heat inactivation, chlorine treatment, and UV exposure.

**Materials and methods**

**Sample collection and culture media**

Twenty-five *Leptospira* DNA samples, including that of eight saprophytes (four for S1 and four for S2) and seventeen pathogens (twelve for P1 and five for P2) (S1 Table) were used to test the performance of the PCR assays. The pathogen *L. interrogans* serovar Manilae strain L495 and the saprophyte *L. biflexa* serovar Patoc strain Patoc 1 were used for inactivation tests. Strains were incubated at 30°C in liquid Ellinghausen, McCullough, Johnson, and Harris (EMJH) medium [37, 38].

For colony numeraion, EMJH medium supplemented with 1.2% agar was used. Bacterial numbers were determined using a Petroff-Hauser chamber and dark-field microscopy. *Leptospira* DNA and strains were provided by the National Reference Center (NRC) for Leptospirosis (Institut Pasteur, Paris, France). Other bacterial strains used to determine the specificity of the pan-*Leptospira* PCR assay were provided by the Eau De Paris Laboratory.

**Extraction**

For inactivation tests, DNA was extracted using the Q400 protocol with a QIAsymphony® DSP Virus/Pathogen Midi Kit (Qiagen) and the MagNA Pure Compact System (Roche®) for environmental monitoring. Nucleic acids were extracted from 200 μL of sample by elution into 50 μL, according to the manufacturer’s protocols.

**PCR assays**

The multiplex qPCR was tested using *Leptospira* strains isolated from patients and environmental strains. The specificity of the qPCR methodology was established by the analysis of *Leptospira* strains and other bacterial species (S1 Table).

The Pan-*Leptospira* PCR was based on the *rrs* (16S) gene sequence alignment of 113 *Leptospira* species, including saprophytes and pathogens. The primers 16S-F267 (5’-GGCACAATTGAACTGAG-3’) and 16S-R336 (5’-CCCCATTTGACAAAGATCTTAAAC-3’), associated with the probe 16S-P286 (5’-FAM-CACGTTCCATACCTCT-3NFQ-MGB-3’), achieve the amplification of a 70-bp fragment. The pathogenic-*Leptospira* PCR was based on the *LilpL32* gene sequence alignment of 30 pathogenic *Leptospira* species. The primers LipL32-F164 (5’-CTGTTGATCACCACTATTACGG-3’) and LipL32-R298 (5’-GGGAAATCATACGAACTC-3’), associated with the probe LipL32-P188 (5’-HEX-TAAAGCCAGGACAAGCGCG-BHQ1-3’), achieve the amplification of a 135-bp fragment.
Sequences from the Genbank database of the National Center for Biotechnology Information (NCBI) were used to design the primers (S2 and S3 Tables). The primers and probes for both PCR assays were designed using AlleleID® software version 7 (http://premierbiosoft.com/bacterial-identification/index.html).

Two plasmids (16S and LipL32) were generated as positive PCR controls using pCR2.1 (Topo TA-cloning, Life Technologies, Carlsbad, CA). An internal positive competitive amplification control (IPC) was used [39] to evaluate the presence of PCR inhibitors. The IPC is composed of a partial sequence of the human β-actin gene and was cloned into the pCR™2.1-TOPO® vector (Life Technologies, Carlsbad, CA) and flanked by the LipL32 primers using an approach similar to that described by Wurtzer et al. [40]. The primers were LipL32_BACT-F1146 (5’-CTGTGATCAAATACGTGttGCAGGAGTATGACGAGT-3’) and LipL32_BACT-R1215 (5’-GGGAACTCATACGAACTCttCAAGAAAGGGTGTAACGCAACTAA-3’). The probe was BACT_P1172 (5’-CCCCCTCCATCGTCCACCGCAATG-3’).

Each PCR reaction was performed using the TaqMan® Fast Virus 1-Step Master Mix (4444434). Unlike other polymerases, this Taq polymerase was less affected by inhibition due to the environmental sample matrix. The RT step was removed to achieve rapid diagnosis. Nevertheless, residual activity of the RT step remained, thus conferring better sensitivity than other kits.

For the pan-Leptospira PCR (16S), the F267 primer was used at 500 nM and the R336 primer and P286 probe at 100 nM. Simultaneously, oligonucleotides targeting pathogenic-Leptospira PCR (LipL32) were used at 600 nM for primer F164 and primer R298, and 200 nM for probe P188. IPC was added to the reaction mixture at 10^4 copies and detected using the IPC probe at 100 nM.

The PCR reaction was performed in a 20-μL reaction volume using a ViiA 7 real-time PCR system (Life Technologies, Carlsbad, CA) in 96-well plates. The thermal profile consisted of an initial denaturation step at 94°C for 20 s, followed by 45 cycles at 94°C for 5 s and 60°C for 30 s. FAM Yakima Yellow and Tamra fluorescence were detected at the end of the elongation step.

Three plasmids, positive controls, and IPC were quantified using an ultra-sensitive fluorescent nucleic acid stain for quantitimating double-stranded DNA (Quant-IT™ PicoGreen® dsDNA reagent), according to the manufacturer’s protocol.

### Propidium monoazide treatment

Before DNA extraction, samples were incubated with propidium monoazide (PMAxx) to ensure bacterial integrity [41–43]. The PMAxx solution was diluted in molecular grade water to obtain a final concentration of 10 mM and aliquots were stored at -20°C. Based on pilot studies (data not shown), the PMAxx dye was used at a final concentration of 0.1 mM to pre-treat the samples. After mixing, samples were incubated on ice, in the dark, for 30 min. Photo-activation was performed for 15 min using the PhaST Blue system (IUL, Barcelona, Spain). In this study, PMAxx-qPCR was also called integrity qPCR and is sometimes wrongly called viability PCR.

### Persistence tests

Persistence tests were simultaneously performed in duplicate on two laboratory strains: Leptospira biflexa serovar Patoc (saprophyte) and Leptospira interrogans serovar Manilae (pathogen).

For each test, bacterial inactivation was modeled using GraphPad Prism version 8 software (GraphPad, La Jolla, CA). After testing several models, the Leptospira reduction data were adjusted using a sigmoidal dose–response model based on the equation:

\[
Y = Ct + \frac{(C_0 - Ct)}{1 + (\frac{Ct}{C_{50}})^2}
\]
In this equation, X was the studied parameter (temperature, CT value, time, UV dose), the variable “CI” the Leptospira concentration at a given time during the assay, “C0” the Leptospira concentration at T0, and IC50 the value of X at which the response was halfway between C0 and C0. Finally, α described the slope of the curve.

As mentioned in every test referenced below, two detection methods were used in this study: integrity qPCR (described above) and culture on specific EMJH medium.

**Heat exposure.** Bacterial cultures were adjusted to a final concentration of approximately 10^4 Leptospira/mL in 1X PBS (pH 7.4). Each sample was aliquoted in duplicate and incubated for 1 h at various temperatures in a thermal cycler (Mastercycler nexus, Eppendorf). Samples tested at 4°C were stored on ice.

Half of each sample was used for plating after resuspension in EMJH medium and half for PMA-qPCR analysis after resuspension in 1X PBS and the addition of PMAxx (100 μM final concentration). Nucleic acids were extracted using a QIAasympohony instrument (QIAGEN).

**Ultraviolet (UV) exposure.** Exponential phase cultures of L. interrogans and L. biflexa were centrifuged at 8,000 x g for 10 min and the pellet resuspended in 1X PBS to a final concentration of 10^9 Leptospira/mL. The bacterial suspension was split into microtubes (450 μL/tube). For each strain, eight UV conditions were tested in duplicate, from 0 mJ/cm² to 40 mJ/cm² (the last is the UV dose applied in drinking-water treatment plants) using a 254 nm UV lamp (Phillips, Amsterdam, Netherlands) at room temperature. A digital UVC radiometer (IL Metronic Sensortechnik GmbH, Germany) was used to monitor UV irradiation.

Samples were subjected to various exposure times according to the tested dose and half used for plating on EMJH agar and half for PMA-qPCR analysis.

**Chlorine treatment.** Before starting the experiments, laboratory glassware was prepared by soaking it in a sodium hypochlorite solution containing 40 mg/L free active chlorine. Glassware was then intensively cleaned with chlorine demand-free (CDF) water. CDF water was used to prepare all experimental solutions and was prepared using a Milli-Q® Purification system with a Biopak® Polisher (Merck, Darmstadt, Germany). A stock solution of sodium hypochlorite (81 g/L) was used to prepare an intermediate solution of 0.5 mg/L with CDF water (pH 6).

As described previously, L. interrogans and L. biflexa were incubated at 30°C in liquid EMJH medium until reaching a concentration of approximately 10^6 Leptospira/mL. Leptospira cultures were centrifuged at 5,000 x g for 15 min, the supernatant discarded, the pellet washed in 0.9% NaCl, and finally resuspended in 0.9% NaCl. Leptospira suspensions were prepared by adding bacteria (final concentration of 10^4 Leptospira/mL) to a chlorine solution (0.5 mg/L). The free chlorine concentration was measured before and after addition of the Leptospira suspension using a Pocket colorimeter II (Hach Lange, Dusseldorf, Germany) after activation of the DPD reagent (N,N-diethyl-p-phenylene-diamine).

Unreacted free chlorine was quenched by the addition of sodium thiosulfate (100 mg/L) to stop the activation reaction. After chlorine treatment, each sample was analyzed by culture, qPCR, and integrity qPCR.

**Environmental sample collection**

Thirty-four surface-water samples were collected in Paris, including an area which includes a controlled bathing section during the summer period (48.885441128096, 2.37411186180 21346). The surface water had not undergone any sanitation treatment.

Samples were analyzed within 24 h of collection. Surface-water samples (500 mL) were concentrated by successive centrifugation steps down to 400 μL. Half of the sample was treated with PMAxx (as described in the section on propidium monoazide treatments) and half remained untreated. All samples (with and without PMAxx) were then extracted and analyzed by qPCR.
Statistical analysis with GraphPad software

In addition to its use in the persistence test to model bacterial inactivation, GraphPad Prism software version 8 (GraphPad, La Jolla, CA) was also used for statistical analysis. Normality of the distribution was assessed using the Shapiro-Wilk test. Paired groups were tested using the nonparametric Wilcoxon matched pairs signed rank test. P-values < 0.05 were considered significant.

Results

Leptosira multiplex qPCR

First designed to operate separately, the three qPCR assays were adapted to be performed in a multiplex assay to obtain a single reaction (effectiveness demonstrated below in the section on the analytical sensitivity of the method). This was made possible through the use of different fluorophores. Total Leptosira, pathogenic Leptosira, and IPC were targeted by different dyes: FAM, Yakima Yellow, and TAMRA, respectively.

Several enzymatic master mixes were tested to optimize the qPCR reaction (Fig 1). The Fast Virus 1-Step Master mix resulted in better detection limits than amplification using two other enzyme mixes, with a difference of approximately 10 CT between qPCRs. In addition, the efficiency and coefficient of determination (R2) showed the Fast Virus 1-step master mix to outperform the others in this study (Table 1).

As indicated previously, the two qPCR assays for detecting Leptosira and the IPC were first designed to operate independently. We compared the performance of the qPCR assays separately (simplex mode) or together (multiplex mode). The simplex and multiplex modes provided the same results for the 16S target (efficiency of approximately 100% and R2 of approximately 0.98) (Fig 2) and these two parameters were slightly higher in the multiplex mode (89% of efficiency of 89% and R2 of 0.987) than the simplex mode (efficiency of and 84% R2 of 0.984) for the LipL32 target (Table 2).

![Fig 1. qPCR tests on range of concentrations of the Leptosira 16S plasmid with various enzymatic mixes (Taqman® Fast Virus 1-Step Master Mix, Quant Nova® Multiplex Master Mix, Taqman® Fast Advanced Master Mix). The colored dotted lines correspond to the number of cycles required to obtain the smallest detectable quantity of genome, shown by the black dotted line.](https://doi.org/10.1371/journal.pone.0251901.g001)
Table 1. Comparison of R² and efficiency between various enzymatic mixes.

|               | TaqMan® Fast Virus 1-Step Master Mix | QuantiNova® | Taqman® Fast Advanced Master Mix |
|---------------|-------------------------------------|-------------|---------------------------------|
| R²            | 0.984                               | 0.996       | 0.994                           |
| Efficacy (%)  | 95.8                                | 81.9        | 71.3                            |

The efficiency and R² were compared between various enzymatic mixes from two suppliers: TaqMan® (Applied Biosystems) and QuantiNova® (Qiagen).

https://doi.org/10.1371/journal.pone.0251901.t001

Ten-fold serial dilutions (ranging from 10⁶ copies/μL to 10³ copies/μL), followed by two-fold serial dilutions (from 10⁵ copies/μL to 1 copy/μL), of L. interrogans serovar Manilae were used to determine the true analytical sensitivity of the detection method. The limit of detection (LoD) was determined as the quantity of plasmid that could be detected in 95% of the replicates. The limit of quantification (LoQ) was the lowest concentration of plasmid that could be properly quantified in a standard range. The LoD, LoQ, and amplification range were separately determined for each target (16S, LipL32, and IPC) to determine their own parameters. The LoD and LoQ of 16S were both at a CT value of 33.09, corresponding to 1 bacterium/well. For LipL32, the LoQ was at a CT value of 37.97, corresponding to 125 bacteria/well (Fig 3), and the LoD at 38.66, corresponding to 86 bacteria/well.

We tested the specificity of the pan-Leptospira PCR on bacterial strains other than Leptospira. The assay was found to be specific for Leptospira spp., as none of the six other pathogenic organisms were amplified. Moreover, we tested the specificity of pathogenic-Leptospira PCR. None of the nonpathogenic Leptospira were detected by the assay. The sensitivity was also measured, and results were mentioned in S1 Table.

**Evaluation of the efficacy of disinfection treatments of Leptospira**

Addition of the integrity assay to the detection by qPCR enabled the specific detection of unaltered bacteria and viable but non-cultivable (VBN) bacteria in the samples [44]. An intercalating agent was added and photoactivated to avoid the amplification of “free” or not protected DNA by the qPCR. This protocol was tested on Leptospira subjected to disinfection treatments (temperature, UV radiation, chlorine). The bacterial concentration was evaluated by three different methods to validate the use of PMAqPCR: microscopic enumeration, colony-forming units by plating, and PMAqPCR (Table 3).

![Comparison between simplex and multiplex qPCR for the two targets: 16S gene and LipL32 gene; using range of plasmids.](https://doi.org/10.1371/journal.pone.0251901.g002)
The titrations obtained by microscopy and plating were similar, giving approximately $10^7$ Leptospira/mL. Quantification of the two genes indicated concentrations 25 to 60 times higher.

Various experiments were independently carried out with fresh Leptospira suspensions. Thus, the initial concentration of bacteria could have differed. Nonetheless, this parameter did not interfere with the interpretation of results because the analysis was based on the log of inactivation.

Heat inactivation was assessed by culture analysis and PMA-qPCR analysis. The pathogenic strain appeared to be more resistant than the saprophytic strain. Indeed, the curve of the slope from the culture analysis was lower for *L. interrogans* (-6.945) than *L. biflexa* (-8.211). However, the temperature which induced a reduction of the concentration of the bacteria by half was broadly similar for both strains (32.12°C for *L. interrogans* and 32.91°C for *L. biflexa*) (Fig 4).

*Leptospira* appeared to not be cultivable on EMJH solid plates beyond 37°C and not detectable by PMA-qPCR beyond 55°C.

The time of exposure to free chlorine was calculated from the kinetics of free chlorine consumption and adjusted to reach a CT value equal to 10 mg.min/L (Fig 5). This CT value corresponded to the concentration of this powerful oxidant (mg/L) multiplied by the time (min) of exposure. In this case, we selected the time at which the area under the free chlorine CT curves

| Table 2. Comparison of $R^2$ and efficiency between the simplex and multiplex qPCR assays developed in this study. |
|---------------------------------------------------------------|
| Gene             | simplex | multiplex | Gene             | simplex | multiplex |
| $R^2$            | 0.9827  | 0.9846    | 0.9820           | 0.9873  |
| Efficacy (%)     | 104     | 84        | 104              | 89      |

The efficiency and $R^2$ were compared between the simplex and multiplex modes for two targets: *rrs* (16S) gene for all Leptospira and LipL32 gene for pathogenic strains.

https://doi.org/10.1371/journal.pone.0251901.t002

---

Fig 3. Determination by PMA-qPCR of LoD for the LipL32 gene using serial dilution (eight replicates for each dilution).

https://doi.org/10.1371/journal.pone.0251901.g003
was 10 mg.min/L. The time of exposure ranged from 0 to 27 min. Chlorine-dependent inactivation was assessed by culture and molecular methods. We observed a slight decrease in the concentration of *L. interrogans* in the presence of the hypochlorous acid during the assay by PMAqPCR. The effect was stronger for *L. biflexa*, with 1.5 log removal at CT = 4mg.min/L and up to 2 logs of inactivation at the end of the experiment (CT = 10mg.min/L). Both strains showed strong sensitivity to chlorine treatment by culture assay (Fig 6). No *L. interrogans* grew on the EMJH culture media after exposure to the lowest chlorine dose (CT = 0.001 mg.min/L), whereas total inactivation of *L. biflexa* occurred at CT = 0.1 mg.min/L.

UV$_{254}$ light inactivates microorganisms by targeting their nucleic acids, resulting in the inhibition of DNA replication and thus their growth in culture. Culture analysis showed the same kinetics for the action of UV radiation for both strains (Fig 4). Three logs of removal were achieved at 10 mJ/cm$^2$, whereas 40 J/cm$^2$ is currently used in drinking water treatment plants. UV light kills cells by damaging their DNA and does not usually result in cell lysis; the

### Table 3. Comparison of *Leptospira* detection between microscopy, plating and molecular analysis.

|                      | Microscopic enumeration | Plating enumeration (EMJH) | PMAqPCR (PBS) | PMAqPCR (EMJH) |
|----------------------|-------------------------|-----------------------------|---------------|---------------|
| **L. biflexa Patoc** | n = 9                    | n = 9                       | (165)         | (165)         |
|                      | 1.00E+07                 | 1.66E+07                    | 4.25E+08      | 4.21E+08      |
| **L. interrogans Manilae** | n = 6                 | 1.63E+07                    | 1.11E+09      | 7.10E+08      |
|                      | (LipL32)                 | 7.45E+08                    | 4.65E+08      |

Microscopic enumeration was assessed by dark field microscopy using a Petroff-Hauser chamber. Plating enumeration was performed using EMJH semi-solid medium after seven days of incubation for *L. biflexa* and more than two weeks of incubation for *L. interrogans*. Detection by molecular analysis was performed in PBS and EMJH liquid medium.
lack of decrease for PMAqPCR signal is therefore consistent with the preservation of membrane integrity.

**Proof-of-concept of application for environmental monitoring.** Our PMAxx-qPCR method was tested on 32 surface water samples, with and without PMAxx. The results are summarized in Fig 7. A Wilcoxon signed-rank test was performed to show whether the data with PMAxx were significantly different from those without. The two sets of data were significantly different (p-value < 0.0001). The genome concentration using PMAxx-qPCR was consistently lower than that estimated using conventional qPCR in 100% of cases, signifying that a significant proportion of target DNA was “free” or inside permeable bacteria. Adding PMAxx reduced overestimation by amplifying only the genomes of non-permeable bacteria.

![Fig 5. Free chlorine consumption kinetics for *L. biflexa* serovar Patoc and *L. interrogans* serovar Manilae. The time of exposure to free chlorine was adjusted to maintain a CT-value equal to 10 mg.min*L⁻¹*.](https://doi.org/10.1371/journal.pone.0251901.g005)

![Fig 6. Chlorine inactivation for *L. biflexa* serovar Patoc and *L. interrogans* serovar Manilae by culture and PMAqPCR. Results were gathered for the two strains *L. biflexa* serovar Patoc (blue, red) and *L. interrogans* serovar Manilae (green, orange). The X-axis indicates the CT value: powerful oxidant concentration (mg/L) * time (min) of exposure. The left Y-axis represents the median bacterial culture results (in colony-forming units) after several days of incubation in EMJH semi-solid medium at 30°C (triangle). The right Y-axis represents the median PMAxxqPCR results (in genome units) after extraction and molecular biology assay based on the 16S gene (circle).](https://doi.org/10.1371/journal.pone.0251901.g006)
Discussion

Leptospirosis is an emerging waterborne zoonosis of global importance for both humans and animals. However, there is also an urgent need for a robust and easy-to-use Leptospira detection method for environmental samples. Traditional culture methods are fastidious and Leptospira are slow-growing bacteria and cultures can be contaminated by other microorganisms [28]. Alternative detection methods, such as qPCR, have thus been proposed but no gold-standard has been implemented for environmental monitoring. In general, molecular methods allow a rapid measurement of the pathogen concentration coupled with high sensitivity and specificity. In the present study, we implemented a triplex qPCR based on Taqman technology for the detection and quantification of both pathogenic and saprophytic Leptospira spp. using the rrs gene and specifically pathogenic strains based on LipL32 gene amplification. An internal competitive amplification control was added to the analysis to evaluate the inhibition of amplification resulting from the samples. In addition, we coupled this method with a bacterial integrity assay. A triplex PCR (two genes and an internal control) was designed and used to discriminate Leptospira from subclades P1 and P2 in animal samples [45]. This multiplex qPCR was set up, according the MIQE guidelines [46], to detect all Leptospira and selectively discriminate between pathogenic and saprophytic strains. An internal control was also added as a supplementary monitor to check for inhibition of amplification in environmental samples.

Genome amplification by qPCR also has certain limitations. Amplification of any targeted DNA present in the sample makes it impossible to distinguish between live and dead cells, resulting in potential overestimation of the bacterial concentration and the risk of infection. It was also shown that the use of propidium monoazide to distinguish between living bacteria from dead bacteria was not relevant under certain experimental conditions and in particular to estimate the effect of chlorination [47]. Here, we used propidium monoazide (PMAxx) to avoid
the amplification of DNA from degraded cells, permeable to the dye, and solely amplify DNA from potentially viable bacteria [35] to reduce potential overestimation.

Validation of the qPCR method was performed by comparing the molecular results (using PMAxx coupled with the RT qPCR measurement) with culture-based methods. A higher number of *Leptospira* was counted by the qPCR. This difference can be explained by various factors, such as colonies not issued from one bacteria (i.e. aggregated bacteria), genome multiplication before cells separation, count of viable but non cultivable bacteria and multiple copies of the 16S gene in *Leptospira* [48].

We compared the survival of pathogenic and saprophytic strains of *Leptospira* in several environments. Heat inactivation experiments revealed different kinetics between pathogenic and saprophytic strains, the pathogenic strain being more tolerant to heat. This relative tolerance may explain their higher prevalence in tropical areas as well as the role of these strains in human infections. Other inactivation processes did not show any significant differences in inactivation kinetics between the two strains.

Our results based on bacterial culture methods suggest that *Leptospira* is rapidly inactivated by free chlorine. However, the use of PMAqPCR shows that leptospiral membranes would not be directly damaged by free chlorine. Several studies have shown that free chlorine inactivates *E. coli* without damaging its cell membranes [49–52] further indicating that the use of PMAqPCR is not appropriate to determine the capacity of chlorine to kill bacteria.

In absence of impact of disinfection treatment, it was essential to also consider that the absence of cultivability does not necessarily indicate cell lysis as bacteria could remain as a “viable but non-cultivable” state [53].

Contrary to classical PCR, the integrity PCR approach allowed the use of more adaptable and faster molecular methods to assess inactivation efficiencies. Due to interfering flora or organic matter, the culture is too complicated to implement, especially on complex samples whose matrix negatively impacts the re-cultivation of *Leptospira*.

Certain physicochemical parameters (for example, salty water) can alter the survival of *Leptospira* [24], whereas other parameters can provide a protective effect. For example, the presence of organic matter or biofilms could increase the survival of *Leptospira* survival in aquatic environments [16, 54].

The PMAxx approach showed certain limitations concerning its use to evaluate the efficiency of disinfection treatments of *Leptospira*. Although useful results were obtained after heat or low-level free chlorine exposure, the use of PMAxx was not informative for the analysis of UV treatment. It is possible that treatments or conditions that affect bacterial integrity (temperature, chlorine) allow PMAxx to access the genome, contrary to UV radiation. However, when only DNA was targeted for the inactivation of the microorganism, PMAxx had no effect in improving the determination of *Leptospira* sensitivity (Fig 7). Such an observation has already been reported for a virus assay [55].

To date, *Leptospira* are not considered when investigating microorganisms in water. Our methods allowed us to obtain information on the presence and integrity of such bacteria in Parisian surface-water samples (Fig 7). This proof of concept should be applied to answer other questions, such as the influence of seasonal variations or the impact of rodent control campaigns. The development of a sensitive qPCR method using a rapid reverse-transcription step targeting the *rrs* (16S) or LipL32 genes improved the sensitivity of detection [56]. Within the sampling area, the median concentration was approximately 10³ eq. bacteria/L. Further studies are under way to determine whether it would be relevant and useful to routinely use this method to monitor *Leptospira* in surface water. These results could be considered to establish threshold alerts, leading to restrictions of access, after events that favor bacterial contamination (heavy rain, flooding, etc.). The impact of
various parameters, such as seasons, climatic conditions, and human activities, on Leptospira dynamics needs to be evaluated.

Analysis of the presence of viable Leptospira in the environment and the measurement of the effectiveness of treatments are essential. The advantages of combining PMAxx addition and RT qPCR methods to detect low levels of non-permeable pathogens in water is now accepted [57]. The use of PMAxx combined with qPCR is therefore one possible solution for Leptospira measurement; it is time saving and avoids overestimation. By detecting non-permeable and VBNC bacteria, this method pinpointed and prevented a Leptospira outbreak originating from environmental water [58]. These data could be useful for quantitative microbiological risk assessment (QMRA) approaches.

Although it is now well known that these bacteria can have a high impact on public health in endemic areas [59] or during specific seasons [60, 61], little is known about the environmental concentration in urbanized areas in Europe affected by the proliferation of rodents, which are the main animal reservoir of Leptospira. With ongoing social changes, water-related activities have increased in these regions, with the installation of urban beaches, bathing areas, and aquatic activities in areas with non-treated surface water. Our PMAqPCR method will be further used to better evaluate the presence of pathogenic Leptospira in bathing areas in Paris, France. The monitoring of microbial contamination is a requirement for establishing microbial risk assessment guidelines. Moreover, regular monitoring of Leptospira could help to provide a better description of infection events. Despite an increase in reported cases of leptospirosis and evidence that most cases are due to exposure to contaminated water, regulations are still based on fecal indicators and do not yet include pathogenic Leptospira, probably due to the absence of reliable measurement methods. Indeed, only Escherichia coli and intestinal enterococci are analyzed in France (French Public Health Code-D. 1332-15D1332-15). The only existing recommendations related to the risk of Leptospira exposure are to avoid bathing with skin lesions or in uncontrolled bathing areas. Leptospira monitoring could be implemented in freshwater swimming facilities to improve awareness of Leptospira exposure. This could consist of identifying sources of pollution prone to affect water quality and the health of bathers. By detecting Leptospira in the environmental water during flooding, this assay can also contribute to early warning of potential outbreaks of leptospirosis.

Supporting information

S1 Table. Microorganism strains used for specificity tests and results from the TaqMan real-time multiplex (LipL32 and 16S) PCR assays.
(DOCX)

S2 Table. Leptospira sequences and their NCBI accession numbers required to design the primers and probe for the 16S qPCR.
(DOCX)

S3 Table. Leptospira sequences and their NCBI accession numbers required to design primers and probe for the LipL32 qPCR.
(DOCX)

Acknowledgments

We are very grateful to the sampling department of Eau de Paris for providing samples to the laboratory. We thank the staff of the National Reference Center for Leptospirosis for support and the processing of some of the samples.
Author Contributions
Conceptualization: Mathieu Picardeau, Laurent Moulin.
Formal analysis: Elise Richard.
Funding acquisition: Mathieu Picardeau, Laurent Moulin.
Investigation: Elise Richard, Sébastien Wurtzer.
Methodology: Elise Richard, Pascale Bourhy, Sébastien Wurtzer.
Supervision: Mathieu Picardeau.
Validation: Laurent Moulin, Sébastien Wurtzer.
Writing – original draft: Elise Richard.
Writing – review & editing: Mathieu Picardeau, Laurent Moulin, Sébastien Wurtzer.

References
1. Costa FB, Hagan JE, Calicagno J, Kane M, Torgerson P, Martinez-Silveira MS, et al. Global Morbidity and Mortality of Leptospirosis: A Systematic Review. Small PLC, editor. PLoS Negl Trop Dis. 2015 Sep 17; 9(9):e0003889. https://doi.org/10.1371/journal.pntd.0003889 PMID: 26379143
2. Tique V, Mattar S, Miranda J, Oviedo M, Noda A, Montes E, et al. Clinical and Epidemiological Status of Leptospirosis in a Tropical Caribbean Area of Colombia. BioMed Res Int. 2018 May 29; 2018:1–8.
3. Biscomett L, de Comarmond J, Bibi J, Mavingui P, Delliagi K, Tortosa P. An Observational Study of Human Leptospirosis in Seychelles.: 10.
4. Pijnacker R, Goris MGA, te Wierik MJM, Broens EM, van der Giessen JW, de Rosa M, et al. Marked increase in leptospirosis infections in humans and dogs in the Netherlands, 2014. Eurosurveillance [Internet]. 2016 Apr 28 [cited 2021 Apr 7]; 21(17). Available from: https://www.eurosurveillance.org/content/10.2807/1560-7917.ES.2016.21.17.30211. https://doi.org/10.2807/1560-7917.ES.2016.21.17.30211 PMID: 27168584
5. Bourhy P. DIAGNOSTIC, SURVEILLANCE ET EPIDEMIOLOGIE DE LA LEPTOSPIROSE EN FRANCE / DIAGNOSIS, SURVEILLANCE, AND EPIDEMIOLOGY OF LEPTOSPIROSIS IN FRANCE.: 7.
6. Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA, et al. Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis. 2003 Dec; 3(12):757–71. https://doi.org/10.1016/s1473-3099(03)00830-2 PMID: 14652202
7. Morgan J, Bornstein SL, Karpati AM, Bruce M, Bolin CA, Austin CC, et al. Outbreak of Leptospirosis among Triathlon Participants and Community Residents in Springfield, Illinois, 1998. Clin Infect Dis. 2002 Jun 15; 34(12):1593–9. https://doi.org/10.1086/346165 PMID: 12032894
8. Lau CL, Smythe LD, Craig SB, Weinstein P. Climate change, flooding, urbanisation and leptospirosis: fuelling the fire? Trans R Soc Trop Med Hyg. 2010 Oct; 104(10):631–8. https://doi.org/10.1016/j.trstmh.2010.07.002 PMID: 20613968
9. Munoz-Zanzi C, Groene E, Morawski BM, Bonner K, Costa F, Bertherat E, et al. A systematic literature review of leptospirosis outbreaks worldwide, 1970–2012. Rev Panam Salud Publica. 2020 Jul 15; 44:1. https://doi.org/10.26633/RPSP.2020.78 PMID: 32684917
10. Mwachui MA, Crump L, Hartskeerl R, Zinsstag J, Hattendorf J. Environmental and Behavioural Determinants of Leptospirosis Transmission: A Systematic Review. Small PLC, editor. PLoS Negl Trop Dis. 2016 Sep 17; 9(9):e0003843. https://doi.org/10.1371/journal.pntd.0003843 PMID: 26379035
11. Abgueuen P, Delbos V, Blanvillain J, Chennebault JM, Cottin J, Fanello S, et al. Clinical aspects and prognostic factors of leptospirosis in adults. Retrospective study in France. J Infect. 2008 Sep; 57(3):171–8. https://doi.org/10.1016/j.jinf.2008.06.010 PMID: 18656263
12. Guillois Y, Bourhy P, Ayral F, Privete M, Decors A, Aranda Grau JH, et al. An outbreak of leptospirosis among kayakers in Brittany, North-West France, 2016. Eurosurveillance [Internet]. 2018 Nov 29 [cited 2020 Aug 10]; 23(48). Available from: https://www.eurosurveillance.org/content/10.2807/1560-7917.ES.2018.23.48.1700848.
13. Cacciapuoti B, Ciceroni L, Maffei C, Stanislas FD, Strusi P, Calegari L, et al. A WATERBORNE OUTBREAK OF LEPTOSPIROSIS. Am J Epidemiol. 1987 Sep; 126(3):535–45. https://doi.org/10.1093/oxfordjournals.aje.a114686 PMID: 3618584
14. Ramakrishnan R, Patel MS, Gupte MD, Manickam P, Venkataraman S. An institutional outbreak of leptospirosis in Chennai, South India. J Commun Dis. 2003 Mar; 35(1):1–8. PMID: 15236298

15. Vincent AT, Schiettekatte O, Goarant C, Neela VK, Bernet E, Thibeaux R, et al. Revisiting the taxonomy and evolution of pathogenicity of the genus Leptospira through the prism of genomics. Martins EAL, editor. PLoS Negl Trop Dis. 2019 May 23; 13(5):e0007270. https://doi.org/10.1371/journal.pntd.0007270 PMID: 31120895

16. Thibeaux R, Gerout S, Benezech C, Chabaud S, Soupé-Gilbert M-E, Girault D, et al. Seeking the environmental source of Leptospirosis reveals durable bacterial viability in river soils. Munoz- Zendz C, editor. PLoS Negl Trop Dis. 2017 Feb 27; 11(2):e0005414. https://doi.org/10.1371/journal.pntd.0005414 PMID: 28641042

17. Faine S, Adler B, Bolin C, Perolat P. “Leptospirosis” and leptospirosis [Internet]. MediSci; 1999 [cited 2020 Oct 13]. Available from: https://research.monash.edu/en/publications/leptospirosi-and-leptospirosis

18. Adler B, editor. Leptospirosis and Leptospirosis [Internet]. Berlin, Heidelberg: Springer Berlin Heidelberg; 2015 [cited 2020 Oct 13]. (Current Topics in Microbiology and Immunology; vol. 387). Available from: http://link.springer.com/10.1007/978-3-662-45095-8

19. Goarant C. Leptospirosis: risk factors and management challenges in developing countries. Res Rep Trop Med. 2016 Sep;Volume 7:49–62. https://doi.org/10.2147/RRTM.S102543 PMID: 30050339

20. Schaechter M. Escherichia coli and Salmonella 2000: the View From Here. Microbiol Mol Biol Rev. 2001 Mar; 65(1):119–30. https://doi.org/10.1128/MMBR.65.1.119-130.2001 PMID: 11238988

21. Loureiro AP, Martins G, Pinto P, Nardulle L, Teixeira RC, Lilenbaum W. Usage of a selective media (EMJH-STAFF) in primary culturing of pathogenic leptospiroses from bovine clinical samples. Lett Appl Microbiol. 2015 Dec; 61(6):603–6. https://doi.org/10.1111/lam.12501 PMID: 26408270

22. Chakrabarty A, Miyahara S, Villanueva SYAM, Saito M, Gloriani NG, Yoshida S. A novel combination of selective agents for isolation of Leptospirosis species: Selective medium for Leptospirosis. Microbiol Immunol. 2011 Jul; 55(7):494–501. https://doi.org/10.1111/j.1348-0421.2011.00347.x PMID: 21545510

23. Huang K. A comprehensive insight into bacterial virulence in drinking water using 454 pyrosequencing and illumina high-throughput sequencing. Ecotoxicol Environ Saf. 2014; 8: https://doi.org/10.1016/j.ecoenv.2014.07.029 PMID: 25129220

24. Chang SL, Buckingham M, Taylor MP. Studies on Leptospirosis icterohaemorrhagiae: IV. Survival in Water and Sewage: Destruction in Water by Halogen Compounds, Synthetic Detergents, and Heat. J Infect Dis. 1948 May 1; 82(3):256–66. https://doi.org/10.1093/infdis/82.3.256 PMID: 18864110

25. Andre-Fontaine G, Aviat F, Thorin C. Waterborne Leptospirosis: Survival and Preservation of the Virulence of Pathogenic Leptospira spp. in Fresh Water. Curr Microbiol. 2015 Jul; 71(1):136–42. https://doi.org/10.1007/s00284-015-0836-4 PMID: 26003629

26. Bierque E, Soupé-Gilbert M-E, Thibeaux R, Girault D, Guentas L, Goarant C. Leptospirosis interrogans Retains Direct Virulence After Long Starvation in Water. Curr Microbiol. 2020 Oct; 77(10):3035–43. https://doi.org/10.1007/s00284-020-02128-7 PMID: 32683468

27. Ahmed A, Engelberts MFM, Boer KR, Ahmed N, Hartsekeer RA. Development and Validation of a Real-Time PCR for Detection of Pathogenic Leptospira Species in Clinical Materials. Bereswill S, editor. PLoS ONE. 2009 Sep 18; 4(9):e7093. https://doi.org/10.1371/journal.pone.0007093 PMID: 19763264

28. Podgoršek D, Rukič-Sabljić E, Logar M, Pavlović A, Remec T, Baklan Z, et al. Evaluation of real-time PCR targeting the ipL32 gene for diagnosis of Leptospirosis infection. BMC Microbiol. 2020 Dec; 20(1):59. https://doi.org/10.1186/s12866-020-01744-4 PMID: 32160864

29. Stoddard RA, Gee JE, Wilkins PP, McCaustland K, Hoffmaster AR. Detection of pathogenic Leptospira spp. through TaqMan polymerase chain reaction targeting the lipL32 gene. Diagn Microbiol Infect Dis. 2009; 9: https://doi.org/10.1016/j.diagmicrobio.2009.03.014 PMID: 19395218

30. Mohd Ali MR, Mohd Safiee AW, Ismail NH, Abu Sapiar R, Mat Hussin H, Ismail N, et al. Development and validation of pan-Leptospira Taqman qPCR for the detection of Leptospira spp. in clinical specimens. Mol Cell Probes. 2018 Apr; 38:1–6. https://doi.org/10.1016/j.mcp.2018.03.001 PMID: 29524642

31. Levet PN. Detection of pathogenic leptospiroses by real-time quantitative PCR. J Med Microbiol. 2005 Jan 1; 54(1):45–9. https://doi.org/10.1099/jmm.0.45860-0 PMID: 15591254

32. Gökmen TG, Soyal A, Kalayci Y, Öhlen C, Köksal F. COMPARISON OF 16S rRNA-PCR-RFLP, LipL32-PCR AND OmpL1-PCR METHODS IN THE DIAGNOSIS OF LEPTOSPIOSIS. Rev Inst Med Trop São Paulo [Internet]. 2016 [cited 2020 Jan 2]; 58(0). Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0036-46652016000000248&lng=en&tlng=en

33. Slack AT, Symonds ML, Dohnt MF, Smythe LD. [No title found]. BMC Microbiol. 2006; 6(1):95.

34. Palaniappan RUM, Chang Y-F, Chang C-F, Pan MJ, Yang CW, Harpending P, et al. Evaluation of ligase-based conventional and real time PCR for the detection of pathogenic leptospiroses. Mol Cell Probes. 2005 Apr; 19(2):111–7. https://doi.org/10.1016/j.mcp.2004.10.002 PMID: 15680212
Effect of disinfection agents on Leptospira in water using a high sensitivity integrity-qPCR assay

35. Casanovas-Massana A, Pedra GG, Wunder EA, Diggle PJ, Begon M, Ko AI. Quantification of Leptospira interrogans Survival in Soil and Water Microcosms. Appl Environ Microbiol. 2018 Jun 18; [cited 2020 Oct 13]; 84(13). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6007094/. https://doi.org/10.1128/AEM.00507-18 PMID: 29703737

36. Roussel C, Galia W, Leriche F, Chalancon S, Denis S, Van de Wiele T, et al. Comparison of conventional plating, PMA-qPCR, and flow cytometry for the determination of viable enterotoxigenic Escherichia coli along a gastrointestinal in vitro model. Appl Microbiol Biotechnol. 2018 Nov; 102(22):9793–802. https://doi.org/10.1007/s00253-018-9380-z PMID: 3038141

37. Ellinghausen HC, Mccullough WG. NUTRITION OF LEPTOSPIRA POMONA AND GROWTH OF 13 OTHER SEROYPES: FRACTIONATION OF OLEIC ALBUMIN COMPLEX AND A MEDIUM OF BOVINE ALBUMIN AND POLYSORBATE 80. Am J Vet Res. 1985 Jan; 26:45–51. PMID: 14266934

38. Johnson RC, Harris VG. Differentiation of Pathogenic and Saprothetic Leptospires I. Growth at Low Temperatures. J Bacteriol. 1967; 94(1):27–31. https://doi.org/10.1128/JB.94.1.27-31.1967 PMID: 6027998

39. Abdulmawjood A, Roth S, Bülte M. Two methods for construction of internal amplification controls for the detection of Escherichia coli O157 by polymerase chain reaction. Mol Cell Probes. 2002 Oct; 16 (5):335–9. https://doi.org/10.1006/mcpr.2002.0431 PMID: 12477437

40. Wurtzer S, Prevost B, Lucas F, Moulin L. Detection of enterovirus in environmental waters: A new optimized method compared to commercial real-time RT-qPCR kits. J Virol Methods. 2014 Sep 4; 209. https://doi.org/10.1016/j.jviromet.2014.08.016 PMID: 25196451

41. Karim MR, Fout GS, Johnson CH, White KM, Parashionkar SU. Propidium monoazide reverse transcriptase PCR and RT-qPCR for detecting infectious enterovirus and norovirus. J Virol Methods. 2015 Jul; 219:51–61. https://doi.org/10.1016/j.jviromet.2015.02.020 PMID: 25796356

42. Coudray-Meunier C, Fraisse A, Martin-Latil S, Guillier L, Perelle S. Discrimination of infectious hepatitis A virus and rotavirus by combining dyes and surfactants with RT-qPCR. BMC Microbiol. 2013; 13 (1):216. https://doi.org/10.1186/1471-2180-13-216 PMID: 24083486

43. Prevost B, Goulet M, Lucas FS, Joyeux M, Moulin L, Wurtzer S. Viral persistence in surface and drinking water: Suitability of PCR pre-treatment with intercalating dyes. Water Res. 2016 Mar; 91:68–76. https://doi.org/10.1016/j.watres.2015.12.049 PMID: 26773484

44. Nocker A, Sossa-Fernandez P, Burr MD, Camper AK. Use of Propidium Monoazide for Live/Dead Distinction in Microbial Ecology. Appl Environ Microbiol. 2007 Aug 15; 73(16):5111–7. https://doi.org/10.1128/AEM.02967-06 PMID: 17596667

45. Pérez JJ, Lanka S, DeShambo VJ, Frederickson RL, Maddox CW. A Validated Multiplex Real-Time PCR Assay for the Diagnosis of Infectious Leptospira spp.: A Novel Assay for the Detection and Differentiation of Strains From Both Pathogenic Groups I and II. Front Microbiol. 2020 Mar 20; 11:457. https://doi.org/10.3389/fmicb.2020.00457 PMID: 32265882

46. Bustin SA, Benes V, Garson JA, Hellermans J, Huggett J, Kubista M, et al. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clin Chem. 2009 Apr 1; 55(4):611–22. https://doi.org/10.1373/clinchem.2008.112797 PMID: 19246189

47. Virto R, Mañas P, Álvarez I, Condon S, Raso J. Membrane Damage and Microbial Inactivation by Chlorine in the Absence and Presence of a Chlorine-Demanding Substrate. Appl Environ Microbiol. 2005 Sep; 71(9):5022–8. https://doi.org/10.1128/AEM.71.9.5022-5028.2005 PMID: 16151082

48. Fukunaga M, Mifuchi I. Unique organization of Leptospira interrogans rRNA genes. J Bacteriol. 1989; 171(11):5763–7. https://doi.org/10.1128/jb.171.11.5763-5767.1989 PMID: 20982097

49. Mizooze Otaki, Aikawa. The Mechanism of Chlorine Damage Using Enhanced Green Fluorescent Protein-Expressing Escherichia coli. Water. 2019 Oct 16; 11(10):2156.

50. Bridges DF, Lacombe A, Wu VCH. Integrity of the Escherichia coli O157:H7 Cell Wall and Membranes After Chlorine Dioxide Treatment. Front Microbiol. 2020 May 15; 11:888. https://doi.org/10.3389/fmicb.2020.00888 PMID: 32499765

51. Cho M, Kim J, Kim JY, Yoon J, Kim J-H. Mechanisms of Escherichia coli inactivation by several disinfectants. Water Res. 2010 Jun; 44(11):3410–8. https://doi.org/10.1016/j.watres.2010.03.017 PMID: 20427068

52. Kazama S, Otaki M. Quantitative Analysis of the Inactivation Mechanisms of Escherichia coli by a Newly Developed Method Using Propidium Monoazide. J Water Environ Technol. 2013; 11(6):507–17.

53. Xu L, Zhang C, Xu P, Wang XC. Mechanisms of ultraviolet disinfection and chlorination of Escherichia coli: Culturability, membrane permeability, metabolism, and genetic damage. J Environ Sci. 2018 Mar; 65:356–66.
54. Barragan VA, Mejia ME, Trávez A, Zapata S, Hartekeert RA, Haake DA, et al. Interactions of Leptospira with Environmental Bacteria from Surface Water. Curr Microbiol. 2011 Jun; 62(6):1802–6. https://doi.org/10.1007/s00284-011-9931-3 PMID: 21479795

55. Prevost B, Lucas FS, Goncalves A, Richard F, Moulin L, Wurtzer S. Large scale survey of enteric viruses in river and waste water underlines the health status of the local population. Environ Int. 2015 Jun; 79:42–50. https://doi.org/10.1016/j.envint.2015.03.004 PMID: 25795193

56. Waggoner JJ, Balassiano I, Abeynayake J, Sahoo MK, Mohamed-Hadley A, Liu Y, et al. Sensitive Real-Time PCR Detection of Pathogenic Leptospira spp. and a Comparison of Nucleic Acid Amplification Methods for the Diagnosis of Leptospirosis. Lin B, editor. PLoS ONE. 2014 Nov 7; 9(11):e112356. https://doi.org/10.1371/journal.pone.0112356 PMID: 26379890

57. Gensabeger ET, Polt M, Konrad-Köszer M, Kinner F, Sessitsch A, Kostić T. Evaluation of quantitative PCR combined with PMA treatment for molecular assessment of microbial water quality. Water Res. 2014 Dec; 67:367–76. https://doi.org/10.1016/j.watres.2014.09.022 PMID: 25459225

58. Golpayegani A, Douraghi M, Rezaei F, Alimohammadi M, Nodehi RN. Propidium monoazide–quantitative polymerase chain reaction (PMA-qPCR) assay for rapid detection of viable and viable but non-culturable (VBNC) Pseudomonas aeruginosa in swimming pools. J Environ Health Sci Eng. 2019 Jun; 17(1):407–16. https://doi.org/10.1007/s40201-019-00359-w PMID: 31297217

59. Pappas G, Papadimitriou P, Siozopoulou V, Christou L, Akritidis N. The globalisation of leptospirosis: worldwide incidence trends. Int J Infect Dis. 2008 Jul; 12(4):351–7. https://doi.org/10.1016/j.ijid.2007.09.011 PMID: 18055245

60. Bouscaren N, Benoît de Coignac C, Lastère S, Musso D, Teissier Y, Formont J, et al. Leptospirosis in French Polynesia: 11 years of surveillance data, 2007–2017. New Microbes New Infect. 2019 May; 29:100518. https://doi.org/10.1016/j.nmni.2019.100518 PMID: 30899522

61. Joshi YP, Kim E-H, Cheong H-K. The influence of climatic factors on the development of hemorrhagic fever with renal syndrome and leptospirosis during the peak season in Korea: an ecologic study. BMC Infect Dis. 2017 Dec; 17(1):406. https://doi.org/10.1186/s12879-017-2506-6 PMID: 28592316