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# Detection of *Legionella* spp. in Natural and Man-made Water Systems Using Standard Guidelines

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**Abstract** Infections caused by *Legionella* spp. are considered at the present time, an emerging public health problem and are linked to high rates of mortality and morbidity, if not properly treated. In this study were analyzed 54 samples of water from 8 counties at Northern Portugal, with the aim of obtaining a collection of strains of the genus *Legionella* and to characterize them genetically and phenotypically. Another objective of this study was to evaluate the effectiveness of the technique of cultivation, a standard method according to International Organization for Standardization ISO 11731:1998, for detection and enumeration of species of *Legionella*. For laboratory processing, after the filtration of samples (1 L), the filtrate was resuspended in sterile distilled water (5 ml). Heat treatment for selective inhibition of non-*Legionella* bacteria was performed. Subsequently, 100 µl of the suspension was spread in GVPC selective agar medium, and incubated (7 to 10 days) at 37 °C. Colonies that were morphologically characteristic of the genus were sub-cultured onto BCYE agar and blood agar for verification. According to the procedure recommended by the standard method, only the colonies which grew in BCYE agar and not on blood agar were considered as suspected *Legionella* strains. The identification of these initially selected colonies was performed by sequencing the 16S rRNA gene, which revealed that none of the isolates were identified as belonging to the genus *Legionella*. However, through the ISO 11731:1998 they were interpreted as positive, corresponding therefore to "false-positive" results. The methods used in this study allowed the isolation of a number of isolates (40), which form an independent group of all genus of the family *Chitinophagaceae* outlined so far, and that by their phylogenetic distance might be a genus not yet described and therefore a new species. The results obtained, highlighted the importance of using culture and genetic methods in parallel for the proper identification of microorganisms.

**Keywords** Water, *Legionella*, ISO 11731:1998, False positives, 16S rRNA gene sequencing

## 1. Introduction

*Legionella* is an organism of public health interest due to its ability to cause infection in susceptible humans and it's near ubiquitous presence in heated water systems[1]. The genus *Legionella* comprises 71 distinct serogroups from more than 50 known species[2-4] and new species are frequently described[5-7]. *Legionella pneumophila*, was first recognized in 1977 following an epidemic of acute pneumonia in Philadelphia[8], is the etiological agent of the majority of cases of legionellosis, and the best part of cases have been attributed to *L. pneumophila* serogroup 1[3,9]. In addition, at least 21 other species of *Legionella* have been related with human infections[2,10]. These organisms may be a cause of nosocomial and community-acquired infections, principally in immunocompromised patients[10-12]. Legionellosis outbreaks are often associated with high mortality rates[13].

Bacteria have been found in watery soils, natural or man-made aquatic environments, such as rivers, lakes, ponds, mud, and cooling towers or water distribution systems[2,14-17]. *Legionella* bacteria do exist as free-living planktonic forms in the environment, intracellular parasites of protozoans, and/or inhabitants of mixed community biofilms[1,18]. Thus, the diversity of types and the ubiquitous occurrence of legionellae in water environments or moist soil make it difficult to identify epidemic strains, and outbreaks of legionellosis have been associated mainly with contamination of man-made aquatic environments[19].

Legionnaires' disease can be acquired by the inhalation of contaminated droplet aerosols or by microaspiration of contaminated water[10,15]. However transmission of *Legionella* from person to person has never been observed, and the prevention needs to focus on the elimination of this pathogen from water and systems that produce aerosol. Thus, rapid and accurate detection of *Legionella* in water systems is extremely importante for hazard prediction and the elimination of *Legionella* from potential infection sources[20]. The detection of water contaminated by *Le-*

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*gionella* has been usually performed using culture-based methods, approved by the International Organization for Standardization[21] and the French organization for standardization[22]. Culture is important for identifying and typing *Legionella* strains during epidemics but have several limitations, namely long incubation times. This problem makes culture inappropriate for preventive actions and rapid response in emergency situations. Another weakness is the occurrence of viable but nonculturable (VBNC) bacteria. Furthermore, the sensitivity of *Legionella* detection based on culture methods depends largely on the physiological state of the cells[20,23].

Therefore, it is important to improve the current testing for *Legionella* and develop rapid and accurate tests. In recent years, different PCR-based methods (based on 16S rRNA, 5S rRNA and 23S rRNA genes and on the *mip* gene encoding the macrophage infectivity potentiator gene of *L. pneumophila*) for detection and quantification of *Legionella* in water samples have been described and can moderate the main drawbacks of culture-based methods[24-28]. The development of more rapid, culture-independent methods able to discriminating between live and dead cells is very important for assessing *Legionella* infection risks and preventing legionellosis[23].

The aim of the present study was to characterize presumptive bacteria from the genus *Legionella* isolated from water samples from various sources, and also to evaluate the efficiency of detection of *Legionella* species by the technique of culture, according to the recommendations of the International Standard ISO 11731:1998.

## 2. Materials and Methods

**Sample collection**

A total of 54 water samples were collected at different natural aquatic environmental sources (fountains, springs, rivers, wells and lakes), and from man-made systems (decorative fountains, tap water, water cylinder and the water pipe condensation of the cooling towers of air conditioning) of eight counties from Northern Portugal (Table 1), for a period of 10 months (January and October of 2008). Water samples were collected in sterile 1000 ml polyethylene bottles and samples were delivered to the laboratory within one day.

**Isolation of legionellae from water samples**

Isolation of *Legionella* from water samples was performed by culture according to the recommendations of the International Standard method ISO 11731:1998 (Water quality - Detection and enumeration of *Legionella*)[21], based on filtration procedure and culture of bacteria on selective media. Samples of 1000 ml of water were concentrated by filtration on a 0.2 µm pore-diameter polycarbonate membrane (Isopore, Millipore, Ireland). After filtration, bacteria collected on the membranes were resuspended in 5 ml of water and shaken vigorously. Heat treatment for selective inhibition of non-*Legionella* bacteria were performed as described previously[21,24,29,30]. Briefly, 1 ml of the concentrate was treated at 50 ± 1°C for 30 ± 1 min. Subsequently 0.1 ml of the suspension was spread on a 90-mm Petri dish containing BCYE agar supplemented with glycine, vancomycin, polymyxin B and cycloheximide (GVPC selective agar medium) (Becton, Dickinson and Company, USA). The inoculated plates were then incubated for 7 to 10 days at 37±1°C under aerobic conditions and humidified atmosphere. Colonies were counted after 3, 5, and 10 days. Smooth colonies showing a yellowish or sometimes a yellow-green or grayish-white color were counted as suspicious legionellae to be confirmed. Up to 5 to 7 colonies of suspected *Legionella* were subcultured onto BCYE agar (without antibiotics) (Becton, Dickinson and Company, USA), and blood agar (alternatively we can use BCYE agar without L-cysteine) for confirmation. The isolated colonies growing only on BCYE agar but not on blood agar were considered to be *Legionella* colonies. No further confirmatory tests, namely direct or indirect immunofluorescence and latex agglutination, for cysteine-dependent colonies, were carried out. A positive control with *L. pneumophila* CECT 7109 was used.

**Table 1.** Origin of samples, the sampling locations, number of samples and number of isolates

| Source of samples | Sampling sites | Number of samples collected | Number of isolates |
|---|---|---|---|
| Vila Real (VR) | Fountains | 12 | 18 |
| | Springs | 4 | 0 |
| | Wells | 2 | 0 |
| | Rivers | 3 | 22 |
| | Lakes | 1 | 1 |
| | Recreational sources | 1 | 8 |
| | Tap | 1 | 0 |
| | Cylinders | 2 | 1 |
| | Water of the cooling towers of air conditioning | 11 | 59 |
| Peso da Régua (PR) | Fountains | 1 | 0 |
| Lamego (L) | Springs | 3 | 2 |
| | Wells | 1 | 1 |
| Marco de Canaveses (MC) | Rivers | 1 | 8 |
| Alijó (A) | Wells | 2 | 3 |
| | Rivers | 1 | 5 |
| Vila Pouca de Aguiar (VPA) | Wells | 1 | 0 |
| Ribeira de Pena (RP) | Fountains | 4 | 0 |
| Mondim de Basto (MB) | Fountains | 2 | 0 |
| | Springs | 1 | 0 |

**Biochemical test**

Biochemical tests for gelatinase, urease, and catalase as well as for hippurate hydrolysis and nitrate reduction were performed as described previously[31-35]. Oxidase expriment was developed in a test paper containing tetramethyl-phenylenediamine dihydrochloride-oxidase reagent (Merck, Germany). The use of glucose and capacity to ferment or oxidize some carbohydrates was also examined as described previously[36].

**PCR amplification and sequencing of the 16S rRNA gene**

For PCR amplification single colony from fresh cultures was resuspended in 100 µl of TE buffer and 200 µl Chelex (BioRad) were added. The tube was vortexed at high speed for 1 minute; incubated at 96 ℃ for 10 minutes, kept at -20℃ for 10 minutes, and this process was repeated three consecutive times. The tube was again vortexed and centrifuged for 5 min at 12000 g. The supernatant was transferred to a fresh tube and stored at –20℃. Procedures and characteristics of oligonucleotide primers for the amplification and PCR-sequencing of the 16S rRNA gene were as previously described[37].

**Phylogenetic data analysis**

The nucleotide sequences were aligned by the Clustal X program version 1.8[38]. For alignments, previously published reference sequences[37,39-41], were used. Genetic distances were obtained by Kimura's 2 parameter model [42] and evolutionary trees were constructed by the Neighbour-Joining method[43] with the Mega program[44].

**Genetic typing analysis**

**Table 2.** Primers used in RAPD reactions

| Primer | Sequence (5`--- 3`) | Reference |
|---|---|---|
| OPA 16 | AGC CAG CGA A | [45] |
| OPA 20 | GTT GCG ATC C | [45] |
| ERIC-1R | ATG TAA GCT CCT GGG GAT TCA C | [46] |
| ERIC-2 | AAG TAA GTG ACT GGG GTG AGC G | [46] |

Genetic typing of the isolates was carried out by the Randomly Amplified Polymorphic DNA (RAPD) technique, using primers OPA 16, OPA 20 and ERIC (ERIC-1R and ERIC-2) (Table 2). The mixture of PCR was performed to a final volume of 25 µl containing 75 mM Tris HCl (pH 9.0), 2 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH4)_2SO_4$, 2.5 µl genomic DNA, 2.5 mM of each dNTP (BIOTOOLS), 100 pmol of primer OPA 16, OPA 20 and ERIC (ERIC-1R and ERIC-2) for each reaction, respectively, and 1 U Taq polymerase (ULTRATOOLS- BIOTOOLS). The amplification conditions were: one cycle of initial denaturation temperature of 94℃ for 4 minutes, followed by 40 cycles of amplification (denaturation at 94 ℃ for 1 minute, annealing at 36℃ for 1 minute and extension at 72℃ for 2 minutes) and finally one cycle of final extension at 72℃ for 7 minutes. The PCR products were subjected to electrophoresis on agarose gel.

## 3. Results

Strains isolated during this work were obtained from 54 water samples, collected from 8 counties of Northern Portugal and 9 different sampling sites (fountains, springs, wells, rivers, lakes, recreational sources, tap water, cylinders and air conditioning) (Table 1). From a total of 128 isolated bacteria, 49 grew only on BCYE agar and the remaining 79 grew in both culture media (BCYE agar and blood agar). The first group was then considered presumptive *Legionella* species, in accordance with ISO 11731:1998. Biochemical tests were carried out in two isolates ($VR_{17}a_4$ and $VR_{17}a_5$) of this group. All results were the same for the two isolates. The tests for oxidase, catalase and hippurate hydrolysis were positive. However, tests for gelatinase, urease, nitrate reduction and utilization of glucose and other carbohydrates were negative. The suspected colonies (49) were then directly identified by molecular methods, performed by sequencing the 16S rRNA gene (Table 3). Gene sequencing results revealed that none of the isolates were identified as belonging to the genus *Legionella*. However, through the indications of the ISO 11731:1998 they were interpreted as positive, corresponding therefore to "false-positive" results. This occurrence emphasizes the importance of the use of methods at the molecular level, for unambiguous identification of strains. In Table 3 are shown the results of the identification of presumptive *Legionella* species, by 16S rRNA gene sequencing. The isolate $F_1L$ was obtained from a water sample of a fountain, and identified as *Bacillus pumilus*. The isolates $VR_{26}a_1$, $VR_{27}a_1$ and $VR_{27}a_1F$ were identified as *Burkholderia fungorum*. The identification of the bacteria $VR_{27}a_3$ provided two different species, *Sphingomonas melonis* and *Sphingomonas aquatilis*. Those species belong to the same bacterial genus, based on the partial sequence.

**Table 3.** Isolates considered presumptive *Legionella* spp. according to the ISO 11731:1998 and identified by 16S rRNA gene sequencing

| Sampling sites | Strain Reference | Identification |
|---|---|---|
| Fountains | $F_1L$ | *Bacillus pumilus* |
| Water of the cooling towers of air conditioning | $VR_{26}a_1$ | *Burkholderia fungorum* |
| | $VR_{27}a_1$ | *Burkholderia fungorum* |
| | $VR_{27}a_1$ F | *Burkholderia fungorum* |
| | $VR_{27}a_3$ | *Sphingomonas aquatilis* / *Sphingomonas melonis* |
| | $VR_{29}a_2$ | *Novosphingobium* sp. |
| | $VR_{29}a_3$, $VR_{29}a_4$, $VR_{29}a_5$ | *Bacillus cereus* |
| | $VR_{27}a_2$, $VR_{27}a_2F$, $VR_{17}a_2$, $VR_{17}a_3$, $VR_{17}a_4$, $VR_{17}a_5$, $VR_{25}a_1$, $VR_{25}b_1$, $VR_{25}a_2$, $VR_{25}a_4$, $VR_{25}a_5$, $VR_{25}a_6$, $VR_{25}a_7$, $VR_{25}a_8$, $VR_{25}b_2$, $VR_{25}b_3$, $VR_{25}b_4$, $VR_{25}b_5$, $VR_{25}c_2$, $VR_{25}d_2$, $VR_{25}e_2$, $VR_{25}a_1F$, $VR_{25}a_2F$, $VR_{25}a_3F$, $VR_{25}a_4F$, $VR_{25}a_{10}$, $VR_{25}a_{23}$, $VR_{25}a_{24}$, $VR_{25}a_{25}$, $VR_{25}a_{26}$, $VR_{25}a_{27}$, $VR_{25}a_{28}$, $VR_{25}a_{29}$, $VR_{25}a_{30}$, $VR_{25}a_{31}$ $VR_{25}a_{32}$, $VR_{25}a_{35}$; $VR_{25}a_{37}$, $VR_{25}a_{38}$, $VR_{25}a_{39}$ | *Chitinophagaceae* |

It is possible that there is some controversy, because they only differ in one nucleotide in its complete sequence. The isolate $VR_{29}a_2$ belongs to the genus *Novosphingobium*,

where the nearest species is the *Novosphingobium stygium* with 98.33% of similarity and 8 nucleotides of difference, based on partial sequence. The species *Bacillus cereus* was identified in three isolates, the $VR_{29}a_3$, $VR_{29}a_4$ and $VR_{29}a_5$.

Isolates listed in Table 3 as belonging to the family *Chitinophagaceae* (40) were acquired from samples of air conditioning and are included in a genus not described and therefore a new species. This family is included in the phylum Bacteroidetes, class Sphingobacteria and order *Sphingobacteriales*, with currently 8 genera, including the gender *Balneola*, *Chitinophaga*, *Flavisolibacter*, *Niabella*, *Niastella*, *Sediminibacterium*, *Segetibacter* and *Terrimonas*.

The group of isolates that are part of not described genus have a yellowish and sometimes yellow/green or gray color in GVPC agar and/or BCYE agar. Those isolates had identical partial sequence of 16S rRNA. Analysis of genetic typing by RAPD using the primers OPA 16, OPA 20 e ERIC (ERIC-1R e ERIC-2) was performed with 25 of the 40 isolated strains belonging to the family *Chitinophagaceae*, in order to identify identical RAPD patterns.

Through the analysis of Figure 1 with primer ERIC it is possible to observe that this group of isolates is homogeneous, since all have the same banding pattern, and therefore the same clonic origin.

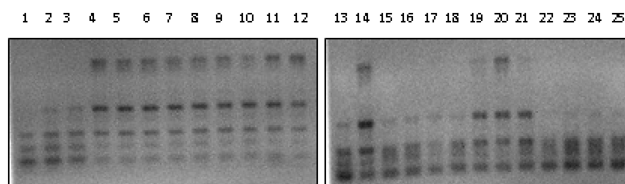


**Figure 1.** RAPD image with the primer ERIC (ERIC-1R and ERIC-2). The numbers in the image correspond to the isolates: 1 - $VR_{17}a_2$, 2 - $VR_{17}a_3$, 3 - $VR_{17}a_4$, 4 - $VR_{17}a_5$, 5 - $VR_{25}a_1$, 6 - $VR_{25}b_1$, 7 - $VR_{25}a_2$, 8 - $VR_{25}a_4$, 9 - $VR_{25}a_5$, 10 - $VR_{25}a_6$, 11 - $VR_{25}a_7$, 12 - $VR_{25}a_8$, 13 - $VR_{25}b_2$, 14 - $VR_{25}b_3$, 15 - $VR_{25}b_4$, 16 - $VR_{25}b_5$, 17 - $VR_{25}c_2$, 18 - $VR_{25}d_2$, 19 - $VR_{25}e_2$, 20 - $VR_{25}a_1F$, 21 - $VR_{25}a_2F$, 22 - $VR_{25}a_3F$, 23 - $VR_{25}a_4F$, 24 - $VR_{27}a_2$, 25 - $VR_{27}a_2F$.

With primers OPA 20 and OPA 16 (Figure 2), it was found that these isolates exhibit identical RAPD profiles, thus corresponding to isolates phylogenetically very similar or even the same bacterial strain. The isolate $VR_{17}a_5$ was selected as representative strain, as all isolates have the same pattern, corresponding to a bacterial clone.

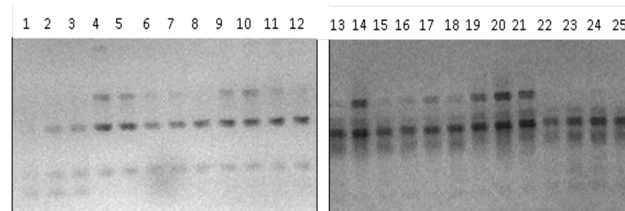


**Figure 2.** RAPD image with primers OPA 20 (1 -$VR_{17}a_2$, 2 - $VR_{17}a_3$, 3 - $VR_{17}a_4$, 4 - $VR_{17}a_5$, 5 - $VR_{25}a_1$, 6 - $VR_{25}b_1$, 7 - $VR_{25}a_2$, 8 - $VR_{25}a_4$, 9 - $VR_{25}a_5$, 10 - $VR_{25}a_6$, 11 - $VR_{25}a_7$, 12 - $VR_{25}a_8$) and OPA 16 (13 - $VR_{25}b_2$, 14 - $VR_{25}b_3$, 15 - $VR_{25}b_4$, 16 - $VR_{25}b_5$, 17 - $VR_{25}c_2$, 18 - $VR_{25}d_2$, 19 - $VR_{25}e_2$, 20 - $VR_{25}a_1F$, 21 - $VR_{25}a_2F$, 22 - $VR_{25}a_3F$, 23 - $VR_{25}a_4F$, 24 - $VR_{27}a_2$, 25 - $VR_{27}a_2F$).

A phylogenetic tree (Figure 3) was elaborated by selecting the microorganisms phylogenetically closest, i.e., with higher homology with that strain. In the phylogenetic tree (Figure 3) are presented two families of the order *Sphingobacteriales*, family *Chitinophagaceae* and *Saprospiraceae*, as well as some of the genera and species belonging to these two families. Through the analysis of the phylogenetic tree obtained, it was observed a separation of $VR_{17}a_5$ strain from the other genera of the family *Chitinophagaceae* presented, forming an independent cluster.

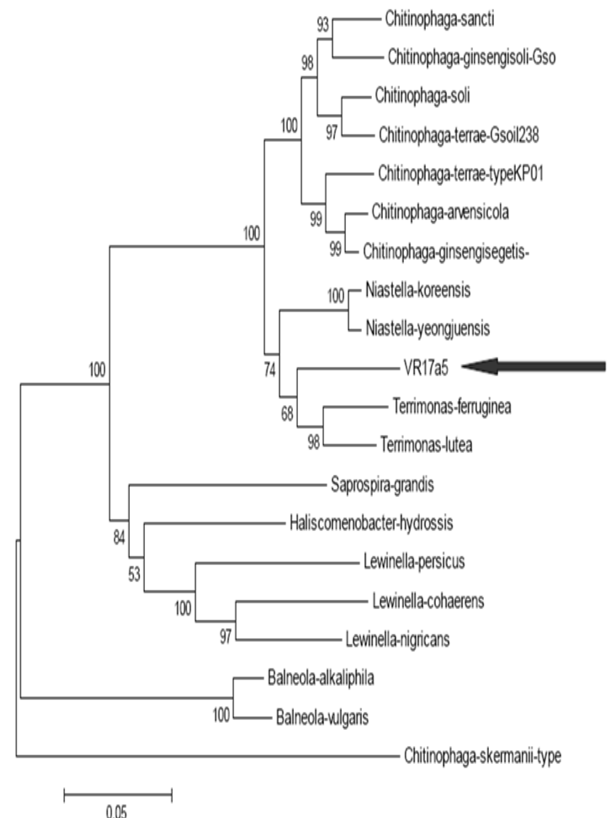


**Figure 3.** Phylogenetic tree based on the sequence of the 16S rRNA, showing the phylogenetic position of strain $VR_{17}a_5$ (representative strain)

The species phylogenetically closest of this strain are *Terrimonas ferruginea* and *Terrimonas lutea*, both from the genus *Terrimonas*. This strain seems to form a cluster entirely independent of all currently recognized genera in this family, so it may represent a new genus and therefore a species undescribed to date.

This is supported by the value of "bootstrap" (68), in the branch of separation of these two groups, that evidence the distance of the phylogenetic branches and formation of another branch of evolution.

## 4. Discussion

*Legionella* species have growth requirement for L-cysteine and without this amino acid, they are unable to grow. Usually colonies with typical morphology on selective media are subcultured onto BCYE and BCYE without L-cysteine (or blood agar). The isolates that grow on BCYE but fail to grow on BCYE without L-cysteine, can be presumptively identified as *Legionella* spp.[24,47]. Closer identification can be carried out using latex agglutination,

direct immunofluoresence or molecular techniques. Some phenotypic tests have lack of sensitivity, and inability for accurately detect all clinically important *Legionella* species and serogroups, and should not be used as a single tool[48].

Using the traditional biochemical tests, bacteria of the genus *Legionella* cannot be identified with any degree of certainty. Many of the species can only be correctly identified using molecular methods. There is a broad panel of biochemical tests whose usefulness has been demonstrated in identification, but these methods have not been widely used or validated after their initial description[49]. Tests for nitrate reductase and urease are negative for all species. Most strains liquefy gelatin and are reported to be catalase positive and oxidase negative (although they may provide positive results). *L. pneumophila* and a few other species hydrolyze hippurate[16,19,50]. The legionellae are chemoorganotropic and do not possess a glucose transport system, nor ferment or oxidize other carbohydrates[49,51]. These biochemical characteristics were observed for two selected isolates ($VR_{17}a_4$ and $VR_{17}a_5$). The results obtained are a further indication, in addition to the L-cysteine growth dependence, that these isolates may belong to the genus *Legionella*.

In this study, no additional confirmatory tests, for cysteine-dependent colonies, were performed and the presumptive *Legionella* spp. were identified by 16S rRNA gene sequencing. The positive results of the cultivation method were not confirmed by genetic identification. At this context, the results obtained can create a problem of economic nature, since, one of the measures to be implemented, after the detection of *Legionella*, would be the cleaning and disinfection of all contaminated systems, in order to avoid potential cases of infection. It should be noted that sometimes the opposite occurs, i.e. false negatives are obtained, since the technique of cultivation does not detect the presence of *Legionella* and genetically this microorganism is detected[48,52].

*Legionella* species have been isolated from a wide variety of water types, such as potable water of hospital, industries and hotels, ground and surface water and biofilms[53]. Moreover, several authors[1,3,50,54] have described the isolation of *Legionella* spp. from showers, cooling towers and boilers. In contrast with those reports, in this work *Legionella* spp. were not detected in the aquatic environments studied.

Despite advances in medium formulations and pretreatment techniques, recovery of *Legionella* species from water samples can still be quite low, difficult and time consuming[48,52]. They are generally present at very low or undetectable concentrations in freshwater and moist natural aquatic environments. Thus, when working with environmental samples, it is usually necessary to use a concentration technique (centrifugation and/or filtration) of the microflora. However, for clinical or environmental samples it is necessary to eliminate or suppress competitive flora during primary culture. To reduce the growth of unwanted bacteria, the samples can be subjected to a heat treatment (50 ℃ for 30 min) or acid (pH 2.2 for 5 min). This treatment of the samples is performed according to the sampling criteria specified by ISO 11731:1998[21] and was already used in several studies[24,29,51].

The water temperature is assumed as the prime factor affecting their incidence[55,56]. Legionellae appear to have a predilection for the warm water encountered in artificial environments, such as man-made systems. Looking for better recovery results, Sanden *et al*.[57] observed that preincubation of water samples with free-living amoebae for several days (seven days) increased the recovery of *Legionella* spp., which was related with proliferation of amoebae in the samples. The same observations were done by Bartie *et al*.[30], who showed that re-incubation of water samples concentrates with autochthonous amoebae improved the culturability of legionellae in a selective medium. Although these approaches are quite selective, due to the improvement of limit of detection or interference with other bacteria, these methods can be time expensive.

Culturing is generally accepted as the "golden standard" for *Legionella* detection in the environment, but the lack of standardization of culturing methods by some laboratories, especially for environmental legionellae, complicates the interpretation of results[58]. This method allows the isolation and the quantification of cultivable *Legionella* species from environmental and clinic samples but it does have limitations[13,59]. The cultivation method has also the disadvantage of being a very time-consuming technique, requiring selective media and several days of incubation (7 to 10 days) to obtain results[25,59]. In addition, although there is an international consensus standard for culture detection and enumeration of *Legionella* in water (ISO 11731:1998), the overgrowth of other accompanying bacteria, the presence of viable but non-culturable (VBNC) cells, loss of viability of bacteria after collection and during the sample treatment (concentration stage followed by decontamination with heat or acid), reduced recoveries by the use of antibiotics in the medium and low concentration of legionellae in the samples limit the use of this method[16,24,52,59,60].

Bacteria exposed to potentially lethal environmental conditions including nutrient restriction, oxidative stress, heat, UV irradiation, osmotic stress, or sublethal concentrations of antibacterial compounds undergo physiological or morphological alterations that complicate the detection and accurate enumeration of such stressed bacteria using available culture methods[24,58]. We can conclude, for all these reasons, that the populations of *Legionella* are underestimate by culture methods.

There is even the occurrence of "false-positives", as found in this work, which highlights the importance of using faster genetic methods for the proper identification of microorganisms. Thus, there is a need for improved detection methods.

PCR methods, such as quantitative real-time PCR (qPCR), are an alternative tool to the conventional culture method for the detection of slow-growing and fastidious bacteria such as *Legionella* species[61-63]. Amplification of *Legionella* DNA by PCR can provide results within a short time. It also has the potential to detect infections caused by any *Le-*

*gionella* species and serogroups, in addition to detect non-culturable legionella and allow the manipulation of a large number of samples[3,9,59,64]. The detection of other species besides *L. pneumophila* is relevant to the diagnosis of Legionnaires' disease, since this disease can be caused by other species, such as *L. longbeachae* and *L. bozemanii*. The correct identification of species is also important for epidemiological studies and identification of sources of infection[65]. However, the major disadvantage of PCR it is the inability to evaluate the viability, in other words, by the PCR technique it is not possible to distinguish between viable and nonviable microorganisms, detecting only their presence or absence, while only viable bacteria are able to cause infections in human and represent an interest for public health[20,23,47]. The qPCR is an important complement, but not a substitute, for the standard culture-based methods[66].

The development of new and rapid assays that combine both specific detection and viability criteria is essential for monitoring water quality and legionellosis prevention[3,24]. A rapid, sensitive, and specific method for the detection of *Legionella* is clearly important to identify infected individuals and to expedite cleanup of contaminated water systems (reduction in disinfecting treatment cost) in order to prevent additional cases of infection[25,47].

## 5. Conclusions

In conclusion, elucidation of the properties of the *Legionella* species is needed to assess their potential public health significance and explain the conditions favoring their growth in aquatic environments. The detection tools for *Legionella* species have been commonly based on culture method investigations. However, this method is not conveniently sensitive and is time-consuming (most *Legionella* spp. colonies being detected within 7 days). From this study, we can conclude that the utilization of the technique of culture, according to the International Standard ISO 11731:1998, it is possible to have false positives results in the detection of presumptive *Legionella* species. Therefore, no *Legionella* spp. were detected in water samples analyzed during this work. Prospective studies using the culture method and molecular methods should be performed in parallel, to provide better and more comprehensive approach to detection of *Legionella* species. The method used in this study, allowed obtaining a number of isolates, which form an independent group of all genus of the family *Chitinophagaceae* described so far, and that by their phylogenetic distance may be a genus and therefore a species not yet described.


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## REFERENCES


[1] Taylor, M., Ross, K., Bentham, R. 2009. *Legionella*, protozoa, and biofilms: Interactions within complex microbial systems. Microb Ecol 58:538-547.

[2] Kuroki, H., Miyamoto, H., Fukuda, K., Iihara, H., Kawamura, Y., Ogawa, M., Wang, Y., Ezaki, T., Taniguchi, H. 2007. *Legionella impletisoli* sp. nov. and *Legionella yabuuchiae* sp. nov., isolated from soils contaminated with industrial wastes in Japan. Syst Appl Microbiol 30:273-279.

[3] Diederen, B.M.W., Kluytmans, J.A.J.W., Vandenbroucke-Grauls, C.M., Peeters, M.F. 2008. Utility of real-time PCR for diagnosis of legionnaires' disease in routine clinical practice. J Clin Microbiol 46:671-677.

[4] Tronel, H., Hartemann, P. 2009. Overview of diagnostic and detection methods for legionellosis and *Legionella* spp. Lett Appl Microbiol 48:653-656.

[5] Park, M.Y., Ko, K.S., Lee, H.K., Park, M.S., Kook, Y.H. 2003. *Legionella busanensis* sp. nov., isolated from cooling tower water in Korea. Int J Syst Evol Microbiol 53:77-80.

[6] La Scola, B., Birtles, R.J., Greub, G., Harrison, T.J., Ratcliff, R.M., Raoult, D. 2004. *Legionella drancourtii* sp. nov., a strictly intracellular amoebal pathogen. Int J Syst Evol Microbiol 54:699-703.

[7] Pourcel, C., Visca, P., Afshar, B., D'Arezzo, S., Vergnaud, G., Fry, N.K. 2007. Identification of variable-number tandem-repeat (VNTR) sequences in *Legionella pneumophila* and development of an optimized multiple-locus VNTR analysis typing scheme. J Clin Microbiol 45:1190-1199.

[8] Fraser, D.W., Tsai, T.R., Orenstein, W. 1977. Legionnaires' disease. Description of an epidemic of pneumonia. N Engl J Med 297:1189-1197.

[9] Jarraud, S., Reyrolle, M., Meugnier, H., Forey, F., Etienne, J. 2007. Legionnaires disease. Presse Med 36:279-287.

[10] Kümpers, P., Tiede, A., Kirschner, P., Girke, J., Ganser, A., Peest, D. 2008. Legionnaires' disease in immunocompromised patients: A case report of *Legionella longbeachae* pneumonia and review of the literature. J Med Microbiol 57:384-387.

[11] Stojek, N.M., Szymańska, J., Dutkiewicz, J. 2008. Gram-negative bacteria in water distribution systems of hospitals. Ann Agric Environ Med 15:135-142.

[12] Liguori, G., Bagattini, M., Gallè, F., Quartucci, V., Di Onofrio, V., Negrone, M., Triassi, M. 2009. Microbiological evaluation of the efficacy of two new biodetergents on multidrug-resistant nosocomial pathogens. Ann Clin Microbiol Antimicrob 8:1-6.

[13] Yaradou, D.F., Hallier-Soulier, S., Moreau, S., Poty, F., Hillion, Y., Reyrolle, M., André, J., Festoc, G., Delabre, K., Vandenesch, F., Etienne, J., Jarraud, S. 2007. Integrated real-time PCR for detection and monitoring of *Legionella*

*pneumophila* in water systems. Appl Environ Microbiol 73:1452-1456.

[14] Alp, E., Voss, A. 2006. Ventilator associated pneumonia and infection control. Ann Clin Microbiol Antimicrob 5:1-11.

[15] Diederen, B.M.W. 2008. *Legionella* spp. and Legionnaires' disease. J Infect 56:1-12.

[16] Bartram, J., Chartier, Y., Lee, J.V., Pond, K., Surman-Lee, S. 2007. *Legionella* and the prevention of legionellosis. World Health Organization (WHO). Geneva. SBN: 9241562978.

[17] Fields, B.S., Ed. 2008. *Legionella* in the environment. In: *Legionella pneumophila*: pathogenesis and immunity. Hoffman, P., Friedman, H., Bendinell, M. (Ed.), Springer.

[18] Declerck, P., Behets, J., Van Hoef, V., Ollevier, F. 2007. Replication of *Legionella pneumophila* in floating biofilms. Curr Microbiol 55:435-440.

[19] Fields, B.S., Benson, R.F., Besser, R.E. 2002. *Legionella* and legionnaires' disease: 25 Years of investigation. Clin Microbiol Rev 15:506-526.

[20] Mérault, N., Rusniok, C., Jarraud, S., Gomez-Valero, L., Cazalet, C., Marin, M., Brachet, E., Aegerter, P., Gaillard, J.L., Etienne, J., Herrmann, J.L., Lawrence, C., Buchrieser, C. 2011. Specific real-time PCR for simultaneous detection and identification of *Legionella pneumophila* serogroup 1 in water and clinical samples. Appl Environ Microbiol 77:1708-1717.

[21] International Standards Organisation. 1998. Water quality - detection and enumeration of *Legionella*. International standard ISO 11731. International Standards Organisation (International Organization for Standardization), Geneva, Switzerland.

[22] Association Franc¸aise de Normalisation. 2003. Water quality. Detection and enumeration of *Legionella* spp. and *Legionella pneumophila*. Method by direct inoculation and after concentration by membrane filtration of centrifugation. AFNOR NF T90-431. Association Française de Normalisation, La Plaine Saint-Denis, France.

[23] Delgado-Viscogliosi, P., Solignac, L., Delattre, J.M. 2009. Viability PCR, a culture-independent method for rapid and selective quantification of viable *Legionella pneumophila* cells in environmental water samples. Appl Environ Microbiol 75:3502-3512.

[24] Delgado-Viscogliosi, P., Simonart, T., Parent, V., Marchand, G., Dobbelaere, M., Pierlot, E., Pierzo, V., Menard-Szczebara, F., Gaudard-Ferveur, E., Delabre, K., Delattre, J.M. 2005. Rapid method for enumeration of viable *Legionella pneumophila* and other *Legionella* spp. in water. Appl Environ Microbiol 71:4086-4096.

[25] Nazarian, E.J., Bopp, D.J., Saylors, A., Limberger, R.J., Musser, K.A. 2008. Design and implementation of a protocol for the detection of *Legionella* in clinical and environmental samples. Diagn Microbiol Infect Dis 62:125-132.

[26] Wellinghausen, N., Frost, C., Marre, R. 2001. Detection of *Legionellae* in Hospital Water Samples by Quantitative Real-Time LightCycler PCR. Appl Environ Microbiol 67:3985-3993.

[27] Villari, P., Motti, E., Farullo, C., Torre, I. 1998. Comparison of conventional culture and PCR methods for the detection of *Legionella pneumophila* in water. Lett Appl Microbiol 27:106-110.

[28] Van der Zee, A., Verbakel, H., De Jong, C., Pot, R., Bergmans, A., Peeters, M., Schneeberger, P., Schellekens, J. 2002. Novel PCR-probe assay for detection of and discrimination between *Legionella pneumophila* and other *Legionella* species in clinical samples. J Clin Microbiol 40:1124-1125.

[29] Lück, P.C., Helbig, J.H., Schuppler, M. 2002. Epidemiology and laboratory diagnosis of *Legionella* infections. J Lab Med 26:174-182.

[30] Bartie, C., Venter, S.N., Nel, L.H. 2003. Identification methods for *Legionella* from environmental samples. Water Res 37:1362-1370.

[31] Benson, R.F., Thacker, W.L., Daneshvar, M.I., Brenner, D.J. 1996. *Legionella waltersii* sp. nov. and an unnamed *Legionella* genomospecies isolated from water in Australia. Int J Syst Bacteriol 46:631-634.

[32] Fox, K.F., Brown, A. 1989. Application of numerical systematics to the phenotypic differentiation of legionellae. J Clin Microbiol 27:1952-1955.

[33] Hebert, G.A. 1981. Hippurate hydrolysis by *Legionella pneumophila*. J Clin Microbiol 13:240-242.

[34] Orrison, L.H., Cherry, W.B., Fliermans, C.B. 1981. Characteristics of environmental isolates of *Legionella pneumophila*. Appl Environ Microbiol 42:109-115.

[35] Pine, L., Hoffman, P.S., Malcolm, G.B. 1984. Whole-cell peroxidase test for identification of *Legionella pneumophila*. J Clin Microbiol 19:286-290.

[36] Weaver, R.E., Feeley, J.C., (Eds.), 1979. Cultural and biochemical characterization of Legionnaires' disease bacterium, in: G.L. Jones, G.A. Herbert (Eds.), Legionnaires', the Disease, the Bacterium, and Methodology, Centers for Disease Control, Atlanta, GA, pp. 20-25.

[37] Martinez-Murcia, A.J., Benlloch, S., Collins, M.D. 1992. Phylogenetic interrelationships of members of the genera *Aeromonas* and *Plesiomonas* as determined by 16S ribosomal DNA sequencing: Lack of congruence with results of DNA-DNA hybridizations. Int J Syst Bacteriol 42:412-421.

[38] Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G. 1997. The CLUSTAL X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876-4882.

[39] Martínez-Murcia, A.J. 1999. Phylogenetic positions of *Aeromonas encheleia*, *Aeromonas popoffii*, *Aeromonas* DNA hybridization Group 11 and *Aeromonas* Group 501. Int J Syst Bacteriol 49:1403-1408.

[40] Saavedra, M.J., Figueras, M.J., Martínez-Murcia, A.J. 2006. Updated phylogeny of the genus *Aeromonas*. Int J Syst Evol Microbiol 56:2481-2487.

[41] Saavedra, M.J., Perea, V., Fontes, M.C., Martins, C., Martínez-Murcia, A. 2007. Phylogenetic identification of *Aeromonas* strains isolated from carcasses of pig as new members of the species *Aeromonas allosaccharophila*. Antonie van Leeuwenhoek 91:159-167.

[42] Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111-120.

[43] Saitou, N., Nei, M. 1987. The Neibhbour-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406-425.

[44] Kumar, S., Tamura, K., Jakobsen, I.B., Nei, M. 2001. MEGA2: Molecular evolutionary genetics analysis software. Bioinformatics 17:1244-1245.

[45] Nkongolo, K.K., Nsapato, L. 2003. Genetic diversity in *Sorghum bicolor* (L.) Moench accessions from different ecogeographical regions in Malawi assessed with RAPDS. Genet Resour Crop Evol 50:149-156.

[46] Davin-Regli, A., Bollet, C., Chamorey, E., Colonna D'Istria, V., Cremieux, A. 1998. A cluster of cases of infections due to *Aeromonas hydrophila* revealed by combined RAPD and ERIC-PCR. J Med Microbiol 47:499-504.

[47] Cooper, I.R., Meikle, S.T., Standen, G., Hanlon, G.W., Santin, M. 2009. The rapid and specific real-time detection of *Legionella pneumophila* in water samples using Optical Waveguide Lightmode Spectroscopy. J Microbiol Methods 78:40-44.

[48] Murdoch, D.R. 2003. Diagnosis of *Legionella* infection. Clin Infect Dis 36:64-69.

[49] Edelstein, P.H., Cianciotto, N.P. 2006. *Legionella* Species and Legionnaires' Disease. Prokaryotes 6:988-1033.

[50] Muder, R.R., Yu, V.L. 2002. Infection due to *Legionella* species other than *L. pneumophila*. Clin Infect Dis 35:990-998.

[51] Murray, P.R., Rosenthal, K.S., Pfaller, M.A., (Eds.), 2005. *Legionella*. In: Medical Microbiology, 5th edn. Elsevier Science, pp. 391-340.

[52] Blanco, S., Prat, C., Sánchez, M.D., Ferrer, D., Pellicer, T., Haba, L., Latorre, I., Vilaplana, C., Ausina, V., Domínguez, J. 2008. Evaluation of a *Legionella* urinary antigen enzyme immunoassay for rapid detection of *Legionella pneumophila* in water samples. Int J Hyg Environ Health 211:168-171.

[53] Carvalho, F.R.S., Foronda, A.S., Pellizari, V.H. 2007. Detection of *Legionella pneumophila* in water and biofilm samples by culture and molecular methods from man-made systems in São Paulo - Brazil. Brazil J Microbiol 38:743-751.

[54] Delaedt, Y., Daneels, A., Declerck, P., Behets, J., Ryckeboer, J., Peters, E., Ollevier, F. 2008. The impact of electrochemical disinfection on *Escherichia coli* and *Legionella pneumophila* in tap water. Microbiol Res 163:192-199.

[55] Lee, J.V., West, A.A. 1991. Survival and growth of *Legionella* species in the environment. J Appl Bacteriol 121S-129S.

[56] Ozlem Sanli-Yurudu, N., Kimiran-Erdem, A., Cotuk, A. 2007. Studies on the efficacy of Chloramine T trihydrate (N-chloro-p-toluene sulfonamide) against planktonic and sessile populations of different *Legionella pneumophila* strains. Int J Hyg Environ Health 210:147-153.

[57] Sanden, G.N., Morrill, W.E., Fields, B.S., Breiman, R.F., Barbaree, J.M. 1992. Incubation of water samples containing amoebae improves detection of legionellae by the culture method. Appl Environ Microbiol 58:2001-2004.

[58] Kozioł-Montewka, M., Magryś, A., Stojek, N., Palusińska-Szysz, M., Danielak, M., Wójtowicz, M., Niewiedzioł, J., Koncewicz, R., Niedźwiadek, J., Paluch-Oleś, J., Trzeciak, H., Drozański, W., Dutkiewicz, J. 2008. Monitoring *Legionella* species in hospital water systems. Link with disease and evaluation of different detection methods. Ann Agric Environ Med 15:143-147.

[59] Diederen, B.M.W., de Jong, C.M.A., Aarts, I., Peeters, M.F., van der Zee, A. 2007. Molecular evidence for the ubiquitous presence of *Legionella* species in Dutch tap water installations. J Water Health 5:375-383.

[60] Allegra, S., Berger, F., Berthelot, P., Grattard, F., Pozzetto, B., Riffard, S. 2008. Use of flow cytometry to monitor *Legionella* viability. Appl Environ Microbiol 74:7813-7816.

[61] Dusserre, E., Ginevra, C., Hallier-Soulier, S., Vandenesch, F., Festoc, G., Etienne, J., Jarraud, S., Molmeret, M. 2008. A PCR-based method for monitoring *Legionella pneumophila* in water samples detects viable but noncultivable legionellae that can recover their cultivability. Appl Environ Microbiol 74:4817-4824.

[62] Joly, P., Falconnet, P.A., André, J., Weill, N., Reyrolle, M., Vandenesch, F., Maurin, M., Etienne, J., Jarraud, S. 2006. Quantitative real-time *Legionella* PCR for environmental water samples: Data interpretation. Appl Environ Microbiol 72:2801-2808.

[63] Behets, J., Declerck, P., Delaedt, Y., Creemers, B., Ollevier, F. 2007. Development and evaluation of a Taqman duplex real-time PCR quantification method for reliable enumeration of *Legionella pneumophila* in water samples. J Microbiol Methods 68:137-144.

[64] Wilson, D.A., Yen-Lieberman, B., Reischl, U., Gordon, S.M., Procop, G.W. 2003. Detection of *Legionella pneumophila* by real-time PCR for the mip gene. J Clin Microbiol 41:3327-3330.

[65] Bencini, M.A., Van Den Brule, A.J.C., Claas, E.C.J., Hermans, M.H.A., Melchers, W.J.G., Noordhoek, G.T., Salimans, M.M.M., Schirm, J., Vink, C., Van Der Zee, A., Jansen, R. 2007. Multicenter comparison of molecular methods for detection of *Legionella* spp. in sputum samples. J Clin Microbiol 45:3390-3392.

[66] Morio, F., Corvec, S., Caroff, N., Le Gallou, F., Drugeon, H., Reynaud, A. 2008. Real-time PCR assay for the detection and quantification of *Legionella pneumophila* in environmental water samples: Utility for daily practice. Int J Hyg Environ Health 211:403-411.