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# Occurrence of *Legionella* in showers of recreational facilities

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## ABSTRACT

Critical environments, including water systems in recreational settings, represent an important source of *Legionella pneumophila* infection in humans. In order to assess the potential risk for legionellosis, we analyzed *Legionella* contamination of water distribution system in 36 recreational facilities equipped with swimming pools. One hundred and sixty water samples were analyzed from shower heads or taps located in locker rooms or in bathrooms. By culture method and polymerase chain reaction, 41/160 samples were positive for *Legionella* from 12/36 recreational centers. Hotels (57.1%) and sports centers (41.2%) were the most contaminated. *L. pneumophila* serotypes 2–14 (25/41) were more frequently found than serotype 1 (10/41). Samples at temperature $\geq$30 °C were more frequently positive than samples at temperature <30 °C ($n = 39$ vs $n = 2$, $p < 0.00001$). The presence of *L. pneumophila* was investigated by comparison with heterotrophic plate count (HPC), an indicator of water quality. The presence of *L. pneumophila* was associated more frequently with high and intermediate HPC load at 37 °C, therefore should be considered a potential source when HPC at 37 °C is >10 CFU/mL. Maintenance, good hygiene practices, interventions on the hydraulic system and regular controls must be implemented to minimize exposure to *L. pneumophila* infection risk.

**Key words** | hot water system, *Legionella*, recreational and sport facilities, shower

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## INTRODUCTION

*Legionella* is a fastidious Gram-negative bacterium living in natural aquatic environments (lakes, rivers, etc.). It can be found at high concentrations in artificial habitats such as warm man-made water systems. Temperatures between 25 and 43 °C, the inorganic and organic contents of water, and the presence of protozoa play key roles in *Legionella*'s replication (Steinert *et al.* 2002; Laganà *et al.* 2014). Also, the presence of dead-end loops, stagnation in plumbing systems and service intervals, have been shown to be important risk factors (Exner *et al.* 2005).

Some species belonging to the *Legionella* genus, mostly *L. pneumophila*, may cause the serious pulmonary infection 'Legionnaires' disease' (LD). Known risk factors for LD include increasing age, male sex, smoking, chronic lung disease, diabetes, and various conditions associated with immunodeficiency (ECDC 2016). There are no reported cases of interhuman transmission and the environment may represent the only source of infection (ECDC 2016).

The transmission of *Legionella* takes place by inhalation of contaminated aerosols which can be produced by air conditioning systems, cooling towers, whirlpools, spas, ice machines, dental devices, and shower heads (Castiglia *et al.* 2008; Mouchtouri *et al.* 2010). Others sources of *Legionella* exposure, such as hotel fountains, have been recently

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documented regarding outbreaks of legionellosis (Smith *et al.* 2015).

The incidence of the disease has been significantly increasing in recent years. In the USA, a 192% increase in the crude national incidence of *Legionella* disease has been observed, rising from 3.9 cases per million inhabitants in 2000, to 11.5 cases per million inhabitants in 2009 (Cunha *et al.* 2016). Similarly, in Europe, the latest report on ECDC data of 2014 indicates the highest incidence reported so far in Europe, with 6,941 cases of Legionnaires' disease notified (ECDC 2016). Although the majority of cases (up to 74% in the ECDC report) were community acquired, 18% of cases were travel associated (ECDC 2016), indicating the need for particular attention to recreational facilities. Similarly, in Italy, the latest Legionnaires' disease prevalence data, reported for the year 2014 and a total of 1,497 cases, show a slight increase over the previous year. Interestingly, 10% of these are associated with travel (hotels, camping, ships, etc.) and 1% is related to people who attend sports facilities (Rota *et al.* 2015).

The use of contaminated hot water distribution systems via showers and display pools has been identified as a potential health hazard for legionellosis development, especially for populations who attend sports centers (Bonadonna *et al.* 2009). Often these centers organize courses, or are attended not only by children and adults but also by the elderly, pregnant women, and people undergoing rehabilitative treatments, all of whom may have a weakened immune system (Leoni *et al.* 2001). This condition, combined with individual factors and their concomitant pathologies, contributes to subjects' susceptibility to develop legionellosis from the same source of *Legionella* exposure (Berrington & Hawn 2013).

In this context, monitoring critical environments that create favorable conditions for the excessive growth of *Legionella*, including various water systems in recreational settings, plays a critical role in disease control.

The first Italian guidelines concerning the control and prevention of legionellosis were published in 2000 (Linee Guida 2000). In 2005 they were followed by the instructions for tourist accommodation and spas (Linee Guida 2005). In May 2015, a new document was approved with the aim to gather, update, and integrate in a single text all the instructions reported in the previous national guidelines and regulations (Linee Guida 2015). The instructions recommend that critical factors for *Legionella* growth and diffusion must be taken into account during designing and maintaining water systems. Although it cannot be guaranteed that the bacteria will be completely eradicated, such measures reduce a possible contamination.

In this study, we have evaluated *Legionella* contamination in the hot water distribution systems of some recreational facilities in order to assess possible risks of legionellosis outbreaks. Also, we investigated the relationship between the presence of *Legionella* and heterotrophic plate count (HPC). This parameter is an indicator of general water quality within the distribution systems.

## METHODS

### Study area and sampling

From May 2014 to June 2015, water samples were collected from hot water distribution systems of 36 recreational centers equipped with swimming pools. These facilities are located in the southern area of Rome, under territorial responsibility of the Local Health Units (ASL) Roma 6 ex H (according to the Italian National and Regional Health Plan, the ASL ensures health protection of the population, by providing health care services and performing health checks in facilities within their territory). All the buildings were supplied from the public network that use groundwater. In detail, the samples were taken from the following recreational centers: 17 sports centers, 8 camp sites, 5 hotels and 2 holiday farmhouses, 3 beach resorts and 1 amusement park. Two to 18 water samples were taken from each facility according to the size of the building.

A total of 160 water samples were taken from shower heads or taps located in locker rooms or bathrooms. In agreement with the Italian guidelines for hot water sampling in common use conditions (namely, instantaneous samples to simulate the possible exposure by a user), all the samples were taken without flaming the outlet point and without previously running the water (Linee Guida 2015). The temperature was measured during the sampling. Although all samples were collected by turning on the hot water, not all samples exceeded 30 °C.

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Samples were placed in 1 L sterile bottles, containing 10% sodium thiosulfate to neutralize the chlorine (able to neutralize up to 5 mg/L of residual free and combined chlorine) (ISO 19458; Manuali e Linee Guida 29/2003; Rapporti ISTISAN 07/5). Then, they were transported at room temperature, protected by direct light, to the laboratory for microbiological analysis. Some water samples, collected in those centers where corrective actions had been recently made, were included in the analysis.

The samples were taken as part of a microbiological risk monitoring campaign for control of the territory carried out in collaboration with Local Health Authorities as required by law for these types of building.

### Microbiological analysis

*Legionella* isolation was performed in accordance with the selective procedure described in the Italian guidelines (Linee Guida 2015). One liter of water was filtered through a membrane with 0.20 μm diameter pores (cellulose nitrate filter, Sartorius). Each membrane was aseptically inserted into test tubes containing 5 mL of the original water sample and then shaken with vortex for 2 minutes to detach the bacteria. In order to reduce contamination by interfering microorganisms (ISO 11731; Linee Guida 2015), the sample was held at 50 °C for 30 minutes. Then, an aliquot of 0.5 mL was spread on *Legionella* CYE agar base (Oxoid) with an addition of BCYE growth supplement and GVPC selective supplement (Oxoid). The inoculated plates were incubated at 37 ± 1 °C in 2.5% $CO_2$ for 10 days and read every day. Suspected colonies were counted from each sampling and subsequently confirmed by their inability to grow on CYE agar base without BCYE growth supplement and by the final agglutination with *Legionella* Latex Test Kit (Oxoid). The test allows a separate identification of *L. pneumophila* serogroup 1 and serogroups 2–14 and detection of seven *Legionella* (polyvalent) species, which have been implicated in human disease: *L. longbeachae*, *L. bozemanii* 1 and 2, *L. dumoffii*, *L. gormanii*, *L. jordanis*, *L. micdadei*, and *L. anisa*.

Isolated positive *Legionella* colonies were also confirmed by polymerase chain reaction (PCR) assay, according to the protocol of Van der Zee *et al.* (2002). The primer set used, LEG1 (5′TACCTACCCTTGACATACAGTG-3′) and LEG2 (5′-CTTCCTCCGGTTTGTCAC-3′), was derived from the 16S rRNA gene sequence and used to amplify a 200 bp DNA fragment specific for all *Legionella* species. The PCR reaction mixture, 25 μL final volume, contained 10 pmol of each primer, LEG1 and LEG 2, 200 μM of each dNTP, 3 mM MgCl2, and 2 U AmpliTaq Gold polymerase in 1 × PCR buffer (Promega). Samples were preheated for 10 min at 95 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C, with a final extension of 5 min at 72 °C. A negative and positive control was included in each PCR run. Amplified DNA was detected by agarose gel electrophoresis and ethidium bromide staining.

The results were expressed in CFU/L and the detection limit, based on the concentration factor and the volume of the inoculum, was 10 CFU/L. Accuracy of method is monthly checked through internal titered control.

The HPC at 22 °C and 37 °C was determined in duplicate by the pour plate method, by using standard plate count agar (Oxoid). The plates were incubated at 37 °C for at least 48 h and at 22 °C for at least 72 h. The results were expressed in CFU/mL.

### Statistical analysis

Data are expressed as median and range and as percentage, as appropriate. Comparison between groups was made: (i) for 2 × 2 tables by using Fisher's exact test or chi-square with Yates' correction, as appropriate; (ii) for all the other tables by chi-square or chi-square for trend when data in the cells were below 5. Bonferroni's correction for multiple comparisons was applied when appropriate. A $p$ value below 0.05 was considered significant. All analyses were performed by using the GraphPad Prism 5.0 (Graphpad software, San Diego, CA, USA) software package.

## RESULTS

Table 1 shows the distribution of positive *Legionella* samples among the different recreational facilities. From a total of 160 water samples taken from 36 recreational facilities, 41 (25.6%) samples were positive for *Legionella*, among them 12 (33.3%) recreational centers were found to be positive. Hotels and sports centers were found to be the most

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**Table 1** | Number of facilities/samples monitored and positive for *Legionella pneumophila*

| | Facilities Number | Total samples Number | Samples per facility Median (min–max) | Facilities positive for *L. pneumophila* Number (%) | Number of samples positive for *L. pneumophila* Number (%) | Samples positive for *Legionella* per facility Median (min–max) |
|---|---|---|---|---|---|---|
| Sports centers | 17 | 92 | 3 (2–18) | 7 (41.2) | 32 (34.8) | 0 (0–11) |
| Camping sites | 8 | 22 | 3 (2–3) | 1 (12.5) | 2 (9.1) | 0 (0–2) |
| Hotels and holiday farmhouses | 7 | 34 | 3 (2–17) | 4 (57.1) | 7 (20.6) | 1 (0–3) |
| Beach resort and amusement park | 4 | 12 | 3 (3–3) | 0 (0) | 0 (0) | 0 (0–0) |
| Total | 36 | 160 | 3 (2–18) | 12 (33.3) | 41 (25.6) | 0 (0–11) |

affected by *L. pneumophila* colonization, namely 4/7 (57.1%) and 7/17 (41.2%) positive facilities, respectively.

All isolated positive *Legionella* colonies were confirmed by PCR alongside a sub-set of *Legionella* negative cultures (all negative). The structures in which *Legionella* has been found were homogeneously distributed in the districts of the reference area (ASL Roma 6, formerly called ASL Roma H).

Table 2 shows the relationship between the presence of *Legionella* and the temperature of the samples collected from hot water distribution systems. Most of the water samples positive for *L. pneumophila* colonization were related to temperature higher than 30 °C (samples >30 °C $n = 39$, samples <30 °C $n = 2$, $p < 0.00001$). In particular, while this observation is generally valid for sports centers and camp sites, both cold and hot samples collected from hotels showed the same level of contamination by *Legionella*.

Table 3 shows the concentrations of *L. pneumophila* load found in all monitored facilities. In nearly all samples, they ranged from $\geq 10^3$ to $<10^5$ CFU/L, but in three samples, taken in sports centers, the contamination was $>10^5$ CFU/L.

The distribution of the *L. pneumophila* load in the analyzed samples did not display differences among the various analyzed locations ($p > 0.05$ all comparisons, Table 3). The *L. pneumophila* serotypes 2–14 were found more frequently than serotype 1. Finally, in 134 of 160 water samples, the presence of *L. pneumophila* and its load have been compared to the total bacterial load (Table 4). The HPC at 22 °C did not correlate with *L. pneumophila* in the analyzed samples ($p > 0.05$). However, the high ($>10^3$ CFU/mL) and intermediate ($10^1$–$10^3$ CFU/mL) total bacterial load of the HPC at 37 °C is generally correlated to a higher frequency of positivity for *L. pneumophila* ($p < 0.005$ vs total bacterial load $>10^3$ CFU/mL and $10^1$–$10^2$ CFU/mL).

**Table 2** | Distribution of samples positive for *Legionella pneumophila* in relationship with the temperatures measured during the sampling

| | Total samples Number | Thermal characterization of samples[b] | | Samples positive for *L. pneumophila* by thermal characterization at the sampling | | |
|---|---|---|---|---|---|---|
| | | < 30 °C | ≥ 30 °C | < 30 °C, number (%) | ≥ 30 °C, number (%) | *p*-value[a] |
| Sports centers | 92 | 8 | 84 | 0 (0%) | 32 (38%) | 0.0471 |
| Camping sites | 22 | 17 | 5 | 0 (0%) | 2 (40%) | 0.0433 |
| Hotels and holiday farmhouses | 34 | 12 | 22 | 2 (17%) | 5 (23%) | 1.000 |
| Beach resort and amusement park | 12 | 12 | 0 | 0 (0%) | n.a | n.a |
| Total | 160 | 49 | 111 | 2 (4%) | 39 (35%) | <0.00001 |

[a]*p*-value as for Fisher's exact test for comparison in each facility type studied the <30 °C vs >30 °C samples for the identification of *L. pneumophila*.
n.a.: not applicable.
[b]Temperature of the samples collected from hot water distribution systems.

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**Table 3** | Distribution of samples positive for *Legionella* by bacterial load and serotype

| | Samples positive for *L. pneumophila* Number | *L. pneumophila* load (CFU/L) | | | *L. pneumophila* serotype | | |
|---|---|---|---|---|---|---|---|
| | | $\geq 10^3 - < 10^4$ | $\geq 10^4 - < 10^5$ | $\geq 10^5$ | Ser 1 (%) | Ser 2–14 (%) | Mixed (%) |
| Sports centers | 32 | 13 | 16 | 3 | 6 (18.8) | 21 (65.6) | 5 (15.6) |
| Camping sites | 2 | 0 | 2 | 0 | 2 (100) | 0 (0) | 0 (0) |
| Hotels and holiday farmhouses | 7 | 5 | 2 | 0 | 2 (28.6) | 4 (57.1) | 1 (14.3) |
| Beach resort and amusement park | 0 | n.a | n.a | n.a | n.a | n.a | n.a |
| *p-value*[a] | | 0.3218 | | | 0.1457 | | |
| Total | 41 | 18 | 20 | 3 | 10 | 25 | 6 |

[a]Chi-square for trend for all groups' comparison.
n.a.: not applicable.

**Table 4** | HPC load (22 °C and 37 °C) related to the presence of *L. pneumophila* detected using culture

| HPC 22 °C CFU/mL | Number of samples Number | Number of samples positive for *L. pneumophila* Number (%) | HPC 37 °C CFU/mL | Number of samples Number | Number of samples positive for *L. pneumophila* Number (%) |
|---|---|---|---|---|---|
| $>10^3$ | 28 | 5 (17.8) | $>10^3$ | 36 | 14 (38.9) |
| $10^2$–$10^3$ | 10 | 4 (40) | $10^2$–$10^3$ | 18 | 5 (27.8) |
| $10^1$–$10^2$ | 19 | 7 (36.8) | $10^1$–$10^2$ | 21 | 12 (57.1) |
| <10 | 77 | 21 (27.3) | <10 | 59 | 7 (11.8)* |
| *p-value*[a] | | *0.4049* | *p-value*[a] | | *0.0009* |

[a]Chi-square test for all groups' comparison.
*$p$ <0.005 (Fisher's exact test) for subgroup <10 vs $10^1$–$10^2$ and <10 vs $>10^3$.

## DISCUSSION

It was previously demonstrated by Leoni *et al.* (2001) that the potential risk of becoming infected by *Legionella* in sport and recreational facilities equipped with swimming pools is not connected to the use of the pool, but to the showers. Indeed, the greater concentration of chlorine, the mixed type of contamination, and the lower temperatures in the pool might contribute to preventing *Legionella* from reproducing and proliferating, thus reducing health risk (Leoni *et al.* 2001). Conversely, biofilms on surfaces within water distribution pipes can create a biological niche suitable for *Legionella* growth and persistence (Declerck *et al.* 2009).

The infection with *Legionella* is known to be associated with the inhalation of aerosols containing the bacteria. Therefore, the aerosol created in the showers has been identified as a potential pathway for exposure to *Legionella* from colonized pipes (Cowen & Ollison 2006; Schoen & Ashbolt 2011).

In recreational centers with a swimming pool, it is mandatory to shower before diving in the pool, therefore it is clear how the risk of exposure is greater if the water system is contaminated with *Legionella*.

According to the above considerations, the survey presented here has been performed on water samples mainly collected from showers of the hot water distribution system of recreational centers, monitored during an environmental surveillance.

In line with literature reports (Steinert *et al.* 2002; Borella *et al.* 2005; Leoni *et al.* 2005), we have found a widespread contamination of *Legionella*. The bacteria were found in one-third of the facilities and in one-fourth of the samples analyzed, again clearly indicating the limited efficacy in controlling *Legionella* colonization in

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recreational facilities even though guidelines have been enforced for the last ten years (Linee Guida 2005). In addition, considering that in recent years the number of people who play sport has significantly increased and also the people who may have a higher chance of becoming ill from *Legionella* (the elderly or young children), the environmental control and surveillance for *Legionella* is increasingly important.

Although *L. pneumophila* is an ubiquitous environmental microorganism, the real risk to public health is represented by its concentration. A high *Legionella* load in some microenvironments, such as hot water distribution systems that produce aerosols, might pose a strong risk of contracting the disease. In this study, about 14.4% of the examined samples showed a concentration of *Legionella* >10,000 CFU/L. In agreement with Italian guidelines, such contamination level, even in the absence of cases of disease, requires the immediate implementation of appropriate disinfection measures (Linee Guida 2005, 2015).

Even though the samples were taken from the hot water distribution systems, the temperature detected at the time of sampling was not always ≥30 °C: in fact, 30% of them were <30 °C. However, the temperatures detected were never higher than 40 °C. The majority of the samples that were positive for *L. pneumophila* are generally related to water with temperatures higher than 30 °C, as also suggested by Borella *et al.* (2005) and Leoni *et al.* (2005).

It is interesting to note that the larger structures showed greater contamination, probably due to the complexity of the hydraulic system, characterized by a high number of distribution points and by the occurrence of stagnation points in pipes, where the disinfection treatment could be more complex and less effective (Borella *et al.* 2005).

In six sport facilities, a notice to perform appropriate sanitizing actions was issued and subsequently controls were performed to evaluate the absence of *Legionella*, or at least the reduction of the bacterial load (data not shown here).

In some structures, a repeated treatment has been required to remove *Legionella* by changing the cleaning mode in order to increase its effectiveness. In our practical experience, we have observed that the most effective solution has been using in combination two treatments of decontamination: hyper-chlorination and heating to 60 °C, especially for the complex water distribution systems.

The *L. pneumophila* serotype distributions were found, as expected, to belong to serotypes 2–14 more frequently than serotype 1 (Bonadonna *et al.* 2009; Napoli *et al.* 2010). In fact, more than half of the samples were contaminated by *L. pneumophila* serotypes 2–14 (61.0%), while only 24.4% were positive for serotype 1, and in 14.6% of samples, *L. pneumophila* serotype 1 and serotypes 2–14 were found (mixed cultures).

Finally, in a subgroup of samples, the *Legionella* contamination was investigated in comparison with the HPC at 22 and 37 °C in the colonization of water system pipes. This research aimed to look in depth into the relationship between heterotrophic bacteria and *Legionella* in hot water systems, which has begun to be investigated recently (Bargellini *et al.* 2011).

HPCs were among the first parameters used to monitor drinking water and have become an indicator of water quality within distribution systems (Bartram *et al.* 2003). HPCs are, in fact, used to follow biofilm development in both drinking and hot water distribution systems (Moritz *et al.* 2010). The presence of these bacteria could be correlated with the colonization of water systems by *Legionella*. Indeed, it is important to consider that *Legionella* can survive not only as isolated bacteria but also as intracellular parasites of amoebae, ciliated protozoa, or slime molds, all conditions found in naturally occurring microbial communities that form biofilms (Cunha *et al.* 2016).

The results obtained show a possible association between the presence of *Legionella* and high values of HPC at 37 °C. This might suggest the use of this parameter as a preliminary assessment of the possible presence of *Legionella*. Therefore, high values of HPC at 37 °C in a potability analysis may also suggest searching for *Legionella*, particularly in those cases where there is a high risk for the population to become ill.

## CONCLUSIONS

Epidemiological studies show that potable water systems contaminated by *Legionella* are a significant cause of sporadic cases of legionellosis acquired in the community; in this

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context, recreational and sports facilities are also included (Delia *et al.* 2007; Bonadonna *et al.* 2009).

Although in recent years attention regarding the risks of all the population has increased, in these establishments microbiological surveillance should be more frequent, in order to control the environmental spread of *Legionella*.

Facilities' administrators and operators should be responsible for operations and management, including the water distribution system. Proper planning, design, installation, and management of the hydraulic system must be followed by specialized operators and maintained by qualified personnel, because good general hygiene practices and interventions to minimize exposure to specific risks are the foundation of all the prevention activities. In particular, the presence of *Legionella* should be considered and investigated in potential sources when high loads of heterotrophic bacteria or its variations are present.

Based on the results obtained, we consider it important to examine in depth the relationship between HPC and *Legionella* and to extend the study to other analytical parameters that could facilitate the spread of *Legionella* in hot water distribution circuits.

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