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Environmental Surveillance of *Legionella* spp. in an Italian University Hospital Results of 10 Years of Analysis

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Abstract: The occurrence of *Legionella* spp. in the water distribution systems of large hospitals and other healthcare facilities is considered particularly dangerous, due to the critical nature of the hospitalized patients. The aim of this study is to present a pluri-annual environmental surveillance in a large university hospital assessing the prevalence of *Legionella* spp. and underlining its variability over the years. The samples of water were collected in accordance with the Italian National Guidelines and the sampling sites considered in this study were selected favoring wards with very high-risk patients and with patients at increased risk. The laboratory analyzed a total of 305 water samples deriving from 24 different sampling points. *Legionella* spp. were detected in 39.4% of samples, the majority of which were contaminated by *Legionella pneumophila* serogroups 2–14 (68.7%). Statistically significant differences were found among different seasons with a linear trend in positive proportion from summer to spring. Several experimental interventions to prevent and reduce *Legionella* colonization were attempted, but there is no a definitive method for the complete eradication of this microorganism. The permanent monitoring of hospital water distribution systems is fundamental to preventing the potential risk of nosocomial Legionellosis and to implementing procedures to minimize the risk of *Legionella* spp. colonization.

Keywords: *Legionella* spp.; hospitals; environmental surveillance; water systems; Italy

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1. Introduction

Legionella spp. are Gram-negative waterborne pathogens known to be the causative agents of Legionnaires' disease, a severe atypical pneumoniae mainly acquired through aspiration of contaminated water, and Pontiac fever, a flu-like syndrome [1,2].

The genus *Legionella* currently includes 61 species and more than 70 serogroups, of which about half have been recognized as responsible for opportunistic infections in human beings. *Legionella* spp. are intracellular parasites of freshwater protozoa and they use a similar mechanism to multiply within human phagocyte cells. *Legionella pneumophila (L. pneumophila)*, the most common cause of Legionnaires' disease cases, is subdivided into 16 different serogroups, of which the serogroup 1 accounts for around 85% of all cases of legionellosis worldwide [3,4].

Legionella, a ubiquitous bacterium commonly found in various aquatic environments, both natural and human-made, can survive for long periods and multiply in low or undetectable numbers. Water systems of large public buildings, households, and industrial plants are suitable environments for the growth and replication of *Legionella* spp. and other Gram-negative bacteria. If favorable conditions are created (areas of stagnation, parasitism of amoebas, sludge formation), it may result in the acquisition of infection through aspiration of contaminated water or direct inhalation of aerosols [5,6]. The probability of infection

depends on the rate of multiplication of bacteria in water installations, the formation of water–air aerosol, as well as the susceptibility of the subject [7].

Particularly dangerous, due to its high lethality, is the occurrence of *Legionella* spp. in the water distribution systems of large hospitals and other healthcare facilities since these often present several risk factors such as the presence of dead-legs, storage tanks, low flow, and old pipelines. Therefore, water systems represent a potential risk for the hospital population, primarily if *Legionella* spp. is found in certain wards such as intensive care, hematology, cardiology, hemodialysis, and pulmonology due to the critical nature of patients hospitalized in these units [8–10].

According to the latest available data collected by the European Centre for Disease Prevention and Control (ECDC), 10,672 confirmed cases of Legionellosis were reported in 2018, 6% of which were acquired in a hospital [11]. In Italy, in 2019, there were 3199 cases of Legionellosis, 3.8% were hospital acquired [12].

In order to prevent and control *Legionella* infection sourced from the colonization of water systems, many countries have developed guidelines or regulations. The Italian National guidelines include several concepts in common with other international guidelines, e.g., giving importance to the correct construction and maintenance of water systems, the use of disinfection methods and environmental investigations and the creation of risk assessments for exposure to *Legionella*. Furthermore, corrective interventions include short-term measures such as replacement of shower heads and taps or long-term measures such as filtration, thermal shock, hyperchlorination, and disinfection with chlorine dioxide [13,14].

Moreover, according to the guidelines of the World Health Organization (WHO) and the recommendations of the European Legionnaires' Disease Surveillance Network (ELDSNet), especially in healthcare settings, periodic monitoring of the presence of *Legionella* in the water network in conjunction with control measures is thought to be the most successful preventive approach to avoid possible exposure of patients and health professionals. Proactive environmental surveillance is a useful strategy for preventing nosocomial Legionnaires' disease [15,16], as well as other nosocomial infections [17–19].

In accordance with the Italian National guidelines, in healthcare settings, specific interventions must be planned to ensure the absence of colonization of the air treatment systems and the absence of *Legionella* (not detectable in relation to the analytical method used and in any case always <100 CFU/L) in the supplied water [14].

The aim of this study is to present a pluri-annual environmental surveillance conducted with the aim to assess the *Legionella* spp. prevalence in a large university hospital in Sassari (Italy) from 2010 to 2020, underlining its variability over the years and evaluating the potential risk for hospitalized patients and healthcare workers.

2. Materials and Methods

2.1. Study Setting

The study was conducted in the University Hospital of Sassari, the main hospital in Sardinia for the number and heterogeneity of its technological and professional resources (861 beds and 3044 employees). The hospital, which carries out multi-specialist activities of care, teaching, and research for all of northern Sardinia, is comprised of seven pavilions in total.

At the beginning of the 10-years study, a sampling program was prepared which provided for an equal number of samplings every year, at least once a month, even if, due to various contingencies (e.g., restructuring of departments, detection of problems in portions of the water network) some samplings were delayed or more frequently implemented, both between years and between seasons within a specific year.

The laboratory analyzed a total of 170 scheduled samples deriving from 24 different sampling points. Including the control samples carried out following the decontamination procedures performed after the finding of a positive sample, there were 305 water samples (scheduled + control samples). The statistical analysis was performed on the scheduled samples, being all the control samples negative.

The water samplings were performed by a member of the Laboratory of the Hygiene and Hospital Infection Control Operative Unit of the hospital. Sampling sites considered in this study were selected favoring wards with very high-risk patients and with patients at increased risk [14].

The wards analyzed were located in 5 of the 7 hospital pavilions: Gynecology and Obstetrics, Neonatology, Hematology and Operating Rooms (pavilion 2), Intensive Care Unit (pavilion 3), Pulmonology and Operating Rooms (pavilion 4), Infectious Diseases (pavilion 5) and Operating Rooms, Cardioanesthesia, Burn Center and Intensive Care Unit (pavilion 7).

2.2. Sampling

In accordance with the 2015 Italian National Guidelines, the samples of heated water were collected, without running the water, in sterile 1-L bottles containing 0.01% sodium thiosulfate concentration in order to neutralize any residual presence of chlorine [14].

The collected samples were taken to the laboratory and processed within 24 hours according to ISO 11731: 1998 and ISO 11731-2: 2004, in order to be able to compare the results during the years of the study. The 1-L water samples were filtered through sterile membranes of polycarbonate or nylon or nitrocellulose with porosity equal to 0.22 µm or with several membranes in succession if the sample was particularly contaminated. At the end of filtration, the membrane was placed in a closed Falcon container, containing 10 mL of sample water. The detachment of the microorganisms which have been retained was then carried out scraping the membrane by means of glass sticks. After a vigorous stirring with the vortex, the samples obtained represented the concentrate to be used for inoculation.

To minimize the growth of other microbial flora, the concentrated samples were divided into three aliquots: inoculum without treatment, inoculum with heat treatment for 30′ ± 1 at 50 ± 2 °C in an oven, and inoculum with acid treatment in which 1 mL of sample was centrifuged at 3000× *g* for 30′. Half of the supernatant was removed and 0.5 mL of a solution of HCl-KCl at pH 2.2 ± 0.2 was added to the other half, leaving it at room temperature for 5′. The samples were then sown (0.1 mL) distributing them homogeneously with a sterile spatula. They were then incubated at 36 ± 1 °C in a thermostat, in a humid environment with an atmosphere enriched with CO_2 for 10 days.

The suspected colonies were isolated and confirmed as *Legionella* spp. after verifying the presence of growth on a BCYE (buffered charcoal yeast extract) plate with cysteine and their inability to grow on the culture medium without cysteine. A significant number of *Legionella*-positive colonies, at least 10 if suspicious colonies were present, were tested from each plate and then identified as *L. pneumophila* serogroup 1, *L. pneumophila* serogroups 2–14, or other *Legionella* species implicated in human diseases using the latex agglutination test (*Legionella* Latex Test, Oxoid Ltd., Cheshire, UK).

The number of *Legionella* was derived from the count of the highest number of bacteria observed in the three samples into which we divided our concentrate and results were presented as the number of colony-forming units (CFU) of *Legionella* per liter (L). Their absence was denoted with the statement "not found". Samplings were divided into three groups according to the latest national guidelines: (1) 100–1000 CFU/L; (2) 1001–10,000 CFU/L; (3) > 10,000 CFU/L. Samples with a bacterial load lower than 100 CFU/L were not taken into consideration as no intervention was required.

In the event of contamination, in compliance with the Italian National Guidelines, the colonized pipeline was immediately subjected to decontamination procedures and then resampled after 1 and 3 months. Remedial actions, in accordance with ELDSNet recommendations, were thermal shock treatment at 70–80 °C and hyperchlorination.

In accordance with the Italian National Health Institute Technical Report, water temperature and residual chlorine of the sample were determined at the time of collection [20]. The samples were also searched for *Pseudomonas aeruginosa* as it is believed there is a negative correlation with the finding of *Legionella* [21].

In this latter case, the sampling, generally equal to 250 mL, was carried out after fluxing the water to check the contamination of the system. Then, in accordance with the UNI EN 12780: 2002 standard, we proceed with filtration of a portion of the sample with a 47-mm diameter cellulose ester membrane with filtration characteristics equivalent to a nominal pore diameter of 0.45 µm. The membrane is placed on the surface of the selective medium Cetrimide Agar and, after incubation at 36 ± 1 °C for 40–48 hours, typical colonies are counted and confirmed. Blue-green colonies that produce pyocyanin are considered confirmed.

The RT-PCR analysis was performed by iQ-Check™ Quanti *Legionella* spp., according to the manufacturer's instructions (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.3. Statistical Analysis

The collected data were archived in a specific database. Results were calculated in terms of percentage positivity of the water outlets which tested positive for *Legionella*. The W Shapiro-Wilk test was used to assess normality of distribution of the analyzed quantitative parameters. Differences among proportions of positive samples calculated for each tertile in relation to water temperature and residual chlorine were tested with z test. Linear trend in proportions (Mantel-Haenszel extension of the chi-square test for trend) was tested, too. Positivity rates were compared using the Chi-squared test. Cuzick's nonparametric test was used to study the trend across ordered groups. Results were considered statistically significant at $p \leq 0.05$. The statistical computations were performed using STATA 16 (StatCorp., Austin, TX, USA).

3. Results

During the 10-years-study, the laboratory analyzed a total of 305 water samples representing 24 different sampling points from wards with very high-risk patients and with patients at increased risk. Excluding the control samples carried out following the decontamination procedures, there were 170 scheduled analyses. Among these, *Legionella* spp. were detected in 67 samples (39.4%); *L. pneumophila* was present in 61 samples, which accounted for 91.0%.

Of all samples, 7 (10.5%) were simultaneously positive for different *Legionella* serotypes. The study showed that in 50.8% (n = 34) of water samples the levels of *L. pneumophila* were 100–1000 CFU/L, while in 29 samples (43.3%) the levels were 1001–10,000 CFU/L; only a small number of water isolates (n = 4) exceeded 10,000 CFU/L (Table 1).

Table 1. Summary of the results characterizing the contamination of water systems by *Legionella* spp. in the examined pavilions and in the different sampling points. Positive water samples, divided for the various levels of CFU/L, are presented in whole numbers and percentages.

Pavilions	Sampling Points	Positive Water Samples	CFU/L 100–1000			CFU/L 1001–10,000			CFU/L >10,000		
			Number of Samples	%	Range	Number of Samples	%	Range	Number of Samples	%	Range
2	7	18 (28.6%)	10	55.6	100–900	7	38.9	1200–8300	1	5.5	15,200
3	1	5 (17.9%)	3	60.0	100–400	-	-	-	2	40.0	36,000–38,000
4	7	20 (80.0%)	9	45.0	100–900	10	50.0	1100–8100	1	5.0	60,000
5	1	-	-	-	-	-	-	-	-	-	-
7	8	24 (50.0%)	12	50.0	100–800	12	50.0	1000–9200	-	-	-
Total	24	67 (39.4%)	34	50.8	100–900	29	43.3	1000–9200	4	5.9	15,200–60,000

In particular, as shown in Figure 1, the majority of the positive water samples were contaminated by *L. pneumophila* serogroups 2–14 (68.7%), whereas *L. pneumophila* serogroup 1 accounted for 17.9%; a percentage of samples (14.9%) was positive for other *Legionella* spp.

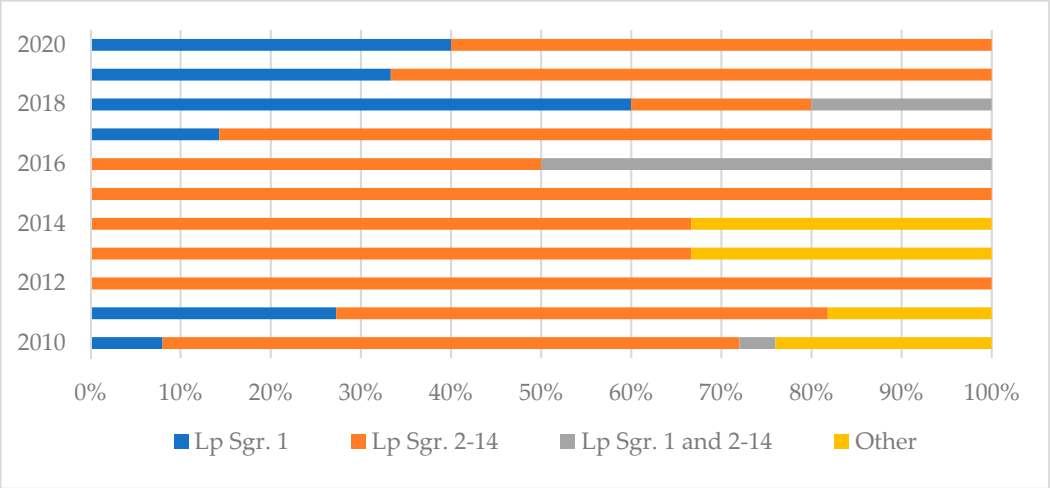

Figure 1. Bar chart showing percentages of *Legionella pneumophila* (Lp) Sgr. 1, Sgr. 2-14, Sgr. 1, and 2-14, and *Legionella* spp. (other) detected in each year of our survey.

The highest number of *L. pneumophila* were detected in the operating room (60,000 CFU/L) and in the intensive care unit (38,000 CFU/L) posing a risk for nosocomial infection and indicating that legionellosis might be a common cause of pneumonia in these settings, as these patients undergo intubation and extubation maneuvers.

Water samples were also analyzed for the presence of *Pseudomonas aeruginosa*. Although there was no evidence of growth on *Legionella* positive samples, it was not possible to draw conclusions as only four samples, detected in the intensive care unit and in the operating room, were found to be positive for *Pseudomonas aeruginosa*.

The majority of *Legionella* positive samples were detected in 2010 (32.8%), while the lowest percentage was identified in 2016 (3%). There is a rather constant presence of positive samples during the years considered, but a decreasing trend is evident in the most recent years and after the application of the corrective actions (Figure 2).

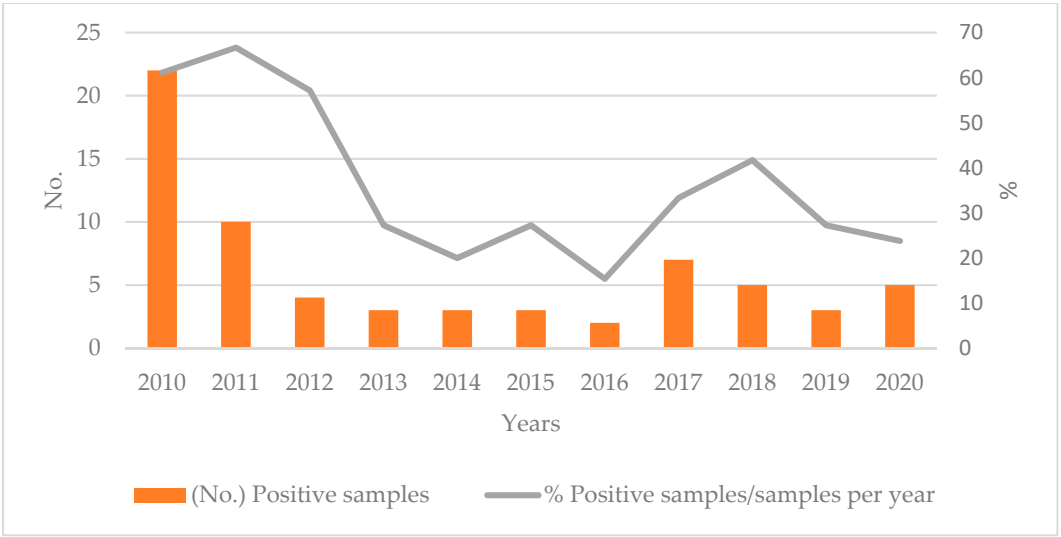

Figure 2. Trend of the number of positive samples detected during the years considered shown both as absolute numbers (orange bars referred to the left scale) and as a trend of the normalized data (grey line referred to the right scale).

Statistically significant differences ($X^2 = 9.549$, $p = 0.0228$) were found among different seasons. The higher rates of isolation of *L. pneumophila* were observed in May (60.0%) and March (53.8%). Furthermore, in contrast to what emerges in other studies, the lowest percentage of positive samples was found during the summer season (Figure 3) with a

statistically significant linear trend in positive proportion from summer to spring (summer 27.9%, autumn 32.7%, winter 41.7%, spring 57.1%) (Z = 2.96, $p = 0.003$).

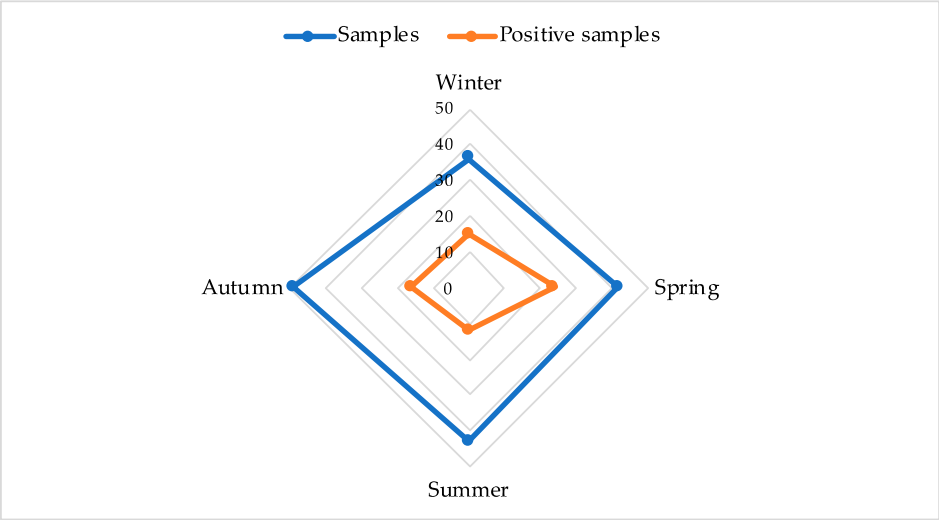

Figure 3. Distribution of total (blue line) and positive samples (orange line) in the seasons of the year. Data are shown as absolute numbers.

The mean temperature for all the samples collected was 39.5 °C (range 12–68 °C), while average residual chlorine was 0.1 mg/L (range 0.02–0.47). Despite the low chlorine content found in all samples, no sample above 0.2 mg/L was found positive for *Legionella* spp. According to the Italian National Guidelines, the residual chlorine concentration at the distal points of the water supply must remain within the range of 0.5–1.0 mg/L, while according to the Legislative Decree that establishes the standards of drinking water (D.Lgs. n. 31/2001), to which we comply, it must be higher than 0.2 mg/L.

Regarding the chemical and physical parameters of the water samples, we divided the temperature and residual chlorine ranges found during the sampling into tertiles (1st tertile $\leq$ 32.0 °C; 2nd tertile = 32.1–47.2 °C; 3rd tertile $\geq$ 47.3 °C). A statistically significant trend of decrease in positivity with increasing temperature was detected, while for the residual chlorine no statistical significance was shown. In particular, as regards temperature, the proportions of positive samples were 63.9% for the first tertile, 47.1% for the second tertile, and 5.3% for the third tertile ($p < 0.05$) (Figure 4).

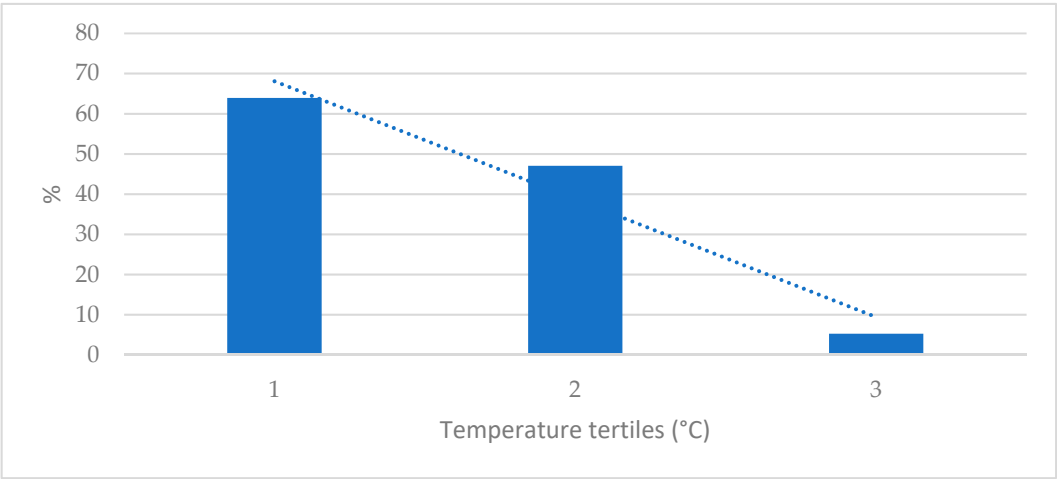

Figure 4. Proportions of positive samples for each tertile of temperature (1st tertile $\leq$ 32.0 °C; 2nd tertile = 32.1–47.2 °C; 3rd tertile $\geq$ 47.3 °C). The dashed blue line shows the decreasing trend of positive samples.

Experimentation of Corrective Interventions

During the 10 years in which the study was carried out, several experimental interventions to prevent and reduce *Legionella* colonization were attempted. One of these methods consisted in the application, in 2015, of sterile disposable filters (Pall Corporation, Port Washington, New York, USA), equipped with a 0.2-µm membrane, to the dispensing taps of five different sampling points. The investigations carried out following the application of these filters demonstrated, both with cultural examination and with real-time PCR, their effectiveness in reducing the high bacterial load detected at time zero, showing neither bacterial growth nor DNA of the microorganism. Filters can therefore be an essential element in reducing *Legionella* spread by colonized pipelines. However, in view of their routine adoption, a careful evaluation of the economic aspect is necessary. Moreover, the water must be suitably pre-filtered as otherwise the filters become blocked quickly.

Another prevention method adopted was a device (Quantum Hospital Freebioenergy-QHFBE) which, operating with electromagnetic waves, is able to create a volumetric sphere with a radius of 60 m within which the flow of the water in the system improves, increasing the internal surface and decreasing the surface tension by about 20%. The instrument, through a negative ionization of the water, is able to physically transform the limestone ($CaCO_3$) into an allotropic form which greatly reduces the new encrustations of the thermo-hydraulic systems, cleaning, within a few weeks, even the old encrustations [22]. The quantum device was applied at a point considered significant within the hospital's complex water network that could allow the greatest possible distribution of water to be achieved. After the start of treatment in 2012, three sampling points have been identified and monthly samplings were performed for approximately a year. To facilitate the collection and management of the information acquired, a specific data collection form has been prepared which reports the environmental variables observed during the sampling and the results obtained. The preliminary results, as can be seen from Figure 2, suggested a decrease in positive samples of *Legionella* of between 60% and 37%. As part of a broader Water Safety Plan process, the results obtained with these methods have been very encouraging and it is believed that, if combined with the classic prevention procedures, they can significantly reduce the presence of *Legionella* and the related risk of infection. Unfortunately, the high costs associated with the management of the device did not allow a constant or repeated application of this tool over time.

4. Discussion

Legionella spp. have their natural habitat in freshwater environments, where they occur in relatively low numbers. However, industrialization has made it possible for them to colonize hot and cold water pipelines in residential buildings, hospitals, and hotels, where they can reproduce owing to the presence of biofilm in the inner walls of the pipes. In hot water distribution networks, a temperature ranging from 20 °C to 45 °C is the optimum temperature for the replication of these bacteria. Further favorable factors facilitating the colonization of water distribution systems are the lengths and the winding formats of the pipelines, the presence of dead legs with water stagnation, corrosion, and biotic factors (presence of other microorganisms, biofilm) [23]. Frequently, the colonization of a water system by *Legionella* is the result of an incorrectly designed system, the use of inappropriate materials, and improper maintenance of the water system [24,25].

The results of the present study identified that 39.4% of the analyzed water samples were positive for *Legionella* spp. It is difficult to make comparisons with the findings reported in the literature resulting from other studies conducted in Italian hospitals as some results are considerably higher [26], while others are substantially similar [27] or lower [28]. Despite this, according to various international environmental surveys, hospital water systems have shown a significantly high percentage of colonization by *Legionella* [29].

Almost all *Legionella* isolates were *L. pneumophila* (91.0%), with most belonging to serogroups 2-14 (68.7%). This is congruent with the fact that *L. pneumophila* strains from environmental samples have been reported to belong mainly to the pooled serogroups

2–14, in contrast to clinical isolates which are almost exclusively serogroup 1 [30]. On the contrary, in other countries, the most commonly isolated serogroup of *L. pneumophila* is the serogroup 1 [31]. In 50.8% of water samples collected in this study the levels of *L. pneumophila* were 100–1000 CFU/L, in 43.3% of samples 1001–10,000 CFU/L levels were detected, and 5.9% of samples had levels of *L. pneumophila* higher than 10,000 CFU/L.

The present study shows an association between *L. pneumophila* levels and hot water temperature; the increase in water temperature is linked to the decrease in the number of bacteria. Similarly, the same results were obtained by other researchers [32,33]. The influence of temperature has been identified not only on the survival, but also on the virulence of *Legionella* [34].

Otherwise, no statistically significant correlation was found for residual chlorine. In this regard we can assume that the low levels of residual chlorine are not totally attributable to a low concentration in the distributed water since the contamination inside the network could have led to an increased reactivity of the chlorine with the organic substances present in the network. Moreover, there was seasonal variability in the positive samples with a statistically significant linear trend in positive proportion from summer to spring, even though recent studies suggest that Legionnaires' disease occurs most frequently in summer [35–37].

The presence of *Legionella* in water systems poses a potential risk of infection for humans, especially for hospitalized patients, considering that, the infective dose has not yet been precisely identified [38,39]. According to the estimated data, the disease may occur sporadically if the water contamination by *Legionella* is 1001–10,000 CFU/L, while Legionnaires' disease can be expected when the number exceeds 10,000 CFU/L [1,32]. However, despite the level of water contamination, once the pipeline has been contaminated there is a latent risk of illness due to the presence of a source of infection.

As the Italian National guidelines suggest, the hospital authorities adopted remedial interventions whenever the bacterial load exceeded the limits and the sample points corresponding to water contaminated by *Legionella* spp. were subjected to hyper-chlorination and equipped with water heaters and then resampled after 1 and 3 months. Considering that, at present no biocide disinfection methods guarantee the complete eradication of *Legionella* spp. in water systems, several possible interventions were attempted during the study period. These included the application of antibacterial filters to taps and the use of a gravitational magnetic wave emission device (Quantum). Following the application of these corrective actions there were reductions in trends especially after the use of Quantum in 2012 and until reaching the lowest peak after the use of filters in 2015 (Figure 2). Although these systems have made it possible to detect a certain improvement in the reduction of *Legionella*, their availability was discontinued due to the economic cost involved.

To prevent and reduce *Legionella* colonization and nosocomial cases of legionellosis, national and international guidelines recommend environmental surveillance along with remedial measures to control *Legionella* spp. water contamination. In accordance with the World Health Organization (WHO), one of the best approaches to assessing the health risks associated with *Legionella* colonization is the development of a water safety plan (WSP). This is a dynamic tool that identifies the risks and dangers involved in the colonization of water systems and indicates the most appropriate control measures as well as the possible obstacles to their implementation [40].

In Sardinia, due to the water characteristics, there is a strong variability in the quality of water supplied, as reported by the authors for various parameters [41–43]. For this reason, we currently carry out checks on the potability of the distributed water on a monthly basis. Moreover, a system of additional disinfection units based on chlorine dioxide is being installed in each hospital pavilion at our university hospital.

In our study, despite the number of *Legionella* isolated over 10 years of constant environmental surveillance, no hospital-acquired cases have been recorded. This may suggest that clinical surveillance was, in fact, not carried out properly, possibly because

physicians are unaccustomed to considering *Legionella* as a possible cause of nosocomial pneumonia. Consequently, it can be argued that there may still be a considerable number of undocumented cases of *Legionella* infections in our hospital settings. However, in various prospective studies the identification of *Legionella* colonization led to the identification of hospital-acquired legionellosis [44,45].

Although this study highlights continuous environmental surveillance as a strong point, some limitations are present. First, the impossibility of showing data prior to 2010 as the surveillance was unsystematic due to the lack of national and international guidelines in that period. Second, since no cases of hospital-acquired pneumonia have been recorded, it was not possible to determine a link between the isolation of *Legionella* and nosocomial pneumonia.

5. Conclusions

This study underlines the importance of the permanent monitoring of hospital water distribution systems to prevent the potential risk of nosocomial legionellosis and to implement procedures to minimize the risk of *Legionella* spp. colonization. Since many recent studies recommend concurrent research on air to increase the probability of finding *Legionella* [46,47], it will be important in the future to increase the sensitivity of surveillance by introducing this type of research as well.

Numerous disinfection measures for the eradication of *Legionella* spp. are available, such as heat and flush, hyper-chlorination and point-of-use filters, but there is no a definitive method for the complete eradication of this microorganism [48–51]. However, to evaluate the efficacy of any disinfection method, monitoring over a prolonged period is required. Furthermore, active clinical surveillance might substantiate the evidence of its role as a potential nosocomial pathogen in a hospital environment.

Based on our findings, we have promoted awareness to formulate *Legionella* risk management in our university hospital and advised physicians to recommend *Legionella* diagnostic testing for patients with suspected nosocomial pneumonia. Indeed, underdiagnosed hospital-acquired Legionnaires' disease may become evident as the awareness of clinicians increases.

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