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Occurrence of *Legionella* spp. in thermal environments: Virulence factors and biofilm formation in isolates from a spa☆,☆☆

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ABSTRACT

The aim of the study was to evaluate the occurrence of *Legionella* spp. in the water system of a spa in the city of Naples by analyzing water, air and surface samples. On the whole, 312 samples were collected and analyzed in the course of 10 months. Legionella CYE Agar Base and Legionella Latex Test (Oxoid©) were used to identify and serotype presumptive *Legionella pneumophila* strains. A further identification was carried out by rDNA16S and ITS region amplification followed by a sequence analysis by DNA Sequencing Analysis software (Applied Biosystems). Similarity search was performed using BLAST algorithm against the GenBank database (NCBI GenBank). Specific in-vitro tests aimed to evaluate the production of esoenzymes (hemolysins, collagenases, mucinases, lipases, proteinases, DNAses, elastases) on GC-FC Agar were also carried out. Finally, a crystal violet staining method (absorbance at 570 nm) was used to evaluate the ability of the strains to produce biofilm in a 96-multiwell polyethylene plate. All samples were negative for *L. pneumophila*. Six different Legionella strains were isolated from water samples and identified as *Legionella londiniensis* and *Legionella* spp. A significant (from 1000 to 10,000 CFU/L) and a low to moderate (from 100 to 1000 CFU/L) contamination were detected respectively in the 5% and 4% of samples; 91% of water samples showed a *Legionella* spp. amount less than 100 CFU/L. Two *Legionella londiniensis* isolates showed collagenases, caseinases, proteinases and gelatinases activities, being classified as potentially pathogenic bacteria. None of the isolates were classified as strong biofilm producer but they showed a moderate to weak ability to form biofilm on polyethylene. This result is significant because large part of the spa pipelines is plastic-coated.
The highest frequency of isolation of *Legionella* spp. was detected in the unit for Thermal Mud Therapy, which showed a relative risk value equal to 1.69 (CI 95% 0.60–4.70).
Although our results proved a moderate contamination in different water samples, the presence of potentially pathogenic environmental strains of *Legionella* spp. should not be underestimated because most part of costumers attending the spa are old and sick people, and Legionella strains can represent a real risk.

1. Introduction

Legionella spp. is a ubiquitous microorganism which can grow at temperatures of 25 to 42 °C in natural and artificial aquatic environments. *Legionella pneumophila* serogroup 1 is the most frequently reported aetiological agent in community-acquired legionellosis [1–3].

Legionnaires' disease (LD) is a form of interstitial pneumonia that is normally transmitted through the inhalation of droplets containing the bacteria. The aerosol containing *Legionella* can be produced by contaminated water sources such as cooling towers, swimming and spa pools, fountains, respiratory therapy equipment, and other devices. Many factors, such as temperature, hardness and chemical element concentration of the water, flow rate, type of surface, concentration of nutrients and disinfectants, can influence the accumulation of bacteria on pipeline surfaces and biofilm formation [1,2].

In Italy, Legionnaires' disease shows an incidence of 16.6 cases per million [4]. The number of cases reported by the Italian Surveillance System for Legionnaires' disease increased since the year 2000, and 73% of them were community-acquired [3].

As for community settings, thermal water systems, including hot tubs and natural spas, have been responsible for large outbreaks of Legionnaires' disease. In these kinds of facilities, the risk is mainly due to

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the warm water temperature, the high bather density, the presence of areas not properly disinfected, and the therapeutic use of aerosols [5].

Nowadays, the periodic environmental monitoring of critical points is the best strategy for legionellosis prevention [6].

The Italian guidelines in force for the control of *Legionella* contamination in thermal and travel/accommodation facilities recommend the adoption of preventive measure and the periodic risk analysis to highlight critical situations [7].

In line with this, the aim of this study was to evaluate the level of *Legionella* contamination in a spa and to characterize the virulence of the isolates. Furthermore, we determined the risk of contamination for users attending the different units of the facility.

2. Materials and methods

The surveillance was carried out for ten months (January–October 2011) in a spa center of Naples' District. Sampling procedures, as well as the choice of sampling points, were in accordance with the guidelines by the Italian Health Ministry [8].

The guidelines consider the value of 10^3 CFU/L as a limit for *Legionella* spp. concentration in water.

2.1. Detection and quantification of Legionella spp.

Spa plumbing scheme was examined in order to identify critical points for sampling procedures on the basis of structural characteristics and potential exposure of the customers. Reserve tanks of cold and hot water were sampled as well as the more distant distribution points. Water taps, showers, saunas, and sulfur water for aerosol therapy were sampled monthly.

A total amount of 220 water samples was collected. One liter of water was collected from each sampling point and sodium thiosulfate was added to a final concentration of 0.01%. Flushing and flaming of the tap were carried out before the collection of samples for *Legionella* quantification. Water temperature was measured by a Brannan thermometer.

The detection of *Legionella* in water samples was performed on the base of the ISO protocol 11731:1998 [9]. Briefly, samples were filtered through a cellulose acetate membrane with a pore size of 0.22 μm. Then, particles entrapped by filters were re-suspended in 10 mL of the analyzed water. 0.1 mL of the suspension was seeded on Legionella CYE Agar Base + BCYE-α Growth Supplement (OXOID©) and incubated in a moist chamber with 2.5% CO_2 at 36 ± 1 °C for ten days. The results were expressed as CFU/L. Microbial count values were also used to evaluate the temporal trend of *Legionella* occurrence.

Sterile swabs were used to detect the presence of *Legionella* inside water pipes, taps, filters, and shower faucets. Swabs were stored in 2 mL of water coming from the same tap.

The Surface Air System (International PBI) active sampler with RODAC plates filled with Legionella CYE Agar was used to collect air samples (3000 L each) from the "Inhalation" and "Nebulization" units. The air was sampled before and after the aerosol production in the dedicated areas. Surfaces and aerosols were analyzed before the opening of the spa and every 3 months. Overall, 20 air samples and 72 surface swabs were analyzed.

The isolates were serotyped through the Legionella Latex Test (OXOID©). Six strains isolated from samples with higher levels of *Legionella* contamination were identified through 16S rDNA amplification by universal primers and qualitative Polymerase Chain Reaction (PCR). Amplified DNA sequences were aligned using DNA Sequencing Analysis software, version 5.2 (Applied Biosystems) and BioEdit software, version 7.0.9.0 [10].

Table 1
Enzymatic substrates.

Enzyme	Substrate
Mucinase	Mucin 1%
Collagenase	Hide azure powder 0,2%
Proteases	Skim milk 1%
Caseinase	Caseine 1%
Lecithinase	Egg yolk emulsion 10%
Lipase	Tween 80 1%
Amylase	Soluble starch 0,5%
Gelatinase	Gelatine 1%
Hemolysis	Red blood cells

2.2. Study of virulence factors

In order to evaluate the production rate of eso-enzymes, a suspension of the six strains of *Legionella* spp. above mentioned was plated in spots on GC-FC Agar, with the addition of specific substrates (Table 1). Strains positive for two in-vitro virulence tests at least, were considered potentially pathogenic bacteria [11].

Plates were incubated for seven days at 36 ± 1 °C in a moist chamber with 2.5% CO_2 and examined daily to check the diameter of degradation halo [11,12].

2.3. Biofilm production

The ability to produce biofilm was also investigated in the same isolates through the colorimetric assay by Stepanovic et al. [13], modified by Piao et al. [14].

For each isolate, 20 μL of a microbial suspension (0.2–0,3 $O.D._{600}$) in Yeast extract added with L-cysteine was inoculated in a 96-well polyethylene microtiter plate (Beckton-Dickinson©). 230 μL of culture medium was, then, added. A negative control made of 250 μL of the sole culture medium was included. The multiwell plates were incubated at 36 ± 1 °C in a moist chamber with 2.5% CO_2 atmosphere. After 4 days, the wells were washed with sterile water, fixed with ethanol 96% and colored with 250 μL of crystal violet 2%; after a further rinse with distilled water, glacial acetic acid 33% was added to each well. Then, the absorbance at 570 nm was read at a spectrophotometer. The values were used to classify the isolates as non-biofilm-producing, weak, moderate- or strong-biofilm-producing [13]. Cut-off values were calculated as the mean OD value + 3 standard deviation (SD) of the negative controls.

2.4. Risk analysis

The relative risk (RR) of *Legionella* contamination was calculated by comparing the incidence of contamination in the unit with the highest number of positive samples with that of the other units.

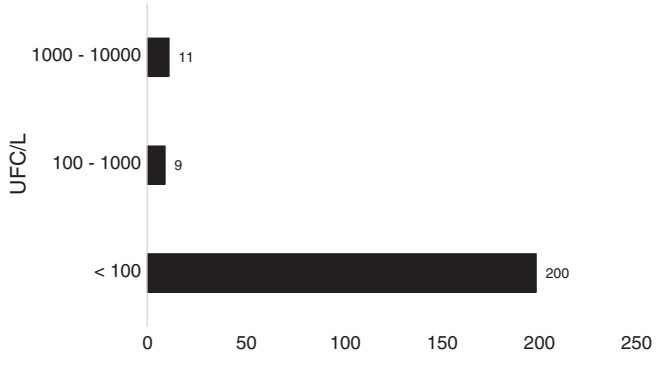

Fig. 1. Number of water samples with low (<10^2 CFU/L), moderate (>10^2 CFU/L, <10^3 CFU/L) and significant (>10^3 CFU/L) concentration of *Legionella* spp.

3. Results

3.1. Detection and quantification of Legionella spp.

Legionella spp. resulted always absent in thermal water, mainly used to treat respiratory tract diseases. Fig. 1 reports the number of tap water samples which showed a *Legionella* concentration higher than the limit value of 10^3 CFU/L, together with that of samples with a contamination between 10^2 and 10^3 CFU/L or lower than 10^2 CFU/L.

Only 5% of these samples showed a *Legionella* contamination higher than the limit value established by the Italian guidelines (10^3 CFU/L). The higher concentration registered was 9.1×10^3 CFU/L.

Serotyping showed the absence of *L. pneumophila* among the isolates. DNA sequencing confirmed this result, by showing the presence of three "*Legionella* spp." (similarity coefficient 95.3%), two "*Legionella londiniensis*" (96.8%) and a strain of "*Uncultured Legionellales bacterium clone MP9B72*" (99.4%).

The temporal trend showed the highest number of positive samples during Summer and Autumn (data not shown).

Legionella was always absent in water used for mud and aerosol therapy as well as in air samples, and in surface samples from inhalation and ventilation systems.

3.2. Study of virulence factors

Both *L. londiniensis* strains showed eso-enzymatic activity due to the production of collagenases, proteases, caseinases and gelatinase. As they were positive for more than two in-vitro virulence tests, they can be considered potentially pathogenic strains [11].

3.3. Biofilm production

Among the six isolates analyzed, *Legionella* spp. strains were moderate-biofilm-producing, *L. londiniensis* were weak-biofilm-producing while the strain identified as *Uncultured Legionellales bacterium clone MP9B72* was classified as non-biofilm-producing.

3.4. Risk analysis

The highest number of positive samples was registered in the unit for Thermal Mud Therapy, then we calculated the risk of contamination by considering "exposed" the users attending this unit. The relative risk was calculated through the ratio between the incidence of contamination in this unit ($6/26 = 0.230$) and that registered in other units ($6/44 = 0.136$). The value of RR was 1.69 (CI 95% 0.60–4.70).

4. Discussion

The results of the study prove a moderate risk of *Legionella* contamination in the examined spa.

In fact, the limited number of water samples which showed a critical concentration of *Legionella* spp. testifies the effectiveness of control measures adopted in the spa, in line with the Italian guidelines in force [7]. Control measures seem to be effective enough to maintain generally contamination values below 10^3 CFU/L, which accounts for a real infective risk in exposed people [8].

The absence of *Legionella* in water used for therapies as well as in air and surfaces samples could be explained with the chemical composition of the water used in such therapies, rich in sulfurs, and with the low temperature of water supplied (16–17 °C) [2].

The higher frequency of isolation registered in Summer and Autumn could be related to the increase in water use due to the higher number of persons attending the facility during these seasons. Nevertheless, further investigations are needed in order to explain this finding.

Although microbial challenge test carried out with a strain of *L. pneumophila* (ATCC 33153) proved the ability of this microorganism to grow in the same spa tap water (data not shown), the identification methods used showed the absence of *L. pneumophila* in all the samples.

However, the presence of *L. londiniensis* should not be underestimated, especially in the light of its ability to produce extracellular enzymes and biofilm as virulence factors. The ability of *Legionella* spp. and *L. londiniensis* strains to produce biofilm on a polyethylene surface could indicate the potential colonization of both plastic-coated pipelines and human tissues. Moreover, it is well known that a microbial biofilm is the ideal micro-environment for *L. pneumophila* proliferation [15]. So, the presence of *Legionellaceae* in pipelines should be considered as a possible "alarm bell" of a *L. pneumophila* colonization.

Furthermore, in line with literature, our study confirms the convenience of molecular methods to characterize the isolates, which is useful in environmental routine monitoring, but becomes fundamental when an outbreak occurs [16]. Molecular methods offer unquestionable advantages (mainly linked to their rapidity and precision) in comparison to traditional culture methods for the analysis of samples, usually contaminated with microorganisms other than *Legionella*. Then, molecular technologies are of crucial importance to identify infected subjects and to expedite cleanup of contaminated water systems. However, rapid methods require equipment and cost that should not be underestimated. Then, the combination of both conventional and molecular methods might be the best approach to test clinical and environmental samples [17].

5. Conclusions

Our study highlights the importance of a continuous maintenance and monitoring of water distribution systems, even in community settings such as spa centers. The evaluation of *Legionella* spp. amount in pipelines appears to be an essential step in the control of contamination. As showed by other experiences [18], considering that the emission of *Legionella* from water systems is not constant over time, risk analysis and microbiological surveillance should be frequent to control the environmental spread of *Legionella* spp. Moreover, other control measures such as disinfection or thermal shock should be carried out, even in the presence of low levels of contamination. This is valid not only for hospitals, where people are more susceptible to infection, but also for other facilities which supply therapies to persons with frail health conditions.

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