Environmental surveillance and molecular investigation of *Legionella* spp. in Apulia, in the years 2008-2011

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**Key words:** *Legionella* spp., environmental contamination, water system, AFLP

**Parole chiave:** *Legionella* spp., contaminazione ambientale, rete idrica, AFLP

**Abstract**

*Legionella* spp. is considered an emerging microorganism involved in aquatic environments contamination and cause of Legionnaires’ disease.

The aims of the study are to evaluate the level of contamination of *Legionella* spp. in the water system of the largest Hospital of Apulia region during a 4-year surveillance and to establish, by molecular method, the presence of a predominant genotype of *L. pn.* sg 1.

The results showed that *Legionella* spp. was present in 36% of water samples with *Legionella pneumophila* serogroup 1 (*L. pn.* sg 1) the most prevalent species and serogroup and the wards most contaminated are the high risk units. In addition, despite four main clones of *L. pn.* sg 1 were identified, a predominant genotype existed.

In conclusion the study demonstrates the necessity for periodic evaluation on hospitals water system to assess the potential contamination of *Legionella* spp., performing decontamination in the presence of bacterial contamination, even low, in particular in high risk wards. Moreover, the switching of the disinfection methods may be suggested in order to prevent resistance phenomenon by some *L. pn.* sg 1 clones.

**Introduction**

*Legionella* spp. is a Gram-negative bacteria, which normally inhabits aquatic environments. It causes a form of pneumonia known as Legionnaires’ Disease (LD), usually involving hosts with an impaired immune system (1).

Since LD can be acquired by inhalation of aerosols contaminated by *Legionella* spp. (2-4), sources of infection are hot water systems (in domestic, touristic and hospital facilities), cooling towers and other water disseminators as medical equipment where often *Legionella* spp. presence reaches high level of microbial count (5-10).

*Legionella pneumophila* (*L. pn.*) is the aetiological agent of approximately 90% of Legionellosis cases, and serogroup (sg) 1 is the most frequent cause of infections followed by sg 6 (11-13).

The characterization of both clinical and environmental isolates by molecular typing methods is essential for epidemiological investigations. The European Working Group on *Legionella* Infections (EWGLI), recently renamed

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European Study Group for *Legionella* Infections (ESGLI), has provided a description of standardized protocols for the molecular identification of *Legionella* spp. by amplified fragment length polymorphism (AFLP) (14) and by sequence-based typing (SBT) (15, 16).

The present study reports the results of a four-year environmental surveillance for *L. pn. sg 1* in the water system of the largest Hospital of Apulia region (Southern Italy), including the genotyping of strains isolated.

**Materials and methods**

During the period January 2008 - December 2011 an environmental surveillance for the detection of *Legionella* spp. was carried out on the water system of a hospital of Apulia (Southern Italy) which is composed up of 32 separate buildings with 22 wards-buildings, for a total bed capacity of about 1,400.

All 22 wards-buildings have been examined. Six sampling points by each wards-buildings were monitored 2 times per year, for a total of 1,056 samples (1,012 sink taps and 44 showers).

According to the procedures described in the Italian Guidelines, water samples of 1 liter were collected in sterile containers with sodium thiosulphate (0.01%) (17).

The *Legionella* strains were serologically identified by agglutination tests polyvalent latex (Oxoid spa, Milan) and with monovalent antisera (Biogenetics s.r.l.) and the *L. pn. sg 1* strains were molecularly analyzed through AFLP, using the standard protocol of the EWGLI version 1.2 (http://www.ewgli.org). The AFLP profiles were phylogenetically evaluated by the software GelCompare II (Applied Maths, Belgium) using as a cluster analysis the Dice coefficient and the method “unweighted pair group method with averages” (UPGMA). In agreement with Fry et al. (18) have been considered homologous strains those which showed a homology greater than 90%.

**Results**

*Legionella* spp. was found in 384 (36.4%) samples, of which 304 (79.2%) were contaminated by one single species or serogroup, while 80 (20.8%) resulted mixed cultures. During the 4-year environmental surveillance the percentage of positive samples decreased from 2008 to 2009 (27.8% and 14.1%, respectively) and than increased to 16.4% in 2010 and 41.7% in 2011.

A total of 464 strains of *Legionella* spp. were isolated; the most frequent was *L. pn. sg 1* (32.0%), followed by *L. pn. sg 6* (30.0%), *L. pn. sg 10* (29.2%), *L. pn. sg 8* (2.8%), *L. pn. sg 3* (1.9%), *L. pn. sg 7* (1.7%), *L. species* (1.1%), *L. pn. sg 12* (0.9%) and *L. pn. sg 14* (0.4%) (Figure 1). Of the 22 wards-buildings examined, 15 were positive for *L. pn. sg 1* as shown in Figure 2.

*Legionella* spp. count was <1,000 (47.0%), 1,000-10,000 (37.5%), >10,000 cfu/L (15.5%) (range 100 – 180,000 cfu/L). The distribution of *L. pn. sg 1* percentage of positivity by year accounted at 33.8% in 2008, 12.8% in 2009, 21.6% in 2010 and 31.8% in 2011.

All *L. pn. sg 1* isolates (n = 148) underwent a further molecular investigation through AFLP and electrophoresis profiles obtained were phylogenetically analyzed (Figure 3). Four main groups of AFLP patterns were identified and arbitrarily named as A1, A2, A3, A4:

- group A1 includes 14 isolates from 5 wards-buildings (N. 9-14-16-25-30);
- group A2 includes 85 isolates from 11 wards-buildings (N. 3-4-6-7-8-9-14-15-17-19-30);
Figure 1 - Distribution (%) of 464 strains of Legionella spp. by species and serogroups.

Figure 2 - Distribution (%) of L. pn. sg 1 by wards-buildings.
showing a widespread environmental contamination by *Legionella* spp. The most common species isolated was represented by *L. pn.* sg 1 and the most contaminated wards-buildings are those which include high risk units, N. 14 (20.4%), 17 (18.3%) and 19 (14.4%). Moreover, despite the treatment of disinfection (i.e., hyper-chlorination) performed as suggested by the Italian Guidelines (25), the 4-year environmental surveillance trend shows that the percentage of positive samples for *Legionella* spp. is always changing from 27.8% in 2008 to 41.7% in 2011, showing the persistence of the microrganism in the hospital water system.

In addition, the genetic diversity of the colonizing *Legionella* flora was examined using genotyping methods highlighting that, despite the variability of *L. pn.* sg 1 genotypes not only among the different wards-buildings, but also within the same wards-buildings, there is a predominant genotype of *L. pn.* sg 1 (85 strains from 11 ward-buildings represented by the group A2 in the dendrogram). So the Authors hypothesized that in the water systems can circulate more clones of *L. pn.* sg 1 some of which are able to withstand the continuous treatments of disinfection (26). The resistance to chlorination of such clones of *L. pneumophila* sg 1 could be due to their ability to adapt to different habitats, as biofilm, that facilitates a higher tolerance to the disinfectant as recently demonstrated (27, 28).

Hospital-acquired legionnaires’ disease arises from the presence of *Legionella* spp. in hospital water systems. *Legionella* spp. not only persists in hot water tanks but it was also found in the biofilm throughout the entire water distribution system. Conditions within water systems that promote *Legionella* colonization include water temperature, configuration and age of the hot water tank, physicochemical constituents of the water, plumbing

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**Discussion**

Several studies describe the frequency of *Legionella* spp. contamination in health care and spa facilities (4, 19-23) demonstrating a wide environmental diffusion of the microorganism and the association between the strains isolated from the environment and the biological specimens of infected humans (24). The present study confirmed previous findings,
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Materials, and commensal microflora. Hospital-acquired LD has been prevented by instituting control measures directed at the water distribution system. A successful *Legionella* prevention program requires cooperation and communication among hospital administrative personnel, engineers, and infection control staff. Routine environmental surveillance cultures for *Legionella* are the critical component for successful long-term disinfection. Culture results document the efficacy of the disinfection method and alert the hospital staff to consider *Legionella* in hospitalized patients with pneumonia. It has shown the persistence capacity of *Legionella pneumophila* sg 1 species in such an ecosystem. Finally, it also pointed out the biodiversity of *Legionella pneumophila* in their natural environment. These results suggest that the strains better adapted to the environment will manage to displace the others and will be able to spread and infect humans. The adaptation to the environmental conditions could play an important role in the pathogenesis of the strains.

Genotypic variability and clonal persistence are concepts of enormous importance in molecular epidemiology as they facilitate the search for the source of sporadic cases or outbreaks of legionellosis (29).

Conclusions

This study demonstrates the necessity for periodic evaluation on hospitals water system to assess the potential contamination of *Legionella* spp., with the aim to drive decontamination efforts in the presence of bacterial load, even low, in particular in high risk units. In addition, the switching of the disinfection methods may prevent resistance phenomenon by some *L. pn.* sg 1 clones.

Riassunto

Sorveglianza ambientale e indagine molecolare di *Legionella* spp. in Puglia negli anni 2008-2011

*Legionella* spp. è un microrganismo ubiquitario che proliferà soprattutto in ambienti acquatici caldi e causa polmonite definita la malattia del legionario.

Gli obiettivi dello studio sono valutare il livello di contaminazione di *Legionella* spp. nel sistema idrico di un grande ospedale pugliese durante un periodo di sorveglianza di 4 anni, e stabilire la presenza di genotipi predominanti di *L. pneumophila* sierogruppo 1 (*L. pn.* sg 1).

I risultati hanno mostrato che il 36% dei campioni di acqua era positivo per *Legionella* spp. e che *L. pn.* sg 1 rappresentava la specie ed il sierogruppo prevalente. Inoltre i reparti risultati maggiormente contaminati erano rappresentati dalle unità ad alto rischio. Lo studio molecolare evidenziava quattro principali cloni di *L. pn.* sg 1, di cui un genotipo predominante.

In conclusione, lo studio dimostra la necessità di eseguire periodici controlli ambientali per la ricerca di *Legionella* spp. negli ospedali ed eseguire la bonifica della rete idrica anche in presenza di cariche microbiche basse, in particolare nei reparti ad alto rischio. Inoltre, è consigliabile non adottare sempre lo stesso intervento di disinfezione in modo da evitare fenomeni di resistenza da parte di particolari cloni di *L. pn.* sg 1.

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