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Serological and molecular identification of *Legionella* spp. isolated from water and surrounding air samples in Italian healthcare facilities

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ABSTRACT

Background: *Legionella* is an intracellular microorganism living in natural and artificial aquatic environments. Although its transmission to humans is linked to the inhalation of contaminated aerosols, there is no validated air sampling method for the control and prevention of the disease. The aim of the present study was to provide more information on the distribution of *Legionella* spp. in indoor environments and to determine whether the same *Legionella* strains are isolated from air and water samples.

Methods: Ten healthcare facilities located in seven regions of Italy were enrolled. The serological typing of *Legionella* spp. from water samples and the surrounding air by active and passive sampling was assessed using polyvalent and monovalent antisera. Subsequently, the strains identified as *Legionella pneumophila* (*Lpn*) underwent molecular typing by sequence-based typing (SBT) using seven genes (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA*). The allelic profile number was assigned using the European Working Group for *Legionella* Infections–SBT database.

Results: *Lpn* serogroup 6 was the most prevalent serogroup; it was found simultaneously in the air and water samples of three different healthcare facilities. In the remaining seven hospitals, *Lpn* serogroups 1, 6, 7, 9, and 12 were isolated exclusively from water samples. The molecular investigation showed that *Lpn* strains in the water and air samples of each positive healthcare facility had the same allelic profile. Strains, identified as sequence types (STs) 728 and ST 1638+ST 1324, were isolated in two respective healthcare facilities, and a new strain, identified as ST 1989, was obtained in one healthcare facility.

Abbreviations: *Lpn*, *Legionella pneumophila*; SBT, sequence-based typing; STs, sequence types; CFUs, colony-forming units; EWGLI, European Working Group for *Legionella* Infections

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Conclusion: The application of the SBT method allowed to verify the homology among *Legionella* strains from water samples and the surrounding air. The results showed that the same *Lpn* strains were present in the air and water samples, and a new *Legionella* strain was identified.

1. Introduction

Legionella is the etiologic agent of various clinical manifestations, including a pneumonia known commonly as Legionnaires' disease. Individual factors and predisposing conditions are the basis for the different susceptibilities of individuals exposed to the same infection source (Fonseca and Swanson, 2014). Currently, the genus *Legionella* comprises 58 different bacterial species and 70 serogroups (sgs) that live in aquatic environments, both natural (*e.g.*, rivers, lakes, and ponds) and artificial (*e.g.*, potable water systems, faucets, showers, cooling towers, fountains, and medical equipment). The microorganism prefers temperatures ranging from 25 to 50 °C, especially if the water is stagnant and rich in sediments (Napoli et al., 2010; Spagnolo et al., 2013). Although *Legionella pneumophila* (*Lpn*) sgs 1 and 6 are the main causes of disease in humans (Messi et al., 2013), other species, including *Legionella longbeachae* (3.9% incidence rate) and *Legionella bozemanii* (2.4% incidence rate), have recently been associated with cases of legionellosis (Lee et al., 2010).

Efficient air sampling combined with water surveillance is beneficial for preventing legionellosis (Chang and Hung, 2012). Monitoring the air around aerosol-producing devices may assist in tracking the greatest potential for *Legionella* spp. aerosolization (Blatny et al., 2008), identifying plausible infection sources, and assessing the distance that *Legionella* has spread (Nguyen et al., 2006). However, difficulties in detecting *Legionella* spp. in air samples, as well as differing air sampling methods, have been reported widely in the scientific literature (Chang and Chou, 2011; Napoli et al., 2012; Pasquarella et al., 2008, 2012).

Based on this scientific background and on experiences with *Legionella* spp. contamination in healthcare environments (Castiglia et al., 2008; Montagna et al., 2006; Napoli et al., 2010), the Italian study group on hospital hygiene (GISIO) of the Italian Society of Hygiene, Preventive Medicine, and Public Health (SItI), in collaboration with the Italian Association of Aerobiology (AIA), promoted a multicenter study that aimed to identify a sensible sampling protocol to detect airborne contamination coming from water sources contaminated with *Legionella* spp. Ten Italian healthcare facilities enrolled voluntarily in the study after a bathroom was identified as having a water supply contaminated with >1000 colony-forming units (CFUs)/L of *Legionella* (Montagna et al., 2014).

The objectives of the present investigation were: (i) to verify the distribution of *Lpn* sgs in water samples from the 10 enrolled centers; and (ii) to determine, via molecular investigations, whether the same *Lpn* strains were isolated from the air and water samples.

2. Materials and methods

2.1. Environmental sampling

Ten healthcare facilities located in seven regions throughout Italy (Campania, Lazio, Liguria, Apulia, Veneto, Sardinia, and Sicily) participated in the air and water sampling from March to May 2014 (Montagna et al., 2014).

For the isolation of *Legionella* spp. were used plates containing GVPC (Glycine-Vancomycin-Polymyxin-Cycloheximide medium, Liofilchem Srl, Teramo, Italy). After incubation at 36 °C for 10 days in a humid environment and CO_2 at 2.5%, the suspect colonies were subcultured on Charcoal Yeast Extract medium (CYE, Liofilchem Srl, Teramo, Italy) without L-cysteine and Buffered Charcoal Yeast Extract medium (BCYE, Liofilchem Srl, Teramo, Italy) with L-cysteine. Colonies that only grew on BCYE agar plates were ascribable to the *Legionella* genus.

2.1.1. Air sampling

Air contamination was assessed by the active and passive sampling for a total period of 8 h (from 9 a.m. to 17 p.m.).

Active sampling was performed by Surface Air System (SAS, PBI International, Milan, Italy), located at 1 m from the floor and 50 cm from the tap. The flow rate was set to 180 L/min, following a predetermined time schedule: every 12 min and after flushing water for 2 min, 200 L of air were aspirated, for a total of 1000 L/h, taking care to change the plate at the end of each sampling hour. Altogether, 40 aspirations were performed on a total of 8 plates (5 aspirations/plate/h). The number of CFUs was adjusted using the conversion table provided by the manufacturer, and the value was expressed in CFU/m^3. The sampler was placed immediately beside the plates employed for passive sampling.

Passive sampling was performed to determine the Index of Microbial Air Contamination (IMA) (Pasquarella et al., 2000). This index corresponds to the number of CFU counted on a Petri dish with a diameter of 9 cm placed according to the 1/1/1 scheme (for 1 h, 1 m above the floor, about 1 m away from walls or any major obstacles). In our study, two plates/h were placed at 1 m from the floor and 50 cm from the selected tap water. The result was an average of values measured on 16 plates/8 h and expressed as CFU/plate.

2.1.2. Water sampling

During the eight hours required by the study protocol, the hot tap water was sampled three times: T_0 = before starting the first air sampling; T_1 = after 4 h; T_2 = 8 h after the end of the air sampling. Water contamination was monitored according to the procedures reported in the Italian Guidelines for the Prevention and Control of Legionellosis (Linee, 2000).

2.2. Serological identification of Legionella pneumophila

Colonies ascribable to the *Legionella* genus were subjected to identification using a latex agglutination test with polyvalent antisera (Oxoid Spa, Milan, Italy). Of the *Lpn* strains, which were maintained at −80 °C, a total of 126 isolates (all the seven strains from air sampling and other 119 selected randomly from water samples) were serologically identified using a latex agglutination test with monovalent antisera (Biogenetics Srl, Tokyo, Japan).

2.3. Molecular investigation

A molecular study was conducted on 17 *Lpn* strains (seven from air samples and 10 from water samples) from three healthcare facilities. Genotyping was performed via the standard sequence-based typing

(SBT) method of the European Working Group for *Legionella* Infections (EWGLI) using seven genes (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA*) (Gaia et al., 2005; Ratzow et al., 2007).

For each gene sequence, a distinct allele number was assigned through the EWGLI–SBT database for *Lpn* (http://www.hpa-bioinformatics.org.uk/legionella/legionella_sbt/php/sbt_homepage.php). The combination of these allele numbers defines an allelic profile to which a sequence type (ST) is attributed using the EWGLI–SBT database.

For strains from which *neuA* could not be amplified, primers targeting *neuAh* were used, as suggested by the European Study Group for *Legionella* Infections (Farhat et al., 2011). The *neuAh* gene is present in some *Lpn* non-sg 1 strains, and it is functionally equivalent to the *neuA* gene of the *Lpn* subsp. *pneumophila* Philadelphia-1 strain.

3. Results

Lpn sg 6 was isolated from 78.6% of the water samples from five healthcare facilities located in Campania, Lazio, Apulia, and Sicily (two locations). *Lpn* sg 9 was found in the water samples from Liguria (9.5%); *Lpn* sg 1 was found in the water samples from Campania (5.5%, two locations); *Lpn* sg 7 was found in the water samples from Sardinia (5.5%); and *Lpn* sgs 1 and 12 were found in the water samples (0.8%) from Veneto. Overall, *Lpn* sg 6 was the prevalent serogroup, especially in southern Italy.

Lpn sg 6 was detected in air and water of three of 10 examined bathrooms: the first, positive via passive sampling (1 CFU/plate), showed a water contamination of 1100 CFU/L (T_0), 400 CFU/L (T_1), and 800 CFU/L (T_2); the second, positive via passive sampling (1.85 CFU/plate), showed a water contamination of 40,000 CFU/L (T_0), 500 CFU/L (T_1), 700 CFU/L (T_2); the third, positive via active sampling (2 CFU/m^3), showed a water contamination of 43,000 CFU/L (T_0), 140,000 CFU/L (T_1), 160,000 CFU/L (T_2).

Regarding the molecular investigation, a match between the *Legionella* strains from the water and air samples was confirmed by SBT. Specifically, ST 728 (allelic profile 2, 10, 3, 28, 9, 4, 3) was found in the air and water samples of the first healthcare facility, while ST 1638 (allelic profile 2, 3, 6, 10, 51, 1, 218) and ST 1324 (allelic profile 5, 1, 22, 30, 6, 10, 203) were isolated simultaneously from the air and water samples of the second healthcare facility. In the third healthcare facility, which is located in southern Italy, a new ST that had never been isolated and typed in Europe was obtained from the air and water samples. This ST had the allelic profile 3, 10, 14, 28, 21, 14, 9. This strain has been included in the EWGLI–SBT database and identified as ST 1989.

Isolates identified in this study (ST 728, ST 1638, ST 1324, and ST 1989) show different strings of the individual allele numbers; anyway ST 1989 show the same ST 728 pilE and mip alleles.

To date, there are not other reports in EWGLI SBT database regarding ST 1989 from other European or extra-european countries.

4. Discussion

Some researchers (Blatny et al., 2008; Chang and Hung, 2012) have reported air and water sampling methods for *Legionella* spp. detection, but to date, only one report has examined the correlation between *Legionella* strain contamination in water sources and the surrounding air (Crimi et al., 2006), although no genetic analysis was performed. To our knowledge, ours is the first Italian multicenter study regarding the serological and molecular identification of *Legionella* spp. in water and surrounding air samples from hospital water systems. Several epidemiological studies have reported that *Lpn* sg 1 is the predominant strain in environmental water sources in facilities such as buildings, public baths, hospitals, factories, and hotels (Casini et al., 2008; Iatta et al., 2013; Lee et al., 2010). *Lpn* sg 1 is the most frequent serogroup associated with disease as well, followed by *Lpn* sg 6 (Napoli et al., 2010), which is the second most virulent serogroup (Helbig et al., 2002) and the second most frequently isolated strain in hospitals (Mavridou et al., 2008).

Our study showed that *Lpn* sg 6 was the most frequent serogroup in the water samples from all of the enrolled healthcare facilities, and it was the only serogroup isolated from the air samples. Although these results proved a contamination in only three air samples, they represent a good contribution for further investigation aimed at assessing the degree of *Legionella* air contamination.

This finding does not establish a clear correlation between the microbe's concentration in the water and in the surrounding air, but it highlights the importance of monovalent serotyping, as it allows a more accurate assessment of the spread of *Lpn* sgs in the environment. In this regard, some authors (Marchesi et al., 2011) have demonstrated that the use of some biocides for water treatment may select for resistant *Lpn* sgs. Thus, microbial culture studies, supported by monovalent serotyping, would avoid problems associated with improper disinfection methods. Additionally, molecular typing aids in the accurate assessment of strains' interrelationships, as well as the appearance of new STs (Fontana et al., 2014). Molecular typing also enables understanding the evolutionary response of pathogens to the environment when disinfection procedures are undertaken (Casini et al., 2008).

5. Conclusions

To date, there are no specific indications with regard to the protocol to be used in air sampling. Previous studies have not given consistent results due to the different samplers used, the different places sampled and/or the different parameters applied (volume of air sampled, sampling time protocol, point of sampling, etc.) (Napoli et al., 2012).

At the moment, our objective is to plan further studies to improve the air sampling methods employing alternative samplers (*e.g.* liquid impingement technique). These additional studies, supported by molecular investigations, could increase the knowledge about *Legionella* spp. air contamination.

Conflict of interest

The authors declare they have no actual or potential competing financial interests.

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