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Review article

Environmental surveillance and in vitro activity of antimicrobial agents against *Legionella pneumophila* isolated from hospital water systems in Campania, South Italy: a 5-year study

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ABSTRACT

Background: Legionellosis' treatment failures have been recently reported showing the possibility of resistance development to traditional therapy, especially in healthcare related disease cases. Environmental impact of antibiotic residues, especially in hospital waters, may act on the resistome of *Legionella* resulting in developing resistance mechanisms.

Objectives: In this study we investigate the antibiotic susceptibility of environmental *Legionella pneumophila* (Lpn) strains isolated from hospital water systems in Campania, a region located in Southwest Italy.

Methods: 5321 hospital water samples were investigated for the presence of Lpn. Among positive samples, antibiotic susceptibility was tested for a random subset of 125 Lpn strains (25 Lpn isolates from each of the following serogroups: 1, 3, 5, 6, 8).

Susceptibility testing was performed, using the E-test on buffered charcoal yeast extract agar supplemented with α-ketoglutarate, for 10 antimicrobial drugs: azithromycin, cefotaxime, clarithromycin, doxycycline, erythromycin, rifampicin, tigecycline, ciprofloxacin, levofloxacin and moxifloxacin. Non parametric tests were used to determine and assess the significant differences in susceptibility to the different antimicrobics between the serogroups.

Results: Among the isolated strains, none showed resistance to the antibiotics tested. Rifampicin was the most active antibiotic against overall *Legionella* strains, followed by levofloxacin. Between the macrolides the clarithromycin was overall the most active drug, instead the azithromycin was the less active. Analyzing the different serogroups a significant difference was found between serogroup 1 and non-1 serogroup isolates for doxycycline and tigecycline.

Conclusions: Antibiotic susceptibility of environmental isolates of *Legionella* spp. might be useful for the early detection of resistance to antibiotics that directly impacts on mortality and length of hospital stay.

1. Introduction

Legionellosis is an infectious disease caused by the Gram-negative bacilli belonging to the *Legionellaceae* family. These bacteria are found ubiquitously in aquatic habitats, where they grow in multispecies natural biofilms and replicates intracellularly in various protozoa, mainly amoeba but also ciliates (Eisenreich and Heuner, 2016). In particular, healthcare facilities, including hospitals, health centers, hospices, residential care dental settings, and dialysis units, represent an at-risk environment for Legionnaires' disease (LD) transmission because of the frequently old plumbing systems and the use of medical devices from immunocompromised patients (Cristina et al., 2009; Spagnolo et al., 2013; Montagna et al., 2017a, 2017b).

Among the *Legionella* genus, that consists of 61 species and more than 70 serogroups (sgs) (LG, 2015), the *Legionella pneumophila* (*Lpn*) is the aetiological agent causing approximately 90% of reported legionellosis cases (SepinÖzen et al., 2017). Among the 16 sgs of *Lpn* identified up-to-date, serogroup (sg) 1 is the most prevalent in clinical isolates and most frequent cause of human infections, followed by sg 6 and sg 4 (Montagna et al., 2014, 2016; De Giglio et al., 2015).

Legionella infection mainly causes two distinct illnesses: Pontiac fever, an acute febrile and self-limiting illness that doesn't require any

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treatment and is often underdiagnosed and underreported; and the LD, an important cause of community-acquired and hospital-acquired atypical pneumonia, potentially fatal (Hashmi et al., 2017). The exact incidence of legionellosis worldwide is difficult to quantify and compare, because countries differ greatly in the methods of defining and reporting the cases (WHO, 2007).

In 2015, in 30 European countries, 6573 cases of LD have been confirmed from the data of the European Legionnaires' Disease Surveillance Network (ELDSNet), with a case fatality of 8% (ECDC, 2017). In the same period in Italy, the National Surveillance System has estimated about 1548 cases of confirmed LD, out of a total of 1569 cases notified, 5% of which have been reported as acquired in healthcare facilities. Case-fatality ratio was 9% for community-acquired cases and 44% for hospital-acquired cases (ISS, 2016; Montagna et al., 2017a).

Time-series analysis of LD incidence demonstrates an increasing burden of the disease in Italy and worldwide making LD being an important cause of potentially preventable morbidity and mortality (Parr et al., 2015; ISS, 2016; ECDC, 2017). This concept of preventable illness has resulted in a number of guidelines and new control strategies aimed at reducing the risk of legionellosis in building water systems. In fact, however the factors that lead to outbreaks or cases of LD are not completely understood, the presence of the bacterium in an aquatic environment is constantly considered prerequisite for the infection (Phin et al., 2014; Soda et al., 2017).

Thus, the correct water management quality practices, included sanitation procedures, and the rapid methods for analyzing *Legionella* species in environmental water are the key point in the prevention of LD outbreaks (Fontana et al., 2014; De Giglio et al., 2015). Furthermore, it has been shown that the environmental impact of antibiotic residues in soil and water acts on the resistome of the bacteria and results in developing resistance mechanisms (D'Costa et al., 2006; Hilbi, 2010). In literature, the development of *Lpn* has been described particularly in hospital-acquired LD because probably the *Legionella* spp. that colonizes and persists in healthcare water facilities, despite harsh physical and chemical treatments, can be exposed to antibiotics from medical or veterinary practices (Almahmoud et al., 2009; Berjeaud et al., 2016).

Successful treatment of LD requires that antimicrobial agents reach therapeutic intracellular concentrations because *Lpn* is an intracellular pathogen residing within tissue and alveolar macrophage (Bruin et al., 2012). The antibiotics most commonly used are macrolides, fluoroquinolones and tetracyclines families (Sabrià et al., 2005; LG, 2015), however failures treatment have been recently reported in literature showing the possibility of development of resistance to traditional therapy (Erdogan et al., 2010).

Routine susceptibility testing of *Legionella* spp. is not recommended because of difficulties in determining standard minimal inhibitory concentration values (MICs) due to high nutritional necessities of legionellae and inactivation of some antibiotics (for example: sulfonamide, tetracycline, polymyxin B) by charcoal which is necessary for the proliferation of the species (Nielsen et al., 2000; Sikora et al., 2017). Moreover LD is usually a non-productive pneumonia, and it is difficult to obtain respiratory secretions for culturing before the patient undergoes antibiotic therapy (De Giglio et al., 2015).

Therefore, several studies dealing with the antibiotic susceptibility of environmental *Legionella* strains have been reported in the literature (Nielsen et al., 2000; Alexandropoulou et al., 2013; De Giglio et al., 2015; Sikora et al., 2017). Given the disparity in the results, although a study has been already conducted in Southern Italy (De Giglio et al., 2015) we are led to believe that the resistances are closely related to the geographic area. For this reason we decided to carry out this study in Campania, a region located in Southwest of Italy, analyzing different *Lpn* strains isolated from hospital water systems during a 5-year environmental surveillance campaign.

2. Methods

From 2012 to 2016, the Department of Public Health of the University Federico II of Naples collected 5321 samples of water for the environmental surveillance of *Legionella* spp. from 52 hospitals in Campania region, Italy. Water samples were collected and processed according to the procedures described in the national standard UNI EN ISO 11731-2:2008.

Samples were considered positive if more one or more colonies grew on the media. *Legionella* strains in water samples were first serologically identified by the latex agglutination test using a polyvalent commercial kit (Oxoid S.p.A., Milan, Italy), and then by a panel of monovalent antisera (Biogenetics S.R.L., Denka Seiken, Ponte San Nicolò, Italy). The strains were frozen at − 80 °C.

The antibiotic susceptibility was tested only for serotypes with a percentage of isolation greater than 1%.

25 *Lpn* strains for each of the leading sg were randomly selected. Antibiotic sensitivity to ten drugs was performed using E-tests on Buffered Charcoal Yeast Extract (BCYE-α) (BioMérieux, Marcy l'Etoile, France). Antimicrobial drugs tested were: azithromycin (AZ), cefotaxime (CT), clarithromycin (CH), doxycycline (DC), erythromycin (EM), rifampicin (RI), and tigecycline (TGC) (ranging from 0.016 to 256 mg/L each); as well as ciprofloxacin (CI), levofloxacin (LE), and moxifloxacin (MX) (ranging from 0.002 to 32 mg/L each).

Legionella strains were subcultured on BCYE-α plates and incubated for 48 h at 37 °C in a humidified atmosphere. Colonies were suspended in sterile water, and the turbidity was adjusted to an optical density equivalent to 0.5 McFarland units. Suspensions, approximately 10^7 colony-forming units (CFU)/mL, were swabbed onto BCYE-α plates, and the surfaces of the plates were allowed to completely dry (15 min at room temperature). Then, antimicrobial strips were applied to each inoculated plate. The plates were incubated at 35 °C (without CO_2) for 48 h before reading the MICs; if no growth was detected, the plates were incubated for an additional 24 h. The lowest concentration of antibiotic at which the zone of inhibition intersected the E-test strip was taken as the MICs. ECOFF ware determined in according to EUCAST guidelines (EUCAST, 2016) for all antibiotics, for cefotaxime were used the ECOFF values of Bruin et al. (2012).

Lpn sg 1 American Type Culture Collection (ATCC) 33152 was used as the reference strain as previously described by Marques and Piedade (1997), to determine the influence of charcoal (present in buffered charcoal yeast extract agar supplemented with α-ketoglutarate) on the activity of the antimicrobials, we selected *Staphylococcus aureus* ATCC 6538 for susceptibility testing. For *S. aureus* ATCC 6538, the E-test was performed on Mueller-Hinton agar (MH) (Biolife, Milan, Italy) and on BCYE-α, and the MICs ware read after 24 h of incubation at 35 °C.

Interpretation criteria of MICs values were based on the EUCAST Clinical Breakpoint Tables (EUCAST, 2017) and on the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2012).

Nonparametric tests were used to determine and assess the significant differences in susceptibility to the different antimicrobic between the sgs. The Mann–Whitney U was applied to test statistical significance in antimicrobic susceptibility between *Lpn* sg1 isolates and *Lpn* non-sg 1 isolates, while the Kruskal–Wallis test, followed by the Dunn's test using Benjamini-Hochberg (BH) correction for multiple comparisons, was applied between the different *Lpn* non-sg 1 isolates. Analyses were performed using R 3.3.1 version, using the PMCMR and ggplot2 libraries. Results were considered statistically significant if BH corrected p-values fell below the threshold of 0.05.

3. Results

A total of 1197 over 5321 (22.5%) water samples collected were found positive for *Legionella* spp. The *Legionella* strains isolated from the water samples were distributed as follow: *Lpn* sg 1 (35.0%), *Lpn* sg 6 (23.2%), *Lpn* sg 8 (20.1%), *Lpn* sg 3 (18.8%), *Lpn* sg 5 (2.2%), *Lpn* sg 10