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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

Table 1
MIC50, MIC90, geometric mean MIC, MIC range and ECOFF values of the 10 antibiotics tested for all the *Legionella* strains isolated.

Antibiotic	MIC50	MIC90	GM	Range	ECOFF
***AZ*-Azithromycin**	0.064	0.19	0.093	0.016–0.190	1
***CH*-Clarithromycin**	0.016	0.023	0.019	0.016–0.032	0.5
***EM*-Erytromycin**	0.047	0.064	0.031	0.016–0.094	0.5
***CI*-Ciprofloxacin**	0.047	0.094	0.058	0.023–0.094	1
***LE*-Levofloxacin**	0.008	0.016	0.009	0.004–0.023	1
***MX*-Moxifloxacin**	0.047	0.094	0.050	0.032–0.094	1
***CT*-Cefotaxime**	0.125	0.250	0.120	0.016–0.380	1
***TGC*-Tigecycline**	4.000	6.000	1.887	1–16	16
***DC*-Doxycycline**	1.000	2.000	1.691	1–6	8
***RI*-Rifampicin**	0.006	0.008	0.005	0.003–0.012	0.032

MIC50: MICs required to inhibit the growth of 50% of organisms.
MIC90: MICs required to inhibit the growth of 90% of organisms.
GM: geometric mean.
ECOFF: epidemiological cut-off value.

(0.4%), *Lpn* sg 4 (0.1%), *Lpn* sg 14 (0.1%), *Lpn* sg 9 (0.1%).

Table 1 shows MIC50 and MIC90 (respectively, the MICs required to inhibit the growth of 50% and 90% of organisms), geometric mean MIC, MIC range and ECOFF values of the 10 antibiotics tested for the totality of the *Legionella* strains isolated.

Overall the most active antibiotic was RI followed by LE, TGC and DC were the least active antibiotics, all the other antibiotics exhibited intermediate susceptibility (Fig. 1). None of the strains isolated showed resistance to the antibiotics tested (Table 1).

Results from the analyses of antibiotic susceptibility for each *Legionella* strains (namely 1, 3, 5, 6 and 8) are presented separately in the Table 2.

The Fig. 2A shows the comparison of geometric mean MIC values of *Lpn* sg 1 versus all non-sg 1 isolates. A statistically significant difference was found for four out of ten antibiotics tested (AZ pval = 7.3e-11, EM pval = 5.6e-05, TGC pval = 4.7e-09 and DC pval = 0.01). In particular, the geometric mean MIC values of AZ, EM and TGC were significantly higher for *Lpn* sg 1 isolates compared to *Lpn* non-sg 1 isolates, while DC showed significantly lower MIC values for *Lpn* sg 1 isolated than *Lpn* non-sg 1. We further analyzed the difference in the geometric mean MICs of each antibiotic among sgs other than *Lpn* sg 1 (Fig. 2B). For RI and TGC we didn't detect any significant difference. AZ and CH were shown to be significantly less active on *Lpn* sg 5 than on all the others *Lpn* non-sg 1, while for LE was demonstrated the opposite, further LE was also shown to be significantly more active on *Lpn* sg 6 than

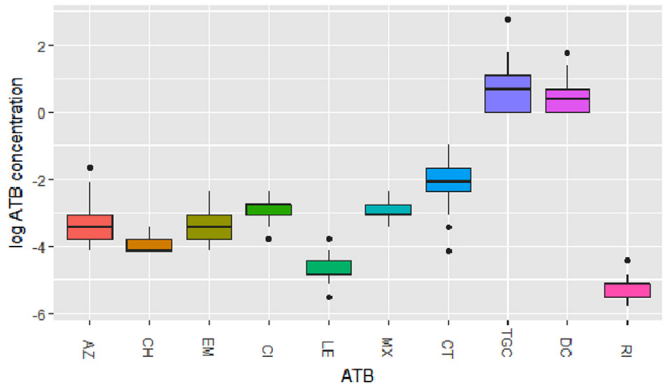

Fig. 1. Antibiotic susceptibility of all the *Legionella* strains isolated. Boxplots show on the y-axis the (log) concentrations (mg/L) of the ten targeted antibiotics on the x-axis identified by colors. Boxes extend from the 25th to the 75th percentile, solid horizontal lines represent the median of each distribution. ATB: Antibiotic; AZ: Azithromycin; CH: Clarithromycin; EM: Erytromycin; CI: Ciprofloxacin; LE: Levofloxacin; MX: Moxifloxacin; CT: Cefotaxime; TGC: Tigecycline; DC: Doxycycline; RI: Rifampicin.

Table 2
MIC50, MIC90, geometric mean MIC and MIC range of the ten antibiotics tested for the *Legionella* sgs 1, 3, 5, 6, and 8.

	Lnp sg 1				Lnp sg 3				Lnp sg 5				Lnp sg 6				Lnp sg 8			
ATB	**MIC 50**	**MIC 90**	**GM**	**Range**	**MIC 50**	**MIC 90**	**GM**	**Range**	**MIC 50**	**MIC 90**	**GM**	**Range**	**MIC 50**	**MIC 90**	**GM**	**Range**	**MIC 50**	**MIC 90**	**GM**	**Range**
AZ	0.064	0.190	0.082	0.047–0.19	0.023	0.032	0.029	0.023–0.094	0.016	0.047	0.046	0.016–0.094	0.023	0.047	0.28	0.016–0.047	0.023	0.047	0.028	0.016–0.047
CH	0.016	0.023	0.018	0.016–0.023	0.016	0.023	0.019	0.016–0.032	0.016	0.032	0.025	0.016–0.032	0.016	0.023	0.018	0.016–0.023	0.016	0.023	0.018	0.016–0.032
EM	0.047	0.064	0.044	0.023–0.094	0.032	0.064	0.035	0.023–0.064	0.023	0.016	0.023	0.016–0.032	0.023	0.047	0.028	0.016–0.047	0.032	0.047	0.031	0.016–0.064
CI	0.047	0.094	0.052	0.023–0.094	0.047	0.064	0.056	0.047–0.094	0.032	0.064	0.067	0.032–0.094	0.064	0.064	0.053	0.032–0.094	0.064	0.094	0.064	0.047–0.094
LE	0.008	0.016	0.010	0.006–0.016	0.008	0.012	0.010	0.006–0.016	0.004	0.004	0.006	0.004–0.012	0.008	0.023	0.009	0.004–0.023	0.012	0.016	0.013	0.006–0.023
MX	0.047	0.094	0.049	0.032–0.094	0.047	0.064	0.053	0.032–0.094	0.032	0.047	0.043	0.032–0.094	0.047	0.064	0.049	0.032–0.094	0.047	0.094	0.056	0.032–0.094
CT	0.125	0.250	0.117	0.016–0.38	0.094	0.190	0.109	0.047–0.19	0.047	0.190	0.170	0.047–0.380	0.125	0.19	0.131	0.047–0.38	0.064	0.125	0.087	0.047–0.25
TGC	4.000	6.000	3.786	1.5–16	2.000	3.000	1.663	1–6	1.000	1.500	1.451	1–3	1.5	2	1.451	1–3	2.000	3.000	1.803	1–4
DC	1.000	2.000	1.345	1–3	2.000	6.000	2.352	1–6	1.000	1.000	1.226	1–2	1.5	3	1.714	1–4	2.000	4.000	2.081	1–4
RI	0.006	0.008	0.005	0.003–0.012	0.006	0.006	0.005	0.003–0.008	0.003	0.004	0.004	0.003–0.008	0.006	0.008	0.005	0.004–0.008	0.004	0.008	0.005	0.003–0.008

ATB: Antibiotic.
MIC50: MICs required to inhibit the growth of 50% of organisms.
MIC90: MICs required to inhibit the growth of 90% of organisms.
GM: geometric mean.
AZ: Azithromycin; CH: Clarithromycin; EM: Erytromycin; CI: Ciprofloxacin; LE: Levofloxacin; MX: Moxifloxacin; CT: Cefotaxime; TGC: Tigecycline; DC: Doxycycline; RI: Rifampicin.

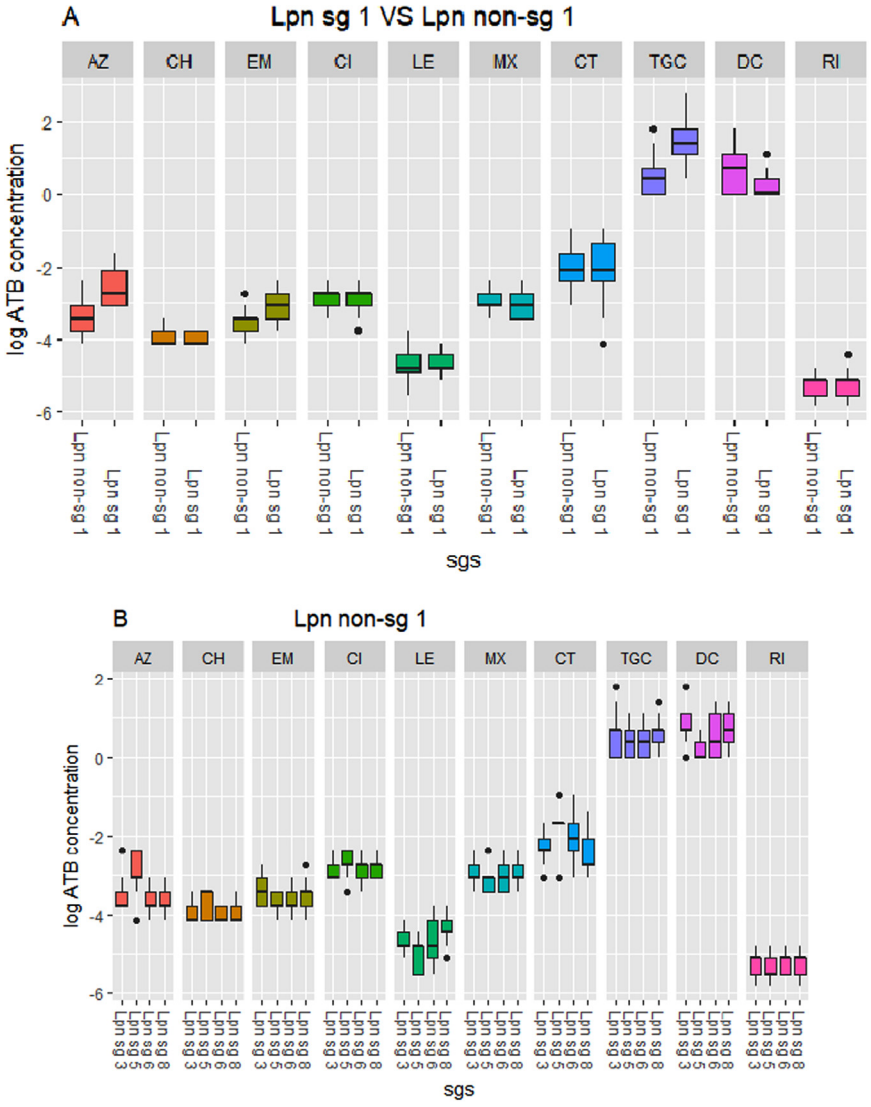

Fig. 2. Antibiotic susceptibility of the *Legionella pneumophila* sg 1 versus non-sg 1 isolates (Fig. 2A), and among the non-sg 1 isolates namely 3, 5, 6 and 8 (Fig. 2B). Boxplots show on the y-axis the (log) concentrations (mg/L) of ten targeted antibiotics identified by colors and split by each of the sgs of *Lpn* on the x-axis. Boxes extend from the 25th to the 75th percentile, solid horizontal lines represent the median of each distribution. ATB: Antibiotic; LP: *Legionella pneumophila*; AZ: Azithromycin; CH: Clarithromycin; EM: Erytromycin; CI: Ciprofloxacin; LE: Levofloxacin; MX: Moxifloxacin; CT: Cefotaxime; TGC: Tigecycline; DC: Doxycycline; RI: Rifampicin.

on *Lpn* sg 8. EM and MX were shown to be significantly more active on *Lpn* sg 5 than on *Lpn* sgs 3 and 8. CI was shown to be less active on *Lpn* sg 5 < sg 8 < sg 3 < sg 6 (in decreasing order of their MICs). CT was shown to be more active on *Lpn* sg 8 > sg 3 > sg 6 > sg 5 (increasing order of their MICs). DC was demonstrated to be significantly less active on *Lpn* sg 5 than on all the other non-sg1 isolates.

Results from control strains testing are reported in Table 3. The mean values of MICs of the reference strain *Lpn* sg 1 ATCC 33152 (one repetition) showed similar sensitivities to the environmental isolates, apart for CI, LE, MX, CT and TGC that showed lower sensitivities for the reference strain. Comparing the results obtained from *S. aureus* ATCC 6538 (one repetition) on the two different media, the MICs of AZ, CH, CI, LE, MX and TGC exhibited higher values (2–11 folds increase) in BCYE-α, instead the MICs of CT, DC, EM and RI were similar in both media. *S. aureus* resulted sensible for all antibiotics in both media, except for AZ which was resistant in BCYE-α agar.

Table 3
MICs values (mg/L) for the reference strains *Lpn* sg 1 ATCC 33152, and *S. aureus* ATCC 6538 in BCYE-α and MH, *S. aureus* ATCC 6538 MICs ratio in the two different media (BCYE-α over MH). For *S. aureus* MIC breakpoint values are also reported.

Antibiotic	*Lpn* sg 1 ATCC 33152	*S. aureus* ATCC 6538				BCYE-α/MH
		BCYE-α	MH	MIC breakpoint		
				S	R	
***AZ*-Azithromycin**	0,032	2	0,75	≤ 1	> 2	2,67
***CH*-Clarithromycin**	0,38	0,75	0,38	≤ 1	> 2	1,97
***EM*-Erytromycin**	0,094	0,25	0,25	≤ 1	> 2	1
***CI*-Ciprofloxacin**	0,38	0,75	0,38	≤ 1	> 1	1,97
***LE*-Levofloxacin**	0,094	0,38	0,19	≤ 1	> 1	2
***MX*-Moxifloxacin**	0,19	0,25	0,012	≤ 0.25	> 0.25	20,83
***CT*-Cefotaxime**	0,75	0,75	0,75	≤ 8	≥ 64	1
***TGC*-Tigecycline**	1,5	0,38	0,032	≤ 0.5	> 0.5	11,88
***DC*-Doxycycline**	1,5	0,125	0,125	≤ 1	> 2	1
***RI*-Rifampicin**	0,006	0,006	0,006	≤ 0.06	> 0.5	1

MH: Muller-Hinton agar.
BCYE-α: buffered charcoal yeast agar.
S: sensible.
R: resistant.

4. Discussion

Environmental surveillance offers the opportunity to identify antibiotic resistance in the environment before it becomes evident in clinical specimens. In literature, several studies evaluated the variation in sensitivity to antibiotics of environmental isolates of *Legionella* spp. (Erdogan et al., 2010; Sandalakis et al., 2014; De Giglio et al., 2015;

Xiong et al., 2016). To date, although several studies have examined the environmental reservoirs of *Legionella* spp. (Pasquarella et al., 2012; Montagna et al., 2014; Torre et al., 2014) in Italy, only one study, performed by the University of Bari, described the antimicrobial susceptibility of *Legionella* spp. isolated from hospital water systems in Apulia (De Giglio et al., 2015).

The present study raises from a 5-year environmental surveillance carried out with the purpose to evaluate the antibiotic sensitivity changes of *Legionella* strains in Campania region.

In our study non-sg 1 isolates were most frequent than sg 1. Our results are in accordance with previous descriptions Italy (De Giglio et al., 2015), although a reverse pattern was detected in other countries (Sikora et al., 2017; SepinÖzen et al., 2017). In community LD is mainly described as caused by sg 1 but in hospitals, because of the high susceptibility of the hosts, other sgs may cause cases or outbreaks of LD (Montagna et al., 2017a, 2017b). In this regard we didn't collect any clinical strain, but as the sgs 6, 8 and 3 have been described as among the most virulent sgs (Helbig et al., 2002), we are prone to believe possible the transmission of these sgs to humans.

None of the isolates was resistant to the antibiotics tested. In literature, failure treatment of legionellosis has been reported despite using appropriate antibiotic therapy (Pedro-Botet and Yu, 2006; Jespersen et al., 2010; Chidiac et al., 2012; Burdet et al., 2014), and acquired resistance to first-line antibiotics has been described both in vitro (Nielsen et al., 2000; Almahmoud et al., 2009) and more recently in vivo (Bruin et al., 2012; Shadoud et al., 2015). A previous study in Poland found azithromycin resistance in one out of 28 isolates of *Legionella* from public water systems (Sikora et al., 2017).

In our study, in accordance to previous reports (Marques and Piedade, 1997; Erdogan et al., 2010; Bruin et al., 2012; Al-Matawah et al., 2012; Sandalakis et al., 2014; De Giglio et al., 2015), rifampicin was the most active antibiotic against overall *Legionella* strains and showed no difference in activity toward *Lpn* sg 1 and non-sg 1 isolates. Despite this result in vitro, we acknowledge that in the clinical practice the use of rifampicin in monotherapy is not recommended because of the rapid appearance of resistance (Nielsen et al., 2000). In Italy, according the latest guideline released from the Superior Institute of Health, combined treatments of rifampicin with a macrolide is recommended only as third-line treatment in immunocompromised patients or with severe LD (LG, 2015).

Macrolides (especially azithromycin) and the fluoroquinolones (especially levofloxacin) are first choice antibiotics for legionellosis treatment. The broad-spectrum activity and the reduced adverse effects actually favor fluoroquinolones over macrolides (Erdogan et al., 2010).

As described in literature, we found that fluoroquinolones have better activity than macrolides (Sabrià et al., 2005; Pedro-Botet and Yu, 2006; Dunbar and Farrell, 2007), however mainly driven by levofloxacin.

In fact, we found that levofloxacin was the second most active antibiotic, the most active drug among all the fluoroquinolones and showed similar activity in both *Lpn* sg 1 and non-sg 1 isolates in accordance to previous reports (Stout et al., 2005; Dunbar and Farrell, 2007; De Giglio et al., 2015). Moreover, we confirmed that clarithromycin was overall the most active drug among the macrolides as found in previous reports (Pedro-Botet and Yu, 2006; Bruin et al., 2012; De Giglio et al., 2015). Azithromycin, instead, was the less active macrolide and its activities toward *Lpn* sg 1 was significantly higher than in *Lpn* non-sg 1 isolates as also reported in another Italian study (De Giglio et al., 2015).

Tigecycline was the least active antibiotic, followed by doxycycline. Analyzing the different sgs, for these two antibiotics a significant difference was found between sg 1 and non-sg 1 isolates, with doxycycline being less active and tigecycline more active for non-sg 1 than sg 1 isolates. Currently, evidence does not support the use of tigecycline in the treatment of legionellosis (Bopp et al., 2011). Doxycycline is instead recommended as second choice treatment in not immunocompromised patient with minor pneumonia, however attention should be paid as different studies found doxycycline as the least active antimicrobial among the antibiotic usually used in legionellosis (De Giglio et al., 2015; Xiong et al., 2016).

Only few other studies analyzed the sensitivities of *Lpn* non-sg 1 isolates (Al-Matawah et al., 2012; De Giglio et al., 2015). In this study, we found significant differences in susceptibility between sg 5 isolates and various other non-sg 1 isolates for all the antibiotics except for rifampicin and tigecycline. Similar susceptibility was found for the other sgs, as also detected in the previous reports (Al-Matawah et al., 2012; De Giglio et al., 2015), however both levofloxacin and clarithromycin were significantly more active on *Lpn* sg 6 than on *Lpn* sg 8 isolates.

In regard to the differences observed in susceptibility of *S. aureus* ATCC 6538 to TGC, MX and AZ according to the different media used, several studies have indicated that charcoal or other components of BCYE-α can inhibit various antibiotics, including tetracyclines, fluoroquinolones, and macrolides (Marques and Piedade, 1997; Erdogan et al., 2010; Bruin et al., 2012; Sandalakis et al., 2014). In particular in our study, *S. aureus* resulted resistant to AZ in BCYE-α and susceptible in MH according to EUCAST clinical breakpoint table (EUCAST, 2017).

Various methods can be used to determine values of MIC: E-Test, broth and agar dilution, disk diffusion methods in vivo and in vitro. None of this method is considered a gold standard (Bruin et al., 2012). In the present study, despite the E-test on BCYE-α agar yielded elevated MICs compared to MH, we decided to use it because it is a simple, accurate and easily available method to determine the antibiotic susceptibility of *Legionella* spp. In fact, the E-test can be used in many laboratories, thus avoiding sending the strains to reference laboratories for susceptibility testing. The high costs of the tests used represent the main limitation for routinely examination of the susceptibility of strains isolated during environmental surveillance. However, in hospital settings the evaluation could be conducted every 3–5 years or at occurrence, during periods when it is likely the development of antibiotic resistance (Jonas et al., 2003).

Furthermore, we recommend caution in interpreting the results because is necessary to consider that experimentation in vitro of antimicrobial agents is poorly correlated to the clinical effectiveness (Marques and Piedade, 1997).

5. Conclusions

To our knowledge, this is the second study to investigate variation in antibiotic sensibility in Italy (De Giglio et al., 2015) and the first study to investigate the epidemiologic scenery of Campania region. We detect differences in sensitivity to antibiotics and between the different sgs of the same species that may provide important information for guiding the clinics to select the best treatment option. Environmental surveillance of *Legionella* spp. might be also useful for the early detection of *Lpn* non-sg 1 circulating in the hospitals, that in turn may cause LD in susceptible hosts.

Conflict of interest

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

References

Alexandropoulou, I.G., Parasidis, T.A., Konstantinidis, T.G., Constantinidis, T.C., Panopoulou, M., 2013. Antibiotic susceptibility surveillance of environmental *Legionella* strains: application of the E-test to bacteria isolated from hospitals in Greece. J. Infect. Dis. Ther. 2, 1. http://dx.doi.org/10.4172/2332-0877.1000e103.

Almahmoud, I., Kay, E., Schneider, D., Maurin, M., 2009. Mutational paths towards increased fluoroquinolone resistance in *Legionella pneumophila*. J. Antimicrob. Chemother. 64, 284–293.

Al-Matawah, Q.A., Al Zenki, S.F., Qasem, J.A., Al Waalan, T.E., BenHeji, A.H., 2012.

Detection and quantification of *Legionella pneumophila* from water systems in Kuwait residential facilities. J. Pathog. 2012, 138389. http://dx.doi.org/10.1155/2012/138389.

Berjeaud, J.M., Chevalier, S., Schlusselhuber, M., Portier, E., Loiseau, C., Aucher, W., Lesouhaitier, O., Verdon, J., 2016. *Legionella pneumophila*: the paradox of a highly sensitive opportunistic waterborne pathogen able to persist in the environment. Front. Microbiol. 7, 486. http://dx.doi.org/10.3389/fmicb.2016.00486.

Bopp, L.H., Baltch, A.L., Ritz, W.J., Michelsen, P.B., Smith, R.P., 2011. Activities of tigecycline and comparators against *Legionella pneumophila* and *Legionella micdadei* extracellularly and in human monocyte-derived macrophages. Diagn. Microbiol. Infect. Dis. 69 (1), 86–93.

Bruin, J.P., Ijzerman, E.P., denBoer, J.W., Mouton, J.W., Diederen, B.M., 2012. Wild- type MIC distribution and epidemiological cut-off values in clinical *Legionella pneumophila* serogroup 1 isolates. Diagn. Microbiol. Infect. Dis. 72, 103–108.

Burdet, C., Lepeule, R., Duval, X., Caseris, M., Rioux, C., Lucet, J.C., Yazdanpanah, Y., 2014. Quinolones versus macrolides in the treatment of legionellosis: a systematic review and meta-analysis. J. Antimicrob. Chemother. 69 (9), 2354–2360.

Chidiac, C., Che, D., Pires-Cronenberger, S., Jarraud, S., Campèse, C., Bissery, A., Weinbreck, P., Brun-Buisson, C., Sollet, J.P., Ecochard, R., Desenclos, J.C., Etienne, J., Vanhems, P., 2012. French Legionnaires' disease study group. Factors associated with hospital mortality in community-acquired legionellosis in France. Eur. Respir. J. 39 (4), 963–970.

CLSI, 2012. Clinical and Laboratory Standards Institute. Approved Standard M100-S22 Twenty-Second Informational Supplement. Clinical and Laboratory Standards Institute, Wayne, PA.

Cristina, M.L., Spagnolo, A.M., Sartini, M., Dallera, M., Ottria, G., Perdelli, F., Orlando, P., 2009. Investigation of organizational and hygiene features in dentistry: a pilot study. J. Prev. Med. Hyg. 50 (3), 175–180.

D'Costa, V.M., McGrann, K.M., Hugues, D.W., Wright, G.D., 2006. Sampling the antibiotic resistome. Science 311 (5759), 374–377.

De Giglio, O., Napoli, C., Lovero, G., Diella, G., Rutigliano, S., Caggiano, G., Montagna, M.T., 2015. Antibiotic susceptibility of *Legionella pneumophila* strains isolated from hospital water systems in Southern Italy. Environ. Res. 142, 586–590.

Dunbar, L.M., Farrell, D.J., 2007. Activity of telithromycin and comparators against isolates of *Legionella pneumophila* collected from patients with community acquired respiratory tract infections: protekt years 1–5. Clin. Microbiol. Infect. 13 (7), 743–746.

Eisenreich, W., Heuner, K., 2016. The life stage-specific pathometabolism of *Legionella pneumophila*. Fed. Eur. Biochem. Soc. Lett. 590, 3868–3886.

Erdogan, H., Can, F., Demirbilek, M., Timurkaynak, F., Arslan, H., 2010. In vitro activity of antimicrobial agents against *Legionella* isolated from environmental water systems: first results from Turkey. Environ. Monit. Assess. 171 (1–4), 487–491.

EUCAST, 2016. Antimicrobial susceptibility testing of *Legionella pneumophila*. 〈http://www.eucast.org〉, (Accessed 10 August 2017).

EUCAST, 2017. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 7.1. 〈http://www.eucast.org〉.

European Centre for Disease Prevention and Control, 2017. Introduction to the annual epidemiological report for 2015. In: ECDC. Annual epidemiological report for 2015. ECDC, Stockholm.

Fontana, S., Scaturro, M., Rota, M.C., Caporali, M.G., Ricci, M.L., 2014. Molecular typing of *Legionella pneumophila* serogroup 1 clinical strains isolated in Italy. Int. J. Med. Microbiol. 304 (5–6), 597–602.

Hashmi, H.R.T., Saladi, L., Petersen, F., Khaja, M., Diaz-Fuentes, G., 2017. Legionnaires' disease: clinicoradiological comparison of sporadic versus outbreak cases. Clin. Med. Insights Circ. Respir. Pulm. Med. 11, 1–8. http://dx.doi.org/10.1177/1179548417711941.

Helbig, J.H., Bernander, S., Castellani Pastoris, M., Etienne, J., Gaia, V., Lauwers, S., Lindsay, D., Lück, P.C., Marques, T., Mentula, S., Peeters, M.F., Pelaz, C., Struelens, M., Uldum, S.A., Wewalka, G., Harrison, T.G., 2002. Pan-European study on culture-proven Legionnaires' disease: distribution of *Legionella pneumophila* serogroups and monoclonal subgroups. Eur. J. Clin. Microbiol. Infect. Dis. 21 (10), 710–716.

Hilbi, H., 2010. Update on Legionnaires' disease: pathogenesis, epidemiology, detection and control. Mol. Microbiol. 76 (1), 1–11.

Istituto Superiore di Sanità, 2016. Rapporto Annuale sulla legionellosi in Italia nel 2015. 29. Notiziario ISS, Rome, pp. 3–10.

Jespersen, S., Søgaard, O.S., Schønheyder, H.C., Fine, M.J., Ostergaard, L., 2010. Clinical features and predictors of mortality in admitted patients with community and hospital acquired legionellosis: a Danish historical cohort study. BMC Infect. Dis. 21 (10), 124. http://dx.doi.org/10.1186/1471-2334-10-124.

Jonas, D., Engels, I., Hartung, D., Beyersmann, J., Frank, U., Daschner, F.D., 2003. Development and mechanism of fluoroquinolone resistance in *Legionella pneumophila*. J. Antimicrob. Chemother. 51, 275–280.

LG, 2015. Guidelines for Prevention and Control of Legionellosis. Italian Health Ministry. http://www.salute.gov.it/imgs/C_17_pubblicazioni_2362_allegato.pdf.

Marques, T., Piedade, J., 1997. Susceptibility testing by E-test and agar dilution of 30 strains of Legionella spp. isolated in Portugal. Clin. Microbiol. Infect. 3 (3), 365–368.

Montagna, M.T., De Giglio, O., Napoli, C., Cannova, L., Cristina, M.L., Deriu, M.G., Delia, S.A., Giuliano, A., Guida, M., Laganà, P., Liguori, G., Mura, I., Pennino, F., Rossini, A., Tardivo, S., Torre, I., Torregrossa, M.V., Villafrate, M.R., Albertini, R., Pasquarella, C., 2014. *Legionella spp*. contamination in indoor air: preliminary results of an italian multicenter study. Epidemiol. Prev. 38 (6 Suppl. 2), S62–S65.

Montagna, M.T., Cristina, M.L., De Giglio, O., Spagnolo, A.M., Napoli, C., Cannova, L., Deriu, M.G., Delia, S.A., Giuliano, A., Guida, M., Laganà, P., Liguori, G., Mura, I., Pennino, F., Rossini, A., Tardivo, S., Torre, I., Torregrossa, M.V., Villafrate, M.R., Albertini, R., Pasquarella, C., 2016. Serological and molecular identification of Legionella spp. isolated from water and surrounding air samples in italian healthcare facilities. Environ. Res. 146, 47–50. http://dx.doi.org/10.1016/j.envres.2015.12.015.

Montagna, M.T., De Giglio, O., Cristina, M.L., Napoli, C., Pacifico, C., Agodi, A., Baldovin, T., Casini, B., Coniglio, M.A., D'Errico, M.M., Delia, S.A., Deriu, M.G., Guida, M., Laganà, P., Liguori, G., Moro, M., Mura, I., Pennino, F., Privitera, G., Romano Spica, V., Sembeni, S., Spagnolo, A.M., Tardivo, S., Torre, I., Valeriani, F., Albertini, R., Pasquarella, C., 2017a. Evaluation of *Legionella* air contamination in healthcare facilities by different sampling methods: an Italian multicenter study. Int. J. Environ. Res. Public Health 14 (7). http://dx.doi.org/10.3390/ijerph14070670.

Montagna, M.T., De Giglio, O., Cristina, M.L., Albertini, R., Pasquarella, C., GISIO-SItI Working Group, AIA Working Group, SIMPIOS Working Group, 2017b. *Legionella* indoor air contamination in health care environments. Springer Briefs in Public Health Issue 9783319491592, pp. 63–71.

Nielsen, K., Bangsborg, J.M., Hoiby, N., 2000. Susceptibility of Legionella species to five antibiotics and development to resistance by exposure to erythromycin, ciprofloxacin, and rifampicin. Diagn. Microbiol. Infect. Dis. 36 (1), 43–48.

Parr, A., Ellen, A., Whitney, E.A., Berkelman, R.L., 2015. Legionellosis on the rise: a review of guidelines for prevention in the United States. J. Public Health Manag. Pract. 21 (5), E17–E26.

Pasquarella, C., Veronesi, L., Napoli, C., Castiglia, P., Liguori, G., Rizzetto, R., Torre, I., Righi, E., Farruggia, P., Tesauro, M., Torregrossa, M.V., Montagna, M.T., Colucci, M.E., Gallè, F., Masia, M.D., Strohmenger, L., Bergomi, M., Tinteri, C., Panico, M., Pennino, F., Cannova, L., Tanzi, M., SItI Working Group Hygiene in Dentistry, 2012. Microbial environmental contamination in Italian dental clinics: a multicenter study yielding recommendations for standardized sampling methods and threshold values. Sci. Total Environ. 15 (420), 289–299. http://dx.doi.org/10.1016/j.scitotenv.2012.01.030.

Pedro-Botet, M.L., Yu, V.L., 2006. Legionella: macrolides or quinolones? Clin. Microbiol. Infect. 12 (3), 25–30.

Phin, N., Parry-Ford, F., Harrison, T., Stagg, H.R., Zhang, N., Kumar, K., Lortholary, O., Zumla, A., Abubakar, I., 2014. Epidemiology and clinical management of Legionnaires' disease. Lancet Infect. Dis. 14 (10), 1011–1021. http://dx.doi.org/10.1016/S1473-3099(14)70713-3.

Sabrià, M., Pedro-Botet, M.L., Gómez, J., Roig, J., Vilaseca, B., Sopena, N., Baños, V., 2005. Fluoroquinolones vs macrolides in the treatment of Legionnaires disease. Chest 128 (3), 1401–1405.

Sandalakis, V., Chochlakis, D., Goniotakis, I., Tselentis, Y., Psaroulaki, A., 2014. Minimum inhibitory concentration distribution in environmental Legionella spp. isolates. J. Water Health 12 (4), 678–685.

SepinÖzen, N., Tuğlu Ataman, Ş., Emek, M., 2017. Exploring the *Legionella pneumophila* positivity rate in hotel water samples from Antalya, Turkey. Environ. Sci. Pollut. Res. 24, 12238–12242.

Shadoud, L., Almahmoud, I., Jarraud, S., Etienne, J., Larrat, S., Schwebel, C., Timsit, J.F., Schneider, D., Maurin, M., 2015. Hidden selection of bacterial resistance to fluoroquinolones in vivo: the case of *Legionella pneumophila* and humans. EBioMedicine 2 (9), 1179–1185.

Sikora, A., Gładysz, I., Kozioł-Montewka, M., Wójtowicz-Bobin, M., Stańczak, T., Matuszewska, R., Krogulska, B., 2017. Assessment of antibiotic susceptibility of *Legionella pneumophila* isolated from water systems in Poland. Ann. Agric. Environ. Med. 24 (1), 66–69.

Soda, E.A., Barskey, A.E., Shah, P.P., Schrag, S., Whitney, C.G., Arduino, M.J., Reddy, S.C., Kunz, J.M., Hunter, C.M., Raphael, B.H., Cooley, L.A., 2017. Vital signs: health care-associated Legionnaires' disease surveillance data from 20 states and a large metropolitan area-United States, 2015. Am. J. Transplant. 17 (8), 2215–2220.

Spagnolo, A.M., Cristina, M.L., Casini, B., Perdelli, F., 2013. *Legionella pneumophila* in healthcare facilities. Rev. Med. Microbiol. 24, 70–80.

Stout, J.E., Sens, K., Mietzner, S., Obman, A., Yu, V.L., 2005. Comparative activity of quinolones, macrolides and ketolides against Legionella species using in vitro broth dilution and intracellular susceptibility testing. Int. J. Antimicrob. Agents 25 (4), 302–307.

Torre, I., Diana, M.V., Iervolino, C., Borriello, T., Imperato, O.C., Maccarino, S., Pennino, F., 2014. Legionella contamination in hospitals of the Campania region: five years of environmental surveillance results. Ann. Ig. 26 (1), 89–96.

Xiong, L., Yan, H., Shi, L., Mo, Z., 2016. Antibiotic susceptibility of *Legionella* strains isolated from public water sources in Macau and Guangzhou. J. Water Health 14 (6), 1041–1046.

World Health Organization, 2007. *Legionella* and the Prevention of Legionellosis. World Health Organization, Geneva, Switzerland.