



Antibiotic susceptibility of *Legionella pneumophila* strains isolated from hospital water systems in Southern Italy



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ABSTRACT

Objectives: The purpose of this study was to describe the susceptibility of environmental strains of *Legionella* spp. to 10 antimicrobials commonly used for legionellosis therapy. A study of environmental strains could be useful to timely predict the onset of antibiotic resistance in the environment before it is evidenced in clinical specimens.

Methods: The minimum inhibitory concentrations (MICs) of 100 environmental *Legionella pneumophila* (*Lpn*) strains belonging to serogroups (sgs) 1, 6, 8, and 10 were tested using the E-test methodology on buffered charcoal yeast extract agar supplemented with α -ketoglutarate. The most frequent sgs were selected from those obtained during microbiological surveillance conducted in 2014 in a hospital in Southern Italy. The MICs were read after 2 days of incubation at 35 °C in a humidified atmosphere without CO₂.

Results: All isolates were inhibited by low concentrations of fluoroquinolones and macrolides. Rifampicin was the most active drug against the isolates in vitro. All *Lpn* isolates were inhibited by the following drugs (in decreasing order of their MICs): doxycycline > tigecycline > cefotaxime. The MICs of azithromycin, ciprofloxacin, levofloxacin, moxifloxacin, and tigecycline were significantly lower for *Lpn* non-sg 1 than *Lpn* sg 1 isolates.

Conclusions: Susceptibility testing of *Legionella* strains to appropriate antibiotics should be performed often to evaluate the possible emergence of resistance, to improve the outcomes of patients, and to reduce the direct costs associated with hospitalization.

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1. Introduction

Legionella pneumophila (*Lpn*) is a ubiquitous, intracellular microorganism that causes nosocomial and community pneumonia. *Lpn* infections are acquired by the inhalation of aerosols produced from natural and artificial aquatic environments, and they have serious consequences, especially in immunocompromised patients (Neil and Berkelman 2008; Napoli et al., 2009; Lin et al., 2011). Although a total of 59 different species and more than 70 serogroups (sgs) have been identified (Euzéby, 2015), *Lpn* sg 1 is

Abbreviations: ATCC, American Type Culture Collection; AZI, azithromycin; BCYE- α , buffered charcoal yeast extract agar supplemented with α -ketoglutarate; BYE- α , buffered yeast extract agar supplemented with α -ketoglutarate; CEF, cefotaxime; CIP, ciprofloxacin; CLA, clarithromycin; DOX, doxycycline; ERY, erythromycin; LEV, levofloxacin; *Lpn*, *Legionella pneumophila*; MH, Mueller-Hinton; MIC, minimum inhibitory concentration; MIC₅₀, MIC causing inhibition of 50% of isolates; MIC₉₀, MIC causing inhibition of 90% of isolates; MOX, moxifloxacin; RIF, rifampicin; sg, serogroup; TIG, tigecycline

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considered to be responsible for up to 80–90% of human infections, followed by *Lpn* sgs 4 and 6 (Fontana et al., 2014).

Environmental and anthropogenic factors play a key role in the spread of *Legionella* spp., as ecological factors, geographical area, and the efficacy of disinfection methods influence their multiplication and survival. In addition, it has been shown that the presence of antibiotics in the environment may promote the evolution of microbial resistance mechanisms (D'Costa et al., 2006). This is particularly important for *Legionella* spp. that colonize environmental water systems, where they may be exposed to antibiotics from medical or veterinary practices, or even to those secreted by other microorganisms (Almahmoud et al., 2009).

Although several studies have examined the environmental reservoirs of *Legionella* spp. (Montagna et al., 2006; Napoli et al., 2010; Pasquarella et al., 2012; Montagna et al., 2014), their antimicrobial susceptibility has received less attention (Gómez-Lus et al., 2001; Erdogan et al., 2010; Al-Matawah et al., 2012; Sandalakis et al., 2014). The study of antibiotic susceptibility in clinical strains is complicated by different factors: Legionnaires' disease is usually a non-productive pneumonia, and it is difficult to obtain

respiratory secretions for culturing before the patient undergoes antibiotic therapy. In addition, the *in vitro* activity of commonly employed antibiotics is difficult to interpret because the minimum inhibitory concentration (MIC) distributions of wild-type strains, as well as epidemiological cut-off values, have not been described (Bruin et al., 2012).

Because of the ability of *Legionella* spp. to survive and multiply in human macrophages, they are susceptible to antimicrobial agents that are active intracellularly (Bruin et al., 2012). Macrolides, fluoroquinolones, and rifampicin are the antimicrobials most commonly used in the treatment of legionellosis (Sabrià et al., 2005), although some studies have reported treatment failure in patients receiving therapy with these antibiotics (Blázquez Garrido et al., 2005; Erdogan et al., 2010).

The aim of this study was to describe the antibiotic susceptibility of environmental strains of *Legionella* spp. that were selected from those obtained during microbiological surveillance conducted in 2014 in a hospital in Southern Italy. A study of environmental strains could be useful, not only for epidemiological purposes (such as verifying the distribution of susceptibility/resistance of strains in a geographical area), but also for timely prediction of the onset of antibiotic resistance in the environment before it is evidenced in clinical specimens.

2. Material and methods

2.1. Setting

The study was conducted in a university hospital in the Apulia region (Southern Italy), which is composed up of 32 buildings, including 22 ward buildings, with a total bed capacity of about 1400. Since 2000, the hospital water system has been under environmental surveillance for the detection of *Legionella* spp. For the last 7 years, according to the procedures described in the Italian Guidelines (LG, 2000), a standardized protocol of surveillance has been adopted in all 22 ward buildings. At least six sampling points for each ward building were monitored twice per year.

2.2. *Legionella* strains selection

During the last 7 years of environmental surveillance, the most commonly isolated *Legionella* strains were *Lpn* sg 1 (29.41%), *Lpn* sg 10 (29.23%), *Lpn* sg 6 (20.54%), *Lpn* sg 8 (3.18%), *Lpn* sg 3 (0.75%), *Lpn* sg 7 (0.47%), *Lpn* sg 12 (0.37%), *L. micdadei* (0.28%), *Lpn* sg 14 (0.09%), *L. gormanii* (0.09%), *L. bozemanii* (0.09%), and mixed cultures (15.5%). All the isolates were first serologically identified by the latex agglutination test using a polyvalent commercial kit (Oxoid S.p.A., Milan, Italy), then by a panel of monovalent antisera (Biogenetics S.R.L., Denka Seiken, Ponte San Nicolò, Italy). No nosocomial cases were reported in the same period from the hospital and, therefore, no clinical strains were isolated.

During the last year of surveillance (January–December 2014), *Lpn* sgs 1 (31.27%), 10 (29.09%), 6 (24.36%), and 8 (8.73%) were the most frequently isolated strains according to reports of human legionellosis cases (Fendukly et al., 2007; Kawanami et al., 2011; Zhang et al., 2014). For this reason, a sample of 25 *Lpn* strains from each of the four most frequently isolated sgs was selected, which yielded a total of 100 *Lpn* strains.

Lpn sg 1 American Type Culture Collection (ATCC) 33152 was used as the reference strain. To determine the influence of charcoal (present in buffered charcoal yeast extract agar supplemented with α -ketoglutarate, BCYE- α) on the activity of the antimicrobials, *Staphylococcus aureus* ATCC 29213 was also selected for susceptibility testing. The selected strains were frozen at -80°C prior to analysis.

2.3. Susceptibility testing

Susceptibility testing was performed using E-tests on BCYE- α (Liofilchem S.R.L., Teramo, Italy). Ten antimicrobial drugs were tested: azithromycin (AZI), cefotaxime (CEF), clarithromycin (CLA), doxycycline (DOX), erythromycin (ERY), rifampicin (RIF), and tigecycline (TIG) (ranging from 0.016 to 256 mg/L each), as well as ciprofloxacin (CIP), levofloxacin (LEV), and moxifloxacin (MOX) (ranging from 0.002 to 32 mg/L each). The MIC of each antibiotic was taken as the lowest concentration of antibiotic at which the zone of inhibition intersected the E-test strip.

Legionella strains were sub-cultured 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 (AB-BIODISK, BioMérieux, Marcy l'Etoile, France) were applied to each inoculated plate. The plates were incubated at 35°C (without CO_2) for 48 h before reading the MIC value; if no growth was detected, the plates were incubated for an additional 24 h.

For *S. aureus* ATCC 29213, the E-test was performed on Mueller-Hinton agar (MH, Biolife, Milan, Italy) and on BCYE- α , and the MIC was read after 24 h of incubation at 35°C .

2.4. Analysis of results

MIC data are presented as the range, geometric mean, MIC₅₀ (the MIC causing inhibition of 50% of isolates), and MIC₉₀ (the MIC causing inhibition of 90% of isolates) for each sg. The Mann-Whitney test was applied to evaluate the likely significance of differences in antimicrobial susceptibility between *Lpn* sg 1 and non-sg 1 isolates. Moreover, the differences in antimicrobial susceptibility patterns between *Lpn* non-sg 1 isolates were evaluated using the Kruskal-Wallis test, followed by a Dunn's test for multiple comparisons. Statistical analyses were conducted with GraphPad Prism version 5.0 for Windows (San Diego, CA, USA), and statistical significance was defined as $p < 0.05$.

3. Results

The cumulative percentages of 100 environmental *Lpn* isolates inhibited by different concentrations of the 10 antimicrobials tested, as well as the MIC₅₀ and MIC₉₀ values, are shown in Table 1. Overall, the MIC₅₀ and MIC₉₀ values for the macrolides (AZI, CLA, and ERY) ranged from 0.016 to 32. In particular, they were 0.047 and 0.25 mg/L, respectively, (range 0.016–4) for AZI, 0.032 and 0.125 mg/L, respectively, (range 0.016–32) for CLA, and 0.094 and 0.125 mg/L, respectively, (range 0.032–16) for ERY.

The MIC₅₀ and MIC₉₀ values for the fluoroquinolones (CIP, LEV, and MOX) ranged from 0.023 to 3. Specifically, they were 0.19 mg/L (range 0.047–2) for CIP (both values), 0.047 and 0.094 mg/L, respectively, (range 0.023–1) for LEV, and 0.19 and 0.25 mg/L, respectively, (range 0.125–3) for MOX. The MIC₅₀ and MIC₉₀ values for CEF were 0.38 and 1 mg/L, respectively, (range 0.016–1), and 1 and 1.5 mg/L, respectively, for TIG (range 0.19–4). DOX was the least active drug, with an MIC₅₀ = 1.5 mg/L and an MIC₉₀ = 2 mg/L, with MICs ranging from 0.032 to 8 mg/L. RIF was the most active drug, with an MIC₉₀ of 0.016 mg/L (range 0.016–4).

The activity of ten antimicrobial agents tested against *Lpn* sg 1, 6, 8 and 10 is shown in Table 2. When comparing the MIC values of *Lpn* sg 1 versus non-sg 1 isolates, no differences were found for five antibiotics (CEF, CLA, DOX, ERY, and RIF). In contrast, the MIC values of AZI, CIP, LEV, MOX, and TIG were significantly lower for

Table 1
Cumulative distribution of the MICs of 100 environmental *Legionella pneumophila* isolates.

Drug	Cumulative % of strains inhibited at indicated concentrations (mg/L)																				
	0.016	0.023	0.032	0.047	0.064	0.094	0.125	0.19	0.25	0.38	0.5	0.75	1	1.5	2	3	4	8	16	24	32
Azithromycin	8	19	39	71	82		83	89	91	94	97					99	100				
Cefotaxime	2				3	8	20	25	38	54	69	87	100								
Ciprofloxacin				2	3	6	38	90	95	97			98		100						
Clarithromycin	1	5	58	75	78	88	94	97			98									99	100
Doxycycline			1					2		3	6	9	26	58	95	97	98	100			
Erythromycin			2	10	34	71	91	97		98									100		
Levofloxacin		1	24	59	87	97					98		100								
Moxifloxacin							8	65	95	97		98				100					
Rifampicin	97		98											99			100				
Tigecycline							1			6	15	34	72	92	93	94	100				

MIC₅₀ and MIC₉₀ values can be read directly from this table.

Lpn non-sg 1 than sg 1 isolates.

An analysis of the MICs of each antibiotic against serogroups, other than *Lpn* sg 1, revealed some differences. For example, the MIC value of CEF for *Lpn* sg 10 was the lowest; for TIG, there was no significant difference between the MIC values of the *Lpn* sg 6 and sg 8 isolates, while *Lpn* sg 10 isolates were the most susceptible.

Regarding the control strains (Table 3), *Lpn* sg 1 ATCC 33152 (one repetition) generally showed lower sensitivities, with the exception of CIP (MIC=0.38 mg/L) and LEV (MIC=0.094 mg/L), which were similar to the sensitivities of the environmental isolates. Comparing the results obtained from *S. aureus* ATCC 29213 (one repetition) on two different media, BCYE- α and MH, AZI exhibited the highest MIC (2 mg/L), and the MICs of AZI, TIG, and MOX were the most influenced by charcoal.

Table 3
MICs (mg/L) of the two reference strains.

Drug	<i>L. pneumophila</i> sg ATCC 12821	<i>S. aureus</i> ATCC 29213	
	BCYE- α	BCYE- α	MH
Azithromycin	0.032	2	0.75
Cefotaxime	0.75	0.75	0.75
Ciprofloxacin	0.38	0.75	0.38
Clarithromycin	0.032	0.38	0.19
Doxycycline	1.5	0.125	0.125
Erythromycin	0.094	0.25	0.25
Levofloxacin	0.094	0.38	0.19
Moxifloxacin	0.19	0.25	0.012
Rifampicin	0.006	0.012	0.004
Tigecycline	1.5	0.38	0.032

MH, Mueller-Hinton agar; BCYE- α , buffered charcoal yeast agar.

4. Discussion

Environmental surveillance of *Lpn* contamination, apart from having a great role in risk assessment, can be of great help in identifying changes in antibiotic susceptibility. With regard to this issue, to date, there have been few observational reports, especially regarding clinical isolates (Blázquez Garrido et al., 2005;

Sabrià et al., 2005; Gómez-Lus et al., 2001). To our knowledge, ours is the first survey of the antimicrobial susceptibility of *Legionella* spp. isolated from hospital water systems in Italy.

Our study confirms that our *Lpn* isolates are inhibited by low concentrations of macrolides and fluoroquinolones (Al-Matawah et al., 2012). Among the macrolides, CLA is the most active drug for *Lpn* sg 1, and its activities toward *Lpn* sg 1 and non-sg 1 isolates

Table 2
MIC values (mg/L) for *L. pneumophila* sg 1, 6, 8, and 10.

Drug	<i>L. pneumophila</i> sg 1			<i>L. pneumophila</i> sg 6			<i>L. pneumophila</i> sg 8			<i>L. pneumophila</i> sg 10		
	MIC ₅₀	MIC ₉₀	GM	MIC ₅₀	MIC ₉₀	GM	MIC ₅₀	MIC ₉₀	GM	MIC ₅₀	MIC ₉₀	GM
Azithromycin	0.19	0.5	0.101	0.047	0.064	0.052	0.032	0.064	0.035	0.032	0.19	0.054
Cefotaxime	0.5	1	0.369	0.75	1	0.643	0.38	0.75	0.32	0.19	0.38	0.173
Ciprofloxacin	0.19	0.38	0.187	0.125	0.19	0.151	0.19	0.19	0.167	0.19	0.25	0.184
Clarithromycin	0.032	0.125	0.046	0.047	0.125	0.060	0.032	0.064	0.037	0.047	0.125	0.067
Doxycycline	1.5	3	1.542	1.5	2	1.267	2	2	1.706	1.5	2	1.352
Erythromycin	0.094	0.019	0.098	0.094	0.125	0.102	0.094	0.125	0.085	0.094	0.125	0.101
Levofloxacin	0.064	0.094	0.063	0.047	0.064	0.051	0.047	0.064	0.047	0.047	0.094	0.058
Moxifloxacin	0.25	0.25	0.227	0.19	0.25	0.214	0.19	0.25	0.202	0.19	0.38	0.225
Rifampicin	0.016	0.016	0.016	0.016	0.016	0.019	0.016	0.016	0.016	0.016	0.016	0.020
Tigecycline	1.5	4	1.527	1	1.5	0.930	1	1.5	1.007	0.75	1.5	0.699

GM=geometric mean

were not significantly different (Pedro-Botet and Yu, 2006; Bruin et al., 2012).

The MIC₉₀ value for AZI is consistent with the results of other studies that investigated the susceptibility of clinical isolates (Dunbar and Farrell, 2007). Overall, the MIC₅₀ and MIC₉₀ values of AZI were significantly lower for *Lpn* non-sg 1 than sg 1 isolates; this highlights that significant differences in antibiotic susceptibility can be reported among different sgs of the same specie with possible implication in the choice of the best therapy.

In contrast, Gómez-Lus et al. (2001) reported that the MIC values of macrolides are comparable for *Lpn* sg 1, 6, 8, and 10 isolates. These results differ from those of our study probably because of the different geographical origin, different methodology used, and the different origins of the strains (nosocomial or community vs. clinical or environmental).

In our study, the activities of the fluoroquinolones toward *Lpn* were similar to those of the macrolides based on MIC₉₀ values (0.25 mg/L). In contrast, other studies reported that quinolones have greater activities toward *Lpn* compared with macrolides, with a decreased time to defervescence, reduced length of stay, and reduced time to clinical resolution; however, these differences probably result from differences in the susceptibility testing methods used (Blázquez Garrido et al., 2005; Pedro-Botet and Yu, 2006; Sabrià et al., 2005; Dunbar and Farrell, 2007). In fact, various methods have been used to determine MIC values: the *E*-test, broth and agar dilution, disk diffusion methods, in vivo animal studies, and in vitro cell culture models. None of these methods is considered to be a gold standard (Bruin et al., 2012), and some studies showed that using a different methodology resulted in some variability in the range of MIC values (García et al., 2000). Overall our results are comparable with studies that used the same *E*-test method on BCYE- α agar (García et al., 2000; Bruin et al., 2012).

Thus far, in the treatment of lower respiratory tract infections, fluoroquinolones have become the most widely used agents because of their broad-spectrum coverage, their ease of administration, and their comparatively fewer adverse effects (Erdogan et al., 2010). As reported by others (Dunbar and Farrell 2007; Stout et al., 2005), in our study, LEV was the most active quinolone against *Lpn* sg 1 and non-sg 1 isolates despite the use of different susceptibility tests and differences in the origins of the strains (clinical vs. environmental).

In accordance with other studies (Marques and Piedade, 1997; Dubois and St-Pierre, 1999; Erdogan et al., 2010; Bruin et al., 2012; Al-Matawah et al., 2012; Sandalakis et al., 2014), in our study, RIF was the most active drug in vitro against both *Lpn* sg 1 and non-sg 1 isolates. With regard to this antimicrobial, it should be noted that resistance develops rapidly upon RIF exposure, precluding its use as a monotherapy for *Lpn* (Nielsen et al., 2000), although Varner et al. (2011) reported that RIF combination therapy should be considered for patients with severe disease or significant comorbidity conditions (e.g., uncontrolled diabetes, smoking, or obstructive lung disease), including those who were refractory to conventional monotherapy regimens.

According to other studies (Marques and Piedade, 1997; Al-Matawah et al., 2012; Bruin et al., 2012; Sandalakis et al., 2014), all *Lpn* isolates were inhibited by (in decreasing order of their MICs) DOX > TIG > CEF, although they all exhibited higher MICs than the other antimicrobial drugs tested.

Although only a few studies (Gómez-Lus et al., 2001; Al-Matawah et al., 2012) have compared the sensitivities of *Lpn* non-sg 1 isolates to each antibiotic, our study yielded the same results. Differences in susceptibility between sg 1 and non-sg 1 isolates were detected only for CEF and TIG, whereas DOX inhibited all sgs equally.

In our hospital, CLA and LEV are the most used macrolides and fluoroquinolones, respectively, in treatment of pneumonia. RIF is not used because of its adverse effects, especially when in

combination with other drugs (Varner et al., 2011).

Regarding to differences observed in susceptibility of *S. aureus* ATCC 29213 to TIG, MOX and AZI according to the different media used, several studies have indicated that charcoal or other components of BCYE- α agar can inhibit various antibiotics, including tetracyclines, fluoroquinolones, and macrolides (Marques and Piedade, 1997; García et al., 2000; Gómez-Lus et al., 2001; Erdogan et al., 2010; Bruin et al., 2012; Sandalakis et al., 2014). In particular in our study, *S. aureus* resulted resistant to AZI in BCYE- α agar and susceptible in MH according to EUCAST clinical breakpoint table (EUCAST, 2015). As reported by some authors (Marques and Piedade, 1997; Gómez-Lus et al., 2001; Erdogan et al., 2010), the use of BYE- α (without charcoal) instead of BCYE- α may be a good option to choose for the in vitro testing of susceptibility of *Legionella* spp. to antimicrobials.

Although performing the *E*-test on BCYE- α agar may yield elevated MICs, we used the *E*-test on BCYE- α because it represents a simple, readily available, and accurate method for routine susceptibility testing of *Legionella* spp. (Bruin et al., 2012). The *E*-test has proven itself over the years, and it can be easily used in many laboratories, precluding the need to send the strains to reference laboratories for susceptibility testing. Further studies should be specifically addressed to better investigate the antibiotic resistance of *Legionella* using BCYE- α versus BYE- α .

Some limitations of this study are that the high costs of these susceptibility tests make it prohibitively expensive to examine the antibiotic susceptibility of all strains isolated during routine environmental surveillance; however, a periodical assessment (e.g., every 3–5 years) should be conducted, especially in hospital environments where resistance is likely (Jonas et al., 2003). Moreover, caution should be taken when interpreting the results. It should be considered that in vitro testing of antimicrobial agents sometimes poorly correlates with clinical efficacy, as the intracellular location of the microorganisms may protect them from an otherwise effective antimicrobial therapy (Marques and Piedade, 1997).

Finally, we did not investigate all sgs found in our hospital, some of which are reported as being pathogenic for humans (e.g., *Lpn* sgs 3 and 7).

5. Conclusions

Susceptibility testing of strains to appropriate antibiotics should be performed often to evaluate the possible emergence of resistance, to improve the outcomes of patients, and to reduce the direct costs associated with hospitalization. In some cases, unfortunately, the difficulties in isolating clinical strains preclude these kinds of studies. Therefore, for pathogens with environmental reservoirs, such as *Legionella* spp., an antibiotic susceptibility study of environmental strains, which are more easily detected, can be used to provide an early warning of the onset of antibiotic resistance, although results should be interpreted with due caution.

Conflict of interest

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

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