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Original Article MEDICAL MICROBIOLOGY

Assessment of macrolide susceptibility of *Legionella pneumophila* isolated from public buildings' water systems in Turkiye

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ABSTRACT

OBJECTIVE: *Legionella pneumophila* (*Lp*) is aerobic, non-spore forming Gram-negative bacteria, which is ubiquitous in freshwater habitats, such as rivers and hot springs, as well as colonizing artificial aquatic environments. The ability of *Lp* to grow intracellularly within pulmonary macrophages is a prerequisite for the development of infection. Therefore, macrolides can achieve appropriate therapeutic concentrations in eukaryotic cells, such as azithromycin. This study aimed to investigate the macrolides susceptibility of *Lp*.

METHODS: Pre-flash water samples (n=143) were collected from the public buildings (hospitals and hotels) water system in Istanbul. Colonies were confirmed as *Lp* ST1, ST2-14, and non-pneumophila *Lp* using latex agglutination kit.

RESULTS: 30 *Lp* were detected in hospital (n=23) and hotel (n=7) water systems using latex agglutination. Regardless of serotype and excluding strains without zone formation (≥256 mg/L), the main MIC values of azithromycin, erythromycin and clarithromycin were 0.61 mg/L (range 0.047–1 mg/L), 0.47 mg/L (range 0.047–1 mg/L) and 0.44 mg/L (range 0.047–1 mg/L), respectively. The MIC_{50} and MIC_{90} values for macrolides were 0.5 and 3 mg/L for azithromycin, respectively; 0.38 and 1 mg/L for erythromycin, respectively; and 0.5 and 1 mg/L for clarithromycin, respectively. We compared the MIC values of the strains for all antimicrobial agents tested without serotype discrimination. We did not find a significant difference between the MIC values of the antibiotics (p=0.16).

CONCLUSION: Although the data obtained from our study do not fully reflect the breakpoints, further epidemiological studies are needed to standardize MIC values.

Keywords: E test; Legionnaires' disease; Legionella pneumophila; macrolides; susceptibility.

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Legionella pneumophila (Lp) is a Gram-negative, aerobic bacteria, which are colonizing in water habitats, such as pools and man-made aquatic environments [1, 2]. Thanks to their survivability in biofilm, *Lp* can colonize man-made water systems, from households, hospitals, and hotels to tap water [1]. Currently, the *Legionella* genus comprises 59 species (spp.) and more than 70 serotypes (ST) [3]. Among *Legionella* spp., *Lp* ST1 bacteria are responsible for the majority (80%) of all reported cases of legionellosis [4]. *Lp* most commonly causes Legionnaires' disease, which can result in lung damage or death [5–7]. The ability of *Lp* to grow intracellularly within pulmonary macrophages is important for becoming infection. Therefore, macrolides that can reach suitable therapeutic levels in eukaryotic cells are used in treat-

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ment [8]. Due to the difficulty of isolating bacteria from the patient sample and the lack of antibiotic susceptibility screening methods, susceptibility testing is not performed [9]. The purpose of this study was to study the macrolide sensitivity of *Lp* isolated from hospitals and hotels in Istanbul.

MATERIALS AND METHODS

Sample collection and bacterial strains

Pre-flash water samples (n=143) were collected from the public buildings' (hospitals and hotels) water systems (16 cooling towers, 27 water tanks, 69 tap water, 21 shower heads) in January-July 2019 in Istanbul. All samples were taken into sterile thiosulfate-free bottles. 1 L of water sample was filtered (Sartorius, Germany), and the filters were placed on GVPC (Glycine Vancomycin Polymyxin Cyclohexmide Agar) agar (Liofilchem, Italy) containing L-cysteine and Fe3+ and incubated at 36±1°C for seven days. The colonies thought to be *Lp* were passaged on BCYE (Buffered Charcoal Yeast Extract) agar (Liofilchem, Italy) and Columbia Blood Agar (Liofilchem, Italy). After another 48-hour incubation period at 36±1°C, colonies that grew on BCYE agar were included in our study. Gray to white, frosted-glass colonies with a diameter of 3–4 mm were confirmed as *Lp* ST1, ST2-14, and non-pneumophila *Lp* using a latex agglutination kit (Microgen, UK).

Minimum inhibitory concentrations determination and Statistical analysis

We used the E-test (gradient strip) to determine minimum inhibitory concentrations (MICs). (bioMérieux, France) using BCYE agar. Colonies were suspended in sterile water at 0.5 McFarland. Gradient strips were plated on BCYE agar and incubated for 48 hours at 35±1°C, 2.5% CO_2, and 70% humidity. The data sets were compared with the epidemiological cut-off values (ECOFF) of the studies referred to in the technical document published in 2021 [10]. For control, we also selected *Legionella pneumophila* (ATCC 33153) and *Staphylococcus aureus* (ATCC 25923). Statistical calculations were performed using SPSS Statistics 26 (IBM, USA). Antimicrobial susceptibility differences among *Lp* strains were evaluated by the Kruskal-Wallis test ($p<0.05$).

Highlight key points

- Antibiotic susceptibility tests are not routinely performed in the clinic for *Legionella pneumophila*.
- There is no MIC value that can be described as resistant worldwide.
- *Legionella pneumophila* colonizing the systems of communal living spaces poses a serious threat to human health.

TABLE 1. Distributions of isolated strains

	Lp ST1 (n)	*Lp* ST2-14 (n)	*Legionella* spp. (n)
Hospitals (n=23)			
Cooling tower	3	0	1
Tap water	6	3	3
Water tank	8	3	4
Hotels (n=7)			
Shower head	0	1	1
Tap water	2	3	2
Water tank	1	0	2

RESULTS

43 *Legionella* spp. were isolated in 143 water specimens (30.06% of all water samples). 30 *Lp* were detected in hospital (n=23) and hotel (n=7) water systems using latex agglutination. Taps were the primary isolation area of *Legionella* spp. (n=19), while shower heads were the least *Legionella*-isolated area (n=2). In addition, the area with the highest *Legionella* spp. isolation rate according to the number of samples is the water tanks (18/27, 66.7%) (Table 1).

Among these isolates, 15, 15, and 13 *Lp* ST1, ST2-14, and non-pneumophila *Legionella* were identified, respectively. Three of the fifteen *Lp* ST1 strains showed reduced susceptibility to macrolides, and for ST2-14 strains, three of fifteen showed reduced susceptibility to macrolides. (No zone of inhibition around the gradient strip.) The cumulative percentages for the 30 *Lp* isolates inhibited at different concentrations are shown in Table 2.

Regardless of serotype and excluding strains without zone formation (≥256 mg/L), the main MIC values of azithromycin, erythromycin and clarithromycin were 0.61 mg/L (range 0.047–1 mg/L), 0.47 mg/L (range

Table 2. Cumulative percentage (%) of strains inhibited at indicated antimicrobials concentrations (mg/L)

Drugs	0.047	0.064	0.094	0.19	0.25	0.38	0.5	0.75	1	2	3	≥256
Azithromycin	3.33	3.33	9.99	0	16.65	13.32	13.32	9.99	13.32	3.33	3.33	9.99
Clarithromycin	6.67	0	0	13.32	6.67	16.65	23.31	19.98	3.33	0	0	9.99
Erythromycin	3.33	3.33	0	13.32	16.65	13.32	16.65	16.65	6.67	0	0	9.99

0.047–1 mg/L) and 0.44 mg/L (range 0.047–1 mg/L), respectively. Regardless of serotype and excluding strains without zone formation (≥256 mg/L), the MIC_{50} and MIC_{90} values for macrolides were 0.5 and 3 mg/L for azithromycin, respectively; 0.5 and 1 mg/L for erythromycin, respectively; and 0.38 and 1 mg/L for clarithromycin, respectively. We compared the MIC values of the strains for all antimicrobial agents tested without serotype discrimination. We did not find a significant difference between the inhibition values of the antibiotics (p=0.16). Reduced susceptibility to azithromycin 6/30 (20%), erythromycin 10/30 (33.3%), and clarithromycin 10/30 (33.3%) were observed in the present study in and of tested strains all belonging to the ST1 and ST2-14, respectively.

DISCUSSION

In industrialized countries, *Lp* causes atypical pneumonia that originates in communal living spaces such as hospitals, hotels, and dormitories [11]. If not treated for community-acquired diseases, the mortality rate can reach 16–30%, and the mortality rate can reach 50% with the wrong drug treatment [12, 13]. Treatment failure has been demonstrated in patients with pneumonia caused by Lp, which may be due to resistance in clinical *Lp* isolates [14]. Macrolide resistance may be related to many factors. Although in the literature, macrolide resistance was associated with mutations in the L4 (*rplD*) and L22 (*rplV*) ribosomal proteins, 23S rRNA gene (*rrl*), *phosphotransferase-esterase* encoding genes (*ereA*, *ereB*, and *mphA*), and efflux pumps synthesis [15, 16]; the efflux pumps synthesis and mutations in the genes encoding the L4 and L22 ribosomal proteins are frequently associated with macrolide resistance of *Legionella* spp. [17].

The *lpeAB* genes are similar to the *Escherichia coli acrAB* genes and are arranged in an operon. A tripartite efflux pump of the RND (Resistance-Nodulation-Division) family is made up of the proteins *AcrA* in the periplasm, *AcrB* in the inner membrane, and *TolC* in the outer membrane [18]. All ST1 bacteria had the efflux pump subunit *lpeAB*, which had decreased azithromycin susceptibility [19]. In addition, it has been shown that the presence of *lpeAB* genes is associated with decreased azithromycin sensitivity of ST1 [20].

In a previous study, mutations in the *rplD* and *rplV* genes, which encode the L4 and L22 ribosomal proteins, respectively, were shown to cause a conformational change in the ribosome that prevents macrolides from binding to the peptide exit tunnel [21]. Similar resistance levels suggested that the 2- to 32-fold increase in erythromycin and azithromycin MICs in *Haemophilus influenzae* isolated from clinical samples was caused by L4/L22 substitutions [22].

Although not documented with molecular data, we found a reduction in macrolide susceptibility in 6 *Lp* strains in our study. In addition, *Lp* thrives only in cysteine-containing environments such as BCYE. This medium contains the charcoal necessary to neutralize the toxic metabolites produced during bacterial growth [23]. In our study, we used BCYE-α for MIC value determination. The efficacy of drugs used in the treatment of *Lp* is determined using micro-broth dilution, agar dilution, gradient test, and cell culture [12, 24]. However, none of these methods is considered a gold standard for *Lp* susceptibility testing [14].

The absence of clear guidelines for interpreting *Lp* susceptibility precludes identification as susceptible or resistant to each of the three drugs tested, thus increasing the need to determine the ECOFF value. Working on this subject, Massip et al. [25] recently reported an erythromycin MIC of 0.25 mg/L for *Lp* Paris. Also, Xiong et al. [26] reported lower MIC_{50} values for azithromycin and erythromycin which were 0.062 mg/L and 0.0125 mg/L, respectively. Our findings (MIC_{50} values for azithromycin and erythromycin were 0.5 mg/L and 0.38 mg/L, respectively) do not match those

of previous works' results. The MIC_{90} values for both azithromycin and erythromycin are higher than the reported isolates (for azithromycin MIC_{90}: 3 mg/L and for erythromycin MIC_{90}: 1 mg/L) [27, 28]. Differences in these results may be due to different strains of origin and MIC determination methods. Although our data differ from the studies performed, they are equivalent to ECOFF values [10].

Azithromycin showed lower MIC_{90} values against ST1 isolates compared to ST2-14. Of all *Lp* isolates tested, six *Lp* strains showed MIC values of 256 mg/L for macrolides, which is higher than the MIC levels reported for macrolides in the literature [3, 27, 28]. Also, in our study, contrary to the previous report, clarithromycin was not the most effective drug among macrolides [13]. The Azithromycin MIC_{50}=0.5 mg/L and MIC_{90}=3 mg/L values reported in our study differed from those reported by Vandewalle-Capo et al. [20] for ST1. Also, for ST2-14, Portal et al. [29] detected higher MIC_{90} values.

Conclusion

In this study, we showed that the macrolide susceptibility of six strains decreased compared to other isolates. Macrolide resistance can develop in three ways: ribosome modification, efflux pump activity, and drug inactivation by the enzyme [30]. Further studies at the molecular level are needed to determine the mechanisms leading to resistance. We have performed *in vitro* sensitivities of *Lp* to macrolides. There is no standard method for determining the MIC of antibiotics for *Lp*. Therefore, treatment is started directly without antimicrobial susceptibility testing [11]. In this study, several drug groups could not be tested due to the high cost of susceptibility testing. Our study data can be used as epidemiological study results and can also be used to raise awareness about bacteria colonizing water systems.

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