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ORIGINAL ARTICLE

Antibiotic sensitivity of environmental Legionella pneumophila strains isolated in Poland

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Abstract

Introduction and Objective. Legionella bacteria are commonly found in natural aquatic environments such as rivers, lakes, ponds and hot springs. Legionella infection occurs through the inhalation of water-air aerosol generated, for example, by showers or hot tubs. The most common species responsible for infection is Legionella pneumophila, which can cause Pontiac fever, and Legionnaires' disease, as well as a rare extrapulmonary form. The aim of the study's is to assess the susceptibility of Legionella pneumophila bacteria isolated from water systems of public buildings in Poland to antibiotics and chemotherapeutic agents used in the treatment of Legionellosis pneumonia.

Materials and method. A total of 100 L. pneumophila strains isolated from public buildings, such as hospitals and water recreation facilities, were used for the study. The drug sensitivity of the following antibiotics was determined: erythromycin, azithromycin, ciprofloxacin, levofloxacin, rifampicin, trimethoprim-sulfamethoxazole and tetracycline. Mean MIC50 and MIC90 values were read using accepted standards.

Results. The highest mean MIC value was obtained for tetracycline 6,130+/-0,353 µg/ml (with a range from 1,500 µg/ml to 16,000 µg/ml. In contrast, the lowest MIC was recorded with rifampicin: 0.020+/-0.037 µg/ml (with a range from 0.016 µg/ml to 0.380 µg/ml).

Conclusions. The lowest biocidal concentration was found for levofloxacin, the highest for tetracycline. The highest MIC50 and MIC90 values were found for tetracycline and the lowest for rifampicin. The highest biocidal values were found for azithromycin and the lowest for tetracycline.

Key words

E-test, minimum inhibitory concentration, water systems, Legionella pneumphila, antimicrobial sensitivity testing

INTRODUCTION

Legionella bacteria are commonly found in natural water environments such as rivers, lakes, ponds and hot springs [1]. *Legionella* spp. also colonise water systems in public facilities, such as hotels, hospitals or nursing homes, where they pose a serious risk to immunocompromised persons [2]. *Legionella* infection occurs through inhalation of water-air aerosol generated, for example, by showers or whirlpool baths [3, 4]. The most common species responsible for infection is *Legionella pneumophila*, which can cause Pontiac fever (a mild, self-limiting form with flu-like symptoms), and Legionnaires' disease (a severe atypical pneumonia with high mortality in immunosuppressed patients), as well as a rare extrapulmonary form [5, 6]. The determining factor in the development of Legionnaires' disease is the ability of the bacteria to invade and survive inside the lung macrophages. For this reason, antimicrobial drugs that reach therapeutic concentrations in eukaryotic cells, e.g. erythromycin, rifampicin or fluoroquinolones, are important in the treatment of infections and are most effective in the treatment of Legionnaires' disease. The drugs of choice are azithromycin and levofloxacin [5, 7, 8]. Treatment of infections with *Legionella* aetiology is often empirical. This is due to the difficulty in isolating the pathogen from clinical specimens, the lack of routinely performed drug sensitivity testing of *Legionella* to antibiotics/chemotherapeutics, and the lack of EUCAST recommendations for drug sensitivity assessment. There is also no 'epidemiological cut-off' value (ECOFF) for *Legionella* spp., defined as an *in vitro* MIC threshold that would distinguish wild-type (WT) strains from those with acquired resistance mechanisms. To date, several cases of failure of macrolide and/or fluoroquinolone therapy have been reported. Further, resistance to ciprofloxacin has been reported, due to a single mutation in the *gyr*A gene and reduced sensitivity to azithromycin (MIC 0.125–2 mg/l), which is related to the active pumping of the drug out of the cell (the so-called *efflux* mechanism). Thus, bacterial drug resistance is currently a major public health problem worldwide [9, 10].

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OBJECTIVE

The aim of the study was to assess the sensitivity of *Legionella pneumophila* bacteria isolated from water systems of public buildings in Poland to antibiotics and chemotherapeutic agents used in the treatment of legionellosis pneumonia.

MATERIALS AND METHOD

100 strains of *L. pneumophila* were isolated from public buildings such as hospitals and water recreation facilities. The *L. pneumophila* strains were sourced from the collection of the National Institute of Public Health – National Research Institute (NIZP- PIB) in Warsaw, Poland. The strains were isolated between January and December 2021 and stored at -70°C in Microbank® Microorganism Storage Systems until testing was performed. All *L. pneumophila* isolates tested were thawed, then transferred onto a special buffered agar medium with charcoal, yeast extract and L-cysteine (BCYE-α) designed for *Legionella* culture. The media were then incubated at 37°C in a humid atmosphere with 5% CO_2 for 48–72 hours. After incubation, *L. pneumophila* serogroup membership (SG 1, SG 2–14) was confirmed using a latex agglutination test (Legionella Latex Test, Oxoid, Ireland). The reference strain used in the study was *L. pneumophila* (Philadelphia-1) ATCC 33152 (LGC Standards, USA). From the bacteria grown on the media, a suspension was prepared in 0.85% NaCl at a McFarland density of 0.5 (1.5×10^8 CFU/ml). The density of the suspension was measured using a densitometer (Densimat, bioMérieux, France). With a sterile swab, the suspension was inoculated evenly onto BCYE-α medium. E-test strips were then applied with sterile tweezers. The media were incubated at 35 °C in a humid atmosphere in 5% CO_2 for 48 hours. The MIC values were read at the intersection of the ellipse with the E-test strip.

Minimal inhibitory concentration (MIC) determination. The value of the minimum inhibitory concentration was determined using the E-test method. Blotting paper strips with a concentration gradient of erythromycin, azithromycin, ciprofloxacin, levofloxacin, rifampicin, trimethoprim-sulfamethoxazole and tetracycline were used. The MIC values read for the tested isolates were compared with the MIC distributions contained in the Tables of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) 'Guidance Document on Antimicrobial Susceptibility Testing of *Legionella pneumophila*'.

Statistical analysis. The obtained results were statistically analysed with the Statistica programme. Mean values for individual antibiotics were compared, and MIC50 and MIC90 values determined. Differences in biocidal properties between individual antibiotics were determined using Shapiro-Wilk and Kruskal-Wallis tests.

RESULTS

The highest mean MIC values were obtained for tetracycline: 6.130+/-0.353 μg/ml (with a range of 1,500 μg/ml to 16,000 μg/ml), erythromycin: 0.232+/-0.181 μg/ml (with a range from 0.016 μg/ml to 0.750 μg/ml) ciprofloxacin: 0.213+/-0.145 μg/ml (with a range from 0.016 μg/ml to 0.750 μg/ml) and trimethoprim/sulfamethoxazole: 0.203+/-0.134 μg/ml (with a range from 0.016 μg/ml to 0.750 μg/ml). In contrast, the lowest MICs were recorded for rifampicin: 0.020+/-0.037 μg/ml (with a range from 0.016 μg/ml to 0.380 μg/ml), levofloxacin: 0.068+/-0.078 μg/ml (with a range from 0.012 μg/ml to 0.380 μg/ml) and azithromycin: 0.170+/-0.202 μg/ml (with a range from 0.016 μg/ml to 0.750 μg/ml) (Tab. 1).

Table 1. Mean bacteriostatic concentration of the tested antibiotics (μg/ml)

Tested antibiotic	Mean	Minimum	Maximum	Standard deviation (SD)	Standard error (SE)
erythromycin	0.232	0.016	0.750	0.181	0.018
azithromycin	0.170	0.016	0.750	0.202	0.020
ciprofloxacin	0.213	0.016	0.750	0.145	0.015
levofloxacin	0.068	0.012	0.380	0.078	0.008
rifampicin	0.020	0.016	0.380	0.037	0.004
trimethoprim/ sulfamethoxazole	0.203	0.016	0.750	0.134	0.013
tetracycline	6.130	1.500	16.000	3.525	0.353

The minimum inhibitory concentration of 50% (MIC50) is shown in Table 2 and was: 0.016 μg/ml for rifampicin, 0.032 μg/ml for levofloxacin, 0.094 μg/ml for azithromycin, 0.190 μg/ml for erythromycin, ciprofloxacin and trimethoprim/ sulfamethoxazole and 6,000 μg/ml for tetracycline. The minimum growth inhibitory concentration of 90% (MIC90) of L. *pneumophila* was: 0.016 μg/ml for rifampicin, 0.190 μg/ml for levofloxacin, 0.380 μg/ml for azithromycin, ciprofloxacin and trimethoprim/sulfamethoxazole, 0.5000 μg/ml for erythromycin and 12,000 μg/ml for tetracycline (Table 2).

Table 2. Minimum MIC50 and MIC90 concentrations of the tested antibiotics

Antibiotic	*MIC50 (μg/ml)*	*MIC90 (μg/ml)*
erythromycin	0.190	0.500
azithromycin	0.094	0.380
ciprofloxacin	0.190	0.380
levofloxacin	0.032	0.190
rifampicin	0.016	0.016
trimethoprim/sulfamethoxazole	0.190	0.380
tetracycline	6.000	12.000

Comparison of MIC values of antibiotics

The mean MIC value of rifampicin (0.020 μg/ml) was significantly lower than that all the other antibiotics. In turn, the mean MIC value of levofloxacin (0.068 μg/ml) was significantly higher than that of rifampicin (0.020 μg/ml) and significantly lower than that of the other antibiotics. The mean MIC of azithromycin (0.170 μg/ml) was significantly higher than that of rifampicin (0.020 μg/ml) and levofloxacin (0.068 μg/ml) and significantly lower than that of the other antibiotics. There was no significant difference in the mean MIC values of trimethoprim/sulfamethoxazole (0.203 μg/ml), ciprofloxacin (0.213 μg/ml) and erythromycin (0.232 μg/ml).

Table 3. Comparison of biocidal properties of the antibiotics

Antibiotic (mean inhibitory concentration)	ERYTROMYCIN	AZITHROMYCIN	CIPROFLOXACIN	LEVOFLOXACIN	RIFAMPICIN	TRIMETHOPRIM/ SULFAMETHOXAZOLE	TETRACYCLINE
ERYTROMYCIN (0.232 µg/ml)	---	1.130*	0.035	2.22*	3.47*	0.175	2.34*
AZITHROMYCIN (0.170 µg/ml)		---	1.165*	1.09*	2.340*	0.955*	3.470*
CIPROFLOXACIN (0.213 µg/ml)			---	2.255*	3.505*	0.21	2.305*
LEVOFLOXACIN (0.068 µg/ml)				---	1.25*	2.045*	4.560*
RIFAMPICIN (0.020 µg/ml)					---	3.295*	5.810*
TRIMETHOPRIM/SULFAMETHOXAZOLE (0.203 µg/ml)						---	2.515*
TETRACYCLINE (6.130 µg/ml)							---

* Using Shapiro-Wilk and Kruskal-Wallis tests, significant variation was determined at $p<0.05$

In contrast, they all had mean MIC values significantly higher than rifampicin (0.020 µg/ml), levofloxacin (0.068 µg/ml) and azithromycin (0.170 µg/ml), and significantly lower than tetracycline (6.130 µg/ml), which had significantly the highest mean MIC value relative to all other antibiotics tested (Tab. 3).

DISCUSSION

The development of bacterial resistance to antibiotics and chemotherapeutics is a major public health problem worldwide, reducing the effectiveness of antimicrobial treatment in clinical practice. Water systems are an important reservoir for the transmission of *L. pneumophila* infections, despite which antimicrobial sensitivity testing of wild-type isolates is not usually performed.

Legionella bacteria are among the atypical pathogens that constitute one of the aetiological agents of pneumonia. They cause about 0.4–5% of out-of-hospital pneumonias treated at home among adults [8].

Legionella bacteria can colonise water systems, cooling towers, fountains, water storage tanks, air-conditioning and humidification equipment, as well as medical equipment [11]. The mortality rate for pneumonia of legionellosis aetiology is quite high, ranging from 16% to as high as 33% in adults [12].

Early appropriate antibiotic therapy is crucial to reduce mortality among patients with legionellosis. Typical bacterial pathogens classically respond to antibacterial therapy with β-lactam antibiotics because they have a cell wall susceptible to disruption by β-lactams. The mechanism of action of this group of drugs involves inhibition of cell wall synthesis and bacterial cell death (bactericidal effect). Most atypical pathogens do not have a cell wall, some are intracellular (e.g. Legionella) and some are paracellular (e.g. *M. pneumoniae*) [13, 14].

L. *pneumophila* causes severe out-of-hospital pneumonia (OP), often requiring treatment in an intensive care unit (ICU). The incidence is most common in summer and early autumn and can take the form of localised epidemics. The risk is increased by recent travel and exposure to plumbing infrastructure [5].

According to the Polish recommendations for the treatment of out-of-hospital pneumonia, the antibiotic of choice is levofloxacin. As an alternative treatment, azithromycin is also proposed [15]. The recommendations also specify the duration of treatment, which should be between 10–14 days and, in immunocompromised patients, the treatment can be as long as 21 days [16]. Due to the lack of standardised MIC determination methods for antibiotics used to treat *Legionella* spp. infections, it is not recommended to determine sensitivity [17].

In this study, the *in vitro* sensitivity of *L. pneumophila* to antibiotics was analysed used in the treatment of Legionellosis pneumonia, i.e. to erythromycin, azithromycin, ciprofloxacin, levofloxacin, rifampicin, trimethoprim-sulfamethoxazole and tetracycline [18].

Currently, there are no defined cut-off values for *L. pneumophila*, as there is no reference method for testing/defining drug sensitivity. The use of different sensitivity testing methods and different microbiological media results in different MIC values. *Legionella* do not grow on the reference medium for sensitivity testing, i.e. Mueller-Hinton medium, due to the high nutritional requirements. Charcoal in *Legionella* spp. culture media has been shown to increase the MIC of most drugs. However, one of innovative methods of drug microdilution in solid media without charcoal and in liquid media ensures an optimal bacterial growth and appropriate drug sensitivity testing results. It uses drug-soaked strips in a concentration gradient (E-tests) on buffered charcoal agar, yeast extract with α-ketoglutarate to determine MIC values. The method is accessible to microbiological laboratories, easy to perform and reproducible. Another method is microdilution of the drug in liquid medium without the addition of charcoal. This method however is time- and labour-intensive [19, 20, 21, 22].

The results of this study indicate resistance of one strain to azithromycin. The same strain showed sensitivity with increased exposure to ciprofloxacin [23]. Resistance to macrolide antibiotics is due to ribosomal modification, efflux mechanism and enzymatic inactivation. The exact mechanism of resistance requires further research. Analysing the results of the study (without the azithromycin-resistant strain), the MIC values were contained within the following ranges: erythromycin 0.190–0.500 µg/ml, azithromycin 0.094–0.380 µg/ml, ciprofloxacin 0.190–0.380 µg/ml, levofloxacin 0.032–0.190 µg/ml, rifampicin 0.016–0.016 µg/ml, trimethoprim/sulfamethoxazole 0.190–0.380 µg/ml, tetracycline 6,000–12,000 µg/ml.

In the conducted studies, the MIC50 for azithromycin, ciprofloxacin, levofloxacin, rifampicin, trimethoprim/sulfamethoxazole were respectively: 0.190 µg/ml,

0.094 µg/ml 0.190 µg/ml, 0.032 µg/ml, 0.016 µg/ml, 0.190 µg/ml, 6,000 µg/ml. These results are similar to those obtained in previous studies, although it shou6ld be noted that the range of MICs for antimicrobials may vary slightly in different geographical regions [24, 25]. Torre *et al*. assessed the antimicrobial sensitivity of *Legionella* spp. isolated from hospital water systems in the south-western region of Italy. None of the isolates were resistant to the tested antibiotics (azithromycin, clarithromycin, erythromycin, ciprofloxacin, levofloxacin, moxifloxacin, cefotaxime, tigecycline, doxycycline, rifampicin) [9].

Other authors showed that omadacycline showed potent *in vitro* activity against *L. pneumophila* serogroups 1 to 6. Based on MIC 90 values, omadacycline was 4-fold more potent by weight than doxycycline and erythromycin; omadacycline MIC90 values were 2-fold lower by weight than azithromycin. Omadacycline was 10 times weaker by weight than the telithromycin and tested fluoroquinolones. Noteworthy was the activity of omadacycline against serogroup 1 of *L. pneumophila*, the most common serotype isolated from hospital-acquired or out-of-hospital respiratory tract infections [26].

CONCLUSIONS

1) The lowest biocidal concentration was found for levofloxacin and the highest for tetracycline.
2) The highest MIC50 and MIC90 values were found for tetracycline and the lowest for rifampicin.
3) The highest biocidal values were found for azithromycin.

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