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ORIGINAL ARTICLE

Assessment of antibiotic susceptibility of *Legionella pneumophila* isolated from water systems in Poland

Agnieszka Sikora[1], Iwona Gładysz[2], Maria Kozioł-Montewka[2], Małgorzata Wójtowicz-Bobin[1], Tomasz Stańczak[1], Renata Matuszewska[3], Bożena Krogulska[3]

[1] *Department of Medical Microbiology, Medical University of Lublin, Poland*
[2] *Pope John Paul II State School of Higher Education of Biała Podlaska, Poland*
[3] *National Institute of Public Health - National Institute of Hygiene, Department of Environmental Hygiene, Warsaw, Polandd*

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Abstract

Introduction and objective. Several studies have reported therapy failures in patients with legionnaires'disease; however, antimicrobial resistance of clinical and environmental isolates of *Legionella* spp. has not yet been documented. Routine susceptibility testing of *Legionella* spp. is not recommended because of difficulties in determining standard minimal inhibitory concentration values. The purpose of this study was to analyze the antimicrobial susceptibility of *Legionella pneumophila*. strains isolated from a water supply system.

Materials and method. Twenty-eight isolates of *L. pneumophila* (16 – *L. pneumophila* SG 1, 12 – *L. pneumophila* SG 2–14) obtained from water systems in public buildings in Poland were tested. Susceptibility testing was performed using the E-test method. The tested antibiotic were azithromycin, ciprofloxacin, and rifampicin. The medium used for the susceptibility testing was BCYE-, a special medium for *Legionella* cultivation.

Results. Among the tested strains, *L. pneumophila* was the only one resistant to azithromycin. It was a strain of *L. pneumophila* SG 2–14 isolated from the water system in a sanitorium. All isolates were found to be sensitive to ciprofloxacin and rifampicin. However, the azithromycin-resistant strain exhibited higher ciprofloxacin and rifampicin MIC (1.5 μg/ml, and 0.19 μg/ml, respectively). The MIC_{50} for azithromycin, ciprofloxacin, and rifampicin were 0,032, 0,125, and 0,003 μg/ml, respectively. The MIC_{90} for azithromycin, ciprofloxacin, and rifampicin were 0,032, 0,125, and 0,003 μg/ml, respectively.

Conclusions. Azithromycin resistance was found in one strain of *L. pneumophila* SG 2–14, but the resistance mechanism is unknown and needs further study. It is possible that therapeutic failures in Legionnaires' disease may be associated with bacterial resistance which should be taken into account. The antibiotic sensitivity testing described in this study could be helpful in detecting the resistance of clinical *L. pneumophila* isolates. Ciprofloxacin and rifampicin have good *in vitro* activity against environmental *L. pneumophila* SG 1 and SG 2–14 in Poland.

Key words

Legionella pneumophila, susceptibility, E-test, minimum inhibitory concentration

INTRODUCTION

Legionella species are responsible for legionellosis, which may occur in two clinical forms -pneumonia (also known as legionnaires' disease) and Pontiac fever (influenza-like, mild illness). The infection is acquired through aspiration of contaminated water [1]. *Legionella* can proliferate in hot water distribution systems of large buildings, such as health care facilities, or in domestic water systems [1, 2].

Currently, more than 50 species of *Legionella* have been identified, some of which have been associated with human disease, for example, *Legionella pneumophila*, *Legionella micdadei*, *Legionella longbeachae*, *Legionella dumoffii*, and *Legionella bozemanii* [3, 4]. *L. pneumophila* is the most important etiological agent of legionellosis, and serogroup 1 (SG 1) accounts for more than 90% of reported human infections [5]. The mortality rate in patients with legionnaires'disease varies and depends on the clinical settings, patient population, and antimicrobial treatment [5]. The antimicrobial agents commonly used for treatment of *Legionella* pneumonia are macrolides and fluoroquinolones. These agents are active against intracellular *Legionella* spp., which can survive and proliferate in human macrophages [5, 6]. Pontiac fever does not require antibiotics because it resolves spontaneously.

Erythromycin is the drug of choice in the treatment of Legionnaires' disease. Currently, the use erythromycin has been limited by the high incidence of side-effects, for example, phlebitis, disorders of the gastrointestinal tract, and drug interactions (e.g. immunosuppressive medications). Moreover, reversible ototoxicity was found with the use of higher doses recommended in patients with Legionnaires' disease [7].

At present, the newer macrolides (such as azithromycin) and fluoroquinolones (e.g. ciprofloxacin, levofloxacin) are recommended for the treatment of Legionnaires' disease [8]. They have a superior *in vitro* activity and greater intracellular penetration [8, 9]. Also, rifampicin is used in severe cases of *Legionella* infection, especially in immunocompromised patients and patients with comorbidities (e.g. obstructive lung disease, or diabetes mellitus) [10]. In clinical practice, this drug is used most often in combination with other

Address for correspondence: Agnieszka Sikora, Department of Medical Microbiology, Medical University of Lublin, Chodźki 1, 20-093, Lublin, Poland
E-mail: agnieszka.sikora@umlub.pl

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antimicrobial drugs because of the possibility of developing resistance [8, 9].

Several studies have reported therapy failures in patients with legionnaires'disease; however, the antimicrobial resistance of clinical and environmental isolates of *Legionella* spp. has not been yet documented [9, 11]. Only *in vitro* studies showed antibiotic-resistance variants [12].

Routine susceptibility testing of *Legionella* spp. is not recommended because of difficulties in determining standard minimal inhibitory concentration values (MICs). This is associated with high nutritional requirements of these bacteria and inactivation of some antibiotics (for example: sulfonamide, tetracycline, polymyxin B) by charcoal which is necessary for the of *Legionella* species [13].

OBJECTIVES

Susceptibility testing of environmental *Legionella* isolates has never been published, in Poland. Therefore, the purpose of this study was to analyze the antimicrobial susceptibility of *L. pneumophila* strains isolated from a water supply system using the E-test.

MATERIALS AND METHOD

The E-test methods and BCYE-α agar for susceptibility testing of *L. pneumophila* were based on previous studies [14, 15, 16].

Bacterial strains. Twenty-eight isolates of *L. pneumophila* obtained from the water systems in hospitals (15), sanatoriums (2), hotels (8) and other public buildings (3) in Poland were tested. Of these isolates, 12 strains *L. pneumophila* serogroup 2–14 (SG 2–14) came from own collection, and 16 strains *L. pneumophila* serogroup 1 (SG 1) from the National Institute of Public Health – National Institute of Hygiene (NIPH-NIH) in Warsaw, Poland. *L. pneumophila* were isolated during the period from January 2009 – March 2010 in Lublin Province (SG 2–14) and Mazowieckie Province (SG 1).

The isolates were stored at –70 °C in nutrient broth with 20% glycerol. All isolates were subcultured on buffered charcoal yeast extract agar with L-cysteine (BCYE-α) which is a special medium for *L. pneumophila* cultivation. Plates were incubated at 37°C in a humidified atmosphere for 48–72 hours. Serogroups *L. pneumophila* (SG 1, SG 2–14) were confirmed (after incubation) with the use of the agglutination latex test (*Legionella* Latex Test, Oxoid). As reference strains, *L. pneumophila* (Philadelphia-1) ATCC 33152 and *Staphylococcus aureus* ATCC 25923 (LGC Standards, United Kingdom) were used as the control [17].

The media used for susceptibility testing were BCYE-α for *L. pneumophila* and Mueller-Hinton agar for control *S. aureus* strain ATCC 25923. Susceptibility testing was performed using the E-test method (bioMérieux, France). The tested antibiotics were azithromycin (range 0.016–256 μg/ml), ciprofloxacin (range 0.002–32 μg/ml), and rifampicin (range 0.002–32 μg/ml).

The colonies of *L. pneumophila* (from BCYE-α medium) were suspended in sterile saline buffer (0.5 McFarland standard) and were swabbed in 3 directions on BCYE-α medium. The strips of azithromycin, ciprofloxacin, and rifampicin were applied to the agar surface. The same procedure was performed for the control strains. *S. aureus* ATCC 25923 was inoculated in parallel onto antimicrobial agent containing Mueller-Hinton agar plates as well as BCYE-α plates, to determinate whether BCYE-α inhibited the activity of the antimicrobial agents. All procedures were undertaken according to the manufacturer's instructions (bioMérieux, France). *L. pneumophila* strains were culture for 48 hours at 35 °C with an increased humidity on BCYE-α agar before reading the MIC values. Plates with slow-growing *L. pneumophila* strains were incubated for another 24 h. *S. aureus* was incubated at 35 °C for 24 h.

MIC determination. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of antimicrobial that inhibited the visible growth of a microorganisms. MICs were read at the point at which the zone of complete inhibition intersects the MIC scale. The MIC_{50} and MIC_{90} were defined as the lowest of the antimicrobial concentrations that inhibited growth of 50 and 90% of the isolates, respectively.

Statistical analysis. MIC values between different groups (SG 1 vs. SG 2–14 *L. pneumophila*, hospital vs. non-hospital strains) were analyzed by Mann-Whitney U-test using STATISTICA version 10 (StatSoft, Poland). A p-value of <0.05 was considered to be statistically significant.

RESULTS

The antimicrobial susceptibility of *L. pneumophila* strains isolated from the water supply system of different large public buildings was analyzed. This is the first such study performed in Poland. Among the tested strains of *L. pneumophila* (n=28), only one was resistant to azithromycin (no zone of inhibition around the E-test strip). This was a strain of *L. pneumophila* SG 2–14 isolated from the water system in one of the 2 sanatoria included in the study. All isolates were found sensitive to ciprofloxacin and rifampicin; however, the azithromycin-resistant strain exhibited higher ciprofloxacin and rifampicin MIC (1.5 μg/ml, and 0.19 μg/ml, respectively). Further analysis during calculations are not included in the results of the strain on azithromycin resistance because it was determined the MIC value.

MICs were in the following ranges: azithromycin 0.016–0.32 μg/ml, ciprofloxacin 0.004–1.5 μg/ml, and rifampicin 0,002–0.19 μg/ml. The minimum inhibitory concentration required to inhibit the growth of 50% of *L. pneumophila* (MIC_{50}) were 0.032 μg/ml for azithromycin, 0.125 μg/ml – ciprofloxacin, and 0.003 μg/ml – rifampicin. The minimum inhibitory concentration required to inhibit the growth of 90% of *L. pneumophila* (MIC_{90}) for azithromycin, ciprofloxacin, and rifampicin were 0.032, 0.125, and 0.003 μg/ml, respectively.

The MIC values of *L. pneumophila* SG 1 were compared with MIC values for strains of *L. pneumophila* SG 2–14 for all tested antimicrobial agents (Tab. 1). There was no significant difference between MIC values of strains SG 1 and SG 2–14 (azithromycin p=0.65; ciprofloxacin p=0.42; rifampicin p=0.07). Comparison of MIC values for isolates *L. pneumophila* from hospital versus non-hospital environments showed no significant difference in any of the tested antibiotics (azithromycin p=0.12; ciprofloxacin p=0.27; rifampicin p=1.0).

Table 1. Comparison of MIC values between strains of *L. pneumophila* SG 1 and SG 2–14

Antibiotics	*L. pneumophila* SG 1			*L. pneumophila* SG 2–14		
	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}
AZITHROMYCIN	0.016[a]-0.32	0.032	0.25	0.016–0.064	0.032	0.047
CIPROFLOXACIN	0.006–0.5	0.125	0.19	0.004–1.5	0.125	0.25
RIFAMPICIN	0.002–0.006	0.003	0.004	0.002–0.19	0.003	0.008

[a] without azithromycin-resistant strain (no zones of inhibition)

Reference strain *S. aureus* ATCC 25923 was investigated on 2 media: Mueller-Hinton (reference agar to perform susceptibility testing) and BCYE-α in order to determine the influence of components contained in BCYE-α on the MIC value. All the tested antibiotics were inhibited on BCYE-α. The MIC values were higher (4-fold increase) in BCYE-α, compared with MIC values on Mueller-Hinton agar (MIC value BCYE-α/Mueller-Hinton agar: azithromycin – 3.94; ciprofloxacin – 4; rifampicin – 4) (Tab. 2).

Table 2. MIC values for reference strains

		MIC (μg/ml)		
		AZITHRO-MYCIN	CIPROFLO-XACIN	RIFAMPI-CIN
ATCC 33152 *L. pneumophila* SG 1	BCYE-α agar	0.023	0.19	0.004
ATCC 25923 *S. aureus*	Mueller-Hinton agar	0.19	0.125	0.008
	BCYE-α agar	0.75	0.5	0.032

The mean values of MICs for the tested antibiotics did not differ considerably from the MIC values for the reference strain (ATCC 33152 *L. pneumophila* SG 1).

DISCUSSION

L. pneumophila is the most common pathogenic species of the genus *Legionella*. responsible for community-acquired and nosocomial atypical pneumonia (hospital acquired pneumonia) in industrialized countries [1, 18]. It is estimated that *L. pneumophila* causes from <1–5% of cases of community-acquired pneumonia in adults, dependent on the geographic area [18]. This bacterium is ubiquitous in water environments worldwide. *L. pneumophila* can colonize tap water, cooling towers, hot water distribution systems, fountains, air conditioning systems, and medical equipment containing water [1].

The mortality of community-acquired legionnaires' disease ranges from 16–30% if untreated, or when treated with inactive antimicrobial drugs, the mortality for nosocomial legionnaires' disease can approach 50%, given the underlying disease of the patient [7, 19]. Early appropriate antibiotic therapy is crucial to reduce mortality in patients with legionellosis. However, several studies have shown treatment failure in patients with pneumonia caused by *Legionella,* which may be due to resistance in clinical isolates of *L. pneumophila*. This has not yet been documented [12]; therefore, the *in vitro* activity of antibiotic against *L. pneumophila* (environmental and clinical isolates) should be tested in order to monitor the possible resistant strains in different regions [12].

The intracellular location of the *Legionella* spp. is relevant for the efficacy of the antibiotic. The use of antibiotics in the therapy capable of achieving intracellular concentration higher than the MIC were more clinically effective than antibiotics with poor intracellular penetration [7]. Antibiotics with intracellular penetration include the macrolides, fluoroquinolones, tetracyclines, and rifampicin. These antimicrobial drugs are used in the therapy for legionnaires' disease [7, 20], and their effectiveness has been confirmed by clinical experience and retrospective analysis. It should be emphasized that β-lactam antibiotics are ineffective in the treatment of *Legionella* infections because the bacteria produce β-lactamases which inactivate this group of drugs [21].

In Poland, according to recommendations of proceedings in community-acquired respiratory tract infections, the drug of choice in *Legionella* infection is azithromycin. Alternatively doxycyclin, moxyfloxacin, ciprofloxacin, and clarithromycin may be used. Duration of treatment is 7–10 days, depending on the severity of the disease. Antibiotic therapy may be extended in patients with lung abscesses, endocarditis, and extrapulmonary infection [7]. Moreover, in immunocompromised patients with severe legionnaires' disease, the therapy period may be up to 21 days [7].

In the presented study, *in vitro* susceptibilities of *L. pneumophila* against commonly used antibiotics (azithromycin, ciprofloxacin, rifampicin) were performed. Antimicrobial susceptibility testing for *Legionella* spp. is not generally recommended for routine microbiology, because a standard method for the determination of MICs of antibiotics for *Legionella* spp. is not available [12].

In vitro or *in vivo* efficacy of antibiotics against *Legionella* spp. has usually been based on the determination of MIC values by micro-broth dilution, agar dilution, E-test methods, cell culture models (e. g. human monocytes), and animal models. Another method is the disc diffusion method [7, 9, 22]. None of these methods, however, are a gold standard for the susceptibility testing of *L. pneumophila* [12].

The use of agar and broth dilution methods are limited because of the intracellular location of the bacteria of the genus *Legionella* [9]. Another limitation of the susceptibility testing methods are the specific nutritional requirements of this microorganism [14]. *Legionella* spp grows only on BCYE-α medium. This medium contain charcoal which is necessary for absorbing toxic metabolites produced during growth of *Legionella* [14]. In the current study, BCYE-α medium was used in the susceptibility test. The effect of medium components on the activity of antibiotics tested was studied. All the antibiotics tested were inhibited on BCYE-α in comparison with Mueller-Hinton medium. The charcoal did not cause significant changes which would affect the interpretation of the test results. Furthermore, in comparison with other tests, in this study a lower increase was obtained in the MIC values for the reference strain, and growth was constant for all study drugs [5, 13]. In the current study, comparing the sensitivity of the reference strain with strains isolated from the water systems, there were no significant differences in the MIC values.

Some researchers report that an alternative medium used to determine the susceptibility of *Legionella* spp. is a charcoal-free medium – washed buffered yeast extract (WBYE), [13, 14, 16, 17, 23]. However, not all *Legionella* strains grow on this medium. In addition, a considerable decrease in growth

has also been reported in tested strains of *L. pneumophila* [13]. This confirms necessity to use a conventional BCYE medium for susceptibility testing.

In the presented study, one strain was resistant to azithromycin, which also showed intermediate susceptibility to ciprofloxacin, and a much lower sensitivity to rifampicin in comparison with other tested isolates. Resistance to macrolides may be due to 3 mechanisms: ribosomal modification, efflux mechanism and drug inactivation [23]. Accurate determination of the mechanism of resistance requires further investigation.

In analyzing the results of this study (without azithromycin-resistant strain), MIC values came within in the following ranges: azithromycin – 0.016–0.32 μg/ml, ciprofloxacin – 0.004–1.5 μg/ml, and rifampicin – 0.002–0.19 μg/ml. In the currentr study, MIC_{50} for azithromycin, ciprofloxacin, and rifampicin were 0.032 μg/ml, 0.125 μg/ml, and 0,003 μg/ml, respectively. These results are similar to those obtained in previous studies, but it should be emphasized that the MIC range for antimicrobial agents may vary somewhat in different geographic regions [12, 13, 25].

Erdogan et al. evaluated the antimicrobial susceptibility of *Legionella* spp. isolated from the hotel and hospital water supply system in different regions of Turkey. The researchers used a microdilution method and buffered yeast extract medium supplemented with 0.1% α-ketoglutarate (BCE-α). MIC values were 0,001–0.5 mg/L for azithromycin, 0,001–0,125 mg/L for ciprofloxacin, and 0,001–0.5 mg/L for rifampicin. MIC_{50} for azithromycin, ciprofloxacin, and rifampicin were 0,015 mg/L, 0.03 ml/L, and 0,001 mg/L, respectively [9]. In another study evaluating the drug sensitivity of *Legionella* isolated in Portugal, the MIC values for ciprofloxacin and rifampicin were 0,250–1 μg/ml (MIC_{50} 0.5 μg/ml), and 0.16–0.5 μg/ml (MIC_{50}–0,023 μg/ml), respectively [17].

When isolates from hospital environments were compared in the presented study, there was no significant difference in the MIC values. The results are similar to those obtained by other researchers [11]. Also, in the presented study there were no differences in susceptibility between serogroups of *L. pneumophila* (SG1 and SG 2–14).

CONCLUSIONS

Azithromycin resistance occurred in one strain of *L. pneumophila* SG 2–14. The resistance mechanism is unknown and needs further study. It is possible that therapeutic failures in Legionnaires' disease may be associated with bacterial resistance, which should be taken into account. The antibiotic sensitivity testing described in this study could be helpful in detecting the resistance of clinical *L. pneumophila* isolates. Ciprofloxacin and rifampicin have good *in vitro* activity against environmental *L. pneumophila* SG 1 and SG 2–14 in Poland.

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