Antibiotic susceptibility of *Legionella* strains isolated from public water sources in Macau and Guangzhou

Lina Xiong, He Yan, Lei Shi and Ziyao Mo

ABSTRACT

The purpose of this study was to investigate the susceptibility of waterborne strains of *Legionella* to eight antimicrobials commonly used in legionellosis therapy. The minimum inhibitory concentrations (MICs) of 66 environmental *Legionella* strains, isolated from fountains and cooling towers of public facilities (hotels, schools, and shopping malls) in Macau and Guangzhou, were tested using the microdilution method in buffered yeast extract broth. The MIC_{50}/MIC_{90} values for erythromycin, cefotaxime (CTX), doxycycline (DOC), minocycline (MIN), azithromycin, ciprofloxacin, levofloxacin (LEV), and moxifloxacin were 0.125/0.5 mg/L, 4/8 mg/L, 8/16 mg/L, 4/8 mg/L, 0.125/0.5 mg/L, 0.031/0.031 mg/L, 0.031/0.031 mg/L, and 0.031/0.062 mg/L, respectively. *Legionella* isolates were inhibited by either low concentrations of macrolides and fluoroquinolones, or high concentrations of CTX and tetracycline drugs. LEV was the most effective drug against different *Legionella* species and serogroups of *L. pneumophila* isolates. The latter were inhibited in decreasing order by MIN > CTX >DOC, while non-*L. pneumophila* isolates were inhibited by CTX> MIN >DOC. In this study, we evaluated drug resistance of pathogenic bacteria from the environment. This may help predict the emergence of drug resistance, improve patient outcomes, and reduce hospitalization costs.

Key words | antibiotic susceptibility, environmental *Legionella* strains, fluoroquinolones, macrolides, minimum inhibitory concentrations, tetracycline drugs

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INTRODUCTION

Legionella species are intracellular microorganisms ubiquitous in man-made water systems, and the causative agents of Legionnaires' disease and Pontiac fever (Cunha *et al.* 2016). *Legionella*-related illnesses originate upon inhaling contaminated aerosols from natural and artificial aquatic environments, following which bacteria colonize alveolar macrophages and trigger severe pneumonia (Phin *et al.* 2014). Currently, the genus *Legionella* comprises 59 species and more than 70 distinct serogroups (De Giglio *et al.* 2015). Among them, *L. pneumophila* serogroup (sg) 1 bacteria are the primary culprits of all reported cases of legionellosis (Xiong *et al.* 2015).

Recent studies have confirmed that some outbreaks of legionellosis were linked to aerosol-producing devices, including fountains and cooling towers of public facilities (Quinn *et al.* 2015; Smith *et al.* 2015). As a result of disinfection and other practices, *Legionella* spp. in these artificial water environments are subjected to multiple adverse factors. In addition, it is widely accepted that the presence of antibiotics leads to selective pressure toward acquisition of resistance in initially susceptible bacteria (Andersson & Hughes 2014). Little is known about the influence of antibiotics and other disinfectants on *Legionella* spp. that colonize these water systems. Several studies have indicated that biofilms and numerous protozoan genera contribute to *Legionella* spp. colonization and persistence in the natural environment (Molmeret *et al.* 2005; Abdel-Nour *et al.* 2013). However,

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little is known about *Legionella* spp. susceptibility to antibiotics.

More than 97% of Legionnaires' disease cases are diagnosed by urinary antigen testing, while only 5% are confirmed by culture (Cunha *et al.* 2016). This, and the lack of a standardized *Legionella* antimicrobial test, make it difficult to study drug susceptibility of clinical *Legionella* isolates. Two methods, microdilution and E-test, are currently used in extracellular susceptibility testing (Edelstein 1995). Since *Legionella* spp. can colonize and proliferate in human macrophages (Phin *et al.* 2014), intracellularly active antimicrobial agents, such as macrolides, fluoroquinolones, and tetracycline drugs, are always used in the treatment of illnesses caused by *Legionella* (Bruin *et al.* 2012; De Giglio *et al.* 2015).

The purpose of this study was to investigate the antimicrobial susceptibility of *Legionella* spp. isolated from the water sources of public facilities in Macau and Guangzhou. The results could not only contribute to improved disinfection against *Legionella* in water systems, but would also provide an epidemiological survey (such as a geographical distribution of susceptible/resistant strains) and a timely warning about the occurrence of antibiotic resistance in environmental *Legionella* spp., before it becomes evident in clinical samples.

MATERIALS AND METHODS

Bacterial strains

Legionella strains were isolated from fountains and cooling towers of public facilities (hotels, schools, and shopping malls) in Macau and Guangzhou from 2007 to 2010 (Lu *et al.* 2011; Xiong *et al.* 2015). All *Legionella* strains were cultured on buffered charcoal yeast extract (BCYE) agar plates supplemented with 0.04% L-cysteine, 0.025% ferric pyrophosphate, and 0.1% α-ketoglutarate (BCYE-α) at 37 °C in 5% CO_2. *Legionella* spp. were identified by serological agglutination using the Legionella Latex Agglutination Kit (PRO-LAB, Weston, FL, USA), fatty acid analysis (MIDI software version 6.0; Microbial ID, Inc., Newark, DE, USA), and sequence-based typing, following the European Working Group for *Legionella* infections guidelines (Ratzow *et al.* 2007).

L. pneumophila (ATCC 33153) and *L. jordanis* (ATCC 33623) were used as reference strains. *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 29213) were also selected for testing.

Susceptibility testing

Eight antimicrobial drugs were tested: erythromycin (ERM), cefotaxime (CTX), doxycycline (DOC), minocycline (MIN), azithromycin (AZM), ciprofloxacin (CIP), levofloxacin (LEV), and moxifloxacin (MOX).

Antimicrobial susceptibility was assessed by microdilution in buffered yeast extract (BYE) broth supplemented with 0.04% L-cysteine, 0.025% ferric pyrophosphate, and 0.1% α-ketoglutarate in 96-well microtiter plates, following the National Committee for Clinical Laboratory Standards guidelines. A single colony of *Legionella* was picked from a BCYE-α plate and suspended in sterile water. The turbidity of the bacterial suspension was adjusted to an optical density equivalent to 0.5 McFarland units. Antibiotic serial dilutions (8–0.004 mg/L for AZM, CIP, ERM, LEV, and MOX; 32–0.016 mg/L for CTX, DOC, and MIN) were prepared in 50 μL BYE broth per well, to which 50 μL of bacterial solution (1×10^6 CFU/mL) was added. After incubation in 5% CO_2 at 37 °C for 72 h, the minimum inhibitory concentration (MIC) was defined as the first well with no visible growth.

Pseudomonas aeruginosa and *Staphylococcus aureus* susceptibility tests were performed in both Mueller-Hinton (MH) and BYE broths, and MICs were read after incubation at 37 °C for 24 h.

Statistical analysis

Statistical analyses were conducted using SPSS Statistics 21.0 software (SPSS Inc., Chicago, IL, USA), and statistical significance was defined as $p \leq 0.05$. Differences in antimicrobial susceptibility between *L. pneumophila* and non-*L. pneumophila* isolates, or between *L. pneumophila* sg 1 and non-sg 1, were evaluated using the Kruskal–Wallis test, followed by a Dunn's test.

RESULTS

Legionella strains used in this study are listed in Table 1; they include 40 *L. pneumophila* and 26 non-*L. pneumophila* strains. *L. gormanii* and *L. wadsworthii* isolates resulted in no visible growth after 96 h incubation in BYE broth. The cumulative percentages, MIC_{50}, and MIC_{90} values for the other 60 *Legionella* isolates, inhibited by different concentrations of the tested antimicrobials, are shown in Table 2. MIC_{50} and MIC_{90} values for macrolides were 0.062 and 0.25 mg/L, respectively, for AZM (range 0.031–0.5 mg/L); and 0.125 and 0.5 mg/L, respectively, for ERM (range 0.031–0.5 mg/L). MIC_{50} and MIC_{90} values for fluoroquinolones were 0.031 and 0.031 mg/L, respectively, for CIP and LEV (range 0.004–0.062 mg/L); and 0.031 and 0.062 mg/L, respectively, for MOX (range 0.004–0.125 mg/L).

Table 1 | List of *Legionella* strains used in this study

***L. pneumophila* (40)**		**Non-*L. pneumophila* (26)**	
Serogroup	**No. of strains**	**Species**	**No. of strains**
1	12	*L. adelaidensis*	1
2	1	*L. rubrilucens*	3
3	1	*L. gormanii*	3
5	5	*L. shakespearei*	5
6	1	*L. feeleii*	6
8	1	*L. wadsworthii*	3
9	2	*L. quateirensis*	5
14	17		

MIC_{50} and MIC_{90} values for tetracycline drugs were 8 and 16 mg/L, respectively, for DOC (range 0.5–16 mg/L); and 4 and 8 mg/L, respectively, for MIN (range 0.5–8 mg/L). MIC_{50} and MIC_{90} values for CTX were 4 and 8 mg/L, respectively (range 0.062–16 mg/L). With a MIC_{90} of 0.031 mg/L, CIP and LEV were the most active drugs, while DOX (MIC_{90} of 16 mg/L) was the least effective.

The susceptibilities of *L. pneumophila* sg 1, *L. pneumophila* non-sg 1, total *L. pneumophila*, and non-*L. pneumophila* isolates to the eight tested antimicrobials are shown in Table 3. No differences were found between *L. pneumophila* sg 1 and non-sg 1 isolates for any of the eight antibiotics. In addition, no differences were found between *L. pneumophila* and non-*L. pneumophila* for six of the antibiotics (AZM, CIP, ERM, MIN, LEV, and MOX). In contrast, MIC values for CTX and DOC were significantly lower in non-*L. pneumophila* than in *L. pneumophila* isolates.

As shown in Table 4, the susceptibilities of *Legionella* strains isolated from Guangzhou and Macau to the eight tested antimicrobials were compared. No differences were found between Guangzhou isolates and Macau isolates for three of the antibiotics (CTX, DOC, and MIN). In contrast, MIC values for AZM, CIP, ERM, LEV, and MOX were significantly lower in Guangzhou isolates than in Macau isolates.

With the exception of CTX (MIC of 8 mg/L), reference strains *L. pneumophila* sg 1 and *L. jordanis* showed similar susceptibilities to those of the environmental isolates (Table 5). In the case of *P. aeruginosa* and *S. aureus*, DOC

Table 2 | Cumulative distribution of MICs for 60 environmental *Legionella* isolates

	Cumulative % of strains inhibited at indicated concentrations of antimicrobials (mg/L)												
Drugs	**0.004**	**0.008**	**0.016**	**0.031**	**0.062**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**
AZM				30.0	55.0	58.3	78.3	100					
CTX					8.3	10.0		11.7	16.7	30.0	68.3	95.0	100
CIP	1.7	8.3	40.0	91.7	100								
DOC								1.7	3.3		33.3	83.3	100
ERM				1.7	5.0	60.0	88.3	100					
LEV	1.7	15.0	45.0	96.7	100								
MIN						1.7		6.7	16.7	33.3	71.7	100	
MOX	1.7	5.0	36.7	68.3	98.3	100							

Table 3 | MIC values (mg/L) for *L. pneumophila* sg 1, *L. pneumophila* non-sg 1, total *L. pneumophila*, and non-*L. pneumophila*

	L. pneumophila sg 1			*L. pneumophila* non-sg 1			*L. pneumophila*			non-*L. pneumophila*		
Drugs	**MIC_{50}**	**MIC_{90}**	**GM**	**MIC_{50}**	**MIC_{90}**	**GM**	**MIC_{50}**	**MIC_{90}**	**GM**	**MIC_{50}**	**MIC_{90}**	**GM**
AZM	0.062	0.25	0.129	0.062	0.5	0.093	0.062	0.25	0.103	0.062	0.5	0.166
CTX	4	8	3.364	4	16	4.203	4	8	3.931	4	8	1.495
CIP	0.031	0.031	0.023	0.031	0.062	0.026	0.031	0.031	0.025	0.031	0.031	0.027
DOC	8	16	5.993	8	16	8.406	8	16	7.595	4	8	5.278
ERM	0.125	0.25	0.151	0.25	0.5	0.205	0.125	0.5	0.187	0.125	0.5	0.219
LEV	0.031	0.031	0.020	0.016	0.031	0.020	0.016	0.031	0.020	0.031	0.031	0.029
MIN	2	8	2.378	4	8	3.534	4	8	3.138	4	8	3.364
MOX	0.031	0.062	0.025	0.031	0.062	0.032	0.031	0.062	0.030	0.031	0.062	0.035

GM, geometric median.

Table 4 | MIC values (mg/L) for *Legionella* from Guangzhou and Macau

	Guangzhou			Macau		
Drugs	**MIC_{50}**	**MIC_{90}**	**GM**	**MIC_{50}**	**MIC_{90}**	**GM**
AZM	0.034	0.068	0.049	0.25	0.5	0.148
CTX	4	8	4	4	8	2.639
CIP	0.017	0.034	0.018	0.034	0.068	0.027
DOC	8	16	9.075	8	16	6.290
ERM	0.125	0.25	0.142	0.25	0.5	0.212
LEV	0.017	0.034	0.017	0.034	0.034	0.024
MIN	4	8	3.526	4	8	3.145
MOX	0.017	0.068	0.022	0.034	0.068	0.034

GM, geometric median.

showed the highest MIC (>32 mg/L); however, it should be noted that MICs for DOC and MIN were influenced by the type of medium (BYE or MH) (Table 5).

DISCUSSION

Legionella is a widespread and potentially pathogenic microorganism. To date, there have been limited reports about the antibiotic susceptibility of *Legionella* environmental isolates (Mallegol *et al.* 2014; Nagel *et al.* 2014; Gershengorn *et al.* 2015). In this study, we confirm that *Legionella* isolates are inhibited by low concentrations of macrolides and fluoroquinolones, but high concentrations of tetracycline drugs and CTX (Bruin *et al.* 2012; De Giglio *et al.* 2015). Among these antibiotics, LEV is considered to be the most effective drug against different species of *Legionella* and serogroups from *L. pneumophila* isolates (Pedro-Botet & Yu 2006; De Giglio *et al.* 2015).

MIC_{90} values relative to AZM and ERM are slightly higher than previously reported for environmental and

Table 5 | MIC (mg/L) values for reference strains in their corresponding media

	L. pneumophila ATCC 33153	*L. jordanis* ATCC 33623	*P. aeruginosa* ATCC 27853		*S. aureus* ATCC 29213	
Drugs	**BYE**	**BYE**	**BYE**	**MH**	**BYE**	**MH**
AZM	0.016	0.016	>8	>8	0.5	0.5
CTX	8	8	>16	>16	1	1
CIP	0.008	0.016	0.5	0.125	0.25	0.25
DOC	2	4	>32	32	0.5	0.125
ERM	0.062	0.062	>8	>8	0.5	0.5
LEV	0.004	0.004	1	0.5	0.031	0.016
MIN	4	2	16	4	1	0.125
MOX	0.016	0.016	4	4	0.031	0.016

clinical isolates (Mallegol *et al.* 2014; De Giglio *et al.* 2015). Moreover, the susceptibility of *L. pneumophila* sg 1, non-sg1, and non-*L. pneumophila* isolates to these antimicrobials did not differ significantly, as opposed to a previous study (Gómez-Lus *et al.* 2001). These discrepancies may be accounted for by a different origin of the strains, geographical environment, and methodology used.

In accordance with other reports (Blázquez Garrido *et al.* 2005; Sabrià *et al.* 2005; Pedro-Botet & Yu 2006), MIC_{50} and MIC_{90} values in this study confirmed the far superior efficacy of fluoroquinolones over macrolides in inhibiting *Legionella*. Thanks to their wide antimicrobial spectrum, strong disinfection effect, and fewer adverse effects, fluoroquinolones have been broadly used in the treatment of respiratory tract infections (Galstian *et al.* 2014). In line with other reports (De Giglio *et al.* 2015), MIC_{90} values in our study indicate that *L. pneumophila* isolates were most susceptible to LEV, while non-*L. pneumophila* isolates were most susceptible to CIP.

Previous studies (Reda *et al.* 1994; Bruin *et al.* 2012) reported that *L. pneumophila* isolates were inhibited in decreasing order by MIN > CTX > DOC, while non-*L. pneumophila* isolates were inhibited by CTX > MIN > DOC, and all three antibiotics showed higher MIC values than macrolides and fluoroquinolones. Here, MIC_{50} and MIC_{90} values for DOC were significantly higher than previously observed (De Giglio *et al.* 2015), which may be explained by the use of different susceptibility testing methods. This possibility is reinforced by control experiments on *P. aeruginosa* and *S. aureus*, whereby significant differences were observed between MIC values–for MIN and DOC–in BYE and MH broths. None of the three methods used in *Legionella* extracellular susceptibility testing, such as standard dilution in agar, broth, or E-tests, offers a golden standard. Some studies suggest that using a different methodology leads to variability in the range of MIC values (García *et al.* 2000). In general, the results from this study are comparable with previous works where the same standard dilution testing in broth was used (Stout *et al.* 1998; Erdogan *et al.* 2010).

In this study, susceptibility to DOC and CTX were significantly lower in *L. pneumophila* than in non-*L. pneumophila* ($p \leq 0.5$). This may indicate better environmental adaptability of *L. pneumophila*. It may also provide useful information when choosing the best therapy against a *Legionella* outbreak. In addition, susceptibility to AZM, CIP, ERM, LEV, and MOX were significantly lower in Guangzhou isolates than in Macau isolates ($p \leq 0.5$). This might be due to the geographical correlation, which could lead to the emergence of these results. It may be helpful to create a database of drug resistance of local *Legionella* strains.

Previous studies suggested that MIC values for DOC were influenced by components of the BCYE-α medium (García *et al.* 2000; Gómez-Lus *et al.* 2001; Erdogan *et al.* 2010; Bruin *et al.* 2012; De Giglio *et al.* 2015); however, our study indicates that charcoal in BCYE-α medium may not be the main source of variability.

Finally, we did not investigate all *Legionella* spp. because of the specific growth requirements of *L. gormanii* and *L. wadsworthii* isolates.

CONCLUSIONS

Susceptibility testing is often used in evaluating drug sensitivity of pathogenic bacteria, which helps predict the emergence of drug resistance, improve patient outcomes, and reduce the costs associated with hospitalization. As a result of the intracellular proliferation ability of *Legionella* spp., there is a lack of clinical samples for susceptibility testing. In addition, *Legionella* spp. are widely distributed in the environment. Thus, an antibiotic susceptibility study of environmental isolates, which are easier to investigate, can be used for predicting the occurrence of antibiotic resistance.

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