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 (MOX) were 0.125/0.5 mg/L, 4/8 mg/L, 8/16 mg/L, 0.125/0.5 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

**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) I 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. 2015). However,
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% CO2. *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 × 106 CFU/mL) was added. After incubation in 5% CO2 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 ≤ 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<sub>50</sub> and MIC<sub>90</sub> values for the other 60 Legionella isolates, inhibited by different concentrations of the tested antimicrobials, are shown in Table 2. MIC<sub>50</sub> and MIC<sub>90</sub> 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<sub>50</sub> and MIC<sub>90</sub> 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).

MIC<sub>50</sub> and MIC<sub>90</sub> 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<sub>50</sub> and MIC<sub>90</sub> values for CTX were 4 and 8 mg/L, respectively (range 0.062–16 mg/L). With a MIC<sub>90</sub> of 0.031 mg/L, CIP and LEV were the most active drugs, while DOX (MIC<sub>90</sub> 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
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).

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

<table>
<thead>
<tr>
<th>Drugs</th>
<th>MIC90 (mg/L)</th>
<th>MIC90 (mg/L)</th>
<th>GM (mg/L)</th>
<th>MIC90 (mg/L)</th>
<th>MIC90 (mg/L)</th>
<th>GM (mg/L)</th>
<th>MIC90 (mg/L)</th>
<th>MIC90 (mg/L)</th>
<th>GM (mg/L)</th>
<th>MIC90 (mg/L)</th>
<th>MIC90 (mg/L)</th>
<th>GM (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZM</td>
<td>0.062</td>
<td>0.25</td>
<td>0.129</td>
<td>0.062</td>
<td>0.25</td>
<td>0.103</td>
<td>0.062</td>
<td>0.25</td>
<td>0.166</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTX</td>
<td>4</td>
<td>8</td>
<td>3.364</td>
<td>4</td>
<td>16</td>
<td>4.203</td>
<td>4</td>
<td>8</td>
<td>1.495</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIP</td>
<td>0.031</td>
<td>0.031</td>
<td>0.023</td>
<td>0.031</td>
<td>0.062</td>
<td>0.026</td>
<td>0.031</td>
<td>0.031</td>
<td>0.027</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DOC</td>
<td>8</td>
<td>16</td>
<td>5.993</td>
<td>8</td>
<td>16</td>
<td>8.406</td>
<td>8</td>
<td>16</td>
<td>5.278</td>
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<tr>
<td>ERM</td>
<td>0.125</td>
<td>0.25</td>
<td>0.151</td>
<td>0.25</td>
<td>0.5</td>
<td>0.205</td>
<td>0.125</td>
<td>0.5</td>
<td>0.219</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LEV</td>
<td>0.031</td>
<td>0.031</td>
<td>0.020</td>
<td>0.016</td>
<td>0.031</td>
<td>0.020</td>
<td>0.016</td>
<td>0.031</td>
<td>0.029</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIN</td>
<td>2</td>
<td>8</td>
<td>2.378</td>
<td>4</td>
<td>8</td>
<td>3.534</td>
<td>4</td>
<td>8</td>
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<tr>
<td>MOX</td>
<td>0.031</td>
<td>0.062</td>
<td>0.025</td>
<td>0.031</td>
<td>0.062</td>
<td>0.032</td>
<td>0.031</td>
<td>0.062</td>
<td>0.035</td>
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</tr>
</tbody>
</table>

GM, geometric median.

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

<table>
<thead>
<tr>
<th>Drugs</th>
<th>MIC90 (mg/L)</th>
<th>MIC90 (mg/L)</th>
<th>GM (mg/L)</th>
<th>MIC90 (mg/L)</th>
<th>MIC90 (mg/L)</th>
<th>GM (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZM</td>
<td>0.034</td>
<td>0.068</td>
<td>0.049</td>
<td>0.25</td>
<td>0.5</td>
<td>0.148</td>
</tr>
<tr>
<td>CTX</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>8</td>
<td>2.639</td>
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</tr>
<tr>
<td>CIP</td>
<td>0.017</td>
<td>0.034</td>
<td>0.018</td>
<td>0.034</td>
<td>0.068</td>
<td>0.027</td>
</tr>
<tr>
<td>DOC</td>
<td>8</td>
<td>16</td>
<td>9.075</td>
<td>8</td>
<td>16</td>
<td>6.290</td>
</tr>
<tr>
<td>ERM</td>
<td>0.125</td>
<td>0.25</td>
<td>0.142</td>
<td>0.25</td>
<td>0.5</td>
<td>0.212</td>
</tr>
<tr>
<td>LEV</td>
<td>0.017</td>
<td>0.034</td>
<td>0.017</td>
<td>0.034</td>
<td>0.034</td>
<td>0.024</td>
</tr>
<tr>
<td>MIN</td>
<td>4</td>
<td>8</td>
<td>3.526</td>
<td>4</td>
<td>8</td>
<td>3.145</td>
</tr>
<tr>
<td>MOX</td>
<td>0.017</td>
<td>0.068</td>
<td>0.022</td>
<td>0.034</td>
<td>0.068</td>
<td>0.034</td>
</tr>
</tbody>
</table>

GM, geometric median.

**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. 2018). 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. 2018). 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).

MIC90 values relative to AZM and ERM are slightly higher than previously reported for environmental and pathogens.
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. 2003). 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; Sabrìa et al. 2005; Pedro-Botet & Yu 2006), MIC50 and MIC90 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), MIC90 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, MIC50 and MIC90 values for DOC were significantly higher than previously observed (De Giglio et al. 2013), 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 ≤ 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 ≤ 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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