Prevalence and characterization of quinolone resistance in Laribacter hongkongensis from grass carp and Chinese tiger frog

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Laribacter hongkongensis is a food-borne bacterium associated with community-acquired gastroenteritis and diarrhoea. Quinolone resistance was recently reported in bacterial isolates from aquatic products, but the molecular mechanisms for resistance were still unknown. In this study, a total of 157 L. hongkongensis strains were isolated from grass carp (n=443) and Chinese tiger frogs (n=171). Twenty-one ciprofloxacin-resistant strains were analysed for mutations in quinolone resistance-determining regions (QRDR), acquired quinolone resistance (AQR) genes and the role of efflux pumps in resistance. All QRDR mutations in gyrA (codons 85 and 89) and parC (codons 83 and 231) were found to be closely associated with ciprofloxacin resistance. The AQR gene aac(6’)-Ib-cr was found in 42.9 % (9/21) of the resistant strains, but qnrA, qnrB, qnrC, qnrD, qnrS and qepA were not detected. No significant change of MICs to ciprofloxacin was observed in the presence of an efflux pump inhibitor, indicating the role of efflux pump was probably absent. All 21 ciprofloxacin-resistant strains showed different electrophoretic patterns, which suggested they were not genetically related. These data highlight the importance of QRDR mutations and the AQR gene aac(6’)-Ib-cr during the development of quinolone resistance in a heterogeneous population of L. hongkongensis.

INTRODUCTION

Laribacter hongkongensis, a novel genus and species of the family Neisseriaceae, was first isolated from the blood and empyema pus of a Hong Kong patient in 2001 (Yuen et al., 2001). Subsequent reports showed that the bacterium was associated with community-acquired gastroenteritis and traveller’s diarrhoea. Isolation of L. hongkongensis has been reported in patients from Asia, Europe, Africa and Central America, suggesting that L. hongkongensis is of global importance (Ni et al., 2007; Woo et al., 2004). The clinical syndrome of patients positive for L. hongkongensis in stools is similar to those of Salmonella- or Campylobacter-infected patients. Most patients have watery or bloody diarrhoea, and some of them may have systemic symptoms (Woo et al., 2005), but there have been no reported deaths for Laribacter-associated gastroenteritis so far. In 2009, the full genome sequence of L. hongkongensis was published and a series of genes were predicted to be associated with intestinal colonization, host defence evasion and bacterial virulence (Lau et al., 2011; Woo et al., 2009). However, experimental evidence addressing these issues is still largely absent. The research on pathogenesis and immunology related to L. hongkongensis has also been greatly hampered by the lack of genetic manipulation systems and animal models for this bacterium.

Eating fish was found to be a risk factor for Laribacter-associated gastroenteritis, and most infection cases were reported from southern China, where freshwater fish and frogs were commonly consumed. Evidence from epidemiological studies showed that the intestines of freshwater fish and frogs were natural reservoirs for the bacterium, with recovery rates of more than 50 % from these aquatic products (Lau et al., 2009a; Teng et al., 2005). These data strongly suggest that contaminated aquatic products have the potential to infect the human host.
L. hongkongensis strains isolated from both humans and fresh water animals have been found to be resistant to multiple antibiotics (Feng et al., 2011; Lau et al., 2009b). Resistance to β-lactams, including penicillins and cephalosporins, has been detected in all isolates, which was due to the presence of a novel chromosomal class C β-lactamase (Lau et al., 2005). Tetracycline resistance was also detected in 10–20% of the isolates, with plasmid-encoded tetA being found in these strains (Lau et al., 2008). This is the only study so far describing plasmid-mediated antibiotic resistance in this bacterium, although the carriage rate for plasmids was greater than 30% in a collection of 21 clinical L. hongkongensis isolates (Woo et al., 2007). The emergence of ciprofloxacin-resistant L. hongkongensis of animal origin has raised concern, as fluoroquinolones are recommended for patients who need antimicrobial therapy (Feng et al., 2011; Lau et al., 2005). However, the molecular mechanism for quinolone resistance in this bacterium is still unknown. The objective of this study was to investigate the major known mechanisms for quinolone resistance, including quinolone resistance-determining region (QRDR) mutations, acquired quinolone resistance (AQR) genes and the involvement of active efflux pumps in L. hongkongensis isolates from grass carp and Chinese tiger frog.

**METHODS**

**Samples and bacterial strains.** Two kinds of farm-raised aquatic food animals with a high recovery rate of L. hongkongensis, grass carp (Ctenopharyngodon idella) and Chinese tiger frog (Hoplobatrachus chinensis), were sampled for bacterial isolation. A total of 443 grass carp and 171 Chinese tiger frogs were taken from seven retail food markets in Guangzhou City, China, between March and September in 2011. After transporting to a microbiology laboratory, live animals were sacrificed and intestinal samples obtained. A standard swab and selective culture technique was applied for bacterial isolation, which enabled fast and accurate detection of the samples (Lau et al., 2003; Teng et al., 2005). The intestinal swabs from each sample were streaked onto MacConkey agar plates containing 32 μg cefoperazone ml⁻¹. Lactose-negative colonies were picked from the plates after incubation at 37°C for 48 h. Biochemical tests for bacterial identification were carried out as described previously (Yuen et al., 2001). Gram-negative bacterial isolates positive for catalase, cytochrome oxidase, urease and arginine dihydrolase, and negative for sugar utilization, were subjected to a specific 16S rRNA gene PCR for further identification (Lau et al., 2009a). Bacterial isolates were identified as L. hongkongensis when they were in accordance with the reported biochemical profile and positive for the 16S rRNA gene PCR. The first L. hongkongensis isolate in mainland China was ZSU1 (Chen et al., 2004), and this provides clear information for the biochemical profile, antibiotic susceptibility and 16S rRNA gene sequences; this was used as a positive control in biochemical identification and PCR analysis.

**Antimicrobial susceptibility testing.** Susceptibility of L. hongkongensis to quinolones was evaluated by the disc diffusion method. Discs (Oxoid) with the following concentrations were used: nalidixic acid (NAL), 30 μg; ciprofloxacin (CIP), 5 μg; levofloxacin (LEV), 10 μg; enrofloxacin (ENR), 10 μg; norfloxacin (NOR), 10 μg. MICs to NAL, CIP and efflux pump inhibitor carbonyl cyanide m-chlorophenylhydrazine (CCCP; Sigma) were detected by the agar dilution method. The Clinical and Laboratory Standards Institute tentative standard for Enterobacteriaceae was used to interpret the disc diffusion zone diameters and MICs for susceptibility as described previously (Lau et al., 2009b). *Escherichia coli* ATCC 25922 was used as a control strain for susceptibility testing. To evaluate the role of efflux pumps affecting quinolone resistance, MICs to CIP were further detected in the presence of CCCP in CIP-resistant strains. A fourfold reduction in the MIC following CCCP addition was considered significant (Alcaide et al., 2010).

**Detection of QRDR mutations in gyrA and parC.** To determine the relationship between QRDR mutations and quinolone resistance in L. hongkongensis, CIP-resistant strains were analysed by PCR and sequencing for gyrA and parC sequences using the primers listed in Table 1. Both genes were also amplified and sequenced in 10 quinolone susceptible L. hongkongensis strains as controls. The primers were designed according to the published genome sequence of L. hongkongensis (Woo et al., 2009). PCR was performed in a 20 μl reaction volume containing 10 μl PCR Master Mix (Takara), 1 μl bacterial DNA and 0.5 μM each primer. PCR consisted of initial denaturation at 94°C for 5 min, 35 cycles of 1 min at 94°C, 40 s at 52°C (for gyrA) or 63°C (for parC) and 1 min at 72°C, plus a final extension at 72°C for 5 min. PCR products were purified by QIAquick PCR Purification kits (Qiagen). Both strands of the PCR product were sequenced with an ABI 3730 sequencer according to the manufacturer’s instructions. All sequencing data were analysed by the BLASTX program and compared with the gyrA and parC sequences of *HLHK9*, a clinical L. hongkongensis isolate sensitive to CIP (Lau et al., 2009b).

**Detection of AQR genes.** AQR genes qnrA, qnrB, qnrC, qnrD, qnrS, aac(6’)-Ib-cr and qepA were detected by PCR amplification as described previously (Cavaco et al., 2009; Kim et al., 2009). Primers used for PCR are listed in Table 1. PCR products were purified, sequenced and analysed as described above.

**Repertive sequence-based PCR (Rep-PCR).** The genetic diversity among 21 CIP-resistant L. hongkongensis strains was determined by Rep-PCR as described previously (Feng et al., 2013) with minor modifications. Briefly, bacterial total DNA was purified by DNA extraction kits (Qiagen). Rep-PCR was performed with 0.5 μM each primer (Rep1: 5’-ATGTAAGCTCCTGGGGATTCAC-3’ and Rep2: 5’-AAGTTAAGTACGTTGGGATGAGC-3’). PCR reaction mixture (20 μl) contained 10 μl PCR Master Mix (Takara) and 1 μl DNA sample. PCR amplification was carried out by 35 cycles of 94°C for 30 s, 48°C for 45 s, and 72°C for 4 min, and a final extension at 72°C for 10 min. PCR products were electrophoresed in 2% agarose gel in TBE buffer. The gel was stained with ethidium bromide and photographed under UV light illumination. The gel images were analysed visually and with Quantity One software (version 4.6.2; Bio-Rad Laboratories). The Rep-PCR patterns were subjected to cluster analysis by the unweighted-pair group method with arithmetic mean (UPGMA) linkages with 1% tolerance and 0.5% optimization settings.

**Statistical analyses.** A χ² test was used to compare the quinolone resistance rates of L. hongkongensis between fish and frog isolates. \( P<0.05 \) was considered statistically significant.

**RESULTS AND DISCUSSION**

**Quinolone resistance in L. hongkongensis**

Of the 614 intestine samples (443 from grass carp and 171 from Chinese tiger frogs), 157 (25.6%) were positive for L.
hongkongensis (82 from grass carp and 75 from Chinese tiger frogs). Although several colonies were selected and identified for each positive sample, only one isolate for an individual sample was used for susceptibility testing to avoid possible clonality of resistant bacteria in the same animal. Therefore, a total of 157 unique L. hongkongensis isolates from the positive samples were collected in this study, and the disc diffusion method and agar dilution method were carried out to evaluate the susceptibility to quinolones in these strains. Only 13.4% of the fish isolates were resistant to NAL and none was resistant to fluoroquinolones using disc diffusion. However, a significantly higher resistance rate to fluoroquinolones was detected for frog isolates (\( P < 0.05 \) by \( x^2 \) test), with 32, 8, 34.5 and 17.3% of the strains resistant to CIP, LEV, NOR, and ENR, respectively. A similar resistance pattern was detected by the agar dilution method for MICs to NAL and CIP, although an increase in resistance was observed by agar dilution (Table 2). As a validated interpretative criterion for quinolone resistant L. hongkongensis has not yet been established, 21 isolates resistant to CIP by both disk diffusion and agar dilution were selected for further mechanistic study.

The MIC\(_{50}\) values for NAL and CIP for L. hongkongensis were 32 and 0.25 µg ml\(^{-1}\), respectively. The MIC\(_{50}\) of CIP showed an eightfold increase (0.25 vs 0.03 µg ml\(^{-1}\)) compared with a previous study (Lau et al., 2009b). As the aquatic animals selected for this study were from freshwater fish aquaculture, the increased resistance may be related to the excessive application of quinolones during fish culture in China. Interestingly, all fish isolates were sensitive to fluoroquinolones, whilst a significant proportion of frog isolates exhibited a resistant phenotype. It is largely unknown why resistance to these antibiotics is acquired more easily in frog isolates. A possible explanation is that horizontal transfer of resistant genes is more active among gut flora in frogs. Feng et al. (2011) reported that integron-positive L. hongkongensis were mostly isolated from frogs, and CIP resistance was commonly found in these strains. This suggests that integron-associated genetic elements may play an important role in the development of resistance.

### Table 1. PCR primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequences (5(\rightarrow)3())</th>
<th>Size (nt)</th>
<th>Reference</th>
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<td>gyrA</td>
<td>gyrA-F</td>
<td>GTGCCGTCCGTGCTAGAATC</td>
<td>819</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>gyrA-R</td>
<td>CCGATGTCTTGCAGTGGGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>parC</td>
<td>parC-F</td>
<td>AAGCAGGCGCCAGGCTTTAT</td>
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<td>parC-R</td>
<td>ACTTCCTACGGGACGTGAAC</td>
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<td>Kim et al. (2009)</td>
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<tr>
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<td>476</td>
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<td></td>
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<td>qnrC-F</td>
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<td>307</td>
<td>Kim et al. (2009)</td>
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<td>qnrD</td>
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<td></td>
<td>qnrD-R</td>
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<td></td>
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<tr>
<td>qnrS</td>
<td>qnrS-F</td>
<td>GCAAAGTCAATTTATGGAACGGT</td>
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<td>Kim et al. (2009)</td>
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<td>qnrS-R</td>
<td>TCTAAACCGTGAGTGGGGG</td>
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<td>aac(6(^9))-Ib-cr</td>
<td>aac(6(^9))-IbF</td>
<td>TTGGATGAATCTATGAGTGGGTA</td>
<td>482</td>
<td>Kim et al. (2009)</td>
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<td>aac(6(^9))-IbR</td>
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<td>qepA</td>
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<td>qepA-R</td>
<td>GTCTACGGAATGGCCTCAC</td>
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</table>

### Table 2. Distribution of quinolone resistance in 157 L. hongkongensis isolates

<table>
<thead>
<tr>
<th>Origin</th>
<th>Percentage (( n )) of antibiotic resistance in isolates</th>
<th>Disc diffusion</th>
<th>Agar dilution</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>NAL</td>
<td>CIP*</td>
<td>LEV</td>
</tr>
<tr>
<td>Grass carp (( n=82 ))</td>
<td>13.4 (11)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Chinese tiger frog (( n=75 ))</td>
<td>70.7 (53)</td>
<td>32.0 (24)</td>
<td>8 (6)</td>
</tr>
</tbody>
</table>

*Twenty-one isolates that were resistant to CIP by both disc diffusion and agar dilution were used for further mechanistic study.
fluoroquinolone resistance in frogs, and merits further investigation.

**QRDR mutations in ciprofloxacin-resistant *L. hongkongensis***

QRDR mutations of *gyrA* and *parC* were analysed by PCR and sequencing in 21 ciprofloxacin-resistant isolates. Ten quinolone-sensitive isolates, including ZSU1, were also sequenced as controls. As shown in Table 3, more than 70% of the resistant strains had two mutations in *gyrA*, with T85I and D89N in nine isolates, and T85I and D89G in six isolates. Two QRDR mutations, S83R and R231C, were also identified in *parC* among resistant strains. It is noteworthy that none of these mutations was detected in all 10 sensitive strains in this study and clinical isolate HLHK9, suggesting that these mutations are associated with CIP resistance in *L. hongkongensis*. Furthermore, 90% (19/21) of the strains carried at least two mutations in these genes, but a correlation between higher levels of CIP MICs and stepwise accumulation of mutations was not observed.

Substitutions within the regions of codons 80–90 in *gyrA* and *parC* have been reported to confer quinolone resistance in many Gram-negative bacteria (Fábrega et al., 2008; Weigel et al., 1998). Mutations within codons 85 and 89 in *gyrA*, and codon 83 in *parC* were also found to be closely associated with CIP resistance in *L. hongkongensis*. Interestingly, a novel mutation (R231C) was identified in six resistant strains, but was absent in sensitive strains. Although the exact impact of this mutation is unclear, our results highlight the possible role of amino acids beyond the traditional QRDR region of *parC* in drug-target interaction.

**Detection of AQR genes**

The presence of AQR genes in *L. hongkongensis* isolates was investigated by a PCR detection theme. Nine strains with CIP resistance were positive for *aac(6')-Ib-cr* (Table 3). Sequencing of the PCR products showed that all *aac(6')-Ib-cr* amplicons demonstrated 100% protein sequence identity to the *aac(6')-Ib-cr* gene from *E. coli* plasmid pEC_L8 (Smet et al., 2010). No other AQR genes (*qnrA, qnrB, qnrC, qnrD, qnr* and *qepA*) were detected in this study. All *L. hongkongensis* strains positive for AQR gene *aac(6')-Ib-cr* were resistant to CIP and NOR, but most (8/9) were sensitive or intermediate to LEV and enrofloxacin (Table 3). This is consistent with the enzyme activity of *aac(6')-Ib-cr*, which modifies CIP and NOR but not other fluoroquinolones. This AQR gene was widely disseminated in clinical *Enterobacteriaceae* isolates and always associated with extended-spectrum *ß*-lactamase genes (Yang et al., 2008). The current report represents the first identification of *aac(6')-Ib-cr* in bacteria of the family *Neisseriaceae*. In addition, the QRDR mutation D89G in the *gyrA* gene was identified in 66.7% (6/9) of *L. hongkongensis* isolates.
carrying \textit{aac(6')-Ib-cr} but not in any (0/12) \textit{aac(6')-Ib-cr}-negative isolates (Table 3). Although the possibility that the D89G mutation in \textit{gyrA} facilitates the acquisition of \textit{aac(6')-Ib-cr} could not be excluded, it is more likely that expression of \textit{aac(6')-Ib-cr} in \textit{L. hongkongensis} induces D89G mutation in \textit{gyrA}, since the presence of \textit{aac(6')-Ib-cr} is known to increase the frequency of selection of chromosomal mutants upon exposure to CIP (Robicsek \textit{et al.}, 2006).

**Involvement of active efflux pumps**

Most CIP-resistant strains exhibited high sensitivities to CCCP with MICs that ranged from 0.32 to 1.25 \(\mu\text{g}\text{ml}^{-1}\). A fixed CCCP concentration of 0.1 \(\mu\text{g}\text{ml}^{-1}\) was selected and the MICs of CIP were determined in the presence of the efflux pump inhibitor. As shown in Table 3, although one isolate (W59) exhibited a twofold reduction in CIP MIC, no significant (twofold) decrease of CIP MICs could be observed in the isolates tested, suggesting that efflux pumps are not likely to be involved in CIP resistance.

**Rep-PCR patterns**

Rep-PCR has proven to be a fast and effective technique for molecular typing of \textit{L. hongkongensis} (Feng \textit{et al.}, 2013), and was applied here to investigate whether the CIP-resistant isolates in our collection were genetically related. As shown in Fig. 1, 21 different banding patterns were revealed by Rep-PCR analysis of the 21 CIP-resistant \textit{L. hongkongensis} strains. CIP-sensitive strain ZSU1 was also loaded for reference. Molecular typing by Rep-PCR produced 6–12 bands ranging from 200 to 3000 bp. Using cut-off values of 60 and 80\%, the 21 genotypes could be further grouped into eight clusters and 15 subtypes. No precise correlation could be detected among genotypes and resistance characteristics, including CIP MICs, QRDR mutations and AQR genes. This suggests that, in accordance with previous studies (Feng \textit{et al.}, 2011, 2012), CIP-resistant \textit{L. hongkongensis} strains from aquatic products from China exhibit highly diverse genetic relations.

In this study, two CIP-resistant isolates (W66 and W68) did not carry any QRDR mutations in \textit{gyrA} and \textit{parC}, and neither AQR genes nor efflux-pump-mediated mechanisms were present in both strains. Furthermore, they were genetically diverse by Rep-PCR analysis (Fig. 1). It was possible that such a resistant phenotype was conferred by mechanisms not investigated in this study, including QRDR mutations in \textit{gyrB} and \textit{parE}, a lack of or lower expression of porins, and elevated levels of efflux pumps. However, as these resistance mechanisms rarely act as major factors leading to quinolone resistance (Hernández \textit{et al.}, 2011), the possible existence of unknown mechanisms with a strong effect on resistance development could not be ruled out. Further studies may be required to elaborate the possible resistance mechanisms in these \textit{L. hongkongensis} isolates.

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**REFERENCES**


