High Prevalence and Mechanism Associated With Extended Spectrum Beta-Lactamase-Positive Phenotype in Laribacter hongkongensis

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In this study, we reported the prevalence and mechanism associated with the extended-spectrum beta-lactamase (ESBL)-positive phenotype in Laribacter hongkongensis isolated from patients and fish. Using the inhibition zone enhancement test, 20 (95.2%) of the 21 patient strains and 8 (57.1%) of the 14 fish strains were tested ESBL-positive. However, ESBL genes, including SHV, TEM, CTX-M, GES, and PER, were not detected in all of these 28 L. hongkongensis isolates. No ESBL gene could be detected in either the complete genome of L. hongkongensis HLHK9 or the draft genome of PW3643. PCR and DNA sequencing revealed that all the 35 L. hongkongensis isolates (showing both ESBL-positive and ESBL-negative phenotypes) were positive for the ampC gene. When the AmpC deletion mutant, HLHK9 ΔampC, was subject to the zone enhancement test, the difference of zone size between ceftazidime/clavulanate and ceftazidime was less than 5 mm. When boronic acid was added to the antibiotic disks, none of the 28 “ESBL-positive” isolates showed a ≥ 5 mm enhancement of inhibition zone size diameter between ceftazidime/clavulanate and ceftazidime and between cefotaxime/clavulanate and ceftaxime. A high prevalence (80%) of ESBL-positive phenotype is present in L. hongkongensis. Overall, our results suggested that the ESBL-positive phenotype in L. hongkongensis results from the expression of the intrinsic AmpC beta-lactamase. Confirmatory tests should be performed before issuing laboratory reports for L. hongkongensis isolates that are tested ESBL-positive by disk diffusion clavulanate inhibition test.

Keywords: ESBL, Laribacter hongkongensis, AmpC beta-lactamase, prevalence, mechanism

INTRODUCTION

Laribacter hongkongensis is a globally distributed Gram-negative, facultative anaerobic, motile, S-shaped, asaccharolytic, urease-positive bacillus in the Chromobacteriaceae family of beta-proteobacteria (Woo et al., 2003, 2004; Ni et al., 2007; Kim et al., 2011; Beilfuss et al., 2015). L. hongkongensis causes highly fatal bacteremic sepsis in patients with underlying liver diseases
AmpC was only distantly related to other chromosomal- or plasmid-encoded class C beta-lactamases, sharing <50% amino acid sequence identities, but the kinetic properties of this novel AmpC was compatible with those of a class C beta-lactamase (Lau et al., 2005). To test the hypothesis, we deleted the ampC gene of a strain of *L. hongkongensis* and studied its change in phenotype. Finally, we examined the use of a boronic acid-based phenotypic test on all the *L. hongkongensis* strains with ESBL-positive phenotype.

**MATERIALS AND METHODS**

**Bacterial Strains**

This study was approved by the Institutional Review Board (IRB) of The University of Hong Kong/Hospital Authority Hong Kong West Cluster, Hong Kong East Cluster Research Ethics Committee. The index isolate refers to the first *L. hongkongensis* strain, PW3643, from which we observed the ESBL-positive phenotype. It was isolated from the stool sample of a patient with gastroenteritis in Hong Kong in 2018. In addition to strain PW3643, the present study also included 34 non-duplicated *L. hongkongensis* strains, 20 of which were previously isolated from patients (Woo et al., 2003, 2004) and 14 from grass carps. For the 20 patient strains, one (HLHK1) was isolated from the blood culture and empyema pus of a patient with bacteremic empyema thoracis (Yuen et al., 2001). The other 19 strains (HLHK2 to HLHK20) were recovered from the stool of patients with community-acquired gastroenteritis (Woo et al., 2003, 2004). For the 14 fish strains, they were isolated from grass carps which were purchased during June to August 2018 from retail markets at three different locations in Hong Kong. The identities of all 35 *L. hongkongensis* strains were confirmed by standard conventional biochemical methods, and 16S ribosomal RNA gene sequencing (Lau et al., 2003; Woo et al., 2004).

**Inhibition Zone Enhancement Test for ESBLs**

Since no existing CLSI guidelines were available for ESBL testing of *L. hongkongensis*, ESBL phenotypic tests were performed and interpreted according to methods for testing *Enterobacteriaceae* (CLSI, 2019). Briefly, the inhibition zone enhancement test was carried out by inoculating a 0.5 McFarland standard suspension of overnight culture on Mueller-Hinton agar (Bio-Rad, United States) and applying antibiotic disks (BD BBL™ Sensi-Disc™, Benton Dickinson, United States) of cefotaxime (CTX-30 µg) and ceftazidime (CAZ-30 µg) alone and in combination with clavulanic acid (CTX-CLA 30/10 µg or CAZ-CLA 30/10 µg) following the standard procedure (CLSI, 2019). The plates were incubated at 37°C for 18 h. Upon incubation, the diameter of inhibition zone around each disk was measured following the CLSI guideline. An ESBL-positive phenotype was confirmed when a ≥ 5 mm enhancement of the zone of inhibition was observed in CTX-CLA or CAZ-CLA compared with the diameter of the zone of inhibition of the respective antimicrobial disk alone. *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were used as positive and negative controls, respectively.
The modified CLSI ESBL phenotypic test developed by Poulou's group was also performed using the same procedures as described in the previous paragraph with one modification, in which 10 µl of boronic acid (30 mg/ml) was added to the antimicrobial disk before they were deposited onto the inoculated Mueller-Hinton agar (Poulou et al., 2014).

Bacterial RNA Extraction
*L. hongkongensis* cultures grown to mid-log phase were first stabilized with the RNAProtect bacterial reagent (QIAGEN, Germany) followed by isolation of total RNA using RNeasy Mini kit (QIAGEN, Germany) according to the manufacturer's protocol. The extracted RNA was treated with RNase-free DNase I (Roche, Switzerland) at 37°C for 45 min to degrade the genomic DNA (Xiong et al., 2017).

PCR Detection of ESBL and AmpC Beta-Lactamase Genes
Bacterial DNA extraction was performed as described previously (Woo et al., 2004). DNA extracts of all *L. hongkongensis* isolates with the ESBL phenotype were subject to PCR and sequencing with conditions as described by Dalenne et al. (2010) for the detection of ESBL genes. They were also subject to PCR for detection of the *ampC* gene, which encodes the AmpC beta-lactamase, following a previously published protocol with newly designed PCR and DNA sequencing primers (5′-CCAGATTCCGGGACATGGC-3′; 5′-CCATCGAGCCATGCTCGG-3′) (Lau et al., 2005). The reaction was performed in a GeneAmp PCR System 9,700 with the following conditions: 10 min at 95°C, followed by 40 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min. Standard precautions were taken to avoid PCR contamination, and no false-positive result was observed for negative controls.

Real-Time Quantitative RT-PCR (qRT-PCR)
qRT-PCR was performed using an ABI7900HT Fast Real Time PCR machine (Applied Biosystems, United States) with FastStart DNA Master SYBR Green I Mix reagent kit (Roche), as described by the manufacturer. The mRNA levels of *ampC* in *L. hongkongensis* isolates with and without ESBL phenotype were measured, respectively, by quantitation of cDNA. The calculated threshold cycle (CT) corresponding to the target gene was calculated as 2^((CtTarget–CtReference)) , normalized to the rpoB gene (Xiong et al., 2017). The sequences of the primers used for qRT-PCR are listed in Table 2.

Genome Sequencing and Assembly
The draft genome sequence of the index isolate PW3643 was determined by high-throughput sequencing using the Illumina Hi-Seq 1500 platform as described previously (Teng et al., 2017). Genomic DNA was extracted from overnight cultures grown on 5% horse blood agar (BD Benton Dickinson, United States) at 37°C using the genomic DNA purification kit (Qiagen, Germany). Extracted DNA was then sequenced by 151 bp paired-end reads with mean library size of 350 bp. *De novo* assembly was performed using SPAdes (Bankevich et al., 2012).

Genome Annotation and Phylogenetic Analysis of AmpC Gene
To confirm the species identity at the genome level, intergenomic distances [i.e., average nucleotide identity (ANI) values] between HLHK9 (GenBank accession number NC_012559.1), PW3643 (GenBank accession number LZOW00000000), and the type strain HLHK1 (GenBank accession number NZ_AUHR01000001.1) were calculated using web services available at http://enve-omics.ce.gatech.edu/ (Goris et al., 2007). Prediction of protein coding regions and automatic functional annotation of the *L. hongkongensis* genome for strain HLHK9 we published previously (Woo et al., 2009) and that of PW3643 was performed using RAST (Rapid Annotations using Subsystem Technology) server version 2.03 (Aziz et al., 2008). Identification of ESBL-encoding genes was performed by blasting the genomes using the ResFinder 3.1 with default parameters (Zankari et al., 2012).

Phylogenetic tree was constructed based on a 123-aa AmpC protein fragment in MEGA X using the maximum likelihood method based on the Jones-Taylor-Thornton model with uniform rates and bootstrap values calculated from 1,000 replicates (Kumar et al., 2018).

Construction of AmpC Deletion Mutant of *L. hongkongensis* HLHK9
Unmarked, non-polar deletion of *ampC* was constructed by homologous recombination using the suicide plasmid pCVD442 as described previously (Xiong et al., 2014). Bacterial strains and plasmids and the primers used for the deletion mutagenesis are listed in Tables 1, 2, respectively. Briefly, the in-frame deletion arrangement of *ampC* containing its 5′- and 3′-flanking regions was generated using the overlap PCR method and subcloned into pCVD442. The resulting plasmid was transferred into HLHK9 by bacterial conjugation from *E. coli* SM10 λ pir. The selection of allelic replacement was performed as described previously (Xiong et al., 2015) and the mutant strain was further confirmed by PCR using primers (LPW31555/31556) specific for the deleted sequence (Table 2). All mutant strains were confirmed by DNA sequencing.

Antimicrobial Susceptibility Testing of AmpC Deletion Mutant and Wild Type *L. hongkongensis* HLHK9
*In vitro* antimicrobial susceptibility testing was performed in triplicate using the broth microdilution method according to CLSI guidelines (CLSI, 2019). Freshly prepared in-house 96-well microtiter panels were used to test the antimicrobial agents at their respective concentration ranges [ampicillin: 0.05–128 mg/L; amoxicillin/clavulanate: 0.03125–128 mg/L (at 2:1 ratio as recommended by CLSI); cefotaxime: 1–256 mg/L; ceftriaxone: 0.25–512 mg/L; cefuroxime: 2–128 mg/L]. The minimum inhibitory concentration of antimicrobial agents was
defined as the lowest concentration that inhibited visible growth of the microorganism and results were interpreted as susceptible, defined as the lowest concentration that inhibited visible growth.

Inhibition Zone Enhancement Test for ESBLs
Using the inhibition zone enhancement test, 20 (95.2%) of the 21 L. hongkongensis patient strains, including the index isolate, were tested ESBL-positive (Figures 1A, 2A and Table 3). One patient isolate (HLHK1T) was negative for the ESBL-positive phenotype (Table 3). For these 20 patient isolates with ESBL-positive phenotype, 10 (50%; HLHK7, HLHK8, HLHK10- HLHK12, HLHK14, and HLHK16-HLHK19) had a ≥ 5 mm enhancement of inhibition zone around both CTX-CLA and CAZ-CLA when compared with the inhibition zone of the respective antimicrobial disk alone (CTX or CAZ). The other 10 isolates (50%; PW3643, HLHK2-HLHK6, HLHK9, HLHK13, HLHK15, and HLHK20) had a ≥ 5 mm enhancement of inhibition zone around CAZ-CLA but not CTX-CLA when compared with the inhibition zone diameter of the respective antimicrobial disk alone (Table 3). As for the 14 L. hongkongensis fish isolates, 8 (57.1%) were tested ESBL-positive (Table 3). Six (75%; F2, F4, F6, F10, F12, and F13) of them had a ≥ 5 mm enhancement of inhibition zone around both CTX-CLA and CAZ-CLA, while the remaining 2 (25%; F3 and F14) had a ≥ 5 mm enhancement of inhibition zone around CAZ-CLA but not CTX-CLA (Table 3).

PCR Detection of ESBL Genes
Detection of ESBL genes, including SHV, TEM, CTX-M, GES, and PER types, by PCR was negative in all the 28 (20 from patients and 8 from fish) L. hongkongensis isolates with ESBL-positive phenotypes.

Genome Analysis of L. hongkongensis HLHK9 and Index Isolate PW3643
In both the complete genome of L. hongkongensis HLHK9 and the draft genome of the index isolate PW3643, no known ESBL gene was observed. On the other hand, a putative ampC gene encoding an AmpC beta-lactamase was identified in both genomes.

PCR Detection and Sequencing of AmpC Beta-Lactamase in L. hongkongensis Isolates
We have confirmed the presence of the ampC gene in all L. hongkongensis isolates, including the patient isolates HLHK1- HLHK20 (Lau et al., 2005), the index isolate PW3643, and all the fish isolates (8 ESBL-positive and 6 ESBL-negative isolates) (Table 3). Sequence analysis showed high similarities between the patient and fish isolates and they shared 95–100% amino acid identities among each other. The topology

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**TABLE 1** | Bacterial strains and plasmids used in this study.

| Strains or plasmids | Relative characteristics | Source or references |
|---------------------|-------------------------|---------------------|
| **Strains**         |                         |                     |
| E. coli SM10(ompT)  | Donor strain for conjugation | Xiong et al. 14     |
| HLHK9               | Patient isolate, Cef<sup>+</sup>, Sm<sup>+</sup> | Xiong et al. 15     |
| HLHK9ampC           | HLHK9 derivative with ampC deletion | This study          |
| **Plasmid**         |                         |                     |
| pCVD442             | Suicide plasmid; R6K ori mob RP4 bis sacB | Xiong et al. 15     |

**TABLE 2** | Primers used in this study.

| Primers | Sequence (5’–3’)<sup>3</sup> |
|---------|-------------------------------|
| **For mutagenesis of ampC** |             |
| LPW38006 (ampC-1F) | GCTCTAGAGTCATTACGCAGGATGTCCTTCCTGGATGTCATTACGGCATGTGCACTGTATG |
| LPW38007 (ampC-1R) | CAATTCTAGATGAGCCTGTTTCTCCGTATG |
| LPW38008 (ampC-2F) | CGGAAAACACGGGCTACATGACTTGATGGTTTGGATG |
| LPW38009 (ampC-2R) | CGCATTGCTCGAGAACTTTCGCTGCA |
| LPW36155 (ampC-nip-F) | CATGCGCACTACGCGAATGCTCCGGG |
| LPW36156 (ampC-nip-RF) | GTCCGTCTCGCTAGGAG |
| **For qRT-PCR of ampC** |             |
| LPW21635 (rpoB-F) | TCTAGA |
| LPW21636 (rpoB-R) | GCATGC |

1<sup>3</sup>Restriction sites in the primer sequences appear in bold.

results

**RESULTS**

Identification of L. hongkongensis
All suspected L. hongkongensis isolates exhibited the same phenotypic properties and biochemical profiles: All appeared as lactose-negative colonies on cefoperazone MacConkey agar (a selective agar medium for isolation of L. hongkongensis), seagull-shapes, motile, Gram-negative, bacteria positive for catalase, cytochrome oxidase, urease and arginine dihydrolase, and negative for sugar oxidation/fermentation. Sequence analysis of the 1,318 bp 16S rRNA gene fragment (nucleotide positions 18–1,335 corresponding to the 16S rRNA gene sequence of HLHK1<sup>T</sup>) among these isolates demonstrated ≥ 99% nucleotide identities to that of L. hongkongensis HLHK1<sup>T</sup>. *In silico* genome-to-genome comparisons showed that ANI values between the type strain HLHK1<sup>T</sup> and PW3643 was 97.8%, while between the type strain HLHK1<sup>T</sup> and HLHK9 was 98.9%, which fall in ANI threshold range (95–96%) for species demarcation.

**PCR Detection and Sequencing of AmpC Beta-Lactamase in L. hongkongensis Isolates**
We have confirmed the presence of the ampC gene in all L. hongkongensis isolates, including the patient isolates HLHK1–HLHK20 (Lau et al., 2005), the index isolate PW3643, and all the fish isolates (8 ESBL-positive and 6 ESBL-negative isolates) (Table 3). Sequence analysis showed high similarities between the patient and fish isolates and they shared 95–100% amino acid identities among each other. The topology...
of the phylogenetic tree based on the amino acid sequences of AmpC did not reveal any distinct cluster of *L. hongkongensis* isolates with ESBL-positive phenotype (Figure 3). Multiple alignment of the AmpC amino acid sequences also did not reveal any specific mutation associated with each ESBL phenotype (Figure 4). qRT-PCR showed that expression levels of ampC in *L. hongkongensis* isolates with ESBL-negative phenotype were significantly lower than those with ESBL-negative phenotype (Figure 5).

**Antimicrobial Susceptibility and ESBL Phenotype of AmpC Deletion Mutant of *L. hongkongensis* HLHK9**

To examine the possible role of AmpC in causing the ESBL-positive phenotype in *L. hongkongensis*, an AmpC deletion mutant, HLHK9ΔampC, was generated and subject to the zone enhancement test in parallel with wild type HLHK9 (Figure 2). The in vitro activities of eight beta-lactams or beta-lactam/beta-lactamase inhibitor combinations against the AmpC deletion mutant and wild type HLHK9 were also tested. Results showed that the AmpC deletion mutant, HLHK9ΔampC, exhibited significant reduction in resistance to ampicillin (MIC from 64 to 1 mg/L), amoxicillin/clavulanate (MIC from 2 to 0.0625 mg/L), ceftazidime (MIC from 128 to 32 mg/L), ceftriaxone (MIC from 128 to 16 mg/L), and cefuroxime (MIC from 32 to 4 mg/L) when compared to the wild type HLHK9. No reduction in resistance was observed on cefepime, imipenem, and cefoperazone/sulbactam, to which the wild type HLHK9 was susceptible. The difference of zone size between CAZ/CLA and CAZ was reduced to less than 5 mm (Figure 2), suggesting that the ESBL-positive phenotype in *L. hongkongensis* was due to the AmpC beta-lactamase.

**Effect of AmpC Beta-Lactamase on Phenotypic Testing of ESBL**

Given that boronic acid has been shown to be an effective inhibitor for AmpC beta-lactamase (Song et al., 2007b; Poulou et al., 2014), a modified CLSI phenotypic confirmatory test based on boronic acid was employed to study the effect of AmpC on the ESBL-positive phenotypes in *L. hongkongensis*. Results showed that upon the addition of boronic acid, all the 28 isolates demonstrated a significant increase in susceptibility to CAZ or CTX, with 15 of them demonstrated an increase in susceptibility.

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**FIGURE 1** | CLSI ESBL confirmatory test of the index isolate PW3643. Results of (A) the CLSI ESBL disk diffusion test and (B) its modification using antibiotic disks containing boronic acid for the index isolate PW3643. CTX, cefotaxime; CTX/CLA, cefotaxime and clavulanic acid; CAZ, ceftazidime; CAZ/CLA, ceftazidime and clavulanic acid; BA, boronic acid.
inhibition zone diameter from 0 to > 20 mm (Table 3). Paper disk impregnated with boronic acid only did not result in growth inhibition (Figure 1B).

DISCUSSION

A high prevalence (80%) of ESBL-positive phenotype is present in *L. hongkongensis* isolated from both patients and fish. ESBL genes are most prevalent in bacteria from the gastrointestinal tract, such as *E. coli* and *K. pneumoniae*. In grass carp, bighead carps, and mud carps, *L. hongkongensis* is mainly isolated from their gutts (Teng et al., 2005). As for human, *L. hongkongensis* is most commonly associated with freshwater fish borne community-acquired gastroenteritis (Woo et al., 2004). In patients with underlying liver diseases, it translocates through the intestinal wall to the blood stream, causing highly fatal bactereemic sepsis (Yuen et al., 2001; Kim et al., 2011; Hung et al., 2020). In patients undergoing peritoneal dialysis, it can also lead to peritonitis presumably through the same route of infection (Woo et al., 2016). When we first received the index *L. hongkongensis* isolate PW3643 with ESBL-positive phenotype, we thought it might have acquired a plasmid with an ESBL gene from *E. coli*, *K. pneumoniae*, or other members of the *Enterobacteriaceae* family, as we have previously shown that shuttle plasmids that were able to replicate efficiently and propagate in both *E. coli* and *L. hongkongensis* can be easily constructed using plasmids originated from *L. hongkongensis* (Woo et al., 2005). However, PCR detection of the commonly encountered ESBL genes was negative in the index isolate. Therefore, we carried out this study to examine the prevalence and mechanism associated with ESBL-positive phenotype in *L. hongkongensis*. Our results showed that 95.2 and 57.1% of the patient and fish strains, respectively were positive for the ESBL phenotype by the disk diffusion clavulanate inhibition test recommended by CLSI for the detection of ESBL in *Enterobacteriaceae*. However, no known ESBL gene(s) were observed in any of these *L. hongkongensis* strains with ESBL-positive phenotypes. We retrieved the complete genome of *L. hongkongensis* HLHK9 which we have manually annotated more than 10 years ago and re-annotated using more sophisticated bioinformatics tools 7 years ago (Woo et al., 2009; Guo et al., 2013). In addition, we also performed whole genome sequencing of the index isolate. In both *L. hongkongensis* genomes, no known ESBL gene(s) were observed. It is indeed intriguing why ESBL genes were claimed to be detected in the previous studies from China to India (Raja and Ghosh, 2014; Wang et al., 2019), however, since no ESBL gene sequences were published, we cannot compare it with our genome sequence. A possibility is that their detected ESBL genes may contain variable genomic
| L. hongkongensis isolate | PCR detection of the ampC gene | Without boronic acid (inhibitory zone size in mm) | With boronic acid (inhibitory zone size in mm) | ESBL phenotype |
|-------------------------|--------------------------------|--------------------------------|--------------------------------|----------------|
|                         | CTX | CTX/CLA | ≥5 mm difference between CTX and CTX/CLA | CAZ | CAZ CLA | ≥5 mm difference between CAZ and CAZ/CLA |
| Human isolates (n = 21) |     |        |                                       |     |         |                                           |
| PW3643                  | +   | 17     | 21                                   | x   | 14      | 21                                         | ✓              | +         | 30 | 27 |
| HLUHK1 (Type strain)    | +   | 0      | 0                                    | x   | 0       | 23                                         | ✓              | +         | 26 | 28 |
| HLUHK2                  | +   | 9      | 9                                    | x   | 0       | 25                                         | ✓              | +         | 26 | 28 |
| HLUHK3                  | +   | 7      | 7                                    | x   | 0       | 23                                         | ✓              | +         | 30 | 29 |
| HLUHK4                  | +   | 24     | 24                                   | x   | 20      | 28                                         | ✓              | +         | 27 | 28 |
| HLUHK5                  | +   | 22     | 22                                   | x   | 15      | 24                                         | ✓              | +         | 30 | 30 |
| HLUHK6                  | +   | 15     | 24                                   | ✓   | 7       | 24                                         | ✓              | +         | 28 | 30 |
| HLUHK7                  | +   | 13     | 25                                   | ✓   | 0       | 25                                         | ✓              | +         | 25 | 26 |
| HLUHK8                  | +   | 21     | 23                                   | x   | 17      | 24                                         | ✓              | +         | 33 | 30 |
| HLUHK9                  | +   | 14     | 25                                   | ✓   | 0       | 24                                         | ✓              | +         | 28 | 27 |
| HLUHK10                 | +   | 15     | 25                                   | ✓   | 0       | 22                                         | ✓              | +         | 27 | 25 |
| HLUHK11                 | +   | 6      | 20                                   | ✓   | 0       | 9                                          | ✓              | +         | 26 | 28 |
| HLUHK12                 | +   | 27     | 27                                   | x   | 18      | 25                                         | ✓              | +         | 30 | 30 |
| HLUHK13                 | +   | 22     | 30                                   | ✓   | 20      | 26                                         | ✓              | +         | 28 | 30 |
| HLUHK15                 | +   | 21     | 25                                   | x   | 17      | 24                                         | ✓              | +         | 27 | 25 |
| HLUHK16                 | +   | 21     | 29                                   | ✓   | 18      | 25                                         | ✓              | +         | 29 | 26 |
| HLUHK17                 | +   | 21     | 28                                   | ✓   | 14      | 24                                         | ✓              | +         | 32 | 30 |
| HLUHK18                 | +   | 17     | 23                                   | ✓   | 15      | 21                                         | ✓              | +         | 32 | 31 |
| HLUHK19                 | +   | 17     | 25                                   | ✓   | 0       | 25                                         | ✓              | +         | 28 | 28 |
| HLUHK20                 | +   | 25     | 26                                   | x   | 19      | 25                                         | ✓              | +         | 28 | 26 |
| Fish isolates (n = 14)  |     |        |                                       |     |         |                                           |               |           |    |    |
| F1                      | +   | 29     | 32                                   | x   | 24      | 27                                         | x              | –         | 33 | 27 |
| F2                      | +   | 16     | 22                                   | ✓   | 0       | 21                                         | ✓              | +         | 29 | 26 |
| F3                      | +   | 19     | 23                                   | x   | 0       | 22                                         | ✓              | +         | 27 | 27 |
| F4                      | +   | 10     | 21                                   | ✓   | 0       | 21                                         | ✓              | +         | 25 | 24 |
| F5                      | +   | 30     | 32                                   | x   | 24      | 25                                         | x              | –         | 32 | 28 |
| F6                      | +   | 10     | 20                                   | ✓   | 0       | 20                                         | ✓              | +         | 23 | 23 |
| F7                      | +   | 27     | 28                                   | x   | 24      | 22                                         | x              | –         | 31 | 28 |
| F8                      | +   | 25     | 25                                   | x   | 23      | 22                                         | x              | –         | 29 | 26 |
| F9                      | +   | 27     | 30                                   | x   | 22      | 25                                         | x              | –         | 31 | 26 |
| F10                     | +   | 12     | 22                                   | ✓   | 0       | 21                                         | ✓              | +         | 23 | 23 |
| F11                     | +   | 27     | 29                                   | x   | 25      | 23                                         | x              | –         | 31 | 28 |
| F12                     | +   | 11     | 21                                   | ✓   | 0       | 20                                         | ✓              | +         | 24 | 23 |
| F13                     | +   | 11     | 22                                   | ✓   | 0       | 20                                         | ✓              | +         | 25 | 25 |
| F14                     | +   | 24     | 25                                   | x   | 18      | 24                                         | ✓              | +         | 30 | 27 |
**FIGURE 3** | Phylogenetic analysis of the AmpC of “ESBL-positive” and “ESBL-negative” *L. hongkongensis* isolates detected in the present study and their relationship with AmpC sequences of other *L. hongkongensis* isolates. The tree was constructed using the maximum likelihood method and *Pseudomonas oleovorans* (LR130779.1) as the root. Numbers at nodes indicated level of bootstrap support calculated from 1,000 replicates and the scale bar indicates the number of amino acid substitutions per site. Sequences determined in the present study are shown in bold.

**FIGURE 4** | Multiple alignment of amino acid sequences of the AmpC protein from *L. hongkongensis* with different ESBL phenotypes.
differences to known ESBL genes, hence our inability to detect it conventionally, or they may be false-positive PCR results. More sequence data from these two studies would be useful to validate their observations.

Our results suggested that the ESBL-positive phenotype in *L. hongkongensis* results from the expression of the intrinsic AmpC beta-lactamase. As it has been shown previously that the disk diffusion clavulanate inhibition test may give rise to ESBL-positive phenotype, with positive rates ranging from 2.2 to 47.4%, in clinical isolates of *Enterobacteriaceae* that produce chromosomal or plasmid-mediated AmpC beta-lactamase, such as *E. coli*, *Klebsiella* species, and *Enterobacter* species (Tzelepi et al., 2000; Song et al., 2007a; Jeong et al., 2008; Robberts et al., 2009; Poulou et al., 2014), we hypothesized that the ESBL-positive phenotype in *L. hongkongensis* could be due to its *ampC* gene (Lau et al., 2005), which is indeed present in all *L. hongkongensis* isolates tested. The hypothesis was first confirmed by comparing the results of the disk diffusion clavulanate inhibition test for HLHK9 and HLHK9Δ*ampC*. For HLHK9, the diameters of the inhibitory zone for CTX, CTX/CLA, CAZ, and CAZ/CLA were 21, 23, 17, and 24 mm, respectively (Figure 2A and Table 3). When the *ampC* gene of HLHK9 was deleted, the corresponding sizes were 37, 37, 34, and 36 mm, respectively (Figure 2B), suggesting that the *ampC* gene of HLHK9 was responsible for its ESBL-positive phenotype. This fact was further confirmed by a boronic acid-based phenotypic test. Since boronic acid is an inhibitor of AmpC beta-lactamase, the inhibitory zones for CTX and CAZ will become larger when boronic acid is added to the corresponding disks. For HLHK9, the diameters of the inhibitory zone for CTX and CAZ with boronic acid were 33 and 30 mm, respectively, as compared to 21 and 17 mm when there was no boronic acid (Figure 2A and Table 3). This phenomenon of inhibitory zones enlargement was observed in all the other 27 human and fish *L. hongkongensis* isolates with the ESBL-positive phenotypes. It is notable that *ampC* gene was in fact present in all *L. hongkongensis* isolates, but only 80% of them showed the ESBL-positive phenotype (Table 3). This may be due to the differential expression of *ampC* in *L. hongkongensis* as the qRT-PCR results showed that the expression of *ampC* in the remaining 20% of *L. hongkongensis* isolates (i.e., ESBL-negative phenotype) were lower than those with the ESBL-positive phenotype (Figure 5). We also noted that *L. hongkongensis* isolates exhibited variability in susceptibility profiles against CAZ and/or CTX, with more isolates resistant to CAZ (Table 3). Given the whole genome sequencing as well as the *ampC* deletion were only performed in a few *L. hongkongensis* isolates, further studies are required to determine whether the variations in resistance profiles of different *L. hongkongensis* isolates is due to the presence of other or unrecognized antimicrobial resistance genes.

Confirmatory tests should be performed before issuing laboratory reports for *L. hongkongensis* isolates that are tested ESBL-positive by disk diffusion clavulanate inhibition test. For severe infections caused by ESBL producing bacteria, the standard treatment is an intravenous carbapenem. Alternatively, piperacillin-tazobactam or the newer beta-lactam-beta-lactamase inhibitor combinations such as ceftazidime-avibactam or ceftolozane-tazobactam can be used if the isolate is susceptible. Cephalosporins alone should be avoided. For *L. hongkongensis*, although there is no study on its optimal treatment, *in vitro* susceptibility testing showed that it has variable susceptibilities to the cephalosporins. For the four cases of severe bacteremia sepsis reported in the literature, the two patients who survived received intravenous cefuroxime and cefotaxime, respectively (Yuen et al., 2001; Kim et al., 2011); whereas the two who succumbed were treated with meropenem (Tse et al., 2014; Hung et al., 2020). Although no statistical conclusions can be drawn, it suggests that cephalosporins can be considered if the isolate is susceptible, which is in line with the absence of ESBL production. As for infection control, recovery of an ESBL producing bacterium from any site requires contact precautions. However, for *L. hongkongensis*, contact precaution is only necessary if the patient has gastroenteritis. Due to the implications on the choice of antibiotics and infection control measures, *L. hongkongensis* with positive ESBL phenotypes that are picked up by disk diffusion clavulanate inhibition test should be at least confirmed with the boronic acid based modified CLSI ESBL phenotypic test. In laboratories with resources in molecular testing, the presence of known ESBL genes can also be confirmed by PCR sequencing.

**FIGURE 5** The ampC expression in selected *L. hongkongensis* strains. Expression of the ampC gene was determined by qRT-PCR in representative strains with different ESBL phenotypes. Error bars represent means ± SEM of three independent experiments. Student's t-test was performed to determine the difference between the ampC expression of the selected strains and that of HLHK9. An asterisk indicates a significant difference (ns, not significant; *p* < 0.05).
DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, LZOW00000000; https://www.ncbi.nlm.nih.gov/genbank/, MT640029–MT640042.

ETHICS STATEMENT

This study was approved by the Institutional Review Board (IRB) of The University of Hong Kong/Hospital Authority Hong Kong West Cluster, Hong Kong East Cluster Research Ethics Committee. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest: PW has provided scientific advisory/laboratory services for Gilead Sciences, Incorporated; International Health Management Associates, Incorporated; Merck & Corporation, Incorporated and Pfizer, Incorporated. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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