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ORIGINAL ARTICLE

A novel MLST sequence type discovered in the first fatal case of *Laribacter hongkongensis* bacteremia clusters with the sequence types of other human isolates

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Laribacter hongkongensis **is a gram-negative, facultative anaerobic, motile, S-shaped, asaccharolytic, urease-positive bacillus in the *Neisseriaceae* family of β-proteobacteria. To date, all patients with *L. hongkongensis* infection have survived, including the two patients with *L. hongkongensis* bacteremia and patients with *L. hongkongensis* gastroenteritis. In this study, we describe the clinical, microbiological and molecular characterization of the first fatal case associated with *L. hongkongensis* bacteremia in a patient with colonic carcinoma that metastasized to the liver. The identity of the isolate was confirmed via phenotypic tests and 16S rRNA gene sequencing. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI–TOF MS), using the Bruker database extended with *L. hongkongensis* reference strains, also identified the isolate as *L. hongkongensis*, with a top match score of 2.473. Multilocus sequence typing revealed a new sequence type (ST), and phylogenetic analysis and eBURST demonstrated unambiguously that the ST of the isolate was clustered with two other STs found exclusively in human patients, consistent with the theory that some clones of *L. hongkongensis* could be more virulent than others. Underlying liver diseases and ascites potentially represent distinct risk factors for invasive *L. hongkongensis* infection. More widespread use of MALDI–TOF MS for identification and improvements of selective media should facilitate the identification of more cases of *L. hongkongensis* infection.**

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Keywords: bacteraemia; infections

INTRODUCTION

Laribacter hongkongensis is a gram-negative, facultative anaerobic, motile, S-shaped, asaccharolytic, urease-positive bacillus in the *Neisseriaceae* family of β-proteobacteria.[1,2] This species was first isolated from the blood and thoracic empyema of an alcoholic liver cirrhosis patient in Hong Kong.[3] This species was also recently recovered from a blood culture from a Korean patient with liver cirrhosis caused by Wilson's disease.[4] These cases suggest that chronic liver disease is a risk factor for invasive *L. hongkongensis* infections. In addition to invasive bacteremic infections, *L. hongkongensis* is also associated with community-acquired gastroenteritis and traveler's diarrhea.[2] *L. hongkongensis* is likely to be globally distributed, as travel histories from patients suggest that this bacterium is present on at least four continents: Asia, Europe, Africa and Central America.[2,5–7] *L. hongkongensis* has been found in up to 60% of intestines from commonly consumed freshwater fish of the carp family.[2,8] This species has also been isolated from drinking water reservoirs, Chinese tiger frogs and little egrets.[9–11] Pulsed-field gel electrophoresis and multilocus sequence typing (MLST) demonstrated that the fish and patient isolates fall into separate clusters, suggesting that some clones could be more virulent or adapted to humans.[8,12] The complete genome of *L. hongkongensis* was recently sequenced, and this bacterium's mechanism of acid resistance was characterized.[1,13]

To date, all patients with *L. hongkongensis* infection have survived, including the two patients with *L. hongkongensis* bacteremia and the patients with *L. hongkongensis* gastroenteritis. In this article, we report the first fatal case associated with *L. hongkongensis* bacteremia. In addition to phenotypic tests and 16S rRNA gene sequencing, the isolate was also characterized using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI–TOF MS) and MLST. The antibiotic resistance mechanisms of this isolate were also determined via sequencing of the *ampC* and *tetA* genes.

MATERIALS AND METHODS

Patient and microbiological methods

All clinical data were collected prospectively, as described in our previous publication.[14] The BacT/ALERT 3D blood culture system (Biomerieux, Marcy L'Etoile, Frace) was used. Phenotypic identification was performed using standard conventional biochemical methods.[12] All tests were performed in triplicate with freshly prepared media on

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separate occasions. Antimicrobial susceptibility was tested using the Kirby Bauer disk diffusion method, and the results were interpreted according to the Clinical and Laboratory Standards Institute interpretive criteria for *Enterobacteriaceae*.

16S rRNA gene sequencing

16S rRNA gene sequencing was performed as described previously.[7,10] To extract bacterial DNA, 80 μL of 0.05 M NaOH were added to 20 μL of bacterial cells suspended in distilled water, and the mixture was incubated at 60 ℃ for 45 min, followed by the addition of 6 μL of Tris-HCl (pH 7.0), achieving a final pH of 8.0. The resultant mixture was diluted ×100, and 5 μL of the diluted extract was used for polymerase chain reaction (PCR).

For PCR amplification, DNase I-treated distilled water and PCR master mix, which contains deoxynucleoside triphosphates, PCR buffer and *Taq* polymerase, were used in all PCR reactions by adding 1 U of DNase I (Pharmacia, Uppsala, Sweden) to 40 μL of distilled water or PCR master mix, incubating the mixture at 25 ℃ for 15 min and subsequently incubating the mixture at 95 ℃ for 10 min to inactivate the DNase I. The bacterial DNA extract and the control were amplified using 0.5 μM primers (LPW57 (5′-AGT TTG ATC CTG GCT CAG-3′) and LPW58 (5′-AGG CCC GGG AAC GTA TTC AC-3′)) (Sigma-Proligo, Singapore). The PCR mixture (50 μL) contained bacterial DNA, PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$ and 0.01% gelatin), 200 μM of each deoxynucleoside triphosphate and 1.0 U of *Taq* polymerase (Boehringer Mannheim, Mannheim, Germany). The mixtures were amplified in an automated thermal cycler (Perkin-Elmer Cetus, Gouda, The Netherlands) via 40 cycles of 94 ℃ for 1 min, 55 ℃ for 1 min and 72 ℃ for 2 min and a final extension at 72 ℃ for 10 min. DNase I-treated distilled water was used as the negative control. A total of 10 μL of each amplified product was electrophoresed in a 1.0% (w/v) agarose gel, with the Lambda DNA *Ava*II digest molecular size marker (Boehringer Mannheim) electrophoresed in parallel. Electrophoresis was performed in Tris-borate-EDTA buffer at 100 V for 1.5 h. The gel was stained with 0.5 μg/mL of ethidium bromide for 15 min, rinsed and photographed under ultraviolet light illumination.

The PCR product was gel-purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). Both strands of the PCR product were sequenced twice with an ABI 377 automated sequencer, according to the manufacturers' instructions (Perkin-Elmer, Foster City, CA, USA), using the PCR primers LPW57 and LPW58.

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)

MALDI-TOF MS was performed as described in our previous publication, with 21 *L. hongkongensis* strains added as reference strains.[15] The bacterial isolate was grown on sheep blood agar at 35℃ for 18–24 h and analyzed using the direct transfer method. The sample was processed in a Bruker Daltonik MALDI–TOF MS spectrometer with 1 μL of Bruker α-cyano matrix solution. The spectrum was obtained with an accelerating voltage of 20 kV in linear mode and analyzed

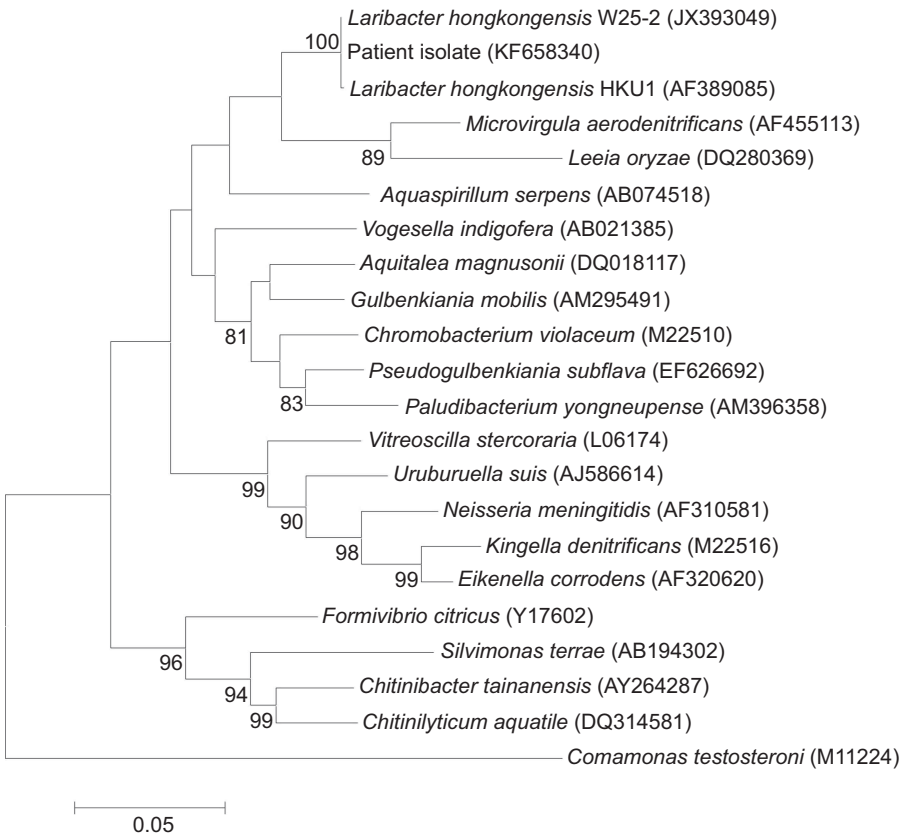

Figure 1 Phylogenetic tree showing the relationship of the patient isolate to other related members of the β-subclass of *Proteobacteria*, inferred from 16S rRNA gene sequences. The tree was constructed using the neighbor-joining method and rooted using *Comamonas testosteroni* (M11224). The bootstrap values calculated from 1000 trees are shown when they are ⩾70%. The scale bar indicates the estimated number of substitutions per 20 bases. Names and accession numbers are presented as cited in the GenBank database.

within an *m*/*z* range 2000–20 000 Da. The spectrum was analyzed using MALDI Biotyper 3.0 and Reference Library v3.1.2.0 software (Bruker Daltonik, Bremen, Germany).

ampC and *tetA* gene sequencing

The *ampC* genes of the isolate were amplified and sequenced using primers LPW663 (5′-CGG ATT ACC CCA TTT TCC CG-3′) and LPW664 (5′-CAT GTC TGC CCG GAA TGT GC-3′), and the *tetA* genes of the isolate were amplified and sequenced using primers LPW1015 (5′-ATG TCC ACC AAC TTA TCA-3′) and LPW1697 (5′-TCA GCG ATC GGC TCG TTG-3′). The conditions used were those that were described in our previous publications.[14,16]

Multi-locus sequence typing (MLST)

MLST was performed according to the protocol described in our previous publication.[12] Seven housekeeping genes (i.e., *rho*, *acnB*, *ftsH*, *trpE*, *ilvC*, *thiC* and *eno*) were amplified and sequenced using previously described primers.[12] The results were analyzed with MLST data from other *L. hongkongensis* isolates from humans and fish that were described in our previous publication.[12] The Sequence Type Analysis and Recombinational Tests version 2 program was used for Unweighted Pair Group Method with Arithmetic Mean dendrogram construction, using the nucleotide sequences of the seven gene loci. In addition, the eBURST program was used for lineage analysis.[17,18]

RESULTS

Patient

A 74-year-old Chinese man was admitted to the hospital due to non-specific chest discomfort and dizziness. The patient had hypertension, diabetes mellitus and chronic renal failure and a history of ischemic heart disease with percutaneous intervention to the left anterior descending artery eight years prior to admission and right middle cerebral artery infarct with a recurrent cerebrovascular accident one year prior to admission. On admission, the patient's temperature was 37.9 ℃. The patient also exhibited pallor and ascites. The patient developed right upper quadrant abdominal pain and septic shock, with a blood pressure of 80/40 mmHg and a heart rate of 130 beats/min, two days after admission. The total white cell count was 16.3×10^9/L, with a neutrophil count of 15.2×10^9/L, a lymphocyte count of 0.2×10^9/L and a monocyte count of 1.0×10^9/L. The hemoglobin level was 6.7 g/dL, and the platelet count 328×10^9/L. The serum urea level was 29.3 mmol/L, the creatinine level was 447 μmol/L, the albumin level was 16 g/L, the globulin level was 39 g/L, the total bilirubin level was 27 μmol/L, the alkaline phosphatase level was 165 IU/L, the alanine aminotransferase level was 8 IU/L and the aspartate aminotransferase level was 30 IU/L. Blood culture was performed, and empirical intravenous meropenem at a dose of 500 mg daily was initiated. On day 2 post-incubation, the aerobic blood culture bottle turned positive for a gram-negative bacillus. Ultrasound examination of the abdomen revealed gross ascites, two isoechoic masses of 4.2 cm×2.5 cm×2.5 cm and 8.0 cm×3.4 cm×6.1 cm in the peritoneal cavity anterior to the right hepatic lobe, and multiple vague hypoechoic masses of up to 3.7 cm in the liver. Serum tests for hepatitis B surface antigen and anti-hepatitis C virus antibody were negative. The serum α-fetoprotein level was <1 μg/L, and the carcinoembryonic antigen level was 1841 μg/L. A probable diagnosis of metastatic carcinoma of the colon was made, but the patient refused further investigation. The patient succumbed on day 10 after admission.

Phenotypic characteristics

The blood culture isolate was a gram-negative, S-shaped and motile bacillus. The isolate grew on horse blood agar as non-hemolytic, gray colonies with a 1 mm diameter after 24 h of incubation at 37 ℃ in ambient air. The isolate also grew on MacConkey agar and in an anaerobic environment. The bacillus produced catalase, cytochrome oxidase, urease and arginine dihydrolase and reduced nitrate. However, the isolate failed to ferment, oxidize or assimilate any sugar tested. The isolate

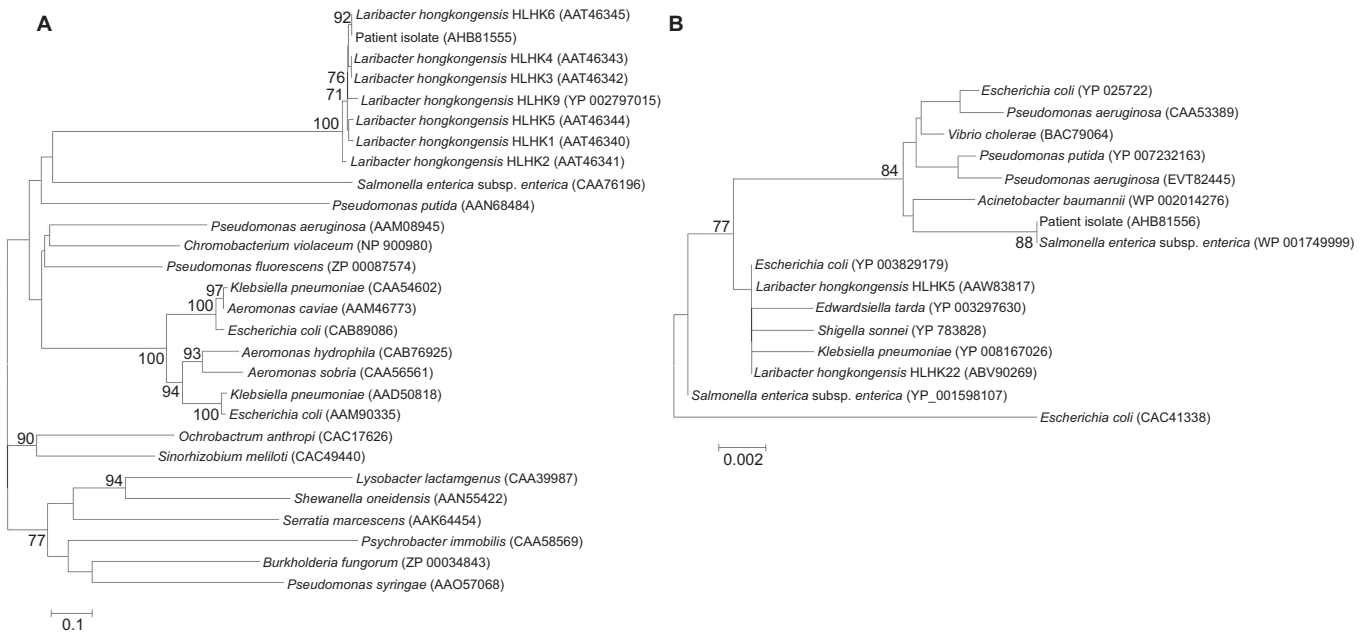

Figure 2 Phylogenetic tree showing the relationships of the amino acid sequences of the (**A**) AmpC and (**B**) TetA proteins from the patient isolate with other related species. The tree was constructed using the neighbor-joining method with Jukes–Cantor correction. The bootstrap values calculated from 1000 trees are shown when they are ⩾70%. The scale bars indicate the estimated number of substitutions per 10 and 500 amino acids. Names and accession numbers are presented as cited in the GenBank database.

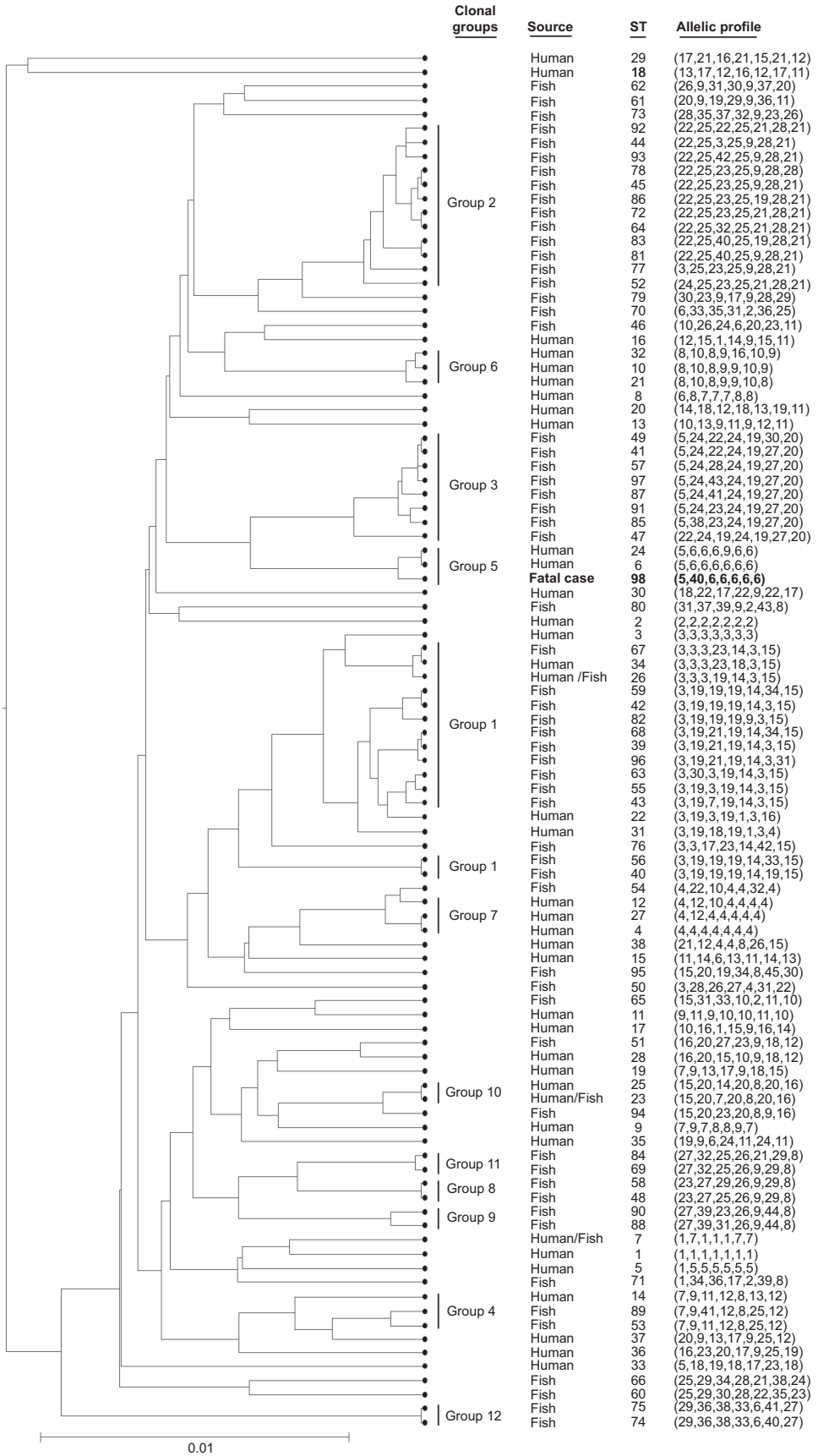
Clonal groups
Source
ST
Allelic profile
Group 2
Group 6
Group 3
Group 5
Group 1
Group 1
Group 7
Group 10
Group 11
Group 8
Group 9
Group 4
Group 12
0.01
Human 29 (17,21,16,21,15,21,12)
Human 18 (13,17,12,16,12,17,11)
Fish 62 (26,9,31,30,9,37,20)
Fish 61 (20,9,19,29,9,36,11)
Fish 73 (28,35,37,32,9,23,26)
Fish 92 (22,25,22,25,21,28,21)
Fish 44 (22,25,3,25,9,28,21)
Fish 93 (22,25,42,25,9,28,21)
Fish 78 (22,25,23,25,9,28,28)
Fish 45 (22,25,23,25,9,28,21)
Fish 86 (22,25,23,25,19,28,21)
Fish 72 (22,25,23,25,21,28,21)
Fish 64 (22,25,32,25,21,28,21)
Fish 83 (22,25,40,25,19,28,21)
Fish 81 (22,25,40,25,9,28,21)
Fish 77 (3,25,23,25,9,28,21)
Fish 52 (24,25,23,25,21,28,21)
Fish 79 (30,23,9,17,9,28,29)
Fish 70 (6,33,35,31,2,36,25)
Fish 46 (10,26,24,6,20,23,11)
Human 16 (12,15,1,14,9,15,11)
Human 32 (8,10,8,9,16,10,9)
Human 10 (8,10,8,9,9,10,9)
Human 21 (8,10,8,9,9,10,8)
Human 8 (6,8,7,7,7,8,8)
Human 20 (14,18,12,18,13,19,11)
Human 13 (10,13,9,11,9,12,11)
Fish 49 (5,24,22,24,19,30,20)
Fish 41 (5,24,22,24,19,27,20)
Fish 57 (5,24,28,24,19,27,20)
Fish 97 (5,24,43,24,19,27,20)
Fish 87 (5,24,41,24,19,27,20)
Fish 91 (5,24,23,24,19,27,20)
Fish 85 (5,38,23,24,19,27,20)
Fish 47 (22,24,19,24,19,27,20)
Human 24 (5,6,6,6,9,6,6)
Human 6 (5,6,6,6,6,6,6)
Fatal case 98 (5,40,6,6,6,6,6)
Human 30 (18,22,17,22,9,22,17)
Fish 80 (31,37,39,9,2,43,8)
Human 2 (2,2,2,2,2,2,2)
Human 3 (3,3,3,3,3,3,3)
Fish 67 (3,3,3,23,14,3,15)
Human 34 (3,3,3,23,18,3,15)
Human /Fish 26 (3,3,3,19,14,3,15)
Fish 59 (3,19,19,19,14,34,15)
Fish 42 (3,19,19,19,14,3,15)
Fish 82 (3,19,19,19,9,3,15)
Fish 68 (3,19,21,19,14,34,15)
Fish 39 (3,19,21,19,14,3,15)
Fish 96 (3,19,21,19,14,3,31)
Fish 63 (3,30,3,19,14,3,15)
Fish 55 (3,19,3,19,14,3,15)
Fish 43 (3,19,7,19,14,3,15)
Human 22 (3,19,3,19,1,3,16)
Human 31 (3,19,18,19,1,3,4)
Fish 76 (3,3,17,23,14,42,15)
Fish 56 (3,19,19,19,14,33,15)
Fish 40 (3,19,19,19,14,19,15)
Fish 54 (4,22,10,4,4,32,4)
Human 12 (4,12,10,4,4,4,4)
Human 27 (4,12,4,4,4,4,4)
Human 4 (4,4,4,4,4,4,4)
Human 38 (21,12,4,4,8,26,15)
Human 15 (11,14,6,13,11,14,13)
Fish 95 (15,20,19,34,8,45,30)
Fish 50 (3,28,26,27,4,31,22)
Fish 65 (15,31,33,10,2,11,10)
Human 11 (9,11,9,10,10,11,10)
Human 17 (10,16,1,15,9,16,14)
Fish 51 (16,20,27,23,9,18,12)
Human 28 (16,20,15,10,9,18,12)
Human 19 (7,9,13,17,9,18,15)
Human 25 (15,20,14,20,8,20,16)
Human/Fish 23 (15,20,7,20,8,20,16)
Fish 94 (15,20,23,20,8,9,16)
Human 9 (7,9,7,8,8,9,7)
Human 35 (19,9,6,24,11,24,11)
Fish 84 (27,32,25,26,21,29,8)
Fish 69 (27,32,25,26,9,29,8)
Fish 58 (23,27,29,26,9,29,8)
Fish 48 (23,27,25,26,9,29,8)
Fish 90 (27,39,23,26,9,44,8)
Fish 88 (27,39,31,26,9,44,8)
Human/Fish 7 (1,7,1,1,1,7,7)
Human 1 (1,1,1,1,1,1,1)
Human 5 (1,5,5,5,5,5,5)
Fish 71 (1,34,36,17,2,39,8)
Human 14 (7,9,11,12,8,13,12)
Fish 89 (7,9,41,12,8,25,12)
Fish 53 (7,9,11,12,8,25,12)
Human 37 (20,9,13,17,9,25,12)
Human 36 (16,23,20,17,9,25,19)
Human 33 (5,18,19,18,17,23,18)
Fish 66 (25,29,34,28,21,38,24)
Fish 60 (25,29,30,28,22,35,23)
Fish 75 (29,36,38,33,6,41,27)
Fish 74 (29,36,38,33,6,40,27)

Figure 3 UPGMA-generated dendrogram showing the relationships of the strain isolated from the fatal case with other *L. hongkongensis* strains isolated from humans and fish.[12] The ST of the isolate from the fatal case is displayed in bold. The genetic relatedness among the STs was generated using START2 software (http://pubmlst.org/software/analysis/start2/).[18] The clonal group displayed was assigned according to the eBURST clonal clusters to which the STs belong. START2, Sequence Type Analysis and Recombinational Tests version 2; UPGMA, Unweighted Pair Group Method with Arithmetic Mean.

was susceptible to gentamicin, amikacin, levofloxacin, cefuroxime, amoxicillin-clavulanate and imipenem but resistant to ampicillin, cefalothin, cefotaxime, ceftazidime, ceftriaxone, ceftibuten and tetracycline.

16S rRNA gene sequencing

PCR amplification of the 16S rRNA gene of the isolate revealed a band with a size of approximately 1300 bp. Sequencing and comparative analysis revealed no differences between the 16S rRNA gene sequence of the blood culture isolate and that of *L. hongkongensis* (GenBank accession NO JX393049), and a single base differed between the 16S rRNA gene sequence of the blood culture isolate and that of *L. hongkongensis* HKU1^T (GenBank accession NO AF389085). These findings indicated that the isolate was a strain of *L. hongkongensis* (Figure 1).

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)

Using the Bruker database extended with *L. hongkongensis* reference strains, the isolate was identified as *L. hongkongensis*, with a top match score of 2.473.

ampC and *tetA* gene sequencing

PCR amplification of the *ampC* and *tetA* genes of the isolate revealed bands of approximately 1350 bp and 1230 bp, respectively. Sequencing and comparative analysis demonstrated that the *ampC* gene of the isolate possessed 100% nucleotide and amino-acid identities to the *ampC* gene of *L. hongkongensis* strain HLHK-6 and that the *tetA* gene of the isolate possessed >99% nucleotide and amino-acid identities to the *tetA* genes of *L. hongkongensis* strains HLHK5 and HLHK22, as well as those of other bacteria, such as *Salmonella enterica* subsp. *Enterica*. These results indicated that the mechanisms of β-lactam and tetracycline resistance in the isolate were identical to those that we previously reported (Figure 2).[14,16]

Multi-locus sequence typing (MLST)

Sequence analysis demonstrated that the sequence of the isolate constituted a new sequence type (ST): ST-98 (5,40,6,6,6,6,6). Phylogenetic analysis demonstrated that the isolate was clustered with ST-6 (5,6,6,6,6,6,6) and ST-24 (5,6,6,6,9,6,6), forming clonal group 5 (Figure 3). eBURST also grouped ST-6, ST-24 and ST-98 into a single lineage, consistent with results of the phylogenetic analysis (Figure 4).

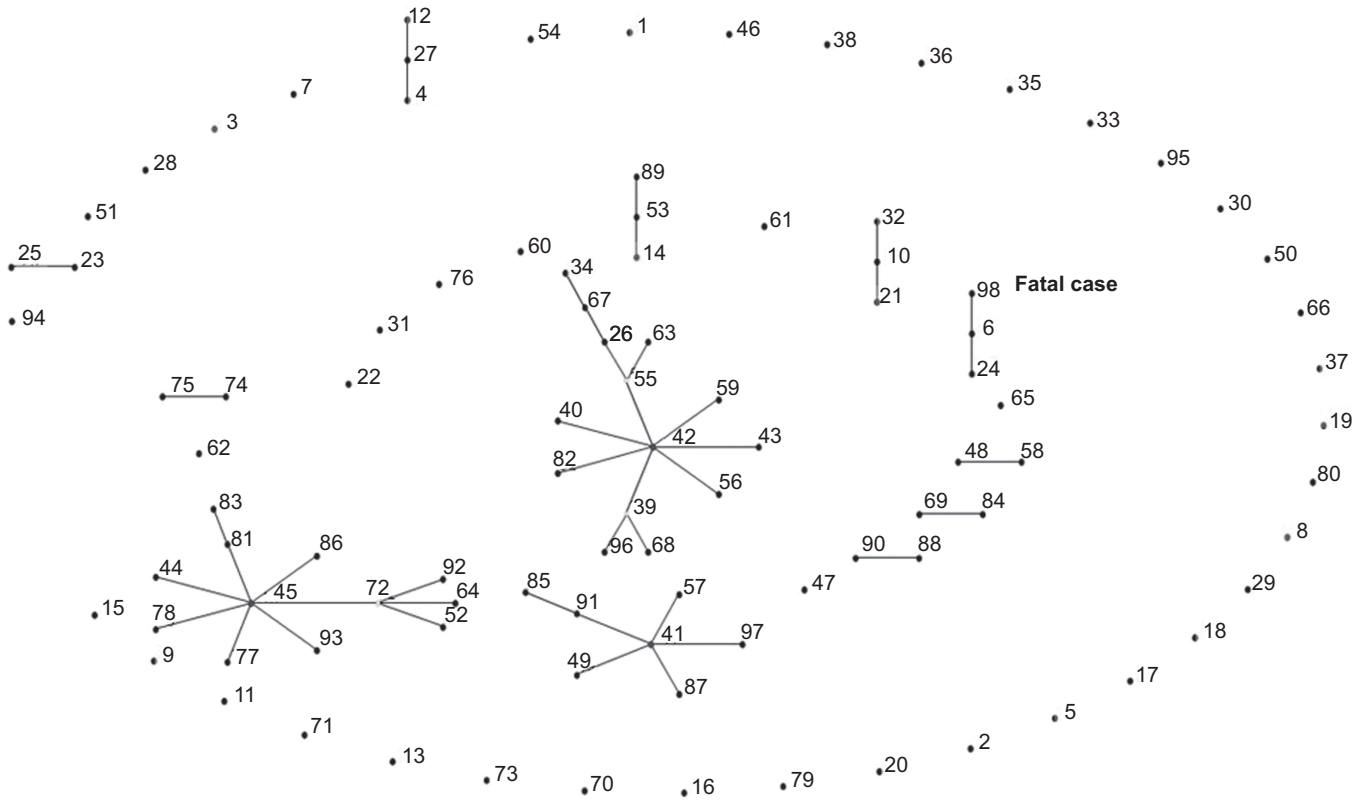

Figure 4 BURST analysis of the strain isolated from the fatal case in comparison to other *L. hongkongensis* strains isolated from humans and fish.[12] The relationships between STs and clonal complexes are displayed. Each number represents an ST, and each line connects STs that are identical for at least six of the seven MLST loci. Boxed numbers represent STs that are found only in humans. The isolate from the fatal case belongs to ST 98 and is displayed in bold. The clonal cluster was generated using eBURST software version 3 (http://eburst.mlst.net/).

Nucleotide sequence accession number

The 16S rRNA, *ampC* and *tetA* gene sequences of the *L. hongkongensis* strain isolated in this study were deposited in the GenBank sequence database under accession numbers KF658340, KF658341 and KF658342, respectively.

DISCUSSION

In this study, we report the first case of fatal infection associated with *L. hongkongensis* bacteremia. To date, the most serious infection associated with *L. hongkongensis* was the first case of thoracic empyema and bacteremia.[3] In this case, the patient required hospitalization for more than one month, with prolonged intravenous antibiotic treatment and chest drainage.[3] With respect to the cases of community-acquired gastroenteritis and traveler's diarrhea associated with *L. hongkongensis*, approximately 20% and 80% of patients developed bloody and watery diarrhea, respectively, and systemic upset and vomiting occurred in approximately 30% of patients.[2] In the most severe cases, patients can have diarrhea up to 30 times per day or the diarrhea can linger for up to three months.[2] MLST, phylogenetic analysis and eBURST demonstrated unambiguously that the ST of the isolate in the present study was clustered with two other STs that were found exclusively in human patients. This result is consistent with the findings of our previous studies, which demonstrated clustering of isolates from humans and fish into different groups, suggesting that some clones of *L. hongkongensis* could be more virulent than others.[8,12]

Underlying liver diseases and ascites potentially represent distinct risk factors for invasive *L. hongkongensis* infection. Both patients with reported *L. hongkongensis* bacteremia had underlying liver cirrhosis and ascites due to alcoholism and Wilson's disease.[3,4] The patient in the present study also had liver metastasis and ascites. Similar to the other reported cases of invasive and non-invasive *L. hongkongensis* infection, the gastrointestinal tract was likely the portal of entry in the present patient. Although a complete and functional urease cassette is present in the genome of *L. hongkongensis*, we recently found that the urease gene is of limited importance for the survival of *L. hongkongensis* in the acidic environment of the stomach.[13] In contrast, the bacterium's *arc* gene cassettes, which each encode three enzymes and a membrane bound arginine-ornithine antiporter of the arginine deiminase pathway, are far more important than urease for acid resistance in *L. hongkongensis*, possibly due to the duplication of the *arc* gene cassette in the *L. hongkongensis* genome.[13] We speculate that after surviving the acidic environment of the stomach and reaching the intestine, intestinal mucosal edema and local immunosuppression secondary to portal venous congestion vasculopathy caused by liver disease predisposed the patients to *L. hongkongensis* invasion through the gastrointestinal mucosa, resulting in patients with *L. hongkongensis* bacteremia. In conclusion, both microbial factors, as demonstrated by molecular typing, and patient factors, as demonstrated by the patients' underlying disease, are likely to make important contributions to the development of *L. hongkongensis* bacteremia.

More widespread use of MALDI–TOF MS for identification and improvements in selective media should help identify more cases of *L. hongkongensis* infection. In all previous cases of human *L. hongkongensis* infection and in reports of the detection of this species in animal specimens, the strains were identified using a combination of phenotypic tests and 16S rRNA gene sequencing.[2,7–11,19–21] In recent years, MALDI–TOF MS has emerged as a revolutionary technique for rapid bacterial identification at a low cost. Using an expanded Bruker database with *L. hongkongensis* reference strains, we recently found that all 240 *L. hongkongensis* isolates could be correctly identified using MALDI–TOF MS, demonstrating the importance of expanding the MALDI–TOF MS database with bacteria endemic to different localities.[15] This finding is also consistent with the correct identification of the present isolate by MALDI–TOF MS. With respect to the selective medium, it was recently observed that the *L. hongkongensis* strain recovered from the blood culture of the Korean patient was cefoperazone-susceptible.[4] Further, a recent study from Guangzhou reported that 45.2% of the *L. hongkongensis* strains recovered from fish and frogs were susceptible to cefoperazone.[21] These findings imply that a significant number of *L. hongkongensis* cases may have been missed in studies that used cefoperazone MacConkey agar as the selective medium and that the incidence of *L. hongkongensis* infection could have been underestimated.[2,20] With bacterial identification becoming easier and more rapid via MALDI–TOF MS, the use of a less selective medium, such as MacConkey agar, in combination with picking more colonies for rapid identification via MALDI–TOF MS, could represent a strategy for diagnosing more cases of *L. hongkongensis* infection of the gastrointestinal tract.

ACKNOWLEDGMENTS

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