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Identification of a novel *Providencia* species showing multi-drug-resistant in three patients with hospital-acquired infection

Weiwei Yang[a,b,†], Jing Chen[e,†], Fengzhen Yang[c], Ping Ji[d], Siquan Shen[a,b], Dandan Yin[a,b], Fupin Hu[a,b,*]

[a] *Institute of Antibiotics, Huashan Hospital, Fudan University, Shanghai, China*
[b] *Key Laboratory of Clinical Pharmacology of Antibiotics, Ministry of Health, Shanghai, China*
[c] *Clinical Laboratory, Yuhuangding Hospital, Qingdao University, Yantai, China*
[d] *Clinical Laboratory, The First Hospital of Xinjiang Medical University, Urumqi, China*
[e] *Hangzhou Matridx Biotechnology Co., Ltd, Hangzhou, China*

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ABSTRACT

Providencia species are important opportunistic pathogens for humans and are associated with several infectious diseases. In this study, we found three clinical strains belonging to a novel *Providencia* species, namely *Providencia huashanensis*, including strains CRE-3FA-0001^T, CRE-138-0026, and CRE-138-0111. These strains were recovered from three patients, and all of them were associated with nosocomial infections, including incision infection, urinary tract infection, and intracranial infection. The three strains showed high-level resistance to many types of antimicrobials, including amikacin, aztreonam, ceftazidime, cefepime, ciprofloxacin, colistin, polymyxin B, imipenem, meropenem, ceftazidime-avibactam, imipenem-relebactam. Investigation of the resistance mechanism revealed that acquired resistance genes such as bla_{KPC}, bla_{NDM}, bla_{PER}, bla_{OXA}, *aac, ant*, and *qnrD*, played an important role in the multidrug-resistant phenotype for the three strains. The phylogenetic trees were reconstructed based on the 16S rRNA gene sequences, multi-locus sequence analysis, and core single nucleotide polymorphisms. The genome sequence of the strains had a range of 83.5%–85.8% average nucleotide identity and 21%–25.5% in silico DNA-DNA hybridization scores with other *Providencia* type strains. The average nucleotide identity and in silico DNA-DNA hybridization values and the phylogenetic trees indicated that the strains CRE-3FA-0001^T, CRE-138-0026, and CRE-138-0111 strains should be considered as a novel species of the genus *Providencia*, for which the name *P. huashanensis* sp. nov. is proposed. The type strain is CRE-3FA-0001^T = China Center for Type Culture Collection AB 2023186^T = Korean Collection for Type Cultures 8373^T.

1. Introduction

Providencia is a genus of g-proteobacteria in the family *Morganellaceae*, a genus of Gram-negative, non-spore-forming bacteria. The taxonomic genus *Providencia* is currently divided into 14 recognized species, including *Providencia stuartii* (1975), *Providencia alcalifaciens* (1979), *Providencia rettgeri* (1979), *Providencia rustigianii* (1983), *Providencia heimbachae* (1986), *Providencia vermicola* (2006), *Providencia sneebia* (2009), *Providencia burhodogranariea* (2009), *Providencia thailandensis* (2013), *Providencia huaxiensis* (2019), *Providencia entomophila* (2019), *Providencia wenzhouensis* (2021), *Providencia manganoxydans* (2022), *Providencia hangzhouensis* (2023) [1–13]. Some of these species are of medical importance as opportunistic pathogens causing travellers' diarrhoea, food-borne infections, urinary tract infections (UTI), bacteraemia, etc., such as *P. alcalifaciens, P. rettgeri, P. heimbachae, P. stuartii* [14–16]. In recent years, more and more researchers have reported the emergence of multi-drug-resistant clinical isolates of *Providencia* spp., and these isolates usually exhibited high-level resistance to the most clinically used antimicrobial agents due to the acquired resistance genes [17,18]. In this study, we identified a novel *Providencia* species and characterized it in terms of phenotype, genotype, physiology, and biochemistry.

* Correspondence: Fupin Hu, Institute of Antibiotics, Huashan Hospital, Fudan University, 12 M. Wulumuqi Road, Shanghai 200040, China.
E-mail address: hufupin@fudan.edu.cn (F. Hu).
† These authors contributed equally.

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2. Methods and materials

2.1. Case information

The first patient, a 70-year-old woman, suffered from persistent dull pain in the upper abdomen accompanied by nausea and vomiting and was soon admitted to the Yuhuangding Hospital of Qingdao University on 15 December 2020. Together with the results of the abdominal computed tomography (CT) scan, she was diagnosed with acute diffuse peritonitis complicated by upper gastrointestinal perforation and subsequently underwent laparoscopic exploration, abdominal adhesiolysis, abdominal irrigation, and percutaneous catheter drainage. Incisional infection occurred after the surgery, the strain CRE-3FA-0001 (initially identified as *P. rettgeri* by MALDI-TOF-MS) was isolated from the abdominal cavity drainage fluid on 25 December 2020. Cefoperazone-sulbactam (3g q12h), imipenem (0.5g q12h), metronidazole (0.5g q12h), piperacillin-tazobactam (4.5g q8h), moxifloxacin (0.4g qd) were given as anti-infective treatment for intra-abdominal infection. The patient was then discharged with a positive series of anti-infective, symptomatic, and supportive treatments and improved after 16 days of hospitalization.

The second patient, a 67-year-old man, had a sudden onset of left limb weakness, inability to stand, and urinary incontinence with nausea and vomiting and was admitted to the First Hospital of Xinjiang Medical University on 8 January 2021. The CT scan of his brain showed abnormal changes in the right frontotemporal lobe and basal ganglia. He was then diagnosed with a cerebral infarction complicated by a haemorrhage in the right frontotemporal lobe and basal ganglia. A series of symptomatic treatments were administered, including blood pressure lowering haemostasis and fluid replacement. During treatment, the strain CRE-138-0026 (initially identified as *P. rettgeri* by MALDI-TOF-MS) was detected in a urine sample on 15 February 2021. Piperacillin-tazobactam (4.5g q8h), amikacin (0.4g qd), and cefoperazone (3g q12h) were then used sequentially to treat UTI. Complicated by poor baseline conditions and advanced age, the patient was transferred to the local hospital with an unfavourable prognosis after 55 days of hospitalization.

The third patient, a 46-year-old woman, had a sudden onset of headache accompanied by altered consciousness, nausea, and vomiting. She was immediately admitted to the First Hospital of Xinjiang Medical University on 8 June 2021. The brain CT scan showed that the patient had intraventricular haemorrhage and subarachnoid haemorrhage. Meanwhile, the patient's chest CT scan showed several large areas of increased density in her right lung, which was considered to be the right lung infection. Due to the critical condition, the patient underwent surgery for "removal of intracranial haematoma, ventricular trepanation, and drainage, tracheotomy" on the same day of admission, and was then transferred to the intensive care unit ward for monitoring and further treatment. After surgery, the patient developed the intracranial infection, and the strain CRE-138-0111 (initially identified as *P. rettgeri* by MALDI-TOF-MS) was isolated from the cerebrospinal fluid sample. Cefuroxime (1.5g q12h), cefoperazone-sulbactam (3g q12h), and meropenem (1g q8h) were used sequentially for anti-infective treatment. With timely anti-infective and supportive treatment, the patient was improved. However, due to other underlying conditions, she was discharged and transferred to the local hospital for further management.

2.2. Specimen collection and bacterial culture

In the clinical laboratory, the three strains were recovered and purified on the Columbia blood agar (CBA) and incubated aerobically at 37°C for 48 h. The primary species identification was performed by MALDI-TOF MS (bioMérieux).

2.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the broth microdilution method recommended by the Clinical and Laboratory Standards Institute. The three strains were grown aerobically on CBA at 35°C for 24 h. Quality control and MIC results were interpreted according to the Clinical and Laboratory Standards Institute breakpoints for all agents except tigecycline and aztreonam-avibactam [19]. The tigecycline breakpoint was interpreted according to the guidelines of FDA (susceptibility: ≤2 mg/L; intermediate: 4 mg/L; resistance: ≥8 mg/L) [20]; the aztreonam-avibactam breakpoint was interpreted according to aztreonam (susceptibility: ≤4 mg/L; intermediate: 8 mg/L; resistance: ≥16 mg/L). *E. coli* ATCC 25922 was used for quality control.

2.4. Genomic DNA extraction, genome sequencing, and bioinformatic analysis

Genomic DNA of the three strains was extracted using the TIANamp bacterial DNA kit according to the manufacturer's recommendations (Tiangen Biotech Co., Ltd., Beijing, China). Genomic DNA was sequenced using Illumina short-read sequencing (150 bp paired-end reads) (Illumina, San Diego, CA, USA). Sequencing data were assembled using Spades (version 3.13.0). The final genome sequence was annotated using Prokka (version 1.14.6) and scored using BUSCO (version 4.1.2). Resistance genes and virulence genes were aligned to the Comprehensive Antibiotic Resistance Database [21] and Virulence Factor Database [22] using the DIAMOND (version 2.0.4.142), respectively. The identity and coverage thresholds for detection resistance genes and virulence genes were 90%. The reference genome sequences ofr *Providencia* spp. were obtained from NCBI (accession number as shown in Table 3), and the comparison of antimicrobial resistance genes and virulence genes was performed using Roary (version 3.13.0). The Roary process used only the cd-hit tool and Prokka software to compare gene components, resulting in the removal of redundant gene sets and clustering of gene components. According to the Roary clustering results, the entire cluster protein was labelled.

2.5. Average nucleotide identity (ANI), in silico DNA-DNA hybridization (isDDH), and phylogenetic tree reconstruction

The ANI between the genomes was calculated using the Python module pyani based on MUMmer (ANIm) algorithms [23], and the isDDH similarities were calculated using the Genome-to-Genome Distance Calculator [24]. The phylogenetic trees were reconstructed based on single nucleotide polymorphism (SNP), 16S rRNA gene, and multi-locus sequence analysis (MLSA). The SNP information for the reference genome of the *Providencia* type strains was obtained using MUMmer software (version 3.1) [25]. The 16S rRNA gene was amplified by PCR using the universal primers of 27F (5′-AGAGTTTGGATCMTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′), and then sequenced [26]. The markers encoding translation elongation factor EF-G (*fusA*), DNA gyrase subunit B (*gyrB*), isoleucyl-tRNA synthetase (*ileS*), translation elongation factor EF-4 (*lepA*), and leucyl-tRNA synthetase (*leuS*) was selected for MLSA and assembled from the whole genome sequence data. Phylogenetic trees based on SNPs, 16S rRNA, and MLSA were reconstructed using neighbour-joining, maximum-likelihood, and maximum-parsimony methods in mega X software. Evolutionary distances were calculated using Kimura's two-parameter and gamma-distributed with invariant sites models. Bootstrap analysis,

based on 1000 replicates, was used to obtain confidence levels for the branches.

2.5.1. Physiological and chemotaxonomic analysis

Gram staining was performed as previously described by Gregersen [27]. The motility of the three strains was observed by light microscopy using cells grown at 37°C for 24 h on Luria-Bertani agar (LBA). The absence of spores was assessed by modified Schaeffer-Fulton staining [28]. Growth was assessed on trypticase soy agar (TSA), MacConkey agar (MAC), CBA, LBA, blood-heart infusion (BHI) agar, and Müller-Hinton (MH) agar (all from Oxoid). Growth at different temperatures (4°C, 25°C, 30°C, 35°C, 37°C, 42°C, and 46°C), different pH values (pH 4.0–10.0, at intervals of 1.0 pH unit), and different salt concentrations (0%–10%, w/v, NaCl, at intervals of 1%) was determined in trypticase soy broth (TSB) after incubation for 24 h in the thermostatically controlled water bath. Anaerobic growth was determined by incubating cultures on BHI agar for 48 h in the anaerobic bag (bioMérieux). Metabolic profiles were determined using API 20E, API 50CHE test strips, and VITEK 2 Gram-negative Identification cards according to the manufacturer's instructions (bioMérieux). Catalase activity was determined by bubble formation after dropping 3% (v/v) H_2O_2 on fresh biomass grown for 24 h on nutrient agar. Oxidase activity was determined using oxidase reagents.

2.6. Accession numbers

The genome sequences of CRE-3FA-0001^T, CRE-138-0026, and CRE-138-0111 in this study have been deposited in the NCBI database under accession numbers JARRYG000000000.1, JAUQTF000000000.1, and JAUQTG000000000.1. The 16S rRNA sequences of CRE-3FA-0001^T, CRE-138-0026, and CRE-138-0111 have been deposited in the NCBI database under accession numbers OR378508, OR378509, OR378510. The type strain of *Providencia huashanensis* CRE-3FA-0001^T was submitted to the Korean Collection for Type Cultures (KCTC) and the China Center for Type Culture Collection (CCTCC) with accession numbers of KCTC 8373^T and CCTCC AB 2023186^T.

3. Results

3.1. Phenotypic and genetic characteristics of the clinical isolates

Antimicrobial susceptibility testing revealed that the three *P. huashanensis* strains were resistant to most first-line antimicrobials, including cephalosporins, carbapenems, monobactams, fluoroquinolones, aminoglycosides, and even new β-lactam-β-lactamase inhibitor combinations (Table 1). Not surprisingly, all the three *P. huashanensis* strains were predicted to be intrinsically resistant to colistin, polymyxin B. Genetic analysis revealed that the *P. huashanensis* strains carried several acquired resistance genes, including those encoding ESBLs (bla_{PER}), carbapenemases (bla_{NDM}, bla_{OXA}, bla_{KPC}), aminoglycoside modifying enzymes (*aac, aad, ant*), 16S rRNA methyltransferase (*armA*), plasmid-mediated quinolone resistance determinant (*qnrD*), etc. The virulence profiles of the three *P. huashanensis* strains showed that they carried type III secretion system and type VI secretion system (Table 2).

From the above analysis of the three *P. huashanensis* strains, it was found that the novel species exhibited high levels of multidrug resistance (MDR) and carried a variety of resistance and virulence genes. To explore the difference in antimicrobial and virulence genotypic profiles between *P. huashanensis* and other *Providencia* species, the distribution of resistance genes and virulence genes was investigated using 115 genomes of the genus *Providencia* retrieved from the NCBI database. As shown in Figure 1A, the resistance-nodulation-cell division antibiotic efflux pump encoding gene, CRP, was commonly found throughout the genus *Providencia*. However, not all *Providencia* species harboured a diverse array of antimicrobial resistance genes. *P. huashanensis* and its evolutionary branches, including *P. rettgeri, P. vermicola*, and *P. huaxiensis*, were genetically similar and generally carried different resistance genes. However, it was clear that *P. huashanensis* had a unique combination of *tetB*+*tetR*+*tet59* genes in its tetracycline-related genotypes, which was different from any other *Providencia* species. On the other hand, *P. stuartii* and *P. thailandensis* represented another evolutionary branch that was also rich in antibiotic resistance genes and displayed distinct characteristics in their aminoglycoside-resistance-related genotype combinations. Furthermore, using stringent protein alignment as shown in Figure 1B, *P. huashanensis* was found to possess two sets of virulence secretion system-related genes, *hcp/tssD* and *ssaG*. This feature was only observed in only the subset of *P. rettgeri* species.

Table 1
Antimicrobial susceptibility profiles of *Providencia huashanensis* CRE-3FA-0001^T, CRE-138-0026, CRE-138-0111.

Antimicrobial agents	MIC (mg/L)		
	CRE-3FA-0001^T	CRE-138-0026	CRE-138-0111
Amikacin	>128 (R)	16 (R)	64 (R)
Aztreonam	>128 (R)	>128 (R)	>128 (R)
Aztreonam-avibactam	>128 (R)	≤0.06 (S)	2 (S)
Ceftazidime	>128 (R)	>128 (R)	>128 (R)
Ciprofloxacin	>128 (R)	8 (R)	16 (R)
Colistin	>128 (R)	>128 (R)	>128 (R)
Ceftazidime-avibactam	>128 (R)	>128 (R)	>128 (R)
Cefepime	>128 (R)	64 (R)	>128 (R)
Imipenem-relebactam	8 (R)	>128 (R)	>128 (R)
Imipenem	>128 (R)	>128 (R)	>128 (R)
Meropenem	>128 (R)	128 (R)	>128 (R)
Polymyxin B	>32 (R)	>32 (R)	32 (R)
Trimethoprim-sulfamethoxazole	>128 (R)	>128 (R)	>128 (R)
Tigecycline	1 (S)	1 (S)	2 (S)

S: susceptible; R: resistant.

3.2. ANI and isDDH

The pairwise ANI and isDDH between the strains CRE-3FA-0001^T, CRE-138-0026, CRE-138-0111 and the type strain of the known *Providencia* species were shown in Table 3. It was found that three *P. huashanensis* strains shared only 83.5%–85.8% identity, which was below the ANI cut-off of 95%–96% to define a bacterial species [29]. The isDDH relatedness between the three *P. huashanensis* strains and the known *Providencia* species ranged from 21% to 25.5%, which was below the 70% cut-off. The results of the ANI and isDDH analyses suggested that the three *P. huashanensis* strains represented a novel *Providencia* species.

3.3. Phylogenetic reconstruction

The phylogenetic tree based on core SNPs showed that the three *P. huashanensis* strains were located on separate evolutionary branches and were distinct from all known *Providencia* species (Fig. 2A). The bootstrap percentages of the tree were all above 90%, indicating that the phylogenetic tree was credible. What's more, the number of SNP sites among the three *P. huashanensis* strains was less than 10, suggesting that the three strains may have originated from single clone. As shown in Figure 2C, the phylogenetic position of *P. huashanensis* at the 16S rRNA sequence level suggested a separate branch within the genus *Providencia*, although the partial bootstrap percentages of the tree were less than 70%. The phylogenetic tree based on five housekeeping genes (*fusA, lepA,*

Table 2
Genetic analysis of *Providencia huashanensis* CRE-3FA-0001^T, CRE-138-0026, and CRE-138-0111.

Genetic analysis	Agents	CRE-3FA-0001^T	CRE-138-0026	CRE-138-0111
Antimicrobial resistance	β-Lactam	bla_{KPC-2} bla_{OXA-1} bla_{PER-4}	bla_{OXA-10} bla_{NDM-1}	bla_{OXA-1} bla_{OXA-10} bla_{NDM-1}
	Aminoglycoside	*aac(6′)-Ib-cr6* *aad-A16* *ant(3)-IIa* *aph(3)-Ia* *armA*	*aac(6′)-Ib3* *aac(6′)-Ib-cr* *aadA1* *ant(2″)-Ia*	*aac(6′)-Ib3* *aadA1* *ant(2″)-Ia* *aac(6′)-Ib-cr*
	Macrolide	*msr(E), mph(E)*		
	Quinolone	*qnrD1* *aac(6′)-Ib-cr6*	*qnrD1* *aac(6′)-Ib-cr*	*qnrD1* *aac(6′)-Ib-cr*
	Rifamycin	*arr-3*		*arr-3*
	Folate pathway antagonist	*sul1* *dfrA1* *dfrA27*	*sul1* *dfrA1*	*sul1* *dfrA1*
	Tetracycline	*tetB, tet59, tetR*	*tetB, tet59*	*tetB, tet59, tetR*
Virulence		*hcp/tssD,ssaG*		

Table 3
ANI and isDDH values between strains CRE-3FA-0001^T, CRE-138-0026, CRE-138-0111, and the type strains of *Providencia* species.

Species	Strain	Accession no.	CRE-138-0026		CRE-138-0111		CRE-3FA-0001^T	
			ANI (%)	isDDH (%)	ANI (%)	isDDH (%)	ANI (%)	isDDH (%)
Providencia burhodogranariea	DSM 19968^T	AKKL00000000	83.7	21.5	83.7	21.5	83.7	21.5
Providencia sneebia	DSM 19967^T	AKKN00000000	83.5	21.1	83.5	21.1	83.6	21.2
Providencia alcalifaciens	DSM 30120^T	ABXW00000000	83.8	21.2	83.8	21.3	83.8	21.2
Providencia huaxiensis	KCTC 62577^T	NQWB00000000	85.2	25.4	85.3	25.4	85.4	25.6
Providencia rettgeri	NCTC 11801^T	NZ_CP017671	85.2	25.5	85.2	25.5	85.2	25.4
Providencia thailandensis	KCTC 23281^T	BMYH00000000	83.6	21	83.7	21.1	83.6	21
Providencia vermicola	DSM 17385^T	JAGSPI000000000	84.5	23.7	84.5	23.7	84.5	23.7
Providencia stuartii	DSM 4539^T	UGUC00000000	84.3	21.3	84.3	21.4	84.5	21.5
Providencia rustigianii	DSM 4541^T	NZ_UGTY00000000	83.7	21.1	83.7	21.2	85.8	22.7
Providencia heimbachae	DSM 3591^T	NZ_LS483422	83.7	22.3	83.8	22.3	83.8	22.3
Providencia manganoxydans	KCTC 92091^T	CP067099.1	84.3	21.7	84.6	21.9	85.3	22.6
Providencia wenzhouensis	R33^T	CP072453	84.6	23.9	84.6	23.9	84.5	23.8
Providencia hangzhouensis	PR-310	CP135052.1	85.4	25.8	85.5	25.9	85.8	26.4
Providencia huashanensis	CRE-138-0026	JAUQTF000000000	100	100	99.9	100	99.9	99.1
Providencia. huashanensis	CRE-138-0111	JAUQTG000000000	99.9	100	100	100	99.9	99.4
Providencia huashanensis	CRE-3FA-0001^T	JARRYG000000000	99.9	99.4	99.9	99.1	100	100

ileS, leuS, gyrB) of the genus *Providencia* also confirmed the taxonomic position of the novel species and bootstrap values ranging from 90% to 100%, suggesting that *P. huashanensis* belonged to a novel species in *Providencia* and that the clade topology around *P. huashanensis* was sufficiently supported (Fig. 2B).

3.3.1. Physiology and chemotaxonomy

Through a series of growth tests and bioassays, we established the profiles of physiological and metabolic characteristics. In terms of culture media, the *P. huashanensis* strains could grow on TSA, MAC, CBA, LBA, BHIA, and MHA at 35°C under air conditions. For the three *P. huashanensis strains*, the optimal growth condition was on CBA at 35°C under the air environment. The colony of the bacteria growing on CBA was white, opaque, shiny, convex, and circular, with sharp edges and characteristic odour (Fig. 3). For the growth temperature, the bacteria could grow at 25°C–37°C with the optimum temperature at 35°C–37°C, but not at 4°C, 42°C or 46°C. For salt, acid, and alkali tolerance, the bacteria could grow in the presence of 0%–7% (w/v) NaCl in TSB; growth occurred in between pH 5.0–9.0, and the optimum pH was 7.0–8.0. The bacteria grew under both aerobic and anaerobic conditions but better under aerobic conditions. Light microscopy revealed that the bacteria were Gram-negative, non-motile, and non-spore-forming. Comparisons of the physicochemical characteristics tested by API 50CHE and API 20E strips between three strains of *P. huashanensis* strains and other model species of the genus *Providencia* are shown in Table 4. The biochemical profiles of three *P. huashanensis* strains were different from the other *Providencia* species. We also performed the oxidase test and catalase test, which was positive for catalase and negative for oxidase. The overall results of API 20E, API 50CHE, and VITEK 2 Gram-negative identification cards were shown in Supplementary Tables 1 and 2.

3.4. Description of P. huashanensis

P. huashanensis (hua.shan.en'sis. N.L. fem. adj. huashanensis referring to the Huashan Hospital, Shanghai, PR China, where the type strain was recovered). Cells are Gram-negative, non-motile, non-gas producing, non-spore-forming, facultatively anaerobic, and capable of growing on media including CBA, TSA, MAC, LBA, BHIA, and MHA. Colonies on CBA after 24 h of incubation at 37°C are white, opaque, shiny, convex, and circular, with sharp edges and characteristic odour. The bacteria can grow at 25°C–37°C with optimal growth temperature at 35°C–37°C. Cells grow between pH 5.0–9.0 and optimal pH is 7.0–8.0. Cells grow at 35°C in the presence of 0%–7% (w/v) NaCl in TSB. It is catalase-positive and oxidase-negative. Acid is produced from glycerol, ribose, ribitol, galactose, glucose, fructose, mannose, inositol, and mannitol, but

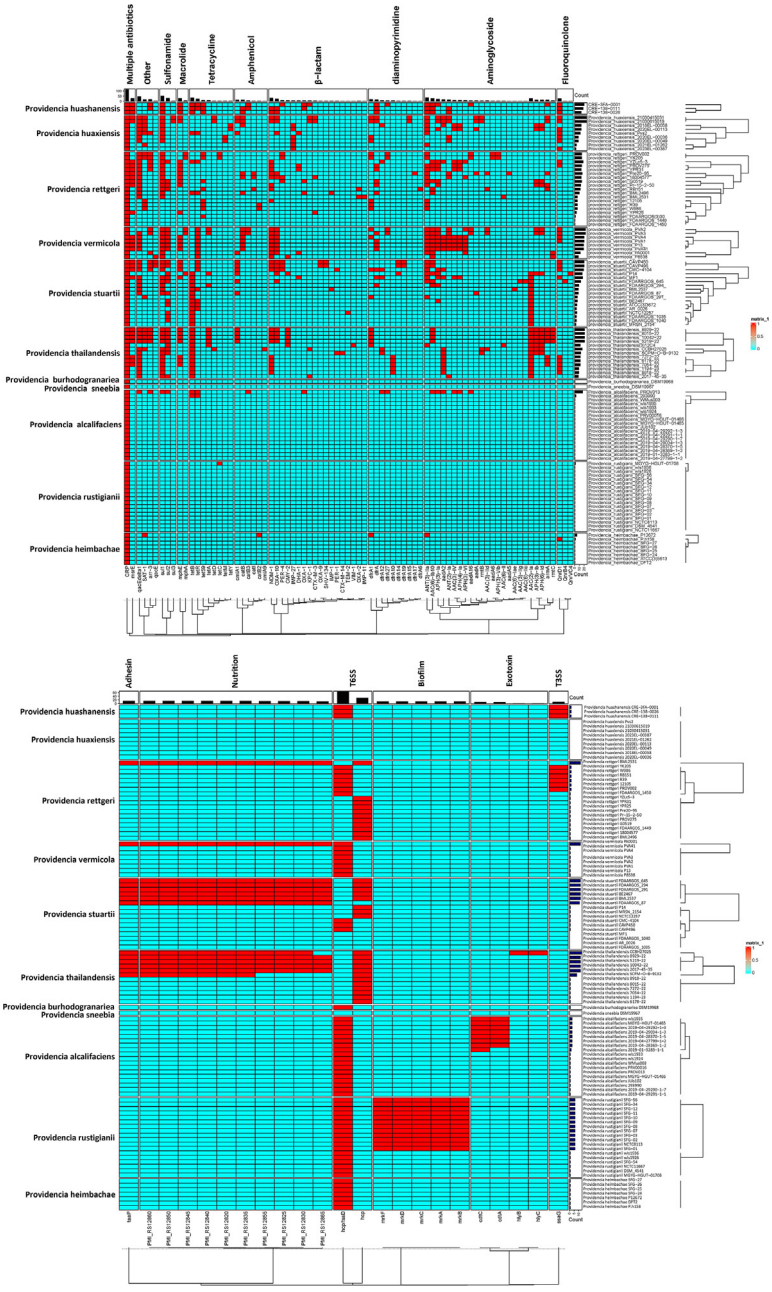

Figure 1. A cluster heatmap of virulence and antibiotic resistance genes for 115 sequences in the genus *Providencia*. Following the order of the core-SNP phylogenetic tree, with hierarchical clustering using Manhattan distance and complete linkage. The length of the bars represents the number of non-zero values in each row or column. (A) The different distribution of antimicrobial genotypic profiles between *Providencia huashanensis* and other *Providencia* species. (B) The different distribution of virulence genotypic profiles between *P. huashanensis* and other *Providencia* species. Legend, copies of resistance genes or virulence genes ranged from 0 to 1.

not from erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, β-methyl-D-xyloside, sorbinose, rhamnose. The G+C% content of the strain CRE-3FA-0001^T, CRE-138-0026, CRE-138-0111 is 40.6%, 40.4%, 40.4%, respectively. The type strain is CRE-3FA-0001^T = KCTC 8373^T = CCTCC AB 2023186^T.

4. Discussion

Bacteria of the genus *Providencia* are Gram-negative opportunistic pathogens, belonging to the *Proteae* of the family *Enterobacteriaceae*, which have been isolated from a wide range of organisms and environments [30]. *Providencia* species include *P. thailandensis* (2013; Thailand; seafood processing wastewater), *P. entomophila* (2019; Tunisia; olive pest), *P. burhodogranariea* (2009; the United States; Drosophila melanogaster), *P. sneebia* (2009; the United States; Drosophila melanogaster), *P. manganoxydans* (2022; China; soil), *P. wenzhouensis* (2021; China; rabbit) have only been isolated from environments or insects [1,2,4,5,12], and the remaining species of the genus *Providencia* play a pathogenic role to humans and could cause infectious diseases, including *P. vermicola* (2006; India; insects) and *P. heimbachae* (1986; the United States; penguin), *P. alcalifaciens* (1979; Canada; human), *P. huaxiensis* (2019; China; human), *P. hangzhouensis* (2023; China; human), *P. stuartii* (1975; the United Kingdom; human), *P. rettgeri* (1979; Canada; human), and *P. rustigianii* (1983; the United States; human) [3,6–11,13,30,31].

According to the previous research, the above pathogenic *Providencia* species were mainly obtained from diarrhoeal patients

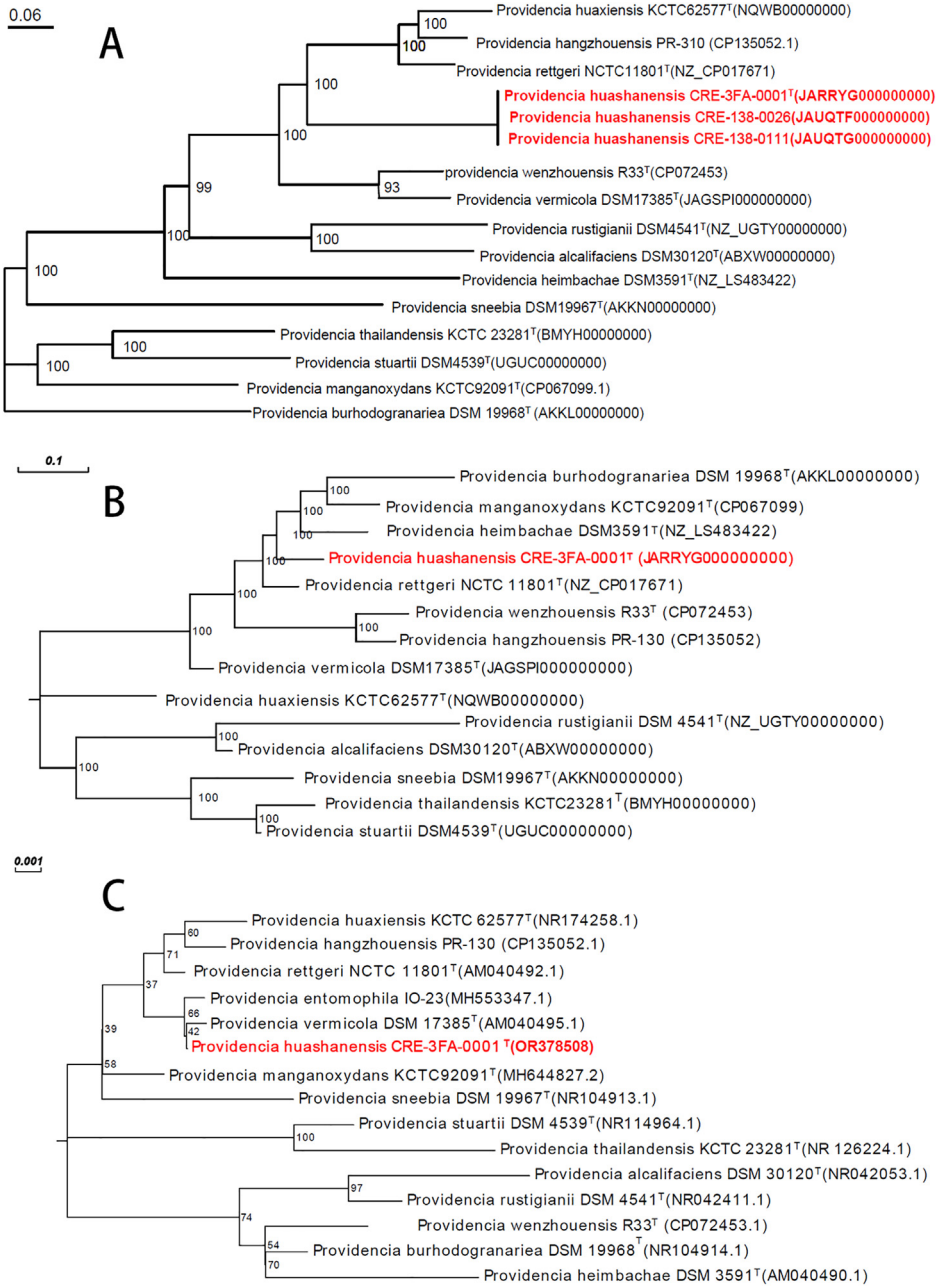

Figure 2. Phylogenetic reconstruction. (A) The phylogenetic tree of the three *Providencia huashanensis* strains CRE-3FA-0001^T, CRE-138-0026, CRE-138-0111, and model species of the genus *Providencia* based on 20046 SNPs from 91 core genes (100%). (B) Phylogenetic tree based on multi-locus sequence analysis concatenating the complete *fusA* (2127 bp), *lepA* (1692 bp), *leuS* (2583 bp), *gyrB* (2415 bp), and *ileS* (2811 bp) gene sequences. Bar, 0.1 substitutions per nucleotide position. *Providencia entomophila* IO-23 was not included in this tree due to the lack of its genome sequence. (C) Phylogenetic reconstruction based on the 16S rRNA gene (1516bp) and the 16S rRNA gene sequence identity values between *P. huashanensis* and other species of genus *Providencia*. Bar, 0.001 substitutions per nucleotide position. The numbers at each node represent bootstrap values. *: Since the sequence of the 16S rRNA gene and five housekeeping genes of the three strains was identical, *P. huashanensis* was represented by strain CRE-3FA-0001^T strain in panels (B and C).

and retained relatively high susceptibility rates to most antimicrobial agents such as cephalothin, β-lactams, quinolones, chloramphenicol, aminoglycosides, and tetracycline [7,8,14,32,33]. However, in recent years, some *Providencia* species, particularly *P. rettgeri* and *P. stuartii,* have developed high-level MDR and caused widespread hospital-acquired infections, including UTI, pneumonia, bacteraemia, neonatal sepsis, ocular infections, meningitis, endocarditis, and diarrhoea [34–36]. The MDR phenotype was primarily driven by relevant antimicrobial resistance genes located either on the chromosome or on plasmids, encoding β-lactamases (NDM, OXA, PER, IMP, CTX-M), 16S rRNA methyltransferases (RmtC), aminoglycoside-modifying enzymes (ANT, AAC) [18,34,37,38]. Unlike other Gram-negative bacteria (e.g. *Acinetobacter baumannii, Klebsiella pneumoniae*), some *Providencia* species, such as *P. stuartii* and *P. rettgeri*, were also notorious for the intrinsic resistance to several antimicrobials including last-resort drugs such as colistin and tigecycline [18,35,37,39]. The emergence of MDR *Providencia* species posed the significant threat to public health. It is essential to monitor the spread of these strains and develop new strategies for their control and treatment.

In this study, we isolated three strains from hospitalized patients, named *P. huashanensis*, belonging to a novel species of the

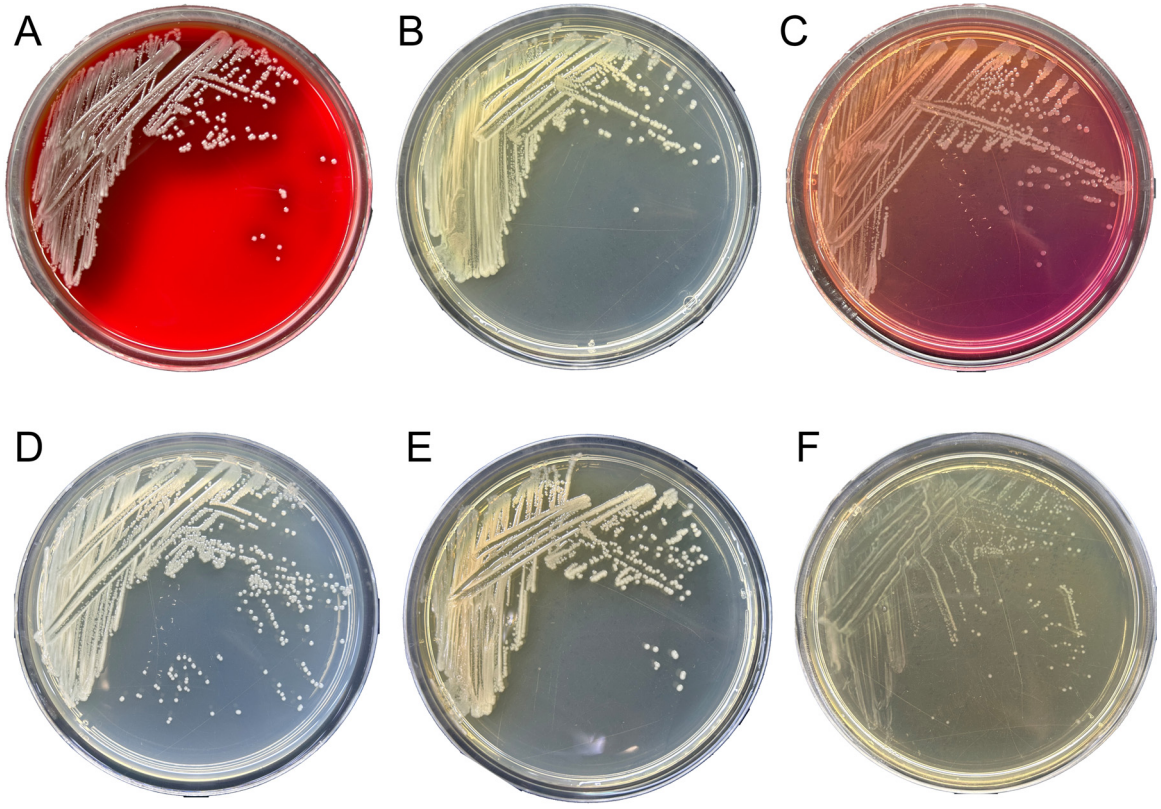

Figure 3. The morphology of *Providencia huashanensis*' colony in different culture mediums. (A) Columbia blood agar, (B) Luria-Bertani agar, (C) MacConkey agar, (D) Müller-Hinton agar, and (E) trypticase soy agar. (A–E, aerobic condition) (F) Blood-heart infusion agar (anaerobic condition).

Table 4
Differentiation of *Providencia* species based on biochemical reactions[a] and genomic information.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Citrate utilization	+	+	−	−	−	−	−	−	+	+	+	−	−	−	+
Urea	−	−	−	−	−	+	−	+	−	+	+	+	+	+	+
Indole production	−	+	+	+	+	+	+	+	−	+	+	−	−	−	+
Gelatinase	−	+	−	+	+	+	+	+	+	−	−	−	−	−	−
Acid from															
D-Lyxose	−	+	−	−	−	−	−	−	−	−	+	−	−	−	NA
D-Mannitol	+	−	+	−	+	−	−	+	−	+	+	+	+	+	+
Raffinose	+	−	−	−	−	+	−	−	−	−	NA	−	−	−	NA
D-Xylose	+	−	−	−	−	−	−	+	−	−	−	−	−	−	NA
L-Arabinose	+	−	+	−	−	+	−	−	−	−	−	−	−	−	−
L-Rhamnose	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+
2-Keto-gluconate	+	−	+	−	−	−	−	−	−	+	−	−	−	−	NA
Arbutin	+	−	−	−	+	−	−	+	+	+	−	+	+	+	NA
Cellobiose	+	−	−	−	−	−	−	−	−	−	−	−	−	−	NA
Esculin	+	−	−	+	+	+	+	+	+	+	−	+	+	+	NA
Glycerol	+	+	−	−	−	+	−	−	−	−	+	+	+	+	NA
Mannitol	+	+	−	−	−	−	−	−	−	+	+	+	+	+	+
Salicin	+	−	−	−	+	−	−	+	−	+	−	+	+	+	NA
Sorbitol	+	−	−	−	+	+	−	+	−	−	−	−	−	−	−
Sucrose	+	+	−	−	−	−	−	−	−	−	+	−	−	−	−
Draft genome size (Mb)	4.3	4.5[b]	4.2	4.0[b]	4.6	4.5[b]	4.0	3.9	4.3[b]	4.8[b]	4.3	4.7	4.7	4.7	4.6[b]
No. of contigs	63	4	22	1	59	2	163	17	1	6	48	43	42	54	3
N50 (bp)	220300	2500000	309600	4000000	140100	4300000	249100	315000	4300000	4800000	4300000	421236	231312	259733	4500000
No. of protein-coding genes	3913	3022	3741	3587	3732	3980	3513	2964	3749	4290	4218	4392	4321	4401	4245
DNA G+C content (mol%)	41.0	41.0	41.0	41.5	39	40.5	41.0	38.0	40.0	41.0	40.0	40.6	40.4	40.4	40.5

Strains: 1. *P. thailandensis* KCTC 23281^T; 2. *P. stuartii* DSM 4539^T; 3. *P. vermicola* DSM 17385^T; 4. *P. alcalifaciens* DSM 30120^T; 5. *P. burhodogranariea* DSM 19968^T; 6. *P. rettgeri* NCTC 11801^T; 7. *P. rustigianii* DSM 4541^T; 8. *P. sneebia* DSM 19967^T; 9. *P. heimbachae* NCTC 12003^T; 10. *P. huaxiensis* WCHPr000369^T; 11. *P. manganoxydans* LLDRA6^T; 12. *P. huashanensis* CRE-3FA-0001^T;13. *P. huashanensis* CRE-138-0026; 14. *P. huashanensis* CRE-138-0111; 15. *P. hangzhouensis* PR-310^T.
NA: not available.
[a] The biochemical reactions of *P. wenzhouensis* were not available.
[b] Genome sequences are complete.

genus *Providencia*. Antimicrobial resistance profile analysis showed that the three strains of *P. huashanensis* were highly resistant to most of the first-line antimicrobials used in clinical practice, including cephalosporins, carbapenems, aminoglycosides, quinolones, sulphonamides, and even polymyxin, colistin, and β-lactam-β-lactamase-inhibitor combinations (ceftazidime-avibactam, imipenem-relebactam, aztreonam-avibactam). The genetic profiles revealed that the co-production of multiple resistance genes was the common feature of the three strains. For the novel *Providencia* species, there were three β-lactamase gene combinations which were bla_{KPC-2}+bla_{OXA-1}+bla_{PER-4}, bla_{OXA-10}+bla_{NDM-1}, bla_{OXA-1}+bla_{OXA-10}+bla_{NDM-1}. Accompanied by other resistance genes, such as aminoglycoside resistance genes (*aac, ant, aph, aad, armA*), quinolone resistance determinant (*qnrD1*), and sulphonamide resistance genes (*sul* and *dfrA*), the *Providencia* strains tended to be resistant not only to common first-line antimicrobials but also to the novel β-lactam-β-lactamase-inhibitor combinations (ceftazidime-avibactam), posing a significant threat to public health. And tigecycline would be an alternative to treat infections against *P. huashanensis*. In addition, the coexistence of type III and VI dual secretion systems allowed the strains to facilitate the establishment of infection and evade the host immune response [40]. Analysis the antimicrobial and virulence genotypic profiles revealed that *P. huashanensis* exhibited diverse and MDR and virulence compared to other species of genus *Providencia*.

To further understand the characteristics of *P. huashanensis*, we used methods of phenotypic testing, ANI and isDDH values, SNP analysis, 16S rRNA gene sequence identity, MLSA, and MALDI-TOF MS to identify species and determined the phylogenetic relationship between *P. huashanensis* and other known *Providencia* species. At the genomic level, the values of ANI and isDDH showed superior accuracy in distinguishing *P. huashanensis* from other known *Providencia* species, with ANI ranging from 83.5% to 85.8% and isDDH ranging from 21% to 25.6%. According to the phylogenetic reconstruction, *P. huashanensis* species represented an independent evolutionary branch in the genus *Providencia* and showed an outstanding evolutionary distance from the species discovered in the last 5 years. The biochemical and physiological tests were also used to characterize the *P. huashanensis* species.

There were a number of limitations to this study. For one thing, the three strains belonged to a single clone, while they were isolated at three different times and in two distant hospitals. This phenomenon needed more further testing and evidence to explain the widespread prevalence of *P. huashanensis* species. On the other hand, from the analysis of patient cases, *P. huashanensis* seemed to be an opportunistic pathogen for humans, the underlying and exact pathogenic mechanism needed further investigation.

5. Conclusion

This case highlights the potential health threat posed by the novel *Providencia* species, named *P. huashanensis*. In this study, all three *P. huashanensis* were associated with hospital-acquired infections and were highly resistant to most first-line antimicrobials. Increased surveillance for the novel species and improved identification methods are essential.

Declarations

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Ethical approval: Not required.

Sequence information: I have deposited the sequence of plasmids to NCBI database, with deposit ID (OR805036, OR805037, OR0805038).

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention (CDC). The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. All experiments involving *Providencia* and other microorganisms in this article were carried out under standard biosecurity and institutional safety standards. All experiments were conducted in biosafety level 2 laboratories and strictly performed following laboratory biosafety regulations.

CRE-3FA-0001^T are preserved in the Korean Collection for Type Cultures (https://kctc.kribb.re.kr/en) and China Center for Type Culture Collection (http://cctcc.whu.edu.cn/portal/Index/index) (CRE-3FA-0001^T =KCTC 8373^T=CCTCC AB 2023186^T). The raw sequence data for CRE-3FA-0001^T, CRE-138-0026^T, CRE-138-0111^T identified in this study have been deposited in the NCBI database (accession number: CRE-3FA-0001^T =JARRYG000000000, CRE-138-0026^T =JAUQTF000000000, CRE-138-0111^T =JAUQTG000000000). The type strain, *P. huashanensis* CRE-3FA-0001^T (=KCTC 8373^T =CCTCC AB 2023186^T), was isolated from the abdominal cavity drainage fluid specimens of a patient at the Yuhuangding Hospital of Qingdao University in Yantai, Shandong, China.

Author contributions

Article design and writing: Weiwei Yang, Jing Chen, Fupin Hu; Clinical sample and case collection: Fengzhen Yang, Ping Ji; In vitro experiment: Weiwei Yang, Siquan Shen, Dandan Yin; Data processing and analysis: Weiwei Yang, Jing Chen; All authors contributed to the acquisition, analysis, or interpretation of the data and reviewed and approved the final version of the manuscript.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2024.107211.

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