Clinical and molecular characterisation of Vibrio furnissii isolates from patients with diarrhoea

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Research Article

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Abstract

Background: *Vibrio furnissii* is an emerging human pathogen closely related to *Vibrio fluvialis* that causes acute gastroenteritis. However, the distribution of virulence factors, antibiotic resistance and molecular epidemiological features of *V. furnissii* isolates in China remain unknown.

Results: We collected seven *V. furnissii* strains from 1,985 stool samples from people over 14 years old with acute diarrhoea at a general hospital in Beijing, China between April and October 2018. Blood agar plates with 20 μg/mL ampicillin helped to isolate more *V. furnissii* compared with only using TCBS (thiosulfate-citrate-bile salts-sucrose agar). Phylogenetic analysis based on SNPs (single-nucleotide polymorphisms) was used to assess the relatedness of isolates. Virulence genes *T2SS*, *T3SS*, *vfh*, *hupO* and *vfp* were detected in all *V. furnissii* strains, and *T6SS* also occurred at a high frequency of 85.7%. High resistance to cefazolin and tetracycline was found in the *V. furnissii* isolates at respective rates of 100.0% and 57.1%, and intermediate resistance to ampicillin/sulbactam and imipenem was found at respective rates of 85.7% and 85.7%. Of the tested strains, 42.9% showed resistance to at least three antibiotics. Transposon islands with concentrations of resistance genes occurred in two strains. Phylogenetic analysis separated the *V. furnissii* strains into three major clades.

Conclusion: This study provided detailed bioinformatic, antibiotic susceptibility and clinical features of *V. furnissii* in China. However, continued and enhanced laboratory-based surveillance is needed for prevention and control of *V. furnissii* infection.

Background

Members of the genus *Vibrio* are abundant in marine environments [1] and in inland rivers where seawater intrusion occurs [2]. Although *Vibrio furnissii* is closely related to *Vibrio fluvialis*, it differs by its ability to produce gas through carbohydrate fermentation [3]. *V. furnissii* is a potential pathogen of European eel (*Anguilla anguilla*) [4], and is also regarded as an emerging human pathogen that can spread through the consumption of contaminated seafood products. *V. furnissii* infection causes acute gastroenteritis with symptoms including diarrhoea, abdominal cramps, nausea, and vomiting [3]. Like *V. fluvialis*, *V. furnissii* has rarely been reported in China and detailed bioinformatic and clinical information remains unknown. We therefore studied *V. furnissii* strains isolated from the stool of patients with diarrhoea and revealed information on the virulence and drug resistance genes and antibiotic resistance of the collected strains as well as the clinical characteristics of the sampled patients.

Results

Clinical features

We collected 1,985 stool samples from people over 14 years old with acute diarrhoea at a general hospital in Beijing, China between April and October 2018. An epidemiological questionnaire was completed by the participants to assess clinical history and physical fitness. There were ultimately 349 diarrhoea cases related to bacteria, which included seven cases (2.0%) caused by seven different strains of *V. furnissii*. This was less than the numbers of cases caused by *Aeromonas* spp. (165, 47.3%), *Vibrio parahaemolyticus* (93, 26.6%), *Salmonella* spp. (30, 8.6%), *Vibrio fluvialis* (25, 7.2%), *Vibrio cholerae* (20, 5.7%) and *Plesiomus shigelloides* (8, 2.3%), but more than the single case caused by *Shigella* spp. (1, 0.3%). The use of agar base with sheep erythrocytes and AMP also helped isolate more strains of *V. furnissii* and other *Vibrio* species (Table 1).

The clinical and epidemiological characteristics of the seven patients with diarrhoea caused by *V. furnissii* strains are shown in Table 2. The sex ratio (male:female) was 2.5. Among these patients, one also had vomiting, two had abdominal pain, three had watery stool and none had fever. Erythrocytes and leukocytes were present under high magnification (× 40) in respectively one and two of the stool samples collected from these seven patients.

Table 1
Comparison of *Vibrio* numbers isolated from TCBS and AMP blood agar

Group	*V. parahaemolyticus* (n = 93)	*V. fluvialis* (n = 25)	*V. cholerae* (n = 20)	*V. furnissii* (n = 7)
A	25 (26.9%)	2 (8.0%)	6 (30.0%)	0 (0.0%)
B	28 (30.1%)	16 (64.0%)	6 (30.0%)	3 (42.9%)
C	40 (43.0%)	7 (28.0%)	8 (40.0%)	4 (57.1%)

A: Strains were isolated only from TCBS, B: Strains were isolated only from AMP blood agar, C: Strains could be isolated from both TCBS and AMP blood agar

Table 2
Clinical characteristics of seven patients with diarrhoea caused by seven different strains of *V. furnissii*

Strain	Sex[a]	Age	Month	Fever[b]	Abdominal pain	Stool consistency	Vomiting	Erythrocytes No./Hp[c]	Leukocytes No. /Hp[c]
VFBJ01	M	45	May	-	-	watery	-	0	0
VFBJ02	F	65	June	-	-	loose	-	0	0
VFBJ03	M	15	July	-	-	watery	+	0	1
VFBJ04	M	62	July	-	-	loose	-	0	0
VFBJ05	F	36	July	-	+	loose	-	3	30
VFBJ06	M	64	July	-	-	watery	-	0	0
VFBJ07	M	72	August	-	+	loose	-	0	0

[a] M, male; F, female.

[b] Defined as axillary temperature > 37.7°C.

[c] Erythrocyte or leukocyte number in stool identified under high-magnification field.

Bioinformatic analysis

As shown in Table 3, the seven *V. furnissii* strains contained chromosomes of 4,802,519 bp to 5,109,544 bp in length with GC contents of 50.46–50.78% and 4,594 to 4,727 genes. They contained no plasmids, but had 8 to 15 GIs (Genomic islands) and 1 to 5 prophages in the genome at the time of sequencing.

The genome of *V. furnissii* contained several virulence factor-encoding genes and antibiotic resistance genes. As shown in Table 4, all seven genomes contained the following virulence operons: polar flagella and lateral flagella gene clusters, *T2SS* (type II secretion system), *T3SS* (type III secretion system), *VFH* (*V. fluvialis* haemolysin) [5], *HupO* (hemin-binding outer membrane protein) [6] and metalloprotease *VFP* (*V. fluvialis* protease)[7]. In addition, all strains other than VFBJ06

contained a *T6SS* (type VI secretion system). VFBJ07 had a series of antibiotic resistance genes, including those for aminoglycoside phosphotransferase (*strA* and *strB*), SUL resistance (*sul1* and *sul2*), TET resistance (*tetA* and *tetB*), florfenicol/CHL resistance (*floR*) and quinolone resistance *aac(6')-Ila*. VFBJ05 also had these antibiotic resistance genes except for *sul1*, *tetB* and *aac(6')-Ila*, and VFBJ01 had the antibiotic resistance genes *strA*, *strB* and *sul2*. Furthermore, we found an insert sequence (IS) through BLAST in the corresponding database and obtained the transposon islands containing the antibiotic resistance genes in VFBJ05 and VFBJ07. As shown in Figs. 1 and 2, one transposon island in VFBJ05 contained *strB*, *strA*, *tetA* and *sul2* and another contained *floR*, while one island in VFBJ07 contained *strB*, *strA*, *tetA*, *sul1*, *sul2* and *aac(6')- a* and another contained *tetB*.

Table 3
Genome characteristics of seven *V. furnissii* strains

Sample ID	Genome size (bp)	Sequence GC%	No. of Genes	No. of GIs	No. of Prophages	No. of tRNA	No. of rRNA	No. of sRNA
VFBJ01	5109544	50.46	4727	15	2	74	1	16
VFBJ02	4906640	50.75	4596	8	4	86	7	15
VFBJ03	5062567	50.78	4725	12	3	61	4	14
VFBJ04	4904255	50.58	4594	13	2	95	6	21
VFBJ05	4965951	50.56	4656	15	2	99	8	17
VFBJ06	4802519	50.74	4489	11	1	71	6	15
VFBJ07	5016530	50.56	4686	12	5	87	6	20

Table 4
Genomic presence/absence of main CDSs of toxins and antibiotic resistance in seven *V. furnissii* strains

Putative protein CDS [a]	VFBJ01	VFBJ02	VFBJ03	VFBJ04	VFBJ05	VFBJ06	VFBJ07
Appendages							
Polar flagellum	+	+	+	+	+	+	+
Lateral flagellum	+	+	+	+	+	+	+
Toxins and exoenzymes							
T2SS	+	+	+	+	+	+	+
T3SS	+	+	+	+	+	+	+
T6SS	+	+	+	+	+	-	+
VFP	+	+	+	+	+	+	+
VFH	+	+	+	+	+	+	+
HupO	+	+	+	+	+	+	+
Antibiotic resistance							
StrA	+	-	-	-	+	-	+
StrB	+	-	-	-	+	-	+
Sul1	-	-	-	-	-	-	+
Sul2	+	-	-	-	+	-	+
tetA	-	-	-	-	+	-	+
tetB	-	-	-	-	-	-	+
floR	-	-	-	-	+	-	+
aac(6')-Ila	-	-	-	-	-	-	+

[a] CDS: coding sequence

Comparative genome analysis and phylogenetic tree

V. furnissii was first identified mass spectrometrically using VITEK MS and then identified biochemically using VITEK II (BioMerieuX, France). We found that the seven *V. furnissii* strains were misidentified as *V. fluvialis* at a rate of 85–99% by VITEK II, so we further identified these strains by whole-genome sequencing.

A phylogenetic tree of the seven isolates of *V. furnissii* was constructed based on SNPs and aligned with *V. furnissii* reference isolates CIP102972, NCTC13120 and NCTC11218 and *V. fluvialis* reference isolate ATCC33809. This revealed three major clades, with clades I, II and III consisting of one, two and eight isolates, respectively (Fig. 3). Clade I solely contained *V. fluvialis* ATCC33809, Clade II contained *V. furnissii* strains CIP102972 and NCTC13120, and Clade III contained all seven collected *V. furnissii* isolates as well as reference isolate NCTC11218.

Susceptibility to antibiotics

The resistance profiles of the seven isolates of *V. furnissii* to 19 antibiotic agents are shown in Table 5. High resistance to CFZ and TET was found in respectively 100.0% and 57.1% of the isolates, and intermediate resistance to AMS and IMI occurred at respective rates of 85.7% and 85.7%. Only three strains, VFBJ02, VFBJ05 and VFBJ07, exhibited multiple-drug resistance patterns against more than one of the 19 antibiotic agents: VFBJ02 was resistant to AMP, CFZ, IMI, MEM and TET, and VFBJ05 and VFBJ07 were resistant to CFZ, TET and STR.

Table 5

Antibiotic susceptibility[a] patterns of all *V. furnissii* strains

Antibiotic[1]	VFBJ01	VFBJ02	VFBJ03	VFBJ04	VFBJ05	VFBJ06	VFBJ07
AMP	I[2]	R	I	I	I	R	I
AMS	I	I	I	I	S	I	I
CFZ	R	R	R	R	R	R	R
CAZ	S	S	S	S	S	S	S
FEP	S	I	S	S	S	S	S
AZM	S	I	S	S	S	S	S
AMI	S	S	S	S	S	S	S
GEN	S	S	S	S	S	S	S
CIP	S	S	S	S	S	S	S
LEV	S	S	S	S	S	S	S
IMI	I	R	I	I	I	I	I
MEM	S	R	S	S	S	S	S
TET	R	R	S	S	R	I	R
DOX	I	S	S	S	S	S	I
CHL	S	S	S	S	I	S	S
SUL	S	S	S	S	S	S	S
SXT	S	S	S	S	S	S	S
AZI	S	S	S	S	S	S	S
STR	R	S	I	I	R	S	R

[1] R: Resistant; I: Intermediate; S: Sensitive.

[2] Most breakpoints for defining susceptibility (μg/mL) are based on the CLSI (clinical and laboratory standards institute) M45-A3 standards for *Vibrio* spp., AMP: S ≤ 8, I = 16, R ≥ 32; AMS: S ≤ 8, I = 16, R ≥ 32; CFZ: S ≤ 2, I = 4, R ≥ 8; CAZ: S ≤ 4, I = 8, R ≥ 16; FEP: S ≤ 2, I = 4–8, R ≥ 16; AMI: S ≤ 16, I = 32, R ≥ 64; GEN: S ≤ 4, I = 8, R ≥ 16; CIP: S ≤ 1, I = 2, R ≥ 4; LEV: S ≤ 2, I = 4, R ≥ 8; IMI: S ≤ 1, I = 2, R ≥ 4; MEM: S ≤ 1, I = 2, R ≥ 4; TET: S ≤ 4, I = 8, R ≥ 16; CHL: S ≤ 8, I = 16, R ≥ 32; SUL: S ≤ 256, -, R ≥ 512; SXT (trimethoprim 2 μg/mL, sulfamethoxazole 38 μg/mL): S ≤ 2/38, R ≥ 4/76. Some breakpoints are based on the CLSIM100-ED31 criteria for *Enterobacteriaceae*, AZM: S ≤ 4, I = 8, R ≥ 16; DOX: S ≤ 4, I = 8, R ≥ 16. Breakpoint of AZI (S ≤ 2) is based on the CLSIM45-A3 standards for *V. cholerae*. Breakpoint of STR (S ≤ 4, I = 8, R ≥ 16) is based on the CLSI M45-A3 standards for *Y. pestis.*

Discussion

Schirmeister reported that biochemical testing was less reliable than MALDI-TOF MS (matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry) and genotyping as a technique to distinguish *V. furnissii* from *V. fluvialis* [8]. This is consistent with the present study, in which the biochemical system VITEK II showed poor ability to identify *V. furnissii*, while VITEK MS identified *V. furnissii* strains as accurately as whole-genome sequencing. Thus, MS has the potential to help clinical labs identify *V. furnissii* more quickly and easily.

Many selective media have been developed to isolate *Vibrio* spp., such as TCBS, which has been widely used for this purpose. However, the recovery of *Vibrio* species on TCBS is less effective than on nonselective media[9]. AMP is frequently added to isolation medium as a selective agent when culturing *Aeromonas* [10]. In this study, as the isolated *V. furnissii* strains caused strong beta haemolysis on blood agar and had intermediate resistance or high resistance to AMP, blood agar plates with 20 μg/mL AMP also helped to isolate more *V. furnissii* compared with only using TCBS.

In a study of the Hun-Tai River and estuary, the abundances of *V. cholerae* and *V. parahaemolyticus* were correlated with water temperature and salinity, and no pathogenic *Vibrio* spp. were identified in April, October or November, when the water temperature was below 15℃ [2]. In our study, during April to October, *V. furnissii* were only isolated from May to August, and four of the seven strains (57.1%) were isolated during July, which also suggests a close relation to temperature.

Bacterial pathogens can secrete proteins to attack other microorganisms, evade host immune systems, cause tissue damage or invade host cells [11]. To date, eight secretory systems have been described, of which three, namely *T2SS*, *T3SS* and *T6SS*, have been found in *Vibrio* spp. [11]. These three secretory systems also occurred in the seven *V. furnissii* strains in this study. Additionally, the strains in this study had the virulence factors *VFH*, *VFP* and *HupO*, which also widely occur in *V. fluvialis* [12]. These factors enhanced the virulence of *V. furnissii*.

Although the STR resistance of VFBJ01, VFBJ05 and VFBJ07 was consistent with the presence of *strA* and *strB* genes, the antibiotic susceptibility patterns of the seven *V. furnissii* strains were not completely consistent with the presence of other antibiotic resistance genes. Similarly, prior studies have shown that resistance genes do not correlate with phenotypic resistance [13–15]. In the present study, the TET resistance of VFBJ05 and VFBJ07 was consistent with the positive detection of *tetA* and *tetB*; however, VFBJ01 and VFBJ02 were also resistant to TET despite having no *tet* genes. Consequently, there are likely to be other unknown genes involved in the relationship between the genotype and phenotype of TET resistance. Ultimately, the detection and bioinformatic analysis of antibiotic resistance genes cannot completely replace antibiotic susceptibility tests, although they provide a useful complement.

Lastly, several mobile genetic elements, such as insertion sequences, transposons and gene cassettes/integrons, can move within or between DNA molecules and transfer between bacterial cells [16]. In our sample, these occurred as transposon islands upon which resistance genes were concentrated in VFBJ05 and VFBJ07, indicating that these two strains could greatly increase the spread of drug resistance among clinical isolates through the process of infection and spread.

Conclusions

In this study, we conducted bioinformatic analysis and examined the drug resistance profiles of *V. furnissii* isolated from patients in China. Virulence genes T2SS, T3SS, *vfh*, *hupO* and *vfp* were detected in all strains, with T6SS also widely carried. The *V. furnissii* isolates had high rates of resistance to CFZ and TET and intermediate resistance to AMS and IMI. In addition, 42.9% of the test strains displayed resistance to at least three antibiotics. Two strains contained transposon islands upon which resistance genes were concentrated. The phylogenetic tree separated the isolated *V. furnissii* strains into three major clades. Overall, the results contribute to the understanding of the bioinformatic and

clinical features of *V. furnissii.* Future academic and clinical efforts should focus on continued and enhanced laboratory-based surveillance to prevent and control *V. furnissii* infections.

Materials And Methods

Isolates of *V. furnissii*

We first enriched stool samples in alkaline peptone water solution (Beijing Land Bridge Technology, China) for 8 h at 37℃, and then further cultured 20 μL of the resulting mixture on Columbia agar (Oxoid, UK) containing 5% sheep erythrocytes and 20 μg/mL ampicillin [10] (Sigma, USA) and TCBS (Oxoid, UK) for 24 h at 37℃, respectively. Next, we performed oxidase tests (BioMerieuX, France) to screen the gram-negative rod colonies from the agar plate. The oxidase tests of *Vibrio* species were positive while those of Enterobacteriaceae colonies were negative. Finally, microorganisms were identified taxonomically using a VITEK MALDI-TOF MS system (BioMerieuX, France) and microbial identification system VITEK II (BioMerieuX, France). *Salmonella* spp., *Shigella* spp., *Aeromonas* spp., *Plesiomus shigelloides* and other *Vibrio* spp. were also routinely detected simultaneously in the same samples. All strains were stored in a LB / glycerol mixture (80:20) at -80°C until identification.

Whole-genome sequencing and annotation

We extracted the total DNA of the *V. furnissii* strains from 1 mL of overnight culture of each strain grown in LB (luria broth) at 37°C with TIANamp Bacteria DNA Kits (Beijing Tiangen, China). The whole genome of *V. furnissii* was sequenced using Illumina NovaSeq PE150 at Beijing Novogene Bioinformatics Technology Co., Ltd. Sequencing libraries of ~350 bp were prepared from 1 μg of total DNA per sample using NEBNext® Ultra™ DNA Library Prep Kits for Illumina (NEB, USA) following the manufacturer's recommendations. PCR products were then purified with an AMPure XP system (Beckman, USA), and libraries were analysed for size distribution with an Agilent 2100 Bioanalyzer and quantified using real-time PCR. We used SOAP denovo [17, 18] (http://soap.genomics.org.cn/soapdenovo.html), SPAdes [19] (http://cab.spbu.ru/software/spades/) and ABySS [20] to assemble paired reads (http://www.bcgsc.ca/platform/bioinfo/software/abyss). All of the genomic sequences are available at the National Center for Biotechnology Information (NCBI) (accession nos. SAMN21988700, SAMN22062944-49).

Bioinformatic analysis

We conducted genome component prediction for coding genes using GeneMarkS [17], then used seven databases to predict gene functions: GO [18] (Gene Ontology), KEGG [20] (Kyoto Encyclopedia of Genes and Genomes), COG [21] (Clusters of Orthologous Groups), NR [22] (Non-Redundant Protein Database), TCDB [23] (Transporter Classification Database) and Swiss-Prot [24]. A whole-genome BLAST [25] search (E-value < $1e^{-5}$, minimal alignment length 40%) was performed against the above seven databases. We used PHI [26] (Pathogen Host Interactions), VFDB [27] (Virulence Factors of Pathogenic Bacteria) and ARDB [28] (Antibiotic Resistance Genes Database) to perform pathogenicity and drug resistance analyses. Lastly, secretory proteins and Type I–VII proteins were respectively predicted with the Signal P [29] database and EffectiveT3 [30] software.

Comparative genome analysis and phylogenetic tree

Phylogenetic analyses based on SNPs in the seven isolates were performed by aligning whole-genome sequencing data of these isolates with the genomic sequences of *V. furnissii* reference isolates CIP102972, NCTC13120 and NCTC11218

and *V. fluvialis* reference isolate ATCC33809 acquired from Genbank. The analyses were performed using MUMmer (Version 3.23) to filter out gaps and SNPs less than 5 bp in length. A phylogenetic tree was then constructed using iqtree2 (Version 2.0.6). Bootstrap values were calculated based on 1,000 replicates.

Antibiotic susceptibility tests

Antibiotic susceptibility tests were performed using the microbroth dilution method according to the guidelines of CLSI. The minimum inhibitory concentrations of the following 19 antibiotics were determined: AMP, AMS, CFZ, CAZ, FEP, AZM, AMI, GEN, CIP, LEV, IMI, MEM, TET, DOX, CHL, SUL, SXT, AZI and STR. *E. coli* ATCC 25922 was used as the quality-control strain for susceptibility testing.

Abbreviations

TCBS
thiosulfate-citrate-bile salts-sucrose agar
MALDI-TOF MS
matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry
LB
Luria broth
SNPs
single-nucleotide polymorphisms
CLSI
Clinical and Laboratory Standards Institute
GIs
genomic islands
IS
insert sequence
CDS
coding sequence
T2SS
type II secretion system
T3SS
type III secretion system
VFH
V. fluvialis haemolysin
HupO
hemin-binding outer membrane protein
VFP
V. fluvialis protease
T6SS
type VI secretion system
AMP
ampicillin
AMS
ampicillin/sulbactam

CFZ
cefazolin
CAZ
ceftazidime
FEP
cefepime
AZM
aztreonam
AMI
amikacin
GEN
gentamicin
CIP
ciprofloxacin
LEV
levofloxacin
IMI
imipenem
MEM
meropenem
TET
tetracycline
DOX
doxycycline
CHL
chloramphenicol
SUL
sulphonamides
SXT
trimethoprim–sulfamethoxazole
AZI
azithromycin
STR
streptomycin

Declarations

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Authors' contributions

YL and SJ designed the study. ZY and NZ performed strain isolation and collection, microbial analysis, antibiotic susceptibility tests and data analysis, and compiled the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

The study obtained ethical approval (2017-P2-095-01) from the Medical Ethics Committee of Beijing Friendship Hospital, Capital Medical University, Beijing, PR China. Consent requirements were waived.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed in this study are included in this article.

Competing interests

The authors declare that they have no competing interests.

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Figures

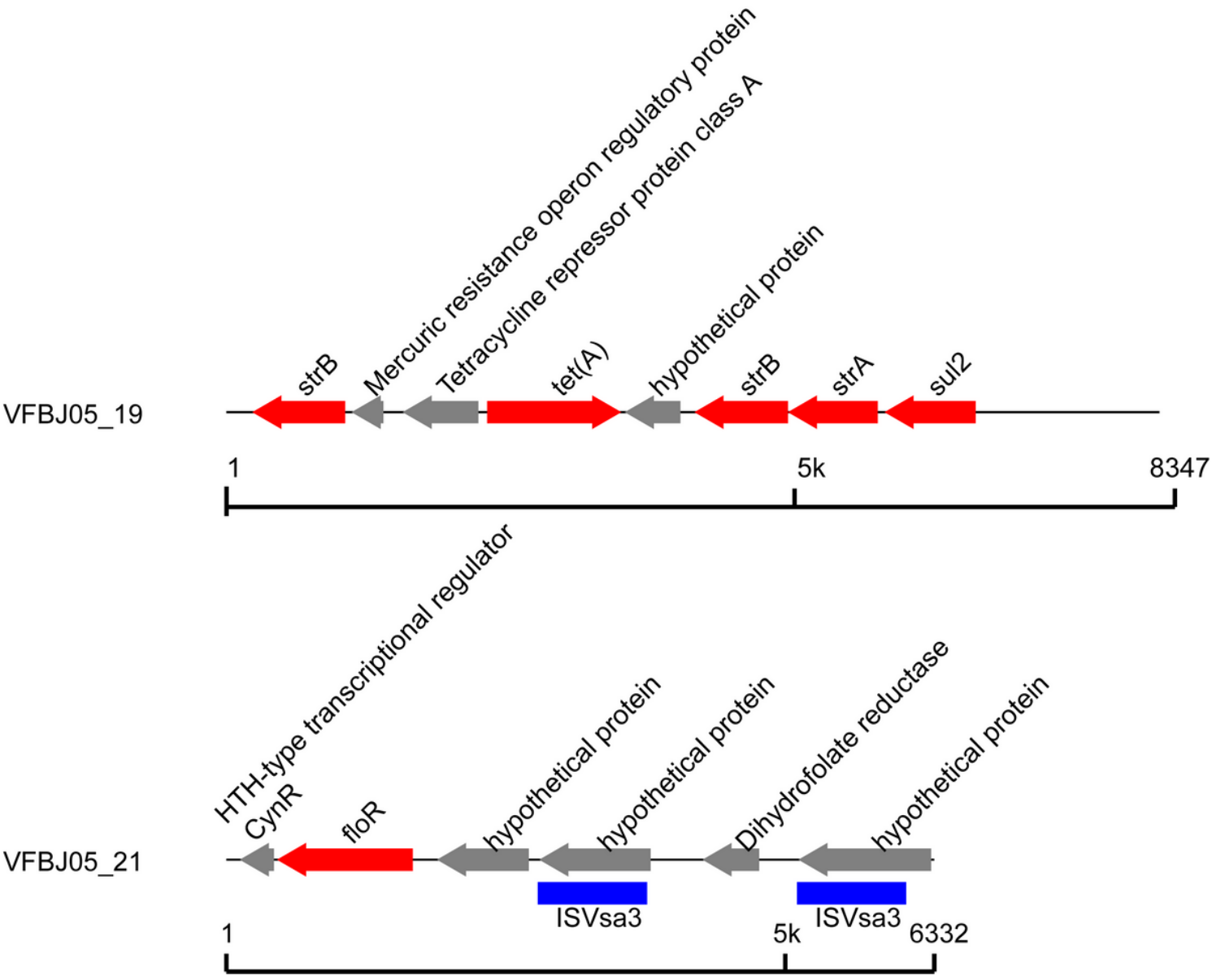

Figure 1

Transposon islands containing the antibiotic resistance genes in VFBJ05. One transposon island contained *strB*, *strA*, *tetA* and *sul2*, and the other contained *floR*.

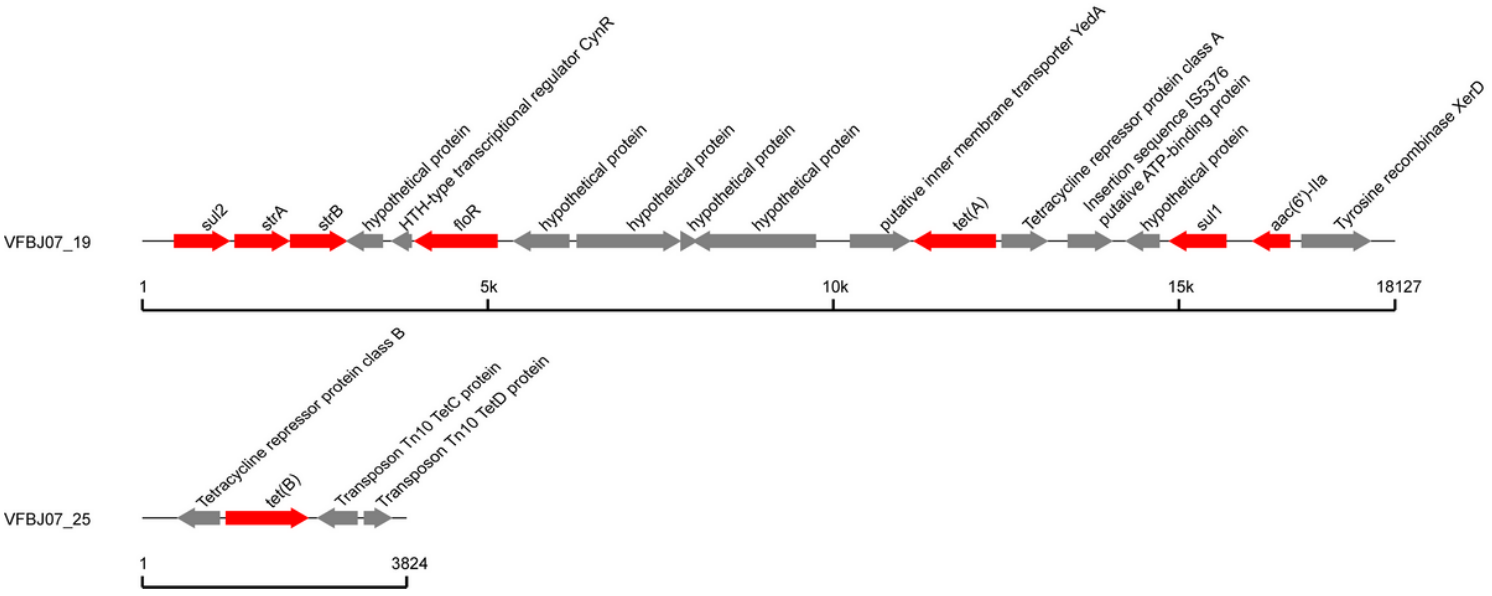

Figure 2

Transposon islands containing the antibiotic resistance genes in VFBJ07. One transposon island in contained *strB*, *strA*, *tetA sul1*, *sul2* and *aac(6')- a*, and the other contained *tetB*.

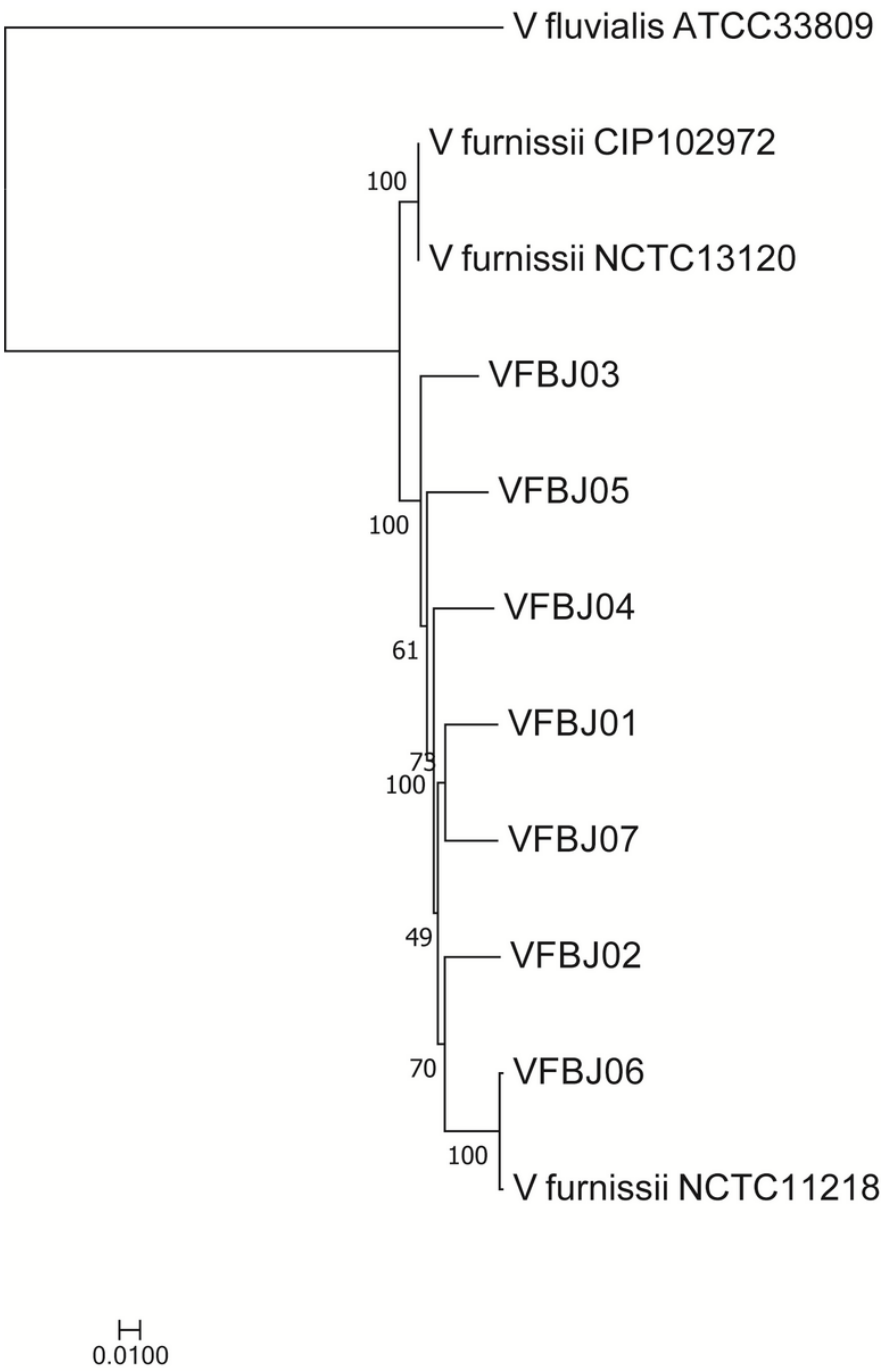

Figure 3

Phylogenetic tree of seven isolates of *V. furnissii* obtained from diarrhoea patients in Beijing. The phylogenetic tree was constructed using iqtree2 based on SNPs and aligned with *V. furnissii* reference isolates CIP102972, NCTC13120 and NCTC11218 and *V. fluvialis* reference isolate ATCC33809. Bootstrap values were calculated based on 1,000 replicates.