Iranian Journal of Microbiology
Volume 18 Number 1 (February 2026) 48-54
DOI: http://doi.org/10.18502/ijm.v18i1.20906

ORIGINAL ARTICLE

Chromosome-borne *bla*$_{CTX-M-65}$ gene in non-O1/O139 *Vibrio cholerae* isolated from bile

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Received: March 2025**, Accepted:** November 2025

ABSTRACT

Background and Objectives: Non-O1/O139 *Vibrio cholerae* (NOVC) has been associated with extraintestinal infections; however, its isolation from bile remains exceedingly rare. This study reports the identification and comprehensive analysis of a NOVC strain isolated from bile, including its antimicrobial resistance profile, virulence gene content, and molecular characteristics.

Materials and Methods: A NOVC isolate was obtained from the bile of a patient with a hepatobiliary tumor. The isolate was identified and subjected to antimicrobial susceptibility testing. Whole-genome sequencing was performed to characterize its molecular features, including antimicrobial resistance genes, virulence genes, and relevant genetic mutations.

Results: The NOVC isolate presents a multi-drug resistance phenotype. Corresponding genomic analysis indicates that this strain belongs to a novel sequence type (ST1736), carrying various drug resistance genes and virulence factors. Moreover, the *bla*$_{CTX-M-65}$ extended-spectrum β-lactamase (ESBL) gene was detected in its chromosomal genome.

Conclusion: This study presents the first report of a multidrug-resistant NOVC strain isolated from bile in mainland China. Notably, the ESBL gene *bla*$_{CTX-M-65}$ was identified chromosomally in NOVC for the first time.

Keywords: *Vibrio cholerae*; Bile; Sequence analysis; DNA; Drug resistance; Virulence factors

INTRODUCTION

Vibrio cholerae is a ubiquitous aquatic bacterium classified into over 200 serogroups based on the O-antigen of its lipopolysaccharide. Among these, only the O1 and O139 serogroups are associated with cholera epidemics and pandemics (1). Strains belonging to other serogroups are collectively termed non-O1/O139 *V. cholerae* (NOVC). While NOVC typically lacks the cholera toxin (CT) and the toxin-coregulated pilus (TCP), it can cause intestinal infections, albeit with milder symptoms. In contrast to the gastrointestinal tropism of O1/O139 strains, NOVC is increasingly recognized as an agent of extraintestinal infections. These include bacteremia, peritonitis, urinary tract infections, skin and soft tissue infections (SSTIs), and hepatobiliary infections such as cholangitis and cholecystitis (2-8).

Despite growing evidence, the burden of NOVC extraintestinal infections remains underappreciated. Although reports of NOVC-related biliary tract infections are scarce, descriptions of its direct isolation from bile are even rarer. Herein, we report the isolation and characterization of a NOVC strain from the

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bile of a patient with a hepatobiliary malignancy in mainland China. To our knowledge, this represents the first such documented case. Whole-genome sequencing revealed that this strain harbors the extended-spectrum β-lactamase (ESBL) gene $bla_{CTX\text{-}M\text{-}65}$ on its chromosome, which, to our knowledge, is the first report of this gene in NOVC.

MATERIALS AND METHODS

Case description. A 69-year-old male with a history of hilar cholangiocarcinoma, for which he underwent radical resection one year prior, was admitted following one week of right upper quadrant abdominal discomfort and fever (38.4°C). Laboratory investigations revealed leukocytosis (16.15 × 10^9/L), elevated C-reactive protein (CRP; 77.34 mg/L), serum gamma-glutamyltransferase (477 U/L), and alkaline phosphatase (320 U/L). Empirical therapy with piperacillin/tazobactam was administered for three days without significant clinical improvement. Ultrasound-guided percutaneous transhepatic cholangial drainage (PTCD) was subsequently performed, yielding 450 mL of brown bile in total. Bacterial culture of the bile identified NOVC as the causative pathogen. The patient's abdominal symptoms and fever initially improved after three days of targeted therapy but recurred on day four (38.1°C). Subsequent blood cultures grew carbapenem-resistant *Klebsiella pneumoniae* (CR-Kp). Given the advanced tumor stage, poor general condition, and grim prognosis, the family opted for palliative discharge.

Strain identification and drug susceptibility testing. Bile specimens were inoculated onto blood agar and MacConkey agar plates (Zhengzhou Autobio Company) and incubated at 35°C. Bacterial identification was performed using MALDI-TOF MS (VITEK MS, bioMérieux; software version V3.2). Antimicrobial susceptibility testing was carried out using the WalkAway-40Plus system with an NC-31 card (Beckman Coulter), interpreted according to CLSI guideline M45 (3rd Edition) (9). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains.

Whole-genome sequencing and analysis. Bacterial genomic DNA was extracted by the boiling method and sequenced by Shanghai Majorbio Biomedical Technology Co., Ltd. Sequencing was performed on both the Illumina HiSeq (150-bp paired-end reads, 200× coverage) and PacBio platforms (100× coverage). Reads were assembled using Unicycler v0.4.8 and polished with Pilon. The complete genome sequence was deposited in the NCBI database (BioProject: PRJNA1127326). Protein-coding genes were predicted using Prodigal 2.6.3. Multilocus sequence typing (MLST) was determined via the PubMLST database (https://pubmlst.org/). Virulence factors were identified using the Virulence Factor Database (VFDB; http://www.mgc.ac.cn/VFs/; version 20230407). Antimicrobial resistance genes were detected using ResFinder 4.1 (https://genepi.food.dtu.dk/resfinder) and the Comprehensive Antibiotic Resistance Database (CARD; https://card.mcmaster.ca; version 3.2.6).

Ethical approval. This study was conducted in accordance with the principles of the Declaration of Helsinki. The Ethics Committee of the First People's Hospital of Lianyungang waived the requirement for informed consent and approved the study (Approval No.: LW20240919001-01), as patient data were anonymized prior to analysis.

RESULTS

Identification and drug susceptibility results. After 24 hours of culture, pale, β-hemolytic, moist colonies were observed on blood agar, and colorless colonies were observed on MacConkey agar. The isolate was identified as *Vibrio cholerae* with 99.9% confidence by MALDI-TOF MS. It did not agglutinate with O1 or O139 antisera and was thus classified as NOVC, designated strain C3616. This identification was confirmed by the local Centers for Disease Control and Prevention (CDC), and antimicrobial susceptibility results are presented in Table 1.

Genomic characteristics and gene analysis. The complete genome of strain C3616 is 4,061,684 bp, comprising two chromosomes (Chr1: 3,058,981 bp; Chr2: 1,011,937 bp) and one plasmid (13,092 bp) (GenBank: CP161854.1, CP161855.1, CP161856.1). MLST analysis assigned it to a novel sequence type, ST1736. GoeBURST analysis indicated that its closest relative is ST278, from which it differs by two alleles (*adk* and *pntA*) (Fig. 1). The presence of *ompW* and

the absence of O1/O139-specific *rfb* genes confirmed its classification as NOVC (10, 11).

Virulence factor profiling against the VFDB revealed the absence of *ctxAB* and *tcp* genes but identified numerous accessory virulence factors and two secretion systems: the type II secretion system (T2SS) and the type VI secretion system (T6SS) (Tables 2A-E).

Analysis with ResFinder and CARD identified multiple antimicrobial resistance genes on Chr1, including the β-lactamase genes (bla_{OXA-1}, $bla_{CTX-M-65}$) aminoglycoside resistance genes (*aadA2, aac(3)-IV, aac(3)-IVa, aph(4)-Ia, aph(3')-Ia, aph(3")-Ib, aph(6)-Id, aac(6')-Ib-cr*), a tetracycline resistance gene (*tet*), a sulfonamide resistance gene (*sul*), and the fosfomycin resistance gene *fosA3*. The plasmid carried the quinolone resistance gene *qnrVC5* (Table 3). Mutations in the quinolone resistance-determining regions (QRDRs) were also identified: GyrA (S83I), ParC (S85L), and ParE (D476N).

Table 1. In Vitro susceptibility results for strain C3616

Antibiotic	MIC	Interpretation
Ampicillin/sulbactam	16/8	I
Amikacin	8	S
Ampicillin	>32	R
Aztreonam	16	–
Chloramphenicol	16	I
Ceftriaxone	>32	–
Ceftazidime	≤1	S
Cefotaxime	>32	R
Cefoxitin	≤8	S
Cefazolin	>16	R
Ciprofloxacin	>2	R
Cefepime	8	I
Cefuroxime	>16	R
Cefotetan	≤16	–
Ertapenem	≤1	–
Gentamycin	8	I
Imipenem	≤1	S
Levofloxacin	4	I
Meropenem	≤1	S
Piperacillin	>64	R
Cotrimoxazole	>2/38	R
Tetracycline	>8	R
Tigecycline	≤1	–
Ticarcillin/clavulanic acid	≤8	–
Tobramycin	>8	–

MIC: minimum inhibitory concentration (μg/mL); S: susceptible; R: resistant; I: intermediate; –: no CLSI interpretation available.

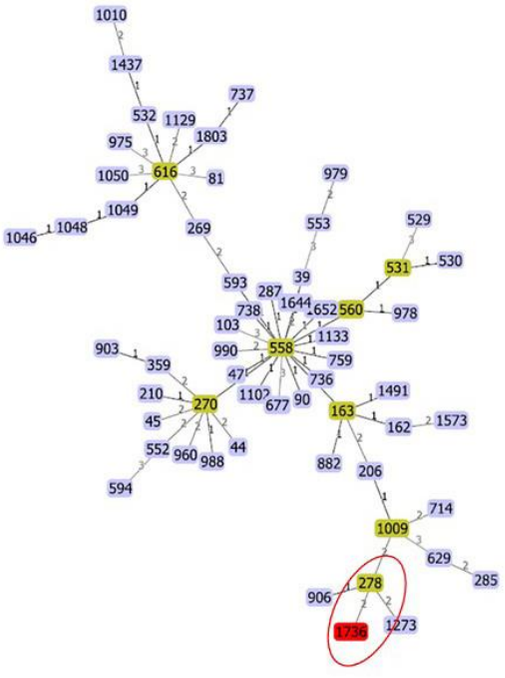

Fig. 1. Population structure of C3616 isolates as determined by GoeBURST analysis.

GoeBURST analysis of *Vibrio cholerae* sequence types (STs). Each node represents a sequence type (ST), and connecting lines indicate the number of allele differences. The novel ST1736 (highlighted in red), identified in this study, differs from its closest relative, ST278, by two alleles.

The $bla_{CTX-M-65}$ gene is integrated into an 85283 bp genomic island within an SXT/R391-family integrative and conjugative element (ICE) on Chr1. Its immediate genetic context is: IS26--ΔISEcp1--$bla_{CTX-M-65}$--IS903B--IS26--IS26--*fosA3*--IS26 (Fig. 2).

DISCUSSION

This study reports the first isolation and genomic characterization of a multidrug-resistant NOVC strain from bile in mainland China. The strain belongs to a novel sequence type (ST1736) and, most notably, carries the ESBL gene $bla_{CTX-M-65}$ on its chromosome, a first for this species.

While NOVC is a known cause of sporadic gastroenteritis and, less commonly, severe extraintestinal infections (2, 12), biliary tract infections are rarely reported (2, 13, 14). Our case highlights that NOVC

Table 2A. Adherence-related virulence factors

VF Category	VFs	Related Genes	Location
Adherence	MSHA	mshBmshCmshDmshEmshFmshGmshHmshImshJmshKmshLmshMmshN	Chromosome
	GbpA	gbpA	PlasmidA
	ChiRP	pilBpilCpilD/vcpD	Chromosome

Note: Annotations from VFDB database.

Table 2B. Biofilm-related virulence factors

VF Category	VFs	Related Genes	Location
Biofilm	VPS	vpsCvpsDvpsEvpsFvpsGvpsHvpsIvpsJvpsPvpsQ	Chromosome
	RbmC	VC_RS04620	Chromosome
	RbmA	VC_RS04610	Chromosome
	CAI-1	cqsA	PlasmidA
	Bap1	VC_RS09110	Chromosome
	AI-2	luxS	Chromosome

Note: Annotations from VFDB database.

Table 2C. Secretory system-related virulence factors

VF Category	VFs	Related Genes	Location
Secretory system	T6SS	VasAvasBvasCvasEvasFvasHvasIVasJvasL VCA0109 vgrG-2 vgrG-3 VvipA/mglAvipB/mglBicmF/vasKclpB/vasG	PlasmidA
		vgrG-1 hcp-1 epsCepsDepsEepsFepsGepsHepsIepsJepsKepsLepsMepsN	Chromosome
	T2SS	VC0395_RS15455	Chromosome
		VC0395_RS15460	

Note: Annotations from VFDB database.

Table 2D. Exotoxin-related virulence factors

VF Category	VFs	Related Genes	Location
Exotoxin	VCC	hlyA tlh	PlasmidA
	TLH	rtxArtxBrtxCrtxD	PlasmidA
	MARTX		Chromosome

Note: Annotations from VFDB database.

Table 2E. Motility-related virulence factors

VF Category	VFs	Related Genes	Location
Motility	Flagella	cheAcheBcheRcheVcheYcheZflaAflaBflaCflaDflaEflaGflaIflgAflgBflgCflgD-flgEflgFflgGflgHflgIflgJflgKflgLflgMflgNflgOflgPflgTflhAflhBflhF	Chromosome
	(VF0519)	fliAfliDfliEfliFfliGfliHfliIfliJfliLfliMfliNfliOfliSfliPfliQfliRflrAfleR/flrCfleS/flrBfleN/flhGcheWmotAmotBmotXmotY	Chromosome

Note: Annotations from VFDB database.

Table 3. Antibiotic resistance genes (ARG) carried by C3616

Gene	Resistance Class	Location
aadA2	Aminoglycoside	Chromosome
AAC(3)-Iva	Aminoglycoside	Chromosome
APH(4)-Ia	Aminoglycoside	Chromosome
APH(3')-Ia	Aminoglycoside	Chromosome
APH(3")-Ib	Aminoglycoside	Chromosome
APH(6)-Id	Aminoglycoside	Chromosome
AAC(6')-Ib-cr6	Aminoglycoside/Fluoroquinolone	Chromosome
OXA-1	Carbapenem; Cephalosporin; Penam	Chromosome
CTX-M-65	Cephalosporin	Chromosome
dfrA32	Diaminopyrimidine	Chromosome
dfrA31	Diaminopyrimidine	Plasmid
rsmA	Diaminopyrimidine/Fluoroquinolone/Phenicol	Chromosome
qacEdelta1	Disinfecting agents and antiseptics	Chromosome
CRP	Fluoroquinolone/Macrolide antibiotic	Chromosome
QnrVC5	Fluoroquinolone	Plasmid
EreA	Macrolide	Chromosome
almE	Peptide	Chromosome
almF	Peptide	Chromosome
almG	Peptide	Chromosome
catB3	Phenicol	Chromosome
floR	Phenicol	Chromosome
FosA3	Phosphonic acid	Chromosome
arr-3	Rifamycin	Chromosome
sul1	Sulfonamide	Chromosome
sul2	Sulfonamide	Chromosome
tet(C)	Tetracycline	Chromosome
tet(59)	Tetracycline	Chromosome

Abbreviations: AAC, aminoglycoside N-acetyltransferase; APH, aminoglycoside O-phosphotransferase; OXA, oxacillinase; CTX-M, cefotaxime-hydrolyzing β-lactamase; dfr, dihydrofolate reductase; qac, quaternary ammonium compound; CRP, cAMP receptor protein; Qnr, quinolone resistance; Ere, erythromycin esterase; cat, chloramphenicol acetyltransferase; flo, florfenicol exporter; Fos, fosfomycin resistance; arr, rifampin ADP-ribosyltransferase; sul, sulfonamide-resistant dihydropteroate synthase; tet, tetracycline resistance.

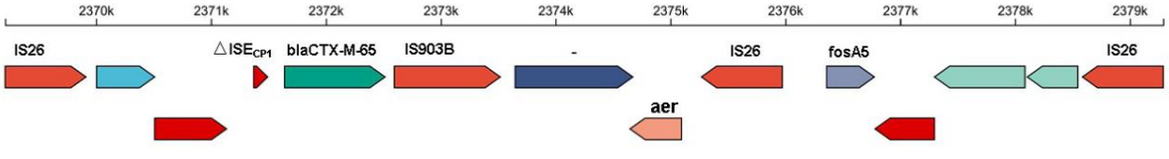

Fig. 2. Genetic environment of $bla_{CTX-M-65}$ in Chromosome of C3616 (GenBank accession no. CP161854.1)
bbreviations: IS26 (Insertion Sequence 26), ISEcp1 (Insertion Sequence Element of *E. coli* plasmid 1), IS903 (Insertion Sequence 903). $bla_{CTX-M-65}$ encodes a CTX–M-65 type β - lactamase, fosA5 encodes a fosfomycin - modifying enzyme, and aer encodes aerobactin biosynthesis - related proteins. The arrow direction indicates the transcriptional orientation of the gene.

should be considered in the differential diagnosis of biliary infections, particularly in patients with underlying hepatobiliary conditions. The patient had no recent history of seafood consumption or water exposure, suggesting possible transmission via undocumented routes or long-term colonization following an asymptomatic intestinal infection, with subsequent reactivation due to immunosuppression. The virulence potential of NOVC is highly strain-dependent and often involves accessory factors (15, 16). Strain C3616 lacks major epidemic markers but possesses a repertoire of genes associated with adhesion, toxin production, and immune evasion, along with T2SS and T6SS, which may collectively facilitate infection and persistence in the biliary tract.

The most concerning finding is the chromosomal integration of $bla_{\text{CTX-M-65}}$ within an SXT/R391 ICE. SXT/R391ICEs are powerful drivers of horizontal gene transfer in Gram-negative bacteria (17-19). The genetic context of $bla_{\text{CTX-M-65}}$ in C3616, flanked by IS26 and other insertion sequences, is highly similar (98.7% identity) to anSXT/R391ICE (GenBank:MZ277866.1) recently reported in *Proteus* (20) (Fig. 3). This strongly suggests horizontal acquisition from an *Enterobacteriaceae* donor, underscoring the role of SXT/R391ICE in disseminating drug resistance among diverse pathogens.

The multidrug-resistant profile of C3616, including resistance to recommended first-line agents like third-generation cephalosporins and fluoroquinolones (21, 22), poses a significant therapeutic challenge. This case underscores the critical need for susceptibility-guided therapy and enhanced surveillance of antimicrobial resistance in NOVC.

CONCLUSION

In conclusion, we report the first biliary isolation of a multidrug-resistant NOVC strain in mainland China, representing a novel sequence type (ST1736). Crucially, this strain harbors the $bla_{\text{CTX-M-65}}$ ESBL gene on a chromosomally integrated SXT/R391ICE, highlighting the potential for interspecies resistance gene dissemination. Clinicians should be aware of NOVC as an emerging cause of invasive infections. Monitoring its evolving resistance patterns is essential for effective patient management and infection control.

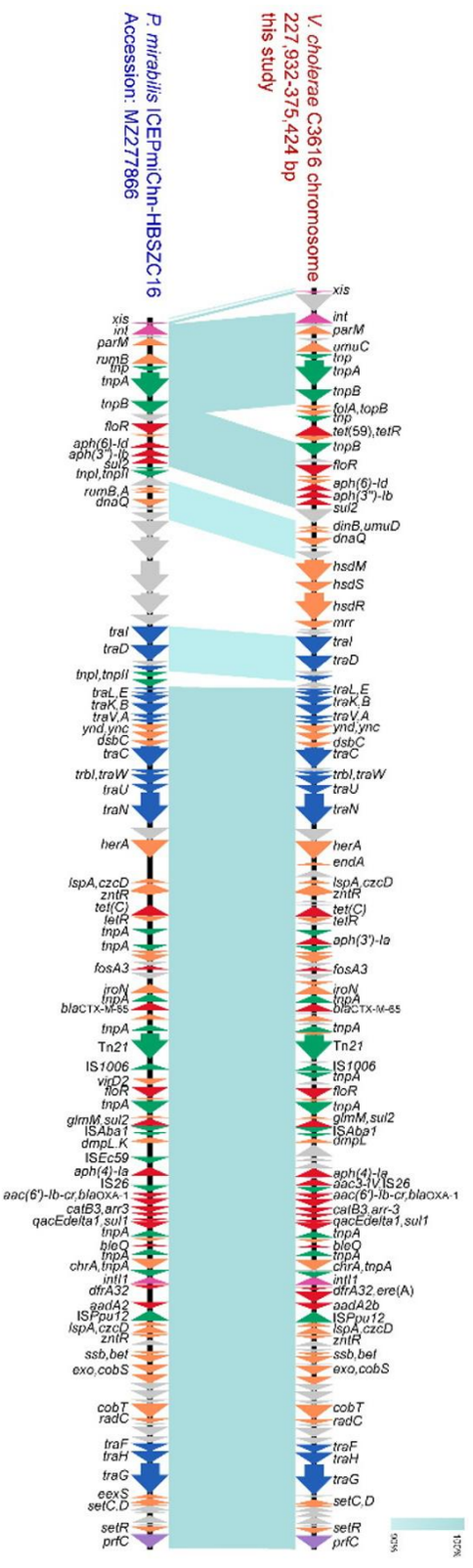

Fig. 3. Genetic structure of SXT/R391 ICE in C3616 compared with ICEPmiChnHBSZC16(MZ277866.1) Pink represents the cleavase/integrase; Green represents insertion sequences or transposases; Resistance genes are represented in red. Type IV secretion system genes (associated with metastasis) are represented in blue; Orange represents other types of genes; ICE insertion site genes are shown in purple. Putative proteins are shown in gray.

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