Identification and genomic analysis of a Vibrio cholerae strain isolated from a patient with bloodstream infection

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HIGHLIGHTS

- VCHL017 belonged to a novel sequence type ST1554 with the allelic profiles of adk-3, gyrB-62, metE-470, mdh-15, pntA-6, purM-1 and pyr-6.
- VCHL017 carried multiple pathogenic factors and was closely related to some V. cholerae strains isolated from aquatic environments.
- Compared to the reference strain, the gyrA gene of VCHL017 has had A171S and A202S missense mutations.

1. Introduction

Vibrio is a bacterial genus that widely exists in aquatic environments. Among them, V. cholerae, V. parahaemolyticus, and V. vulnificus are pathogenic to humans [1]. V. cholerae is the causative agent of the violent infectious disease cholera, which causes disease through various toxins, including cholera toxin (CT). According to the O antigen, V. cholerae can be divided into classical and El Tor biotypes, subdivided into more than 200 groups. Serogroups O1 and O139 are associated with cholera outbreak epidemics [2]. The development of Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and whole genome sequencing (WGS) provided a meaningful way to

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accurately identify *V. cholerae* [3, 4]. Although improvements in personal and environmental hygiene and the application of vaccines have reduced the cholera epidemic, the situation is still not optimistic, especially in some underdeveloped areas [2], where many countries are still facing the threat of cholera and bear the high medical burden. Therefore, it is necessary to continuously and effectively monitor *V. cholerae* to prevent a global cholera epidemic [5].

CT, an important virulence factor of *V. cholerae*, inhibits water and electrolytes reabsorption by intestinal mucosa through activating adenylyl cyclase, thus leading to severe gastrointestinal symptoms [6]. *V. cholerae* strains that do not produce CT are generally in the non-O1/non-O139 serogroup. These non-CT-producing *V. cholerae* strains are still pathogenic and can be isolated from extraintestinal sites such as blood, respiratory tract, urine, and cerebrospinal fluid [6, 7]. Virulence factors such as *ctxABC* (encoding RTX toxin), *hlyA* (encoding *V. cholerae* cytotoxin), *ompW*, and a type III secretion system (TIIISS) are usually identified in non-O1/O139 strains [8, 9]. However, the pathogenic mechanism of non-O1/O139 strains causing extraintestinal infection requires further clarification. This study isolated a *V. cholerae* isolate from a patient with hematologic malignancies. The patient's clinical infectious characteristics were examined, and the genomic characteristics of the isolate were analyzed by whole genome sequencing.

2. Materials and methods

2.1. Isolation and identification of bacteria

Suspected aerobic cultures were inoculated on blood agar plates, chocolate plates and MacConkey plates for subculture and gram stain. Single purified suspected to be *V. cholerae* colonies were selected and inoculated in blood agar and thiosulfate citrate bile salt sucrose (TCBS) agar plates for culture for 24 h. A single fresh colony was selected from the blood plate and identified by MALDI-TOF MS (Bruker, Germany).

2.2. Antimicrobial susceptibility test

The isolate's minimum inhibitory concentration (MIC) for cefazolin, cefazidime, cefepime, meropenem, tetracycline, ciprofloxacin, chloramphenicol, and gentamicin were determined by the microbroth dilution method. The results were interpreted according to the Clinical Laboratory Standards Institute (CLSI) guideline M45 [10].

2.3. Identification of the serogroup and cholera toxin encoding gene ctxA of the isolate

The serogroup based on the O antigen of the isolate was identified according to the reagent manufacturer's recommendations (Tianrun, China). Polymerase Chain Reaction (PCR) was used to detect whether the isolate carried the cholera toxin coding gene ctxA. Total bacterial DNA was crudely extracted by boiling method. Briefly, pick several fresh colonies in 500 μl of distilled water and boil at 100 °C for 10 min. Then the mixture was centrifuged at 12500 rpm/min for 10 min, and the supernatant was aspirated and stored at -20 °C for later use. Primer was designed as previously described (5'-CTCAGCGGGATTGTTAGGCAG-3'; 5'-TCTATCTCTCTG- TAGCCCTATTACG-3') [11]. PCR products were confirmed by 1.5% agarose gel electrophoresis and Sanger sequencing. A non-O1/O139 *V. cholerae* previously derived from the External Quality Assessment Programs (EQA) in Laboratory Medicine was used as a control.

2.4. Whole genome sequencing and analysis

The *V. cholerae* strain was streaked on blood agar and cultured at 37 °C for 18 h. Next, a single purified colony was picked and inoculated in 200 mL of antibiotic-free sterile LB broth, shaking at 37 °C for growth into the logarithmic phase. The total DNA was extracted according to the reagent manufacturer's recommendation (TransGen, China). The draft genome sequence analysis of *V. cholerae* strain was performed using the Hisseq X Ten sequencing platform (MajorBio Co., Shanghai, China). Illumina sequencing libraries were prepared from the sheared fragments using the NEXTflex™ Rapid DNA-Seq Kit. The prepared libraries were then used for paired-end Illumina sequencing (2 × 150 bp) on an Illumina HiSeq X Ten sequencing platform. Raw reads obtained after sequencing were filtered using a fastq format (version 0.19.6) followed by assembly with SOPA de novo version 2.04. Glimmer was used for CDS prediction, tRNA-scan-SE was used for tRNA prediction, and Barmap was used for tRNA prediction [12, 13]. The sequence type of the isolate was analyzed by PubMLST (https://pubmlst.org/) [14]. Virulence factors prediction was performed by the Virulence Factor Database (VFDB, http://www.mgc.ac.cn/VFs/). Acquired antimicrobial resistance genes from the *V. cholerae* strains were identified by ResFinder 4.1 (https://cge.cbs.dtu.dk/services/ResFinder/). Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/) of NCBI, OAT [15], and Mauve software (https://sourceforge.net/projects/mauve/) were used for genome alignment. The final assembled genome was submitted to the NCBI database (https://www.ncbi.nlm.nih.gov/) with the accession number: JALDYW000000000.

2.5. Phylogeny construction

GoEBURST analysis of the sequence types was performed by the PHYLOViZ software (http://www.phyloviz.net/). Other *V. cholerae* genomes were obtained from the NCBI nucleotide database (https://www.ncbi.nlm.nih.gov/, Table S1), and a phylogenetic tree was constructed through the online website service CSI Phylogeny according to the default settings of the webpage (https://cge.cbs.dtu.dk/services/CSIPhylogeny/). *V. cholerae* N16961/ATCC39315 (access number: GCA_000006745.1) served as the reference strain. The phylogenetic tree was polished by iTOL v6 (Interactive Tree Of Life, https://itol.embl.de/).

2.6. Ethics statement

The study was approved by the Institutional Review Board of the Affiliated Hospital of Southwest Medical University (Project No. KY2020043).

3. Results

3.1. Case presentation

In December 2021, an older woman with more than 5 years of multiple myeloma complained of cough and dyspnea and opted for admission. The patient reported a history of hookworm infection, blood transfusions, and long-term use of voriconazole. After admission, she received anti-infective therapy with voriconazole for pulmonary fungal infection. Laboratory investigations revealed pancytopenia and bone marrow suppression. The patient improved after 18 days of this hospitalization and opted to be discharged.

Six days later, the patient deteriorated again with a sudden temperature rise to 39 °C and was admitted to the hospital, immediately after which she underwent blood culture tests and received empiric anti-infective therapy with meropenem and caspofungin. A second blood culture was performed the day after treatment. The patient deteriorated on the third day, and she died due to ineffective rescue due to multiple organ failure. On the fifth day, *V. cholerae*, together with *E. coli* and *S. pneumoniae*, were isolated from the initial blood culture. No microorganisms were isolated from the second blood culture. The detailed clinical features and the laboratory tests performed during this admission are presented in Table 1.

3.2. Isolation and identification of a *V. cholerae* isolate

In January 2022, a *Vibrio cholerae* strain, named VCHL017, was isolated from the blood of an elderly patient in the hematology ward of a
tertiary hospital in Sichuan, China. The single purified colonies were inoculated on a blood agar plate and cultured at 35 °C for 24 h. Smooth, light gray colonies surrounded by long and distinct beta-hemolytic rings could be seen (Figure 1A). Yellow sticky colonies produced by sucrose decomposition were observed on TCBS plates (Figure 1B). Subsequently, VCHL017 was identified as V. cholerae by MALDI-TOF. Antimicrobial susceptibility tests revealed that the VCHL017 was susceptible to tetra-

cycline and chloramphenicol. The MICs for ceftazidime, cefepime, mer-

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Table 1. Clinical characteristics of the patient in the present study.

| Parameters                   | Results               |
|------------------------------|-----------------------|
| Age                          | 70 years              |
| Sex                          | Female                |
| Blood group                  | Type O                |
| Past Medical history         | Hookworm infection, blood transfusion, long-term voriconazole use |
| Underlying diseases          | Multiple myeloma      |
| WBC (×10^9/L)                | 1.84                  |
| NEUT (×10^9/L)               | 1.16                  |
| RBC (×10^12/L)               | 1.44                  |
| PLT (×10^3/μl)               | 24                    |
| PCT (ng/ml)                  | 42.9                  |
| Antimicrobial usage          | Meropenem, caspofungin, voriconazole |
| Invasive procedures          | Blood transfusion     |
| Total hospital stays         | 20 days               |
| Outcome                      | Dead                  |

Table 2. Antimicrobial susceptibility test results for V. cholerae strain VCHL017.

| Antimicrobial agents | MIC(μg/ml) |
|----------------------|------------|
| Cefazolin            | 2          |
| Ceftazidime          | <1         |
| Cefepine             | <1         |
| Meropenem            | <0.25      |
| Tetracycline         | 0.5 (S)    |
| Ciprofloxacin        | 0.125      |
| Chloramphenicol      | 0.25 (S)   |
| Gentamicin           | <1         |

CP049914.1), respectively. Using V. cholerae N16961 as a reference, A171S and A202S missense mutations were identified in the gyrA of VCHL017. The average nucleotide identity (ANI) of this isolate with other Vibrio strains was determined by OAT software. The results showed relatively low ANI between this isolate and other Vibrio strains except V. cholerae. VCHL017 exhibited ANI values ranging from 72% to 74% compared to V. alginolyticus ATCC 17749 (accession number: GCA_000354175.2), V. parahaemolyticus ATCC 17802 (accession number: GCA_001998785.1), V. vulnificus ATCC 27562 (accession number: GCA_00224265.1), and V. harveyi CAIM 1792 (accession number: GCA_000259935.1) (Figure 2). While VCHL017 displayed 98.33 and 98.36% ANI with V. cholerae ATCC14035 (accession number: GCA_000621645.1) and N16961/ATCC39315 (Figure 2). This result suggested that VCHL017 and V. cholerae belong to the same species, consistent with the MALDI-TOF identification [16]. The allele profiles of VCHL017 were adk-3, g6rb-62, metE-470, mdh-15, ptaA-6, purM-1 and pyrC-6 (https://pubmlst.org/) and were assigned a novel sequence type: ST1554. GoeBURST analysis showed that ST1554 belonged to the ST1166 complex and differ by 4 allele differences from ST258 and ST172 (Figure 3). The mause alignment of VCHL017 with other V. cholerae strains divided the genome into multiple Local Colinear Blocks (LCBs), i.e., regions of high homology. The alignment results show that the VCHL017 backbone is consistent with other V. cholerae genomes (Figure 4). Several LCBs of VCHL017 were reversed compared to V. cholerae TSY216 (accession number: GCA_001045415.1), N16961/ATCC39315, and A1552 (accession number: GCA_000621645.1). 3.3. Genome characteristics of V. cholerae isolate

We obtained the completed genome of VCHL017 by whole genome sequencing. The VCHL017 genome was 4,032,676 bp in length with 47% GC content and assembled from 42 scaffolds. Ten genomic islands (GIs), encoding 127 CDSs, were predicted by IslandPath-DIMOB software on seven different scaffolds. Resfinder's analysis revealed that VCHL017 carried a qnrV4-like gene on scaffold 1 and shared a 98% identity with qnrV4. Furthermore, we could not identify any plasmid incompatibility groups by PlasmidFinder 2.1 (https://cge.food.dtu.dk/services/PlasmidFinder/). However, Scaffold 25 and Scaffold 27 showed 93.485 and 90.699% identities to a plasmid isolated from Vibrio sp. HDW18 (GenBank: CP049914.1), respectively. Using V. cholerae N16961 as a reference, A171S and A202S missense mutations were identified in the gyrA of VCHL017. The average nucleotide identity (ANI) of this isolate with other Vibrio strains was determined by OAT software. The results showed relatively low ANI between this isolate and other Vibrio strains except V. cholerae. VCHL017 exhibited ANI values ranging from 72% to 74% compared to V. alginolyticus ATCC 17749 (accession number: GCA_000354175.2), V. parahaemolyticus ATCC 17802 (accession number: GCA_001998785.1), V. vulnificus ATCC 27562 (accession number: GCA_00224265.1), and V. harveyi CAIM 1792 (accession number: GCA_000259935.1) (Figure 2). While VCHL017 displayed 98.33 and 98.36% ANI with V. cholerae ATCC14035 (accession number: GCA_000621645.1) and N16961/ATCC39315 (Figure 2). This result suggested that VCHL017 and V. cholerae belong to the same species, consistent with the MALDI-TOF identification [16]. The allele profiles of VCHL017 were adk-3, g6rb-62, metE-470, mdh-15, ptaA-6, purM-1 and pyrC-6 (https://pubmlst.org/) and were assigned a novel sequence type: ST1554. GoeBURST analysis showed that ST1554 belonged to the ST1166 complex and differ by 4 allele differences from ST258 and ST172 (Figure 3). The mause alignment of VCHL017 with other V. cholerae strains divided the genome into multiple Local Colinear Blocks (LCBs), i.e., regions of high homology. The alignment results show that the VCHL017 backbone is consistent with other V. cholerae genomes (Figure 4). Several LCBs of VCHL017 were reversed compared to V. cholerae TSY216 (accession number: GCA_001045415.1), N16961/ATCC39315, and A1552 (accession number: GCA_000621645.1). 3.4. Multiple virulence factors carried by V. cholerae isolate

WGS confirmed that VCHL017 did not carry the cholera toxin encoding gene ctxA. However, it carried other virulence factors
associated with pathogenicity, including rtxABCD encoding the RTX toxin, tlh encoding thermolabile hemolysin, and hlyA encoding cytolysin. VCHL017 has two main secretion systems: the type II secretion system (T2SS), encoded by epsCEFGHIJKLMN and gspD, and the type VI secretion system (T6SS), encoded by vasABCDEFGHIJK. For iron uptake, VCHL017 carries irgA/vctA (encoding enterobactin receptors), hasR/hutAR (encoding heme receptors), vctCDGCPGP (encoding periplasmic binding protein-dependent ABC transport systems), vibABCDEFH/viuAB (encoding Vibriobactin), and chuWX (encoding heme uptake). VCHL017 also carries hap/vvp and nanH encoding metalloproteinase and neuraminidase. The encoding genes for toxin-coregulated pilus (TCP) and the type III fluctuation system were not identified in the VCHL017 genome. Furthermore, the VCHL017 genome contains a variety of virulence factors associated with adhesion, antiphagocytosis, chemotaxis, and motility, as shown in Supplementary Table 1.

3.5. Phylogenetic analysis of V. Cholerae VCHL017

We retrieved 29 V. cholerae genomes from the NCBI database and constructed a single nucleotide polymorphism (SNP)-based phylogenetic tree. The results indicated that VCHL017 was the closest in genetic relationship to a V. cholerae strain GXFL1-4 (accession number: 11572).
GCA_021431945.1) isolated from *Macrobrachium rosenbergii*. Additionally, VCHL017 belonged to the same phylogenetic lineage as some *V. cholerae* strains isolated from aquatic environments, marked in green, blue, and yellow in Figure 5. VCHL017 is phylogenetically distinct between fecal isolates and previous pandemic strains (E1162, NCTC9420, and M2140) [17].

4. Discussion

This study examined a non-O1/O139 *V. cholerae* (NOVC) strain isolated from the blood of a patient with a bloodstream infection. Despite prompt empiric anti-infective therapy by physicians, polymicrobial bacteremia usually have a poor prognosis and higher mortality than monomicrobial bacteremia [18]. *V. cholerae* is less frequent in the blood than in the digestive tract. However, increasing cases of NOVC bacteremia have been reported in many regions associated with extraintestinal infections [19, 20, 21]. Similar to most bacterial infections, patients with malignancy, hepatic insufficiency, diabetes, immunosuppression, or immunocompromised usually have a higher risk of NOVC infections [22]. The patient in this study had multiple myeloma, received chemotherapy for a long time, was immunocompromised and therefore was at a higher risk of infection (Table 1). Unlike previously *V. cholerae* cases, this patient had a long history of voriconazole use due to fungal infections. It remains unclear whether treatment for antifungal infections increases the risk of *V. cholerae* infections [23]. Notably, this patient did not exhibit gastrointestinal symptoms, which is key in clinical diagnosis and treatment. Gastric acid levels and ABO blood group are also commonly concerned innate host factors for *V. cholerae* infection. A previous study showed that blood type O individuals with are more likely to be infected with O1 ElTor/O139 and show more severe symptoms than those with other blood types [24]. Some studies have suggested that blood type O may promote the response of cholera toxin, but it is mainly limited to El-Tor biotype [25, 26]. Additionally, the shorter blood group H-determinant cluster profile of O-blood individuals differs from other blood group individuals in the binding kinetics to cholera toxin subunits [27]. While NOVC cannot produce cholera toxin, little information is available on the blood type of infected patients in previous studies [28, 29]. In particular, toxin-coregulated pili (TCP) are also necessary for cholera toxin expression, and nontoxigenic strains expressing TCP may obtain cholera toxin genes by lysogenic transformation [30]. More evidence is needed to clarify the link between NOVC infection and blood group O individuals.

The most common route of human infection with *V. cholerae* is through contact with contaminated water and marine animals [31]. However, many patients with NOVC infection do not have exposure-history infections, and the source of infection is unclear [22]. Since the patient in this study died, we could not obtain further contact history of the patient. Notably, this patient self-reported a history of hookworm infection, a widespread parasite in the soil environment [32]. Hookworm infection is associated with *V. cholerae* infection and colonization status in cholera patients [33]. Indeed, it is unclear what role hookworm infection played in this patient’s *V. cholerae* infection. Furthermore, our phylogenetic tree suggests that VCHL017 does not belong to the same lineage as some gastrointestinal-derived and pathogenic *V. cholerae*, and is more evolutionarily related to some environmental-derived strains (Figure 5). The genetically closest strain of VCHL017, GXFL1-4, is pathogenic to fishery organisms [34]. These results also suggest that VCHL017 may be derived from the aquatic environment, but its pathogenicity difference from other lineages needs to be verified through in vitro experiments.

Like most bacteria, *V. cholerae* can develop antimicrobial resistance through efflux pump overexpression, chromosomal loci mutations, and the acquisition of exogenous antimicrobial resistance genes through mobile elements [35]. Currently, the Clinical and Laboratory Standards Institute (CLSI) standards have not defined the resistance breakpoints of *V. cholerae* to fluoroquinolones (Table 2). *qnrVC* also belongs to the
pentapeptide repeat family. *qnrVC1* was initially identified in *V. cholerae*, and subsequently *qnrVC* variants were identified in different species. Studies have shown that the *qnrVC* alleles are generally associated with low-level quinolone resistance, and it can increase the MIC of quinolones several times [36, 37]. Like other gram-negative bacteria, developing quinolone resistance-determining regions (QRDRs) mutations in *V. cholerae* with a high-level quinolone resistance also appears necessary [38]. The A171S missense mutation of *gyrA* is associated with nalidixic acid and ciprofloxacin resistance in the quinolone resistance-determining region VCHL017. In contrast, the contribution of A202S to quinolone resistance remains to be further clarified [39].

The hemolytic properties of NOVC isolated from bloodstream infections may be one of the main reasons for invading these pathogens into the blood [40]. In vitro experiments revealed that hemolysin A (HlyA) contributes to enhanced cytotoxicity and promotes apoptosis [41]. Also, some NOVC can occupy an advantageous ecological niche by secreting HlyA to inhibit the growth of serogroup O1 strains [42]. The *rtxABCD* gene cluster is also associated with cytotoxicity [43]. In addition to cytotoxicity, T6SS contributes to the transport of virulence effector molecules and immune evasion and plays an important role in interspecies competition [44]. The vibABCE gene cluster is homologous to the enterobactin encoding gene *entABCE* and is involved in the early synthesis of siderophores [45]. *hapA* encodes a Zn-dependent metalloprotease that facilitates pathogens’ crossing the intestinal mucosal barrier and enhances their virulence [46]. However, we did not identify genes encoding the third secretion system associated with virulence and environmental fitness commonly found in non-O1/O139 strains in the VCHL017 genome. Overall, extraintestinal infections with NOVC involve multiple virulence factors, and further studies are needed to investigate their exact pathogenic mechanisms.

5. Conclusions

In summary, we reported and sequenced a non-O1/O139 *V. cholerae* strain VCHL017 carrying multiple virulence factors isolated from the blood of an elder patient with a bloodstream infection. VCHL017 is closely related in phylogeny to some *V. cholerae* strains isolated from the environment. Adequate attention to parenteral infections caused by *V. cholerae* still needs to be taken in clinical practice.

Declarations

Author contribution statement

Yinhuan Ding: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data.

Jingchen Hao: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Zhangrui Zeng: Analyzed and interpreted the data; Wrote the paper.

Jinbo Liu: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data associated with this study has been deposited at the NCBI database under the accession number JALDYW00000000.

Declaration of interest’s statement

The authors declare no conflict of interest.

Additional information

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