

Whole Genome Analysis of a Non-O1, Non-O139 *Vibrio cholerae* Detected from Human Blood in China

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Abstract: Non-O1, non-O139 *Vibrio cholerae* (NOVC) can cause cholera-like diarrhea, but it rarely causes extraintestinal infection, so it is easily overlooked. In this report, we present a case of NOVC detected through blood culture in a 58-year-old male patient with cirrhosis, resulting in severe infection. The patient had been diagnosed with cirrhosis seven years prior and was admitted to the hospital due to abdominal distension and gastrointestinal bleeding. Gram-negative bacilli were isolated from blood cultures and identified as *V. cholerae* using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and average nucleotide identity (ANI). Moreover, the serum agglutination test showed that the strain was non-O1/non-O139. Further whole genome sequencing and analysis of the strain showed that the strain mainly carried virulence genes *toxR*, *RTX*, *hlyA*, *T3SS/T6SS*, but no resistant genes such as *sulIII*, *dfrA1*, *strB* were detected. It provides information for the study of the pathogenic mechanism and drug resistance mechanism of *V. cholerae*. The patient had severe symptoms and a poor prognosis, indicating that although the NOVC strain infected in this patient had few virulence genes, it was not weak in pathogenicity. It may be caused by the effect of some virulence genes, which should be paid attention to.

Keywords: non-O1/non-O139 *Vibrio cholerae*, whole genome analysis, cirrhosis, virulence factors

Introduction

Vibrio cholerae is a Gram-negative bacterium that can be classified into different serotypes based on the O surface antigen, with O1 and O139 being responsible for causing cholera in humans. Cholera is a highly infectious and severe diarrheal disease that is prevalent worldwide, particularly in Africa, South Asia, and Southeast Asia.¹ Non-O1, Non-O139 *V. cholerae* can also cause cholera-like diarrhea; however, it does not produce the same severity of symptoms as its counterparts due to the absence of cholera toxin.² NOVC primarily causes intestinal infections but may lead to extraintestinal infections in individuals with weakened immune systems such as those with liver disease or undergoing chemotherapy.³ Studies have shown that NOVC infection is more common in patients with chronic liver disease, but its pathogenesis is still unclear.^{4,5} It may be related to factors such as increased intestinal permeability, weakened liver detoxification function in cirrhosis, and increased serum iron level.⁶ These extraintestinal infections have high mortality and should be paid attention to.⁷

At present, the pathogenic mechanism of NOVC is still unclear. Studies have shown that the main virulence genes of NOVC including *hlyA*, *hapA*, *rtxA*, *nanH*, *stn*, *ompU*, *zot*, *ace*, type VI secretion system (*T6SS*), and type III secretion system (*T3SS*) gene clusters. The distribution characteristics of virulence genes are closely related to their pathogenicity.^{8,9}

This paper reports a Gram-negative bacterium from the blood culture of a patient with liver cirrhosis. It was identified as *V. cholerae* by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and average nucleotide identity (ANI). Furthermore, it was reviewed as NOVC by the Centers for Disease Control (CDC).

Materials and Methods

Isolation and Identification of Strain

The suspected strain was isolated from aerobic and anaerobic blood cultures of a patient with liver cirrhosis. The strain was initially observed under the microscope after Gram staining. It was inoculated into a blood plate culture for 24 hours. Then the strain was identified by MALDI-TOF MS (BioMerieux, Germany) with a 99% confidence level ([Figure S1](#)).

Antibiotic Susceptibility Test

Antimicrobial susceptibility testing were performed by microdilution method and drug-sensitive slips method, according to the Clinical and Laboratory Standards Institute (CLSI) guideline M45,¹⁰ for amikacin, ampicillin, ampicillin/sulbactam, cefazolin, cefepime, ceftazidime, ciprofloxacin, gentamicin, imipenem, levofloxacin, piperacillin/tazobactam, trimethoprim/sulfamethoxazole, meropenem, amoxicillin/clavulanate, cefuroxime, azithromycin, tetracycline, chloramphenicol, polymyxin B, nalidixic acid.

Whole Genome Sequencing and Gene Function Analysis

The DNA of the *V. cholerae* strain was extracted using the VAMNE Magnetic Pathogen DNA/RNA Kit (Vazyme, CHINA) and sequenced on the Illumina MiSeq platforms. The clean reads are filtered using SOAPnuke software¹¹ (version 1.5.2) and then assembled using SPAdes (version 3.11.0) software.¹² The assembled sequences were uploaded to the ribosomal multilocus sequence typing (rMLST) database (<https://pubmlst.org/species-id>) and the JspeciesWS database (<http://jspecies.ribohost.com/jspeciesws>) for strain identification and average nucleotide identity (ANI) analysis, respectively. We used Glimmer software (version 3.02) to compare the predicted target gene sequences with sequences in the KEGG, COG, VFDB, and CARD databases to obtain annotation information. The final assembled genome was submitted to the NCBI database (www.ncbi.nlm.nih.gov) with the accession number: JARYMY010000000.

Results

Case Presentation

A 58-year-old man was diagnosed with cirrhosis decompensation and hypersplenism 7 years ago. Two hours before admission, the patient had no obvious inducement of nausea, abdominal distension, chills, and general fatigue during sleep. He had hematemesis twice, with an unknown amount, obvious blood clots, clear consciousness, and poor spirit. He was admitted to the hospital for treatment for liver cirrhosis and gastrointestinal bleeding. His temperature was 38.6°C. His heart rate, blood pressure, respiration, and oxygen saturation were within the normal range. Laboratory data showed a white blood cell count of $5.17 \times 10^9/L$, with 78.8% polymorphonuclear cells. Hemoglobin (102g/L) was reduced. Procalcitonin (PCT) (0.17ng/mL), total bilirubin (24.7 $\mu\text{mol/L}$), direct bilirubin (8.5 $\mu\text{mol/L}$), total bile acid (25.6 $\mu\text{mol/L}$), and urea (12.1mmol/L) was increased.

The stool was pasty, and no red blood or white blood cells were found. Besides, the patient tested negative for hepatitis B surface antigen (HBsAg).

The CT (computed tomography) results showed no significant abnormalities in both lungs. There were observed spots with slightly increased density, suggestive of blood accumulation or gastric contents, within the stomach, indicating cirrhosis. Esophageal variceal vein ligation and sclerosis were performed under gastroscopy at the bedside while the patient was under general anesthesia with tracheal intubation. However, after 2 days of admission, there was a worsening of abdominal distension accompanied by dyspnea and decreased oxygen saturation. Physical examination revealed generalized abdominal swelling with multiple areas of tenderness and rebound pain, diminished breath sounds in the right lung, and dullness to percussion over the right chest area. Tracheal intubation was performed followed by ventilator-assisted ventilation. A repeat chest CT scan demonstrated a large pleural effusion in the right hemithorax causing compression and collapse of the right lung as well as mediastinal shift towards the left side. Additionally, ground glass opacities were noted in patches within the left lung along with a small amount of fluid accumulation within the left pleural cavity.

The patient was treated with cefoperazone sodium and tazobactam for anti-infection. Because the patient's underlying disease was too serious, the patient's family gave up treatment and asked to be discharged.

Isolation and Identification of Strains

The patient's blood culture was positive after two days of aerobic and anaerobic bottles. The morphology under the microscope (Lens*100) was Gram-negative bacilli (Figure 1A). Transgenic blood agar plate culture occurred within 24 hours β hemolytic colony with metallic luster (Figure 1B), identified as *V. cholerae* by MALDI-TOF MS. The local Center for Disease Control and Prevention (CDC) identified the bacteria as non-O1, non-O139 *V. cholerae*. The strain was named VC1115.

Genome Assembly and Gene Prediction

Quality control, quality assessment, and assembly of VC1115 original reads obtained by sequencing were carried out, and the genomic circle map was mapped by Circos software (Figure 2). It was predicted that the genome size of VC1115 was 4,212,413 bp, the genome encoded 3836 genes, the total length of the encoding genes was 36,344,566 bp, the average length of the encoding genes was 947.49 bp, the gene GC content was 47.49%, and the gene length accounted for 48.36% of the total genome length. The non-coding RNA in the genome of the sequenced strain contained 86 tRNAs, 10 rRNAs, and 72 sRNAs. The assembled sequence was uploaded to the rMLST database of the PubMLST website for strain identification. The results showed that the uploaded sequence was 100% consistent with *V. cholerae*. The sequence was uploaded to the JspeciesWS database for ANI analysis. The ANIb value was 98.10% and the ANIm value was 98.40%. By comparing with various databases, a total of 3113 (81.15%) genes were annotated, of which 3042 (79.3%) genes were annotated by the COG database, 2502 (65.22%) genes were annotated by the KEGG database, and 370 (9.64%) genes were annotated by the VFDB database.

KEGG Pathway Analysis

By comparing with the KEGG database, 2710 genes related to metabolic pathways were found in the VC1115 genome, which can be divided into metabolism, genetic information processing, organic systems, environmental information processing, cell motility, and pathogenicity (Figure 3). There were a total of 1602 metabolic-related genes, among which there were more genes involved in carbohydrate, amino acid, cofactor, and vitamin metabolism. There were 401 genes related to environmental information processing, mainly involved in membrane transport and signal transduction. A total of 333 genes were related to cellular processes, mainly involved in cell movement, cell growth, and death, as well as transport and metabolism. There are 203 genes involved in genetic information processing, such as transcription,

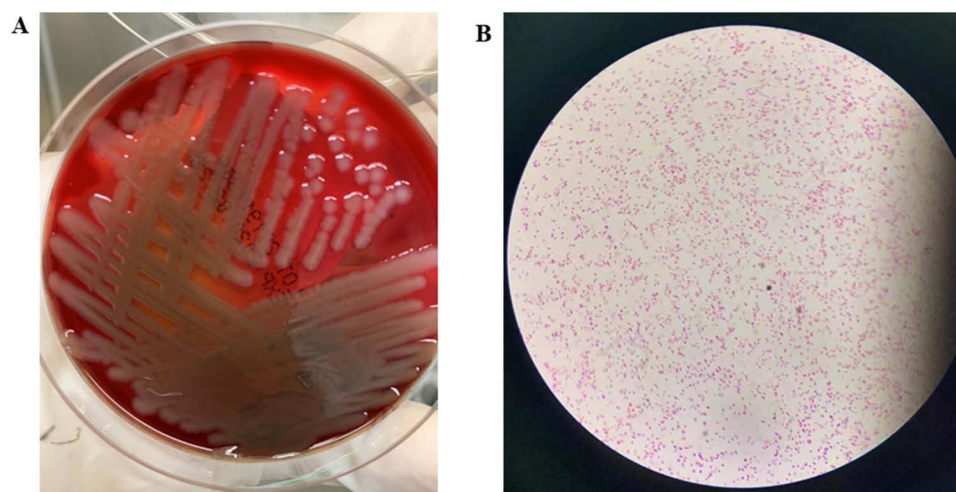


Figure 1 Colony characteristics in blood plate cultured for 24 hours (A) and morphological characteristics were observed under oil microscopy (B) of *V. cholerae* strain VC1115.

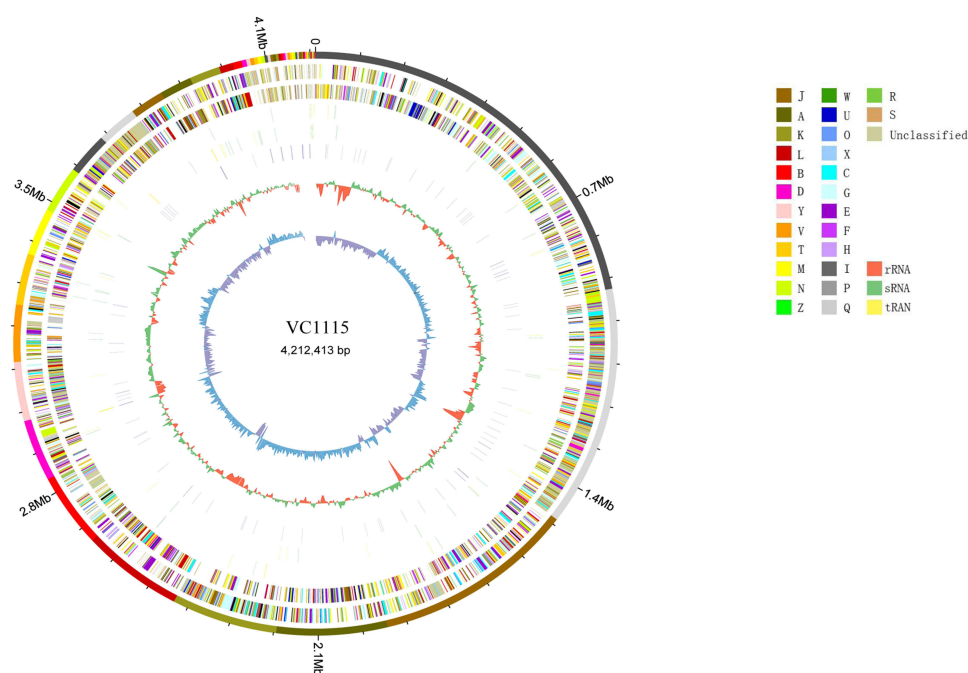


Figure 2 Gene Circle Map of *V. cholerae* VC1115. J: Translation; A: RNA processing and modification; K: Transcription; L: Replication, recombination and repair; B: Chromatin structure and dynamics; D: Cell cycle control, cell division, chromosome partitioning; Y: Nuclear structure; V: Defense mechanisms; T: Signal transduction mechanisms; M: Cell wall/membrane/envelope biogenesis; N: Cell motility; Z: Cytoskeleton; W: Extracellular structures; U: Intracellular trafficking, secretion, and vesicular transport; O: Posttranslational modification, protein turnover, chaperones; X: Mobilome: prophages, transposons; C: Energy production and conversion; G: Carbohydrate transport and metabolism; E: Amino acid transport and metabolism; F: Nucleotide transport and metabolism; H: Coenzyme transport and metabolism; I: Lipid transport and metabolism; ribosomal structure and biogenesis; P: Inorganic ion transport and metabolism; Q: Secondary metabolites biosynthesis, transport and catabolism; R: General function prediction only; S: Function unknown.

translation, protein folding, sorting, and degradation. Notably, 40 genes were associated with “drug resistances”, including 20 cationic antimicrobial peptide resistance genes, 18 beta-lactam resistance genes, and 6 vancomycin resistance genes. Five of the pathogenicity-related genes were involved in the pathogenesis of *V. cholerae* (Table 1).

COG Pathway Analysis

In the COG database, genes were assigned to 24 functional categories and divided into four gene functional types: cellular processes and signals, information storage and processing, metabolism, and poorly characterized (Figure 4).

Toxicity Gene Test Results

The VC1115 genome was examined for genes coding for potential virulence factors using the VFDB database. It lacks CTX virulence factor but carries other 140 related factors to virulence, including *hlyA* virulence factor (Table 2).

Resistance Phenotypes and Resistance Genes

The susceptibility test showed that the strain was resistant to cefazolin, imipenem, and polymyxin B, but sensitive to amikacin, ampicillin, ampicillin/sulbactam, cefepime, ceftazidime, ciprofloxacin, gentamicin, levofloxacin, piperacillin/tazobactam, trimethoprim/sulfamethoxazole, meropenem, amoxicillin/clavulanate, cefuroxime, azithromycin, tetracycline, chloramphenicol, nalidixic acid (Table 3).

Seven drug resistance genes were detected in the genome of strain VC1115, including *CRP*, *catB9*, *almF*, *almG*, *almE*, *MCR-4.3*, and *QnrVC5* (Table 4).

Discussion

NOVC has been considered to have limited pathogenicity due to its lack of CTX toxin production. As NOVC-induced infections have been reported, some of them even lead to serious and life-threatening infections, and it has gradually attracted clinical attention.

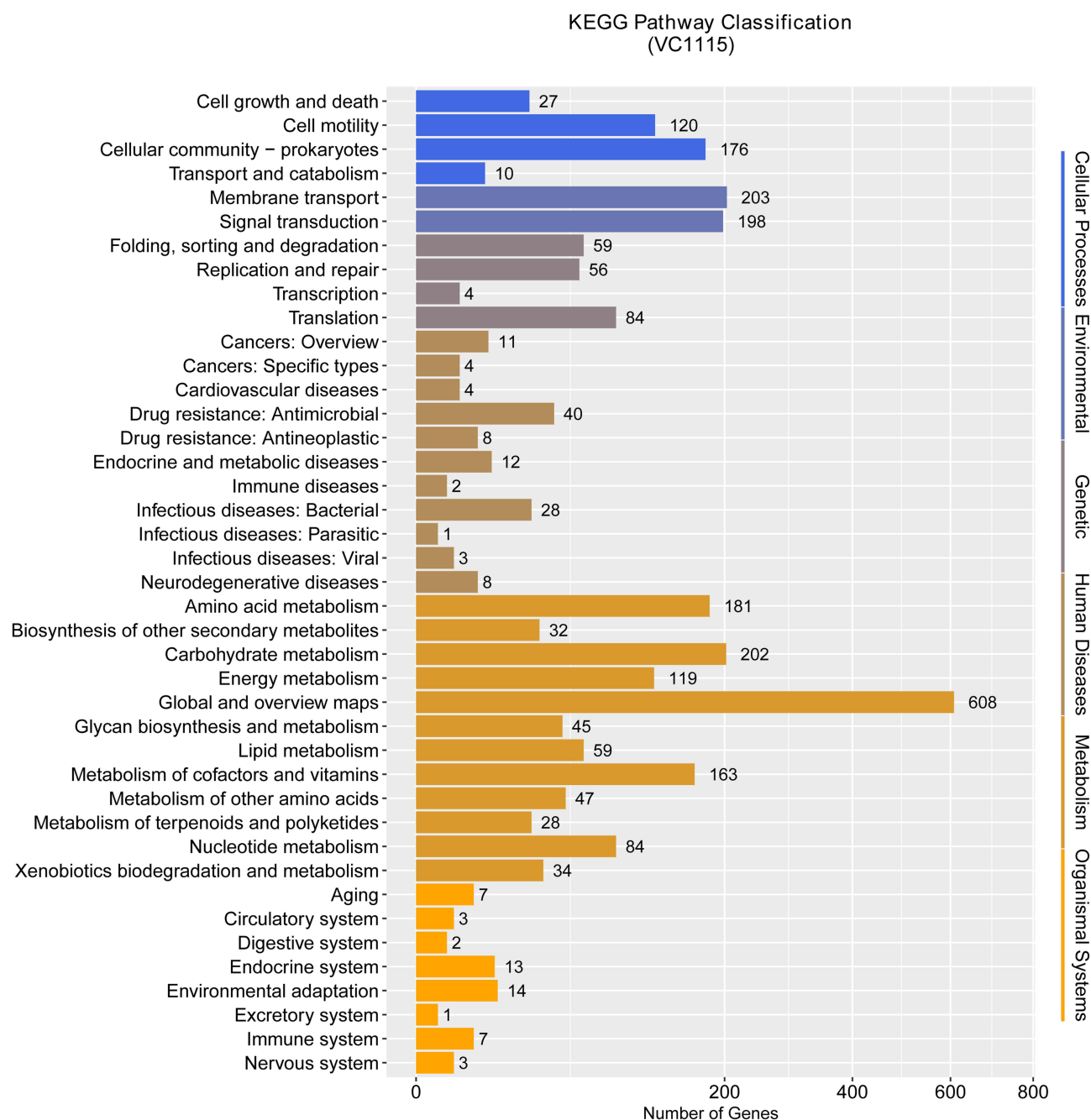


Figure 3 Distribution map of KEGG functional annotations of *V. cholerae* VC1115. The ordinate is the annotated entry and the abscissa is the corresponding entry gene number.

NOVC is prone to gastrointestinal infections, and extraintestinal infections are rare, but this case is an extraintestinal infection. The patient was a 58-year-old man with cirrhosis. It has been reported that men are more susceptible to NOVC infection than women, and liver disease is also a risk factor for NOVC infection.^{13,14} NOVC is more likely to cause infection in patients with low immunity, which can cause extraintestinal infection, bloodstream infection, wound infection, and so on.¹⁵ Tsuruta et al detected NOVC from blood cultures of patients undergoing chemotherapy.¹⁶

The patient had no clear epidemiological history and the cause of infection was unknown. The patient later developed a severe lung infection, which could not be ruled out as being caused by the NOVC strain.

According to gene sequencing of the NOVC strain reported in this paper, the total length of the genome, total length of coding gene, CG content, metabolic pathway, and other aspects of the strain was similar to that of non-toxigenic

Table 1 Pathogenic KEGG Signaling Pathways in *V. cholerae* Strain VC1115

Gene Coding	Gene Name	Functional Description
K08604	<i>hap</i> , <i>nprV</i>	Metalloproteases
K21712	<i>gbpA</i>	N-acetylglucosamine binding protein A
K10953	<i>rtxA</i>	RtxA toxin
K10948	<i>hlyA</i>	erythrocytolysin

V. cholerae.¹⁷ Compared with the CARD database, the drug resistance genes detected in VC1115 strain were *CRP*, *catB9*, *almF*, *almG*, *almE*, *MCR-4.3*, and *QnrVC5*. The drug sensitivity test results indicated that the VC1115 strain exhibited susceptibility to sulfonamides, quinolones, aminoglycosides, and other antibiotics while demonstrating resistance to cefazolin, imipenem, and polymyxin B. Wu et al¹⁸ reported that the resistance rates of 824 clinical NOVC isolates worldwide were ampicillin (44%), streptomycin (40%), cotrimoxazole (27%), and neomycin (27%). It shows that there are some differences in the drug sensitivity of NOVC strains. Bhandari et al¹⁹ reported that among the 56 strains of NOVC isolated from human and animal infections, 17 strains (30%) contained *catB9*, 13 strains (23%) contained *blaCARB-9*, and 1 strain (1.8%) carried *sulIII*, *strA*, and *strB*. Despite its sensitivity to chloramphenicol, quinolones, and aminoglycoside antibiotics, VC1115 still harbors related resistance genes. Therefore, it is imperative to reinforce the integration of in vitro drug susceptibility testing and drug resistance gene monitoring to facilitate disease treatment.

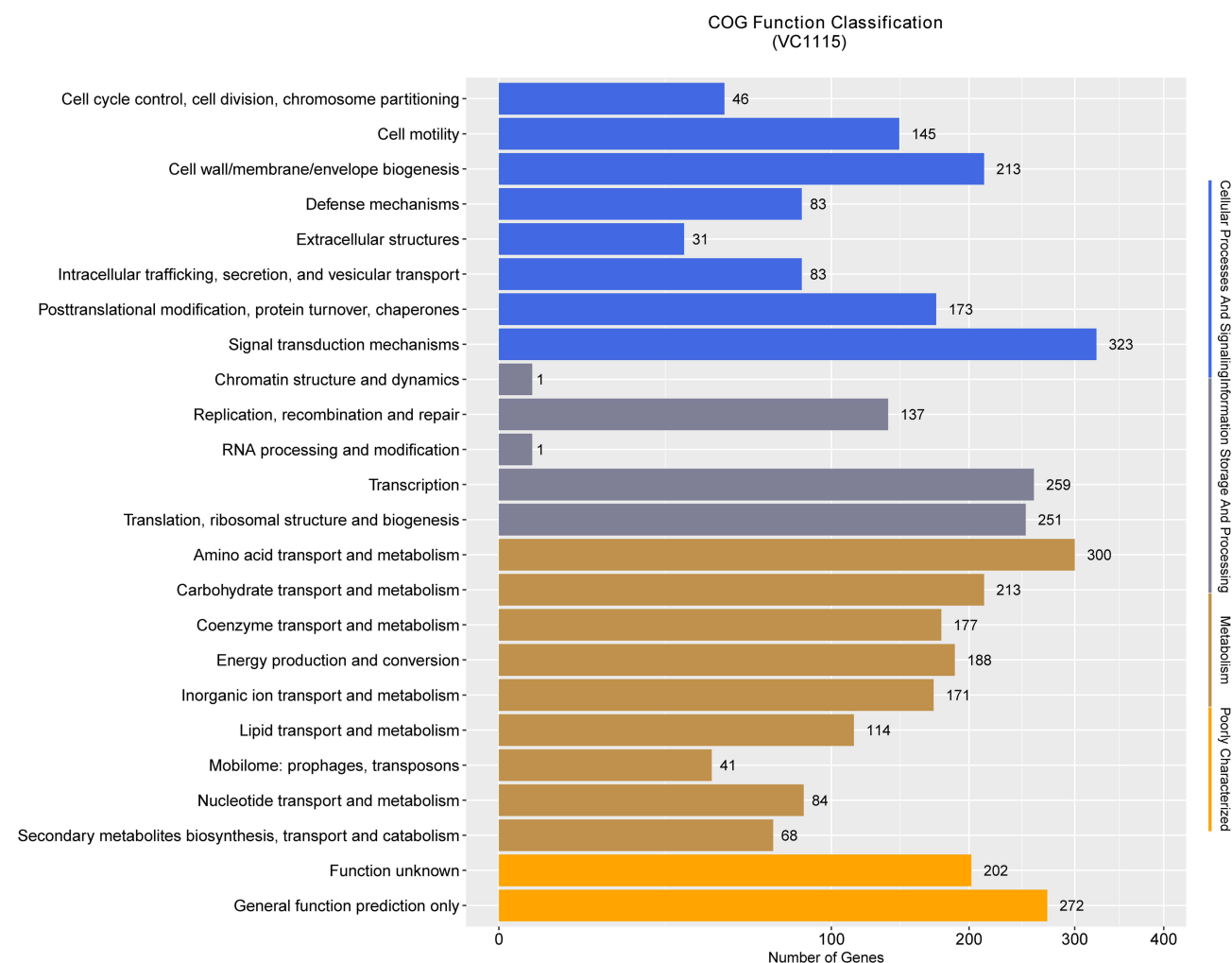


Figure 4 Map of COG functional annotation distribution of *V. cholerae* VC1115. The ordinate is the annotated entry and the abscissa is the corresponding entry gene number.

Table 2 Prediction of Virulence Factor Genes of the *V. cholerae* Strain VC1115

Gene Name	Functional Description
<i>FlgA, flgP, motA, motB, motX, motY, cheA, cheB, cheR, cheV, cheW, cheY, cheZ, flhA, fliD, fliS, flaE, flaD, flaB, flaG, flal, flrA, fleS/flrB, fleR/flrC, flhB, flhA, flhF</i>	Flagella
<i>pilA, pilB, pilC, pilD</i>	Type IV pilin
<i>epsA-epsE, epsG-epsN</i>	EPS type II secretion system
<i>viuC, viuD, vctA</i>	Periplasmic binding protein-dependent ABC transport systems
<i>vgrG-1, vgrG-2, vgrG-3, hcp-2</i>	VAS effector proteins
<i>VasA, vasF, vasH-vasJ, vasL, icmF/vasK, clpB/vasG, VCA0109, vipA/mglA</i>	Type VI secretion system protein
<i>rtxA-rtxD, hlyA, tlh</i>	RTX toxin
<i>hrpH</i>	Type VI secretion system protein
<i>vibA-vibF, vibH</i>	Vibriobactin biosynthesis
<i>viuA, viuD</i>	Vibriobactin utilization
<i>hutA, hasR, hutR</i>	Heme receptors
<i>hap/vvp</i>	Metalloproteases
<i>cqsA</i>	Aminotransferase, class II

Table 3 Antimicrobial Resistance Profile of the *V. cholerae* Strain VC1115

Antimicrobial Agent	KB (mm)	MIC (µg/mL)	Result
Amikacin	/	≤2	S
Ampicillin	/	≤2	S
Ampicillin/Sulbactam	/	≤8/2	S
Cefazolin	/	8	R
Cefepime	/	≤1	S
Ceftazidime	/	≤1	S
Ciprofloxacin	/	≤0.25	S
Gentamicin	/	≤1	S
Imipenem	/	8	R
Levofloxacin	/	≤0.25	S
Piperacillin/Tazobactam	/	≤16/4	S
Trimethoprim/Sulfamethoxazole	/	≤2/38	S
Polymyxin B	/	≥4	R
Azithromycin	/	≤2	S
Meropenem	25	/	S
Amoxicillin/clavulanate	20	/	S
Cefuroxime	26	/	S
Tetracycline	24	/	S
Chloramphenicol	28	/	S
Nalidixic acid	28	/	S

Abbreviations: KB, Kirby-Bauer; MIC, minimum inhibitory concentration; S, sensitivity; R, resistance.

Table 4 The Antibiotic Resistance Genes of the Strain VC1115 Annotated in CARD

Gene Name	Categories
<i>CRP</i>	Macrolide antibiotic, fluoroquinolone antibiotic, penam
<i>catB9</i>	Phenicol antibiotic
<i>almF, almG, almE, MCR-4.3</i>	Peptide antibiotic
<i>QnrVC5</i>	Fluoroquinolone antibiotic

The main toxin of *V. cholerae* causing human diarrhea is CT (cholera toxin).²⁰ CT is mainly encoded by *ctx AB* gene, and the strain carrying *ctx AB* gene is *V. cholerae* toxigenic, which can cause cholera.²¹ *V. cholerae* O1 and O139, which cause cholera, generally have *CTX* genes.²² The virulence genes of the strain reported in this paper are *tox R*, *RTX*, *hlyA*, *T3SS/T6SS*, and lack of *CTX* gene, *ace* gene, and *zot* gene. Compared with O1 or O139, the virulence genes of *V. cholerae* are less, and the strain does not produce cholera toxin. Therefore, there is no obvious diarrhea in the patient. These results are consistent with the results of previous studies on NOVC.^{23,24}

In this study, VC1115 was found to carry more virulence genes. The clinical symptom in the patient was a severe infection. Possible reasons are as follows: (i) The strain carries genes encoding type III secretory systems (T3SS) and type VI secretory systems (T6SS). Studies have found that T3SS/T6SS is widely distributed in multiple lineages of NOVC strains, which may enhance the virulence of NOVC strains lacking *CT* and *TCP* coding genes, thereby enhancing the pathogenic potential of this *V. cholerae*.²⁵ (ii) This strain carries *Hly A* but lacks the *Tag H* gene. Studies²⁶ have shown that *Tag H* negatively regulates the expression of *Hly A* at the transcriptional and translational levels. The absence of *Tag H* enhances the intestinal pathogenicity and extraintestinal invasiveness of *V. cholerae*, which mainly depends on the expression of *Hly A*.

Conclusions

The present study ultimately presents findings on the potential pathogenicity of Non-O1, Non-O139 *V. cholerae*, thereby providing valuable insights into the investigation of both the pathogenic and drug resistance mechanisms associated with *V. cholerae*. Furthermore, it substantiates that certain NOVCs, under harboring specific virulence genes, can induce severe extraintestinal infections and should be accorded due attention by clinicians. Consequently, regular surveillance of NOVC infections and the development of strategies to address such cases are imperative to potentially save lives.

Abbreviations

NOVC, non-O1 non-O139 *V. cholerae*; MIC, minimum inhibitory concentration; CT, cholera toxin; MALDI-TOF MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry; ANI, average nucleotide identity; CDC, Centers for Disease Control; CLSI, the Clinical and Laboratory Standards Institute; KEGG, Kyoto Encyclopedia of Genes and Genomes; COG, Clusters of Orthologous Genes; VFDB, Virulence Factor Database; NCBI, National Center of Biotechnology Information; HBsAg, hepatitis B surface antigen; CT, computed tomography; T3SS, type III secretory systems; T6SS, type VI secretory systems.

Data Sharing Statement

Data associated with this study have been deposited in the NCBI database under the accession number: JARYMY010000000.

Compliance with Ethical Standards

The authors declare that they have compliance with ethical standards. This study has been reviewed and approved by the Research Ethics Committee of the Mianyang Traditional Chinese Medicine Hospital (NO. 2023-013). The patient provided informed consent to publish his case details.

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Disclosure

The authors declare that they have no competing interests for this work.

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