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Virulence-associated genes and molecular characteristics of non-O1/non-O139 *Vibrio cholerae* isolated from hepatitis B cirrhosis patients in China

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ABSTRACT

Objectives: We aimed to report virulence-associated genes and molecular characteristics of non-O1/non-O139 *Vibrio cholerae* isolated from hepatitis B cirrhosis patients in China.
Methods: Patient clinical data including course of disease, laboratory tests, antibiotic treatment and outcomes were collected. Antimicrobial susceptibility testing was performed and virulence-associated genes were detected by PCR. Genetic relatedness among non-O1/non-O139 *V. cholerae* strains was investigated by pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST).
Results: All three strains in this study harbored pathogenicity related genes like *rtxA*, *rtxC*, *toxR*, *hapA*, *hlyA* and *ompW* whereas they lacked *ctxA*, *ctxB*, *tcpA*, *ompU* and *zot* genes. None of them showed resistance to any antibiotic detected. A new allele of *gyrB* was submitted to the MLST database and designated as 97. Two novel sequence types (ST518 and ST519) and ST271 were identified by multilocus sequence typing (MLST). PFGE indicated considerable diversity among three non-O1/non-O139 *V. cholerae* strains.
Conclusions: Three sporadic cases highlight that non-O1/non-O139 *V. cholerae* can cause opportunistic invasiveness infection in cirrhosis patients. Pathogenicity may be related to virulence-associated genes. Timely detection and antibiotic therapy should be paid more attention to in clinic.

Introduction

V. cholerae is the pathogen that causes cholera and is classified into more than 200 serogroups based on the somatic O surface antigen. Only the serogroups O1 and O139 *V. cholerae* can cause epidemic or outbreaks, whereas the non-O1/non-O139 serogroups have also been associated with cholera-like diarrhoea and systematic infections including septicemia, urinary tract infection (Chowdhury et al., 2016), peritonitis (Lan et al., 2014), skin and soft tissue infection (Maraki et al., 2016), meningitis (Hao et al., 2015), bacterial emphysema (Lai et al., 2012). Though *V. cholerae* non-O1/non-O139 serogroups generally lack several major virulence factors such as cholera toxin (*ctx*) and toxin-coregulated pilus (*tcp*), a number of synergistic factors which play roles in the infection process have been identified (Singh et al., 2001). These factors include the toxin regulatory gene (*toxR*), toxin coregulated pilus (*tcp*), repeats-in-toxin (*rtx*), hemolysins (*hly*), outer membrane proteins (*ompU*) and so on (Ceccarelli et al., 2015; Schirmeister et al., 2014; Rajpara et al., 2013; Mathur and Waldor, 2004). Clinical infection cases caused by non-O1/non-O139 *V. cholerae* have been reported in Vietnam (Lan et al., 2014), Germany (Schirmeister et al., 2014), India (Rajpara et al., 2013), China (Luo et al., 2013), USA (Purdy et al., 2010), Italy (Ottaviani et al., 2009), and Mexico (Lizárragapartida and Quilici, 2009).

In this study, we describe clinical data of three patients and genotypic characteristics of three strains. Several discriminative techniques at the species level such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis and *ompW* gene amplification were evaluated. Several virulence genes and synergistic factors were investigated to characterize the pathogenic potential. The heterogeneity of strains was analyzed by Pulsed-field gel electrophoresis (PFGE) and Multilocus Sequence Typing (MLST).

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Materials and methods

Bacterial identification

Three non-O1/non-O139 *V. cholerae* strains were collected from the ascites and blood of three hospitalized patients in the Department of Infectious Diseases at a teaching hospital in Xuzhou, China. Ascites and blood samples were processed using the BacT/Alert automated system (bioMerieux, France) and subcultured on Colombia plate containing 5% sheep blood (KeMaJia, China). Three strains were identified to the species level by VITEK-2 system (bioMerieux, France) and eventually confirmed by MALDI-TOF MS (Bruker Daltonik GmbH, Germany) according to a custom database.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by using the disk diffusion tests (Oxoid) on Mueller Hinton agar (KeMaJia, China) according to Clinical and Laboratory Standards Institute guidelines (Wayne, 2015), with the following antibiotics being included: ampicillin (10 μg), piperacillin (100 μg), amikacin (30 μg), piperacillin-tazobactam (100 μg/10 μg), trimethoprim-sulfamethoxazole (1.25 μg/23.75 μg), levofloxacin (5 μg), ciprofloxacin (5 μg), gentamicin (10 μg), ampicillin-sulbactam (10 μg/10 μg), ceftazidime (30 μg), cefepime (30 μg), chloramphenicol (30 μg), imipenem (10 μg). *E.coli* ATCC25922 was used as the quality control in parallel.

Molecular detection of virulence genes

The polymerase chain reaction (PCR) assays were carried out to check the presence of the virulence genes using appropriate primers. PCR assays were performed using Green Taq Mix (Vazyme Biotech Co.,Ltd, Nanjing, China). The 50 μl reaction mixture contained the following: 25 μl Green Taq Mix, 3 μl template DNA, 2 μl of each primer and 18 μl ddH_2O. Each PCR involved an initial denaturation at 94 °C for 5 min, followed by 30 amplification cycles each consisting of a denaturation at 94 °C for 1 min followed by annealing for 1 min 30 s and an extension at 72 °C for 1 min 30s. Final extension was carried out at 72 °C for 10 min. The primer sequences and temperature of PCR annealing are listed in Table 1. The primer synthesis and positive product sequencing were completed by the GenScript Company (Nanjing, China).

Pulsed-field gel electrophoresis (PFGE)

PFGE was carried out as described previously (Parsons et al., 2007). Total genomic DNA of three strains were digested with *Not*I. For gel electrophoresis, 1% Bio-Rad pulsed field certified agarose gel was made in 0.5X TBE and run in CHEF MAPPER using multialgorithm mode (block1: initial to final time: 2s–10 s for 13 h, block2: initial to final time: 20s–25 s for 4.5 h). The gel was stained with 0.05 mg/ml ethidium bromide for 30 min and destained with sterile water for 1 h. The banding pattern analysis was performed by BioNumerics software (version 6.6). The similarity of banding pattern was calculated using the Dice coefficient. Cluster analysis was performed using the unweighted pairgroup method with arithmetic averages (UPGMA). Strains were considered as the same PFGE type if they possessed ≥85% similarity.

Multiple locus sequence typing (MLST)

MLST was performed to determine these genotypic characterizations. Seven housekeeping genes were targeted for MLST analysis: *adk*, *gyrB*, *metE*, *mdh*, *pntA*, *purM*, and *pyrC*. PCR assays were conducted as previously described. PCR amplifications were carried out under the following conditions: 30 cycles (denaturation at 94 °C for 40 sec, annealing at 54 °C for 40 sec and extension at 72 °C for 40 sec) proceeded by a 5 min initial denaturation at 94 °C and followed by 7 min extension at 72 °C. Purified PCR fragments were sequenced by the GenScript Company (Nanjing,

Table 1
PCR primer sequences, amplicon size and annealing conditions used in this study.

Primer	Sequence (5′-3′)	Amplicon size (bp)	Annealing conditions	References
ctxA-F	CTCAGACGGGATTTGTTAGGCACG	302	55 °C	Nandi et al., 2000
ctxA-R	TCTATCTCTGTAGCCCCTATTACG			
ctxB-F	GGTTGCTTCTCATCATCGAACCAC	460	55 °C	Olsvik et al., 1993
ctxB-R	GATACACATAATAGAATTAAGGATG			
tcpA (classical)-F	CACGATAAGAAAACCGGTCAAGAG	466	60 °C	Rajpara et al., 2013
tcpA (El Tor) R1	GATCAGCGACAGCAGCGAAA	466		
tcpA (classical) R	GATCTGCAAGTGCTACTGCGC			
tox R-F	TTACTACTCACACACTTTGATGGCATCGTT	901	55 °C	Tarr et al., 2007
tox R-R	TTAATGTTCGGATTAGGACACAACTCAAAAG			
hlyA-F	CAATCGTTGCGCAATCGCG	265	50 °C	Rajpara et al., 2013
hlyA-R1	TTGACCTTCAGCATCACT			
ompW-F	CACCAAGAAGGTGACTTTATTGTG	586	55 °C	Nandi et al., 2000
ompW-R	GAACTTATAACCACCCGCG			
ompU-R	CCAAAGCGGTGACAAAGC	655	60 °C	Kumar et al., 2009
ompU-F	TTCCATGCGGTAAGAAGC			
hap-F	GTGAACAACACGCTGGAGAA	700	50 °C	Syngkon et al., 2010
hap R	CGTTGATATCCACCAAAGG			
rtxA VC1451-F	GATTCTTCCGTTCAAGCTCCG	2571	55 °C	Schirmeister et al., 2014
rtxA VC1451-R	TGGTTCAGGCTGTTGCACAC			
rtxC-F	CGACGAAGATCATTGACGAC	265	55 °C	Chow et al., 2001
rtxC-R	CATCGTCGTTATGTGGTTGC			
zot-F	CACTGTTGGTGATGAGCGTTATCG	243	55 °C	Chatterjee et al., 2009
zot-R	TTTCACTTCTACCCACAGCGCTTG			
ace-F	GCTTATGATGGACACCCTTTA	284	55 °C	Chatterjee et al., 2009
ace-R	GTTTAACGCTCGCAGGGCAAA			
*rfb*O139 cluster-F	AGCCTCTTTATTACGGGTGG	449	54 °C	Schirmeister et al., 2014
*rfb*O139 cluster-R	GTCAAACCCGATCGTAAAGG			
rfb O1 cluster-F	GTTTCACTGAACAGATGGG	192	54 °C	
rfb O1 cluster-R	GGTCATCTGTAAGTACAAC			