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Original Article

Clinical characteristics of non-O1/non-O139 *Vibrio cholerae* isolates and polymerase chain reaction analysis of their virulence factors

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Background and Purpose: Non-O1/non-O139 *Vibrio cholerae* can cause invasive extraintestinal disease as well as enteritis. The pathogenesis of invasive non-O1/non-O139 *V. cholerae* infections remains to be determined. This study compared the clinical manifestations and predisposing factors between bacteremic and non-bacteremic non-O1/non-O139 *V. cholerae* infections and examined virulence-associated genes in the pathogenic strains causing invasive disease.

Methods: We retrospectively investigated clinical characteristics of 18 bacteremic patients and 18 non-bacteremic patients, including demographic, laboratory and clinical data. Fourteen clinical isolates (ten isolated from blood and four from stool specimens) were obtained for polymerase chain reaction tests of the presence of virulence-associated genes *ctxA*, *ctxB* and *tcpA*.

Results: There was no difference in age, gender and gastrointestinal symptoms including abdominal pain and diarrhea, laboratory findings including leukocytosis and anemia, or underlying immunocompromised condition, except cirrhosis, between the bacteremic and non-bacteremic groups. Compared to patients with non-bacteremic infections, patients with non-O1/non-O139 *V. cholerae* bacteremia were significantly more likely to have cirrhosis and thrombocytopenia (0.0% vs 77.8% and 5.9% vs 72.2%, respectively; $p<0.001$). The cholera toxin genes (*ctxA* and *ctxB*) were found in only one strain (isolated from the stool specimen of a patient with enteritis) among fourteen clinical strains (7%). The *tcpA* gene, encoding the toxin-coregulated pilus, was present in thirteen of fourteen isolates (93%) [including ten isolates from blood, and three isolates from stool specimens].

Conclusions: Cirrhotic patients with thrombocytopenia were vulnerable to non-O1/non-O139 *V. cholerae* bloodstream invasion. The low prevalence of *ctxA* and *ctxB* genes in stool specimens indicates other toxins could have contributed to diarrhea. The fact that the *tcpA* gene was highly prevalent in clinical isolates in this study could imply an important role of *tcpA* in the pathogenesis of invasive disease caused by non-O1/non-O139 *V. cholerae*.

Key words: Bacteremia; Cholera toxin; *Vibrio cholerae* O139; *Vibrio cholerae* non-O1; Virulence factors

Introduction

Cholera, an epidemic diarrheal disease, is caused by *Vibrio cholerae* serogroup O1 or O139. Similarly, non-O1/non-O139 *V. cholerae* may cause sporadic episodes and occasional outbreaks of diarrheal disease [1]. Gastroenteritis caused by non-O1/non-O139 *V. cholerae* presented clinical symptoms such as diarrhea, abdominal cramps, nausea, vomiting, and fever [2]. The gastroenteritis is often self-limited [3]. Unlike *V. cholerae* serogroup O1 and O139, non-O1/non-O139 *V. cholerae* could lead to extraintestinal diseases, such as bacteremia, invasive soft tissue infections, cholecystitis, and peritonitis [3-6]. The mechanism of invasive non-O1/non-O139 *V. cholerae* infections is not fully understood. Cholera toxin and toxin-coregulated pilus (TCP) are two

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major virulence factors produced by *V. cholerae* during infection. [7-9]. In the pathogenesis of cholera, vibrio colonizes the epithelium of the small intestine by means of TCP and other factors, with the action of cholera enterotoxin leading to the severe diarrhea characteristic of cholera [10]. TCP and cholera toxin have been studied mainly in *V. cholerae* O1 and O139, and it was considered that the majority of non-O1/non-O139 strains do not contain genes for cholera toxin and/or TCP [10, 11]. However, *ctxAB*- and *tcp*-related genes, encoding cholera toxin and TCP respectively, have been found to be present in certain strains of non-O1/non-O139 *V. cholerae* of both clinical and environmental origins [11-13]. Non-O1/non-O139 *V. cholerae* infection is uncommon, especially bacteremia, and identification of virulence genes of non-O1/ non-O139 *V. cholerae* have not been studied in Taiwan. We analyzed the clinical characteristics of patients with non-O1/non-O139 *V. cholerae* infections and performed polymerase chain reaction (PCR) analysis to examine the virulence genes *ctxA* and *ctxB* (encoding cholera toxin) and *tcpA* (encoding TCP) among clinical isolates from blood and stool specimens.

Methods

We reviewed the records, dated from July 1994 to December 2005, from the clinical microbiology laboratories of two medical centers in central Taiwan. There were 39 isolates of non-O1/non-O139 *V. cholerae* identified from 39 patients. Three patients' medical charts were not available. The remaining 36 patients were divided into two groups (bacteremia and non-bacteremia), and there were 18 cases in each group. The demographic, laboratory and clinical data, including age, gender, signs and symptoms, underlying diseases, and clinical outcomes, were reviewed from the medical records. Fourteen clinical isolates, ten isolated from blood, four from stool, were stored and available for PCR tests of virulence genes, *ctxA*, *ctxB*, and *tcpA*.

Identification of species

V. cholerae was identified by automated Vitek by using GNI+ cards (bioMérieux Vitek, Hazelwood, MO, USA) and confirmed by the following biochemical tests: curved Gram-negative bacteria with positive oxidase reactions, beta-hemolysis on a blood agar plate, susceptibility to 10 μg and 150 μg of a vibriostatic agent, O-129 (Oxoid Limited, Hampshire, UK), tolerability to 1% salt solution, lack of tolerability to 10% salt solution, typical biochemistry characteristics including positive nitrate reduction, indole production, citrate utilization, D-glucose utilization with acid production but no gas production, positive Voges-Proskauer test, production of ornithine decarboxylase and lysine decarboxylase, negative arginine dihydrolase production and urea hydrolysis. The isolates that agglutinated with neither O1 nor O139 antisera (Difco, Detroit, MI, USA) were classified as non-O1/non-O139 *V. cholerae* [14].

PCR analysis of virulence genes

The non-O1/non-O139 *V. cholerae* DNA was prepared by guanidine thiocyanate extraction. Each bacterial strain with the size of two rice grains was taken from nutrient agar and dispersed in 100 μL of TE buffer (10 mM Tris-hydrochloride [pH 8.0], 1 mM ethylenediamine tetra-acetic acid). The cells were lysed with 500 μL of GES reagent (5 M guanidine thiocyanate [Sigma Chemical Co., St. Louis, MO, USA], 0.1 M ethylenediamine tetra-acetic acid, 0.5% sarcosyl). After addition of 250 μL of 7.5 mM ammonium acetate, the suspension was kept on ice for 10 min. For deproteination, 500 μL of chloroform-isoamyl alcohol (24:1) was added, and the mixture was centrifuged at 13,000 *g* for 10 min. The DNA was precipitated from the upper phase with 100% ethanol at –20°C for 1 h. The extracted DNA (0.1 μg) was used as the template for amplification. The primers are as follows: for *ctxA*, 5'-CGGGCA GATTCTAGACCTCCTG-3' (F) and 5'-CGATGATCT TGGAGCATTCCCAC-3'(R) [13]; for *ctxB*, 5'-GGTTG CTTCTCATCATCGAACCAC-3'(F) and 5'-GATACAC ATAATAGAATTAAG GAT-3' (R) [12]; for *tcpA*, 5'-CACGATAAGAAAACCGGTCAAGAG-3'(F) and 5'-ACCAAATGCACGCCGAATGGAGC-3'(R) [15] . The PCR tests were set as follows: an initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min; and finally 72°C for 10 min. Amplification was performed in a 50 μL final volume of 5 U of *Taq* polymerase (QIAGEN, Hamburg, Germany), 10 mM Tris (pH 8.3), 50 mM potassium chloride, 2.5 mM magnesium chloride, 0.01% (w/v) gelatin, 250 μM deoxynucleotide triphosphates, and 1 μM of primer. A negative control (sterile distilled water) and a positive control (*V. cholerae* serogroup O1 strain from the Center for Disease Control, Taiwan) were run in each amplification. The amplified products were analyzed by electrophoresis in a 1.8% agarose gel containing ethidium bromide (1 μg/mL) at 120 V for 45 min and were detected by ultraviolet transillumination.

Statistical analysis

Chi-squared test or Fisher's exact test was used to analyze dichotomous variables. Mann-Whitney *U* test was used to analyze continuous variables. A 2-tailed $p<0.05$ was considered statistically significant. All statistical analyses were performed using the Statistical Package for the Social Sciences for Windows (Version 10.0; SPSS, Chicago, IL, USA) software package.

Results

Among the 18 bacteremic cases, the median age was 59.6 years (range, 39 to 83 years), while the median age of the 18 non-bacteremic cases was 55 years (range, 2 to 96 years). There was only one child (two years old) and the remaining cases were older than seventeen years. Old age (mean, 60 and 65 years in the bacteremic and non-bacteremic groups, respectively) and male gender were prominent in both groups (Table 1). Stool cultures were performed in 9 of 18 patients with bacteremia and the results were all negative. In the non-bacteremic group, blood cultures were performed in seven patients. There were 14 patients with cirrhosis in the bacteremic group and none with cirrhosis in the non-bacteremic group (77.8% vs 0.0%, $p<0.01$). Eight of the 14 cirrhotic patients (57.1%) had severe cirrhosis (Child C). Other underlying diseases including malignancy, diabetes mellitus and hematological disease did not predispose

Table 1. Demographic and clinical characteristics of patients with non-O1/non-O139 *Vibrio cholerae* infections

Characteristic	Bacteremia (n = 18) No. (%)	Non-bacteremia (n = 18) No. (%)	*p*
Age (range; years) [mean]	39-83 (60)	2-96 (55)	0.389
Male gender	12 (66.7)	13 (72.2)	
Underlying disease			
Cirrhosis	14 (77.8)	0 (0.0)	<0.001
Malignancy	3 (16.7)[a]	2 (11.1)[f]	1.000
Biliary stones	1 (5.6)	1 (5.6)	1.000
Congestive heart failure	1 (5.6)	0 (0.0)	1.000
Diabetes mellitus	3 (16.7)	3 (16.7)	1.000
Hematologic diseases	1 (5.6)[b]	1 (5.6)[g]	1.000
Other immunocompromised condition	0 (0.0)	2 (11.1)[h]	0.486
Presentation			
Fever or hypothermia	16 (88.9)	8 (44.4)	0.013
Diarrhea	9 (75.0)[c]	12 (66.7)	0.704
Bloody stool	1 (5.6)	3 (16.7)	0.603
Abdominal pain	7 (38.9)	12 (70.6)[d]	0.123
Leukocytosis (>12,000/mm^3)	9 (52.9)[d]	5 (27.8)	0.241
Thrombocytopenia (<100,000/mm^3)	13 (72.2)	1 (5.9)[d]	<0.001
Anemia	6 (33.3)	3 (17.6)[d]	0.443
Diagnoses			
Primary bacteremia	8 (44.4)		
Gastroenteritis	0 (0.0)	12 (66.7)	<0.001
Peritonitis	5 (27.8)[e]	0 (0.0)	0.045
Biliary tract infection	1 (5.6)	2 (11.1)	1.000
Necrotizing fasciitis	3 (16.7)	1 (5.6)	0.603
Cellulitis	1 (5.6)	2 (11.1)	1.000
Pneumonia	0 (0.0)	1 (5.6)	1.000
Hospital days (range) [mean]	3-74 (15.0)	1-53 (8.5)	
Mortality	6 (33.3)	0 (0.0)	0.019

[a]One had hepatoma, one had cholangiocarcinoma, and one had prostate cancer.
[b]Aplastic anemia.
[c]n = 12; 6 cases who had received laxatives were excluded.
[d]n = 17.
[e]All were spontaneous bacterial peritonitis.
[f]One had colon cancer, one had neuroblastoma.
[g]Myelodysplastic syndrome and colon cancer.
[h]One case each of Takayasu's disease and systemic lupus erythematosus.

Table 2. Virulence-associated genes in clinical isolates of non-O1/non-O139 *Vibrio cholerae* from two medical centers (Hospitals A and B) in Taiwan

Number of isolates	Hospital	Diagnoses	Specimen	*ctxA*	*ctxB*	*tcpA*	Outcome
1	A	Bacteremia	Blood	–	–	+	Cured
2	A	SBP	Blood	–	–	+	Cured
3	A	SBP	Blood	–	–	+	Cured
4	A	Septic shock	Blood	–	–	+	Cured
5	A	Fasciitis	Blood	–	–	+	Died
6	A	Bacteremia	Blood	–	–	+	Cured
7	A	Bacteremia	Blood	–	–	+	Cured
8	A	SBP with septic shock	Blood	–	–	+	Died
9	B	Cholangitis	Blood	–	–	+	Cured
10	B	Bacteremia	Blood	–	–	+	Cured
11	A	Enteritis	Stool	+	+	+	Cured
12	B	Enteritis	Stool	–	–	+	Cured
13	B	Enteritis	Stool	–	–	–	Cured
14	B	Enteritis	Stool	–	–	+	Cured

Abbreviations: SBP = spontaneous bacterial peritonitis; – = absent; + = present

to bacteremia (p=1.000). There were five patients with spontaneous bacterial peritonitis in the bacteremic group, and none in the non-bacteremic group (27.8% vs 0.0%, p=0.045). Other invasive infections such as necrotizing fasciitis and biliary tract infection were not significantly more frequent in patients with bacteremia (p=0.603 and 1.000, respectively). The 6 deaths all occurred in patients in the bacteremic group (33.3% vs 0.0%, p<0.001).

There were 16 cases of fever or hypothermia in the bacteremic group and 8 cases in the non-bacteremic group (88.9% vs 44.4%, p= 0.013). Thrombocytopenia was present in 13 cases in the bacteremic group (platelet count, 18,000/mm^3 to 408,000/mm^3; median, 47,000/mm^3), and 1 case in the non-bacteremic group (platelet count, 46,000 to 428,000/mm^3; median, 241,500/mm^3). Among the 13 cases with thrombocytopenia in the bacteremic group, 12 cases had cirrhosis. After excluding patients who used laxatives, 9 of 12 patients with bacteremia and 12 of 18 patients without bacteremia had diarrhea (75.0% and 66.7%, respectively). There was no difference in the presentations of diarrhea, abdominal pain, bloody stool and anemia between the bacteremic and non-bacteremic groups (Table 1). Although a higher proportion of the patients with bacteremia developed leukocytosis compared with those without bacteremia (52.9% and 27.8%, respectively), the difference was not significant (p=0.241). None of the strains isolated from blood carried *ctxA* or *ctxB* (Table 2). Only 1 stool strain carried *ctxA* and *ctxB* concurrently, in a patient who presented with bloody diarrhea without fever and recovered after tetracycline therapy. Ten strains isolated from blood specimens all carried the *tcpA* gene. Three of four isolates from stool specimens carried the *tcpA* gene (Fig. 1).

Discussion

The median age of subjects with non-O1/non-O139 *V. cholerae* infections ranged from 50 to 60 years [16,17]. Morris et al described the predominance of male patients with gastroenteritis (13 of 14 patients) [2]. In our study, patients were relatively old and predominantly male in both groups (bacteremia and non-bacteremia). The majority of the bacteremic and non-bacteremic episodes (83% and 94%, respectively) occurred in warm weather months (from May through October). In this analysis, gastrointestinal symptoms including diarrhea and abdominal pain were observed in other diagnoses such as bacteremia, cellulitis and cholecystitis, but the percentage of fever or hypothermia was significantly higher in bacteremia than in non-bacteremic infections.

In two Taiwanese reports, 30.7% to 33.3% of patients with invasive non-O1 *V. cholerae* infections had diarrhea and 52.4% to 60% of those patients had abdominal pain, although there were some patients with spontaneous bacterial peritonitis in the group [18,19], which may increase the rate of intestinal symptoms. The similarity in the presentations of diarrhea, abdominal pain and bloody stool between bacteremic and non-bacteremic groups may implicate the same portal of entry (gastroenteric tract) but different vulnerability and outcome between the two groups. It is reasonable to use antibiotics in patients whose symptoms mimic

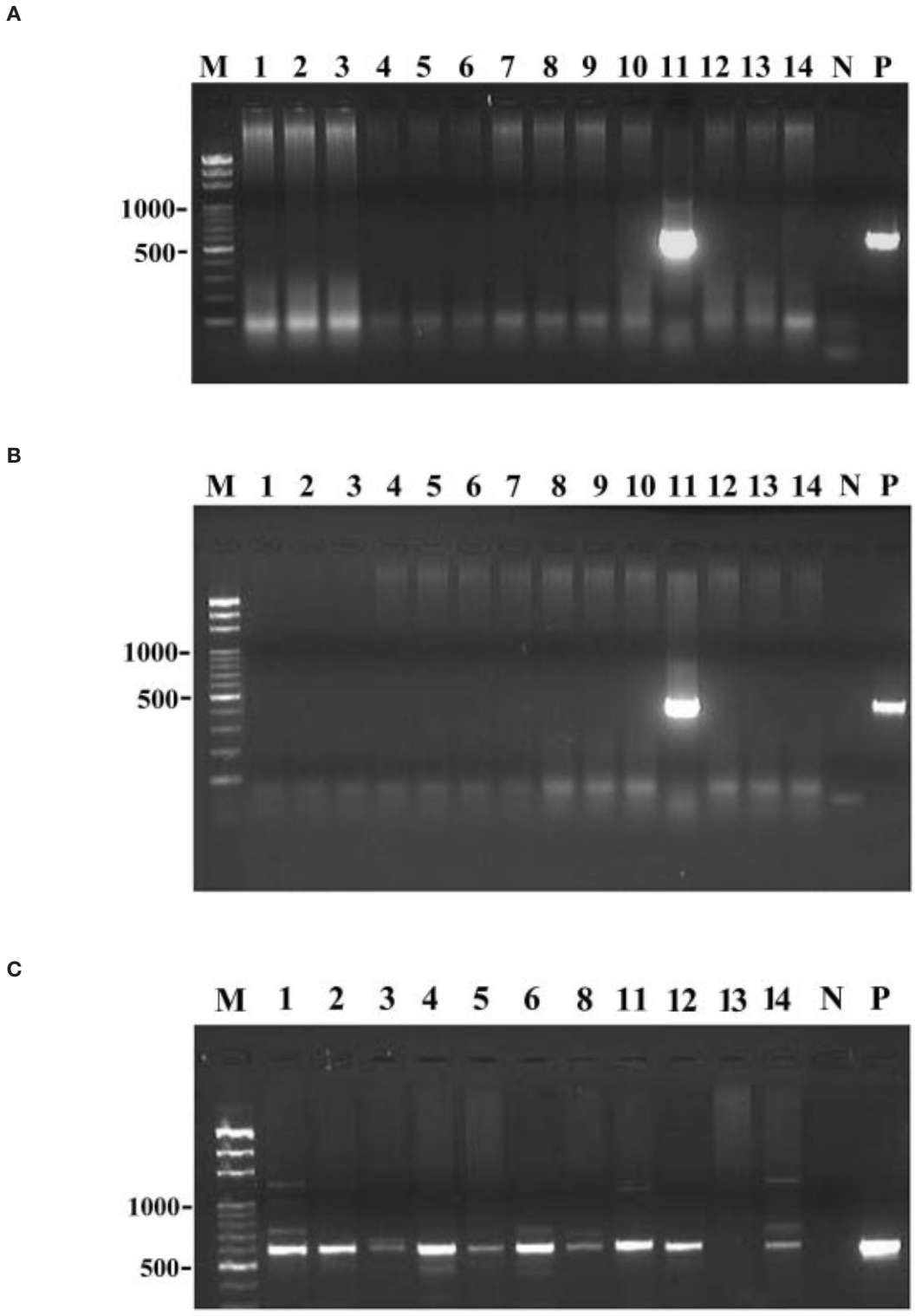

Fig. 1. Amplification of virulence genes from fourteen clinical non-O1/O139 *Vibrio cholerae* isolates; polymerase chain reaction of *ctxA* (A), *ctxB* (B) and *tcpA* (C). M, 100-bp DNA ladder; N, negative control; P, positive control (569B, Center for Disease Control, Taiwan). (A) and (B): lane 1-10, 10 isolates from blood specimens; lane 11-14, 4 isolates from stool specimens. Only one isolate (isolate 11) was positive. (C) All ten isolates from blood specimens were positive, but only 7 isolates (isolate 1-6, and 8) are shown (lane 1-7). Lane 8-11, 4 isolates from stool specimens. Three isolates (isolate 11, 12, and 14) were positive.

gastroenteritis along with fever for probable complicated bacteremia.

Patients with non-O1 *V. cholerae* bacteremia tended to have a high percentage (33.3%) of underlying malignancies in a previous study [18]. However, cirrhosis rather than malignancy was predominant among the patients with non-O1/non-O139 *V. cholerae* bacteremia in this study. It is interesting that cirrhosis did not predispose to non-bacteremic infections in our study. There was a much higher proportion of patients with thrombocytopenia in the bacteremic than the non-bacteremic group (72.2% vs 5.9%, $p<0.001$). The status of thrombocytopenia was associated with cirrhosis in the bacteremic group. It could be assumed that cirrhotic patients with thrombocytopenia easily progress to bacteremia when developing non-O1/non-O139 *V. cholerae* infections.

In this study, bacteremic patients had a high incidence (27.8%) of peritonitis. This is comparable to previous findings that 33.3% of non-O1/non-O139 *V. cholerae* bacteremia was related with peritonitis [3]. The finding may be a direct result of the high prevalence of cirrhosis in our bacteremic group. Other invasive infections, such as necrotizing fasciitis or biliary tract infections, did not complicate bacteremia significantly in this study. However, statistical bias should be considered due to the limited case number.

The gastroenteritis associated with non-O1/non-O139 *V. cholerae* can range from mild illness to profuse watery diarrhea, comparable to that seen in patients with epidemic cholera [2,6,20]. It is well known that cholera toxin is responsible for the induction of massive diarrhea in *V. cholerae* O1 or O139 infections [10]. *ctxA* and *ctxB* genes were found to have low prevalence in clinical and environmental non-O1/non-O139 *V. cholerae* isolates. Jiang et al reported the presence of *ctxA* in 18 of 104 non-O1/non-O139 *V. Cholerae* environmental strains (17.3%) from San Diego Creek and Newport Bay, California [13]. Only 3% of 300 clinical (diarrheal) and environmental non-O1/non-O139 strains carried the *ctxAB* genes in Li et al's study [12]. In our study, only one stool isolate was found to have the *ctxAB* gene. This indicates that other toxins could contribute to diarrhea caused by non-O1/non-O139 *V. cholerae.* That all strains causing bacteremia did not carry the *ctxAB* gene suggested that cholera toxin was not associated with the mechanism of bloodstream invasion caused by non-O1/non-O139 *V. cholerae*.

TCP is a colonization factor and the cholera toxin phage receptor [21]. No environmental non-O1 strains containing *tcpA* genes were recovered in studies in Brazil and Argentina [22,23]. It was reported that only 5% (15 among 300 strains) of clinical and environmental non-O1/non-O139 stains carried the *tcpA* gene [12]. In contrast to those previous studies, *tcpA* was highly prevalent (93%) in clinical isolates in this study. It is possible that *tcpA* plays an important role in the pathogenesis of invasive diseases caused by non-O1/non-O139 *V. cholerae*. However, further studies on environmental isolates in Taiwan were needed to evaluate this.

Non-O1/non-O139 *V. cholerae* could cause invasive infections, especially in cirrhotic patients with thrombocytopenia. In patients with advanced cirrhosis, raw seafood should be avoided because of the susceptibility to non-O1/non-O139 *V. cholerae* bloodstream invasion and high mortality. The mechanism of vulnerability needs further investigation. *TcpA* was highly prevalent in clinical isolates, so surveillance of environmental isolates from Taiwan will help understanding of the evolution of the *tcpA* gene. To delineate the virulence factors of invasive non-O1/non-O139 *V. cholerae* and facilitate the development of adjuvants to standard antimicrobial therapy, further investigations on the mechanism of pathogenesis are required.

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