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Molecular Epidemiology of Newly Emerged *V. cholerae* O139 in Taiwan

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ABSTRACT

A *Vibrio cholerae* O139 strain was isolated from a cholera patient in August 1997 in Kaohsiung County, Taiwan. This was the first case of *V. cholerae* O139 emerging in Taiwan. An epidemiological study showed that the infectious source was turtle eggs. One clinical isolate and fourteen isolates from environmental specimens of the turtle farm were collected. From all isolates, the genes encoded for cholera toxin (*ctxA* and *ctxB*) and for toxin-coregulated pili genes (*tcpA* and *tcpI*) were specifically amplified by polymerase chain reactions. In pulsed-field gel electrophoresis (PFGE) studies, these isolates were categorized to five subtypes using *Sfi*I restriction digestion; whereas three distinct subtypes were identified when *Not*I was used for digestion. The banding pattern of the clinical isolate in PFGE only differed by 1-3 bands from those of the environmental isolates regardless of the restriction enzyme used. The Dice coefficients were found to be in the range of 0.88-1.0 and 0.87-1.0, respectively, by using *Sfi*I and *Not*I for the subtyping. When these isolates were analyzed by plasmid profile analysis, the profile pattern of the clinical strain was identical to 12 of the 14 environmental strains. Based on these results, it was concluded that the fifteen strains studied were descended from the same origin, with the clinical isolate originated from the environmental isolates. The data suggests that the molecular subtyping is a powerful tool for tracing and verifying the infectious sources of *V. cholerae* cases.

Key words: *Vibrio cholerae* O139, polymerase chain reaction, pulsed-field gel electrophoresis, plasmid profile analysis, Dice coefficient

INTRODUCTION

Cholera is a disease caused by the infection of *Vibrio cholerae* from the intake of contaminated food or surface water. According to clinical trials, symptoms such as watery diarrhea occur only when a single intake of *V. cholerae* O1 and O139 amounts to 10^3 and 10^4, respectively. For normal healthy adults, 75% of *V. cholerae* intakes will not demonstrate clinical signs and symptoms. Only around 2% of *V. cholerae* intoxication is serious enough to require hospitalization[(1)]. When the amount of bacteria intake is large, or the secretion of gastric acid is insufficient to kill the germs in the stomach, *V. cholerae* can reach the lower small intestine where it multiplies and releases cholera toxin. Cholera toxin is what causes severe diarrhea and dehydration. On the basis of variations in the O antigenic forms, *V. cholerae* can be differentiated into approximately 193 serogroups[(2)]. Among these, only the O1 and O139 strains are associated with epidemic cholera. All others are called *V. cholerae* non-O1/non-O139. Although certain strains of the non-O1/non-O139 *V. cholera* release cholera toxin, the lack of some coregulating factors such as toxin-coregulated pili genes[(3,4,5)] make it unlikely to be correlated with large epidemics. As a result, *V. cholerae* non-O1/non-O139 strains are considered non-pathogenic for cholera. In Japan, for example, they are only categorized as food-poisoning pathogens[(6)].

Since the seventh pandemic of cholera in 1961, the world has been threatened by cholera epidemic outbreaks, including an outbreak that occurred in Taiwan in 1962. The causative strains were primarily *V. cholerae* O1 biotype E1 Tor. Ten Asian countries, including China, Japan, Thailand, and Malaysia, have detected *V. cholerae* O139[(8)] since its first discovery in India in 1992[(7)]. Additionally, travel-associated imported cases have been reported in the United States since 1993[(9)]. In late August 1997, a domestic case of cholera emerged in Taiwan. There had been no cholera cases for 35 years before the outbreak. Furthermore, the toxigenic *V. cholerae* O139 was new to Taiwan. Therefore, it is necessary to characterize the features of this newly emerged strain of *V. cholerae*. In addition, it has been reported that *V. cholerae* O139 can survive in water bodies for years[(10)]; and consequently, continued monitoring and investigation of cholera cases resulting from this particular strain of *V. cholerae* is required.

In addition to serogroups O1 and O139, the capability of cholera toxin production is also used for discrimination of strains of *V. cholerae*. A total of 15 *V. cholerae* isolates, one clinical isolate obtained from cholera patient who was infected by *V. cholerae* O139-contaminated turtle eggs during 1997 in Taiwan, and 14 environmental isolates collected from turtles and their living environments, were included in the present study. Cholera toxin genes (*ctxA* and *ctxB*)[(11,12,13)] and

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toxin-coregulated pili genes (*tcpA* and *tcpI*)[14,15] were analyzed by polymerase chain reaction (PCR). Pulsed-field gel electrophoresis and plasmid profile analysis were utilized for the comparison purpose of genotyping. In addition, a database of fingerprints of strains was established in order to serve as references for preventive medicine practices in the future.

MATERIALS AND METHODS

I. *Vibrio cholerae O139 Isolates*

A total of 15 *V. cholerae* isolates, including 14 environmental isolates collected from turtles and turtle farms in Kaohsiung and Pingtung Counties in 1997, and one clinical isolate obtained from a patient of a cholera case in 1997, were analyzed in the present study. These isolates were provided by the former National Institute of Preventive Medicine. A clinical isolate of *V. cholerae* O139 from Japan and 2 domestic isolates of *V. cholerae* non-O1/non-O139 were also included as positive and negative controls, respectively. The isolation and serological identification of bacterial strains followed the Diagnostic Procedures for Clinical Specimens published by the National Institute of Preventive Medicine, Taiwan[16].

II. *Polymerase Chain Reaction (PCR)*

The procedure of polymerase chain reaction is briefly described as follows: Bacterial DNA was extracted by the High Pure PCR Template Preparation kit (Boehringer Mannheim, Mannheim, Germany). A 1-μL aliquot of the extracted DNA template was mixed with 49 μL of PCR mixture. The PCR mixture (50 μL) consisted of a 1-fold concentration of reaction mixture (Tris-HCl, 10 mM; $MgCl_2$, 1.5 mM; KCl, 50 mM; Triton X-100, 0.1%, pH 8.8), 2.5 mM of dNTP, 100 μM of primer, and 1 unit of AmpliTaq DNA polymerase (Boehringer Mannheim, Mannheim, Germany). The reactions were carried out in a GeneAmp PCR System 2400 (Perkin Elmer, New Jersey, U.S.A.). Based on previous published literature[11,12,13,14,15], the sequences of the primers were the following: ctxA1, (5'-TCAAACTATATTGTCTGGTC-3'); ctxA2, (5'-CGCAAGTATTACTCATCGA-3'); ctxB1, (5'-CCCAAAGTCTAGGTGTAAAAT-3'); ctxB2 (5'-AAAACGGTTGCTTCTCAT-3'); tcpA1, (5'-TCAAACTATATTGTCTGGTC-3'); and tcpA2, (5'-CGCAAGTATTACTCATCGA-3'). Thermocycle parameters for the PCR assays of the former 2 sets of primers consisted of initial denaturation at 94˚C for 5 min., followed by 30 cycle of amplification, each consisting of 30 sec at 94˚C, 1 min at 53˚C, and 2 min at 72˚C. The synthesis was completed at 72˚C for 10 min. The assay condition for primer set tcpI1 (5'-AACGATAAAGCTGATTTTCAC-3') and tcpI2 (5'-CCAAGGCTTAGAGCCTTTTAT-3') was initially denatured at 95˚C for 5 min, and then subjected to 25 amplification cycles of three steps each, comprised of heating at 94˚C for 2 min, cooling at 50˚C for 2 min, and heating at 72˚C for 3 min. The reaction was concluded at 72˚C for 10 min. A 10-μL aliquot of PCR amplified sample from each of the PCR tubes was electrophoresed through 1.5% agarose gel. The molecular masses of the amplicons were determined by comparison with molecular mass markers of 0.07-12.2 kb (Marker-X; Bushranger Mannheim). Amplification products were visualized by ethidium bromide staining. The identities of the amplification products were further verified by digesting with specific restriction enzymes and determining the sizes of the digestion fragments by agarose electrophoresis, and comparing the sizes of the fragments with known nucleic acids.

III. *Pulsed-Field Gel Electrophoresis (PFGE)*

Pulsed-field gel electrophoresis was performed according to the published manual by the Center for Disease Control, USA[17] with minor modification. After embedded in the agarose blocks, bacteria were lysed in the agarose plugs and incubated overnight. The next day, the plugs were rinsed and sliced to 2.0-2.5 mm thickness, and incubated in reaction solutions containing 20 units of restriction enzymes *Sfi*I or *Not*I (Boehringer Mannheim, Mannheim, Germany) at 50˚C or 37˚C, respectively, overnight with aeration. PGFE was carried out using a CHEF-DRIII system (Bio-Rad, Hercules, CA, USA) through 1.2% agarose with ramp times of 7-12 sec for 11.5 h, followed by 20-65 sec for 12 h for *Sfi*I, and 2-5 sec for 10.5 h, followed by 9-12 sec for 10.5 h for *Not*I. Electrophoresis was performed at 14˚C at 6 volts/cm with a 120˚ reorienting angle. PFGE marker I (Boehringer Mannheim) was used as the molecular mass marker. Gels were then stained with ethidium bromide (0.5 μg/mL) for 30 min, rinsed 3 times for a total of 30 min, and visualized by UV transillumination. Photographs were taken and filed.

The Dice coefficient often is cited as a means of determining the similarity of sets of isolates. With this method, the number of DNA fragments common to both isolates is multiplied by two, and the result is divided by the total number of DNA fragments exhibited by the two isolates. When the Dice coefficient equals 1, the two isolates are identical. When the Dice coefficient equals or is greater than 0.8, the two isolates are considered related[18,19,20,21].

IV. *Plasmid Profile Analysis*

Bacteria was cultured overnight and a single colony was isolated and inoculated into 2 mL of nutrient broth (Difco Laboratories, Detroit, Michigan, USA). After culturing with shaking at 37˚C overnight, the plasmid DNA was prepared by alkaline extraction procedure[22] and analyzed by 0.8 % agarose gel electrophoresis running at 100 V for 2 h with the DNA marker-X (Boehringer Mannheim) being the molecular mass marker. The gels were then stained with ethidium bromide (0.5 μg/mL) for 15 min, rinsed for 1 h, and finally visualized by UV transillumination. Photographs were taken and filed.

RESULTS AND DISCUSSION

I. *Epidemics of ctxA, ctxB, tcpA and tcpI Genes*

Prior to the newly emerged *V. cholerae* O 139 in August 1997, there have been no cholera cases reported in Taiwan for 35 years. This reemergence of cholera is of great concern to disease control institutes at the national level. In order to prevent this new pathogenic strain of *V. cholerae* from spreading and causing epidemics, it is necessary to conduct research on its pathogenic characterization and molecular epidemiology. The detection of cholera toxin has been focused on reversed passive latex agglutination (RPLA) or enzyme-linked immuno-absorbent assay (ELISA)[23]. But these detection methods are limited by the availability of commercial products. Recently, PCR has received increasing interest as a means for the identification of cholera toxin genes. The data generated by PCR are in general agreement with those obtained from RPLA or ELISA[11,12,13]; thus, using PCR for the characterization of cholera toxin is an acceptable alternative. There have been multiple reports using PCR to distinguish *tcpA* and *tcpI* genes[14,15,24,25,26,27]. However, there are no commercial kits for the detection of TcpA protein, although this protein can be analyzed by ELISA[28,29]. RPLA and ELISA, on the other hand, have not been applied in the detection of Tcp I protein. The present study used the PCR technique to identify cholera toxin genes *ctxA* and *ctxB*, and the coregulated pili genes *tcpA* and *tcpI*. The data showed that the 15 domestic isolates tested were all positive for these four genes (Figure 1), as was the Japanese clinical isolate (positive control). In contrast, the two clinical *V. cholerae* non-O1/non-O139 isolates (negative controls) were negative to these genes (Table 1). The manifestations of *V. cholerae* infection are the results of the ability of the bacterium to col-

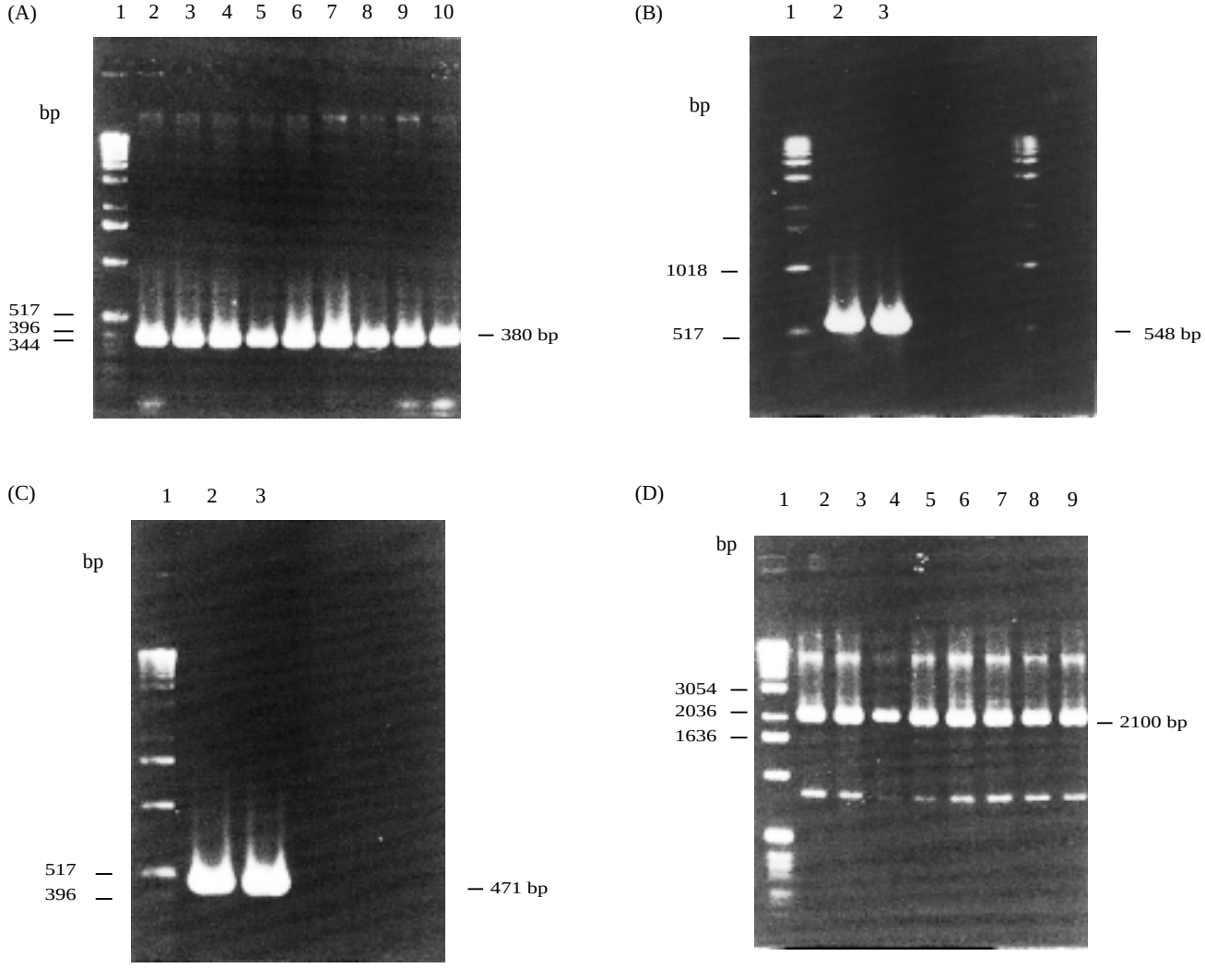

Figure 1. Agarose gel eletrophoresis showing PCR amplification products.
The products for detection of the *ctxA* gene (A), *ctxB* gene (B), *tcpA* gene (C), and *tcpI* gene (D) are shown. (A) Lanes: 2, isolate 8; 3, isolate 9;4, isolate 10; 5, isolate 11; 6, isolate 12; 7, isolate 13; 8, isolate 14; 9, isolate 15; 10, isolate 16;Lane 1, DNA molecular size markers. (B) Lanes: 2, isolate 8; 3, isolate 9;Lane 1, DNA molecular size markers. (C) Lanes: 2, isolate 8; 3, isolate 9;Lane 1, DNA molecular size markers. (D) Lanes: 2, isolate 8; 3, isolate 9;4, isolate 10; 5, isolate 11; 6, isolate 12; 7, isolate 13; 8, isolate 14; 9, isolate 15;Lane 1, DNA molecular size markers.Right number indicate the size of product.

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Table 1. Analysis of genes for cholera toxin (*ctxA* and *ctxB*), toxin-coregulated pili (*tcpA* and *tcpI*) in 18 strains of *V. cholerae* O139 or non-O1 non-O139 by PCR assays

Strain	Years of isolation	Location[a]	Source	No. of isolates	Presence of gene:			
					ctxA	*ctxB*	*tcpA*	*tcpI*
V.cholerae O139	1997	Pingtung	Turtle farm	1	+	+	+	+
	1997	Pingtung	Turtle farm	2	+	+	+	+
	1997	Pingtung	Turtle farm	3	+	+	+	+
	1997	Pingtung	Turtle farm	4	+	+	+	+
	1997	Pingtung	Turtle farm	5	+	+	+	+
	1997	Pingtung	Turtle farm	6	+	+	+	+
	1997	Pingtung	Turtle farm	7	+	+	+	+
	1997	Pingtung	Turtle farm	8	+	+	+	+
	1997	Kaohsiung	Turtle	9	+	+	+	+
	1997	Pingtung	Turtle farm	10	+	+	+	+
	1997	Kaohsiung	Turtle farm	11	+	+	+	+
	1997	Kaohsiung	Turtle farm	12	+	+	+	+
	1997	Pingtung	Turtle farm	13	+	+	+	+
	1997	Pingtung	Turtle farm	14	+	+	+	+
	1997	Kaohsiung	Patient stool	15[c]	+	+	+	+
	1995	Japan[b]	Patient stool	16[c]	+	+	+	+
V. cholerae non-O1	1996	Kaohsiung	Patient stool	17[c]	-	-	-	-
non-O139	1997	Kaohsiung	Patient stool	18[c]	-	-	-	-

[a]County.
[b]Country.
[c]Clinical isolate.

onize the surface epithelium of the small intestine and to produce numerous exotoxins, including the potent enterotoxin cholera toxin. Secretion of cholera toxin alone is not sufficient to become pandemic. Besides, it has been shown that most of the environmental isolates of the serogroup O1 strain do not produce cholera toxin[30,31]. It is therefore suspected that the secretion of cholera toxin is encoded by the genome of the bacteriophage residing within the *V. cholerae*[32,33]. Toxin-coregulated genes *tcpA* and *tcpI*, on the other hand, were only detected in the O1 and O139 serogroups of *V. cholerae*. In addition to serving as a determinant for cell surface pilus biosynthesis for enterocolonization, Tcp A is also shown to be the receptor of the bacteriophages that is closely related with their insertion of cholera toxin genes[33]. The expression of *tcpA* is indirectly regulated by *tcpI*. TcpI is an integral inner membrane protein that plays the role of being an environmental sensory molecule involved in regulating bacterial chemotaxis[15]. TcpI recognizes the environmental signals and elucidates the signal transduction pathway to facilitate the activation of pilus biosynthesis for the colonization of the surface epithelium of the host's small intestine[3,4,5,15]. The expression of TcpI and TcpA is the prereq-

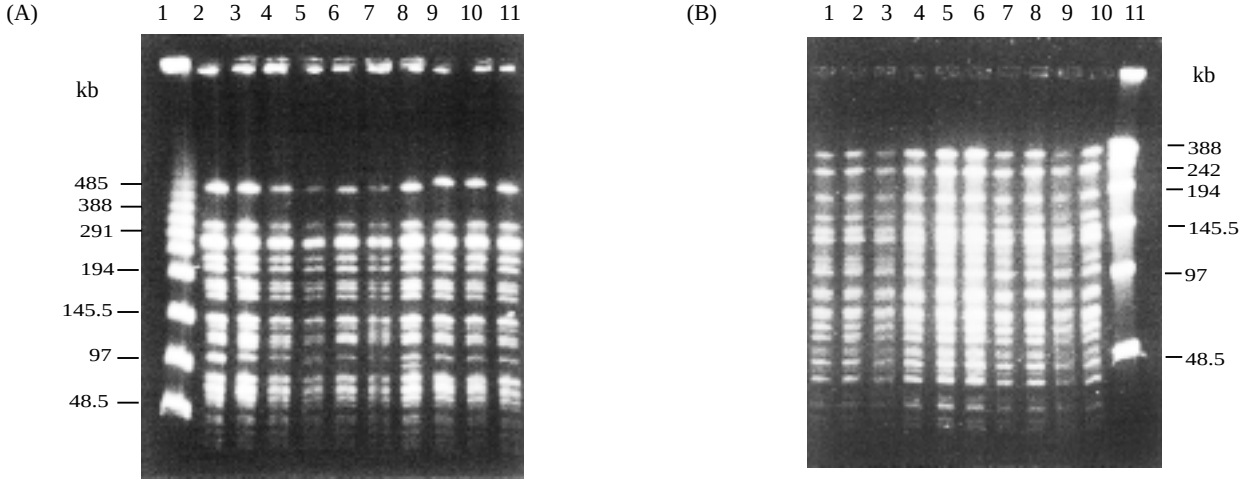

Figure 2. Pulsed-field gel electrophoresis (PFGE) patterns of *Vibrio cholerae* O139 isolates with *Sfi*I (A)or *Not*I (B) digestions. (A) Lanes: 2, isolate 1 (pattern SA); 3, isolate 11 (pattern SA-2); 4, isolate 12 (pattern SA-2); 5, isolate 10 (pattern SA-1); 6, isolate 8 (pattern SA); 7, isolate 9 (pattern SA); 8, isolate 15 (pattern SA-4); 9, isolate 13 (pattern SA-3); 10, isolate 14 (pattern SA-3); 11, isolate 2 (pattern SA); Lanes 1: lamda DNA marker. (B) Lanes: 1, isolate 1 (pattern NA); 2, isolate 2 (pattern NA); 3, isolate 3 (pattern NA); 4, isolate 5 (pattern NA-1); 5, isolate 6 (pattern NA-1); 6, isolate 7 (pattern NA-1); 7, isolate 8 (pattern NA-1); 8, isolate 9 (pattern NA-2); 9, isolate 12 (pattern NA-1); 10, isolate 15 (pattern NA-1); Lanes 11: lamda DNA marker, starting from bottom at 48.5 kb. Letters correspond to the different PFGE patterns listed in the Table 2.

uisite for the pathogenic mechanism; consequently, toxin-coregulated pili genes and cholera toxin genes can be used coordinately as the markers for the identification of toxigenic strains of *V. cholerae*.

II. *Pulse-Field Gel Electrophoresis (PFGE) Patterns of the 15 Domestic V. cholerae Isolates*

It has been shown that the PGFE pattern of the 1992 India epidemic caused by O139 strain is similar to the seventh pandemic-causing O1 E1 Tor biotype, but distinct from the non-O1/non-O139 strains[34]. The toxin-coregulated pili genes *tcpA* and *tcpI* have only been detected from the serogroups O1and O139; and the sequences of the aforementioned genes are very similar between the O139 strain and the O1 El Tor biotype. As a result, it is suggested that O139 is derived from the O1 El Tor biotype[5,35]. *V. cholerae* O139 isolates collected from various areas during various times exhibited very similar, although not entirely identical, PGFE banding patterns which suggest that they are clonal[36]. Bacterial strain typing with PFGE examines the restriction patterns of the digested fragments for comparative analyses[21]. The present study employed the restriction enzymes *Sfi*I and *Not*I to digest DNA. Fifteen domestic isolates were digested by *Sfi*I and 5 patterns were revealed (i.e., SA, SA-1, SA-2, SA-3, and SA-4), or by *Not*I and 3 patterns were obtained (i.e., NA, NA-1, and NA-2) (Table 2). Restricted by *Sfi*I resulted in 22-23 fragments in the range of 40-500 kb (Figure 2), whereas restricted by *Not*I resulted in 20-21 fragments in the range of 40-388 kb (Figure 2). The banding patterns of the domestic clinical isolate and the 14 domestic environmental isolates resulted from digestion by these two restriction enzymes were either identical or differed only by 1-3 fragments. Additionally, the fragment patterns of some isolates were identical regardless the restriction enzyme employed. For instance, isolates No. 1-4 are all SA or NA types, whereas isolates No. 5-8 are all SA or NA-1 types. This phenomena further strengthens the view of their clonal relationship. The numerical similarities between isolates, as defined by Dice coefficients, were in the ranges of 0.88-1.0 and 0.87-1.0, respectively, by using *Sfi*I and *Not*I for subtyping. The high Dice coefficients (greater than 0.8 for all pairs of isolates) represented the high similarities among chromosomal restriction fragment patterns. These isolates are epidemiological related isolates[18-21]. The limited number of band shifts among isolates is likely due to the loss or gain of a restriction site, an insertion, or a deletion during the infection or cultivation periods[21,25]. However, issues regarding high similarity in the PFGE banding patterns of serogroup O139 strains have been raised. Further in-depth epidemiological investigations are required for the assurance of the linkage between strains and epidemic outbreaks.

III. *Plasmid Profile Analysis of the 15 Domestic Isolates*

Plasmid profile analysis that examines the transferable plasmids of chromosomes has been widely used for the subtyping of pathogens[37]. Only the classical biotype of *V. cholerae* O1 has been reported to contain plasmids[38]. *V. cholerae* O139 strains do not contain plasmid for the most part, similar to *V. cholera* O1 of the EI Tor biotype. Only a very small portion of the O139 strains carries a large multiple drug resistant plasmid with a size of approximately 200 kb[39]. The 15 domestic *V. cholerae* O139 isolates analyzed in the present study contained small plasmids with 3 distinct plasmid profiles (Table 2). One of the environmental isolates contained two plasmids with sizes of 3.5 and 7.0 kb, identical to the plasmid profile of the Japanese clinical isolate. Another environmental isolate contained two plasmids with sizes of 4.0 and 8.0 kb. The other isolates, including the domestic clinical isolate, contained only one plasmid with a size of 5.0 kb (Figure 3).

The 15 domestic isolates all tested positive to the four selected toxigenic genes with great similarities in their PFGE banding patterns and plasmid profiles. Epidemiological surveys revealed the infectious source to be raw turtle eggs. Taken together, the data suggest that the clinical isolate was derived from the environmental isolates. The present study demonstrated that the molecular typing is of advantage for tracing and correlating the similarities of isolates at the time of the emergence of clinical cholera cases. Although the virulence of *V. cholerae* is not as potent as some bacilli that are pathogenic and cause bacillary dysentery when the amount of ingestion reaches 10-100[37], the long survival duration in surface waters and the ability to adapt to non-optimal conditions via cell transformation[10,40] have made deaths resulting from *V. cholerae*-mediated diarrhea a long lasting problem in third world countries where personal hygiene, environmental sanitation and quality of medical services are poor[7]. The reemergence of this disease has drawn great concern by health organizations worldwide because that the newly emerged derivatives may be more virulent and/or more resistant to therapeutic agents[41]. As a consequence, the existence of drug resistance genes on the plasmids DNA of the *V.*

Table 2. PFGE patterns and plasmid profiles for *Vibrio cholerae* O139 isolates in Taiwan

No. of Isolates	PFGE patterns		Plasmid
	*Sfi*I	*Not*I	Profiles, Kb
1	SA	NA	5.0
2	SA	NA	5.0
3	SA	NA	5.0
4	SA	NA	5.0
5	SA	NA-1	5.0
6	SA	NA-1	5.0
7	SA	NA-1	5.0
8	SA	NA-1	5.0
9	SA	NA-2	5.0
10	SA-1	NA-1	5.0
11	SA-2	NA-1	5.0
12	SA-2	NA-1	3.5, 7.0
13	SA-3	NA	4.0, 8.0
14	SA-3	NA	5.0
15[a]	SA-4	NA-1	5.0

[a]Clinical isolate.

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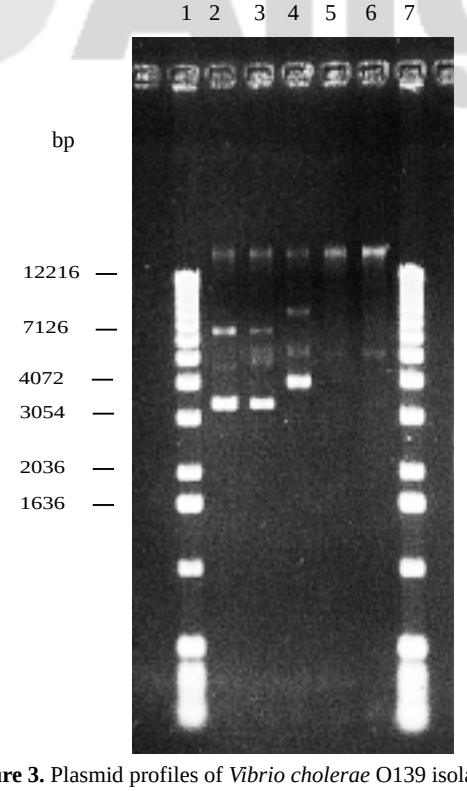

Figure 3. Plasmid profiles of *Vibrio cholerae* O139 isolates. Lanes: 2, isolate No.16; 3, isolate No.12; 4, isolate No.13; 5, isolate No.6; 6, isolate No.7. Lane 1 and 7: linear DNA marker.

cholerae requires further investigation. The development of *V. cholerae* O139 will be under continuous monitoring in Taiwan. Further, more isolates of *V. cholerae* are to be collected domestically and from aboard for future work on the performance of comparative biotypings of the various strains, and thereafter, on the facilitation of the establishment of genetic fingerprint databases and their respective epidemiology.

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新興霍亂弧菌O139型感染事件之分子流行病學分析

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摘　要

1997年8月台灣高雄地區首次發現感染霍亂弧菌（*Vibrio cholerae*）O139型病例，經追查得知因生食甲魚卵而被感染，並從甲魚及甲魚池檢出霍亂弧菌O139型；本菌近年來頗受國內外防疫界之重視。本研究收集當時分別從甲魚及甲魚池環境所分離出之霍亂弧菌O139型菌株共14株，及當時個案病例之1株臨床分離株共15株，以聚合酶鏈反應法（PCR）測試其染色體上霍亂毒素基因cholera toxin gene（*ctxA*和*ctxB*）和共同調控毒素纖毛基因toxin-coregulated pili gene（*tcpA*和*tcpI*）之普及率，發現14株環境和1株臨床本土性菌株對此四種基因均為陽性反應；以脈場膠電泳法（pulsed-field gel electrophoresis, PFGE）進行基因組分型比較，分別以限制酵素*Sfi*I或*Not*I切割，15株菌株以限制酵素*Sfi*I分為5次型，以限制酵素*Not*I分為3次型，臨床分離株與14株環境分離株之PFGE圖譜彼此間或相同或僅相差1~3個帶狀片段，經由*Sfi*I分型其D系數（Dice coefficients）介於0.88至1.0；經由*Not*I分型其D系數介於0.87至1.0。雖然以質體輪廓分析法（plasmid profile analysis）可將15株菌株區分為3型，臨床分離株與其中12株環境分離株質體輪廓分型圖譜相同。綜合以上分析結果，推論這些菌株衍生自相同菌源，即臨床分離株應源自環境分離株。顯示分子分型有利於霍亂感染案件分離株之追查印證。

關鍵詞：霍亂弧菌O139型，聚合酶鏈反應法，脈場膠電泳法，質體輪廓分析法，D系數