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Molecular epidemiologic analysis of *Vibrio cholerae* O1 isolated during the 1997–8 cholera epidemic in southern Thailand

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SUMMARY

An unusually high incidence of *Vibrio cholerae* O1 infection was observed in southern Thailand between late December 1997 and March 1998. Fifty-seven *V. cholerae* O1 strains were isolated in five provinces during this epidemic and were examined. They were El Tor Ogawa strains exhibiting similar antibiograms. All strains were resistant to tetracycline, which had not been reported in Thailand since 1993. The ribotypes, hybridization patterns with *ctx* and *zot* gene probes, arbitrarily primed PCR profiles, and pulsed-field gel electrophoresis profiles of the representative strains were compared with the clinical strains isolated from patients in India and Bangladesh in 1997 and 1998 and from international travellers originating from various Asian countries during the 1992–8 period. All southern Thailand strains and the 1998 international traveller strain of Thai origin showed indistinguishable genetic fingerprinting patterns that were distinct from those of other test strains. The results suggest that a tetracycline-resistant clone newly emerged in late December 1997 caused the large epidemic in southern Thailand and that the variants with a slightly different antibiogram appeared during the course of the spreading epidemic.

INTRODUCTION

The aetiologic agent of cholera usually belongs to *Vibrio cholerae* serovar O1. Cholera outbreaks due to *V. cholerae* serovar O139 were first observed in the Bengal area in 1992 and rapidly spread to other parts of the world. However, *V. cholerae* O139 infection was soon displaced by *V. cholerae* O1 infection but the former has still been reported in the Bengal area [1]. The cholera cases in Thailand in the 1990s were due to *V. cholerae* O1 of El Tor biotype and Ogawa serotype (El Tor Ogawa) and the cases occurred sporadically except that some *V. cholerae* O139 infections were observed between 1993 and 1995 [2–4].

The patients infected with *V. cholerae* O1 were effectively treated with various antibiotics such as tetracycline (Tc), erythromycin, cotrimoxazole, furazolidone and new quinolones [5, 6]. However, the strains have become resistant to one or more antibiotics. Multiply resistant strains were isolated in Africa, Asia, and Latin America [1, 5–7]. Siddique [8] reported that tetracycline and doxycycline were no longer effective for cholera treatment in the outbreak

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in Zaire. Islam [9] reported that 33·3% of EI Tor Ogawa strains were resistant to Tc, ampicillin, cotrimoxazole, nalidixic acid and doxycycline but susceptible to erythromycin and mecillinam in Zaire. The EI Tor Ogawa strains isolated in Bangladesh were susceptible to Tc but resistant to ampicillin [10]. However, 3·8% of the strains of EI Tor biotype and Inaba serotype and 98·1% of the strains of the classical biotype and Ogawa serotype isolated in the same country were resistant to Tc [11].

In the period 1992–6, there were sporadic cholera cases in 14 provinces of southern Thailand according to a report from The Ministry of Public Health, Division of Epidemiology, Bangkok, Thailand. However, an unusually high incidence of cholera cases was noticed in Songkla Province and the neighbouring provinces of southern Thailand in late December 1997. They were caused by *V. cholerae* O1 El Tor Ogawa and it was unusual that the strains were resistant to Tc and other antibiotics. Tc-resistant *V. cholerae* O1 had not been reported since 1993 in Thailand. A high cholera incidence apparently due to the same microorganism was also observed from January to March 1998. To examine the possibility that a new Tc-resistant clone of *V. cholerae* O1 emerged and caused the epidemic, we analysed the strains isolated during this epidemic in southern Thailand and those strains isolated elsewhere by various genetic fingerprinting methods.

MATERIALS AND METODS

Bacterial strains

Stool or rectal swab samples were collected from patients with diarrhoea from the provincial hospitals in southern Thailand between late December 1997 and January 1998. The samples were plated directly onto thiosulfate-citrate-bile salt-sucrose agar (TCBS agar; Eiken Chemical Co., Ltd, Tokyo, Japan). The yellow colonies detected on the medium were examined by standard biochemical tests for identification of *V. cholerae* [12]. The *V. cholerae* strains thus identified were subjected to serotyping by the agglutination tests with polyvalent anti-O1 antiserum and antiserum specific to serogroup Ogawa and Inaba (Difco Laboratories, Detroit, MI, USA) and a slide latex agglutination kit (Vibrio cholerae AD; Denka Seiken Co., Tokyo, Japan). *V. cholerae* O1 El Tor Ogawa strains isolated from the patients in Bangladesh in 1998 and in India in 1997 were provided, respectively, by Dr M. John Albert of International Centre for Diarrheal Diseases Research, Bangladesh and by Dr G. Balakrish Nair of National Institute of Cholera and Enteric Diseases, Calcutta, India. *V. cholerae* O1 El Tor Ogawa strains isolated from international travelers arriving in Japan from various Asian countries between 1992 and 1998 were supplied by Kansai Airport Quarantine Station and Nagoya Airport Quarantine Station, Japan. *V. cholerae* O1 NIH 41 belonging to classical biotype and Ogawa serotype, included as a reference strain, was obtained from Dr Toshio Shimada of National Institute of Infectious Diseases, Tokyo, Japan.

Detection of virulence genes

The presence or absence of the genes encoding cholera toxin (*ctxA*), toxin coregurated pilus (*tcpA*) of El Tor biotype [13, 14] and zonula occludens toxin (*zot*) [15] were examined by PCR methods as previously reported except that differing thermal cycling was used as described below. All PCR experiments were carried out using DNA thermal cycler 480 (Perkin–Elmer, Norwalk, CT, USA) using the following thermal cycling protocol: 94 °C for 4 min for denaturing the template; 30 cycles of 94 °C for 1 min and 60 °C for 1·5 min and 72 °C for 1·5 min and a final extension at 72 °C for 10 min. Five μl of the amplification products were separated by gel electrophoresis in a 1·5% agarose gel using TAE buffer (40 mM Tris–HCl pH 8·5, 5 mM sodium acetate, 1 mM EDTA) and visualized with ethidium bromide staining and u.v. transillumination.

Detection of plasmid

Plasmid extraction using the alkaline lysis method was carried out as previously described by Sambrook [16]. The extracted DNA sample was examined by electrophoresis in a 1% agarose gel followed by staining with ethidium bromide and u.v. transillumination.

Antibiotic susceptibility

The antibiotic susceptibility of test strains was measured by the disk diffusion method of Bauer [17] with the following antibiotics: ampicillin (30 μg), Tc (30 μg), cotrimoxazole (trimethoprim, 1·25 μg; sulfamethoxazole, 23·75 μg), chloramphenicol (30 μg),

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gentamicin (10 μg), nalidixic acid (30 μg), erythromycin (15 μg), ciprofloxacin (5 μg), and norfloxacin (10 μg).

Isolation of total cellular DNA

Total cellular DNA was extracted from *V. cholerae* O1 strains and purified as described previously [18].

Arbitrarily primed PCR

Arbitrarily primed PCR (AP–PCR) was carried out in a volume of 30 μl containing 25 ng of *V. cholerae* total cellular DNA, 25 pmol of a primer (primer number 1, 2, 3, 4, 5 or 6) of the RAPD Analysis Primer set (Pharmacia Biotech, Inc., Uppsala, Sweden), 1 U of Ex-Taq DNA polymerase (Takara Ex Taq; Takara, Shiga, Japan), 25 mM deoxynucleoside triphosphate and 3 μl of ×10 buffer containing 20 mM $MgCl_2$ (Ex Taq buffer; Takara). The sequences of the primers were: 5′-GGTGCGGGAA-3′ (primer 1); 5′-GTTTCGCTCC-3′ (primer 2); 5′-GTAGACCCGT-3′ (primer 3); 5′-AAGAGCCCGT (primer 4); 5′-AACGCGCAAC-3′ (primer 5); and 5′-CCCGTCA-GCA-3′ (primer 6). Each amplification reaction was performed under a drop of mineral oil in a thermal cycler (Zymoreactor II, model AB-1820, Atto Co., Tokyo, Japan) with the following thermal cycling protocol: 95 °C for 4 min to denature the template; 45 cycles of 94 °C for 1 min, 36 °C for 1 min, and 72 °C for 2 min and a final extension at 72 °C for 7 min. Ten μl of the amplification products were separated by gel electrophoresis in a 1·5% agarose gel in TBE buffer (44·5 mM Tris–HCl, 44·5 mM boric acid, 1·0 mM EDTA [pH 8·0]) and visualized by ethidium bromide staining and u.v. transillumination.

Southern hybridization with the *ctxA* and *zot* probes

The purified total cellular DNA was digested with the appropriate restriction endonucleases: *Pst*I or *Xba*I (for the *ctxA* probe); *Pst*I (for the *zot* probe). The DNA digest was separated in 1% agarose gel and transferred to nitrocellulose membrane using the Southern blot method [16]. A *Hin*dIII digest of bacteriophage lambda was included as the molecular weight markers in each gel. The *ctxA* and *zot* probe DNAs were obtained by PCR amplification as described above. The template was the total cellular DNA isolated from *V. cholerae* O1 Vc143 (El Tor biotype Ogawa serotype; a clinical strain isolated during the investigation in Nakhon si Thammarat Province in 1998). The amplicons were isolated by electrophoresis in a 1% agarose gel followed by extraction and purification using an extraction kit (QiaexII gel extraction kit; Qiagen GmbH, Dusseldorf, Germany). The nitrocellulose membrane with immobilized DNA restriction fragments were hybridized with the digoxigenin-labelled DNA probe and probe-positive fragments were visualized by the immunological detection system (DIF DNA labelling and detection kit, Boehringer Mannheim, Germany).

Ribotyping

The purified total cellular DNA was digested with restriction endonuclease *Bgl*I. The fragmented DNA was electrophoresed in a 1·0% agarose gel and transferred to a nitrocellulose membrane using the Southern blot method [16]. Probe DNA was prepared by PCR amplification. The PCR reaction mixture consisted of 5 μl of 10× Thermophilic DNA Polymerase 10× Buffer, (Magnesium Free; Promega Corp., Madison, WI, USA; containing 100 mM Tris–HCl [pH 9·0], 500 mM KCl, 1% Triton X-100), 1·5 mM $MgCl_2$, 0·125 mM deoxynucleoside triphosphate, 0·2 mM each of the primers, 10 μl template solution (10 ng DNA/μl), and 1 U Taq DNA polymerase in storage buffer A (Promega Corp., Madison, WI) in a 50 μl volume. The primer sequences (5′-AGAGTTT-GATCCTGGCTCAG-3′ and 5′-GGTTACCTTGT-TACGACTT-3′) and the conditions of thermal cycling (30 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1·5 min; and finally 72 °C for 2 min) were as described by Shangkuan et al. [19]. The template DNA was total cellular DNA isolated from *V. cholerae* O1 NIH41. A Genius thermal cycler (model FGENO5TY; Techne Ltd., Oxford, Cambridge, England) was used. Labelling of the probe DNA, hybridization and detection of the probe-positive fragments were performed with the digoxigenin-labelling system as described above.

Pulsed-field gel electrophoresis (PFGE)

The test strain was grown to a mid-log phase (optical densities of 0·9–1·0 at 600 nm) in Luria-Bertani broth containing 1% NaCl. Bacterial cells were harvested

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Table 1. *Antibiograms of* V. cholerae *O1 El Tor Ogawa strains isolated in southern Thailand and the reference strains isolated in other countries*

Antibiogram pattern*	Number of the strains tested from:							
	Southern Thailand†							
	SR	NK	PT	SK	NR	India	Bangladesh	International travellers‡
I: $Tc^R Cm^R SXT^R Em^I$	0	0	0	4	1	0	0	0
I: $Tc^R Cm^R SXT^R Em^I Ap^R$	8	7	2	34	1	0	0	2
III: $Cm^R SXT^R Em^I Ap^R NA^R$	0	0	0	0	0	5	10	1
IV: Ap^R	0	0	0	0	0	0	0	4
V: None	0	0	0	0	0	0	0	4

* The antibiotics examined were tetracycline (Tc), chloramphenicol (Cm), cotrimoxazole (SXT), erythromycin (Em), ampicillin (Ap), nalidixic acid (NA), norfloxacin, gentamicin, and ciprofloxacin. Resistance or sensitivity was determined according to the definition by Bauer et al. [17]: resistant (R), intermediate (I), or sensitive. Only resistant and intermediate reactions are listed and sensitive reaction is omitted in these antibiograms. None, sensitive to all tested antibiotics.

† Abbreviations of the provinces: SR, Suratthani; NK, Nakhon si Thammarat; Pt, Phatthalung; SK, Songkla; NR, Narathiwat.

‡ International travellers arriving in Japan (Osaka Airport Quarantine station, Kansai Airport Quarantine Station, and Nagoya Airport Quarantine Station) from various countries.

by centrifugation (3000 ***g***), washed, mixed with 2% agarose, and dispensed into a plug mold as described by Albert et al. [20] except that Low Melting Point Agarose (Bethesda Research Laboratories, Gaithersburg, MD, USA) was used. The agarose plug was treated with lysozyme-RNase solution, deproteinated and digested with 30 U of *Not*I restriction enzyme as described by Albert et al. [20]. The digested DNA fragments were separated by using the contour-clamped homogeneous electric field method on a CHEF-DRIII system (Bio–Rad Laboratories, Hercules, CA, USA) in 1% agarose (Pulse Field Certified Agarose, Bio–Rad Laboratories) using 0·5 × TBE buffer. The running condition (6 V/cm at 14 °C for 22 h at a field angle of 120 °C) and switch times (15–25 for 3 h and 8–25 for 19 h) were adopted from a published procedure [2]. Following electrophoresis, the gel was stained with ethidium bromide (10 μg/ml) for 15 min, destained in distilled water for 1 h, and photographed using the u.v. transilluminator.

RESULTS

Characteristics of the 1997–8 strains isolated from southern Thailand

Fifty-seven strains of *V. cholerae* O1 isolated in clinical specimens from five provincial hospitals in southern Thailand during December 1997 and January 1998 were examined in this study. The number of patients examined and the isolation frequency of *V. cholerae* O1 (given in parentheses) were as follows: 8 (100%) from Suratthani, 7 (100%) from Nakhon si Thammarat, 2 (100%) from Phatthalung, 40 (85%) from Songkla, and 2 (50%) from Narathiwat. All the strains belonged to El Tor biotype and Ogawa serotype. The serotyping was performed using the antisera commercially available from two different sources and the results were the same. The *ctxA* and *zot* genes and the *tcpA* gene of El Tor biotype were detected in all the strains using PCR methods. The antibiograms of the strains except for susceptibility to ampicillin were identical (Table 1). All strains were resistant to Tc, chloramphenicol, cotrimoxazole, and erythromycin, and were susceptible to norfloxacin, gentamicin and ciprofloxacin. No plasmids were detected in any of the strains (data not shown).

Genetic fingerprinting patterns

Based on the antibiogram, place and frequency of isolation, seven representative southern Thailand strains were selected for genetic fingerprinting analyses (Table 2, designations A–G). The following *V. cholerae* O1 El Tor Ogawa strains were obtained as reference strains and were briefly characterized: multiply antibiotic-resistant strains isolated in India in 1997 and 1998 (randomly selected five strains) and in Bangladesh in 1998 (randomly selected 10 strains);

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Table 2. *Characteristics of the representative* V. cholerae *O1 El Tor Ogawa strains based on ribotyping, hybridization with* ctxA *and* zot *probes and AP–PCR*

Designation and name of strain	Isolation: Year	Isolation: Place*	Antibiogram†	Ribotype pattern	Hybridization pattern with the probe: *ctxA* *Pst*I‡	*ctxA* *Xba*I‡	*zot* *Pst*I‡	AP–PCR§ profile	PFGE profile
A: Vc118	1998	S. Thailand (SK)	I	1	a	a	a	*1*	*a*
B: Vc134	1998	S. Thailand (SR)	II	1	a	a	a	*1*	*a*
C: Vc138	1998	S. Thailand (NK)	II	1	a	a	a	*1*	*a*
D: Vc12	1998	S. Thailand (PT)	II	1	a	a	a	*1*	*a*
E: Vc26	1998	S. Thailand (SK)	II	1	a	a	a	*1*	*a*
F: Vc22	1998	S. Thailand (SK)	II	1	a	a	a	*1*	*a*
G: Vc38	1998	S. Thailand (SK)	II	1	a	a	a	*1*	*a*
H: KX-C22	1998	IT-Thailand	II	1	a	a	a	*1*	*a*
I: AS646	1997	India	III	2	b	b	b	*1*	*b*
J: KX-C23	1997	IT-India	III	3	b	b	b	*1*	*b*
K: 2329/98	1998	Bangladesh	III	4	b	b	b	*1*	*b*
L: AQ1061	1992	IT-Thailand	IV	5	c	c	c	*1*	*c*
M: AQ1062	1992	IT-China	V	6	–**	–	–	*2*	*d*
N: KX-C1	1994	IT-India	IV	7	b	d	b	*1*	*e*
O: KX-C3	1994	IT-India	V	1	a	a	a	*3*	*f*
P: KX-C16	1995	IT-India	V	1	a	a	a	*3*	*f*
Q: KX-C17	1996	IT-Vietnam	V	1	a	a	a	*3*	*g*
R: KX-C20	1996	IT-Thailand	IV	1	a	a	a	*3*	*f*
S: KX-C21	1997	IT-Philippines	V	1	a	a	a	*3*	*f*
T: NIH41	1941	India	V	8	d	d	d	*4*	*h*

† See Table 1 for designations.
§ Results obtained with primer 2 (explained in text).
* S. Thailand: southern Thailand; The abbreviation for the provinces (SK, SR, NK or PT) are given in the parentheses. The abbreviations are explained in the footnote of Table 1.
IT: international traveller; the origin of the travel is described after the hyphen.
‡ Restriction enzyme used to digest total cellular DNA.
** No probe-positive band was detected.

11 strains isolated between 1992 and 1998 from international travellers originating in various Asian countries. The *ctx*, *zot* and *tcp* genes were detected using the PCR methods in all strains but one (an isolate from an international traveller). The antibiograms of the strains are summarized in Table 2. The two strains showing the same antibiogram pattern (II) as that of the majority of southern Thailand strains were isolated from the international travelers originating in Thailand in December 1997 and February 1998. Twelve representative strains were selected from these reference strains for comparison by the genetic fingerprinting methods (Table 2, designations H–S). In addition, a *V. cholerae* O1 strain of the classical biotype and Ogawa serotype, NIH41, was included as another reference strain in the genetic fingerprinting analyses (Table 2, designation T).

These 20 representative strains were compared for their genetic fingerprints by ribotyping, Southern hybridization with the *ctxA* and *zot* probes, AP-PCR and PFGE (Table 2). The *Bgl*I-digested total cellular DNA of the test strain was used for ribotyping (Fig. 1, Table 2). With one or more bands being clearly different, the ribotypes were considered different and the ribotypes were arbitrarily designated as shown in Figure 1. All strains isolated in southern Thailand (Table 2, designations A–G) and the strains isolated from the international travellers originating in Thailand, India, Vietnam and Philippines between 1994 and 1998 (Table 2, designations H, and O–S) showed indistinguishable ribotype patterns.

The Southern hybridization of *Pst*I- or *Xba*I-digested total cellular DNA with *ctxA* probe and of *Pst*I-digested total cellular DNA with *zot* probe were performed to examine the restriction fragment length

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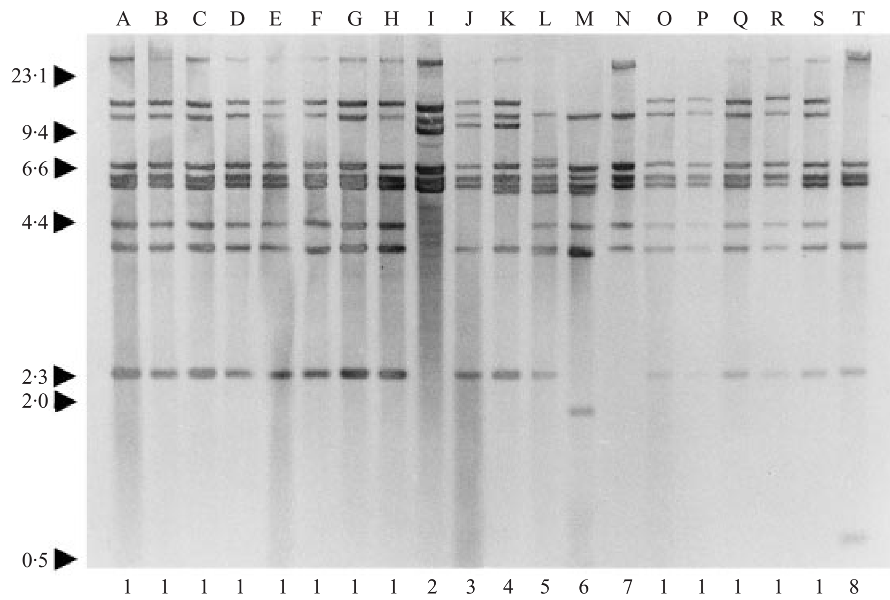

Fig. 1. Ribotype patterns of *V. cholerae* O1 strains. *Bgl*I-digested total cellular DNA was examined. The lane designation (top) corresponds to the strain designation in Table 2. Positions of the molecular weight markers (in kb), phage λ DNA digested with *Hin*dIII, are indicated on the left. Arbitrarily assigned ribotype designations are shown at the bottom.

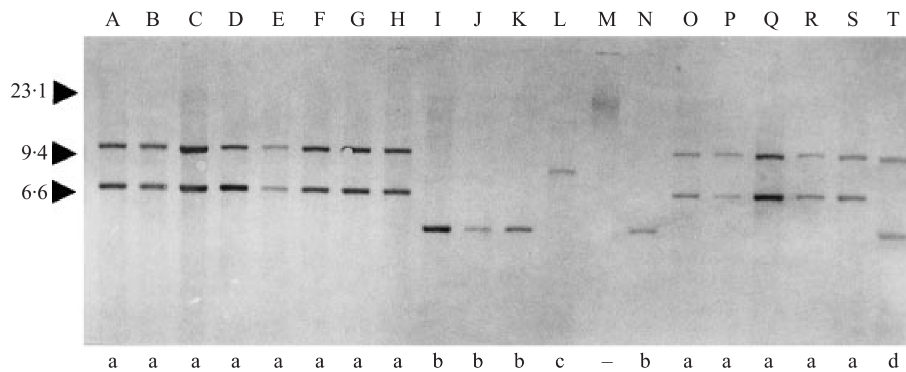

Fig. 2. Southern hybridization patterns of *V. cholerae* O1 strains. *Pst*I-digested total cellular DNA was examined with the *ctx* gene probe. The lane designation (top) corresponds to the strain designations in Table 2. Positions of the molecular weight markers (in kb), phage λ DNA digested with *Hin*dIII, are indicated on the left. Designations of the arbitrarily assigned hybridization patterns are shown at the bottom, –, no probe-positive band detected in this *ctx*- and *zot*-negative strain.

polymorphism of the probe-positive fragments. Different hybridization patterns were given arbitrarily designations in each hybridization test. An example, the *Pst*I-digested total cellular DNA hybridized with the *ctx* probe, is presented in Figure 2. The results of the three hybridizations are summarized in Table 2. Distribution of the hybridization patterns among the test strains were essentially the same in all hybridizations. The hybridization patterns of all southern Thailand strains (designations A–G) were indistinguishable. This pattern was also indistinguishable from those of five strains isolated from international travellers between 1994 and 1998 (designations H and O–S) but were different from those of the rest of the test strains (designations I–N and T).

The AP–PCR profiles of the 20 test strains were compared using a set of commercially available primers. The profiles of all 19 El Tor Ogawa strains (Table 2, designations A–S) were indistinguishable when 5 of 6 primers were used (data not shown). However, when primer 2 was used, four different AP–PCR profiles were observed (Fig. 3, Table 2.) Using this fingerprinting method, the southern Thailand strains (Table 2, designations A–G) could be distinguished from the four strains (Table 2, designations O–S) that were isolated from international

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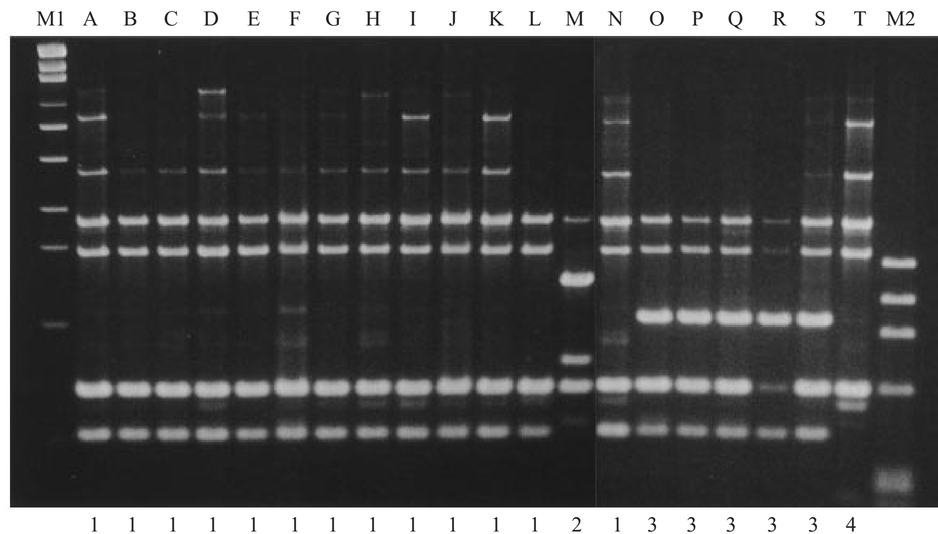

Fig. 3. AP-PCR profiles of *V. cholerae* O1 strains. Primer 2 (explained in the text) was used. The lane designation (top) corresponds to the strain designation in Table 2. Lane designations M1 and M2 indicate the molecular weight markers, phage λ DNA digested with *Eco*T14I and phage øX174 digested with *Hae*III, respectively. Profile designations were arbitrarily given based on the patterns of the major bands and are shown at the bottom.

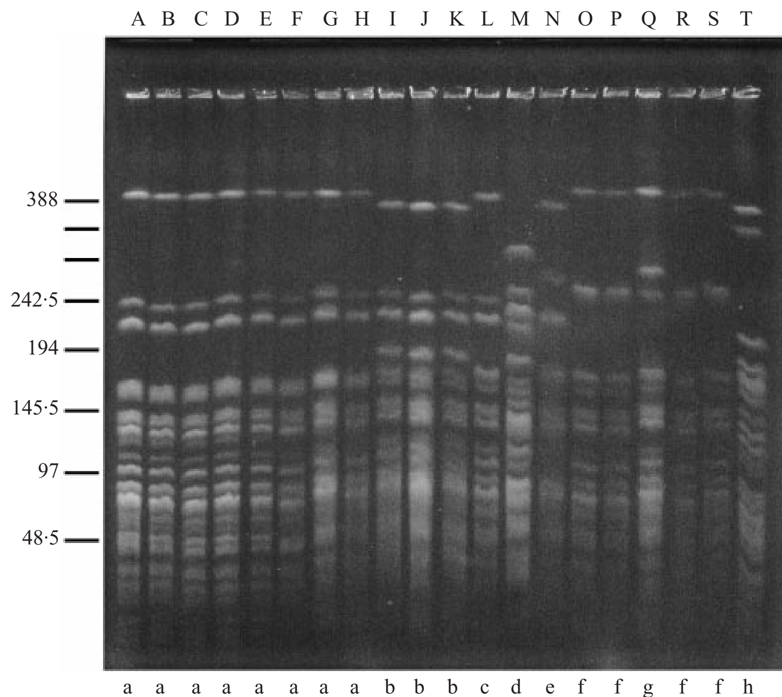

Fig. 4. PFGE profiles of *V. cholerae* O1 strains. *Not*I-digested DNA was examined. The lane designation (top) corresponds to the strain designation in Table 2. Positions of the molecular weight markers (in kb), DNA size standard λ ladder (Bio–Rad Laboratories), determined in a separate experiment are indicated on the left. Designations of the arbitrarily assigned PFGE patterns are shown at the bottom.

travellers between 1994 and 1997 and showed indistinguishable ribotype and hybridization patterns when using the southern Thailand strains. The strain isolated in 1998 from the international traveller of Thai origin (Table 2, designation H) exhibited an indistinguishable profile compared to southern Thailand origin strains using this AP–PCR analysis.

The PFGE analysis of *Not*I-digested DNA that has a high discriminating power for *V. cholerae* O1 strains [21, 22] was performed. With one or more bands being

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clearly different, the PFGE profiles were considered different and the PFGE profiles were given arbitrarily designations as shown in Figure 4. Eight PFGE profiles were detected among the 20 test strains. All strains isolated in southern Thailand (Table 2, designations A–G) and the strain isolated from the international traveller originating in Thailand (Table 2, designation H) showed indistinguishable PFGE patterns (designation *a*) and other strains exhibited different PFGE patterns (designations *b*–*h*).

DISCUSSION

The 57 strains isolated from different provinces in southern Thailand over a 4-month period had identical biochemical and antigenic characteristics. However, some strains showed a slightly different antibiogram compared to the majority of the strains. In addition, two strains isolated from international travellers exhibited identical antibiograms to those of most of the southern Thailand strains (Table 1). The result prompted us to compare the test strains using genetic methods. Plasmid analysis did not distinguish the strains since none of the test strains had a plasmid. The DNA fingerprinting analyses demonstrated the genetic identity of the southern Thailand strains and their relatedness with the strains isolated from other sources. The genetic fingerprints of the southern Thailand strains exhibiting slightly different antibiograms (antibiograms I and II, Table 1) were identical. The results suggest the minor difference in the antibiogram between the two groups is probably due to a mutation in the gene(s) associated with resistance to ampicillin and that the two groups may be derived from the same clone. Of the non-southern Thailand strains compared, the two strains isolated from the international travellers originating in Thailand showed antibiogram II. Both strains were isolated from the international travellers during the period of the epidemic in southern Thailand. The strain representing this group (Table 2, strain H) exhibited the same genetic fingerprint as that of the southern Thailand clone. The results strongly suggest that the southern Thailand clone was carried outside of Thailand by the travellers.

The *V. cholerae* O1 El Tor Ogawa strains isolated before 1997 in Thailand were sensitive to tetracycline (Division of Epidemiology, The Ministry of Public Health, Thailand). In addition, the ribotype of the southern Thailand clone differs from those of the O1 El Tor Ogawa strains previously reported from Thailand [2]. Accordingly, the Tc-resistant southern Thailand clone appears to have newly emerged in December 1997 in Thailand. The incidence of *V. cholerae* O1 infection during the 1994–8 period from the 14 provinces of southern Thailand was compiled from a report from the Division of Epidemiology and is presented in Figure 5. *V. cholerae* O1 infection occurs sporadically throughout the year in southern Thailand. High monthly incidence (> 100 isolations per month) was recorded in March and April in 1994 and 1996. However, it was unusual that high incidence was recorded in not only March but also January and February in 1998. It seems therefore that the new Tc-resistant clone emerged in December 1997 and is responsible for the large epidemic that spread to the entire region of southern Thailand over the 4-month period.

The emergence of a Tc-resistant clone and its rapid spread is important. However, it is not clear whether this clone originated in the environment of southern Thailand or was imported from outside Thailand. This clone was shown to be different from the multiply-antibiotic resistant strains isolated in India and Bangladesh, the endemic area, in 1997 and 1998. Many workers have used *Bgl*I ribotyping for epidemiological analysis of *V. cholerae* infection because of its discriminatory power. The southern Thailand clone belonged to ribotype 1 of our designation. The strains isolated from international travellers originating in various Asian countries between 1994 and 1998 also showed this ribotype (Table 2). In addition, this ribotype resembles the ribotypes B21a [23], V/B2la [24], V [19], and 6a [25] reported by other workers. The strains showing these ribotypes are distributed in various parts of the world. Therefore, an additional genetic fingerprinting method(s) was sought. Analysis of the restriction fragment length polymorphism of *ctx*- and *zot*-bearing DNA fragments, a commonly used fingerprinting method, was less discriminatory than was the ribotyping. The AP-PCR using primer 2 proved to be a useful additional genetic fingerprinting method. The combination of the *Bgl*I ribotype and the AP-PCR profile fingerprinting discriminated the southern Thailand clone and the 1998 international traveller strain of Thai origin from other test strains (Table 2). The PFGE of *Not*I-digested DNA alone had the same discriminating power (Table 2). These genetic fingerprinting methods and examination of antibiogram could be very useful for the further epidemiological investigation of the southern

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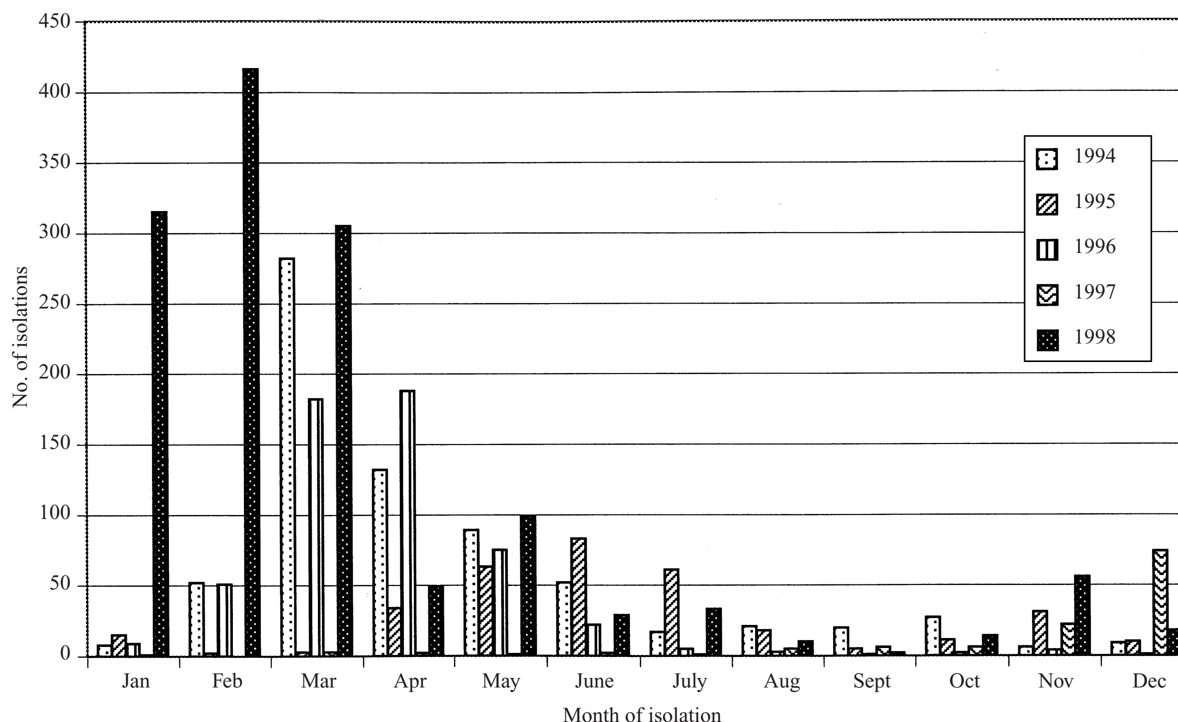

Fig. 5. Surveillance data of monthly isolated of *V. cholerae* O1 in southern Thailand between 1994–8. This figure was prepared by compiling data reported from the Division of Epidemiology, the Ministry of Public Health, Thailand.

Thailand clone. If the Tc-resistant clone had persisted in the environment of southern Thailand and caused the outbreak due to some environmental change, the possibility exists that another outbreak might occur in the same area. This possibility has to be critically studied in the future.

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REFERENCES

1. Faruque SM, Albert MJ, Mekalanos JJ. Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. Microbiol Mol Biol Rev 1998; **62**: 1301–14.
2. Dalsgaard A, Serichantalergs O, Forslund A, Pitarangsi C, Echeverria P. Phenotypic and molecular characterization of *Vibrio cholerae* O1 isolated in Samutsasakorn, Thailand before, during and after the emergence of *V. cholerae* O139. Epidemiol Infect 1998; **121**: 259–68.
3. Dalsgaard A, Forslund A, Bodhidatta L, et al. A high proportion of *Vibrio cholerae* strains isolated from children with diarrhea in Bangkok, Thailand are multiple antibiotic resistant and belong to heterogeneous non-O1, non-O139 O-serotypes. Epidemiol Infect 1999; **122**: 217–26.
4. Hoge CW, Bodhidatta L, Echeverria P, Deesuwan M, Kitporka P. Epidemiologic study of *Vibrio cholerae* O1 and O139 in Thailand: at the advancing edge of the eighth pandemic. Am J Epidemiol 1996; **143**: 263–8.
5 Bennish ML. Cholera: pathophysiology, clinical features, and treatment. In: Wachsmuth IK, Blake PA, Olsvik Ø, eds. *Vibrio cholerae* and cholerae: molecular to global perspective. Washington DC: American Society for Microbiology, 1994; 229–55.
6. Kaper JB, Morris JG Jr, Levine MM. Cholera. Clin Microbiol Rev 1995; **8**: 48–86.
7. O'Grady F, Lewis MJ, Person NJ. Global surveillance of antibiotic sensitivity of *Vibrio cholerae*. Bull W H O 1976; **54**: 181–5.
8. Siddique AK, Salam A, Islam MS, et al. Why treatment

centres failed to prevent cholera deaths among Rwandan refugees in Goma, Zaire. Lancet 1995; **345**: 359–61.
9. Islam MS, Siddique AK, Salam A, et al. Microbiological investigation of the diarrhea epidemics among Rwandan refugees in Zaire. Trans R Soc Trop Med Hyg 1995; **89**: 506.
10. Huq A, Alam M, Parveen S, Colwell RR. Occurrence of resistance to vibriostatic compound O/129 in *Vibrio cholerae* O1 isolated from clinical and environmental samples in Bangladesh. J Clin Microbiol 1992; **30**: 219–21.
11. Siddique AK, Zaman K, Marjumder Y, et al. Simultaneous outbreaks of contrasting drug resistant classic and El Tor *Vibrio cholerae* O1 in Bangladesh. Lancet 1989; **ii**: 396.
12. Kelly MT, Hickman-Brenner FW, Farmer III JJ. Vibrio. In: Balows A, Hausler Jr WJ, Herrmann KL, Isenberg, HD, Shadomy HJ, eds. Manual of clinical microbiology. Washington DC: American Society for Microbiology, 1991; 384–95.
13. Shirai UH, Nishibuchi M, Ramamurthy SK, Pal SC, Takeda Y. Polymerase chain reaction for detection of the cholera toxin operon of *Vibrio cholerae*. J Clin Microbiol 1991; **29**: 2517–21.
14. Keasler SP, Hall RH. Detecting and biotyping of *Vibrio cholerae* O1 with multiplex polymerase chain reaction. Lancet 1993; **341**: 1661.
15. Tamayo M, Koblavi S, Grimont F, Castaneda E, Grimont PAD. Molecular epidemiology of *Vibrio cholerae* O1 isolates from Colombia. J Med Microbiol 1997; **46**: 611–6.
16 Sambrook J, Fritsch EF, Maniatis T, eds. Molecular cloning: a laboratory manual. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press, 1989.
17. Bauer AW, Kirby WMM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol 1966; **45**: 493–6.
18. Nishibuchi M, Kumagai K, Kaper JB. Contribution of the *tdh*1 gene of Kanagawa phenomenon-positive *Vibrio parahaemolyticus* to production of extracellular thermostable direct hemolysis. Microb Pathog 1991; **11**: 453–60.
19. Shangkuan YH, Tsao CM, Lin HC. Comparison of *Vibrio cholerae* O1 isolates by polymerase chain reaction fingerprinting and ribotyping. J Med Microbiol 1997; **46**: 941–8.
20. Albert MJ, Bhuiyan NA, Talukder KA, et al. Phenotypic and genotypic changes in *Vibrio cholerae* O139 Bengal. J Clin Microbiol 1997; **35**: 2588–92.
21. Dalsgaard A, Skov MN, Serichantalergs O, Escheverria P, Meza R, Taylor DN. Molecular evolution of *Vibrio cholerae* O1 isolated in Lima, Peru from 1991–1995. J Clin Microbiol 1997; **35**: 1151–6.
22. Cameron DN, Khambaty FM, Wachsmuth KI, Tauxe RV, Barrett TJ. Molecular characterization of *Vibrio cholerae* O1 strains by pulsed field gel electrophoresis. J Clin Microbiol 1994; **32**: 1685–90.
23. Damian M, Koblavi S, Carle I, et al. Molecular characterization of *Vibrio cholerae* O1 strains isolated in Romania. Res Microbiol 1998; **149**: 745–55.
24. Vadivelu J, Iyer L, Kshatriya K, Puthucheary SD. Molecular evidence of clonality amongst *Vibrio cholerae* O1 biotype El Tor during an outbreak in Malaysia. Epidemiol Infect 2000; **124**: 25–30.
25. Popovic T, Bopp C, Olsvik Ø, Wachsmuth K. Epidemiologic application of a standardized ribotype scheme for *Vibrio cholerae* O1. J Clin Microbiol 1993; **31**: 2474–82.

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