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Phenotypic and molecular characterization of *Vibrio cholerae* O1 isolated in Samutsakorn, Thailand before, during and after the emergence of *V. cholerae* O139

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SUMMARY

Seventy clinical strains of *Vibrio cholerae* O1 isolated from 1982–96 in Samutsakorn, a port city 30 km southwest of Bangkok where cholera occurs at low levels with regular seasonality, were characterized to investigate if there were any differences among the O1 strains isolated before, during and after the O139 epidemic. Pulsed-field gel electrophoresis (PFGE) typing, ribotyping and southern blot hybridization with a cholera toxin probe (CT genotyping) demonstrated several genotypes among O1 strains isolated before the emergence of *V. cholerae* O139. However, O1 strains isolated during and after the advent of O139 showed identical ribotypes which were distinctly different from the types identified in strains isolated before the emergence of O139. Ribotypes identified in strains during and after the advent of O139 were also demonstrated by O1 strains isolated immediately before the emergence of O139. Considering the seasonality of cholera in Samutsakorn, the identical ribotype and CT genotype and the closely related PFGE types shown by all O1 strains isolated during and after the appearance of O139 is remarkable and suggest that the *V. cholerae* O1 strain may reemerge from an environmental source. A subgroup of *V. cholerae* O1 strains isolated before the emergence of the O139 epidemic had a ribotype identical to a type demonstrated by O139 strains isolated in Thailand. Our results support similar findings in Bangladesh and India that a distinct O1 strain appeared during the O139 epidemic. However, compared with the apparent identical strain which replaced O139 in Bangladesh and India, the emerged O1 strain in Samutsakorn showed a different ribotype and CT genotype.

INTRODUCTION

In early 1993, *Vibrio cholerae* O139 Bengal was identified for the first time in Thailand and rapidly spread within the country [1, 2]. Samutsakorn, a major port city located near Bangkok, is one of the few locations in Thailand where cholera occurs with regular seasonality, although at relatively low levels [3]. However, compared to India and Bangladesh were the O139 serotype displaced *V. cholerae* O1 causing an increase in the total number of cholera cases, the O139 serotype established itself in Samutsakorn together with *V. cholerae* O1 with no increase in the total number of cholera cases reported [3–5]. Most surprisingly, just after 10 months of its appearance in Samutsakorn in August 1993 the prevalence of *V. cholerae* O139 decreased dramatically [3, 6]. A similar decrease in the prevalence of *V. cholerae* O139 was reported in Bangladesh where the O1 serotype

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Table 1. *Antibiotic susceptibility patterns, plasmid profiles and ribotypes of 70* V. cholerae *O1 strains isolated from patients in Samutsakorn, Thailand before, during and after the emergence of* V. cholerae *0139*

Strain	Date of isolation (day, month, year)	O-serogroup	Antibiogram*	Plasmid size (kb)	Ribotype†	PFGE type	CT genotype†
Group I	Strains isolated before the epidemic of *V. cholerae* O139						
1075/25	15 Sep 1982	Inaba	Ap; Cl; Cm; Nm; Gm; Km; SXT; Tc	150	RS3	PS6	A
1076/25	15 Sep 1982	Inaba	Ap; Cl; Cm; Sm; Nm; Gm; Km; SXT; Tc	150	RS2	PS7	A
64/26	11 Oct 1982	Inaba	Ap; Cl; Sm; Nm; Gm; Km; SXT; Tc	150	RS2	PS6	E
517/30	15 Jan 1987	Inaba	Cl; Sm_I	—‡	RS3	PS8	A
520/30	15 Jan 1987	Inaba	Ap_I; Cl; Km_I; Nm_I; Sm_I	—	RS2	PS8	A
529/30	15 Jan 1987	Inaba	Cl; Sm_I	—	RS3	PS8	A
572/30	15 Jan 1987	Inaba	Ap_I; Cl; Sm_I	—	RS2	PS8	A
1083/30	27 Feb 1987	Inaba	Ap_I; Cl; Sm_I	—	RS4	PS8	—§
1452/30	12 Mar 1987	Ogawa	Ap_I; Cl; Sm_I	—	RS1	PS12	C
2460/33	5 June 1990	Ogawa	Ap_I; Cl; Sm_I	170	RS5	PS10	A
2590/33	21 June	Ogawa	Cl; Km_I; Nm_I; Sm	—	RS3	PS9	A
2722/33	13 July 1990	Ogawa	Cl; Sm	—	RS5	PS11	A
1540/34	19 Sep 1991	Ogawa	Ap_I; Cl; Sm_I	—	RS3	PS9	A
1546/34	19 Sep 1991	Ogawa	Ap_I; Cl; Sm_I	—	RS2	PS9	A
1548/34	19 Sep 1991	Ogawa	Ap_I; Cl; Sm_I	—	RS3	PS9	A
1722/34	18 Oct 1991	Ogawa	Cl; Km_I; Sm_I; Su	—	RS2	PS9	A
1724/34	18 Oct 1991	Ogawa	Ap_I; Cl; Sm_I	—	RS2	PS9	F
984/35	29 Jun 1992	Ogawa	Cl; Sm_I	—	RS2	PS9	A
1003/35	29 June 1992	Ogawa	Cl; Sm_I	—	RS2	PS9	A
1662/35	6 Nov 1992	Ogawa	Ap_I; Cl; Sm_I	—	RS2	PS9	G
1707/35	13 Nov 1992	Ogawa	Ap_I; Cl; Sm; Su	—	RS1	PS1	C
1837/35	14 Dec 1992	Ogawa	Ap_I; Cl; Sm; Su	—	RS1	PS1	C
326/36	8 Feb 1993	Ogawa	Ap_I; Cl; Sm; Su	—	RS1	PS1	C
1125/36	23 Mar 1993	Ogawa	Cl; Sm; Su	—	RS1	PS1	C
1291/36	23 Mar 1993	Ogawa	Ap_I; Cl; Sm_I	—	RS1	PS9	C
1295/36	23 Mar 1993	Ogawa	Cl; Sm_I	—	RS1	PS8	D
2175/36	22 Apr 1993	Ogawa	Cl; Sm_I	—	RS1	PS13	C
Group II	Strains isolated during the epidemic of *V. cholerae* O139						
SK10	17 Nov 1993	Ogawa	Cl; Sm; Su	—	RS1	PS1	C
SK25	22 Nov 1993	Ogawa	Cl; Sm; Su	—	RS1	PS1	C
SK36	23 Nov 1993	Ogawa	Cl; Sm; Su	—	RS1	PS1	C
SK46	26 Nov 1993	Ogawa	Ap_I; Cl; Sm; Su	—	RS1	PS1	C
SK76	6 Dec 1993	Ogawa	Cl; Sm; Su	—	RS1	PS1	C
SK151	23 Dec 1993	Ogawa	Cl; Sm; Su	—	RS1	PS1	C
SK296	19 Jan 1994	Ogawa	Cl; Sm; Su	—	RS1	PS1	C
SK360	1 Feb 1994	Ogawa	Cl; Sm; Su	—	RS1	PS1	C
SK378	3 Feb 1994	Ogawa	Ap_I; Cl; Sm; Su	—	RS1	PS1	C
SK397	7 Feb 1994	Ogawa	Ap_I; Cl; Nm_I; Sm; Su	—	RS1	PS1	C
SK404	8 Feb 1994	Ogawa	Cl; Sm; Su	—	RS1	PS1	C
SK426	11 Feb 1994	Ogawa	Ap_I; Cl; Sm; Su	—	RS1	PS1	C
SK452	14 Feb 1994	Ogawa	Ap_I; Cl; Sm; Su	—	RS1	PS1	C
SK528	24 Feb 1994	Ogawa	Cl; Sm; Su	—	RS1	PS1	C
SK554	3 Mar 1994	Ogawa	Cl; Sm; Su	—	RS1	PS1	C
SK606	11 Mar 1994	Ogawa	Cl; Sm; Su	6·2; 6·8; 12	RS1	PS2	C
SK645	16 Mar 1994	Ogawa	Ap_I; Cl; Sm; Su	—	RS1	PS1	C
SK680	22 Mar 1994	Ogawa	Cl; Sm; Su	—	RS1	PS2	C
SK726	1 Apr 1994	Ogawa	Cl; Sm; Su	—	RS1	PS1	C
SK753	8 Apr 1994	Ogawa	Cl; Sm; Su	—	RS1	PS1	C
SK781	22 Apr 1994	Ogawa	Cl; Sm; Su	—	RS1	PS1	C

Table 1. (*cont.*)

Strain	Date of isolation (day, month, year)	O-serogroup	Antibiogram*	Plasmid size (kb)	Ribotype†	PFGE type	CT genotype†
SK824	10 May 1994	Ogawa	Ap_1; Cl; Sm; Su	—	RS1	PS1	C
SK842	16 May 1994	Ogawa	Cl; Sm; Su	—	RS1	PS1	C
SK861	20 May 1994	Ogawa	Cl; Sm; Su	—	RS1	PS1	C
Group III	Strains isolated after the epidemic of *V. cholerae* O139						
697/38	2 Dec 1994	Ogawa	Cl; Sm; Su	—	RS1	PS1	C
2110/38	25 Jan 1995	Ogawa	Cl; Sm; Su	—	RS1	PS1	C
2501/38	31 Feb 1995	Ogawa	Cl; Sm; Su	—	RS1	PS1	C
2826/38	8 Mar 1995	Ogawa	Cl; Sm; Su	—	RS1	PS3	C
3649/38	5 Apr 1995	Ogawa	Cl; Sm; Su	—	RS1	PS1	C
4192/38	12 May 1995	Ogawa	Cl; Sm; Su	—	RS1	PS1	C
4490/38	9 Jun 1995	Ogawa	Ap_1; Cl; Sm; Su	—	RS1	PS1	C
4938/38	16 Jun 1995	Ogawa	Cl; Sm; Su	—	RS1	PS1	C
5466/38	17 Jul 1995	Ogawa	Ap_1; Cl; Sm; Su	—	RS1	PS2	C
5854/38	2 Aug 1995	Ogawa	Cl; Sm; Su	—	RS1	PS4	C
6227/38	6 Sep 1995	Ogawa	Ap_1; Cl; Sm; Su	—	RS1	PS1	C
6672/38	27 Sep 1995	Inaba	Cl; Sm; Su	—	RS1	PS1	C
6766/38	11 Oct 1995	Ogawa	Cl; Sm; Su	—	RS1	PS4	C
138/39	26 Oct 1995	Ogawa	Ap_1; Cl; Sm; Su	—	RS1	PS1	C
474/39	22 Oct 1995	Ogawa	Cl; Sm; Su	—	RS1	PS1	C
774/39	19 Dec 1995	Ogawa	Cl; Sm; Su	—	RS1	PS2	C
1570/39	19 Jan 1996	Ogawa	Cl; Sm; Su	—	RS1	PS1	C
2905/39	26 Mar 1996	Ogawa	Cl; Sm; Su	—	RS1	PS5	C
3223/39	9 May 1996	Ogawa	Ap_1; Cl; Sm; Su	—	RS1	PS5	C

* Intermediate resistance; Ap, ampicillin; Cl, colistin; Cm, chloramphenicol; Nm, neomycin; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Su, sulphisoxazole; SXT, trimethoprim/sulphamethoxazole; Tc, tetracycline.
† Ribotype and CT genotype using the restriction enzyme *Bgl*I.
‡ Contained no plasmids.
§ Contained no CT genes.

resumed the role as the dominant cholera strain in early 1994 [7, 8]. The last case of *V. cholerae* O139 associated with the epidemic in Samutsakorn was reported in December 1995.

The factors responsible for the apparent disappearance of *V. cholerae* O139 in Samutsakorn as elsewhere in Thailand and the different pattern of emergence of O139 in Thailand compared with the Indian subcontinent are not well understood [7, 9]. Following the emergence of *V. cholerae* O139, studies in Bangladesh indicated that the O139 serotype survived better than O1 strains in the aquatic environment [10, 11]. It was therefore proposed that the dramatic decrease in the prevalence of *V. cholerae* O139 was associated with changes, for example in colonization factors that determine long-term persistence in the aquatic environment, making the organism less suited for survival in the environment [7]. Alternatively, subsequent to the emergence and peak prevalence of *V. cholerae* O139, *V. cholerae* O1 could have undergone changes that would have enabled it to out compete the O139 serotype and become the predominant strain again [7].

Within this epidemic setting, the objective of the present investigation was to study whether the emergence and rapid decline in the prevalence of *V. cholerae* O139 in Samutsakorn were associated with phenotypic and genotypic changes in O1 strains isolated before, during and after the O139 epidemic.

MATERIALS AND METHODS

Sources of strains and identification procedures

A total of 70 *V. cholerae* O1 strains isolated from 1982–96 were included in the study (Table 1). All strains were recovered from stool specimens and rectal swabs from patients with diarrhoea at the provincial hospital in Samutsakorn [3]. Strains isolated before the emergence of *V. cholerae* O139 in

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August 1993 were collected and identified by the National Institute of Health and by the Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok. The identification of the remaining O1 strains were carried out at AFRIMS or at the hospital laboratory in Samutsakorn using standard procedures, including the culture of faecal specimens on thiosulphate-citrate-bile salts-sucrose agar (Eiken Ltd., Tokyo, Japan) before and after inoculation in alkaline peptone water (pH 8·6). All strains were identified as *V. cholerae* O1 using criteria described by Sakazaki [12] and were tested for agglutination in polyvalent O1, mono-specific Ogawa and Inaba antisera (Denka Seiken, Japan) and O139 antiserum (AFRIMS, Bangkok). In addition to the O1 strains listed in Table 1, *V. cholerae* O139 strain NG 408/36 isolated from a cholera patient in Thailand was included in the ribotyping and pulsed-field gel electrophoresis (PFGE) studies for purposes of comparison [13]. Furthermore, *V. cholerae* O1 strains 1407 and 9868 recovered from patients in Guinea-Bissau in 1987 and 1996 [14], respectively, and strain CHO 467 isolated from a patient in Peru in 1991 [15] were included in restriction fragment length polymorphisms (RFLP) analysis of CT genes for comparison analysis.

The *V. cholerae* O1 strains and positive and negative control strains were examined by the colony hybridization technique for DNA sequences encoding CT with an alkaline phosphatase-labelled oligonucleotide probe as previously described [16, 17].

The O1 strains listed in Table 1 were selected randomly and classified into 3 groups, based on the time of isolation in relation to the emergence of *V. cholerae* O139 in Samutsakorn. Group I comprised 27 strains isolated before the emergence of O139 (September 1982 to April 1993), Group II contained 24 strains isolated during the O139 epidemic (November 1993 to May 1994) while group III included 19 strains isolated after the O139 epidemic (December 1994 to May 1996).

Antibiotic susceptibility testing

Antibiotic susceptibility testing was carried out by disk diffusion on Mueller–Hinton II agar as recommended by the National Committee for Clinical Laboratory Standards [18] with disks (Sensi-Disc BBL, Becton Diskinson, Cockeysville, Md) containing ampicillin (10 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), colistin (10 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), neomycin (30 μg), streptomycin (10 μg), sulfisoxazole (250 μg), tetracycline (30 μg), and trimethoprim/sulfamethoxazole (1·25/23·75 μg). In addition, the O1 strains were also tested for their susceptibilities to the vibriostatic agent O/129 (150 μg) (Neo-Sensitabs, Rosco Diagnostica, Taastrup, Denmark). Isolates were recorded as susceptible, intermediate, or resistant.

Isolation of plasmid DNA

Plasmid preparation was carried out by the method of Kado and Liu [19], modified by incubating the cells at elevated pH (pH 12·75) for 30 min at 56 °C during the lysis step. Following electrophoresis, the plasmids were visualized as described previously [20]. *V. cholerae* O1 1075/25 containing an approximately 150 kb plasmid was used as the control strain [21]. Plasmid sizes were estimated from the migration distance in the agarose gels relative to the migration distance of reference plasmids in *Escherichia coli* strains V517 and 39R861 [22, 23] by the method of Rochelle and colleagues [24]. Repeated extraction of plasmid DNA was carried out for all isolates.

Ribotyping

Total bacterial DNA was extracted from each isolate tested by the method of Pedersen and Larsen [25] followed by restriction with the *Bgl*I enzyme [26]. Ribotyping was performed with digoxigenin-labelled 16S and 23S rRNA probes as previously described [14, 27]. A 1 kb DNA molecular size standard (GIBCO BRL, Gaithersburg, Md.) was used as a size marker. Ribotype patterns were considered to be different when there was a difference of 1 or more bands between isolates. Each ribotype was designated RS followed by an arbitrary number.

PFGE

Each of the *V. cholerae* O1 strains listed in Table 1 were subjected to PFGE as described previously [15, 28]. Briefly, DNA was prepared directly in a solid agarose plug for restriction endonuclease digestion using the enzyme *Not*I (5′-GCGGCCGC-3′) (Amersham, Arlington Heights, Ill.). PFGE types were considered to be different if they differed by 1 or more bands and were designated PS followed by an arbitrary number. PFGE was carried out by using a CHEF-DR III system (Bio-Rad, Richmond, Calif.).

The running conditions were 6 V/cm at 14 °C for 22 h at a field angle of 120°. The electrophoresis was carried out at switch times of 15–25 sec for 3 h and 8–25 sec for 19 h. Multimeric phage lambda (48·5 kb) DNA (Pharmacia LKB, Uppsala Sweden) was used as molecular mass standard. Following electrophoresis, the gels were stained for 15 min in ethidium bromide (2 μg/ml in water; Sigma), destained in distilled water for 15 min, and visualized on a UV light box.

CT genotyping

RFLP of CT genes was performed by hybridization of nylon membranes with *Bgl*I-digested DNA prepared as described for ribotyping with a digoxigenin-labelled CT probe [16, 17]. The restriction enzyme *Bgl*I was selected because it does not have any recognition sequence within the *ctx*A gene, but it has a single cleavage site located upstream and adjacent to the *ctx*A gene [29]. Accordingly, the number of bands comprising each CT genotype pattern represent the possible number of copies of the CTX genetic element harboured by each strain. For purpose of comparison, an alphabet was designated to each CT genotype RFLP pattern.

RESULTS

Bacterial specimens

The emergence of *V. cholerae* O139 in Samutsakorn in August 1993 and the following disappearance of this serotype 10 months later, prompted us to initiate a study to determine if the rapid decline of *V. cholerae* O139 was associated with changes in *V. cholerae* O1 strains isolated before, during and after the O139 epidemic. Each of the 70 strains included in the present study showed biochemical and serological reactions typical of those of *V. cholerae* O1. With the exception of strain 6672/38, which belonged to the serotype Inaba, all O1 strains isolated during and after the O139 epidemic belonged to the El Tor biotype and the Ogawa serotype (Table 1). However, 8 O1 strains isolated from 1982 to 1987 before the O139 epidemic belonged to the Inaba serotype. *V. cholerae* O1 strain 1083/30 was the only strain that did not hybridize with the CT probe.

Antibiotic susceptibility patterns and plasmid analysis

Antibiotic susceptibility testing revealed only limited variations between the three groups of O1 strains with the majority of strains showing resistance to colistin and streptomycin and all strains were susceptible to tetracycline (Table 1). *V. cholerae* O1 strains isolated during and after the O139 epidemic were resistant to sulfisoxazole whereas most strains isolated before the epidemic were susceptible to sulfisoxazole. None of the O1 strains isolated after 1982 were multiple resistant nor did they contain a 150 kb conjugative plasmid which was shown by multiple antibiotic resistant strains (MARV) isolated from an outbreak of cholera among 31 children in 1982 [21] (Table 1). Several strains showed intermediate resistance to ampicillin and streptomycin. Apart from the O1 strains isolated in 1982, only two strains contained plasmids.

In addition to the results of the antibiotic susceptibility testing shown in Table 1, 232 additional *V. cholerae* O1 strains isolated after the O139 epidemic between November 1993 and July 1996 were tested with all strains showing resistance to colistin, streptomycin, and sulfisoxazole and about 50% of the strains showing intermediate resistance to ampicillin.

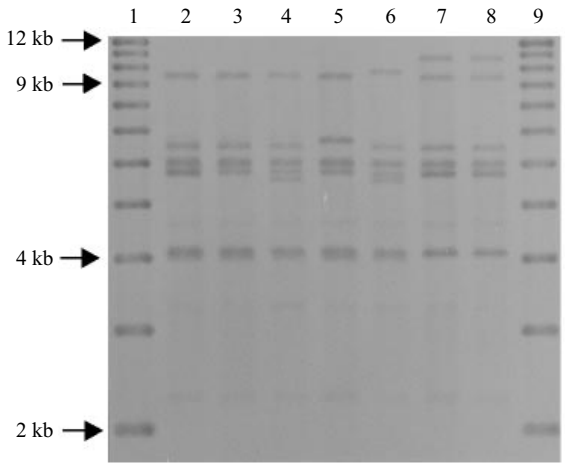

Fig. 1. Examples of *Bgl*I ribotypes of *V. cholerae* O1 recovered from patients with diarrhoea in Samutsakorn, Thailand. Unless indicated otherwise, the following explanations for the contents of the lanes indicate ribotype and strain designation. Lanes: 1, 1 kb molecular mass standard; 2, ribotype RS3, *V. cholerae* O139 strain NG 408/36; 3, type RS3, 1075/25; 4, type RS2, 1076/25; 5, type RS4, 1083/30; 6, type RS5, 2722/33; 7, type RS1, 1452/30; 8, type RS1, 697/38; 9, 1 kb molecular mass standard.

Ribotyping

Ribotyping with the enzyme *Bgl*I produced five different restriction patterns among O1 strains isolated

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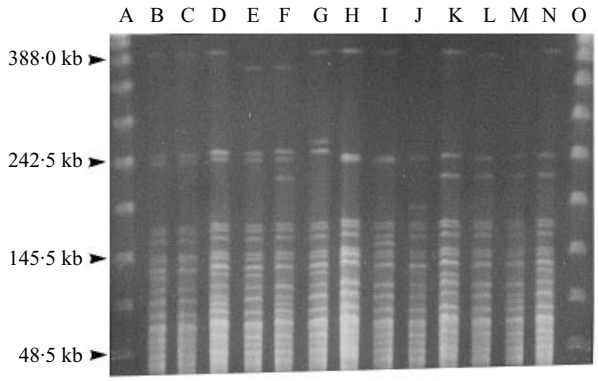

Fig. 2. Thirteen different PFGE banding patterns of *Not*I-digested total cellular DNAs from 70 *V. cholerae* O1 strains isolated in Samutsakorn. Unless indicated otherwise, the following explanations for the contents of the lanes indicate PFGE type and strain designation. Lanes: A, multimers of phage lambda DNA (48·5 kb) as molecular size markers; B, PFGE type PS1, 1707/35; C, type PS2, SK606; D, type PS3, 2826/38; E, type PS5, 2905/39; F, type PS10, 2460/33; G, type PS4, 5854/38; H, type PS6, 64/26; I, type PS7, 1076/25; J, type PS12, 1452/30; K, type PS8, 517/30; L, type PS9, 1546/34; M, type PS11, 2722/33; N, type PS13, 2175/36; O, multimers of phage lambda DNA (48·5 kb) as molecular size markers.

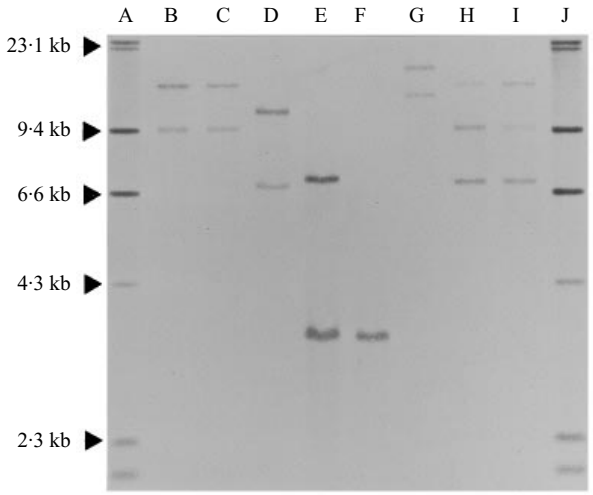

Fig. 3. Examples of Southern hybridization analysis of genomic DNA from *V. cholerae* O1 strains digested with *Bgl*I and probed with a CT probe. Unless indicated otherwise, the following explanations for the contents of the lanes indicate CT genotype and strain designation. Lanes: A, *Hin*dIII digest of phage lambda as molecular size markers; B, CT genotype A, 1407, Guinea-Bissau; C, type A, 2460/33; D, type B, 9868, Guinea-Bissau; E, type C, SK404; F, type D, 1295/36; G, type E, 64/26; H, type F, 1724/34; I, type F, CHO 467; J, *Hin*dIII digest of phage lambda as molecular size markers.

before the emergence of *V. cholerae* O139, while O1 strains isolated during and after the O139 epidemic showed an identical ribotype RS1 (Fig. 1, Table 1). Interestingly, each of seven strains isolated within a 9 months period just before the outbreak of the O139 epidemic showed ribotype RS1 as did strain 1452/30 isolated in 1987 (Table 1). The 3 MARV strains isolated in 1982 showed 2 closely related ribotypes RS2 and RS3 which differed by a single fragment. However, ribotype RS2 and RS3 were also shown by O1 strains that did not contain plasmids and which showed only limited antibiotic resistance. Furthermore, ribotype RS3 was also shown by *V. cholerae* O139 strain NG 408/36 (Fig. 1, Table 1). The identical ribotypes demonstrated by O1 strains showing different antibiotic susceptibility patterns may be explained by a loss of the 150 kb resistance plasmid by the MARV strains. Strain 1083/30, which did not hybridize with the CT probe, showed a unique ribotype RS4. Strain 2460/33, which contained an approximately 170 kb plasmid and was resistant to colistin only, showed an unique ribotype RS5.

PFGE

Based on previous PFGE studies of *V. cholerae* O1 in which *Not*I produced a high discrimination among strains and a suitable distribution of fragments [15, 30], *Not*I was used in the present study. The analysis of the 70 *V. cholerae* O1 strains revealed 13 different but closely related banding patterns (Fig. 2, Table 1). *V. cholerae* O139 strain NG 408/36 showed a unique PFGE type which differed by 6 fragments from the 13 different O1 patterns (results not shown). Group I strains isolated before the O139 epidemic showed PFGE types which differed from types shown by O1 strains isolated during and after the epidemic. Among the 27 strains in Group I, 9 PFGE typing patterns were observed (Fig. 2, Table 1). Four strains isolated from November 1992 to March 1993 before the emergence of *V. cholerae* O139 in August 1993 showed type PS1. Type PS1 contained two fragments of 245 kb and 250 kb sizes which were not observed among the remaining Group I isolates. Furthermore, the four strains were the only Group I strains showing resistance to sulfisoxazole; a trait which was shown by all strains isolated during and after the O139 epidemic.

Among the 24 *V. cholerae* O1 strains isolated during the O139 epidemic, only 2 PFGE types were observed including 22 (92%) strains which showed types PS1 and two PS2 strains (Fig. 2, Table 1). Type

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PS1 contained a 135 kb size fragment which was not seen among PS2 strains.

Five PFGE types (PS1 to PS5) were shown among 19 strains isolated after the disappearance of the O139 serotype (Fig. 2, Table 1). Seventeen (89%) of these strains showed PS types containing the two fragments of 245 kb and 250 kb sizes. The remaining two strains in Group III showed type PS4 which differed from type PS1 by showing a 260 kb size fragment and lacking the 245 kb size fragment.

CT genotypes

Southern blot hybridization of *Bgl*I-digested genomic DNA with the CT probe revealed six CT genotyping patterns among *V. cholerae* O1 strains isolated in Samutsakorn before the O139 epidemic (Fig. 3, Table 1). The majority of these strains and strain 1407 isolated in Guinea-Bissau showed an identical CT genotype A, which demonstrated two 10·0 and 12·8 kb size fragments (Fig. 3). Unique CT genotyping patterns were shown by strains 9868 from Guinea-Bissau, 1295/36, 64/26, 1724/34, and 1662/35 (Fig. 3, Table 1). Except strains 1295/36 and 1724/34, which showed CT genotypes demonstrating a single 3·5 kg fragment and three fragments (12·8, 10·0 and 6·8 kb), respectively, each strain from Samutsakorn showed CT genotypes demonstrating two fragments. All strains isolated during and after the advent of O139 showed ribotype RS1 and an identical CT genotype C demonstrating two fragments of 7·0 and 3·5 kb. Strain 1083/30 did not show a CT genotyping pattern (Table 1).

DISCUSSION

The results of the present study show that the emergence and rapid disappearance of the O139 serotype in Samutsakorn was associated with genotypic changes in *V. cholerae* O1 strains isolated before, during and after the O139 epidemic. Using PFGE typing, ribotyping and CT genotyping we demonstrated several genotypes among O1 strains isolated before the emergence of *V. cholerae* O139 whereas O1 strains isolated during and after the advent of O139 showed identical ribotypes and CT genotypes and closely related PFGE types which were distinctly different from the types demonstrated by strains isolated before the emergence of O139. Thus, an excellent correlation was found between the ribotypes and CT genotypes. However, interestingly the ribotype and CT genotype shown by groups II and III strains were also demonstrated by a single strain isolated in 1987 and by most strains isolated within a 10 month period immediately before the emergence of O139. Thus, the distinct O1 strain which was associated with cholera during and after the O139 epidemic was already being recovered from cholera patients before the advent of O139.

Considering the seasonality of cholera in Samutsakorn, the identical ribotype and CT genotype and the closely related PFGE types shown by all O1 strains isolated after November 1992 is remarkable and suggest that the *V. cholerae* O1 strain reemerges from an environmental source most likely the coastal areas of Samutsakorn. The fact, that the distinct strain was recovered from cholera patients in Samutsakorn before the emergence of O139 could indicate that the strain was present in the coastal environment and that environmental changes have caused the strain to multiply rapidly and to become dominant over existing strains of *V. cholerae* O1 and O139 [8]. However, no information is available about such environmental changes and no studies of *V. cholerae* in the coastal areas of Samutsakorn were conducted before the emergence of O139. A previous study using traditional culture methods of 500 fresh seafood samples collected at markets in Samutsakorn in February and March 1994, which is the peak season for cholera, revealed no *V. cholerae* O1 and O139 [3, 6].

About 70% of the previously described strains of *V. cholerae* O1 El Tor have been reported to carry a single CTX genetic element, with the remaining strains carrying two or more copies arranged in tandem. In comparison, strains of the Classical biotype harbour two copies of the CTX genetic element located in different regions of the chromosome [29, 31]. Thus our findings, that except for two strains, all strains studied carried two copies of the CTX genetic element confirm the results obtained by ribotyping and PFGE typing suggesting that genetic rearrangements are occurring continuously among *V. cholerae* O1 El Tor [32]. Using ribotyping and PFGE typing we have previously demonstrated frequent genetic changes in the epidemic strain responsible for the Latin American cholera outbreak [15].

Comparison of ribotypes in the current study with *Bgl*I ribotypes presented by *V. cholerae* O139 isolated in Thailand, India and Bangladesh [13, 33] revealed that type RS3 which was shown by 6 O1 strains isolated before the advent of O139 is identical to

ribotype 3 which was found to be unique among *V. cholerae* O139 strains isolated in Thailand in 1993 (Fig. 1) [13, 33]. This is to our knowledge the first time identical *Bgl*I ribotypes have been reported for *V. cholerae* O1 and O139. However, the use of PFGE typing of a representative O139 strain revealed a PFGE type very different from the types shown by the O1 strains. These findings underscore the importance of using more than 1 typing method to study the epidemiology of *V. cholerae*.

The finding of 13 different PFGE types compared with 5 different ribotype confirms the higher discriminatory power of PFGE in typing *V. cholerae*. However, genotypic changes among *V. cholerae* O1 isolated before, during and after the O139 epidemic as shown by PFGE was equally shown by ribotyping indicating that there was only limited advantage using both typing methods in this particular study.

None of the O1 strains isolated after 1982 were multiple resistant nor did they harbour the 150 kb conjugative plasmid contained in MARV strains isolated from the cholera outbreak in 1982 [21]. Thus it appears, that the MARV strains did not become established in the Samutsakorn area. The disappearance of the MARV strains is consistent with previous laboratory observations where R-plasmids in strains of *V. cholerae* O1 were found to be unstable and easily eliminated in drug-free conditions [34].

The limited variation in the antibiotic resistance patterns among O1 strains from Samutsakorn is different from the results of a study of changes in phenotypic traits among *V. cholerae* O1 isolated before, during and after the O139 epidemic in Calcutta [9]. *V. cholerae* O1 strains isolated after the O139 epidemic in Calcutta showed an expanding resistance type with resistance to a variety of drugs including co-trimoxazole, nalidixic acid and chloramphenicol as compared to O1 strains isolated before and during the O139 epidemic [9]. It was not reported if the expanding resistance type was associated with the presence of resistance plasmids [9]. The reason(s) for the difference in resistance patterns among O1 strains isolated in Samutsakorn and Calcutta are unclear.

PFGE and ribotyping of *V. cholerae* O1 isolated in Bangladesh and India have demonstrated that the O1 strain which reappeared after being temporarily displaced by *V. cholerae* O139 represent an apparent new clone [8, 32, 35, 36]. Surprisingly, this new clone appears to be identical to the ribotype of the *V. cholerae* O1 strain involved in a cholera outbreak in Guinea-Bissau in 1994 and 1995 [8, 14]. The apparent association of a distinct strain of *V. cholerae* O1 demonstrating a unique ribotype with cholera outbreaks in India, Bangladesh and Guinea-Bissau suggest a large epidemic potential. This new ribotype was not included in the standardized ribotyping scheme proposed by Popovic and colleagues [26] and further studies of these strains are needed to confirm their possibly clonal nature.

Comparison of the ribotypes in the present study with the ribotypes reported from Bangladesh and India showed that the ribotype demonstrated by all O1 strains isolated during and after the O139 outbreak in Samutsakorn appears to lack a 8·5 kb size fragment which was demonstrated by the distinct O1 strain isolated in Bangladesh and India [32, 36]. In fact, the ribotype shown by strains in groups II and III in Samutsakorn appears identical with the ribotype RI demonstrated by O1 strains isolated in India before the advent of O139 [32].

Although the results of the present study support the findings of the studies in Bangladesh and India study that a distinct O1 strain emerged during the O139 outbreak and became established as the cause of cholera, the strain which appeared in Samutsakorn showed a distinct and different ribotype and CT genotype compared with the suggested new clone which replaced the O139 serotype in Bangladesh and India [8, 32].

Since 1996, recent surveillance data on *V. cholerae* from Bangladesh and India revealed a resurgence of *V. cholerae* O139 [7, 9, 36, 37]. In Bangladesh, the current O139 strain appears to have undergone several phenotypic and genotypic changes compared with the original O139 strain [38]. However, at present we have not seen a similar resurgence of O139 in Thailand.

Further studies should determine if the genetic changes of *V. cholerae* O1 and O139 reported in the present study as well as in other recent studies are associated with changes in the structural configuration of the bacteria since such changes may be of importance for an increased survival of these strains in the aquatic environment, and because pre-existing immunity against *V. cholerae* O1 and O139 may only provide a reduced protection against these distinct O1 and O139 strains [7, 15, 35, 36, 38].

The reappearance of distinct *V. cholerae* O1 and O139 strains in South-east Asia as the causes of cholera shows the importance of carefully monitoring O1, O139 and possibly other new emerging O-serogroup infections to keep abreast of the changing traits of the etiologic agent of cholera [9].

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