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A high proportion of *Vibrio cholerae* strains isolated from children with diarrhoea in Bangkok, Thailand are multiple antibiotic resistant and belong to heterogenous non-O1, non-O139 O-serotypes

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SUMMARY

Results of a surveillance on cholera conducted with patients seen at the Children Hospital in Bangkok, Thailand from August 1993 to July 1995 are presented. Annually, isolation rates for *Vibrio cholerae* varied between 1·7 and 4·4% of patients with diarrhoea. *V. cholerae* O1 serotype Ogawa accounted for between 31 and 47% of patients cultured positive for *V. cholerae*, whereas the O139 serotype dominated in early 1994 after which it disappeared. Non-O1, non-O139 strains were isolated at similar rates as serotype O1 in 1993 and 1994, but accounted for 69% of *V. cholerae* culture positive specimens in 1995. However, the annual proportion of the isolation of non-O1, non-O139 strains showed little variation and remained low between 1·0 and 1·3%. Serotyping of 69 epidemiological unrelated non-O1, non-O139 strains produced 37 different O-serotypes. *Bgl*I ribotyping of serotypes containing more than two strains demonstrated a high degree of heterogeneity within and between serotypes, except seven serotype O37 strains which showed an identical ribotype suggesting clonality. None of the 69 strains hybridized with a cholera toxin probe and only two strains hybridized with a heat-stable enterotoxin probe. Susceptibility testing to 12 antibiotics showed that 40 of 69 (58%) non-O1, non-O139 strains were resistant to colistin, streptomycin and sulphisoxazole and 28 of 69 (41%) were multiple antibiotic resistant (MAR; $\geqslant 4$ antibiotics). Although 26 of 69 (38%) strains contained one or more plasmids, the plasmids were of low molecular weights and did not seem to encode antibiotic resistance. The results of the present study showed that a high proportion of heterogenous MAR *V. cholerae* non-O1, non-O139 strains were isolated from children at the hospital. With reference to the emergence of *V. cholerae* O139 in 1992, we suggest that non-O1, non-O139 strains should be monitored carefully to detect new serotypes with a possible epidemic potential, but also to determine the development and mechanism of antibiotic resistance.

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INTRODUCTION

Vibrio cholerae strains of serotypes other than O1 have drawn little scientific attention in the past as possible causes of acute secretory diarrhoea. However, the emergence of the O139 serotype in India in 1992, and the subsequent spread throughout India and Bangladesh [1, 2] and into Thailand in early 1993 [3, 4], sparked interest into the importance of non-O1 serotypes. Although *V. cholerae* O1 and O139 remain the only causes of cholera, several recent reports suggest that non-O1, non-O139 strains are increasingly being associated with diarrhoea, including cholera-like diarrhoea [5–9]. Improved surveillance efforts have been established in India where an approach was taken to monitor *V. cholerae* as a whole with emphasis on no particular serotypes [10]. As a direct response to the emergence of O139 in Thailand in early 1993, a similar strategy to monitor *V. cholerae* was adopted at three different hospitals in Thailand [11].

While non-O1, non-O139 strains are increasingly being associated with diarrhoea and several virulence factors have been studied, the mechanism of virulence is poorly understood. *V. cholerae* non-O1, non-O139 strains rarely harbour cholera-toxin (CT) genes and other virulence genes contained in the so-called virulence cassette or CTX genetic element found in O1 and O139 strains [12, 13]. Following findings by Waldor and Mekalanos [14], it is now generally accepted that the CTX element corresponds to the genome of a filamentous phage, designated CTXθ. The horizontal transmission of the CTXθ phage was demonstrated and depended on the presence of a toxin co-regulated pilus (TCP) colonization factor which also seemed to act as a receptor for the CTXθ phage [14]. Thus, transmission of the CTXθ phage is likely to have a direct bearing on the evolution of toxigenic *V. cholerae* non-O1, non-O139 strains. The production of heat-stable enterotoxin (NAG-ST) and intestinal colonization were reported as important factors among *V. cholerae* non-O1, non-O139 strains causing diarrhoea [15]. However, often NAG-ST and CT negative strains were isolated from outbreaks of diarrhoea associated with non-O1, non-O139 strains [6, 9]. It is possible that the NAG-ST gene may be associated with self-transmissible genetic elements.

Differences and major changes in patterns of antibiotic susceptibility have recently been reported between strains of *V. cholerae* of different serotypes and within strains of a serotype [7, 9, 10, 16–18]. O1 strains have generally been reported as more susceptible than O139 strains, of which the majority show resistance to several antibiotics, including trimethroprim [16, 18]. Plasmids and plasmid encoded resistance are rarely found among *V. cholerae* O1 and O139 strains [19, 20], whereas non-O1, non-O139 strains more often contain low-molecular-weight plasmids of unknown importance [7]. High prevalences of multiple antibiotic resistant non-O1, non-O139 strains isolated from patients with diarrhoea are increasingly being reported [7, 9, 10]. The emergence of such strains resistant to antibiotics commonly used to treat diarrhoea in both adults and children is disturbing.

The present study was performed too determine temporal changes in traits of *V. cholerae* isolated from patients at the Children Hospital in Bangkok from August 1993 to July 1995. In particular, the serotypes and ribotypes of non-O1, non-O139 strains were determined as were antibiotic susceptibility patterns and plasmid content.

MATERIALS AND METHODS

Sources of strains, bacteriology and serology

The Children Hospital, which is situated very close to the Armed Forces Research Institute of Medical Sciences (AFRIMS) in central Bangkok, Thailand, was in 1993 included in a survey programme for *V. cholerae* [11]. From August 1993 to July 1995, all patients seen at the Hospital Out Patient Department (OPD) with watery diarrhoea and loose stools were cultured for *V. cholerae* [11]. Stool or rectal swabs were collected at the hospital and cultured at AFRIMS on thiosulphate-citrate-bile salts-sucrose agar (Eiken Ltd, Tokyo, Japan) before and after pre-enrichment in alkaline peptone water (pH 8·6). Strains were identified as *V. cholerae* using criteria described by Sakazaki [21] and were tested for agglutination in polyvalent O1, mono-specific Ogawa and Inaba antisera (Denka Seiken, Japan) and O139 antiserum (AFRIMS, Bangkok). Faecal specimens were not cultured for other enteric pathogens than *V. cholerae*.

A total of 69 *V. cholerae* strains isolated from 67 patients, which did not agglutinate O1 and O139 antisera, were selected randomly for further analysis. Each strain was examined serologically by the slide agglutination test and designated according to an extended serotyping system (O1 to O140) established by Shimada and colleagues [22]. However, the system is continuously developed and currently includes

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antisera for 193 different O-serotypes (Dr T. Shimada, personal communication). Preparation of O antisera and slide agglutination were performed as previously described [22].

Strains were examined by the colony hybridization technique for DNA sequences encoding NAG-ST and CT using alkaline phosphatase-labelled oligonucleotide probes consisting of 16 and 23 bp, respectively [23–25]. Pre-hybridization and hybridization were performed as reported and the hybridization filters developed colourimetrically [26]. NAG-ST producing *V. cholerae* O14 strain A5 [27] and CT producing *V. cholerae* O1 strain 889 [28] were used as positive controls on all filters.

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 [29] with disks (Sensi-Disc BBL, Becton Dickinson, 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), sulphisoxazole (250 μg), tetracycline (30 μg), and trimethoprim/sulphamethoxazole (1·25/23·75 μg). Isolates were recorded as susceptible or resistant.

Isolation of plasmid DNA and ribotyping

Plasmid preparation was carried out by the method of Kado and Liu [30], 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 [31]. *V. cholerae* O1 1075/25 containing an approximately 150 kb plasmid was used as the control strain [19]. 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 [32, 33] by the method of Rochelle and colleagues [34]. Repeated extraction of plasmid DNA was carried out for all isolates.

Strains belonging to O-serotypes which contained more than two strains were ribotyped to determine a possible clonality within O-serotypes. A total of 28 strains were ribotyped, including 3 serotype O2 strains, 8 serotype O6 strains, 3 serotype O10 strains, 7 serotype O37 strains, 3 serotype O97 strains, and 4 serotype O151 strains. Total bacterial DNA was extracted from each isolate tested by the method of Pedersen and Larsen [35] followed by restriction with the *Bgl*I enzyme [36]. Ribotyping was performed with digoxigenin-labelled 16S and 23S rRNA probes as previously described [37, 38]. A 1 kb DNA molecular size standard (Gibco–BRL, Gaithersburg, MD) and the DNA molecular-weight marker II, λ-DNA *Hin*dIII (Boehringer–Mannheim, Germany) were used as size markers. Ribotype patterns were considered to be different when there was a difference of one or more bands between isolates.

RESULTS

The isolation rate of *V. cholerae* O1, O139 and non-O1, non-O139 strains isolated from patients with watery diarrhoea are shown in Figure 1 and Table 1. Annually, between 1·7 and 4·4% of the samples analysed were found positive for *V. cholerae*. In 1993, 25 of 49 samples positive for *V. cholerae* were of the O1 serotype and 3 specimens only were found positive for O139. The Ogawa serotype was predominant with a total of only 2 Inaba strains being isolated in 1993 (Table 1). Following the introduction of *V. cholerae* O139 into Thailand in early 1993, the first O139 strains were isolated at the Children Hospital in November 1993 (Fig. 1). A pronounced increase in the incidence of O139 was registered in the first 3 months of 1994. However, after the isolation of O139 strains from two patients in April 1994, O139 was subsequently not isolated in the remaining study period.

The annual proportion of the isolates of *V. cholerae* non-O1, non-O139 strains from patients with diarrhoea showed little variation and remained low between 1·0 and 1·3%. Between 29 and 69% of samples cultured positive for *V. cholerae* yielded strains belonging to non-O1, non-O139 O-serotypes with annual variations being associated with variations in the number of O1 and O139 cases. Although a high monthly variation in the incidence of non-O1, non-O139 strains was found throughout the study period, these serotypes were generally, and in particular in 1995, more often isolated from patients than O1 strains (Fig. 1).

Of the 67 patients cultured positive for non-O1, non-O139 strains 22 patients were between 0 and 6 months of age, 15 patients were between 7 and 12 months, 15 patients were between 13 and 18 months,

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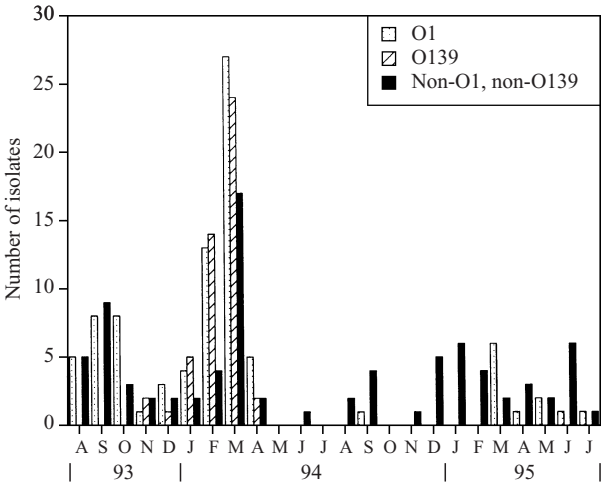

Fig. 1. Monthly isolation of different O-serotypes of *V. cholerae* from patients seen at the Children Hospital in Bangkok, Thailand from August 1993 to July 1995.

and 15 patients were older than 18 months of age. Children with non-O1, non-O139 strains were more often males, as 43 of 67 (64%) patients were males. Clinical data registered at the OPD were available for 40 of 67 (60%) patients. Among these, 58% reported watery diarrhoea and the rest had loose stool. Fever and vomiting were reported to be associated with the diarrhoeal illness in 62 and 40%, respectively. Less than 5% of the patients had abdominal colic. Fifty percent of the patients had mild dehydration, 42% had moderate dehydration and only three patients had severe dehydration and were admitted to the hospital. Antibiotic treatment was reported in 21 of 40 (53%) patients. Nalidixic acid was the most common antibiotic prescribed in 10 of 40 patients. Sulphamethoxazole/trimethoprim was prescribed in three patients.

Serotyping of the 69 *V. cholerae* non-O1, non-O139 strains revealed that 66 strains could be typed, whereas 2 strains showed a rough colony morphology and 1 strain was untypable (Table 2). A total of 37 different O-serotypes were found with 24 serotypes containing 1 strain only. O-serotypes containing more than 2 strains included serotype O2 (3 strains), O6 (8 strains), O10 (3 strains), O37 (7 strains), O97 (4 strains), and O151 (3 strains). One patient yielded two serotype O97 strains with different antibiotic susceptibility patterns (strains VO-3200A and VO-3200B). Another patient yielded a serotype O152 and a O111 strain (VO-6095A and VO-6095B.).

None of the 69 non-O1, non-O139 tested hybridized with the CT probe and only strains VO-2853 and VO-3138 hybridized with the NAG-ST probe. Each of the positive control strains hybridized with the probes yielding purple, dark brown colonies.

Antibiotic susceptibility testing showed that 40 of 69 (58%) non-O1, non-O139 strains were resistant to colistin, streptomycin and sulphisoxazole (Table 2, Fig. 2).) Four strains were resistant to nalidixic acid. In 1993, 6 of 16 strains (38%) were multiple antibiotic resistant (MAR; $\geqslant 4$ antibiotics), 12 of 37 (32%) strains were MAR in 1994, whereas 10 of 16 (63%) strains were MAR in 1995. The majority of the MAR strains were resistant to colistin, streptomycin, sulphisoxazole, tetracycline and trimethoprim/sulphamethoxazole. Temperoral changes in susceptibility were seen for some antibiotics with increased levels of streptomycin resistant found from 1993 to 1995 (Fig. 2). Apart from the seven serotype O37 strains, of which each was resistant to streptomycin, sulphisoxazole, tetracycline and trimethoprim/sulphamethoxazole, limited correlation was found between the remaining serotypes and antibiotic susceptibility patterns.

Repeated plasmids analysis showed that 26 of 69 (38%) strains contained one or more plasmids most often of low molecular weight (Table 2). Although 9 of 57 (16%) strains contained plasmids $>$ 20 kb sizes, MAR strains most often did not contain plasmids or contained a single plasmid of low molecular weight (Table 2). Thus, MAR was not associated with plasmids. The plasmid sizes described in Table 2 are approximations, as the measured sizes of high-molecular-weight plasmids often varied considerably in repeated analysis.

The different *Bgl*I ribotypes of strains belonging to the 6 O-serotypes which contained more than 2 strains are shown in Figure 3. Altogether, 20 different *Bgl*I ribotype patterns were observed among the 28 strains studied. Most ribotypes presented fragments within five major size ranges, namely, 1·5–1·6, 1·8–2·0, 2·4–2·6, 4·0–4·6 and 5·5–7·2 kb, respectively. Fragments within the 7·0–12 kb size range showed a high degree of variability. Limited correlation was found between ribotypes and most serotypes, except for the seven serotype O37 strains which each showed an identical ribotype. Thus, it appears that the O37 strains which were epidemiologically unrelated belong to the same clone as all strains showed identical ribotypes and nearly identical antibiotic susceptibility patterns. Strain VO-675 showed an atypical ribotype for *V. cholerae* (Fig. 3, lane U). VO-675, which contained eight different plasmids, showed biochemical reactions of *V. cholerae*, except that it was lysine

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Table 1. *Isolation rates of* V. cholerae *O1, O139 and non-O1, non-O139 strains from patients with diarrhoea seen at the Children Hospital, Bangkok*

Year (month)	No. of samples analysed	No. (%) of samples: Positive for *V. cholerae*	Of serogroup: O1		O139	Non-O1 non-O139
			Inaba	Ogawa		
1993 (Aug.–Dec.)	2084	49 (2·4)	2 (4·1)	23 (47·0)	3 (6·1)	21 (42·9)
1994	3035	133 (4·4)	0	50 (37·6)	45 (33·8)	38 (28·7)
1995 (Jan.–July)	2082	35 (1·7)	0	11 (31·4)	0	24 (68·6)

negative. Although VO-675 agglutinated O151 antiserum in repeated testing, the atypical ribotype suggest that VO-675 is not a *V. cholerae* strain.

DISCUSSION

V. cholerae non-O1, non-O139 serotypes have previously been isolated at low percentages from patients with watery diarrhoea and therefore been seen as of limited clinical importance [10, 39]. The temporal changes in traits of *V. cholerae* isolated during our surveillance programme conducted at the Children Hospital, Bangkok, from August 1993 to July 1995 showed that non-O1, non-O139 strains were isolated at similar rates as serotype O1 in 1993 and 1994. Although the non-O1, non-O139 strains accounted for 69% of *V. cholerae* culture positive specimens in 1995, the annual proportion of the isolation of non-O1, non-O139 from patients with diarrhoea was only about 1%. These findings are in contrast to the results of a similar study carried out in Calcutta, India from 1993 to 1995, where non-O1, non-O139 serotypes were isolated at much lower rates (2·7–4·9%) compared to O1 and O139 strains from faecal samples positive for *V. cholerae* [10]. The reason(s) for these differences are unclear, however, only children less than 10 years of age were included in the present study whereas both children and adults were included in the Calcutta study [10]. Although clinical information were available for just more than half of the patients, the patients generally showed mild clinical symptoms and only three children were admitted to the hospital because of severe dehydration. Also, it should be noted that stool samples were analysed for *V. cholerae* only. Thus, the diarrhoea of some cases may have had a different aetiology than *V. cholerae*.

After the first case of *V. cholerae* O139 was registered in November 1993 and an epidemic development of both O1 and O139 was registered in early 1994, O139 disappeared in May 1994 with only a few cases caused by O1 strains reported in the remaining part of 1994. A similar development of cholera was reported in Samutsakorn, a major port city located near Bangkok, where the O139 serotype also established itself together with *V. cholerae* O1 to suddenly disappear in July 1994 [40]. These findings are in contrast to the development of cholera in India and Bangladesh where the O139 serotype almost displaced *V. cholerae* O1 [2, 41]. Furthermore, recent surveillance data on *V. cholerae* from Bangladesh and India revealed a resurgence of *V. cholerae* O139 since 1996 [42–45]. Currently, we have not experienced a similar resurgence of O139 in Thailand.

Serotyping of non-O1, non-O139 strains revealed a high degree of heterogeneity with a total of 37 different O-serotypes and only 6 serotypes containing more than 2 strains. *Bgl*I ribotypes of a subset of strains confirmed these findings by demonstrating a high degree of heterogeneity within and between serotypes, except for seven serotype O37 strains which showed an identical ribotype suggesting clonality. Although some O-serotypes were more prevalent than others, these serotypes were not associated with sudden increases in the incidences of diarrhoea. The apparant lack of association between certain O-serotypes and diarrhoea may be caused by the relatively low number of isolates serotyped or that these strains were not the primary cause of disease. A high degree of heterogeneity within and between serotypes was also recently showed by ribotyping of 103 non-O1, non-O139 strains representing 10 O-serotypes commonly isolated from patients with diarrhoea in Japan and received at the National

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Table 2. *Serotypes, plasmid profiles, and antibiotic susceptibility patterns of 69* V. cholerae *non-O1, non-O139 strains isolated from 67 patients seen at the Children Hospital, Bangkok with watery diarrhoea*

Strain no.	Date of isolation	O-sero-type*	Plasmid size (kb)	Antibiogram†
VO-146	23.8.93	O10	5·1; 13	Cl; Sm; Su
VO-163	23.8.93	O14	—‡	Cl; Sm; Su
VO-258	27.8.93	O8	7·9	Cl; Sm; Su; Te
VO-272	30.8.93	O52	—	Am
VO-345	1.9.93	O37	—	Cl; Sm; Su; SXT; Te
VO-479	10.9.93	O19	—	Cl; Su
VO-505	14.9.93	O69	3·0	Cl; Sm; Su
VO-556	17.9.93	O151	3·0	Am; Sm; Su; SXT
VO-557	17.9.93	O151	3·0	Am; Su; SXT
VO-664	22.9.93	O76	—	Cl; Sm; Su
VO-675	23.9.93	O151	2·0; 2·7; 4·2; 5·1; 9·0; 11; 43; 92	Am; Su
VO-742	27.9.93	O2	7·7; 22	Cl; Su; Te
VO-1025	8.10.93	O37	—	Cl; Sm; Su; SXT; Te
VO-1163	19.10.93	O37	—	Cl; Sm; Su; SXT; Te
VO-1523	15.11.93	O6	3·3; 3·9	Cl; Sm; Su
VO-1774	7.12.93	O37	—	Am; Sm; Su; SXT; Te
VO-2339	20.1.94	O62	—	Am; Su; SXT
VO-2400	26.1.94	O41	2·9; 11; 44	Cl; Cm; Sm; Su; SXT; Te
VO-2502	1.2.94	O154	—	Sm
VO-2602	9.2.94	Unknown	2·2; 2·4; 7·7	Am; Su
VO-2612	10.2.94	Rough	—	Am; Cl; Sm; Su
VO-2722	22.2.94	O80	—	Cl; Sm
VO-2841	2.3.94	O56	3·0	Cl; Sm; Su
VO-2867	3.3.94	O107	—	Am; Su
VO-2853§	3.3.94	O110	5·2; 6·9; 20	Cl; Sm; Su
VO-2886	7.3.94	O36	—	Cl; Sm; Su
VO-2921	9.3.94	O2	7·0; 19	Cl; Sm; Su
VO-2922	9.3.94	O19	—	Cl; Sm; Su
VO-2999	15.3.94	O44	4·4; 10	Cl; Sm; Su
VO-3013	15.3.94	O21	—	Cl; Sm; Su
VO-3051	17.3.94	O6	5·1; 14	Am; Cl; Sm; Su
VO-3119	22.3.94	O6	5·1; 14	Cl; Sm; Su; Te
VO-3138§	23.3.94	O6	—	Cl; Su
VO-3148	24.3.94	O10	3·2; 3·4	Na; Am; Cl; Km; Nm; Sm; Su; SXT; Te
VO-3185	24.3.94	O6	—	Cl; Sm; Su
VO-3198	28.3.94	O78	—	Cl; Cm; Sm; Su; SXT; Te
VO-3200A	28.3.94	O97	—	Cl; Sm; Su; SXT; Te
VO-3200B	28.3.94	O97	—	Cl; Sm; Su
VO-3417	22.4.94	O183	6·0; 7·7; 16; 23	Am; Sm; Su; SXT; Te
VO-3414	22.4.94	O97	—	Na; Cl; Sm; Su; SXT; Te
VO-3464	16.6.94	O8	—	Cl; Sm; Su
VO-3946	23.8.94	O34	—	Cl; Nm; Su
VO-4175	19.9.94	O43	3·0	Cl; Su
VO-4208	20.9.94	O6	—	Cl; Su
VO-4233	21.9.94	O6	—	Am; Cl; Sm; Su
VO-4358	30.9.94	O37	—	Sm; Su; SXT; Te
VO-4396	5.10.94	O31	—	Cl; Sm; Su
VO-4827	28.11.94	O2	—	Cl; Sm; Su
VO-4883	2.12.94	O5	3·3; 3·9	Cl; Sm; Su
VO-4913	7.12.94	Rough	—	Cl; Sm; Su
VO-4925	8.12.94	O95	—	Cl; Sm; Su
VO-4967	12.12.94	O110	—	Na; Am; Cl; Km; Nm; Sm; Su; SXT
VO-5040	20.12.94	O41	—	Cl; Sm; Su

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Table 2 (*cont.*)

Strain no.	Date of isolation	O-sero-type*	Plasmid size (kb)	Antibiogram†
VO-5181	5.1.95	O6	—	Cl; Sm; Su
VO-5316	19.1.95	O166	—	Sm; Su; SXT; Te
VO-5398	25.1.95	O13	5·7; 23	Na; Am; Cl; Sm; Su; SXT
VO-5447	30.1.95	O10	—	Cl; Sm; Su
VO-5499	31.1.95	O150	—	Am; Cl; Sm
VO-5602	7.2.95	O13	—	Am; Sm; Su
VO-5758	21.2.95	O176	—	Cl; Sm; Su; Te
VO-5760	21.2.95	O176	—	Cl; Sm; Su; Te
VO-6070	20.3.95	O105	5·0; 7·0; 13; 22; 43; 110	Sm; Su; SXT; Te
VO-6095A	22.3.95	O152	2·0	Am; Sm; Su; SXT
VO-6095B	22.3.95	O111	—	Am; Su
VO-6238	3.4.95	O151	3·2; 5·2	Cl; Su
VO-6305	20.4.95	O107	—	Am; Cl; Sm; Su
VO-6361	1.5.95	O37	4·0	Cl; Sm; Su; SXT; Te
VO-6470	22.5.95	O27	—	Am; Cl; Nm; Sm; Su
VO-6650	13.6.95	O37	—	Cl; Sm; Su; SXT; Te

* O-serotype designations according to Shimada and colleagues [22].
† Am, ampicillin; Cm, chloramphenicol; Cl, colistin; Km, kanamycin; Na, nalidixic acid; Nm, neomycin; Sm, streptomycin; Su, sulphisoxazole; SXT, trimethoprim/sulphamethoxazole; Te, tetracycline.
‡ —, Did not contain plasmids.
§ Strain hybridized with the NAG-ST oligoprobe.

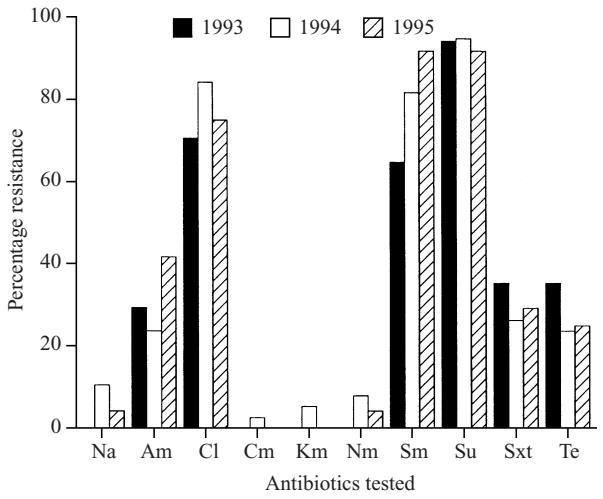

Fig. 2. Temporal changes in antibiotic susceptibility among 69 *V. cholerae* non-O1 non-O139 strains isolated at the Children Hospital in Bangkok, Thailand from August 1993 to July 1995.

Institute of Infectious Diseases in Japan for serotyping [46]. In addition to single cases, diarrhoea outbreaks associated with certain *V. cholerae* non-O1, non-O139 serotypes have been reported including an outbreak associated with O10 and O12 strains in 1994 in Peru [6], an outbreak associated with O6 and O14 strains among Khmers in a refugee camp in Thailand [27, 28], and an outbreak associated with O10 in India [47]. Despite these few outbreaks, in which the virulence mechanism(s) was not determined in most strains, heterogenous non-O1, non-O139 serotypes appear more often associated with non-epidemic cases of diarrhoea [9, 10, 46].

Recently Rubin and colleagues [48] identified a 4·7 kb cryptic plasmid in all *V. cholerae* strains containing the CTX element either as covalently closed circular DNA or as chromosomally integrated DNA. The nucleotide sequence of this plasmid showed similarities to sequences of filamentous phages. The plasmid was designated cryptic plasmid pTLC for toxin-linked cryptic and may play a role in the biology of the CTXθ phage, perhaps facilitating either its acquisition or its replication [48]. Thus, the following hypothesis for the development of toxigenic *V. cholerae* strains was proposed [48]: First, the progenitor strain acquired the TCP gene (via a mechanism that is not yet understood) and thereby became colonization proficient while simultaneously acquiring the receptor for CTXθ. Then, perhaps in the intestine, this TCP$^+$ strain was infected by CTXθ. It also appears that the acquisition of the TLC element is a required intermediate step preceding the acquisition of CTXθ in the evolution of fully pathogenic

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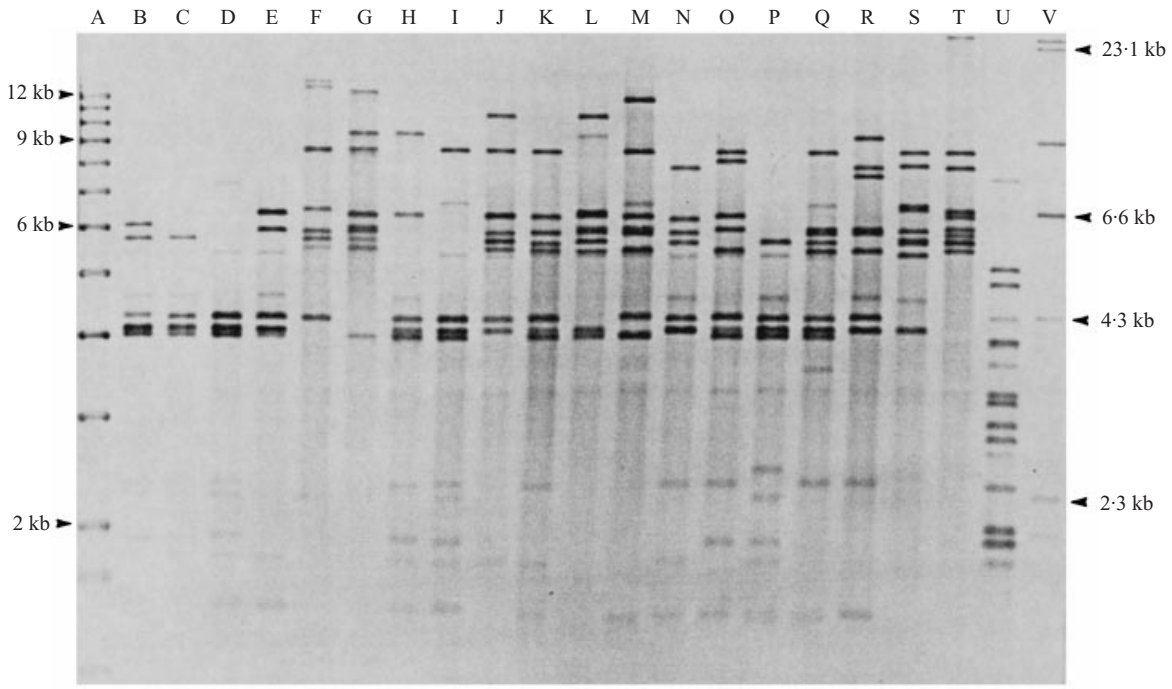

Fig. 3. Examples of *Bgl*I ribotypes of the most common serotypes of *V. cholerae* non-O1, non-O139 strains recovered from patients at the Children Hospital in Bangkok, Thailand. Unless indicated otherwise, the following explanations for the contents of the lanes indicate strain designation and serotype. Lanes: A, 1 kb molecular mass standard; B, VO-742, serotype O2; C, VO-2921, O2; D, VO-4827, O2; E, VO-1523, O6; F, VO-3119, O6; G, VO-3138, O6; H, VO-3185, O6; I, VO-4208, O6; J, VO-4233, O6; K, VO-5181, O6; L, VO-146, O10; M, VO-3148, O10; N, VO-5447, O10; O, VO-345, O37; P, VO-3200A, O97; Q, VO-3200B, O97; R, VO-3414, O97; S, VO-556, O151; T, VO-6238, O151; U, VO-675, O151, V, λ/*Hin*dIII DNA molecular mass standard.

V. cholerae [48]. However, since *V. cholerae* non-O1, non-O139 strains rarely contain the CTX element, it has been suggested that if transfers of genetic elements should occur there would possibly also be attendant changes in the somatic antigen [9, 49]. With reference to this hypothesis, *V. cholerae* non-O1, non-O139 strains, including strains in the present study, should be tested for TCP genes. It should also be investigated if the low-molecular-weight plasmids often detected in non-O1, non-O139 strains are bacteriophages encoding the TLC element.

The results of antibiotic susceptibility testing to 12 antibiotics showed that 58% of the non-O1, non-O139 strains were resistant to colistin, streptomycin and sulphisoxazole and 41% were MAR often showing resistant to trimethoprim/sulphamethoxazole. The high level of antibiotic resistance is disturbing, especially the resistance to sulphisoxazole and trimethroprim/sulphamethoxazole, as these antibiotics are commonly used to treat diarrhoea in children. Similar resistance patterns as shown in the present study were found in non-O1, non-O139 strains isolated in Calcutta from 1993 to 1995 [10]. Ongoing studies in our laboratories indicate that environmental and clinical non-O1, non-O139 strains isolated in Thailand before 1993 were less resistant to sulphonamides than strains isolated after 1993. Interestingly, we recently found similar changes in antibiotic susceptibility patterns among *V. cholerae* O1 isolated in Samutsakorn [17]. In Samutsakorn, O1 strains isolated in 1993 before the emergence of O139 were susceptible to streptomycin and sulphamethoxazole whereas strains isolated after 1993 showed resistance to these antibiotics. Studies in India also revealed major changes in antibiotic resistance patterns among *V. cholerae* serotypes [9, 10]. Although a high percentage of strains in our study contained one or more plasmids, the plasmids were of low molecular weights and did not seem to encode antibiotic resistance. Thus it appears that increased levels of resistance have developed after the emergence of the O139 serotype, however, the exact time as well as the mechanism(s) of antibiotic resistance in the non-O1, non-O139 strains are unknown and remains to be determined. Since resistance to sulphonamides may be associated with the presence of class 1 integrons, we are currently

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investigating if the non-O1, non-O139 strains contain integrons and possible antibiotic resistance gene cassettes [50, 51]. Thus, it could appear that the reports of recent shifts in antibiotic resistance among *V. cholerae*, including increased resistance to sulphonamides, are associated with class 1 integrons [10].

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