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Molecular characterization and antibiotic susceptibility of *Vibrio cholerae* non-O1

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SUMMARY

A collection of 64 clinical and environmental *Vibrio cholerae* non-O1 strains isolated in Asia and Peru were characterized by molecular methods and antibiotic susceptibility testing. All strains were resistant to at least 1 and 80% were resistant to two or more antibiotics. Several strains showed multiple antibiotic resistance ($\geqslant$ three antibiotics). Plasmids most often of low molecular weight were found in 21/64 (33%) strains. The presence of plasmids did not correlate with antibiotic resistance or influence ribotype patterns. In colony hybridization studies 63/64 (98%) *V. cholerae* non-O1 strains were cholera toxin negative, whereas only strains recovered from patients were heat-stable enterotoxin positive. Forty-seven *Bgl* I ribotypes were observed. No correlation was shown between ribotype and toxin gene status. Ribotype similarity was compared by cluster analysis and two main groups of 13 and 34 ribotypes was found. Ribotyping is apparently a useful epidemiological tool in investigations of *V. cholerae* non-O1 infections.

INTRODUCTION

Vibrio cholerae non-O1 serotypes are autochthonous bacteria of aquatic environments [1, 2] and have been associated with cholera-like diseases as well as systemic infections [3–6]. Human infections with non-O1 vibrios are most often associated with seafood consumption, exposure to polluted, brackish water or foreign travel [4, 7]. Until recently, *V. cholerae* non-O1 has not been associated with epidemic cholera. In March 1993, outbreaks of a disease resembling cholera occurred in two countries in Southern Asia. The microorganism responsible for the current epidemic was not *V. cholerae* O1 but a previously unrecognized serogroup of *V. cholerae* designated O139 [8].

Previously described typing techniques for *V. cholerae* non-O1 have included biotyping, serotyping, plasmid profiles, restriction fragment length polymorphisms (RFLP) of chromosomal DNA, and Southern blot analysis.

Cryptic plasmids of low molecular weight are often found in *V. cholerae* non-O1. These plasmids do not normally encode genes for antibiotic resistance [9].

However, conjugal transfer studies have demonstrated that large plasmids harboured by some *V. cholerae* non-O1 strains encoded antibiotic resistance [10, 11].

Determination of rRNA gene restriction (rDNA) fragment polymorphisms (ribotyping) was first described as a taxonomic tool by Grimont and Grimont [12]. The method has proved to be a useful molecular epidemiologic technique in the study of a number of pathogens, including *V. cholerae* O1 [13], *Vibrio vulnificus* [14], and *Vibrio anguillarum* [15].

A collection of 64 *V. cholerae* non-O1 strains was characterized by molecular methods and antibiotic susceptibility testing. Ribotyping, using a digoxigenin-labelled cDNA probe, was performed in order to evaluate the usefulness of this method as an epidemiological tool. A quantitative measure of the genetic similarities between strains was determined on the basis of ribotype patterns.

METHODS

Bacterial strains

A total of 64 *V. cholerae* non-O1 strains isolated in Asia and Peru were examined (Table 1). Forty-one isolates were recovered from stool samples from patients with diarrhoea and 23 isolates were obtained from seafood samples in Thailand. Strains, L48B, D82, C-211, C-677, C-711, IC-210, were unrelated epidemiologically; they were isolated between 1982 and 1985 from seven children, 1–9 years of age, at three different hospitals in metropolitan Bangkok [16]. Strains designated/90 were all isolated between May and June 1990 during an epidemic of a cholerae-like disease among Khmers in a camp in Aranyaprathet, Thailand [6]. NTR36S was isolated from an adult with traveller's diarrhoea in Tokyo [17]. All isolates were characterized and identified as previously described [16, 18–20].

Antibiotic susceptibility testing

All *Vibrio cholerae* non-O1 isolates were tested for antibiotic susceptibility to 20 antibacterial drugs by disk diffusion on Mueller-Hinton II Agar (Difco, Detroit, MI) with disks (Neo-Sensitabs, Rosco, Denmark) containing (μg/disk); ampicillin 30, amoxicillin 33, cephalothin 66, chloramphenicol 10, chloramphenicol 60, erythromycin 78, flumequine 30, furazolidone 50, kanamycin 100, nalidixic acid 130, neomycin 120, novobicin 100, oxolinic acid 10, phosphomycin 40, polymyxin B 50U, rifampicin 30, streptomycin 100, tetracycline 10, tetracycline 80 and trimethoprim/sulfamethiazole 5·2/240. Antibiotic susceptibility testing was carried out by the Bauer–Kirby method [21] and strains were recorded as sensitive, intermediate or resistant.

Conjugal transfer assay

One recipient strain *E. coli* K-12 (185^r) was chosen for mating experiments and *V. cholerae* non-O1 isolates 31/90 (54 kb plasmid), 54/90 (54 kb), SPF-216 (22·5 kb), JBF-59 (36·0 kb, 22·5 kb), and BF-17 (54 kb) were selected for conjugal transfer studies. Mating mixtures were plated on non-selective L agar at 37 °C for 4 h after which growth was harvested, diluted appropriately, and spread on plates of MacConkey agar supplemented with 50 μg/ml of nalidixic acid and adequate concentrations of selected drugs.

Preparation of plasmid DNA

The strains were grown in brain heart infusion (BHI) broth (Difco). After 24 h incubation at 37 °C 1·5 ml of BHI was transferred into an Eppendorf tube. Plasmid preparation was carried out using the method of Kado and Liu [22], modified by incubating the cells at elevated pH (12·75) for 30 min at 64 °C during the lysis step. Electrophoresis and visualization of plasmids was carried out essentially as previously described [23].

Colony hybridization

Vibrio cholerae non-O1 isolates were examined by the colony hybridization technique for DNA sequences encoding cholera toxin (*ctx*) [24] and heat-stable enterotoxin (NAG-ST) [25, 26]. The DNA probes were either labelled with 10^6 cpm of α-^{32}P by the random priming method [27] or with alkaline phosphatase [24]. CT and NAG-ST positive and negative controls were used. Prehybridization and hybridization were performed as earlier described [16, 28]. A part of the colony hybridization results for the Thai clinical isolates has been reported elsewhere [6, 16].

DNA isolation and Southern blotting of enzyme digested DNA fragments

Total bacterial DNA was extracted essentially using the method of Pedersen and Larsen [15]. Based on previous studies [13, 29] and preliminary chromosomal digestion experiments using *Hin*d III, *Eco*R I and *Bgl* I (Promega, Madison, Wis.), *Bgl* I provided the best discrimination among *V. cholerae* non-O1 isolates and was therefore used to digest chromosomal DNA from all strains.

0·1 μg of a 1 kb DNA ladder (GIBCO BRL, Gaithersburg, Md.) was used as a molecular size standard and samples were subjected to electrophoresis. The optimal conditions for electrophoresis, as determined by a uniform fragment distribution, were obtained with an agarose gel length of 20 cm, an agarose concentration of 0·8%, and a voltage of 40 V for 16·5 h. Gels were stained with ethidium bromide (2 μg/ml, Sigma, St Louis, Mo.) for 20 min, destained in distilled water, and photographed at 254 nm UV transillumination. DNA fragments were denatured in the gel using 0·25 M-HCl and then transferred to a nylon membrane [(18 × 20 cm) (Hybond N+, Amersham International plc, Amersham, UK)] by the Southern method [30]. An alkali blotting procedure (0·4 M-NaOH) and a vacuum blotter (LKB Bromma, Sweden) were used. The membranes were air dried prior to hybridization.

Preparation of molecular size standard and cDNA probe for 16S and 23S RNA genes, hybridization and detection

A 1-kb ladder (GIBCO BRL) was digoxigenin-labelled by following the manufacturer's recommendations for the random priming procedure (Boehringer, Mannheim, Germany). *Escherichia coli* 16S and 23S rRNA (Boehringer, Mannheim) was used to prepare the digoxigenin-labelled cDNA by a reverse transcription reaction as previously described [31]. Nylon membranes with immobilized DNA restriction fragments were hybridized and fragments were detected colorimetrically as described by Popovic and colleagues [13].

Table *ıtibiotic susceptibility patterns, plasmid profiles, colony hybridization results and Bgl* I *ribotypes of 64* Vibrio cl ae *non-O1 strains*

	ate	Isolation site	Origin	Antibiogram	RT*	Plasmid	NAG–ST†	C'
N]	;S	Japan	Human	§Em_I Nb_I Pb Rm_I Tc_{AI}	25	—	+	·
L4		Thailand	Human	Nb_I Pb Rm_I Sm_I Tc_{AI}	25	—	+	·
D8		Thailand	Human	Nm_I Nb Pb Rm Sm_I Tc_{AI}	26	—	+	·
C-		Thailand	Human	Em_I Nm_I Nb Pb Rm_I Sm_I Tc_{AI}	26	—	+	·
C-		Thailand	Human	Nb Pb Rm_I Sm_I	26	—	+	·
C-		Thailand	Human	Em_I Nb Pb Rm Sm_I Tc_{AI}	26	—	+	·
IC		Thailand	Human	Nb Pb Rm_I Tc_A	27	—	+	·
29		Thailand	Human	Cm_A Cm_{BI} Nb_I Pb Rm_I Sm Tc_A Tc_{BI} TR	2	—	+	·
31		Thailand	Human	Cm_A Cm_{BI} Pb Sm Tc_A Tc_{BI} TR	1	54	−	·
32		Thailand	Human	Cm_A Cm_{BI} Nb Pb Sm Tc_A Tc_{BI} TR	2	—	+	·
33		Thailand	Human	Cm_A Pb RP Sm Tc_A TR	1	54	−	·
34		Thailand	Human	Cm_A Cm_{BI} Pb Sm Tc_A Tc_B TR	1	54	−	·
35		Thailand	Human	Cm_A Cm_{BI} Nb Pb Rm_I Sm Tc_A Tc_{BI} TR	2	—	+	·
36		Thailand	Human	Cm_A Pb Sm Tc_A Tc_{BI} TR	1	—	−	·
28		Thailand	Human	Cm_A Pb Sm Tc_A Tc_{BI} TR	1	—	−	·
30		Thailand	Human	Cm_A Pb Sm_I Tc_A TR	1	54	−	·
54		Thailand	Human	Cm_A Pb Sm Tc_A Tc_{BI} TR	1	54	−	·
16		Thailand	Human	Pb	3	—	−	·
17		Thailand	Human	Cm_{AI} Cm_{BI} Nb Pb Rm_I Sm Tc_A Tc_B TR	4	10·5; 5·8; 4·9; 3·4	−	·
29		Thailand	Human	Em_I Nb Pb Rm_I Sm_I Tc_A Tc_B TR	2	—	+	·
SI	3	Thailand	Human	Nb Pb Tc_{AI}	5	7·6; 5·7; 4·1; 3·45	−	·
A]		Thailand	Human	Cm_A Nb Pb Sm Tc_A Tc_B TR	7	22·5	−	·
V(		Thailand	Human	Nb Pb Tc_A	8	—	−	·
V(		Thailand	Human	Nb Pb Tc_{AI}	10	—	−	·
V(		Thailand	Human	Nb_I Pb Tc_A	9	12·0; 5·3	−	·
93		Philippines	Human	Ax Ap Pb Tc_A	15	—	−	·
93		Philippines	Human	Nb Pb	16	—	−	·
93		Philippines	Human	Nb Pb Rm_I Tc_A	16	9·0; 8·0; 7·0	−	·
93		Philippines	Human	Nb_I Pb Sm	17	—	+	·
15		Philippines	Human	Nb_I Pb Tc_{AI}	18	—	−	·
15		Philippines	Human	Nb Pb Tc_A	18	—	−	·
15		Philippines	Human	Nb Pb Tc_A Tc_B TR	20	9·4; 6·8; 4·6	−	·

1594	**Philippines**	**Human**	**Pb Tc_{AI}**	**19**	—	—	—
1823	**Philippines**	**Human**	**Nb Pb_I**	**22**	3·5	—	—
2016	Philippines	Human	Ax Ap Ce Cm_A Km Nb Pb Rm Sm Tc_{AI} TR	23	—	—	—
2246	Philippines	Human	Pb Tc_{AI}	24	—	—	—
755	Philippines	Human	Cm_A Nb_I Pb Rm_I Sm_I Tc_{AI} TR	21	—	—	—
ANN-497-2	Peru	Human	Pb_I Tc_{AI}	11	11·9; 5·1	—	—
no. 0248	Peru	Human	Pb	12	6·0; 4·0	—	—
no. 0727	Peru	Human	Cm_A Pb Rm_I TR	13	—	—	—
BAB 2209	Peru	Human	Pb	14	—	—	+
SPH-09	Thailand	Seafood	Ax Ap Cm_A Cm_{BI} Pb Sm Tc_{AI} TR	28	—	—	—
SPF-35	Thailand	Seafood	VB_I Pb Tc_{AI}	29	6·0; 4·0	—	—
SPF-36	Thailand	Seafood	Nm_I Nb Pb Rm_I Tc_{AI}	30	—	—	—
SPF-37	Thailand	Seafood	Pb_I	31	—	—	—
SPF-52	Thailand	Seafood	Nm_I Pb Tc_{AI}	32	—	—	—
SPF-77	Thailand	Seafood	Nb Pb	32	—	—	—
SPF-173	Thailand	Seafood	Nb Pb Rm_I Tc_{AI}	37	—	—	—
SPF-175	Thailand	Seafood	Pb Rm_I Tc_{AI}	38	—	—	—
SPFA-182	Thailand	Seafood	Em_I Pb Rm_I Sm_I Tc_{AI}	40	—	—	—
SPFB-182	Thailand	Seafood	Em_I Pb Tc_{AI}	41	—	—	—
SPF-191	Thailand	Seafood	Ax Ap Em_I Pb Rm_I Sm_I Tc_{AI}	39	—	—	—
SPF-216	Thailand	Seafood	Cm_A Nb Pb Sm Tc_A Tc_B	42	22·5	—	—
SPF-219	Thailand	Seafood	Pb	43	6·0; 3·7	—	—
SPF-281	Thailand	Seafood	Tc_A Tc_B TR	45	22·5; 11·5; 8·0	—	—
PF-410	Thailand	Seafood	Pb_I	46	22·5; 11·5; 5·8; 7·0	—	—
SPF-412	Thailand	Seafood	Nb Pb Tc_{AI}	47	—	—	—
JBF-38	Thailand	Seafood	Pb Rm_I Tc_{AI}	35	—	—	—
JBF-41	Thailand	Seafood	Nb Pb Tc_{AI}	36	—	—	—
JBF-59	Thailand	Seafood	Ax_I Nb_I Fo Pb Tc_{AI}	32	36·0; 22·5	—	—
JBF-108	Thailand	Seafood	Pb	44	—	—	—
BF-16	Thailand	Seafood	Pb Tc_{AI}	33	—	—	—
BF-17	Thailand	Seafood	Pb Rm_I Tc_{AI}	34	54	—	—
1005	Thailand	Seafood	Pb Tc_{AI}	6	—	—	—

* Ribotype.

† Heat-stable enterotoxin.

‡ Cholera toxin.

§ $_I$, Intermediate resistance; Ax, amoxicillin; Ap, ampicillin; Ce, cephalothin; Cm_A, chloramphenicol (10 μg); Cm_B, chloramphenicol (60 μg); Em, erythromycin; Fu, Furazolidone; Km, kanamycin; Nm, neomycin; Nb, novobiocin; Fo, phosphomycin; Pb, polymyxin B; Rm, rifampicin; Sm, streptomycin; Tc_A, tetracycline (10 μg); Tc_B, tetracycline (80 μg); TR, trimethoprim/Sulfamethiazole.

Ribotype data recording and statistical analysis

Molecular size standard, 0·1 μg of a 1 kb DNA ladder, was used as migration references. A matrix was constructed on the basis of presence or absence of ribotyping bands at a given position over the size-range from 1·5 to 19 kb (0, absence of a fragment; 1, presence of a fragment).

A cluster analysis, the unweighted pair group method with arithmetic means (UPGMA clustering) using a simple matching coefficient S_{SM}, of similarity coefficients for all pairs of strains, was carried out and a dendrogram produced with a computer-based taxonomy program (PC-TAXAN, version 1·2; University of Maryland). The S_{SM} coefficient was used since the absence of a particular band is as important as its presence.

Since a number of the *V. cholerae* non-O1 strains included in this study were not necessarily unrelated and randomly selected, the discriminatory power of the ribotyping method was not calculated [32, 33].

RESULTS

The results of the determination of resistance patterns to 20 antibacterial drugs among the 64 strains tested is shown in Table 1. All 64 strains (100%) exhibited resistance to at least one drug. Twenty-five (39%) isolates were multiple resistant ($\geqslant 3$ antibiotics). Resistance to polymyxin B was common (91%) with an additional 8% of the strains showing intermediate resistance to polymyxin B. The following patterns were found: resistance to novobiocin 26%, 10 μg tetracycline 33%, intermediate resistance to 10 μg tetracycline 42%, trimethoprim/sulphamethiazole 28%, 10 μg chloramphenicol 25%, 80 μg tetracycline 8%, ampicillin and amoxicillin both 6%, and cephalothin, phosphomycin and kanamycin all 2%. All isolates were susceptible to oxolinic acid, flumequine, furazolidone and nalidixic acid. In addition, seven and five strains showed intermediate resistance to 60 μg chloramphenicol and erythromycin, respectively.

Four strains, 93–20, 2016, SPH-09, SPH-191, exhibited resistance to both amoxicillin and ampicillin. Only 1 strain, strain 2016, showed resistance to 10 different antibiotics, including cephalothin and kanamycin. Strain JBF-59 was the only strain showing resistance to phosphomycin, whereas SPF-281 was the only strain susceptible to polymyxin B. All 18 strains exhibiting resistance to trimethoprim/sulphamethiazole also showed resistance or intermediate resistance to 10 μg tetracycline. Only two seafood isolates, a multiple antibiotic resistant strain SPH-09 and strain SPF-281, showed resistance to trimethoprim/sulphamethiazole. Out of 15 strains showing resistance to streptomycin 13 strains also exhibited resistance to trimethoprim/sulphamethiazole. Overall, strains recovered from patients in Thailand showed resistance to more antibiotics compared to strains recovered from patients in the Philippines and Peru. The antibiotic resistance patterns of *V. cholerae* non-O1 strains recovered from seafood in Thailand were similar to the patterns seen among clinical isolates from the Philippines and Peru.

Analysis of the plasmid content of the *V. cholerae* non-O1 strains revealed that

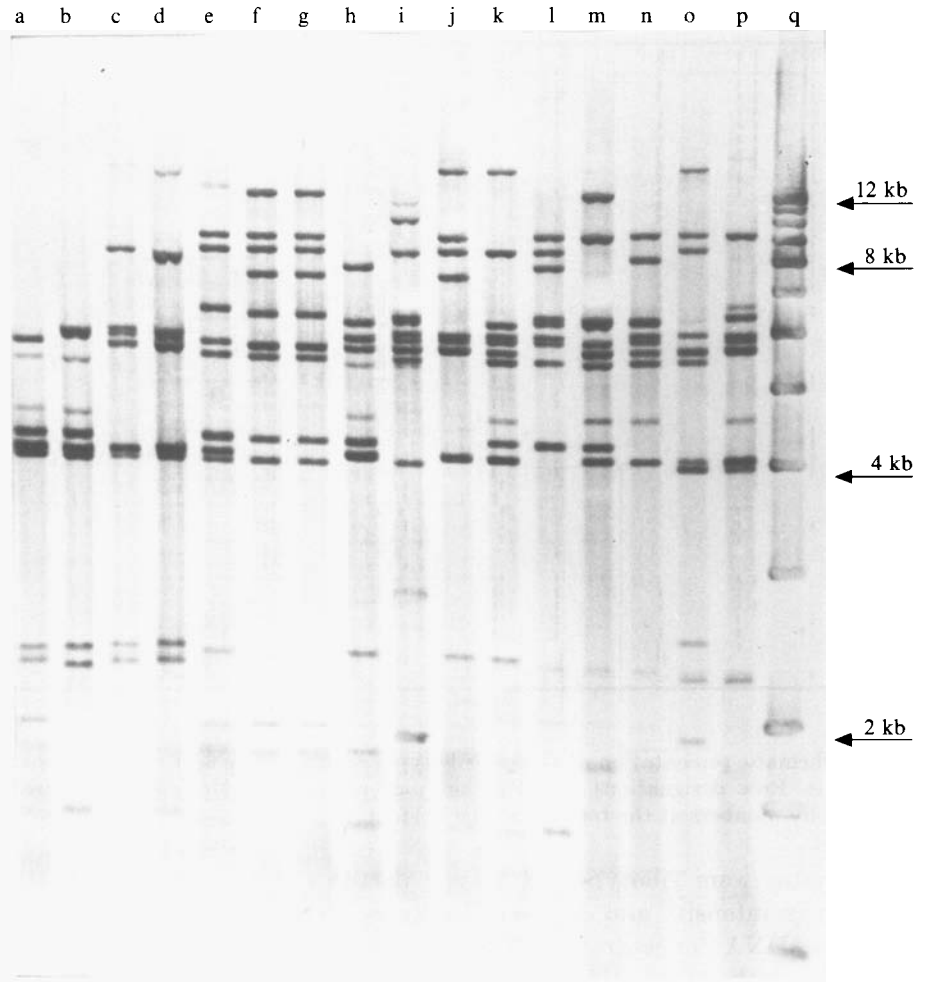

Fig. 1. Examples of *Bgl* I ribotypes of *Vibrio cholerae* non-O1 Lanes: a, strain 28/90, ribotype 1; b, strain 93–23, type 17; c, strain 93–22 type 16; d, strain 93–21, type 16; e, strain 93–20, type 15; f, strain 1571, type 18; g, strain 1577, type 18; h, strain 1594, type 19; i, strain 1595, type 20; j, strain 1755, type 21; k, strain 1823, type 22; l, strain 2016, type 23; m, strain ANN-497-2, type 11; n, strain 0248, type 12; o, strain 0727, type 13; p, strain BAB 2209, type 14; q, 1 Kb DNA ladder.

21/64 (33%) strains carried plasmids of which most were of low molecular weight (Table 1). In addition, 13/64 (20%) strains carried more than one plasmid. Six strains contained a single plasmid of approximately 54 kb.

Using *V. cholerae* non-O1 isolates 31/90, 54/90, SPF-216, JBF-59, and BF-17 for mating experiments no transjugants were obtained.

The results from the colony hybridization studies are shown in Table 1. All *V. cholerae* non-O1 strains tested, except strain BAB 2209, were CT negative. Although strain BAB 2209 contained genes encoding *ctx* the isolate did not agglutinate O139 antisera. All NAG-ST positive strains were recovered from patients in Thailand. All strains, except strain 93-23, isolated from patients in Peru and the Philippines, were NAG-ST negative.

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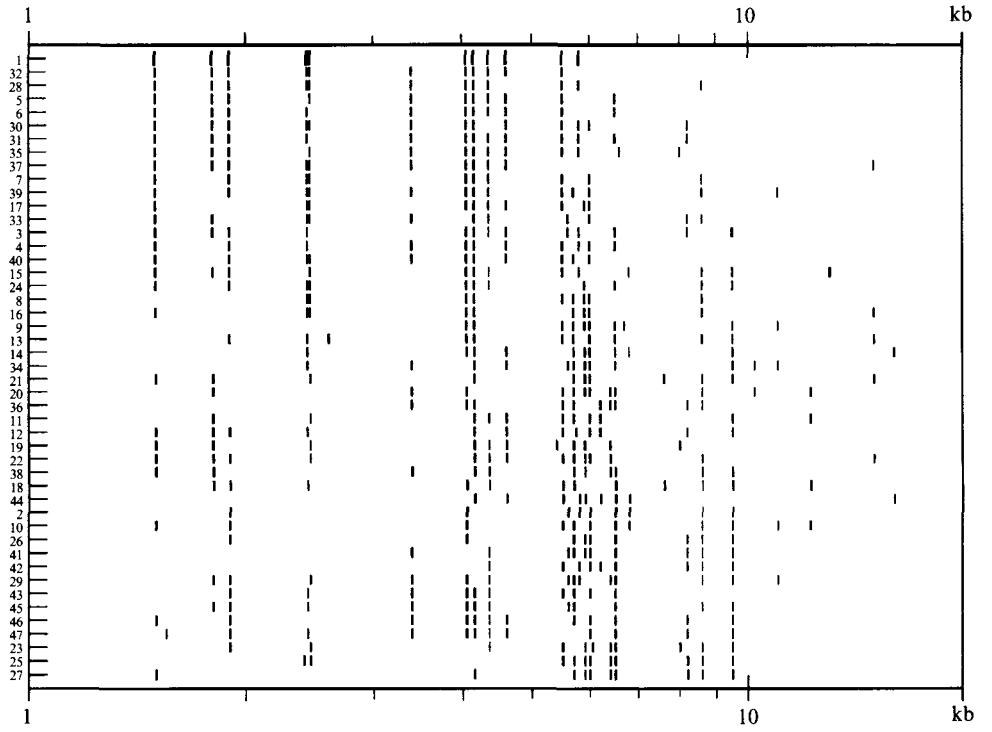

Fig. 2. Schematic presentation of 47 *Bgl* I ribotypes shown among 64 *V. cholerae* non-O1 strains. Row designations indicate the ribotype number. Molecular sizes are indicated by numbers at the top and at the bottom.

All 64 strains were ribotypes. Ribotype patterns were stable and patterns differed only in intensity and degree of background, apparently associated with differences in DNA concentration. The DNA extraction method yielded good quality DNA with no need for further DNA purification. Both digoxigenin-labelled probes, cDNA and the 1 kb ladder were stable for at least 1 year when stored at −20 °C.

Patterns contained 10–12 DNA fragments ranging from 1·5–19 kb and a total of 37 different restriction bands were observed. Fragments with low molecular weight often appeared with low intensity.

Forty-seven different ribotypes patterns were observed after *Bgl* I cleavage. Only seven ribotypes included more than one strain. An example of ribosomal banding patterns of *V. cholerae* non-O1 is shown in Figure 1, and a schematic presentation based on pattern similarities of all ribotypes is shown in Figure 2. Patterns were considered to be different when there was a difference of only one band between isolates and each ribotype was given an arbitrary number.

Five different ribotypes, 2, 17, 25, 26 and 27, were present among 12 NAG-ST positive *V. cholerae* non-O1 strains. None of these five ribotypes included NAG-ST negative strains. Ribotype 25 included one Japanese and one Thai strain. Out of the four strains belonging to ribotype 26, three strains (C-211, C-677, C-711) were recovered from patients at the same hospital.

Among 14 strains recovered from patients at the camp in Aranyaprathet four

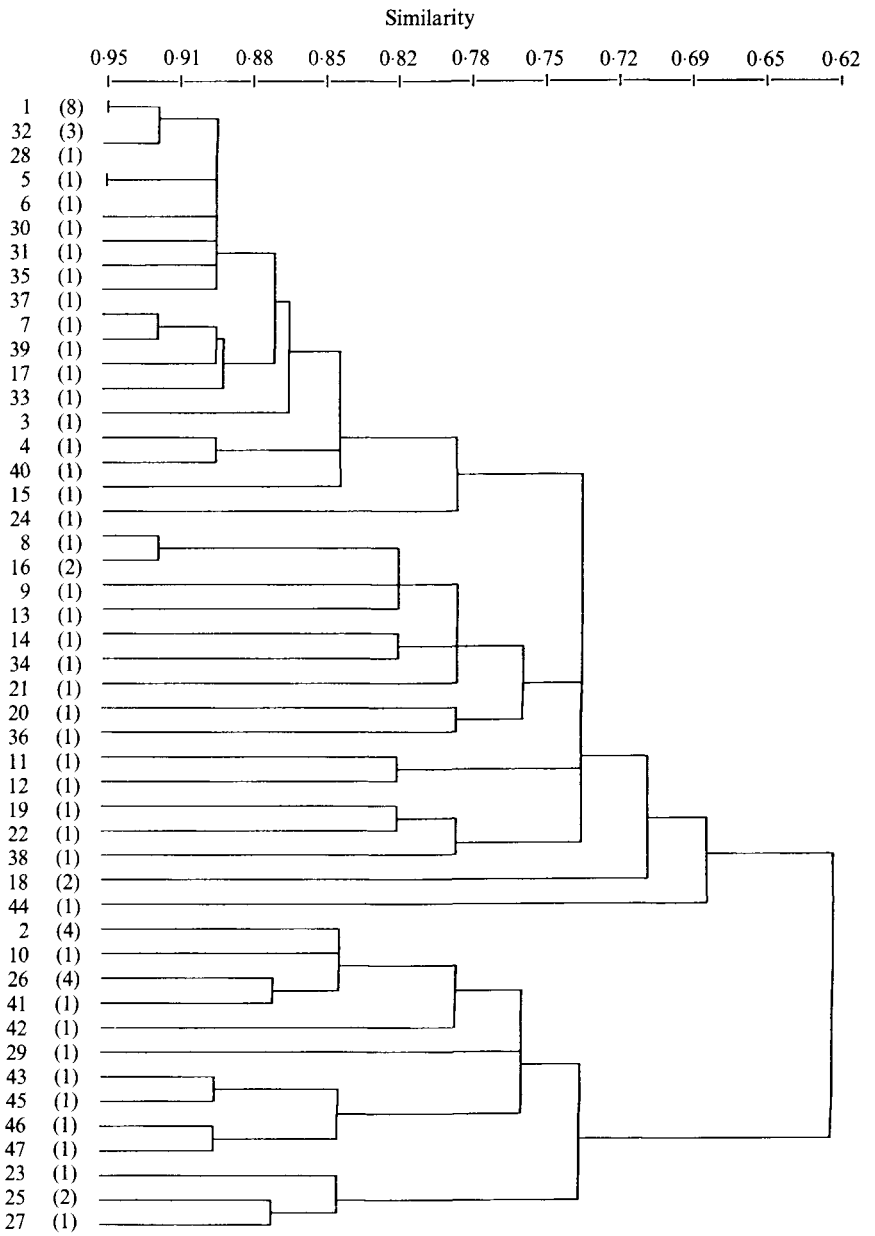

Fig. 3. Clustering of 64 *Vibrio cholerae* non-O1 strains according to similarity of *Bgl* I ribotype patterns. Row designations at the far left of the dendrogram correspond to the ribotype and the number of strains within each ribotype is shown in brackets.

different ribotypes were found, with ribotype 2 and ribotype 1 accounting for four and seven strains, respectively.

Out of the 12 strains isolated from patients in the Philippines ten had different ribotypes, as did all four Peruvian isolates. Twenty different ribotypes were presented among 23 Thai seafood isolates.

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V. cholerae non-O1 strains with the same ribotype most often exhibited similar antibiotic resistance patterns, whereas strains showing the same resistance pattern often had different ribotypes. Strains 93-21 and 93-22 containing no plasmids and three plasmids, respectively, both belonged to ribotype 16. Five out of seven isolates exhibiting ribotype 1 contained a 54 kb plasmid. Strain BF-17 also contained a 54 kb plasmid but exhibited ribotype 34.

A dendrogram was produced from the cluster analysis performed on the basis of the ribotype patterns of the 64 strains (Fig. 3). Two main clusters were found comprising 34 ribotypes and 13 ribotypes, respectively. Both clusters could be further divided into several sub-clusters. Members within the two clusters were 68% and 74% related to each other. Although the 11 NAG-ST positive *V. cholerae* non-O1 belonged to the same cluster, they were only 74% related. Ribotypes 1 and 2, the two major ribotypes shown among strains recovered from patients at the camp in Aranyaprathet, were only 62% related. All the Filipino isolates, except strain 2016, belonged to the same major cluster, as did the four Peruvian isolates.

DISCUSSION

We earlier reported that during the cholera epidemic in Aranyaprathet, Thailand children and pregnant women were treated with trimethoprim/sulphamethoxazole [6]. It is interesting to note that a high percentage (92%) of *V. cholerae* non-O1 isolated during this epidemic showed resistance to trimethoprim/sulphamethiazole and to some degree also showed resistance to tetracycline. Resistance to trimethoprim/sulphamethoxazole is rarely seen among *V. cholerae* O1 but appears to be common among *V. cholerae* O139 [34]. The relatively high prevalence of multiple antibiotic resistance *V. cholerae* non-O1 strains recovered from patients in Thailand stresses the importance of performing antibiotic susceptibility tests. In addition, strains recovered from patients in Thailand were resistant to more antibiotics compared to clinical strains from the Philippines or Peru.

Even though the resistance patterns of *V. cholerae* non-O1 strains recovered from seafood in Thailand were similar to the patterns seen among clinical isolates from the Philippines and Peru, it should be noted that Thai seafood isolates clearly showed resistance to fewer antibiotics in comparison with Thai clinical isolates. Hence, *V. cholerae* non-O1 bacteria excreted from patients with diarrhoea does not seem to constitute a normal part of the microbial flora in marine environments. Whether the resistance patterns found in the Thai seafood isolates can be correlated with the use of antibiotics in aquaculture remains to be studied further.

With the ability of *V. cholerae* strains to produce β-lactamases, it is surprising that only 6% of the strains tested showed resistance to ampicillin and amoxicillin. Amaro and colleagues [10] reported, that of 146 *V. cholerae* non-O1 tested, 63% and 61% exhibited resistance to ampicillin and amoxicillin, respectively. Furthermore, they found 20% of strains tested showed resistance to phosphomycin whereas all strains were susceptible to chloramphenicol, novobiocin, tetracycline, and trimethoprim. These results differ obviously from the results of the present study. Whether these differences could be due to different geographic origins of the

isolates or differences in antibiotic usage in the countries from which the isolates were obtained remains to be studied further.

Previous studies have shown that *V. cholerae* O1, isolated from clinical and environmental sources had a lower frequency of plasmid carriage than clinical and environmental non-O1 strains. Additionally, 46/187 clinical and environmental non-O1 strains were reported to carry plasmids of low molecular weight [9]. Our study provides further evidence that cryptic plasmids of low molecular weight are frequently found in *V. cholerae* non-O1 strains.

Strains that harboured either no plasmids or several different plasmids showed similar antibiotic susceptibility patterns, and indicates that antibiotic resistance is not necessarily plasmid-mediated (Table 1). These results are in agreement with previous studies [11, 35].

Earlier studies have demonstrated plasmid encoded drug resistance and its transferability within *V. cholerae* non-O1 [10, 11]. Amaro and colleagues [10] demonstrated a high transfer frequency among *V. cholerae* non-O1 all containing an approximately 48 kb plasmid, whereas we obtained no transconjugants among five strains that harboured plasmids from 22·5–54 kb in size.

A total of 47 different *Bgl* I ribotypes were shown among 64 *V. cholerae* non-O1 isolates and indicates a high level of genetic diversity within *V. cholerae* non-O1 (Fig. 2 and Fig. 3). We are currently undertaking studies to analyse any correlation between ribotypes and serotypes among *V. cholerae* non-O1.

The ribotyping results of the four NAG–ST positive isolates from Aranyaprathet, Thailand confirm our previous findings, that these strains belonged to a single clone [6]. However, the recovery of seven NAG–ST negative ribotype 1 strains from the same epidemic indicates that more than one clone was involved in the epidemic. This is supported by the results of the cluster analysis where ribotypes 1 and 2 showed only 64% similarity. These results provide further evidence that virulence determinants other than NAG–ST and CT must be present in *V. cholerae* non-O1. A recent study carried out by Rammamurthy and colleagues [36] on virulence patterns of *V. cholerae* non-O1 concluded that the virulence is multifactorial and mediated by several traits functioning in an integrated fashion. The clinical significance of *V. cholerae* non-O1 should therefore be assessed in its totality; the presence of a single factor should not be construed as the cause of enteropathogenicity.

We previously reported that strains D-82, C-211, C-677, and C-711 were epidemiologically unrelated [16]. However, since all strains belonged to ribotype 26 a very close genetic relationship among these strains is likely. It is also interesting that two strains, NTR36S from Japan and L48 B from Thailand, without any known epidemiological relationship, showed an identical ribotype 25.

The cluster analysis based on ribotype patterns did not correlate site of isolation, origin, or toxin gene status but the analysis revealed a high degree of genetic divergence within *V. cholerae* non-O1. In order to be able to compare a large number of ribotype patterns and also to group similar ribotypes a schematic presentation is often produced [13, 37]. We found that the results of the cluster analysis of the 47 different ribotypes were useful when establishing the order of the ribotypes listed in the schematic presentation in Figure 2.

Ribotype schemes have been proposed for various bacteria. Popovic and

colleagues [13] proposed a ribotype scheme for *V. cholerae* O1. In comparison of this scheme with the patterns shown in Figure 2, the major differences are a larger number and greater variation among fragments that ranged from 8–17 kb shown among *V. cholerae* non-O1 isolates. Furthermore, most *V. cholerae* non-O1 strains generated 1–3 fragments between 1·8 and 2·0 kb which were absent or rarely seen among *V. cholerae* O1 strains.

This study shows that ribotyping appears to be a suitable method for differentiating both clinical and environmental *V. cholerae* non-O1 strains for epidemiological purposes.

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