Epidemiol. Infect. (1998), **121**, 535–545. Printed in the United Kingdom © 1998 Cambridge University Press

Ribotypes of clinical *Vibrio cholerae* non-O1 non-O139 strains in relation to O-serotypes

A. DALSGAARD[1]*, A. FORSLUND[1], H. F. MORTENSEN[1]† AND T. SHIMADA[2]

[1] *Department of Veterinary Microbiology, The Royal Veterinary and Agricultural University, Denmark*
[2] *Department of Bacteriology, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162, Japan*

(*Accepted 11 June 1998*)

SUMMARY

The emergence of *Vibrio cholerae* O139 in 1992 and reports of an increasing number of other non-O1 serogroups being associated with diarrhoea, stimulated us to characterize *V. cholerae* non-O1 non-O139 strains received at the National Institute of Infectious Diseases, Japan for serotyping. Ribotyping with the restriction enzyme *BglI* of 103 epidemiological unrelated mainly clinical strains representing 10 O-serotypes yielded 67 different typing patterns. Ribotype similarity within each serotype was compared by using the Dice coefficient (S_d) and different levels of homogeneity were observed (serotypes O5, O41 and O17, S_d between 82 and 90%; serotypes O13 and O141 S_d of 72; and O2, O6, O7, O11, O24 S_d of 62–66%). By cluster analysis, the strains were divided into several clusters of low similarity suggesting a high level of genetic diversity. A low degree of similarity between serotypes and ribotypes was found as strains within a specific serotypes often did not cluster but clustered with strains from other serotypes. However, epidemiological unrelated O5 strains showed identical or closely related ribotypes suggesting that these strains have undergone few genetic changes and may correspond to a clonal line. Surprisingly, 10 of 16 O141 strains studied contained a cholera toxin (CT) gene, including 7 strains recovered from stool and water samples in the United States. This is to our knowledge the first report of CT-positive clinical O141 strains. The closely related ribotypes shown by eight CT-positive strains is disturbing and suggest that these strains may be of a clonal origin and have the potential to cause cholera-like disease. Despite the low degree of correlation found between ribotypes and serotypes, both methods appears to be valuable techniques in studying the epidemiology of emerging serotypes of *V. cholerae*.

INTRODUCTION

The emergence of *Vibrio cholerae* O139 in India in 1992 and the following spread to a number of other countries, mainly in Asia, sparked new interest into the importance of *V. cholerae* non-O1 serotypes as causes of diarrhoeal disease. Non-O1 non-O139 serogroup strains have increasingly been recognized as the causative agents of sporadic cases of cholera-like disease [1, 2] and isolated outbreaks [1, 3]. Dalsgaard and colleagues [4] recently reported an outbreak of diarrhoea associated with *V. cholerae* serotypes O10 and O12 strains among volunteers in a vaccine trial study area in Lima, Peru.

Serotyping has previously been used to study the epidemiology of *V. cholerae* non-O1 by several workers with varying degrees of success, often because several strains did not agglutinate existing antisera [5, 6]. However, the extended serotyping scheme (O1 to O140) established by Shimada and colleagues [7] has reduced the prevalence of non-agglutinating

* Author for correspondence.

Table 1. *Description of* V. cholerae *non-O1 non-O139 strains studied by serotyping and ribotyping*

Strain designation	O-serotype/ Source	Country/ Year of isolation	Country of travel abroad
609-94	02-1/blood	Japan/1994	No history
NQ-4	02-2/stool	Japan/1991	Thailand
NQ-5	02-3/stool	Japan/1991	India
NQ-6	02-4/stool	Japan/1991	Thailand
NQ-10	02-5/stool	Japan/1991	India
NQ-12	02-6/stool	Japan/1991	India
NQ-30	02-7/stool	Japan/1991	Thailand
NQ-36	02-8/stool	Japan/1991	Thailand
NQ-61	02-9/stool	Japan/1991	India
NCTC 4711 (Ref. strain)	02-10	China/1982	—*
1080-93	05-1/stool	Japan/1993	Indonesia
NQ-1	05-2/stool	Japan/1991	Thailand
NQ-11	05-3/stool	Japan/1991	India
NQ-14	05-4/stool	Japan/1991	Thailand
NQ-15	05-5/stool	Japan/1991	Thailand
NQ-16	05-6/stool	Japan/1991	Thailand
NQ-17	05-7/stool	Japan/1991	India
NQ-19	05-8/stool	Japan/1991	Thailand
NQ-29	05-9/stool	Japan/1991	Thailand
218-68 (Ref. strain)	05-10/stool	India/1982	No history
792-94	06-1/blood	Japan/1994	No history
550-94	06-2/stool	Japan/1994	No history
1121-93	06-3/stool	Japan/1993	India
323-93	06-4/stool	Japan/1993	Indonesia
280-943	06-5/stool	Japan/1993	Philippines
278-93†	06-6/stool	Japan/1993	No history
NQ-142†	06-7/stool	Japan/1992	Thailand
NQ-147	06-8/stool	Japan/1992	India
NQ-156†	06-9/stool	Japan/1992	Thailand
7007-62 (Ref. strain)	06-10/stool	India/1962	No history
NQ-33	07-1/stool	Japan/1991	India
NQ-50	07-2/stool	Japan/1991	India
NQ-67	07-3/stool	Japan/1991	India
NQ-132	07-4/stool	Japan/1992	India
NQ-137	07-6/stool	Japan/1992	India
NQ-171	07-7/stool	Japan/1993	Thailand
NQ-175	07-8/stool	Japan/1993	Thailand
NQ-197	07-9/stool	Japan/1993	India
8394-62 (Ref. strain)	07-10/stool	India/1962	No history
NQ-38	011-1/stool	Japan/1991	India
NQ-71	011-2/stool	Japan/1991	India
NQ-114	011-3/stool	Japan/1992	Thailand
NQ-149	011-4/stool	Japan/1992	Philippines
NQ-180	011-5/stool	Japan/1992	Thailand
235-95	011-6/water	Brazil/1995	
1022-94	011-7/stool	Japan/1994	India
737-94	011-8/stool	Japan/1994	India
755-94	011-9/stool	Japan/1994	India
10843-62 (Ref. strain)	011-10/stool	India/1962	No history
NQ-28	013-1/stool	Japan/1991	Thailand
NQ-40	013-2/stool	Japan/1991	Thailand
NQ-42	013-3/stool	Japan/1991	Thailand
NQ-58	013-4/stool	Japan/1991	Thailand
NQ-128	013-5/stool	Japan/1992	Thailand
NQ-177	013-6/stool	Japan/1992	Thailand
73-95	013-7/stool	Japan/1995	Indonesia
74-95	013-8/stool	Japan/1995	Indonesia

Table 1 (*cont.*)

Strain designation	O-serotype/ Source	Country/ Year of isolation	Country of travel abroad
206-95	013-9/stool	Japan/1995	India
11416-62 (Ref. strain)	013-10/stool	India/1962	No history
NQ-83	017-2/stool	Japan/1991	Thailand
NQ-113	017-3/stool	Japan/1992	Thailand
NQ-168	017-4/stool	Japan/1992	India
NQ-173	017-5/stool	Japan/1992	Thailand
NQ-182	017-6/stool	Japan/1992	Thailand
NQ-217	017-7/stool	Japan/1993	Thailand
NQ-224	017-8/stool	Japan/1993	Thailand
NQ-242	017-9/stool	Japan/1993	Thailand
110-71 (Ref. strain)	017-10/stool	India/1971	No history
NQ-21	024-1/stool	Japan/1991	Philippines
NQ-69	024-2/stool	Japan/1991	Philippines
NQ-76	024-3/stool	Japan/1991	India
NQ-111	024-4/stool	Japan/1992	India
NQ-157	024-5/stool	Japan/1992	Philippines
NQ-162	024-6/stool	Japan/1992	Thailand
NQ-178	024-7/stool	Japan/1992	India
NQ-195	024-8/stool	Japan/1992	India
NQ-200	024-9/stool	Japan/1992	Thailand
14438-62 (Ref. strain)	024-10/stool	India/1962	No history
NQ-3	041-1/stool	Japan/1991	Thailand
NQ-7	041-2/stool	Japan/1991	India
NQ-27	041-3/stool	Japan/1991	Pakistan
NQ-39	041-4/stool	Japan/1991	Thailand
NQ-48	041-5/stool	Japan/1991	Hong Kong
NQ-52	041-5/stool	Japan/1991	Thailand
NQ-53	041-7/stool	Japan/1991	Thailand
NQ-72	041-9/stool	Japan/1991	Thailand
284-73 (Ref. strain)	041-10/stool	India/1973	No history
E8498‡	0141-1/water	USA/Louisiana§	
3176-78‡	0141-2/water	USA/Georgia§	
609-84‡	0141-3/stool	USA/New York§	—*
2454-85‡	0141-4/stool	USA/Tennessee§	—*
2466-85‡	0141-5/stool	USA/North Carolina§	—*
2527-86‡	0141-6/stool	USA/Maryland§	—*
2533-83‡	0141-7/stool	USA/California§	—*
F2031‡	0141-8/stool	Spain/1994	—*
574-94	0141-9/water	Bolivia/1992	
1178-96‡	0141-11/stool	Taiwan/1992	—*
930122	0141-12/water	Cambodia/1964	
CH236†	0141-13/shrimp	Germany/1995	
827-95	0141-14/water	Brazil/1995	
834-95	0141-15/water	Brazil/1995	
849-95	0141-16/water	Brazil/1995	
234-93‡	0141-10/stool	India/1993	No history

* No information available.
† Contained genes hybridizing with the NAG-ST probe.
‡ Contained genes hybridizing with the CT probe.
§ No information provided about the year of isolation.

strains and the scheme has successfully been used to study the epidemiology of *V. cholerae* [4].

Among a growing number of gene-based typing methods, determination of rRNA gene restriction (rDNA) fragment polymorphism (ribotyping) has proved to be a useful molecular epidemiologic technique in the study of *V. cholerae* [4, 8–10], although this technique was first described as a taxonomic tool for *V. cholerae* [11]. Dalsgaard and colleagues [10] previously reported a high degree of correlation between ribotypes and serotypes among environmental *V. cholerae* non-O1 strains isolated from shrimp farms in Thailand and among serotypes O10 and O12 strains associated with the outbreak of diarrhoea in Peru [4]. However, the correlation found between serotypes and ribotypes was based on a limited number of strains, including epidemiologically related strains [4, 10]. Thus further studies of other serotypes are needed to confirm this correlation and to determine whether serotypes of *V. cholerae* commonly associated with diarrhoea correspond to clonal lines as defined by ribotyping.

In the present study, the relationships between clinical *V. cholerae* non-O1 non-O139 strains of ten O-serotypes received at the National Institute of Infectious Diseases, Tokyo were analysed by serotyping and ribotyping. The main objective was to determine the variation of ribotype patterns within and between the individual serotypes and to investigate to what extent ribotypes could be used as epidemiological markers of clinical *V. cholerae* non-O1 non-O139 strains. On the basis of computerized analysis of ribotype patterns, a quantitative measure of the genetic relationships between the strains was expressed. Finally, the correlations between serotypes, ribotypes and the presence of CT and heat-stable enterotoxin (NAG-ST) genes were studied.

MATERIALS AND METHODS

Bacterial strains

V. cholerae non-O1 non-O139 strains to be included in the present study were selected among strains received for serotyping at the National Institute of Infectious Diseases in Tokyo. The majority of isolates in the strain collection were recovered from stool specimens of Japanese citizens with diarrhoea associated with travel to a foreign country, although a number of clinical and environmental isolates were received for serotyping from institutions all over the world. For the present study, a total of 103 strains representing 10 O-serotypes commonly associated with diarrhoea were selected randomly (Table 1). Strains were isolated from sporadic cases of diarrhoea which did not appear to be epidemiologically related. Most strains were isolated from 1991 to 1995, although several of the reference strains for each serotype were isolated from patients in India between 1960 and 1975. Two strains isolated from blood specimens and 16 serotype O141 strains isolated from water and stool samples outside Japan, mainly in the United States, were also studied. Each of the clinical O141 strains was isolated from sporadic cases of diarrhoea. Information about whether stool samples were cultured for other important enteric pathogens than *V. cholerae* was not available for the strains studied. For clarity, strain designations used in the text include the O-serogroup number followed by an arbitrarily number assigned in series (Table 1).

Serotyping

Strains studied were identified as *V. cholerae* non-O1 non-O139 based on standard biochemical reactions [12] and negative reactions in the agglutination tests employing polyvalent O1 and polyclonal O139 antisera (National Institute of Infectious Diseases, Japan). Each of the *V. cholerae* non-O1 non-O139 strains listed in Table 1 were examined serologically by the slide agglutination test and designated according to an extended serotyping system (O1 to O140) established by Shimada and colleagues [7]. However, the system has been further developed and currently includes antisera for 193 different O-serotypes (Dr T. Shimada, personal communication). Preparation of O-antisera and slide agglutination were performed as previously described [7].

Colony hybridization with CT and NAG-ST probes

All isolates 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 [13–15]. Prehybridization and hybridization were performed as previously reported and the hybridization filters developed colorimetrically [16]. NAG-ST-producing *V. cholerae* O14 strain A5 [17, 18] and CT-producing *V. cholerae* O1 strain 889 [3] were used as positive controls on all filters.

Ribotyping and data analysis

Each of the *V. cholerae* non-O1 non-O129 strains listed in Table 1 were ribotyped. Total bacterial DNA was extracted by the method of Murray and Thompson [19]. Based on previous studies [4, 10], *BglI* provided the best discrimination among *V. cholerae* non-O1 and was therefore used to digest chromosomal DNA. Ribotyping was performed by the procedure described by Dalsgaard and colleagues [10] with digoxigenin-labelled 16S and 23S rRNA probes A 1 kb molecular-weight standard (Gibco–BRL, Gaithersburg, Maryland) was used as a size marker in every third or fourth lane in the gels. Ribotype patterns were considered to be different when there was a difference of one or more bands between isolates.

Ribotype patterns were analysed visually and by the GelCompar software package (version 3.1; Applied Maths, Kortrijk, Belgium) [20]. In GelCompar, the positions of the 1 kb molecular-weight standard fragments were used as external reference bands. In addition, a number of common fragments shown by the strains were used as internal reference bands. Following alignment, the positions of restriction bands were analysed using fine optimization and a 1·0% position tolerance. The similarity between individual strains was estimated using the Dice coefficient (S_d) as a measure of similarity: $S_d = [2A/(2A+B+C)] \times 100$, where A is the number of matching bands and B and C are the numbers of bands present in one strain but not in the other [21]. Cluster analysis of ribotype patterns based on the similarity matrix obtained was performed using the unweighted paired group method of arithmetic averages (UPGMA) procedure in which a strain joins a group at the average similarity between the strain and all strains of the group [22]. It should be noted that the different restriction fragments in a ribotype may not be independent, as for example when the gain of a restriction site result in the loss of a fragment and the creation of two new fragments. Thus, the assumption that the restriction fragments analysed when producing a dendrogram are independent, may not always be fulfilled. In GelCompar, error flags may be added to the dendrogram representing the standard deviations between the clustering levels and the similarity matrix for each bifurcation in the dendrogram. The mean S_d estimated for each O-serogroup and 95% confidence intervals were calculated as previously described [23].

RESULTS

Serotyping, NAG-ST and CT genes

Each of the strains studied showed typical biochemical reactions of *V. cholerae*. Strains agglutinated antisera of each of the following serotypes: O2 (10 strains), O5 (10 strains), O6 (10 strains), O7 (9 strains), O11 (10 strains), O13 (10 strains), O17 (9 strains), O24 (10 strains), O41 (9 strains), and O141 (16 strains) (Table 1). Three serotype O6 (strains (O6-6, O6-7, and O6-9) and strain O141-13 isolated in Germany from imported shrimp harboured NAG-ST genes. Ten of 16 (63%) O141 strains contained CT genes, including 5 clinical strains and 2 strains isolated from water samples in the United States. No additional information could be obtained about the strains isolated in the United States. The remaining three CT-positive O141 strains were isolated from patients with diarrhoea in Spain, India and Taiwan (Table 1). Repeated serotyping confirmed the serotype of all O141 strains. All NAG-ST- and CT-positive isolates yielded positive colonies on the hybridization filters in repeated testing.

Ribotyping

Altogether, 67 different *BglI* ribotype patterns were observed among the 103 strains studied (Fig. 1). Patterns contained 8–15 DNA fragments ranging from 1·5 to 12 kb in length, although a few strains showed ribotypes with single fragments > 12 kb. However, fragments > 12 kb were not included in the comparison analysis of ribotypes as the 1 kb molecular-weight standard does not contain fragments > 12 kb. Some strains showed variations in colony morphology, including opaque, translucent and dry flat colony types. However, on repeated testing each of these strains showed an identical ribotype. The number of different ribotypes within each serotype and the mean S_d estimated for serotypes, including 95% confidence intervals, are summarized in Table 2.

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 8·0–10·5 kb showed a high degree of variability. A common fragment of approximately 3·5 kb was present in all strains, although this fragment often appeared as a weak band. An example of ribotype patterns is shown in Figure 2, which shows nine

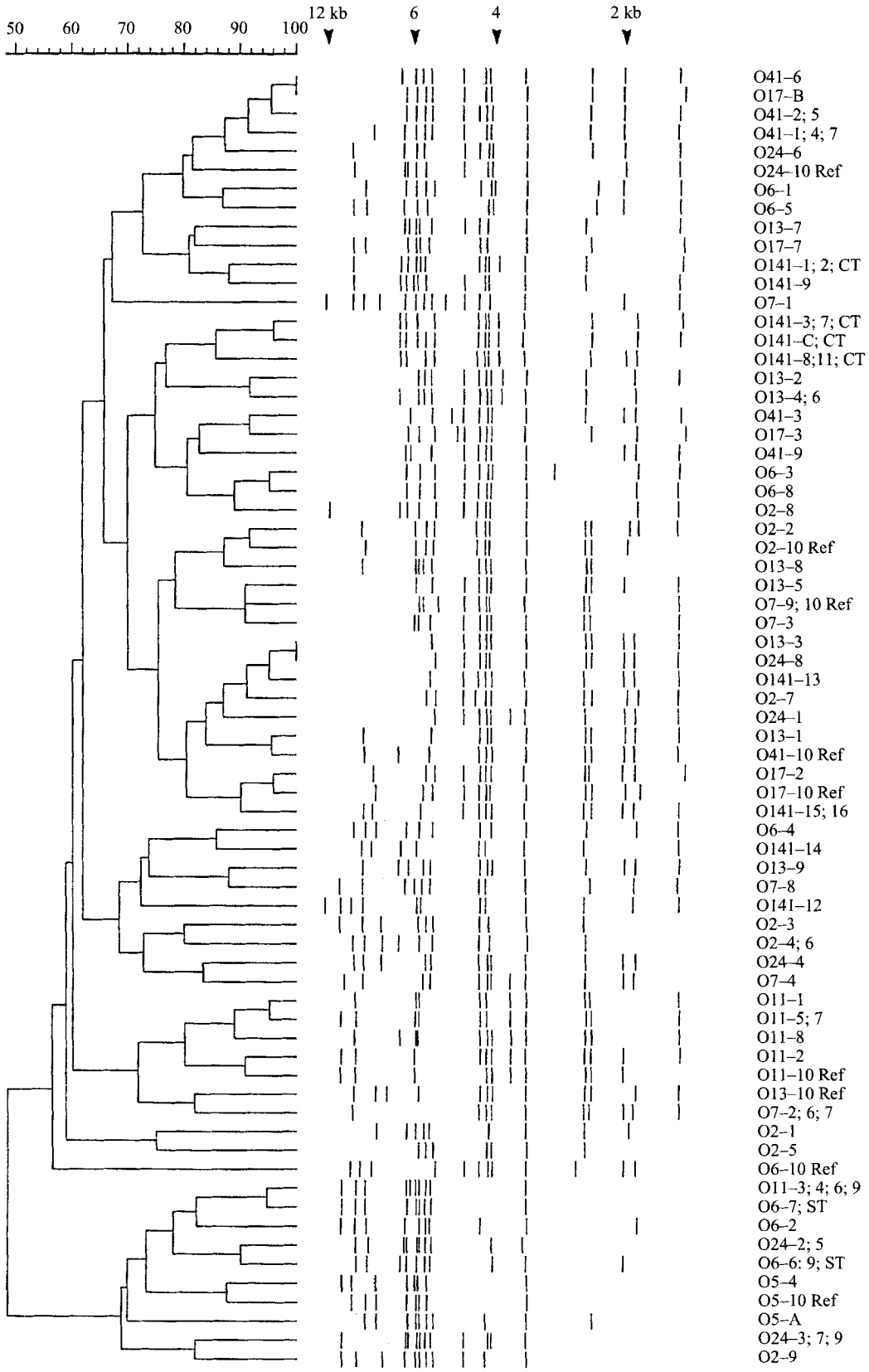

Fig. 1. Ribotype clusters obtained from analysis of 103 *V. cholerae* strains representing ten O-serotypes using fine optimization, 1·0% position tolerance, Dice coefficient of similarity and clustering by UPGMA. Degrees of similarity are shown in percent. The serotype designations of the strains are indicated to the right of the dendrogram. Molecular sizes are

Table 2. *Similarity and number of* BglI *ribotypes of* V. cholerae *non-O1 non-O139 in relation to O-serotypes*

O-serotype	No. of strains tested	No. of ribotypes	S_d* in % with 95% confidence intervals
O2	10	9	64±4
O5	10	3	90±4
O6	10	9	64±4
O7	9	6	66±5
O11	10	6	65±7
O13	10	9	72±3
O17	9	5	82±5
O24	10	7	62±6
O41	9	6	84±4
O141	16	9	72±3

* S_d, Mean Dice similarity coefficient (see Materials and Methods).

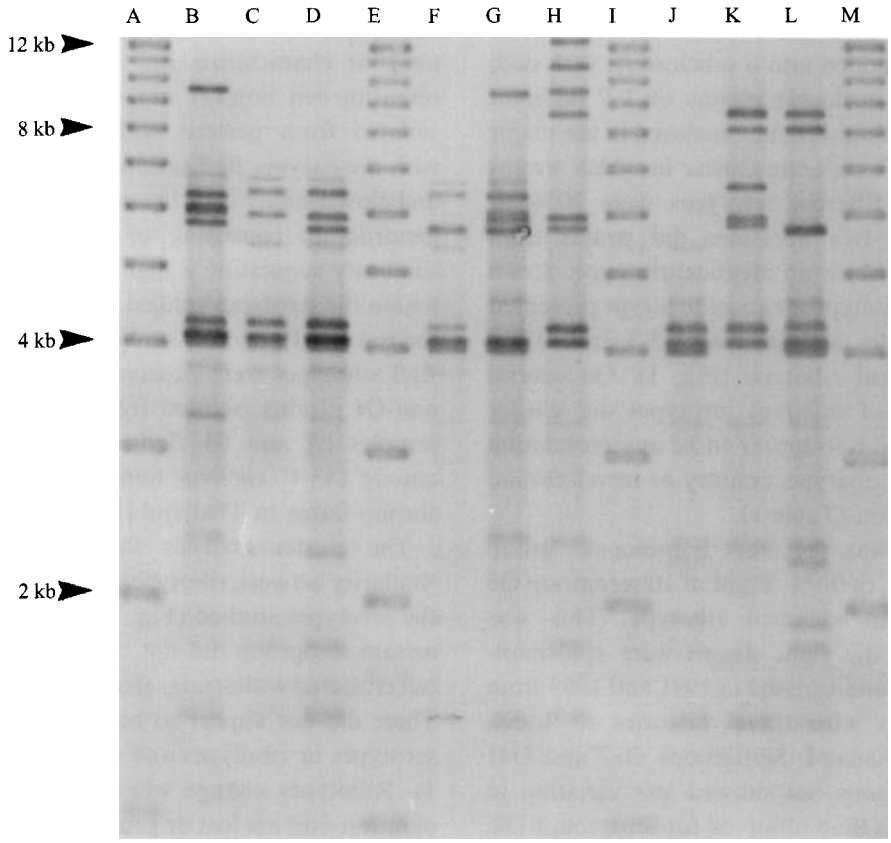

Fig. 2. Examples of *BglI* ribotypes of *Vibrio cholerae* serogroup O141 strains. Lanes: A, 1 kb molecular-weight standard; B, O141-2; C, O141-3; D, O141-6; E, 1 kb molecular-weight standard; F, O141-8; G, O141-9; H, O141-12; I, 1 kb molecular-weight standard; J, O141-13; K, O141-14; L, O141-15; M, 1 kb molecular-weight standard.

indicated by numbers at the top. Notes to serotypes designations: O5-A include strains O5-1, O5-2, O5-5, O5-6, O5-7, O5-8, and O5-9; O17-B include stains O17-4, O17-5, O17-6, O17-8, and O17-9; O141-C include strains O141-4, O141-5, O141-6, and O141-10; CT indicated cholera-toxin positive strains; ST indicates heat-stable enterotoxin (NAG-ST)-positive strains; Ref indicates reference strains.

different ribotypes seen among O141 strains. The dendrogram produced by the cluster analysis, including schematic presentations of the banding patterns, is shown in Figure 1. When error flags were produced for the dendrogram their lengths indicated that there was considerably discrepancy between the calculated similarities between the strains and those displayed in the dendrogram (for purposes of clarification error flags are not shown in Fig. 1). However, carrying out additional cluster analyses after changing the position tolerance in the range from 0·6 to 1·2% produced clusters in which only a few strains changed clusters.

A major cluster, comprising 79 of the 103 *V. cholerae* strains ribotyped, appeared in the dendrogram (Fig. 1; the upper major part of the dendrogram including the reference strain O6-10). Members of this cluster were 55% or more related to each other, as far as ribotypes were concerned. However, this cluster could be further divided into 6 subclusters, with each of the 6 clusters, enclosing strains of 3–7 different serotypes. Strains that were not enclosed in the major cluster comprised a separate cluster in which strains representing five different serotypes were 70% or more related. On two occasions did strains from different serotypes show an identical ribotype. Strain O41-6 showed a ribotype identical to a type presented by five O17 isolates and strains O13-3 and O24-8 showed an identical ribotype (Fig. 1). On several occasions strains of different serotypes did cluster together. There did not appear to be any correlation between serotype, ribotype, country of travel abroad and year of isolation (Table 1).

Serogroup O5 was the most homologous group showing a mean S_d of 90%. Eight of 10 serogroup O5 strains showed an identical ribotype. This was remarkably since the eight strains were epidemiologically unrelated and isolated in 1991 and 1993 from patients in Japan with travel histories to India, Indonesia, and Thailand. Serogroups O17 and O41 were less homologous but showed less variation in ribotyping patterns than observed for serogroups O2, O6, O7, O11, O13, O24 and O141 (Table 2).

Strains O6-6 and O6-9, which were NAG-ST positive and isolated in 1993 and 1992, respectively, showed an identical ribotype. A third NAG-ST positive strain O6-7 showed a ribotype closely related to the type presented by strains O6-6 and O6-9 (Fig. 1).

The CT-positive strains O141-1 and O141-2 isolated from water samples in the United States showed an identical ribotype which showed a similarity of only 66% to ribotypes shown by the remaining CT-positive O141 strains isolated from persons with diarrhoea. Although the clinical strains originated from several different countries and were isolated at different time periods, they showed identical or very closely related ribotypes with similarities in ribotyping patterns of more than 85% (Figs 1, 2). The CT-negative O141 strains that were isolated from environmental samples showed different ribotypes with low similarities to ribotypes observed among the clinical strains.

Comparison of the typing patterns of the ten reference strains by cluster analysis showed a high degree of heterogeneity with a S_d of 58%.

DISCUSSION

In the present study serotyping and ribotyping were used to characterize 103 *V. cholerae* strains representing ten non-O1 non-O139 serogroups mainly isolated from persons with diarrhoea. Ribotyping with the enzyme *BglI* produced 67 different ribotypes and cluster analysis of the typing patterns produced a dendrogram consisting of several clusters of low similarity suggesting a high level of genetic diversity within the serotypes studied. This is in agreement with the results of previous studies where 47 heterogenous *BglI* ribotypes were observed among 64 *V. cholerae* non-O1 strains isolated from patients and seafood samples [9] and 64 *BglI* ribotypes were observed among 143 *V. cholerae* non-O1 strains isolated from shrimp farms in Thailand [10].

The cluster analysis showed a low degree of similarity between ribotypes and serotypes for eight of the serotypes studied (Fig. 1). Often strains within a certain serogroup did not cluster in the dendrogram but clustered with strains from several other serotypes. There did not appear to be any correlation between serotypes or ribotypes and countries of travel (Table 1). Ribotypes change when restriction enzyme recognition sites are lost or gained within or in particular between the rRNA operons. The rate of such genetic changes increases with time, but is also associated with selective pressure from the environment and within the human host. The increasing number of serotypes being reported, especially among environmental strains of *V. cholerae*, suggest that only a fraction of the existing serotypes of *V. cholerae* have been described. Furthermore, *V. cholerae* may also change serotype. It is unknown but unlikely that the

changes in the genetic elements responsible for changes in ribotypes and serotypes should in any way be linked. Thus, a low similarity between ribotypes and serotypes, especially over time, is to be expected.

However, strains in serotype O5 belonged to the same cluster with typing patterns showing a S_d of 90%. Furthermore, the identical ribotype shown by eight epidemiological unrelated serotype O5 strains isolated from persons with travel histories to different countries suggest that O5 strains have undergone few genetic changes and may be of recent origin and correspond to a clonal line. We are planning further studies of additional strains to confirm the high degree of homology among serotype O5 and to determine factors responsible for the apparent clonal origin of this serotype.

The relative high diversity of ribotypes as indicated by a low S_d value within each serotype is in disagreement with the results of a previous study of *V. cholerae* non-O1 isolated from shrimp farms in Thailand, which reported identical *Bgl*I ribotypes within each of four serotypes studied [10]. However, the strains isolated from the shrimp farms were recovered within a 7-month study period from water, sediment and shrimp samples obtained from farms situated in the same geographical area [10]. Thus these relatively few strains, which belonged to different serotypes than the strains included in the present study, may be regarded as epidemiologically related. In outbreaks of diarrhoea associated with *V. cholerae* non-O1, identical or very closely related ribotypes would be expected. This was confirmed by our previous findings in Peru were serotypes O10 and O12 strains associated with an outbreak of diarrhoea showed an identical or very closely related ribotypes, respectively [4].

Although the ribotypes showed a high degree of diversity each type presented fragments within five major size ranges (Fig. 2). These findings corroborate with previous studies of both clinical and environmental *V. cholerae* non-O1 strains [4, 9, 10] and *V. cholerae* O1 [8] which all showed ribotypes presenting restriction fragments within similar size ranges. Thus it appears, that all *V. cholerae* strains show *Bgl*I ribotypes consisting of fragments within similar size ranges. Further studies are needed, including ribotyping of other *Vibrio* spp. and non-vibrio bacteria, to determine if these size ranges of ribotyping fragments are specific for the species *V. cholerae*.

Although, CT and NAG-ST have been described as important virulence factors in *V. cholerae* non-O1 non-O139, these toxins seems to be relatively rare among clinical as well as environmental isolates [24–26]. A previous study of about 2500 *V. cholerae* non-O1 strains isolated in the Louisiana Gulf Coast environment found that only seven strains carried the CT gene [27]. It is apparent that the virulence of non-O1 and non-O139 strains in multifactorial and most likely is mediated by several traits functioning in an integrated fashion [25]. It was therefore expected, as shown in the present study, that a low prevalence of strains contained these virulence genes. However, it was unexpected and quite surprising that strains encoding CT were found among a high percentage of serotype O141 strains. Ten of 16 O141 strains studied contained CT genes, including all seven O141 strains recovered from stool and water samples in the United States. Unfortunately, we were not able to obtain additional information about the O141 strains isolated in the United States. The finding of CT-positive O141 strains with identical or closely related ribotypes, especially among environmental and clinical strains in the United States, is disturbing and suggest that this serotype may be of clonal origin and has the potential to cause cholera-like diarrhoea. This is to our knowledge the first report of CT-positive O141 strains associated with diarrhoea. Studies are in progress to determine if the CT-positive O141 strains contain other important genes commonly found in the virulence cassette of serogroup O1 and O139 strains, the only two serogroups that are currently causing cholera. In a previous study of *V. cholerae* non-O1, CT and toxin co-regulated pili (*tcpA*) genes, with the latter being important for the successful colonization in the small intestine, were found among serotype O23 strains isolated from water samples in Australia [28]. In addition, Echeverria and colleagues [29] reported that five environmental strains isolated in Thailand in 1981, which belonged to serotypes O44, O49 and O8, each contained DNA sequences for the *V. cholerae* virulence gene complex. Thus is appears, that other serogroups than O1 and O139 may have the virulence cassette necessary to cause cholera-like diarrhoea, however, the epidemic potential of such strains is unknown but should be monitored carefully.

Although strains in the present study which contained CT or NAG-ST genes showed unique ribotypes not seen among virulence genes negative strains, it was reported in a previous study that NAG-ST-positive strains showed distant related ribotypes also presented by NAG-ST-negative strains [10]. Future studies should monitor the presence of

virulence genes, in particular CT genes, among clinical isolates of *V. cholerae* non-O1 non-O139 serotypes.

The sudden appearance and explosive spread of *V. cholerae* O139 have stressed the importance of studying *V. cholerae* non-O1 strains associated with diarrhoea, but also their presence in the aquatic environment. Although the prevalence of *V. cholerae* O139 appears recently to have decreased dramatically in South-east Asia, our results, in addition with previous studies, show that other serotypes remain important causes of sporadic cases and outbreaks of diarrhoea [1, 4]. Thus, serotyping together with ribotyping appears to be valuable tools in studying the epidemiology of *V. cholerae* non-O1 non-O139.

ACKNOWLEDGEMENTS

We thank Lise Høi and John E. Olsen at the Royal Veterinary and Agricultural University for help and suggestions in preparation of this manuscript.

This study was supported by a grant to A. Dalsgaard from the Danish Council for Development Research (Danida grant 90810).

REFERENCES

1. Morris JHJ. Non-O1 group *Vibrio cholerae* strains not associated with epidemic disease. In: Wachsmuth IK, Blake PA, Olsvik Ø, eds. *Vibrio cholerae* and cholera. Molecular to globular perspectives. Washington, DC: ASM Press, 1994: 103–15.
2. Finch MJ, Valdespino JL, Wells JG, et al. Non-O1 *Vibrio cholerae* infections in Cancun, Mexico. Am J Trop Med Hyg 1987; **36**: 393–7.
3. Bagchi K, Echeverria P, Arthur JD, Sethabutr O, Serichantalergs O, Hoge CW. Epidemic of diarrhea caused by *Vibrio cholerae* non-O1 that produced heat-stable toxin among Khmers in a camp in Thailand. J Clin Microbiol 1993; **31**: 1315–7.
4. Dalsgaard A, Albert MJ, Taylor DN, et al. Characterization of *Vibrio cholerae* non-O1 serogroups obtained form an outbreak of diarrhea in Lima, Peru. J Clin Microbiol 1995; **33**: 2715–22.
5. Nacescu N, Ciefecu C. Serotypes of Nag vibrios isolated from clinical and environmental sources. Zentralbl Bakteriol Hyg 1978; **A240**: 334–8.
6. Sanyal SC, Singh SJ, Tiwari IC, et al. Role of household animals in maintenance of cholera infection in a community. J Infect Dis 1974; **130**: 575–9.
7. Shimada T, Arakawa E, Itoh K, et al. Extended serotyping scheme for *Vibrio cholerae*. Curr Microbiol 1994; **28**: 175–8.
8. Popovic T, Bopp CA, Olsvik Ø, Wachsmuth K. Epidemiologic application of a standardized ribotype scheme for *Vibrio cholerae* O1. J Clin Microbiol 1993; **31**: 2474–82.
9. Dalsgaard A, Serichantalergs O, Pitarangsi C, Echeverria P. Molecular characterization and antibiotic susceptibility patterns of *Vibrio cholerae* non-O1. Epidemiol Infect 1995; **114**: 51–63.
10. Dalsgaard A, Echeverria P, Larsen JL, Siebeling R, Serichantalergs O, Huss HH. Application of ribotyping for differentiating *Vibrio cholerae* non-O1 isolates from shrimp farms in Thailand. Appl Environ Microbiol 1995; **61**: 245–51.
11. Grimont F, Grimont PAD. Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. Ann Inst Pasteur Microbiol 1986; **137B**: 165–75.
12. Sakazaki R. Bacteriology of vibrio and related organisms. In: Barua D, Greenough WR, eds. Cholera. New York: Plenum Publishing Company, 1992: 37–55.
13. Ogawa A, Kato J, Watanabe H, Nair BG, Takeda T. Cloning and nucleotide sequence of a heat-stable enterotoxin gene from *Vibrio cholerae* non-O1 isolated from a patient with traveller's diarrhea. Infect Immun 1990; **58**: 3325–9.
14. Arita M, Honda T, Miwatani T, Ohmori K, Takao T, Shimonishi Y. Purification and characterization of a new heat-stable enterotoxin produced by *Vibrio cholerae* non-O1 serogroup Hakata. Infect Immun 1991; **59**: 2186–8.
15. Wright AC, Guo Y, Johnson JA, Nataro JP, Morris JG, Jr. Development and testing of a non-radioactive DNA oligonucleotide probe that is specific for *Vibrio cholerae* cholera toxin. J Clin Microbiol 1992; **30**: 2302–6.
16. Oprandy JJ, Thornton SA, Gardiner CH, Burr D, Batchelor R, Bourgeois AL. Alkaline phosphatase-conjugated oligonucleotide probes for enterotoxigenic *Escherichia coli* in travellers to South America and West Africa. J Clin Microbiol 1988; **26**: 92–5.
17. Arita M, Takeda T, Honda T, Miwatani T. Purification and characterization of *Vibrio cholerae* non-O1 heat-stable enterotoxin. Infect Immun 1986; **52**: 45–9.
18. Dalsgaard A, Serichantalergs O, Shimada T, Sethabuthr O, Echeverria P. Prevalence of *Vibrio cholerae* with heat-stable enterotoxin (NAG-ST) and cholera toxin genes; restriction fragment length polymorphisms of NAG-ST genes among *Vibrio cholerae* O serogroups from a major shrimp production area in Thailand. J Med Microbiol 1995; **43**: 216–20.
19. Murray MG, Thompson WF. Rapid isolation of high molecular weight DNA. Nucl Acids Res 1980; **8**: 4321–25.
20. Vauterin L, Vauterin P. Computer-aided objective comparison of electrophoresis patterns for grouping and identification of microorganisms. Eur Microbiol 1992; November/December: 37–42.
21. Römling U, Grothues D, Heuer T, Tümmler B. Physical genome analysis of bacteria. Electrophoresis 1992; **13**: 626–31.
22. Sneath PHA, Sokal RR. Numerical taxonomy. San Francisco: Freeman, 1973.

23. Dunn OJ. Estimation of population means: confidence intervals. Basic statistics: a primer for the biomedical sciences. New York: John Wiley & Sons, 1964: 60–80.
24. Pal A, Ramamurthy T, Bhadra RK, et al. Reassessment of the prevalence of heat-stable enterotoxin (NAG-ST) among environmental *Vibrio cholerae* non-O1 strains isolated from Calcutta, India, by using a NAG-ST DNA probe. Appl Environ Microbiol 1992; **58**: 2485–9.
25. Ramamurthy T, Bag PK, Pal, A, et al. Virulence patterns of *Vibrio cholerae* non-O1 strains isolated from hospitalised patients with acute diarrhoea in Calcutta, India. J Med Microbiol 1993; **39**: 310–7.
26. Morris GJ, R., Takeda T, Tall BD, et al. Experimental non-O group *Vibrio cholerae* gastroenteritis in humans. J Clin Invest 1990; **85**: 697–705.
27. Roberts NC, Siebeling RJ, Kaper JB, Bradford HBJ. Vibrios in the Louisiana Gulf Coat environment. Microb Ecol 1982; **8**: 299–312.
28. Said B, Smith HR, Scotland SM, Rowe B. Detection and differentiation of the gene for toxin co-regulated pili (*tcp*A) in *Vibrio cholerae* non-O1 using the polymerase chain reaction. FEMS Microbiol Lett 1995; **125**: 205–10.
29. Echeverria P, Hoge CW, Bodhidatta L, et al. Molecular characterization of *Vibrio cholerae* O139 isolates from Asia. Am J Trop Med Hyg 1995; **52**: 124–7.