Epidemiol. Infect. (1996), **117**, 51–58

Comparison of pulsed-field gel electrophoresis and ribotyping for subtyping of *Vibrio cholerae* O139 isolated in Thailand

A. DALSGAARD[1], M. N. SKOV[1], O. SERICHANTALERGS[2] AND P. ECHEVERRIA[2]

[1] *Department of Veterinary Microbiology, The Royal Veterinary and Agricultural University, DK-1870 Frederiksberg C, Denmark*
[2] *Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand*

(*Accepted 8 February 1996*)

SUMMARY

Pulsed-field gel electrophoresis (PFGE) of *Cpo* I-digested genomic DNA and ribotyping (*Bgl* I) were applied to 60 *Vibrio cholerae* strains including 48 *V. cholerae* O139 from Thailand to compare their value in differentiating strains of the present *V. cholerae* O139 epidemic. PFGE patterns were divided into groups A and B representing five and four subtypes, respectively, while ribotyping showed four different patterns. PFGE group B subtypes were only presented among O139 isolates from Thailand, whereas four O139 strains from Bangladesh and India showed identical PFGE group A subtypes observed in O139 isolates from Thailand. Two non-toxigenic O139 isolates from Thailand showed different and unique PFGE types as did five *V. cholerae* non-O1 non-O139 isolates containing a gene virulence complex found in *V. cholerae* O139. These results indicate that PFGE (*Cpo* I) can resolve recent evolutionry divergence within *V. cholerae* O139 and offers a useful supplementary tool for following the progressing *V. cholerae* O139 epidemic.

INTRODUCTION

In October 1992, an outbreak of a cholera-like disease occurred in Madras, India, and subsequently spread throughout India and Bangladesh [1, 2]. The causative organism of this outbreak was antigenically unique and was designated *V. cholerae* O139 synonym Bengal [2, 3]. In early 1993, *V. cholerae* O139 was identified for the first time in Thailand and rapidly spread within the country [4–6]. Since the beginning of the outbreak *V. cholerae* O139 has spread to several Asian countries, demonstrating the pandemic potential of this new serotype [7–9].

In recent years, a variety of molecular methods have been applied to *V. cholerae* epidemiology in an effort to obtain a more fundamental genetic assessment of strain interrelationships. Such approaches, aimed at direct genomic comparisons, include the analysis of chromosomal restriction fragment length polymorphisms (RFLPs) [10], ribotyping [11–13], and pulsed-field gel electrophoresis (PFGE) [14, 15].

However, the application of these techniques to differentiate among *V. cholerae* O139 strains has shown little or no variation [10, 16, 17] with some reports suggesting a clonal nature of *V. cholerae* O139 [5, 18]. Studying a collection of 53 *V. cholerae* O139 strains isolated from patients in Bangladesh, India and Thailand we recently demonstrated four different *Bgl* I ribotypes [5]. Furthermore, O139 strains from Thailand showed a unique ribotype compared with strains from India and Bangladesh [5]. Although ribotyping showed variation among *V. cholerae* O139 strains the method did not discriminate strains with no epidemiological relationship. Therefore, a methodology which shows a high discriminatory power is

needed for successful monitoring the spread of *V. cholerae* O139.

In the present study, we applied the PFGE technique testing a number of restriction enzymes to determine whether this technique could be used to differentiate among *V. cholerae* O139 strains mainly isolated in Thailand [19]. In addition, we compared PFGE types with results previously obtained by ribotyping [5] to establish a possible link between the two techniques, and to distinguish strains exhibiting identical ribotypes.

MATERIALS AND METHODS

Bacterial strains

Forty-eight *V. cholerae* O139 strains isolated from patients at hospitals in Thailand between August and October 1993 and four O139 strains from Bangladesh and India were examined. We previously reported that all strains agglutinated O139 specific antisera and contained genes coding for a virulence gene complex, except strains NG 288/36 and NG 653/36 which lacked the virulence complex genes [5, 9]. One specimen that contained the gene complex, a *V. cholerae* O1 serotype Inaba strain, collected in 1993 in Peru from a patient with cholera-like diarrhoea in Lima and a *V. cholerae* O1 strain from Thailand were also studied [5]. In addition, five *V. cholerae* non-O1 non-O139 strains (NWBD 40/1, WBDH 697, WBDV 101E, WBDH 712 and FY2G) previously shown to contain similar virulence genes found in O139 were studied [5]. The five strains were recovered in northeastern Thailand in 1982 from different environmental sources [20, 21]. All strains examined are listed in Table 1.

PFGE

Strains were grown as previously described [22] and DNA was prepared directly in a solid agarose plug (Bio-Rad, Hercules, CA, USA) as described by Cameron and colleagues [15]. For restriction endonuclease digestion thin slices were cut off the agarose plugs, equilibrated in the appropriate nuclease buffer for 1 h, and then digested 4 h with 20 U of enzyme per plug. If strains showed weak DNA fragment bands after electrophoresis and visualization the amount of enzyme was increased to 30 U and incubation was carried out overnight. In addition, new DNA preparations were performed of isolates which showed weak fragment bands. All enzymes screened, including *Cpo* I, *Not* I, *Sfi* I, *Sma* I, *Swa* I, *Xba* I and *Xho* I (Amersham, Arlington Heights, Il, USA), were used according to the manufacturer's recommendation.

The samples were loaded as solid plugs into the wells of a 1·0% agarose (Litex LSL, Denmark) gels prepared in 0·5 TBE buffer (45 mM Tris, 45 mM boric acid, 2 mm Na_2-EDTA, pH 8·3). PFGE was carried out by using a modified contour-clamped homogeneous electric field (CHEF) system [23] (Pulsaphor Plus, Pharmacia LKB, Sweden). The running conditions were 12 V/cm at 14 °C for 22 h. The pulse times were increased as follows: 5 s for 3 h, 9 s for 5 h, 12 s for 5 h, 20 s for 4 h, 25 s for 3 h and 30 s for 2 h. Multimeric phage lambda (48·5 kb) DNA (Pharmacia LKB) was used as size standard. After electrophoresis, gels were stained in ethidium bromide (Sigma) (2 μg/ml) for 15 min, destained in distilled water for 15 min, and photographed with 254-nm UV transillumination.

Ribotyping

Total bacterial DNA was extracted from each isolate tested by the method of Pedersen and Larsen [24] followed by restriction with *Bgl* I enzyme. Ribotyping was performed with digoxigenin-labelled 16S and 23S rRNA probes as previously described [12, 13]. A 1 kb DNA molecular weight standard (0·1 μg; GIBCO BRL, Gaithersburg, MD, USA) was used as a molecular size marker. The ribotyping results have in part recently been published by Echeverria and colleagues [5].

RESULTS

Screening restriction enzymes for PFGE

Genomic DNA from 10 selected *V. cholerae* O139 isolates from Bangladesh, India and Thailand were initially digested with the seven restriction enzymes (data not shown). Restriction with *Xho* I and *Xba* I gave > 25 fragments of < 50 kb with limited discrimination between isolates. *Swa* I and *Sma* I digestion resulted in > 30 fragments of < 150 kb and approximately 25 fragments of < 100 kb, respectively. *Sfi* I and *Not* I gave a suitable distribution of fragments with > 30 fragments of < 550 kb and about 25 fragments of < 440 kb, respectively. However, the two enzymes provided no or little discrimination among isolates tested. Restriction with *Cpo* I gave

https://doi.org/10.1017/S0950268800001126 Published online by Cambridge University Press

Table 1. *V. cholerae* strains typed by PFGE and ribotyping

Strains	Origin*	O Sero-group †	Ribo-type (*Bgl* I)‡	PFGE (*Cpo* I)§	Strains	Origin	O Sero-group	Ribo-type (*Bgl* I)	PFGE (*Cpo* I)
SPH 898	Raj	139	1	A1	IPD 440	Bam	139	1	A5
ADC 1125/9	Bam	139	1	A1	ADC 856/9	Bam	139	2	B2
ADC 1551/8	Bam	139	1	A1	NG 465/36	NIH	139	2	B2
ADC 373/9	Bam	139	1	A1	V 39	CH	139	3	B1
ADC 573/9	Bam	139	1	A1	NIH 178	NIH	139	3	B1
NG 468/36	NIH	139	1	A1	ADC 820/7	Bam	139	3	B1
A4450	Ban	139	1	A1	NG 377/36	NIH	139	3	B1
ADC 711/9	Bam	139	1	A1	NG 392/36	NIH	139	3	B1
ADC 753/9	Bam	139	1	A1	NG 408/36	NIH	139	3	B1
ADC 910/9	Bam	139	1	A1	ADC 2039/6	Bam	139	3	B1
ADC 987/9	Bam	139	1	A1	ADC 978/6	Bam	139	3	B1
ADC 995/9	Bam	139	1	A1	ADC 700/7	Bam	139	3	B1
ADC 1095/9	Bam	139	1	A1	ADC 498/7	Bam	139	3	B1
ADC 1115/9	Bam	139	1	A1	IPD 58	Bam	139	3	B1
ADC 1207/9	Bam	139	1	A1	IPD 935	Bam	139	3	B1
ADC 1221/9	Bam	139	1	A1	IPD 694	Bam	139	3	B2
ADC 1243	Bam	139	1	A1	AD 2441	Bam	139	3	B2
NG 469/36	NIH	139	1	A1	ADC 1/7	Bam	139	3	B2
NG 482/36	NIH	139	1	A1	NG 368/36	NIH	139	3	B2
NG 483/36	NIH	139	1	A1	NG 481/36	NIH	139	3	B2
NG 487/36	NIH	139	1	A1	IPD 145	Bam	139	3	B3
NG 488/36	NIH	139	1	A1	ADC 225/8	Bam	139	3	B4
NG 489/36	NIH	139	1	A1	NG 288/36	NIH	139	4	C
NG 492/36	NIH	139	1	A1	NG 653/36	Thai	139	7	D
NG 493/36	NIH	139	1	A1	BAB 2209	Peru	1	3	E
A1841	Ban	139	1	A2	WBDV 101E	Soon	49	8	F
MO10	Mad	139	1	A2	WBDH 697	Soon	49	9	G
VC 4220/36	NIH	139	1	A3	WBDH 712	Soon	49	9	H
MO45‖	Mad	139	1	A4	FY2G	Soon	8	10	I
VC 889	Thai	1	1b¶	A4	NWBD 40/1	Soon	44	11	J

* All origins are from Thailand unless stated otherwise. Bam, Bamras; Ban, Bangladesh; CH, Childrens Hospital, Bangok; Mad, Madras, India; NIH, National Institute of Health, Bangkok; Raj, Rajburi; Soon, Soongnern; Thai, Thailand.
† According to the O serogroup established by Shimada and colleagues [31].
‡ Designations used by Echeverria and colleagues [5].
§ Only bands of > 100 kb were scored.
‖ Strain MO45 (ATCC 51394).
¶ Ribotype 1b lacked a 2·5 kb DNA fragment present in each isolate belonging to ribotype 1.

approximately 25 fragments of < 550 kb with a suitable fragment distribution. Compared with *Sfi* I and *Not* I, *Cpo* I provided the highest discrimination among the isolates tested. Hence, *Cpo* I was chosen for analyses of strains listed in Table 1.

PFGE

Only > 100 kb bands were scored and PFGE (*Cpo* I) types were considered to be different if they differed by one or more bands. PFGE analysis of 52 *V. cholerae* O139 isolated displayed 11 different patterns (Fig. 1, Table 1). The PFGE types were divided into two major groups arbitrary designated A and B, respectively. All group A isolates lacked a DNA fragment of approximately 235 kb which was present in all group B isolates. Although isolates within PFGE group A showed a very close resemblance, minor differences between the types were evident (Fig. 1). Hence, group A types were further divided into five subtypes, A1–A5. Among 32 *V. cholerae* O139 group A isolates, 24 Thai isolates and one strain A4450 from Bangladesh showed subtype A1. Whereas strain MO10 from India and strain A1841 from Bangladesh both showed subtype A2. Subtype A4 included strains

https://doi.org/10.1017/S0950268800001126 Published online by Cambridge University Press

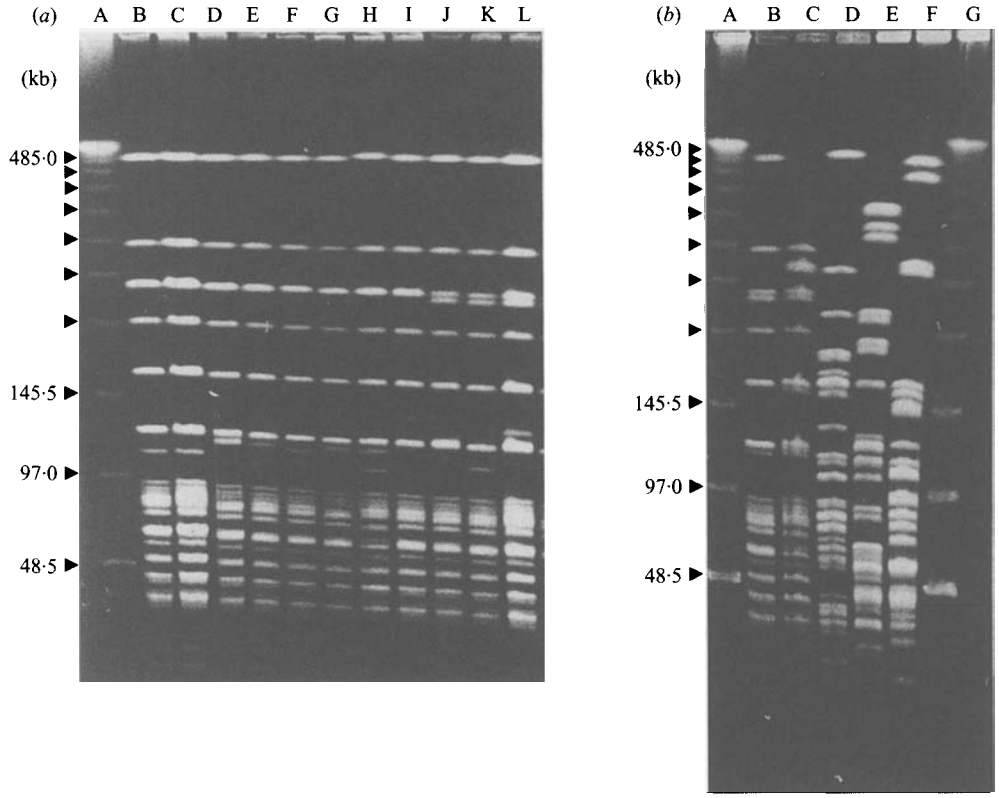

Fig. 1. PFGE types observed among *V. cholerae* O139 and non-O1 strains after DNA digestion with *Cpo* I. (*a*) Lanes: A, multimers of phage lambda DNA (48·5 kb) as molecular size markers; B, A 4450 (type A1); C, ADC 995/9 (type A1); D, MO10 (type A2); E, A 1841 (type A2); F, VC 4220/36 (type A3); G, MO45 (type A4); H, VC 889 (type A4); I, IPD 440 (type A5); J, ADC 2039/6 (type B1); K, NG 368/36 (type B2); L, IPD 145 (type B3). (*b*) Lanes: A and G, multimers of phage lambda DNA (48·5 kb) as molecular size markers; B, ADC 225/8 (type B4); C, BAB 2209 (type E); D, NG 288/36 (type C); E, NG 653/36 (type D); F, NWBD 40/1 F1 (type J).

MO45 from India and *V. cholerae* O1 strain VC 889 from Thailand. Both showed an additional 105 kb fragment which was not shown by other A subtypes. Subtype A5 was observed in a single isolate from Thailand.

Though *V. cholerae* O139 isolates within PFGE group B were closely related differences were clearly visible (Fig. 1, Table 1). Twelve isolates showed subtype B1, which lacked a 105 kb fragment seen among seven subtype B2 isolates. Strain IPD 145 possessed an additional 225 kb fragment compared with subtypes B1 and B2 and was categorized as subtype B3. Group B subtypes were shown only among O139 strains isolated in Thailand.

The non-toxigenic *V. cholerae* O139 strains NG 288/36 and NG 653/36 showed unique PFGE types differing by several fragments compared with toxigenic O139 strains. *V. cholerae* O1 strain BAB 2209, previously shown to exhibit ribotype 3 [5], and the five *V. cholerae* non-O1 non-O139 strains recovered from different environmental sources, all showed unique PFGE types which differed from the types shown by O139 isolates by several band fragments (Fig. 1, Table 1).

Ribotyping

Figure 2 shows examples of the *Bgl* I ribotypes. Thirty isolates belonging to ribotype 1 showed a 5·8 kb fragment which was absent in ribotypes 2, 3, and 4, respectively (data not shown for ribotype 4) [5]. Ribotype 3 contained 19 Thai isolates and one isolate from Peru which all lacked a 2·5 kb fragment seen among ribotypes 1 and 2 isolates. In addition, ribotype 3 isolates exhibited a 6·5 kb fragment which was absent in types 1 and 2 isolates.

DISCUSSION

In the present study, a total of 11 different *Cpo* I PFGE profiles and 4 different *Bgl* I ribotypes were demonstrated among 54 *V. cholerae* O139 isolates studied (Table 1). When ribotype patterns were

https://doi.org/10.1017/S0950268800001126 Published online by Cambridge University Press

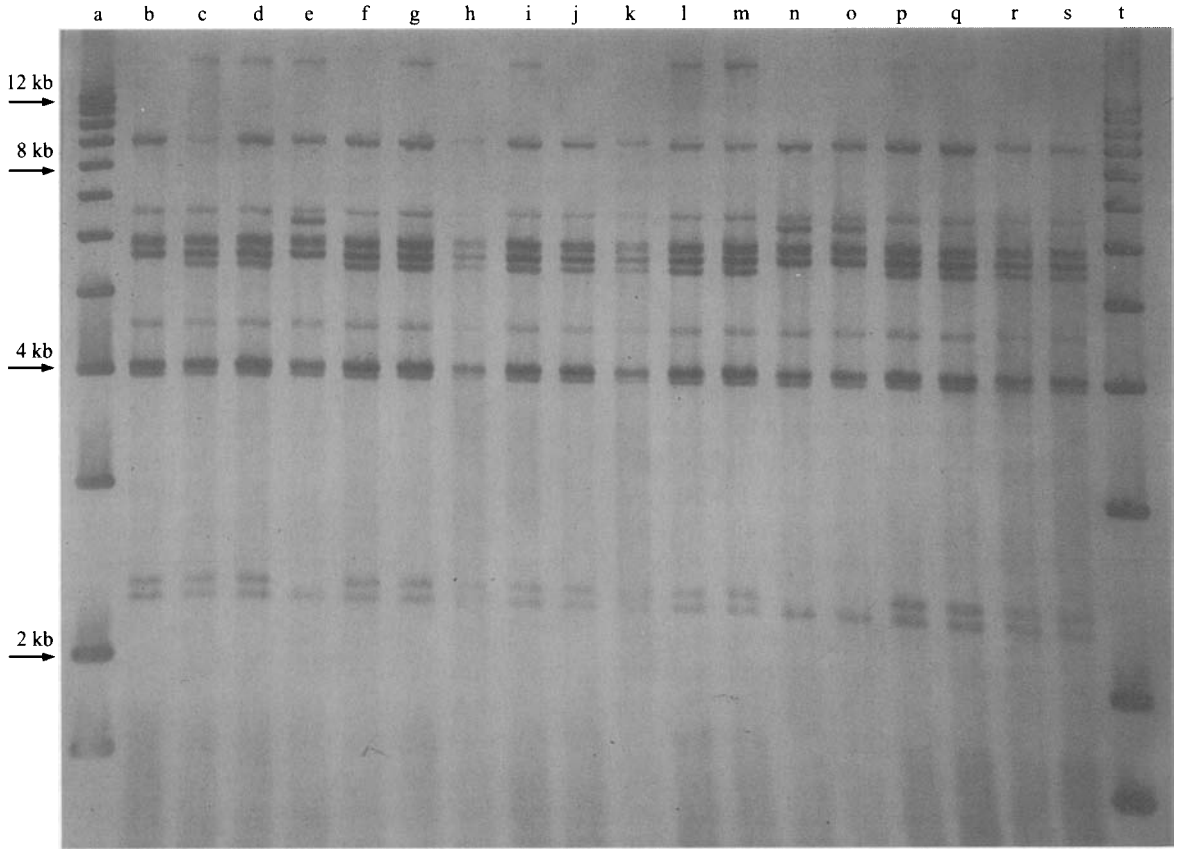

Fig. 2. Example of *Bgl* I ribotypes of *Vibrio cholerae* O139 recovered from patients with diarrhea. Lanes: a, 1 kb DNA ladder; b, NG 465/36, Thailand, type 2; c, NG 468.36, Thailand, type 1; d, NG 469/36, Thailand, type 1; e, NG 481/36, Thailand, type 3; f, NG 482/36, Thailand, type 1; g, NG 483/36, Thailand, type 1; h, NG 487/36, Thailand, type 1; i, NG 488/36, Thailand, type 1; j, NG 489/36, Thailand, type 1; k, NG 492/36, Thailand, type 1; l, NG 493/36, Thailand, type 1; m, VC 4220/36, Thailand, type 1; n, NIH 178, Thailand, type 3; o, V 39, Thailand, type 3; p, SPH 895, Thailand, type 1; q, A 4450, Bangladesh, type 1; r, A 1841, Bangladesh, type 1; s, MO10, India, type 1; t, 1 kb DNA ladder.

compared with PFGE types, ribotyping did not discriminate among isolates belonging to the same PFGE profile. Whereas several PFGE subtypes were often presented within a specific ribotype. PFGE group A subtypes were only demonstrated among ribotype 1 isolates. However, PFGE subtypes A4 was presented by a *V. cholerae* O1 strain (VC 889) which showed a ribotype 1b lacking a 2·5 kb DNA fragment present in each isolate belonging to ribotype 1. The four isolates tested from Bangladesh and India showed identical PFGE group A subtypes demonstrated among O139 isolates from Thailand. Group B subtypes were only exhibited among Thai ribotypes 2 and 3 isolates.

Previous studies have shown patterns produced by PFGE to be reproducible and stable after 40 *in vitro* passages [25–27] and longitudinal recovery from chronically infected patients [26]. In the present study, all strains tested could be typed by PFGE and patterns produced were stable except a few strains which showed weak fragment bands only when inadequate amounts of *Cpo* I was used for DNA digestion.

In PFGE, digested plasmids migrate according to the sizes of fragments, while uncut plasmids are reported to present aberrant mobilities [28, 29]. Preliminary studies of the plasmid content of *V. cholerae* O139 strains studied indicated that they rarely contained plasmids. When present these plasmids were < 10 kb in size as shown in one-dimensional electrophoresis and appeared with sizes < 100 kb in PFGE (unpublished data). Hence, in our study where only fragments > 100 kb were scored, the presence of plasmids in DNA preparations was not anticipated to influence the interpretation and stability of typing results.

The origin of *V. cholerae* O139 has been discussed in several reports [9, 10, 16]. Mechanisms proposed for the emergence of this new serotype include (*a*) acquisition of the virulence gene complex by an

https://doi.org/10.1017/S0950268800001126 Published online by Cambridge University Press

environmental non-toxigenic *V. cholerae* O139 strain and (*b*) *V. cholerae* O139 emerged as a result of mutations in a *V. cholerae* strain already containing the gene complex, e.a. an El Tor vibrio [10]. In this regard, it is interesting that the environmental *V. cholerae* non-O1 strains detected in northeastern Thailand in 1982 contained similar virulence genes found in O139 strains [5]. However, since all strains showed unique and very different PFGE types compared with O139 strains, a serotype conversion to serogroup O139 as a result of mutations in these strains appears unlikely. We recently demonstrated the presence of two clinical non-toxigenic *V. cholerae* O139 strains (NG 288/36 and NG 653/36) [19]. Each of these strains showed a ribotype and a PFGE type very different from the types demonstrated by the toxigenic *V. cholerae* O139 strains studied. If toxigenic O139 strains were likely to have emerged from an environmental non-toxigenic *V. cholerae* O139 strain by acquisition of the virulence gene complex, one would expect very similar ribotypes and PFGE types of such strains. The origin of *V. cholerae* O139 remains to be elucidated. However, there are several cultural, physiological and genetic similarities between O139 strains and *V. cholerae* O1, especially the El Tor biotype [9].

A number of molecular subtyping methods have been applied to *V. cholerae* O139 [10, 18]. However, most of these studies have reported a clonal nature of O139 strains or demonstrated only few subtypes. Faruque and colleagues [10] compared the RFLPs of *ctx*A and rRNA genes of 29 *V. cholerae* O139 isolates from Bangladesh and India and found that the isolates comprised two cholera toxin (*ctx*) genotypes and all belonged to a single ribotype. PFGE of *Not* I digested DNA of nine *V. cholerae* O139 strains isolated in India revealed identical profiles, as did Southern blot hybridization of *Not* I digested DNA with a cholera toxin probe [17]. A clonal nature of *V. cholerae* O139 was also suggested in a PFGE (*Sma* I) study of O139 strains isolated in India, Bangladesh and Thailand as identical patterns were demonstrated [30], although the number of strains studied was not reported [30]. Applying PFGE (*Sfi* I and *Not* I) and an arbitrarily primed polymerase chain reaction technique to 11 *V. cholerae* O139 strains isolated in India, including the reference strain MO45, Berche and colleagues [18] reported identical typing patterns by both techniques.

However, a recent study of 21 *V. cholerae* O139 strains isolated in Bangladesh and India demonstrated two *Bgl* I ribotypes designated 3a and 5a, respectively [11, 16]. Compared with the ribotypes shown in the present study these ribotypes are identical to ribotypes 1 and 2, respectively. The 21 isolates were further subdivided into 4 PFGE (*Not* I) patterns, often differing by a single band, with 3 PFGE patterns shared among isolates belonging to the 2 ribotypes [16]. In comparison, we found none of 11 different PFGE (*Cpo* I) types shared among isolates belonging to 4 different ribotypes, except PFGE subtype B2 which was shared by ribotypes 2 and 3 isolates. Hence, PFGE of *V. cholerae* O139 using the restriction enzyme *Cpo* I appears superior compared with *Not* I in subtyping O139 strains. The discriminatory power of PFGE is dependent, among other factors, on the restriction enzyme in use. Our results emphasize the importance of screening an adequate number of strains with various restriction enzymes before the most appropriate enzyme is chosen.

Although several different PFGE subtypes were demonstrated among *V. cholerae* O139 strains recovered in Thailand, all isolates examined, except the two non-toxigenic O139 strains, exhibited highly conserved restriction fragment patterns closely related to the PFGE types seen among strains from Bangladesh and India, suggesting recent evolutionary divergence from a common isolate. The O139 strain is, however, evolving over time, as indicated by the different PFGE subtypes showed by O139 strains from Thailand. It is likely that these observed differences in PFGE types among Thai strains reflect random mutations that have occurred as the epidemic has spread. Therefore, PFGE might be used in retrospective studies designed to follow the O139 epidemic as it progressed. In this regard, it is interesting that ribotype 3 and PFGE subtypes B1, B2, B3 and B4 were unique for strains isolated in Thailand. Additional data should be obtained to confirm the geographic association of O139 strains showing ribotype 3 and PFGE group B types within Thailand.

Our study illustrates the advantage of using the remarkable resolving ability of the PFGE method to conduct comparative restriction fragment analysis. PFGE could differentiate among epidemiologically unrelated O139 strains that were indistinguishable by ribotyping. In addition, the need for blotting and probing is eliminated, and, unlike ribotyping, essentially the entire genome can be examined, thereby providing a greater degree of strain-specific discrimination. However, our data also show the limitations of PFGE when highly related strains are studied since

we did not demonstrate any correlation between certain PFGE types and the origin of the *V. cholerae* O139 isolates. Hence, it would be difficult to definitely determine by PFGE (*Cpo* I) the exact transmission of a particular *V. cholerae* O139 strain.

ACKNOWLEDGEMENTS

The technical assistance of Charlotte R. Rasmussen and Lasse Smith, the Royal Veterinary and Agricultural University, Denmark, and Chittima Pitarangsi, Apichai Srijan and Pranee Ratarasarn of the Armed Forces Research Institute of Medical Sciences, Bangkok is highly appreciated. We also thank T. Shimada, National Institute of Health, Tokyo, Japan for serotyping *V. cholerae* non-O139 non-O1. This study was supported by the Danish Council for Development Research, Danish International Development Assistance (DANIDA), fellowship no. 104.8/553 and the Plasmid Foundation.

REFERENCES

1. Albert MJ, Siddique AK, Islam MS, et al. Large outbreak of clinical cholera due to *Vibrio cholerae* non-O1 in Bangladesh. Lancet 1993; **341**: 704–5.
2. Cholera Working Group. Large epidemic of cholera-like disease in Bangladesh caused by *Vibrio cholerae* O139 synonym Bengal. Lancet 1993; **342**: 387–90.
3. Ramamurthy T, Garg S, Sharma R, et al. Emergence of novel strain of *Vibrio cholerae* with epidemic potential in southern and eastern India. Lancet 1993; **341**: 703–4.
4. Chongsa-nguan M, Chaicumpa W, Moolasart P, et al. *Vibrio cholerae* O139 Bengal in Bangkok. Lancet 1993; **342**: 430–1.
5. 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.
6. Bodhidatta L, Echeverria P, Hoge CW, et al. *Vibrio cholerae* O139 in Thailand in 1994. Epidemiol Infect 1995; **114**: 71–3.
7. World Health Organization. Epidemic diarrhoea due to *Vibrio cholerae* non-O1. Weekly Epidemiol Rec 1993; **68**: 141–2.
8. Swerdlow DL, Ries AA. *Vibrio cholerae* non-O1-the eight pandemic? Lancet 1993; **342**: 382–3.
9. Albert MJ. *Vibrio cholerae* O139 Bengal. Minireview. J Clin Microbiol 1994; **32**: 2345–9.
10. Faruque SM, Abdul Alim RM, Roy SK, et al. Molecular analysis of rRNA and cholera toxin genes carried by the new epidemic strain of toxigenic *Vibrio cholerae* O139 synonym Bengal. J Clin Microbiol 1994; **32**: 1050–3.
11. 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.
12. 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.
13. 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.
14. Wachsmuth IK, Evins GM, Fields PI, et al. The molecular epidemiology of cholera in Latin America. J Infect Dis 1993; **167**: 621–6.
15. Cameron DN, Khambaty FM, Wachsmuth KI, Tauxe RV, Barrett TJ. Molecular characterization of *Vibrio cholerae* O1 strains by pulsed field gel electrophoresis. J Clin Microbiol 1994; **32**: 1685–90.
16. Popovic T, Fields PI, Olsvik Ø, et al. Molecular subtyping of toxigenic *Vibrio cholera* O139 causing epidemic cholera in India and Bangladesh, 1992–1993. J Infect Dis 1995; **171**: 122–7.
17. Bhadra RK, Choudhury SR, Das J. *Vibrio cholerae* O139 El Tor biotype. Lancet 1994; **343**: 728.
18. Berche P, Poyart C, Abachin E, Lelievre H, Vandepitte J, Dodin A, Fournier J. The novel epidemic strain O139 is closely related to the pandemic strain O1 of *Vibrio cholerae*. J Infect Dis 1994; **170**: 701–4.
19. Pace NR, Olsen GJ, Woese CR. Ribosomal RNA phylogeny and the primary lines of evolutionary descent. Cell 1986; **45**: 325–6.
20. Hanchalay S, Seriwatana J, Echeverria P, et al. Non-O1 *Vibrio cholerae* in Thailand: homology with cloned cholera toxin genes. J Clin Microbiol 1985; **21**: 288–9.
21. Echeverria P, Harrison BA, Tirapat C, McFarland A. Flies as a source of enteric pathogens in a rural village in Thailand. Appl Environ Microbiol 1983; **46**: 32–6.
22. Skov MN, Pedersen K, Larsen JL. Comparison of pulsed-field gel electrophoresis, ribotyping, and plasmid profiling for typing of *Vibrio anguillarum* serovar O1. Appl Environm Microbiol 1995; **61**: 1540–5.
23. Chu G, Vollrath D, Davis RW. Separation of large DNA molecules by contour-clamped homogeneous electric fields. Science 1986; **234**: 1582–3.
24. Pedersen K, Larsen JL. rRNA gene restriction patterns of *Vibrio anguillarum* serogroup O1. Dis Aquat Org 1993; **16**: 121–6.
25. Arbeit RD, Arthur M, Dunn R, Kim C, Selander RK, Goldstein R. Resolution of recent evolutionary divergence among *Escherichia coli* from related lineages: the application of pulsed field electrophoresis to molecular epidemiology. J Infect Dis 1990; **161**: 230–5.
26. Goering RV, Duensing TD. Rapid field inversion gel electrophoresis in combination with an rRNA gene probe in the epidemiological evaluation of staphylococci. J Clin Microbiol 1990; **28**: 426–9.
27. Prévost G, Pottecher B, Dahlet M, Bientz M, Mantz JM, Piémont Y. Pulsed field gel electrophoresis as a new epidemiological tool for monitoring methicillin-resistant *Staphylococcus aureus* in an intensive care unit. J Hosp Infect 1991; **17**: 255–69.

28. Mathew MK, Hui C-F, Smith CL, Cantor CR. High-resolution and accurate size determination in pulsed-field gel electrophoresis of DNA. 4. Influence of DNA topology. Biochemistry 1988; **27**: 9222–6.
29. Baril C, Richaud C, Baranton G, Girons IS. Linear chromosome of *Borrelia burgdorferi*. Res Microbiol 1989; **140**: 507–16.
30. Kurazono H, Okuda J, Takeda Y, et al. *Vibrio cholerae* O139 bengal isolated from India, Bangladesh and Thailand are clonal as determined by pulsed-field gel electrophoresis. J Infect Dis 1994; **170**: 109–10.
31. Shimada T, Arakawa E, Itoh K, et al. Extended serotyping scheme for *Vibrio cholerae*. Curr Microbiol 1994; **28**: 175–8.