PULSED FIELD GEL ELECTROPHORESIS ANALYSIS OF *VIBRIO CHOLERAE* ISOLATES IN SOUTHERN THAILAND

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Abstract. Forty isolates of *V. cholorae* O1, O139 and non-O1/non-O139 collected from outbreaks in Songkhla and Phuket Provinces of southern Thailand during 1999-2001 and sporadic cases from different regions of Thailand during 1993-2002 were characterized using pulsed field gel electrophoresis (PFGE). Digestion of chromosomal DNA of the *V. cholerae* isolates with restriction endonuclease *Not*I, followed by PFGE, generated 10 distinct restriction endonuclease analysis patterns consisting of 8 to 13 bands, ranging in size from 78 to 394 kb. PFGE patterns of O1 Inaba strains from the outbreak in Songkhla were identical (P1) except one isolate (P3). The O1 Inaba outbreak strains from Phuket in the same period belonged to P2 pattern, whereas the O1 Ogawa strain from the outbreak in Phuket isolated in 1999 was of P7 pattern. These patterns of O1 Inaba and Ogawa strains were slightly different suggesting that the isolates were epidemiologically related and therefore the outbreaks were likely due to the same *V. cholerae* clone. Isolates of *V. cholerae* O1 Inaba from sporadic cases in the neighboring area (*eg,* Pattani Province) in a similar period of time of the outbreak in Songkhla Province had very similar patterns, with only one single band different from those of the outbreak isolates. This indicates that the Inaba strains isolated from Songkhla Province during the 2001 cholera outbreak belonging to P1 pattern had not spread to other regions in 2001 and 2002. On the otherhand, the sporadic isolates collected from other regions of Thailand were quite distinct from the outbreak isolates in Songkhla Province, especially those from Chaiyaphum and Chaing Mai Provinces, which belonged to P5 and P6 pattern, respectively. Isolates of *V. cholerae* O139 and non-O1/non-O139 gave different patterns from that of *V. cholerae* O1. This study shows that the PFGE technique is markedly advantageous in distinguishing strains of *V. cholerae* isolates leading to insightful detailed charateristics of these isolates in Thailand.

Key words: *V. cholerae,* PFGE, southern Thailand

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INTRODUCTION

Cholera, caused by *V. cholera* O1 El Tor biotype, has been a global public health problem, especially in developing countries (Glass *et al,* 1992). In Thailand,

this pathogen has been responsible for an increased mortality rate. *V. cholera* O1 strain isolated from Thailand has switched from Inaba to Ogawa serotype during 1980 to 2003 (The Ministry of Public Health Division of Epidemiology, Bangkok, Thailand). However, *V. cholerae* O139 was observed in Thailand between 1993 and 1995 (Hoge *et al*, 1996; Dalsgaard *et al*, 1998, 1999). Cholera has become resistant to antibiotics used for cholera infection, such as cotrimoxazole and tetracycline. The unusual incidence of cholera outbreak due to Ogawa strain in Songkhla Province and the neighboring provinces of southern Thailand in 1997 showed that the isolates are resistant to tetracycline (Kondo *et al*, 2001). Another outbreak in Songkhla Province and sporadic cases in other parts of Thailand occurring in 2001 was due to Inaba strain (Kondo *et al*, 2001). These isolates have not been fully characterized at the molecular level.

In recent years, molecular methods used for genotyping many bacterial pathogens include random amplification of polymorphic DNA (Leal *et al*, 2004), multilocus sequence typing (MLST) (Kotetishvili *et al*, 2003), amplified fragment length polymorphism (Jiang *et al*, 2000), pulsed field gel electrophoresis (PFGE) (Camelon *et al*, 1994) and ribotyping (Koblavi *et al*, 1990; Popovic *et al*, 1993). PFGE method has been used as a general molecular tool in epidemiological tracking (Miettinen *et al*, 1999) and differentiating individual strains of many pathogenic bacteria including *V. cholerae* (Camelon *et al*, 1994), *Salmonella typhi* (Thong *et al*, 1994), *Pseudomonas cepacia* (Anderson *et al*, 1991), *Enterococcus* spp (Miranda *et al*, 1991; Gordillo *et al*, 1993), and *Streptococcus pneumoniae* (Lefevre *et al*, 1993). Epidemiological analysis of *V. cholerae* by PFGE method showed that strains within defined outbreaks were clonal whereas sporadic cases had various profiles (Mahalingam *et al*, 1994). PFGE was also reported to be useful in discriminating non-O1 strains isolated from different sources (Filetici *et al*, 1997). In this study, PFGE was used to investigate genetic characteristics of outbreaks and sporadic cases of cholera in southern Thailand.

MATERIALS AND METHODS

Forty isolates of *V. cholerae* collected from infected individuals and environmental samples, *eg* drinking water, water from well, and cuttlefish, were included in this study. Twenty of the isolates were obtained from Songkhla (n=17) and Phuket (n=3) Provinces of southern Thailand during the 2001 cholera epidemic. The remaining isolates were from sporadic cases occurring either in the neighboring provinces of Songkhla or in other regions of Thailand during the same or different time period (Table 1). All strains were identified by conventional biochemical tests. Serotyping was performed using slide agglutination with polyvalent O1 and monospecific Inaba and Ogawa antisera (Difco Laboratories, Detroit, MI), and a slide agglutination with O139 antisera (Denka Seiken, Tokyo, Japan).

PFGE was performed as previously described with some modifications (Kondo *et al*, 2001). In brief, a colony from an individual *V. cholerae* strain was cultured at 37ºC until the growth in tryptic soy broth (Merck) containing 1% NaCl reached an optical density of 0.9-1.0 at 600 nm. An equal volume of bacterial suspension and 2% low melting point agarose gel (FMC, Bio Products; ME) were prepared and dispensed into a plug mold as described by Albert *et al* (1997). Bacterial cells were lysed with proteinase K in lysis buffer

at 56ºC and washed in TE buffer. Restriction digestion was performed with 20 U *Not*I at 37ºC for 6 hours. Lambda molecular weight fragments ranging from 48.5 to 1,000 kb in size (Bio-Rad Laboratories) were used as standard molecular weight markers. Restriction fragments were separated on a CHEF MAPPER®XA (Bio-Rad Laboratories) in 1% agarose (Pulse Field Certified Agarose, Bio-Rad Laboratories) using 0.5x TBE buffer. Electrophoresis was performed at 6 V/cm and the temperature of the buffer maintained at 14ºC. The pulse time was ramped from 5 to 35 seconds for 27 hours. The gel was stained with ethidium bromide solution (10 µg/ml), destained, and the images were recorded under UV transilluminator. PFGE profiles were analyzed visually. A PFGE pattern number was assigned to each distinctive restriction fragment pattern.

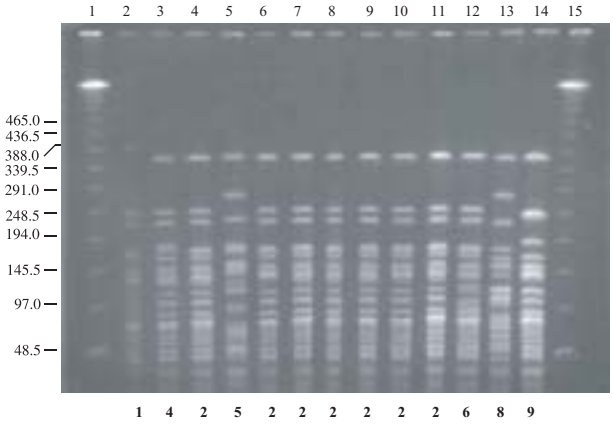

Fig 1–PFGE patterns of *Not*I-digested DNA of representative *V. cholerae* O1 serotype Inaba, *V. cholerae* O139 and *V. cholerae* non-O1/non-O139 strains isolated from patients and environment in Thailand. The designated lanes as indicated on top correspond to the strains in Table 1. Designations of assigned PFGE patterns are shown at the bottom.

RESULTS

PFGE profiles of representative *V. cholerae* strains O1, O139 and non-O1/non-O139 are demonstrated in Fig 1. The PFGE patterns, produced after the chromosomal digestion, were considered different when there is at least one DNA fragment of the PFGE pattern varying between the compared isolates. Each PFGE profile was given an arbitrary designation as shown in Table 2. Of all the test isolates, there were ten distinct PFGE patterns produced (P1-P10). The numbers of restriction fragments generated by *Not*I within the PFGE profiles were 8 to 13 bands, ranging in size from 78 to 394 kb. The common fragments found in most of the PFGE patterns were fragments of 367.4, 250.1, 227.9, 174.4, 161.7, 146.8, 133.0, 115.8, 103.1, 89.0, 78.3 kb in size. The fragments of 394.3, 272.7, 192.6 and 182.7 kb in size were detected only in some PFGE patterns. The latter three fragments appeared to generate varying patterns among the test isolates. Different serotypes generally exhibited different PFGE profiles, except P7 pattern, which was found in all three representative O1 Ogawa outbreak strains from patients and well water in Phuket Province and in one non-O1/non-O139 strain isolated from a patient from Pattani Province (Table 1).

All 17 O1 Inaba outbreak strains isolated from patients and water daily used in a district of Songkhla Province during the year 2001 belonged to P1 and P3 patterns represented by fragment of 394.3 and 367.4 kb, respectively. The P1 pattern was not shared by other Inaba strains from other southern provinces isolated in the same period (Table 1). The PFGE profiles of the O1 Inaba strains from other regions

Table 1
PFGE patterns and characteristics of representative strains of *V. cholerae* examined in this study.

Serotype (no. of strains)	Epidemiological feature	Isolation				
		Province (no. of strains)	Region	Source	Year	PFGE pattern
O1 Inaba (31)	Outbreak	Songkhla (13)	South	Patient	2001	1
		Songkhla (3)	South	Drinking and daily used water	2001	1
		Songkhla (1)	South	Patient	2001	3
		Phuket (1)	South	Patient	2001	2
		Phuket (2)	South	Contact person	2001	2
	Sporadic	Pattani (1)	South	Patient	2001	2
		Chaing Mai (1)	North	Patient	2001	4
		Yala (1)	South	Patient	2002	2
		Chaiyaphum (1)	Northeast	Patient	2002	5
		Chumphon (1)	South	Patient	2002	2
		Bangkok (1)	Central	Patient	2002	2
		Khon Kaen (1)	Northeast	Patient	2002	2
		Nonthaburi (1)	Central	Patient	2002	2
		Pathum Thani (1)	Central	Patient	2002	2
		Lampang (1)	North	Patient	2002	2
		Chiang Mai (1)	North	Patient	2002	6
O1 Ogawa (5)	Outbreak	Phuket (2)	South	Patient	1999	7
		Phuket (1)	South	Water from well	1999	7
	Sporadic	Songkhla (2)	South	Patient	2000	7
non-O1/ non-O139 (2)	Sporadic	Pattani (1)	South	Patient	2001	7
		Samut Sakhon (1)	Central	Cuttlefish	2002	8
O139 (2)	Sporadic	Bangkok (1)	Central	Patient	1993	9
		Songkhla (1)	South	Patient	2001	10

of Thailand belonged to P2, P4, P5 and P6 patterns and were distinct from the cholera epidemic strains. These results revealed that the cholera outbreaks were most likely to have started from a single origin. However, it should be noted that P1, P2 and P3 shared the same bands, except for fragments between the sizes of 367 and 394, and 97 kb. According to the criteria described by Tenover *et al* (1995) these three patterns might have close genetic relationships.

The O1 Ogawa strains isolated from the outbreak in Phuket Province during 1999 had the same PFGE pattern (P7 pattern) as one strain of non-O1/non-O139 isolated from Pattani Province in 2001 (Table 1). This pattern had only one band different from P1. The results revealed that the strains had close genetic intrarelationship. Thus, those isolates from the outbreak in Songkhla Province might be from the same origin. The sporadic case in Songkhla Province, which belonged to P7 pattern,

Table 2
*Not*I-digested fragments in PFGE patterns of *V. cholerae*.

Fragment	Size (kb)	PFGE pattern									
		P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
1	394.3	/						/			
2	367.4		/	/	/	/	/		/	/	/
3	272.7								/	/	/
4	250.1	/	/	/	/		/	/		/	
5	227.9	/	/	/		/	/	/	/		/
6	192.6				/						/
7	182.7					/	/				/
8	174.4	/	/	/	/	/	/	/		/	/
9	161.7	/	/	/	/	/	/		/	/	/
10	146.8	/	/	/	/	/	/	/	/	/	/
11	133.0	/	/	/	/	/	/	/	/		/
12	115.8	/	/	/	/	/	/	/	/	/	/
13	103.1	/	/	/	/		/	/	/		/
14	97	/[a]		/				/			
15	89.0	/	/	/	/	/	/	/			/
16	78.3	/	/	/	/	/	/	/	/	/	/
	Total no. of fragments	11 or 12	11	12	11	10	12	11	9	8	13

[a]The fragment of 97 kb is optional in P1.

could probably have spread from Phuket Province. However, this is not conclusive as the numbers of the test strains were too limited. It seemed that the strains isolated in southern Thailand, belonging to P1, P2, P3 and P7 patterns, have close genetic relationship and had been spreading around the neighboring areas of southern Thailand. In contrast, P2 pattern was detected commonly in all parts of Thailand.

The O139 and non-O1/non-O139 strains isolated in 2001 were different in PFGE profiles from Inaba strains as indicated in Table 1. The PFGE patterns of O139 (P9, P10) and non-O1/non-O139 strain (P8) were distinct from the O1 strains by 4-5 bands. It can be assumed that the strains might possibly be related to the O1 strains examined in this study.

DISCUSSION

Although two outbreaks in 1998 and 2001 in southern Thailand showed differences in serotypes and antibiograms, *V. cholerae* O1 serotype Ogawa isolated from Songkhla and other provinces of southern Thailand in 1998 (Kondo *et al*, 2001) revealed that they belonged to the same PFGE profile as found in the strains of the *V. cholerae* O1 serotype Inaba of the outbreak in 2001. Indistinguishable restriction fragments patterns of Ogawa and Inaba isolates from stool of a single individual in a tour group visiting the Philippines in 2001 were also observed by Kam and colleagues (Kam *et al*, 2003). It was suggested that these strains are most likely from the same clone or very closely related.

The P1 pattern of Inaba strains found in this study had the same pattern designated pattern 8 of Ogawa strains during March 1999 - April 2000 as previously reported (Tapchaisri *et al*, 2008). However, this study illustrated that the P2 and P3 patterns of Inaba isolates from the outbreaks in Phuket and Songkhla Provinces, respectively were newly generated in 2001. Since the P2 pattern seems to be commonly found in the test strains in this study, the strains belonged to P1 and P3 pattern might be genetic modified from the strains that belonged to P2.The variation of subtypes was suggested to have occurred by genetic modification consistent with a point mutation, an insertion or a deletion resulting in an alteration of a restriction site as previously described (Tenover *et al*, 1995). The results of the PFGE patterns found in outbreak strains isolated from patients and environment revealed that they were epidemiologically linked. This confirms that the PFGE technique is a powerful epidemiological tool for outbreak investigation and surveillance.

The five band differences in the PFGE profiles of O139 strains isolated in 1993 and 2001 in this study indicated that the isolates from different periods of time are probably related (Tenover *et al*, 1995). Comparing these results with the O139 strains from many countries, such as Hong Kong, Mainland China, the Philippines and other countries isolated between 1994 to 2000 (Kam *et al*, 2003), it revealed that the O139 isolates from Thailand generated distinctive PFGE profiles from those isolates. In addition, the PFGE pattern of O139 strains isolated in 1993 observed in this study was distinct from the strain isolated from Thailand in the same year as previously reported (Tapchaisri *et al*, 2008). In the same study by Tapchaisri *et al* (2008), the O139 strain from Bangladesh is similar to our test strain. They were considered to be closely related and probably were part of the outbreak in 1993.

The most apparent differences among the strains in this study were restriction fragments ranging from 80 to 395 kb in size while Kam *et al* (2003) noted differences in the range of 80 to 250 kb. However, the results of PFGE patterns of the *V. cholerae* strains in this study could be effectively analyzed and showed the possible relatedness among the strains isolated from outbreaks and sporadic cases. This study also showed that PFGE technique produced several subtypes of the same serotype, which provided more detailed characteristics of the strains. The variation of subtypes would give a remarkable notice to keep an alert of new emerging variants, which would become more virulent giving rise to more difficulties in treatment and control. The finding of P5 pattern in an O1 Inaba strain isolated from Chaiyapum Province in northeast Thailand revealed that this strain might have developed in a different environment resulting in an unique pattern. The awareness of this new emerging strain must be taken in consideration.

The insightful information generated in this study on the particular characteristics of cholera strains isolated in Thailand provides additional epidemiological analysis of cholera cases which can lead to an efficient prevention of outbreaks in the future. It will be useful for tracking the origin of a suspected chlorea outbreak and for control and surveillance of the spread in communities. Moreover, this study of the characterization at the molecular level hopefully will be valuable to facilitate further studies, such as in developing a rapid detection technique and development of an appropriate DNA vaccine for preventing infection of local strains of cholera.

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