Molecular Typing of Vibrio cholerae O1 Isolates from Thailand by Pulsed-field Gel Electrophoresis

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Abstract

The aim of the present study was to genotypically characterize Vibrio cholerae strains isolated from cholera patients in various provinces of Thailand. Two hundred and forty V. cholerae O1 strains, isolated from patients with cholera during two outbreaks, i.e. March 1999–April 2000 and December 2001–February 2002, in Thailand, were genotypically characterized by NotI digestion and pulsed-field gel electrophoresis (PFGE). In total, 17 PFGE banding patterns were found and grouped into four Dice-coefficient clusters (PF-I to PF-IV). The patterns of V. cholerae O1, El Tor reference strains from Australia, Peru, Romania, and the United States were different from the patterns of reference isolates from Asian countries, such as Bangladesh, India, and Thailand, indicating a close genetic relationship or clonal origin of the isolates in the same geographical region. The Asian reference strains, regardless of their biotypes and serogroups (classical O1, El Tor O1, O139, or O151), showed a genetic resemblance, but had different patterns from the strains collected during the two outbreaks in Thailand. Of 200 Ogawa strains collected during the first outbreak in Thailand, two patterns (clones)—PF-I and PF-II—predominated, while other isolates caused sporadic cases and were grouped together as pattern PF-III. PF-II also predominated during the second outbreak, but none of the 40 isolates (39 Inaba and 1 Ogawa) of the second outbreak had the pattern PF-I; a minority showed a new pattern—PF-IV, and others caused single cases, but were not groupable. In summary, this study documented the sustained appearance of the pathogenic V. cholerae O1 clone PF-II, the disappearance of clones PF-I and PF-III, and the emergence of new pathogenic clones during the two outbreaks of cholera. Data of the study on molecular characteristics of indigenous V. cholerae clinical isolates have public-health implications, not only for epidemic tracing of existing strains but also for the recognition of strains with new genotypes that may emerge in the future.

Key words: Cholera; Diarrhoea; Molecular typing; Pulse-field gel electrophoresis; Vibrio cholerae O1; Thailand

INTRODUCTION

Molecular techniques for genomic comparisons of closely-related bacterial species or strains of the same species are extremely valuable for molecular epidemiological surveillance of a particular infectious disease (1). Plasmid profile analysis was among the earliest DNA-based techniques applied to epidemiologic studies (2). However, the discriminatory power of this approach is poor for organisms that lack or possess only one or two plasmid(s), like Vibrio cholerae. For studying the genetic diversity of V. cholerae, various other molecular methods have been extensively used. Multilocus enzyme electrophoresis (MEE) or zymovar analysis was used for classifying strains into multiple electrophoretic types, for distinguishing classical from El Tor strains (3), and for determining
the genetic relationship among and between toxigenic and non-toxigenic
V. cholerae O1 (4). Strains of V. cholerae O1 have shown different patterns of
rRNA restriction fragment length polymorphism (RFLP) (5). Ribotyping was used for differentiating
otherwise phenotypically-indistinguishable V. cholerae
strains, and a standardized ribotyping scheme
has been proposed (6). Amplified fragment length polymorphism fingerprinting (AFLP) was used for
examining the molecular evolution and diversity
in clinical and environmental isolates of V. cholerae
(7). Pulsed-field gel electrophoresis (PFGE) was used,
for example, for studying genetic changes within V.
cholerae strains responsible for epidemics in Latin
America (8), for comparing domestic and imported
V. cholerae strains in Japan (9), and for investigating
an inexplicable upsurge in the incidence of cholera
in Calcutta, India (10).

The seventh cholera pandemic, originating in
Indonesia and caused by V. cholerae biotype El Tor, ar-
rived in Thailand in 1963. Since then, this biotype
has replaced classical vibrios and has established its
endemicity (11). Thailand experienced an outbreak of
cholera, caused by V. cholerae serogroup O139,
during 1993-1995, and the organisms disappeared
thereafter (12,13). Presently, sporadic cases and
transmission of the disease caused by V. cholerae El
Tor still exist in certain groups of the population
living under poor environmental conditions and
reduced personal hygiene, both in urban and rural
areas (11). Focal outbreaks do occur in provinces
with active tourism especially along the coastlines,
such as Phuket Island, the Andaman Sea, and southern
Thailand, and in Chonburi province located on
the eastern coast of the Gulf of Thailand (11). Thus,
surveillance of cholera is still a routine activity of
the Department of Diseases Control, Thailand. Be-
sides, stool samples of patients with watery diar-
rhoea who seek treatment at public hospitals are
intensively investigated for V. cholerae, and if isolat-
ed, the bacteria are subjected to serogrouping and
serotyping, and the evidence must be reported to
the Ministry of Public Health. Despite this routine
activity, the molecular characteristics of the clinical
isolates of V. cholerae and the molecular epidemi-
ology of the disease caused by the bacteria in the
country have never been investigated. It was, there-
fore, our primary objective to genotypically charac-
terize V. cholerae O1 strains isolated from patients
with cholera in various provinces of Thailand, using
NotI macrorestriction digestion and PFGE. Our
data should provide useful epidemiological base-
line information with public-health implications,
such as for epidemic tracing of indigenous strains
and for identifying their genetic relationship with
the strains that may emerge in the future.

MATERIALS AND METHODS

Bacterial strains

Two hundred and forty V. cholerae O1 clinical isolates
were included in this study. Two hundred strains
were isolated from patients with cholera, during an
outbreak from March 1999 to April 2000, who were
admitted to different provincial hospitals in differ-
ent regions of Thailand, i.e. central (Chonburi [18],
Petchaburi [9], and Nakhon Pathom [7] provinces);
northern (Sukhothai province [21]); north-eastern
(Khon-Kaen [39] and Nakhon Panom [13] provinc-
es); and southern Thailand (Phuket province [93]).
All the isolates of the first study period were of the
Ogawa serotype. Forty strains were isolated from
patients with cholera during an outbreak from De-

cember 2001 to February 2002 admitted to hospitals in
Petchaburi (14), Samut Songkhram (2) and Khon-
Kaen (24) provinces. Thirty-nine isolates of the second
period were of the Inaba serotype, and only one iso-
late from Petchaburi was of the Ogawa serotype.
No cholera cases were available from Samut Songkhram
province during the first period and from Chonburi,
Nakhon Pathom, Sukhothai, Nakhon Panom, and
Phuket provinces during the second period. Figure 1
shows the locations of the provinces from where V.
cholerae were isolated. Rectal swabs of patients were
individually placed in Cary-Blair transport medium
and subsequently enriched in alkaline peptone wa-
eter for four hours before plating onto TCBS (Oxoid)
agar. Yellow colonies were subjected to appropriate
biochemical testing (14). The V. cholerae isolates were
tested by agglutination reaction with polyvalent O1
antiserum, and the strains that gave a positive agglu-
tination were serotyped using monovalent Ogawa
and Inaba antisera (Biotech, Thailand). The V. chol-
erae O1 isolates were stored in brain heart infusion
broth (Difco Laboratories, Detroit, USA) containing
15% glycerol at -70 °C until use.

Seventeen V. cholerae strains were used as referenc-
es. They were: one strain of V. cholerae O1 (El Tor,
Ogawa strain 295/33) and one strain of V. cholerae
O139 (strain TH166) isolated in Thailand in 1990
and 1993 respectively; 14 strains of V. cholerae
that were isolated elsewhere (six strains, i.e. three O1 El
Tor, one O1 classical, and one each of O139 and
O151 from India; two O1 and two O139 strains
from Bangladesh; and one strain of O1 each from
Australia, Peru, Romania, and the USA, with un-
known years of isolation, and one O1 laboratory
strain, i.e. classical Inaba 569B (maintained in the
Pulsed-field gel electrophoresis

Genomic DNA for pulsed-field gel electrophoresis (PFGE) was prepared in agarose plugs using a method previously described (15). Slices of agarose plug were digested with 20 units of NotI restriction endonuclease (Promega, Madison, WI, USA). The restriction fragments were separated by the contour-clamped homogeneous electric field method on a CHEF DR III system (Bio-Rad Laboratories, Richmond, CA, USA) under predetermined conditions (27 hours at a temperature of 12 °C, 120° constant angle, 6 V/cm, and with a ramped pulsed time of 3 to 40 s). After the electrophoresis, the gel was stained with 0.5 µg/mL ethidium bromide (Sigma, USA) in 0.5 × TBE for 20 minutes, destained in 0.5 × TBE for 30 minutes, and viewed and photographed under an UV transilluminator (Vilber Lourmat, 77202 Marne-La-Vallée, France, 280 nm). Concatemers of the λ phage DNA ladder (Promega, Madison, WI, USA) starting at 48.5 kb were used as molecular size markers.

Analysis of PFGE patterns

V. cholerae O1 chromosomal restriction PFGE patterns were classified according to Tenover et al. (16) and Arakawa et al. (9). When four or more DNA bands in the PFGE patterns were different from each other, we assigned them as different patterns by Arabic numerals, i.e. patterns 1 to 17. Patterns with less than a four-band difference were considered subtypes, i.e. 2a, 2b, 2c, 2d, 2e, 8a, 8b, 8c, 11a, 11b, 11c, and 11d. The DNA restriction PFGE patterns obtained were also saved as TIFF files for use with Bio-profil (Vilber Lourmat, Marne-La-Vallée, France). For the latter, normalization was done according to the molecular size standards of each gel, with one molecular weight standard being used for 3-4 samples. Construction of similarity matrices was carried out by comparison of Dice coefficients (17). The band-based Dice coefficient is based on a comparison of designated band positions by dividing the number of matching bands between patterns by the total number of bands, thereby emphasizing the matching bands. In all the cases, an un-weighted pair group matching band average
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(UPGMA) at a 1.3% tolerance window was used for clustering the pulsed-field gel electrophoresed (PF) patterns. The experimental variation between duplicate experiments was determined by testing two V. cholerae O1 strains at four different times. The reproducibility of the PFGE was >99%, and each of the DNA banding patterns of the isolates was imported only one time into Bio-Profil.

RESULTS

PFGE analysis with Not I restriction enzyme

In total, 17 PFGE banding patterns, i.e. pattern 1-17, were found among 17 reference strains and 240 clinical isolates of V. cholerae O1. Figure 2 shows the representatives of the individual patterns.

Table 1 shows the details of the PFGE banding patterns of all reference and 240 isolated Thai strains. The reference strains—V. cholerae O1—isolated in 1990 and O139 isolated in 1993 from Thailand gave pattern 1 and 2a respectively; all 6 Indian isolates showed pattern 2b; both O1 isolates from Bangladesh had pattern 2c, while two O139 strains had pattern 2d and 2e; and one isolate each from Australia, Peru, Romania and USA had pattern 3, 4, 5, and 6 respectively. V. cholerae O1, classical Inaba, 569B, which is a laboratory strain, gave pattern 7.

Of the 240 Thailand isolates, 200 Ogawa strains collected during the first period gave six new patterns, i.e. pattern 8-13, which were different from those of the references. The predominate patterns (number of the isolates/total no. of isolates) found in the individual provinces were: 8a (14/18) from Chonburi; 8a (6/9) from Petchaburi; 11a (4/7) from Nakorn Pathom; 11a (19/21) from Sukhothai; 11a (13/39) from Khon Kaen; 8b (7/13) from Nakorn Panom; and 8a (76/93) from Phuket.

Forty isolates (39 Inaba, one Ogawa) of the second period revealed pattern 11a and four new patterns, i.e. pattern 14-17. The predominate patterns (number of the isolates/total no. of isolates) found in these individual provinces were: 11a (8/14) from Petchaburi; 11a (2/2) from Samut Songkhram, and 11a (22/24) Khon Kaen. The remaining eight iso-

Fig. 2. The 17 PFGE banding patterns of V. cholerae isolates in Thailand and other countries

Lane M: Molecular size marker (Lambda phage DNA ladder); Lane 1 and 2: V. cholerae O1 strain 295/33 and O139 strain TH166 isolated in Thailand in 1990 and 1993 respectively; Lane 3-8: V. cholerae O1 strains GP12, GP71, GP156, AS230, AS231 and AS233 respectively, from India; Lane 9 and 10: V. cholerae O1 strains AR-15493 and AR-15425 from Bangladesh; Lane 11 and 12: V. cholerae O139 strains AR-11644 and AR-19467 from Bangladesh; Lane 13: V. cholerae O1 strain 2463-78 from Australia; Lane 14: V. cholerae O1 strain C6706 from Peru; Lane 15: V. cholerae O1 strain C7754 from Romania; Lane 16: V. cholerae O1 strain 2164-88 from the United States; Lane 17: V. cholerae O1, classical, Inaba, strain 569B from stock of University of Adelaide, Australia. Numbers at the far left are DNA molecular sizes in kilobases
### Table 1. Countries and provinces from where *Vibrio cholerae* were isolated and their PFGE banding Patterns

| Country of origin | Biotype | Serogroup | Region | Province | PFGE pattern | No. of isolates |
|-------------------|---------|-----------|--------|----------|--------------|----------------|
| Australia         | El Tor  | O1        | -      | -        | 3            | 1              |
| Bangladesh        | El Tor  | O1        | -      | -        | 2c           | 2              |
|                   |         | O139      | -      | -        | 2d           | 1              |
|                   |         | O139      | -      | -        | 2e           | 1              |
| India             | Classical | O1     | -      | -        | 2b           | 1              |
|                   | El Tor  | O1        | -      | -        | 2b           | 3              |
|                   |         | O139      | -      | -        | 2b           | 1              |
|                   |         | O151      | -      | -        | 2b           | 1              |
| Peru              | El Tor  | O1        | -      | -        | 4            | 1              |
| Romania           | El Tor  | O1        | -      | -        | 5            | 1              |
| Thailand          | El Tor  | O1        | -      | -        | 1            | 1              |
|                   |         | O139      | -      | -        | 2a           | 1              |
| United States     | El Tor  | O1        | -      | -        | 6            | 1              |
| Australia         | Classical, Inaba, 569B | O1 | - | - | 7 | 1 |

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First period (March 1999–April 2000); all 200 isolates were El Tor, Ogawa

| Region          | Province | PFGE pattern | No. of isolates |
|-----------------|----------|--------------|----------------|
| Central         | Chonburi (18) | 8a | 14 |
|                 |          | 8b | 2 |
|                 |          | 11a | 2 |
|                 | Phetchaburi (9) | 8a | 6 |
|                 |          | 11a | 3 |
|                 | Nakhon Pathom (7) | 11a | 4 |
|                 |          | 11b | 2 |
|                 |          | 11c | 1 |
| North           | Sukhothai (21) | 11a | 19 |
|                 |          | 11b | 2 |
| Northeast       | Khon Kaen (39) | 8a | 3 |
|                 |          | 8b | 10 |
|                 |          | 11a | 13 |
|                 |          | 11b | 3 |
|                 |          | 11c | 3 |
|                 |          | 11d | 5 |
|                 |          | 12 | 1 |
|                 |          | 13 | 1 |
|                 | Nakhon Phanom (13) | 8a | 6 |
|                 |          | 8b | 7 |
| South           | Phuket (93) | 8a | 76 |
|                 |          | 8b | 11 |
|                 |          | 8c | 1 |
|                 |          | 9 | 1 |
|                 |          | 10 | 1 |
|                 |          | 11a | 3 |

Second period (December 2001–February 2002); 40 isolates: 39 were Inaba, 1 from Phetchaburi was Ogawa

| Region          | Province | PFGE pattern | No. of isolates |
|-----------------|----------|--------------|----------------|
| Central         | Phetchaburi (14) | 11a | 8 |
|                 |          | 14 | 5 |
|                 |          | 17 | 1 |
|                 | Samut Songkhram (2) | 11a | 2 |
| Northeast       | Khon Kaen (24) | 11a | 22 |
|                 |          | 15 | 1 |
|                 |          | 16 | 1 |

-=Province and region were not known
lates of the second period had new patterns, i.e. pattern 14 (5) and pattern 15-17 (one isolate each).

The PFGE profiles of the 240 Thailand isolates were further grouped by a band-based analysis. The PFGE profiles could be categorized into four major groups, namely PF-I, PF-II, PF-III, and PF-IV, at 95% confidence by the Bio-Profil® software (Fig. 3). Approximately 97% similarity was found between PF-I and PF-II, 94% similarity was found between PF-II and PF-III, and <90% between PF-IV and the other PF clusters (Fig. 3). The DNA banding patterns of the isolates of the first period were clustered into three major PF groups based on their genotypic relationship expressed as Dice coefficient. The DNA banding pattern—8a to 8c—were designated as PF-I; pattern 11a to 11d were PF-II, and pattern 12 and 13 were PF-III. None of the isolates from the second outbreak was grouped as PF-I and PF-III; 32 isolates of the second period with DNA banding pattern 11a were PF-II. Five isolates from Petchaburi showing DNA banding pattern 14 were classified as PF-IV (Fig. 3).

Of the total V. cholerae O1 isolates, the predominant patterns were 8 or PF-I (136/200 isolates) and 11 or PF-II (60/200 isolates) during the first period and 11a or PF-II (32/40 isolates) during the second period. Five isolates (two strains of the first period isolated from Phuket with DNA banding pattern 9 and 10, and three strains of the second period, i.e. two strains from Khon Kaen with DNA pattern 15 and 16 and one strain from Petchaburi with DNA banding pattern 17) were ungroupable under the conditions used.

**DISCUSSION**

In this study, NotI endonuclease was used for generating DNA restriction fragments of the 240 V. cholerae O1 strains isolated from patients with cholera in different regions of Thailand during two different periods using 17 strains isolated in different countries before 1999 as references. The NotI enzyme digestion and the PFGE protocol previously used by many investigators (8-10,18-21) were followed such that our results may be comparable with the previously-reported V. cholerae PFGE patterns. The PFGE pattern 7 (lane 7, Fig. 2) of V. cholerae O1, classical Inaba, strain 569B, found in this study is comparable with the pattern previously reported
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(lane b, Fig. 1) by Majumder et al. (21), indicating reproducibility of the method.

*V. cholerae* reference strains from Australia, Peru, Romania, and the United States showed different PFGE patterns and were dissimilar to the patterns of isolates from Asian countries, such as Bangladesh, India, and Thailand (Table 1). All the Asian reference strains, except one O1 strain isolated from Thailand in 1990, had the same PFGE banding pattern, i.e. pattern 2, although of different subtypes, indicating their close genetic relationship, perhaps clonal in origin (12). The finding that these reference Asian strains, regardless of their biotypes and serogroups (classical versus El Tor O1, O139, or O151), showed genetic resemblance supports the notion that new pathogenic *V. cholerae* clones may be derived from existing serogroups, e.g. serogroup O139, synonym Bengal, as has been previously reported (27). Our findings indicate a sustained appearance of the epidemic *V. cholerae* O1 clone with DNA banding pattern 11 or PF-II, a disappearance of epidemic and non-epidemic clones with other DNA banding patterns (8, 9, 10, 12 and 13), and an emergence of a new epidemic clone pattern 14—PF-IV, and of non-epidemic clones (patterns 15-17) during the two study periods. Similar findings have been reported from other cholera endemic areas such as Bangladesh (31-33). Although *V. cholerae* is naturally a human pathogen, members of this genus constitute part of the normal aquatic flora in estuarine and brackish waters (13,34,35). Virulence genes found in clinical isolates and their homologues are also dispersed among environmental strains of *V. cholerae* belonging to diverse serogroups (32,35). New pathogenic *V. cholerae* clones—either epidemic or non-epidemic—may evolve from multiple O1 or non-toxigenic, non-O1 *V. cholerae* progenitors in environmental sources through horizontal gene transfer like bacteriophage transduction (34,36-38).

Currently, diarrhoeal diseases are receiving less attention and concern from the Ministry of Public Health of Thailand than newly-emerging or re-emerging infectious diseases, such as avian influenza, dengue haemorrhagic fever, HIV/AIDS, and leptospirosis (11). This is because the incidence rates of enteric diseases, especially cholera, have dramatically decreased during the past several years. Nevertheless, in some provinces, sporadic cases are found throughout the year. Small focal outbreaks of cholera occur mostly in provinces along the coastline or on islands, which are main tourist areas, such as Phuket, or in refugee camps along the borders (39). Our data on molecular characteristics of indigenous *V. cholerae* clinical isolates have public-health implications, not only for epidemic tracing of existing strains but also for the recognition of strains with new genotypes that may emerge in the future.

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