A decrease in the proportion of infections by pandemic *Vibrio parahaemolyticus* in Hat Yai Hospital, southern Thailand

Nutthakul Wootipoom,¹ Phuangthip Bhoopong,² Rattana-ruji Pomwised,¹ Mitsuaki Nishibuchi,³ Masanori Ishibashi⁴ and Varaporn Vuddhakul¹

Correspondence

Varaporn Vuddhakul
varaporn.v@psu.ac.th

¹Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Thailand

²Institute of Allied Health Science and Public Health, Walailuk University, Nakhonsithammarat, Thailand

³Center for Southeast Asian Study, Kyoto University, Kyoto, Japan

⁴Osaka Prefectural Institute of Public Health, Higashinari-ku, Osaka, Japan

Infection by the pandemic clone of *Vibrio parahaemolyticus* is prevalent in southern Thailand. This study actively surveyed the incidence of *V. parahaemolyticus* infection in this area. A total of 865 isolates of *V. parahaemolyticus* was obtained from patients at Hat Yai Hospital, the main public hospital in Songkhla Province, Thailand, from 2000 to 2005. The isolates were examined by group-specific PCR (GS-PCR) specific for the pandemic clone, and for the presence of two major virulence genes, *tdh* and *trh*, and the O : K serotype. Representative isolates were also examined by antibiogram pattern and DNA fingerprinting using an arbitrarily primed PCR method to determine the clonal relationships between isolates. The total number of isolates was less in 2000 and more in 2004 and 2005 than in the years 2001–2003. The increase in the numbers of infections in 2004 and 2005 was not due to the emergence of a particular clone having unique characteristics, but was probably due to climate change. From 2000 to 2003, the percentages of pandemic strains of *V. parahaemolyticus*, defined as GS-PCR-positive *tdh*⁺ *trh*⁺, was stable at 64.1, 67.5, 69.7 and 67.7 % of the total isolates each year, respectively. However, in 2004 and 2005, the percentages decreased to 56.1 and 55.5 %, respectively. The O : K serotypes of the pandemic isolates remained unchanged. The proportional decrease in infections caused by the pandemic strains are probably due to the population in this area gradually developing immunity to the pandemic clone whilst continuing to be susceptible to other strains.

Received 9 June 2007

Accepted 9 August 2007

INTRODUCTION

Vibrio parahaemolyticus infections cause acute, self-limiting gastroenteritis, typically characterized by diarrhoea, abdominal cramps, nausea, vomiting, fever and chills, lasting 1–3 days. The onset usually occurs within 24 h of eating contaminated food. Cases are most commonly reported during the warmer months, and are often associated with eating raw or undercooked shellfish or other cooked foods that have been cross-contaminated with raw shellfish (Yeung & Boor, 2004). Not all strains of *V. parahaemolyticus* are considered pathogenic. Most clinical isolates exhibit the Kanagawa phenomenon (KP) (Nishibuchi & Kaper, 1995). KP-positive strains cause β -haemolysis, which is induced by a thermostable direct haemolysin (TDH) in Wagatsuma agar, encoded by the *tdh*

gene. Some KP-negative clinical isolates carry the *trh* gene, encoding a TDH-related haemolysin (TRH). The *trh* gene sequence varies from strain to strain, and can be clustered into two subgroups, *trh1* and *trh2* (Kishishita *et al.*, 1992). Molecular epidemiological studies have shown that clinical isolates possess the *tdh*, *trh* or both genes, but environmental isolates rarely carry these genes (Shirai *et al.*, 1990). Isolates lacking both the *tdh* and *trh* genes have also been isolated from clinical specimens, and possible explanations for their isolation have been presented (Bhoopong *et al.*, 2007). Since 1996, the *V. parahaemolyticus* O3:K6 serotype carrying the *tdh* gene has been confirmed as responsible for infections in many Asian countries, Europe and the USA (Okuda *et al.*, 1997a; Matsumoto *et al.*, 2000). These strains are now considered to be pandemic strains. A molecular typing technique named group-specific PCR (GS-PCR) can detect nucleotide variations within the 1364 bp *toxRS* region that are unique to the pandemic clone (Matsumoto *et al.*, 2000).

Abbreviations: AP-PCR, arbitrarily primed PCR; GS-PCR, group-specific PCR; KP, Kanagawa phenomenon; TDH, thermostable direct haemolysin; TMP/SMX, trimethoprim/sulfamethoxazole.

The use of GS-PCR on recent clinical isolates has shown that some GS-PCR-positive isolates belong to non-O3:K6 serotypes, O4:K68, O1:KUT, O1:K25 and others. It has been reported that these serotypes probably originate from the same clone as O3:K6 (Bhuiyan *et al.*, 2002; Chowdhury *et al.*, 2000, 2004; Matsumoto *et al.*, 2000).

Since the emergence of the pandemic strains, a surveillance programme of *V. parahaemolyticus* has been operating in the southern part of Thailand. In 1998, 87% of 23 isolates from Hat Yai Hospital, the main public hospital located in Songkhla Province, southern Thailand, were pandemic strains (Vuddhakul *et al.*, 2000). In 1999, 76% of 317 isolates from Hat Yai Hospital and Songklanagarind Hospital, a university hospital in Songkhla Province, Thailand, were pandemic strains (Laohaprertthisan *et al.*, 2003). In this study, an investigation of *V. parahaemolyticus* isolates was carried out at Hat Yai Hospital from 2000 to 2005. We examined isolated strains by GS-PCR, toxin gene profiles, O:K serotype, antibiogram and other features. A noteworthy finding was a significant decrease in the percentage of pandemic strains in 2004 and 2005. We discuss a possible reason based on the characteristics of the isolated strains.

METHODS

Isolation and identification of bacterial strains. Stool samples were collected from patients presenting with diarrhoea at Hat Yai Hospital between 2000 and 2005. The samples were plated on MacConkey, *Salmonella*-*Shigella* and thiosulfate-citrate-bile salt-sucrose (TCBS) agar. After overnight incubation at 37 °C, samples showing growth predominantly on TCBS agar were selected. Non-sucrose-fermenting colonies were examined by standard biochemical tests for identification as *V. parahaemolyticus*. In addition, identification was confirmed by PCR targeting the *toxR* gene (Kim *et al.*, 1999). Boiled broth cultures of *V. parahaemolyticus* were used as the source of DNA template for all PCR assays described below.

Detection of *tdh* and *trh* genes. The presence of *tdh* and *trh* in each isolate was determined by PCR. Primer pairs D3 and D5, and R2 and R6 were used to investigate *tdh* and *trh*, respectively, as described previously (Tada *et al.*, 1992).

GS-PCR. GS-PCR to identify pandemic strains was carried out using the technique described by Matsumoto *et al.* (2000).

Determination of O:K serotypes. The O (somatic) and K (capsular) serotypes of isolated strains were determined by agglutination using commercial anti-O and anti-K antisera (Denka Seiken) according to the manufacturer's instructions.

Antibiotic susceptibility tests. Susceptibility to antibiotics was examined using the disc diffusion method (NCCLS, 2000). Antibiotic-loaded paper discs were placed on Mueller-Hinton agar plates inoculated with a bacterial lawn. After incubation at 37 °C for 14–18 h, the diameter of the inhibition zone was recorded and interpreted according to the reference provided by the manufacturer. Seven antibiotic discs were used: ampicillin (10 µg), ciprofloxacin (5 µg), trimethoprim/sulfamethoxazole (TMP/SMX) (1.25 µg), chloramphenicol (30 µg), tetracycline (30 µg), norfloxacin (10 µg) and azithromycin (15 µg). *Escherichia coli* ATCC 25922 was used as a standard strain.

***trh* subgroup investigation.** Genomic DNA from *V. parahaemolyticus* was digested with *Hind*III restriction enzyme. The *trh* subgroup was detected by Southern blot hybridization using digoxigenin-labelled *trh1* and *trh2* probes as described previously (Bhoopong *et al.*, 2007). Hybridization was carried out under high-stringency conditions at 30 °C. The hybridized probes were detected using a DNA detection kit (Roche Diagnostics) according to the manufacturer's instructions.

Arbitrarily primed PCR (AP-PCR). DNA was extracted using a standard phenol/chloroform extraction method (Sambrook *et al.*, 2001). AP-PCR was carried out using primer 2 (5'-GTTTCGCTCC-3') and primer 4 (5'-AAGAGCCCGT-3') as described previously (Matsumoto *et al.*, 2000).

Statistical analysis. Pearson's χ^2 test was used to evaluate significant differences in the results.

RESULTS

Toxin gene profiles and GS-PCR results

A total of 865 isolates of *V. parahaemolyticus* was obtained from stool specimens sent to Hat Yai Hospital from 2000 to 2005. The total number of *V. parahaemolyticus* infections was less in 2000, and more in 2004 and 2005, than in the years 2001–2003 (Table 1). We classified the isolates into four groups based on the presence or absence of the two virulence genes: *tdh*⁺ *trh*[−], *tdh*⁺ *trh*⁺, *tdh*[−] *trh*[−] and *tdh*[−] *trh*⁺. All isolates were also examined by GS-PCR. GS-PCR-positive isolates were detected only in the *tdh*⁺ *trh*[−] group. The *tdh*⁺ *trh*[−] group was therefore divided into two subgroups (Table 1). The most prevalent isolates detected in each year belonged to the *tdh*⁺ *trh*[−] group. They totalled 719 isolates in the 6 years. Within this group, 74.7% (537 isolates) were GS-PCR positive, which were defined as pandemic strains in this study (Table 1). Although the highest percentage of pandemic strains was detected in 2002, there was no significant difference in the percentage of pandemic isolates during 2000–2003 (64.1, 67.5, 69.7 and 67.7% of the total isolates during each consecutive year, respectively). Although the total numbers of GS-PCR-positive isolates were higher in 2004 and 2005 than in 2003, the percentage of GS-PCR-positive strains significantly decreased by 11.6 and 12.2% in 2004 and 2005, respectively, compared with 2003. In contrast, the percentage of non-pandemic isolates in the GS-PCR-negative *tdh*⁺ *trh*[−], *tdh*⁺ *trh*⁺ and *tdh*[−] *trh*[−] groups increased (Table 1), whilst the percentage of non-pandemic isolates in the *tdh*[−] *trh*⁺ group remained the same.

O:K serotype

In each year, the pandemic isolates (GS-PCR-positive *tdh*⁺ *trh*[−]) were predominantly of the O3:K6 serotype (72.8% overall), followed by the O1:K25 and O4:K68 serotypes, except that O4:K68 was not detected among the pandemic isolates in 2002 (Table 2). The serotypes of the isolates belonging to the GS-PCR-negative *tdh*⁺ *trh*[−], *tdh*⁺ *trh*⁺, *tdh*[−] *trh*[−] and *tdh*[−] *trh*⁺ groups varied considerably

Table 1. Characteristics of *V. parahaemolyticus* isolates from Hat Yai Hospital from 2000 to 2005

Year	No. of isolates (%)					
	Total	<i>tdh</i> ⁺ <i>trh</i> ⁻		<i>tdh</i> ⁺ <i>trh</i> ⁺	<i>tdh</i> ⁻ <i>trh</i> ⁻	<i>tdh</i> ⁻ <i>trh</i> ⁺
		GS-PCR positive*	GS-PCR negative			
2000	64	41 (64.1)	12 (18.7)	0	8 (12.5)	3 (4.7)
2001	123	83 (67.5)	25 (20.3)	5 (4.1)	8 (6.5)	2 (1.6)
2002	142	99 (69.7)	18 (12.7)	8 (5.6)	14 (9.8)	3 (2.1)
2003	124	84 (67.7)†	25 (20.2)	6 (4.8)	7 (5.6)	2 (1.6)
2004	205	115 (56.1)‡	50 (24.4)	18 (8.8)	19 (9.3)	3 (1.5)
2005	207	115 (55.5)‡	52 (25.1)	17 (8.2)	20 (9.7)	3 (1.4)
Total	865	537	182	54	76	16

*Pandemic strains.

†Significantly different from ‡ ($P=0.027$).

(Table 3). Many of the isolates belonged to serotypes O4:K8, O3:K29, O4:K45 and O11:KUT. The isolates belonging to serotypes O3:K6 and O1:KUT were

Table 2. Serotypes of GS-PCR-positive *tdh*⁺ *trh*⁻ *V. parahaemolyticus* isolated between 2000 and 2005

Year	No. of isolates per serotype	Serotype
2000	30	O3:K6
	8	O1:K25
	2	O4:K68
	1	O2:K3
2001	63	O3:K6
	11	O1:K25
	8	O4:K68
	1	O1:KUT
2002	74	O3:K6
	18	O1:K25
	5	O1:KUT
	1	O3:K29, O1:K41
2003	60	O3:K6
	12	O1:K25
	6	O1:KUT
	3	O4:K68
	1	O3:K29, O5:KUT, R:KUT
2004	81	O3:K6
	22	O1:K25
	6	O4:K68
	4	O1:KUT
	1	O3:KUT, O3:K46
2005	83	O3:K6
	17	O1:K25
	9	O4:K68
	5	O1:KUT
	1	O4:K4

KUT, K untypeable; R, rough.

also encountered in this non-pandemic group, but their AP-PCR profiles were different from those of O3:K6 and O1:KUT isolates in the pandemic group (data not shown). Of interest was that the K untypeable (KUT) strains accounted for only 4.5 and 11.0% of the total isolates in the pandemic isolates and GS-PCR-negative *tdh*⁺ *trh*⁻ isolates, respectively, whereas KUT strains were detected in 58.2, 73.3 and 68.7% of the isolates belonging to the *tdh*⁺ *trh*⁺, *tdh*⁻ *trh*⁻ and *tdh*⁻ *trh*⁺ groups, respectively (Fig. 1).

Antibiogram patterns

One hundred and eighty-nine isolates randomly selected from five genotype groups were tested for sensitivity to seven commonly used antibiotics. All isolates were susceptible to four of the antibiotics tested: chloramphenicol, tetracycline, norfloxacin and azithromycin (data not shown). Almost all isolates except for two in the *tdh*⁻ *trh*⁻ group were resistant to ampicillin (Table 4). The antibiograms of isolates in the *tdh*⁺ *trh*⁻ group were similar, regardless of the GS-PCR result, but were different from those of the *tdh*⁺ *trh*⁺, *tdh*⁻ *trh*⁻ and *tdh*⁻ *trh*⁺ groups regarding resistance to ciprofloxacin and TMP/SMX. Of the isolates in the *tdh*⁺ *trh*⁺, *tdh*⁻ *trh*⁻ and *tdh*⁻ *trh*⁺ groups, 34–43.8% were resistant to ciprofloxacin, whereas approximately 81% of *tdh*⁺ *trh*⁻ isolates were resistant to this antibiotic. Significantly more isolates in the *tdh*⁺ *trh*⁺, *tdh*⁻ *trh*⁻ and *tdh*⁻ *trh*⁺ groups were susceptible to TMP/SMX than in the *tdh*⁺ *trh*⁻ group.

trh subgroups

To examine whether the *trh* subgroup differed between the *tdh*⁺ *trh*⁺ and *tdh*⁻ *trh*⁺ groups, the *trh* subgroup was determined for 20 of the 55 *tdh*⁺ *trh*⁺ isolates and 12 of the 16 *tdh*⁻ *trh*⁺ isolates. The *trh1* gene predominated among the *tdh*⁺ *trh*⁺ isolates (90.0%), whereas the *trh2* gene predominated among the *tdh*⁻ *trh*⁺ isolates (66.7%).

Table 3. Serotypes of *tdh*⁺ *trh*⁻ (non-pandemic), *tdh*⁺ *trh*⁺, *tdh*⁻ *trh*⁻ and *tdh*⁻ *trh*⁺ *V. parahaemolyticus* isolates during 2000–2005

Year	<i>tdh</i> ⁺ <i>trh</i> ⁻		<i>tdh</i> ⁺ <i>trh</i> ⁺		<i>tdh</i> ⁻ <i>trh</i> ⁻		<i>tdh</i> ⁻ <i>trh</i> ⁺	
	No. of isolates per serotype	Serotype	No. of isolates per serotype	Serotype	No. of isolates per serotype	Serotype	No. of isolates per serotype	Serotype
2000	4	O4:K8	0	–	3	O12:KUT	2	O1:KUT
	3	O4:K9			1	O1:K69, O3:K6, O3:K54, O3:KUT, O11:KUT	1	OUT:KUT
	2	O4:KUT						
2001	1	O2:K3, O3:K6, O4:K13	2	O3:KUT	1	O1:K46, O1:K56, O1:KUT, O3:K6, O7:KUT, O8:KUT, O11:KUT, OUT:KUT	1	O1:KUT, O4:KUT
	11	O4:K8						
	2	O2:K3, O3:K6						
2002	1	O1:K56, O1:KUT, O2:K28, O3:K29, O4:K9, O4:KUT, O5:K17, O8:K41, O8:KUT, R:KUT	1	O1:KUT, O11:K36, O12:KUT				
	9	O3:K29	2	O12:KUT, O8:K74	2	O1:KUT, O10:KUT, O11:KUT, O5:KUT, R:KUT	1	O1:K58, O1:KUT, O11:KUT
	2	O4:K4, O4:K8	1	O1:K69, O1:KUT, O4:KUT, O5:K15	1	O1:K56, O3:KUT, O8:KUT, O12:KUT		
2003	1	O1:K38, O3:K6, O3:K7, O5:K17, O8:KUT	4	O1:KUT	1	O1:K56, O3:KUT, O5:KUT, O10:KUT, O11:KUT, O12:KUT, R:KUT	1	O1:K48, O3:KUT
	14	O4:K8						
	3	O3:K29, O8:K41						
2004	1	O1:KUT, O3:K6, O4:K9, O4:KUT, O8:K21	2	O1:K69				
	17	O4:K8	4	O11:KUT	4	O11:KUT	1	O1:K56, O1:KUT, O3:KUT
	8	O4:K55	2	O3:K56, O5:K15, O5:KUT, O12:KUT	3	O5:KUT		
	7	O3:K29	1	O1:K9, O4:K67, O1:K69, O3:K6, O3:K72, O8:KUT	2	O2:K3, O5:K30, O8:KUT, O12:KUT		
	4	O8:K41			1	O1:KUT, O3:KUT, O3:K54, O5:K17		
	2	O1:K56, O1:KUT, O8:K21						
	1	O2:K3, O3:K5, O3:K7, O3:KUT, O4:K9, O4:KUT, O5:K17, O5:KUT						

Table 3. cont.

Year	$tdh^{+} trh^{-}$		$tdh^{+} trh^{+}$		$tdh^{-} trh^{-}$		$tdh^{-} trh^{+}$	
	No. of isolates per serotype	Serotype	No. of isolates per serotype	Serotype	No. of isolates per serotype	Serotype	No. of isolates per serotype	Serotype
2005	15	O4:K55	4	O1:KUT, O11:KUT	9	O11:KUT	1	O1:KUT, O4:K49, O4:K55
	12	O4:K8	2	O3:K6, O4:KUT	2	O5:KUT		
	5	O2:K3	1	O1:K9, O4:K4, O4:K13, O5:KUT, O8:K20	1	O1:KUT, O2:KUT, O3:K57, O4:K4, O4:K13, O4:KUT, O10:K71, R:K34, OUT:KUT		
	4	O3:K29						
	3	O1:K25						
	2	O3:K7, O3:KUT, O4:K13						
	1	O1:K58, O1:KUT, O3:K5, O4:KUT, O5:KUT, OUT:KUT, O8:K21						

AP-PCR analysis

To investigate whether infections due to the non-pandemic isolates belonging to GS-PCR-negative $tdh^{+} trh^{-}$, $tdh^{+} trh^{+}$, $tdh^{-} trh^{-}$ and $tdh^{-} trh^{+}$ isolates were caused by a specific clone in each group, DNA fingerprints of 139 randomly selected isolates obtained during 2000–2005 were examined using the AP-PCR technique. Except for the $tdh^{-} trh^{+}$ isolates in which all of those tested displayed non-identical AP-PCR profiles (data not shown), isolates from any year with the same serotype within each group mostly produced identical AP-PCR profiles. Two patterns were obtained for GS-PCR-negative $tdh^{+} trh^{-}$ O4:K8; the first pattern comprised nine isolates that gave identical patterns for both primers (Fig. 2a,b, lanes 2–10), whilst the second pattern comprised two isolates that gave identical patterns for both primers (Fig. 2a,b, lanes 13 and 14). For the ten O1:KUT isolates from the $tdh^{+} trh^{+}$ group, eight gave identical patterns (Fig. 2c,d, lanes 3–8, 10 and 11), and three of the twelve O11:KUT isolates in the $tdh^{-} trh^{-}$ group gave identical patterns (Fig. 2e,f, lanes 9–11).

DISCUSSION

In this study, isolates of *V. parahaemolyticus* from the same hospital were investigated continuously for 6 years. The total number of *V. parahaemolyticus* infections was higher in 2004 and 2005 than in other years. We do not know the exact reason for this increase. The number of isolates belonging to four genotype groups increased, except for those belonging to the $tdh^{-} trh^{+}$ group (Table 1). Based on the following results, the increase in each of the groups could not be accounted for by the emergence of a new dominant clone. Analysis of antibiogram patterns (Table 4) did not distinguish among isolates, but the use of serotypes and DNA fingerprints proved to be very useful. The serotypes of the GS-PCR-positive $tdh^{+} trh^{-}$ isolates remained unchanged, being predominantly O3:K6 over the 6 year period (Table 2). In the other groups, the serotypes varied considerably (Table 3) and not all isolates with the same serotype had the same DNA fingerprint (Fig. 2). In each of the GS-PCR-negative $tdh^{+} trh^{-}$, tdh^{+}

Fig. 1. Percentages of K untypeable strains detected in each group.

Table 4. Antibigram patterns of *V. parahaemolyticus* isolates from 2000 to 2005

Antibiogram pattern*	No. of isolates (%)				
	<i>tdh</i> ⁺ <i>trh</i> ⁻		<i>tdh</i> ⁺ <i>trh</i> ⁺	<i>tdh</i> ⁻ <i>trh</i> ⁻	<i>tdh</i> ⁻ <i>trh</i> ⁺
	GS-PCR positive†	GS-PCR negative			
AMP	9 (18.4)	8 (18.6)	33 (66.0)	18 (58.1)	9 (56.2)
AMP CIP	28 (57.1)	28 (65.1)	14 (28.0)	11 (35.5)	7 (43.8)
AMP CIP TMP/SMX	12 (24.5)	7 (16.3)	2 (4.0)	0	0
AMP TMP/SMX	0	0	1 (2.0)	0	0
Total	49 (100)	43 (100)	50 (100)	29 (93.6)‡	16 (100)

*The antibiotics examined were ampicillin (AMP), ciprofloxacin (CIP), trimethoprim/sulfamethoxazole (TMP/SMX), chloramphenicol, tetracycline, norfloxacin and azithromycin. Both resistant and intermediate reactions were judged as resistant in this study and only these reactions are listed in these antibiograms.

†Pandemic strains.

‡Two more isolates in the *tdh*⁻ *trh*⁻ group were examined. They were susceptible to AMP and are not included in this table.

trh⁺ and *tdh*⁻ *trh*⁻ groups, isolates with the same DNA fingerprint pattern and serotype persisted over the study period, but dominance in only 2004 and 2005 was not observed. The increase in the number of the isolates is likely to be related to climate change. Bacteria belonging to the genus *Vibrio* are expected to propagate more rapidly at a higher temperature in their natural habitat of marine and estuarine environments. An increase in ambient temperature and the sea surface temperature of coastal water is associated with an increase in infection by *Vibrio cholerae* (Colwell, 1996; Pascual *et al.*, 2002). This is probably also the case with *V. parahaemolyticus*. The highest mean ambient temperatures around Songkhla during 2000–2005 were 36.1, 37.0, 36.6, 36.5, 37.3 and 36.8 °C, respectively (www.songkhlamet.org). The relatively small number of infections in 2000 (Table 1) may be explained by the lowest temperature in this year. We speculate that the increases in the number of infections in 2001 and 2004 from previous years (Table 1) may have been stimulated by the high temperatures (37.0 °C and above) in these years and that the number of infections may not have decreased after this increase as temperatures did not drop drastically again. Thus, the change in the number of infections may have been mediated by the change in the number of *V. parahaemolyticus* organisms in the environment, although there are no data to support this hypothesis.

The percentage of pandemic isolates (GS-PCR-positive *tdh*⁺ *trh*⁻) decreased considerably and that of non-pandemic isolates increased during the period 2004–2005. The majority of serotypes detected among the pandemic isolates were O3:K6, O1:K25, O1:KUT and O4:K68, and this remained unchanged over the entire 6 year period (Table 2). In contrast, the serotypes of the increased numbers of non-pandemic isolates varied considerably (Table 3). This could be related to immunity acquired by the local people. The pandemic clone emerged around 1995 (Matsumoto *et al.*, 2000). Infection by *V. parahaemolyticus* is prevalent in southern Thailand where seafood is popular,

and previous studies (Bhoopong *et al.*, 2007; Laohaprertthisan *et al.*, 2003; Uddhakul *et al.*, 2000) and this study have revealed that infection has been caused mainly by the pandemic clone at least since 1998 in this area. Infection by *V. parahaemolyticus* can induce a lipopolysaccharide (O antigen)-specific immune response in patients (Qadri *et al.*, 2003). Frequent infections by the pandemic strains with limited O:K serotypes would induce immunity more frequently and specifically than infrequent infections by non-pandemic strains. Individuals previously exposed to pandemic strains may develop immunity to them, but will continue to be susceptible to non-pandemic clones with different serotypes. Such populations may have gradually increased in the last decade. This phenomenon has been described for *V. cholerae*. In Thailand, people infected by *V. cholerae* O1 serotype Ogawa became infected by serotype Inaba after 7–8 years (Supawat & Huttayanant, 1997). The same phenomenon occurred in India, where *V. cholerae* O1 serotype Inaba was predominant until 1989 when it was replaced by serotype Ogawa. It reappeared again almost 10 years later (Garg *et al.*, 2000). This is thought to have been due to the development of the host immune response to the lipopolysaccharide O antigen (Gangarosa *et al.*, 1967; Sack & Miller, 1969; Sheehy *et al.*, 1966). It is likely that human infection with *V. parahaemolyticus* has similar features. Another possible explanation for the decrease in the percentage of the pandemic isolate would be a decrease of the proportion of pandemic strains in their natural habitat caused by environmental changes such as climate change. Pandemic strains carry several unique DNA regions in the genome (Hurley *et al.*, 2006; Okura *et al.*, 2005; Wang *et al.*, 2006; Williams *et al.*, 2004). If any of these DNA regions is associated with survival or propagation of pandemic strains in their natural habitat, the distribution of pandemic strains relative to non-pandemic strains may be influenced by environmental changes. Surveys of specific immunity among local people and of the distribution of pandemic

Fig. 2. AP-PCR profiles of *tdh*⁺ *trh*⁻ *V. parahaemolyticus* non-pandemic isolates (a, b), *tdh*⁺ *trh*⁺ isolates (c, d) and *tdh*⁻ *trh*⁻ isolates (e, f) obtained from 2001 to 2005. The results were obtained using primer 2 (a, c and e) and primer 4 (b, d and f). (a, b) Lanes: 1 and 15, molecular mass markers (1 kb DNA ladder); 2–4, O4:K8 isolates from 2000, 2001 and 2002, respectively; 5 and 6, O4:K8 isolates from 2003; 7 and 8, O4:K8 isolates from 2004; 9–14, O4:K8 isolates from 2005. (c, d) Lanes: 1 and 12, molecular mass markers; 2 and 3, O1:KUT isolates from 2001 and 2002, respectively; 4–7, O1:KUT isolates from 2003; 8–11, O1:KUT isolates from 2005. (e, f) Lanes: 1 and 15, molecular mass markers; 2–5, O11:KUT isolates from 2000, 2001, 2002 and 2003, respectively; 6–8, O11:KUT isolates from 2004; 9–14, O11:KUT isolates from 2005.

versus non-pandemic strains in the environment are needed to examine the above possibilities.

The susceptibilities of our isolates to some of the antibiotics were somewhat different from reports by other workers. Serichantalergs *et al.* (2007) reported that all *V. parahaemolyticus* isolates collected from patients in Bangkok during 2001–2002 were susceptible to TMP/SMX and 52% of isolates were resistant to ampicillin. However, we found that 11.6% (22/189) of isolates we examined were resistant to TMP/SMX and most isolates were resistant to ampicillin. Our results are similar to those reported for pandemic and non-pandemic strains of *V. parahaemolyticus* by Wong *et al.* (2000). They characterized pandemic strains from Asia, including Thailand, and showed that 97.4 and 100% of pandemic strains and

non-pandemic strains, respectively, were resistant to ampicillin. Okuda *et al.* (1997b) reported that their pandemic and non-pandemic strains isolated between 1994 and 1996 in India were sensitive to ciprofloxacin. However, 81.6% of the pandemic isolates were resistant to this antibiotic in our study. In this study, the *tdh*⁺ *trh*⁺ group was more sensitive to antibiotics than the *tdh*⁺ *trh*⁻ group; its antibiotic response pattern was more closely related to the *tdh*⁻ *trh*⁻ and *tdh*⁻ *trh*⁺ groups (Table 4). Although this may indicate that *tdh*⁺ *trh*⁺ and *tdh*⁻ *trh*⁺ groups have existed in similar ecological niches recently, the two groups seem to have different origins, as the *tdh*⁺ *trh*⁺ strains predominantly carried the *trh1* gene (90.0% of total isolates), whilst the majority of the *tdh*⁻ *trh*⁺ isolates had the *trh2* gene (66.7% of total isolates). *V. parahaemolyticus* is found in marine and estuarine

environments. It is important to study how this bacterium, particularly the *tdh*⁺ *trh*⁻ group including the pandemic strains, acquires resistance to antibiotics.

As infections by strains belonging to the *tdh*⁺ *trh*⁺, *tdh*⁻ *trh*⁺ and *tdh*⁻ *trh*⁻ groups are much less frequent compared with infections caused by *tdh*⁺ *trh*⁻ strains, there is little information available on the properties of the strains of the former groups. We noticed that many of the isolates of these groups could not be typed to existing K serogroups (KUT isolates in Fig. 1). A possible reason for detecting KUT isolates at high frequencies is that the O:K typing scheme was established by examining KP-positive strains that produce large amounts of TDH and were mostly clinical specimens isolated in Japan. Although *tdh*⁺ *trh*⁺ strains produce TDH, the amounts of TDH are small and these strains exhibit a KP-negative phenotype (Okitsu *et al.*, 1997). It is only recently that clinical strains possessing the *trh* gene have been included in the serotyping scheme. Therefore, not enough clinical strains belonging to the *tdh*⁺ *trh*⁺ and *tdh*⁻ *trh*⁺ groups have so far been evaluated for adding to the scheme. Isolates belonging to the *tdh*⁻ *trh*⁻ group have been left outside the scheme, even if they were isolated from clinical specimens.

In conclusion, a decrease in the percentage of *V. parahaemolyticus* infections by pandemic isolates was observed towards the end of the 6 year observation period. This is probably due to acquirement of immunity by local people as a result of continued exposure to pandemic strains. This phenomenon has not been reported previously for *V. parahaemolyticus*, although it is known to occur with *V. cholerae*. Continued surveillance would confirm this hypothesis and would be useful for the future control of infections by this pathogen.

ACKNOWLEDGEMENTS

This study was supported by funds from the Government of Thailand and KAKENHI (191010). The authors would like to thank Dr Brian Hodgson for assistance with the manuscript.

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