INTRODUCTION

Vibrio parahaemolyticus, a Gram-negative halophilic bacterium, is responsible for human gastroenteritis worldwide and sporadic cases and outbreaks occur regularly in Asia as well as in other countries (Bag et al., 1999; DePaola et al., 2003; Joseph et al., 1982; Wong et al., 2000; Yeung & Boor, 2004). Virulence of V. parahaemolyticus is associated with the production of a thermostable direct haemolysin (TDH), TDH-related haemolysin (TRH) (Honda et al., 1991; Nishibuchi et al., 1985; Nishibuchi et al., 1989) and urease enzyme (Suthienkul et al., 1995). The genes encoding these factors, tdh, trh and ure, are therefore used as genetic markers indicative of the virulence of strains (Kim et al., 1999; Suthienkul et al., 1995).

As many as 75 different combinations of the somatic (O) and capsular (K) antigens have been identified (Ishibashi et al., 1999). In 1996, the emergence of V. parahaemolyticus serovar O3 : K6 was reported in Calcutta, India (Okuda et al., 1997). Subsequently, O3 : K6 clones have disseminated throughout Southeast Asia, Atlantic and Gulf coasts of the USA (Daniels et al., 2000; Matsumoto et al., 2000; Okuda et al., 1997) and more recently in Europe (Martinez-Urtaza et al., 2004), Africa (Ansaruzzaman et al., 2005) and South America (Gonzalez-Escalona et al., 2005). It has been shown that the group-specific (GS) sequence of the toxRS operon and the presence of ORF8 in the filamentous phage f237 are specific for pandemic O3 : K6 clones, and these genetic markers are used to distinguish between pandemic and non-pandemic traits (Matsumoto et al., 2000; Nasu et al., 2000). Later, several more serovars, O4 : K68, O1 : K25, O1 : K26 and O1 : KUT, were reported to share closely related genetic patterns to pandemic serovar O3 : K6 (Bhuiyan et al., 2002; Chowdhury et al., 2004; Matsumoto et al., 2000; Wong et al., 2000).

In 2001–2002, the Department of Enteric Diseases, AFRIMS, Thailand, conducted a case–control study of acute diarrhoea in expatriates and Thai adults. V. parahaemolyticus was the third leading pathogen isolated from this study. This finding provided the impetus to examine
these V. parahaemolyticus isolates by molecular techniques to understand better the epidemiological relatedness and significance of this pathogen in expatriate and Thai adults presenting with diarrhoea.

**METHODS**

**Enrolment and specimen collection.** After obtaining written informed consent, stool specimens or rectal swabs, and demographic and clinical information were collected from acute diarrhoea patients (417 expatriates and 400 Thai adults) and non-diarrhoea controls at Bumrungrad Hospital in Bangkok from January 2001 to December 2002. Expatriates were travellers and resident foreigners from developed countries seen at Bumrungrad Hospital. Sixty-five per cent (33/51) of the expatriates with V. parahaemolyticus infection were in Thailand for ≤1 month. Controls were patients seen at the hospital with no reported diarrhoea during the previous 2 weeks. One control was selected for each case (417 asymptomatic expatriate samples and 400 asymptomatic Thai adults). All specimens were transported to the laboratory and processed within 2–4 h of collection.

**Isolation and identification.** All samples were examined for V. parahaemolyticus and other enteric pathogens (Salmonella spp., Shigella spp., Vibrio spp., Plesiomonas spp., Aeromonas spp., Campylobacter spp., and pathogenic Escherichia coli) by standard bacteriological methods (Holt et al., 1994) and DNA hybridization assays (Echeverria et al., 1989). For V. parahaemolyticus isolation, fresh stool samples or rectal swabs transported in 10 ml modified Cary-Blair (0.16% agar) medium were suspended in 3 ml normal saline solution and inoculated directly onto selective medium, thiosulphate/citrate/bile salt/sucrose (TCBS) agar. A portion of each faecal suspension was enriched in 5 ml alkaline peptone water containing 0.5% NaCl, followed by overnight incubation at 37 °C, and then subcultured again on TCBS. Presumptive identification of V. parahaemolyticus was determined by the appearance of typical blue–green colonies on TCBS agar. Suspected colonies were picked for further biochemical testing (Holt et al., 1994), including a urease test.

**Antibiotic susceptibility testing.** All V. parahaemolyticus isolates were tested for antimicrobial susceptibility by a standard disc diffusion method on Mueller–Hinton II agar (National Committee for Clinical Laboratory Standards, 2000a). The antibiotic discs (BD Diagnostic Systems) used were: ampicillin, chloramphenicol, kanamycin, gentamicin, tetracycline, trimethoprim–sulfamethoxazole, nalidixic acid, ciprofloxacin and sulfisoxazole. In the absence of Clinical and Laboratory Standards Institute (formerly NCCLS) definitive standards for interpreting V. parahaemolyticus, zone diameters were determined and recorded as sensitive, intermediate or resistant according to interpretive standards established for Vibrio cholera and members of the Enterobacteriaceae. E. coli ATCC 25922 was used as a control organism (National Committee for Clinical Laboratory Standards, 2000b).

**Serotyping.** Confirmed V. parahaemolyticus isolates were serotyped by agglutination using a commercial set of O and K antisera (Denka Seiken) according to the manufacturer’s instructions.

**PCR.** PCR assays for the species-specific toxR gene and two virulence genes (tdh and trh) were performed as described previously using boiled cultures of V. parahaemolyticus as the source of DNA template (Kim et al., 1999; Suthienkul et al., 1995). The GS-PCR and PCR for the orf8 gene were performed using specific primers reported previously to detect toxRS sequences unique to the pandemic O3 : K6 clone of V. parahaemolyticus and the orf8 sequence of phage f237, respectively (Matsumoto et al., 2000; Nasu et al., 2000).

**PFGE.** Not-PFGE was performed on all 95 V. parahaemolyticus isolates as described previously using the PulseNet Protocol (Centers for Disease Control and Prevention, 2004). The images of DNA band patterns were analysed for cluster analysis using BIONUMERICS software version 3.5 (Applied Maths) based on the Dice similarity coefficient and unweighted pair-group method with arithmetic averages.

**RESULTS AND DISCUSSION**

**Epidemiology and clinical data**

Ninety-five isolates of V. parahaemolyticus were recovered. Fifty were from expatriates with diarrhoea and 43 from Thai adults with diarrhoea. Two isolates were identified from non-diarrhoea controls: one from an expatriate and the other from a Thai adult. The finding of similar numbers of expatriates and Thai adults infected with V. parahaemolyticus (51/417 expatriates and 44/400 Thai adults) suggests that both populations are equally susceptible to infection with the species. Epidemiological data showed that all of the expatriates with V. parahaemolyticus stayed in Thailand for at least 48 h before the onset. The incubation period of V. parahaemolyticus is between 4 and 30 h (Besser et al., 2003), suggesting that most expatriates potentially acquired the infection in Thailand. Of the 93 diarrhoea cases with V. parahaemolyticus, 74% reported watery diarrhoea whilst 26% reported loose stools; only one case of bloody diarrhoea was noted. Other symptoms reported included abdominal pain (89%), nausea (77%), vomiting (61%), fatigue (55%) and fever (53%). No significant difference was found between self-reported symptoms and pandemic versus non-pandemic isolates from cases.

**Serotyping**

Twenty-three different combinations of O and K antigens were found and included three previously recognized pandemic group serovars, O3 : K6, O1 : K25 and O1 : KUT, accounting for 54% (51/95), 11% (10/95) and 5% (5/95), respectively (Table 1). One pandemic isolate, O3 : K6, was found from an expatriate in the control group. The non-pandemic isolates belonged to 19 different O : K antigen combinations. These findings confirmed the presence of the same pandemic clone among clinical isolates as described in a previous study from southern Thailand in 2000–2002 (Vuddhakul et al., 2006). However, serovars O4 : K68, O1 : K41 and O4 : K12, which were identified in pandemic clones in 1998 and 1999 from southern Thailand, were not detected in this study (Chowdhury et al., 2000a; Laohaprertthisan et al., 2003). The prevalence of pandemic isolates of serotype O3 : K6 in expatriates and Thai adults was 71% (36/51) and 34% (15/44), respectively. This may be explained by different patterns of food consumption but, unfortunately, dietary information was not collected. Furthermore, little information regarding the existence of pandemic isolates in environmental and food samples is available in Thailand (Vuddhakul et al., 2000).
Fifty-two per cent (49/95) of V. parahaemolyticus isolates were resistant to ampicillin and sulfisoxazole. None of the isolates were resistant to chloramphenicol, kanamycin, gentamicin, tetracycline, trimethoprim–sulfamethoxazole, nalidixic acid or ciprofloxacin. Thus all V. parahaemolyticus isolates tested were susceptible to antibiotics commonly used to treat traveller’s diarrhoea.

**Table 1.** Serotypes, genotypes and PFGE typing of V. parahaemolyticus isolates from Thailand in 2001–2002

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Strains</th>
<th>Total (no. expatriates/Thai adults)</th>
<th>toxR</th>
<th>tdh</th>
<th>trh</th>
<th>GS-PCR</th>
<th>ORF8-PCR</th>
<th>PFGE subtypes*</th>
</tr>
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<tbody>
<tr>
<td>O3 : K6</td>
<td>VPT 8–42/51–65</td>
<td>50 (35/15)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>A1–A5</td>
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<td>O3 : K6</td>
<td>VPTC-1</td>
<td>1 (1/0)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>D</td>
</tr>
<tr>
<td>O1 : K25</td>
<td>VPT 1–4/66–71</td>
<td>10 (4/6)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>A2, A6, A7</td>
</tr>
<tr>
<td>O1 : KUT</td>
<td>VPT 5–7, 44</td>
<td>4 (4/0)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>A2</td>
</tr>
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<td>O1 : KUT</td>
<td>VPT 72</td>
<td>1 (0/1)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>NP</td>
</tr>
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<td>O3 : K46</td>
<td>VPT 82</td>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>O1 : K56</td>
<td>VPT 43/73–75</td>
<td>4 (1/3)</td>
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<td>+</td>
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<td>O2 : K3</td>
<td>VPT 45</td>
<td>1 (1/0)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>NP</td>
<td></td>
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<tr>
<td>O3 : K18</td>
<td>VPT 46</td>
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<td>+</td>
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<td>-</td>
<td>NP</td>
<td></td>
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<tr>
<td>O3 : K29</td>
<td>VPT 76</td>
<td>1 (0/1)</td>
<td>+</td>
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<td>-</td>
<td>-</td>
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<td>O3 : K5</td>
<td>VPT 77</td>
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<tr>
<td>O3 : K57</td>
<td>VPT 47</td>
<td>1 (1/0)</td>
<td>+</td>
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<td>O3 : K7</td>
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<td>+</td>
<td>-</td>
<td>-</td>
<td>NP</td>
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</tr>
<tr>
<td>O3 : KUT</td>
<td>VPT 48</td>
<td>1 (1/0)</td>
<td>+</td>
<td>+</td>
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<td>NP</td>
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<tr>
<td>O4 : K13</td>
<td>VPT 79</td>
<td>1 (0/1)</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>NP</td>
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<tr>
<td>O4 : K4</td>
<td>VPT 80, VPTC 2</td>
<td>2 (0/2)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NP</td>
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<tr>
<td>O4 : K46</td>
<td>VPT 81</td>
<td>1 (0/1)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>NP</td>
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<tr>
<td>O4 : K8</td>
<td>VPT 83, 84</td>
<td>2 (0/2)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>NP</td>
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<tr>
<td>O4 : K9</td>
<td>VPT 49/85–88</td>
<td>5 (1/4)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>NP</td>
<td></td>
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<td>VPT 89</td>
<td>1 (0/1)</td>
<td>+</td>
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<td>-</td>
<td>NP</td>
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</tr>
<tr>
<td>O8 : K41</td>
<td>VPT 50</td>
<td>1 (1/0)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>NP</td>
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<tr>
<td>OUT : K18</td>
<td>VPT 90</td>
<td>1 (0/1)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>NP</td>
<td></td>
</tr>
<tr>
<td>OUT : K46</td>
<td>VPT 91</td>
<td>1 (0/1)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>NP</td>
<td></td>
</tr>
<tr>
<td>OUT : K8</td>
<td>VPT 92</td>
<td>1 (0/1)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>NP</td>
<td></td>
</tr>
<tr>
<td>OUT : KUT</td>
<td>VPT 93</td>
<td>1 (0/1)</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>NP</td>
<td></td>
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<tr>
<td>Total</td>
<td>–</td>
<td>95 (51/44)</td>
<td>95</td>
<td>91</td>
<td>5</td>
<td>66</td>
<td>65</td>
<td>–</td>
</tr>
</tbody>
</table>

*NP, Non-pandemic cluster.

**Antimicrobial susceptibility**

Fifty-two per cent (49/95) of V. parahaemolyticus isolates were resistant to ampicillin and sulfoisoxazole. None of the isolates were resistant to chloramphenicol, kanamycin, gentamicin, tetracycline, trimethoprim–sulfamethoxazole, nalidixic acid or ciprofloxacin. Thus all V. parahaemolyticus isolates tested were susceptible to antibiotics commonly used to treat traveller’s diarrhoea.

**Fig. 1.** Nol-PFGE of selected V. parahaemolyticus isolates with different serovars. Lanes 1, 6, 11, 16 and 21, molecular mass marker of Salmonella serotype Branderup H 9812; lanes 2–4, 8, 9, 15 and 17–20, different serovars of non-pandemic isolates (VPT 77, 85, 86, 92, 83, 75, 81, 87, 91 and 76); lanes 7, 10 and 13, pandemic isolates of serovar O3 : K6 (VPT 63–65); lanes 5 and 12, pandemic isolates of serovars O1 : KUT and O1 : K25 (VPT 72 and 71); lane 14, an isolate of serovar O3 : K46 (VPT 82).
Genotypic traits

Table 1 shows that all V. parahaemolyticus isolates were positive for the species-specific gene toxR, 91 were positive for tdh, five for trh and four for both tdh and trh. All five trh gene-positive isolates were urease positive. Interestingly, three isolates (two from diarrhoea cases and one asymptomatic control) were negative for both tdh and trh. GS-PCR and ORF8-PCR demonstrated that 71% (66/95) of isolates carried the GS sequence of the toxRS operon unique to the pandemic strains of V. parahaemolyticus; these isolates, except for one, were also positive for the orf8 gene. All of the pandemic isolates of the three known serovars O3 : K6, O1 : KUT, O1 : K25 and O3 : K46 were in subtypes A1–A7 (90–100% similarity). All non-pandemic isolates were in the non-pandemic (NP) cluster (<50% similarity).

Fig. 2. Dendrogram of 93 V. parahaemolyticus isolates from Thailand in 2001–2002. All pandemic isolates of serovars O3 : K6, O1 : KUT, O1 : K25 and O3 : K46 were in subtypes A1–A7 (90–100% similarity). All non-pandemic isolates were in the non-pandemic (NP) cluster (<50% similarity).
five isolates carrying the trh gene were positive for GS-PCR and ORF8-PCR.

As previously reported, this study confirmed that the majority of clinical V. parahaemolyticus isolates have the tdh gene and lack the trh gene (Laohapretrthisan et al., 2003; Martinez-Urtaza et al., 2004; Suthienkul et al., 1995; Vuddhakul et al., 2000). Furthermore, isolates from two acute diarrhoea cases were negative for both the tdh and trh genes. Our investigation did not detect any other enteric bacterial pathogens, parasites (Giardia and Cryptosporidium) or viruses (rotavirus, norovirus, adenovirus and astrovirus) in these two acute diarrhoea cases. This suggested the possibility of either other unknown pathogens or other virulence mechanisms in V. parahaemolyticus.

**DNA fingerprint and cluster analysis**

PFGE was performed on all 95 V. parahaemolyticus isolates, but two isolates showed poorly resolved patterns and were omitted from the cluster analysis. Fig. 1 shows NotI-PFGE patterns of selected V. parahaemolyticus isolates. An isolate of serovar O3 : K46 (lane 14) showed two DNA band shifts of approximately 240 and 217 kb compared with serovar O3 : K6 (lane 13). Cluster analysis and a dendrogram combined with serotype results revealed that all of the pandemic isolates of serovars O3 : K6, O1 : K25, O1 : KUT and O3 : K46 had a high degree of similarity (90–100%), as illustrated in Fig. 2. Serotyping and PFGE of the V. parahaemolyticus isolate serovar O3 : K46 was repeated twice with reproducible results. From the dendrogram, the pandemic isolates were classified into seven subtypes (A1–A7). The newly emerged serovar O3 : K46 belonged to subtype A2. All pandemic isolates of serovar O1 : K25 clustered in subtypes A6 and A7 with one exception. As expected, the isolate of serovar O1 : KUT that was negative by GS-PCR and ORF8-PCR was not classified in the pandemic cluster (<50% similarity). The non-pandemic isolates clustered separately from pandemic isolates.

In this study, we combined both genotypic characterization and molecular typing to gain a better understanding of strain diversity of V. parahaemolyticus in Thailand. The combination of serotyping and molecular methods for detection of genetic markers such as virulence genes, GS sequence, orf8 gene and PFGE was valuable for epidemiological surveillance in distinguishing pandemic isolates from non-pandemic isolates. Our results coupled with those of other researchers suggest that serovars of pandemic V. parahaemolyticus are changing over time (Chowdhury et al., 2000b; Laohapretrthisan et al., 2003; Matsumoto et al., 2000), but pandemic isolates of serovar O3 : K6 still maintain the ability to survive in the environment. The detection of a single isolate of V. parahaemolyticus serovar O3 : K46 having pandemic traits should provide the impetus for continued surveillance of V. parahaemolyticus in Thailand and elsewhere.

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