

# The dominance of pandemic serovars of *Vibrio parahaemolyticus* in expatriates and sporadic cases of diarrhoea in Thailand, and a new emergent serovar (O3 : K46) with pandemic traits

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*Vibrio parahaemolyticus* is a major cause of gastroenteritis worldwide. A total of 95 *V. parahaemolyticus* isolates belonging to 23 different serovars were identified in a case–control study of expatriates and Thai adults from 2001 to 2002 in Thailand. Fifty-two per cent of isolates (49/95) were resistant to ampicillin and sulfisoxazole, but all isolates were susceptible to ciprofloxacin and trimethoprim–sulfamethoxazole, two antibiotics commonly used to treat traveller's diarrhoea. All isolates were positive for the species-specific *toxR* gene, and 91 and 5 were positive for the thermostable direct haemolysin (*tdh*) gene and the *tdh*-related (*trh*) gene, respectively. Sixty-five isolates were assigned to the pandemic group of *V. parahaemolyticus* by a group-specific PCR and the presence of the *orf8* gene. The pandemic isolates belonged to three recognized serovars (O3 : K6, O1 : K25, O1 : KUT) and a new serovar, O3 : K46. This new serovar harboured pandemic traits. PFGE analysis revealed that all pandemic isolates including serovar O3 : K46 were closely related and clearly distinct from the non-pandemic isolates. In summary, three well-known serovars of pandemic *V. parahaemolyticus* isolates were identified as a major cause of diarrhoea in Thailand and a new *V. parahaemolyticus* isolate, serovar O3 : K46, with pandemic traits was detected.

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## 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.*, 2000). 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

Abbreviation: GS, group-specific.

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  $\leq 1$  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, thiosulfate/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.** *NotI*-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).

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

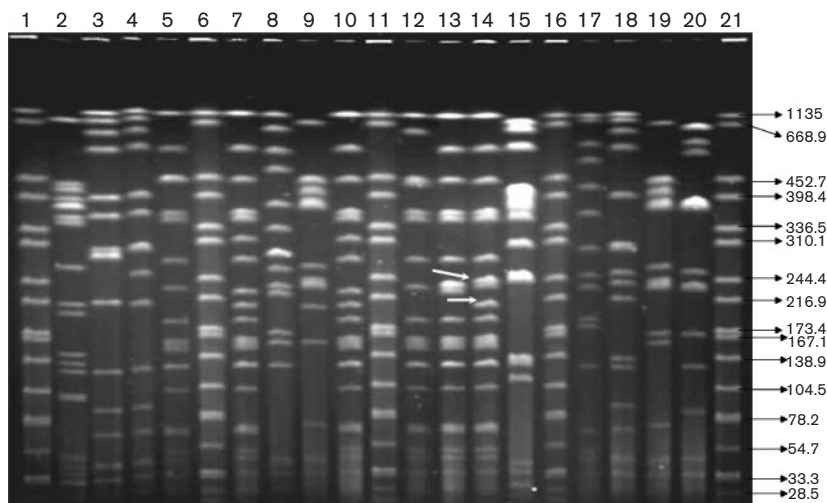
Serotype	Strains	Total (no. expatriates/ Thai adults)	<i>toxR</i>	<i>tdh</i>	<i>trh</i>	GS-PCR	ORF8-PCR	PFGE subtypes*
O3 : K6	VPT 8–42/51–65	50 (35/15)	+	+	–	+	+	A1–A5
O3 : K6	VPTC-1	1 (1/0)	+	+	–	+	–	A4
O1 : K25	VPT 1–4/66–71	10 (4/6)	+	+	–	+	+	A2, A6, A7
O1 : KUT	VPT 5–7, 44	4 (4/0)	+	+	–	+	+	A2
O1 : KUT	VPT 72	1 (0/1)	+	+	+	–	–	NP
O3 : K46	VPT 82	1 (0/1)	+	+	–	+	+	A2
O1 : K56	VPT 43/73–75	4 (1/3)	+	+	–	–	–	NP
O2 : K3	VPT 45	1 (1/0)	+	+	–	–	–	NP
O3 : K18	VPT 46	1 (1/0)	+	+	+	–	–	NP
O3 : K29	VPT 76	1 (0/1)	+	+	–	–	–	NP
O3 : K5	VPT 77	1 (0/1)	+	+	–	–	–	NP
O3 : K57	VPT 47	1 (1/0)	+	+	–	–	–	NP
O3 : K7	VPT 78	1 (0/1)	+	+	–	–	–	NP
O3 : KUT	VPT 48	1 (1/0)	+	–	–	–	–	NP
O4 : K13	VPT 79	1 (0/1)	+	+	–	–	–	NP
O4 : K4	VPT 80, VPTC 2	2 (0/2)	+	–	–	–	–	NP
O4 : K46	VPT 81	1 (0/1)	+	–	+	–	–	NP
O4 : K8	VPT 83, 84	2 (0/2)	+	+	–	–	–	NP
O4 : K9	VPT 49/85–88	5 (1/4)	+	+	–	–	–	NP
O8 : K21	VPT 89	1 (0/1)	+	+	–	–	–	NP
O8 : K41	VPT 50	1 (1/0)	+	+	–	–	–	NP
OUT : K18	VPT 90	1 (0/1)	+	+	+	–	–	NP
OUT : K46	VPT 91	1 (0/1)	+	+	+	–	–	NP
OUT : K8	VPT 92	1 (0/1)	+	+	–	–	–	NP
OUT : KUT	VPT 93	1 (0/1)	+	+	–	–	–	NP
Total	–	95 (51/44)	95	91	5	66	65	–

\*NP, Non-pandemic cluster.

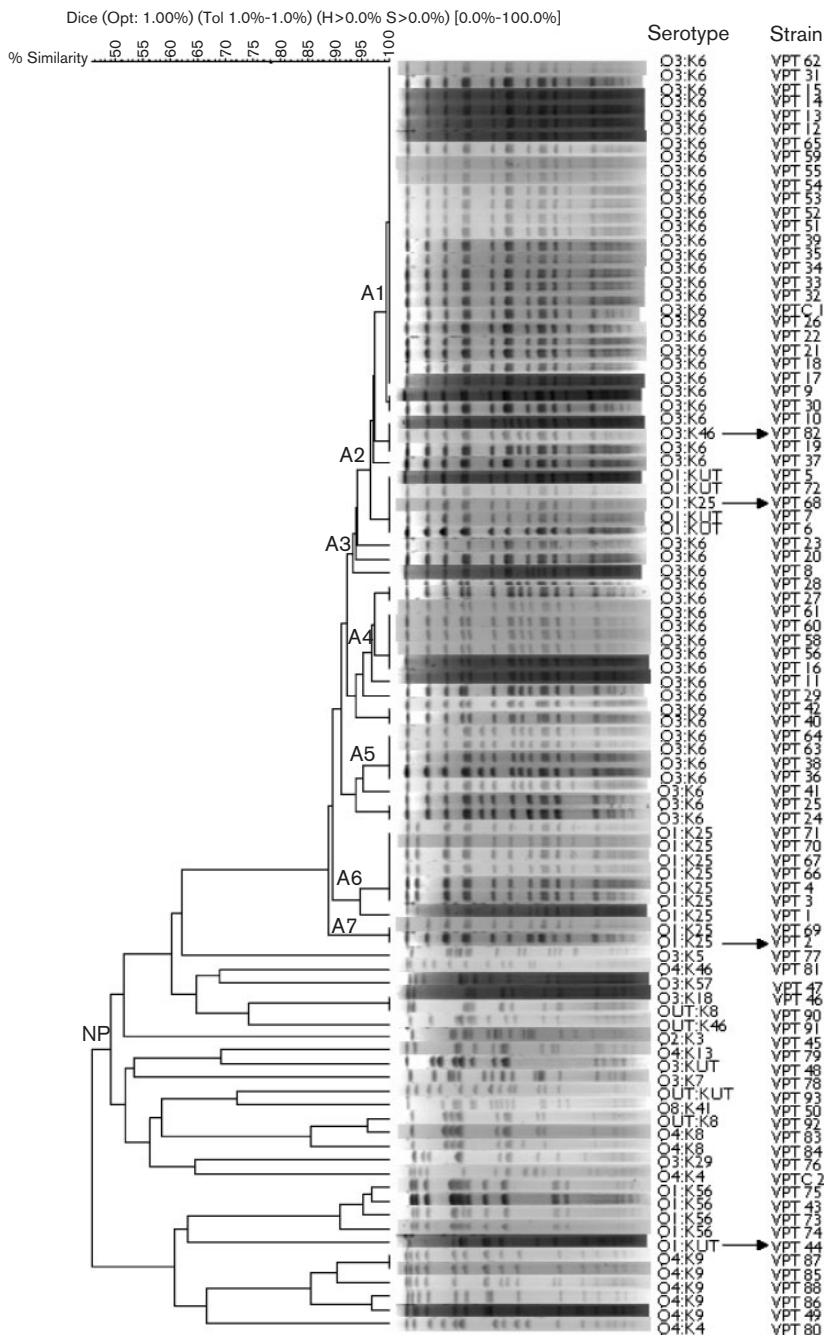
### Antimicrobial susceptibility

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.



**Fig. 1.** *NotI*-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).



**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).

**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 : K25 and O1 : KUT were positive for *tdh*, GS-PCR and ORF8-PCR with the exception of one isolate, serovar O1 : KUT, that was negative for GS-PCR and ORF8-PCR. By definition, this isolate should therefore not be classified as pandemic. Interestingly, one isolate, serovar O3 : K46, from a Thai case, positive for *toxR*, *tdh*, GS-PCR and ORF8-PCR, harboured the pandemic traits. A clinical Thai isolate, serovar O3 : K46, was previously reported as a non-pandemic strain in 1999 (Okura *et al.*, 2003). None of the

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 (Laohaprertthisan *et al.*, 2003; Martinez-Urtaza *et al.*, 2004; Suthienkul *et al.*, 1995; Uddhakul *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; Laohaprertthisan *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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