Epidemiol. Infect. (2003), **130**, 395–406. © 2003 Cambridge University Press
DOI: 10.1017/S0950268803008458 Printed in the United Kingdom

Prevalence and serodiversity of the pandemic clone among the clinical strains of *Vibrio parahaemolyticus* isolated in southern Thailand

V. LAOHAPRERTTHISAN[1], A. CHOWDHURY[2,3], U. KONGMUANG[1], S. KALNAUWAKUL[1], M. ISHIBASHI[4], C. MATSUMOTO[2] AND M. NISHIBUCHI[2]*

[1] *Department of Pathology, Faculty of Medicine, Prince of Songkla University, Hat-Yai, Songkla 90112, Thailand*
[2] *Division of Human Environment, Center for Southeast Asian Studies, Kyoto University, Yoshida, Sakyo-ku, Kyoto 606-8501, Japan*
[3] *Graduate School of Medicine, Kyoto University, Yoshida, Sakyo-ku, Kyoto 606-8501, Japan*
[4] *Division of Food Sanitation, Osaka Prefectural Institute of Public Health, Higashinari-ku, Osaka 537-0025, Japan*

(*Accepted 15 January 2003*)

SUMMARY

We collected diarrhoea specimens in two hospitals in southern Thailand in 1999 to examine whether infection by the *Vibrio parahaemolyticus* pandemic clone is prevalent. *V. parahaemolyticus* was isolated from 317 specimens. Seventy-six per cent of the isolated strains had the pandemic clone-specific characteristics (*tdh*$^+$, *trh*$^-$, and an unique *toxRS* sequence detectable by GS-PCR) and an associated characteristic (the ORF8 sequence of f237 phage). These strains belonged to the three pandemic servovars with the O3:K6 strains being dominant and three other serovars (O1:K25, O1:K41 and O4:K12). We also found O1:K25 and O1:K41 strains with the pandemic clone-specific characteristics among the strains isolated from the international travellers who left Thailand and three other Asian countries between 1998 and 1999, verifying pandemic potential of these strains. The results demonstrate prevalence of infection by the pandemic clone in southern Thailand and suggest emergence of various serovariants in this area and their implication in international spread.

INTRODUCTION

Vibrio parahaemolyticus inhabits the marine environment and it can cause sea food-borne gastroenteritis in humans. However, not all strains are considered virulent strains. The strains capable of producing the thermostable direct haemolysin and thus inducing Kanagawa phenomenon, beta-type haemolysis on a special blood agar medium (Wagatsuma agar), had long been considered clinically significant [1]. The thermostable direct haemolysin is encoded by the *tdh* gene and enterotoxicity of thermostable direct haemolysin was demonstrated using an isogenic *tdh*-defective mutant strain [2, 3]. The *trh* gene that is 68–69% homologous to the *tdh* gene and encodes a thermostable direct haemolysin-related haemolysin was first discovered in a *tdh*-negative clinical strain [4, 5]. Subsequently, molecular epidemiological studies revealed that most clinical strains possess the *tdh* gene, the *trh* gene, or both genes [6, 7]. On the contrary, the *tdh* gene or the *trh* gene was rarely detected in the environmental strains [6–8].

Incidence of *V. parahaemolyticus* infection has increased in many Asian countries and the United States since 1996 and this appears due to a pandemic spread of a particular group of strains [9, 10]. These

* Author for correspondence.

strains were initially characterized by the O3:K6 serovar [10]. These strains could be distinguished from other strains by possession of the *tdh* gene, but not the *trh* gene, by unique profiles in an arbitrarily primed PCR (AP-PCR) analysis [9, 10]. The strains having these characteristics were isolated from seven Asian countries and the United States and from international travellers from 1995 onwards, and thus the strains were called pandemic strains. We subsequently developed a PCR method with which we could easily identify the pandemic clone. This PCR method utilizes the nucleotide sequence variation in the *toxRS* operon encoding a transcriptional regulator [10]. The *toxRS* sequence accumulated enough base changes during evolution and thus it is useful for genetic differentiation of the members of the genus *Vibrio* [11–13]. We found that 1346-bp nucleotide sequences covering 95·4% of the *toxRS* coding region of the pandemic strains of O3:K6 serovar were identical and they differed from those of non-pandemic O3:K6 strains at seven or more base positions. On the basis of this finding, we developed a PCR method to detect two of the nucleotide bases unique to the pandemic strain group and this PCR method was named GS-PCR for group-specific PCR [10]. Examination of various strains by the GS-PCR method lead to the finding that O4:K68 and O1:K untypable (KUT) strains isolated from 1997 onwards have the molecular genetic characteristics of pandemic O3:K6 strains (possession of the *tdh* gene, unique AP-PCR profiles, positive GS-PCR reaction, and 100% identical *toxRS* sequence) [10]. The results of the analyses by pulsed-field gel electrophoresis and ribotyping methods subsequently supported that these strains and the pandemic O3:K6 strains belonged to the same clone [14–17]. Based on the above findings we employ the following simple characteristics to define the pandemic clone at present: possession of the *tdh* gene, but not the *trh* gene, positive reaction in the GS-PCR assay. The PCR assays to examine these characteristics are simple and the results are easy to judge. DNA fingerprinting methods such as AP-PCR, pulsed-field gel electrophoresis, and ribotyping are useful for epidemiological analysis but they are time-consuming and interpretation of the result may need some expertise. These methods could be used for differentiation between pandemic clone and non-pandemic clone and for comparison of the strains belonging to pandemic clone when needed. The O4:K68 and O1:KUT strains with the characteristics of the pandemic clone have been isolated from patients in Bangladesh, India, Japan, and Thailand and from international travellers [10, 15, 16]. We therefore consider that these strains probably diverged from the O3:K6 pandemic clone by alteration of the genes associated with the O:K antigens and followed a spreading pattern similar to that of the pandemic O3:K6 clone [10]. Although future surveillance of clinical strains in various countries is needed to confirm this hypothesis, we regard the O4:K68 and O1:KUT strains with the characteristics of the pandemic clone as pandemic clone and describe them as pandemic strains in this communication.

In addition, these strains belonging to the three serovars seem to share highly homologous genomic sequences of lysogenic filamentous phages [18–21]. The first well-characterized filamentous phages of *V. parahaemolyticus* were the phages named Vf12 and Vf33 [18]. These phages were found in non-pandemic strains and their genomic sequences were related to those of the filamentous phages of *Vibrio cholerae* and *Escherichia coli* [18]. The nucleotide sequences of one genomic region of Vf12 and Vf33 phages (named a distinctive region) were shown to be unique to the *V. parahaemolyticus* filamentous phages [18]. The genomic sequence of the filamentous phage, f237, found in a pandemic O3:K6 strain, KX-V237, contained three open reading frames designated ORF8, ORF9, and ORF10 in the region corresponding to the distinctive region [19]. ORF8, ORF9, and ORF10 hereinafter refer to these sequences of f237 in this paper. The filamentous phages of pandemic O3:K6, O4:K68, and O1:KUT strains appeared to share a unique sequence(s) [20]. The ORF8 sequence, in particular, was shown to be specific to pandemic O3:K6, O4:K68, and O1:KUT strains isolated from international travellers and thus ORF8 was proposed to be a genetic marker for pandemic strains [19, 21]. However, lysogenic phages may not be stably maintained. It would be important to evaluate whether possession of ORF8 can be a characteristic of pandemic clone.

It is not known where in the marine environment the pandemic strains originated, where and how the serovariants emerged, and how these strains spread. Our group was able to isolate an O3:K6 strain having pandemic clone-specific characteristics from a fresh shellfish in Hat Yai City located in southern Thailand in December 1998 [22]. In addition, O3:K6 strains having pandemic clone-specific characteristics had been isolated from clinical specimens in September and October 1998 in this area [22]. The clinical and environmental O3:K6 strains with pandemic clone-specific characteristics showed identical AP-PCR

profiles [22]. It suggested to us a possibility that the pandemic strains may be prevalent in the environment around Hat Yai City and responsible for frequent infection in this area. We therefore conducted a 1-year survey on diarrhoea specimens in this area in 1999. We report the results of the survey and analysis of the isolated *V. parahaemolyticus* strains in this paper. The results demonstrated that *V. parahaemolyticus* infection was prevalent in this area and that the pandemic clone was responsible for majority of *V. parahaemolyticus* infections. In addition, the strains with pandemic clone-specific characteristics but belonging to three serovars other than O3:K6, O4:K48, and O1:KUT were isolated. We also demonstrated that international travellers carried the strains with pandemic clone-specific characteristics and belonging to two of newly recognized three serovars during the 1998–9 period. We then discussed epidemiological implication of the pandemic strains detected in southern Thailand.

MATERIALS AND METHODS

Hospital surveillance

Stool or rectal swab samples were collected from the patients with diarrhoea in Songklanagarind Hospital and Hat Yai Hospital in Hat Yai City from January 1999 to December 1999. The stool specimens and rectal swabs were inoculated into Stuart's transport medium and maintained until bacteriological examination.

Bacteriology

The samples were plated directly onto thiosulphate-citrate-bile salt-sucrose agar (TCBS agar, Eiken Chemical Co. Ltd, Tokyo, Japan). The green colonies detected on the medium were examined by standard biochemical tests for identification of *V. parahaemolyticus* [23]. Strains screened by the biochemical tests were examined by the PCR method for detection of the *V. parahaemolyticus*-specific *toxR* gene sequence [11]. The strain that gave positive result in this PCR test was identified as *V. parahaemolyticus*. *V. parahaemolyticus* strains other than those isolated in Hat Yai City, including KX-V237, were obtained from Kansai Airport Quarantine Station, Osaka, Japan and the identification was confirmed by the PCR method as described above. These strains were isolated from the travellers arriving in Kansai Airport from various Asian countries.

O:K serovar

The test strains were grown and their O:K serovars were determined using specific antisera as described previously [24].

PCR

The presence or absence of the *tdh* and *trh* genes in test strains was determined by the PCR methods using primers D3 and D5 for the *tdh* gene and primers R2 and R6 for the *trh* gene as described previously [25]. GS-PCR to identify the strain belonging to the pandemic clone was performed as described previously [10] with a minor modification. The method to prepare the PCR template was modified to replace the broth culture with the bacterial cells suspended in saline before the boiling step as follows. The test strain was grown in Luria–Bertani (LB) broth medium containing 1% NaCl at 37 °C with shaking (160 rpm) overnight. One millilitre of the culture was centrifuged (5000 rpm) on a tabletop centrifuge (Centrifuge 5415C; Eppendorf, Hamburg, Germany) at room temperature. The supernatant was discarded and the pelleted bacterial cells were suspended in 1 ml of saline (0·85% NaCl) and then boiled for 10 min and transferred onto ice immediately. The supernatant was then obtained by centrifugation (14 000 rpm) at room temperature. The supernatant was diluted 10-fold with distilled water and used as the template solution for PCR.

PCR methods to detect ORF8, ORF9, and ORF10 were established as follows in this study. A forward primer designated VP36RF8U (5′-GCATACAGTTG AGGGGAAAG-3′) and a reverse primer designated VP36RF8L (5′-AGCGCTCTTTGTTTTCTATATG-3′) were used for detection of the ORF8 sequence. For detection of the ORF9 sequence, a forward primer designated VP36RF9U (5′-ACAAGAGCTTAATTC CCTACCC-3′) and a reverse primer designated VP36RF9L (5′-TTAACCGTCGAAGAAGCTGCC-3′) were used. For detection of the ORF10 sequence, a forward primer designated VP36RF10U (5′-TGCG CACTGATATAGGGGTT-3′) and a reverse primer designated VP36RF10L (5′-GCCCGCAAATGATC ACTGAA-3′) were used. The sequences of the primers VP36RF8U, VP36RF8L, VP36RF9U, VP36RF9L, VP36RF10U, and VP36RF10L were identical with (forward primers) or complementary to (reverse primers) the plasmid pO3K6 sequence, the replicative form of the filamentous phage of strain KX-V237 (the base positions correspond to 5241–5260, 6277–6298,

6781–6802, 6895–6915, 7043–7062 and 7289–7308, respectively) (reference [19] and GenBank accession no. AP000581). The ORF9 and ORF10 sequences were examined simultaneously by a multiplex PCR method but the ORF8 sequence was examined by a separate PCR method. The 1:10 diluted supernatant of the boiled broth culture prepared as described above for GS-PCR was used as the template solution. The PCR reaction mixture consisted of 2 μl of 10 × thermophilic DNA polymerase 10 × buffer (Magnesium Free; Promega Corp., Madison, WI, USA; containing 100 mM Tris–HCl [pH 9·0], 500 mM KCl, 1% Triton X-100), 2 mM $MgCl_2$, 0·2 mM deoxynucleoside triphosphate, 0·5 mM (the PCR for ORF8) or 0·25 mM (the multiplex PCR for ORF9 and ORF10) each of the primers, 1·5 μl template solution (supernatant of the boiled culture diluted 1:10), and 0·5 U *Taq* DNA polymerase in storage buffer A (Promega Corp.), in a 20 μl volume. The amplification conditions for the PCR for ORF8 were set at one cycle of 96 °C for 5 min followed by 25 cycles of amplification consisting of denaturation at 94 °C for 1 min, annealing at 53 °C for 1 min, and extension at 72 °C for 1 min, and then followed by one cycle of 72 °C for 7 min. The amplification conditions for the multiplex PCR for ORF9 and ORF10 were set at one cycle of 96 °C for 5 min followed by 35 cycles of amplification consisting of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, and extension at 72 °C for 1 min, and then followed by one cycle of 72 °C for 7 min. The PCR-amplified mixture was resolved by electrophoresis in 2% agarose gel to detect 1058-bp (ORF8), 135-bp (ORF9), or 266-bp (ORF10) amplicons.

DNA colony hybridization tests

The DNA probes for detection of the ORF8, ORF9, and ORF10 were purified by agarose gel electrophoresis from the products of the PCR reactions performed as described above. The template solution was prepared from LB broth culture of KX-V237 strain [19]. DNA colony blots were prepared and hybridization under high-stringency conditions was carried out as described previously [8].

Antibiotic sensitivity

Antibiotic susceptibilities of test strains were measured by the disk diffusion method of Bauer [26] with the following antibiotics: ampicillin (30 μg), tetracycline (30 μg), cotrimoxazole (trimethoprim, 1·25 μg; sulphamethoxazole, 23·75 μg), chloramphenicol (30 μg), and norfloxacin (10 μg).

AP-PCR

Cellular DNA was extracted from the test strain as described previously [27] and was used as the template for AP-PCR. The sequences of the primers were: 5′-GGTGCGGGAA-3′ (primer 1); 5′-GTTTCGCTCC-3′ (primer 2); 5′-GTAGACCCGT-3′ (primer 3); 5′-AAGAGCCCGT (primer 4); 5′-AACGCGCAAC-3′ (primer 5); and 5′-CCCGTCAGCA-3′ (primer 6). These primers were used and the amplification products analysed as described previously [28]. The dendrogram was constructed from the AP-PCR profiles according to an Unweighted Pair-Group Method using Arithmetic Average (UPGMA) clustering analysis by using DENDRON software version 3 (Solltech Inc., Oakdale, LA, USA).

Nucleotide sequence determination

The *toxRS* region of the test DNA was amplified by a PCR method and the nucleotide sequence of the amplicon was determined by a cycle sequencing method as described previously [10].

Nucleotide sequence accession numbers

The nucleotide sequence data of the *toxRS* operon of *V. parahaemolyticus* strains reported in this paper will appear in the DDJB/EMBL/GenBank nucleotide sequence databases with the following accession numbers (the strain number is indicated in parentheses): AB063111 (VPHY191); AB063112 (VPHY197); AB063113 (VPHY216).

RESULTS

Surveillance data

V. parahaemolyticus was isolated from 317 diarrhoea specimens during the 1999 survey period in two hospitals: 276 (3·33%) of 8281 and 43 (1·35%) of 3193 specimens collected in Hat Yai Hospital and Songklanagarind Hospital, respectively. The monthly incidence of *V. parahaemolyticus* infection (those of the two hospitals combined) is presented in Figure 1. *V. parahaemolyticus* was isolated throughout the

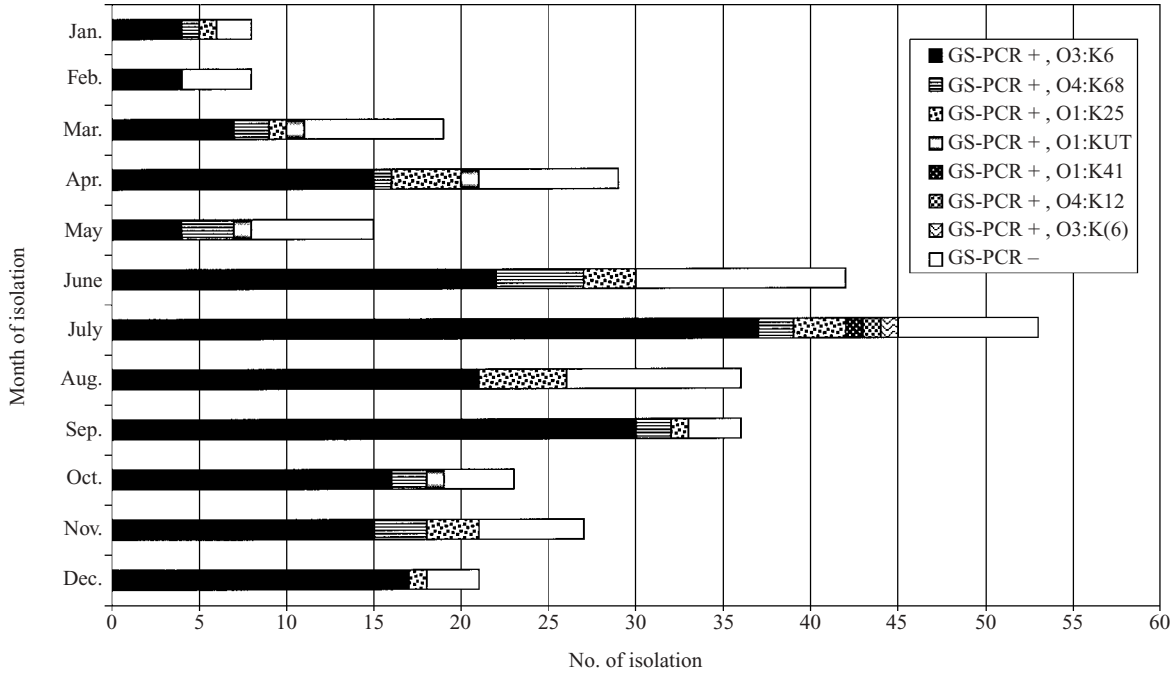

Fig. 1. Monthly incidence of *V. parahaemolyticus* infection in two hospitals in Hat Yai City in 1999. The results of the GS-PCR assay for isolated strains are included. Serovars within GS-PCR+ strains are also indicated. K(6) denotes weak agglutination with anti-K6 serum.

year with the June–September period showing very high isolation frequencies.

Characteristics of the isolates

The 317 strains isolated during the survey period were examined for the reaction in the GS-PCR assay, for the O:K serovar, and for presence or absence of the toxin genes (*tdh* and *trh*), ORF8, ORF9, and ORF10 by PCR methods (Table 1). The GS-PCR-positive (hereinafter abbreviated as GS-PCR+) reaction was selected as the marker for the pandemic clone in this study because all GS-PCR+ strains also showed other pandemic clone-specific characteristics: possession of the *tdh* but not *trh* gene (Table 1). GS-PCR+ strains accounted for 76·3% (242 strains) of total isolates. Of these pandemic strains, 192 (80%), 21 (8·4%), and 4 (1·8%) strains belonged, respectively, to O3:K6, O4:K68, and O1:KUT serovars. In addition, 24 strains belonging to 3 other serovars [O1:K25 (22 strains), O1:K41 (1 strain) and O4:K12 (1 strain)] were included in GS-PCR+ strains. One O3 strain showing weak agglutination with anti-K6, a possible derivative of a pandemic O3:K6 strain, was also detected among the GS-PCR+ strains. The GS-PCR+ strains belonging to O3:K6, O4:K68, or O1:K25 serovar were isolated throughout or almost throughout the survey period (Fig. 1). Four GS-PCR+ O1:KUT strains were isolated in separate months. GS-PCR+ strains of O1:K41, O4:K12, and the possible O3:K6-derivative serovars were isolated in July when monthly incidence was the highest.

GS-PCR-negative (hereinafter abbreviated as GS-PCR−) strains accounted for only 24·6% of the isolates, but they showed various toxin gene profiles and belonged to diverse serovars (Table 1).

All GS-PCR+ strains carried not only ORF8 but also ORF9 and ORF10 and all GS-PCR− strains lacked ORF8 but ORF9, ORF10, or both were detected in 48% of GS-PCR− strains (Table 1), confirming that only ORF8 can be a candidate for an additional characteristic of the pandemic strains.

Thirty-five strains representing the six serovars of GS-PCR+ group, excluding the presumed O3:K6 derivative serovar, were examined for their antibiograms. All strains showed the same antibiograms (sensitivity to chloramphenicol, tetracycline, cotrimoxazole, and norfloxacin, and resistance to ampicillin). Of the serovars detected in the GS-PCR+ group, four serovars (O4:K68, O1:K25, O1:KUT and O4:K12) were also found among the GS-PCR− strains. Four strains selected from the four serovars of the GS-PCR− group also exhibited the same antibiograms.

Table 1. *Characteristics of* V. parahaemolyticus *strains isolated from diarrhoea patients in Hat Yai City in 1999*

No. of strains	Results of GS-PCR†	No. of different O:K serovars	Presence of gene*		Presence of phage sequence‡		
			tdh	*trh*	ORF8	ORF9	ORF10
242	+	6	+	−	+	+	+
1	−	1	+	+	−	+	+
3	−	2	+	+	−	+	−
4	−	4	+	+	−	−	−
4	−	4	+	−	−	+	+
17	−	6	+	−	−	+	−
19	−	10	+	−	−	−	−
1	−	1	−	+	−	+	+
3	−	3	−	+	−	−	−
4	−	4	−	−	−	+	+
6	−	5	−	−	−	+	−
3	−	3	−	−	−	−	+
10	−	6	−	−	−	−	−

* Examined by PCR methods. +, present; −, absent.
† +, positive; −, negative.
‡ Open reading frames in the genome of filamentous phage f237 [19]. Examined by PCR methods as described in the text. +, present; −, absent.

The strains belonging to O1:K25, O1:K41, and O4:K12 serovars (newly recognized three serovars among GS-PCR+ strains) were further characterized to investigate their relationships with previously reported pandemic strains. The *toxRS* sequences of the O3:K6, O4:K68, and O1:KUT pandemic strains contained seven nucleotide bases that were unique to the pandemic clone [10]. We utilized only 2 of the 7 bases to develop the GS-PCR method. We therefore determined the nucleotide sequences of the *toxRS* operons of strains VPHY191, VPHY197, and VPHY216 representing O1:K25, O1:K41, and O4:K12 serovars of the GS-PCR+ group, respectively, to examine whether all the seven unique bases are conserved in these strains. The nucleotide sequences of the 3 strains not only contained the 7 pandemic clone-specific nucleotide bases but also the sequences were 100% identical with those of the O3:K6, O4:K68, and O1:KUT pandemic strains reported previously [10]. Strains VPHY191, VPHY197, and VPHY216 and related strains were also compared by an AP-PCR method. Six different primers were tested and two primers, primer 2 and primer 4, could differentiate the test strains whereas the other four primers could not. The results obtained with the primer 2 and primer 4 were essentially the same (Fig. 2*a*, *b*). The above three strains (lanes 9–11), the GS-PCR+ strains of O3:K6, O4:K68, and O1:KUT serovars isolated in this study (lanes 4, 6, 8), and pandemic strains of O3:K6, O4:K68, and O1:KUT serovars isolated in our previous studies (lanes 3, 5, 7) showed nearly identical profiles whereas GS-PCR− strains of O4:K68, O1:KUT, O1:K41, and O4:K12 serovars isolated in this study (lanes 12–15) manifested the profiles distinct from those of the GS-PCR+ strains.

Strains isolated from international travellers

To examine whether the newly recognized three serovars with pandemic clone-specific characteristics can also be found outside Thailand, we examined the O1:K25, O1:K41, and O4:K12 strains isolated from the international travellers arriving in Osaka, Japan from Asian countries between 1982 and 1999 (Table 2). Sixteen, 8, and 85 strains belonging to O1:K25, O1:K1, and O4:K12 serovars, respectively, were available for examination. Thirteen O1:K25 strains and one O1:K41 strain had pandemic-clone specific characteristics (GS-PCR positive, *tdh* gene positive, and *trh* gene negative). The GS-PCR+ O1:K25 strains were isolated from the travellers originated in Vietnam (6 strains), Thailand (5 strains), Laos (1 strain), and Hong Kong (1 strain) in 1998 and 1999 and the GS-PCR+ O1:K41 strain in Vietnam in 1999. These GS-PCR+ strains except for four O1:K25 strains had ORF8 as examined by the PCR method (Table 2). Absence of ORF8 in the

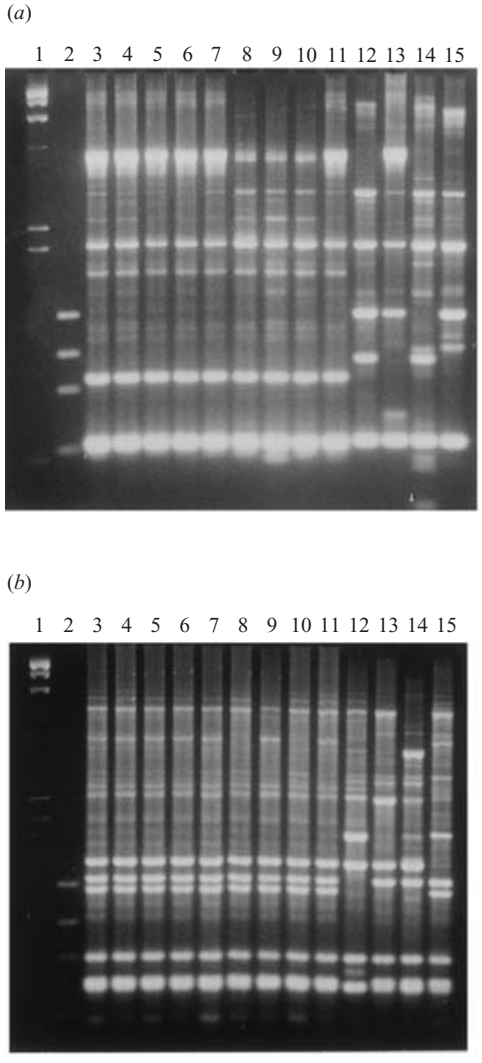

Fig. 2. Results of AP-PCR assay for *V. parahaemolyticus* strains isolated from diarrhoea patients in Hat Yai City. The result obtained with primers 2 and 4 is presented in panels (*a*) and (*b*), respectively. The test strains (lanes 4, 6, 8–15), other than the controls (lanes 3, 5, 7), were isolated from diarrhoea specimens in Hat Yai City in this study. Lane 1, molecular size markers (phage λ DNA digested with *Hin*dIII, 2·0–23·1 kb bands are clearly visible); lane 2, molecular size markers (phage ϕX174 DNA digested with *Hae*III, 0·60–1·4 kb bands are clearly visible); lane 3, strain VP 81 (a control: a pandemic O3:K6 strain isolated in India [9]); lane 4, strain VPHY67 (a GS-PCR+ O3:K6 strain); lane 5, strain AN5034 (a control: a pandemic O4:K68 strain isolated in Bangladesh [10]); lane 6, strain VPHY145 (a GS-PCR+ O4:K68 strain); lane 7, strain VP 187 (a control, a pandemic O1:KUT strain isolated in India [10]); lane 8, strain VPHY123 (a GS-PCR+ O1:KUT strain); lane 9, strain VPHY191 (a GS-PCR+ O1:K25 strain); lane 10, strain VPHY197 (a GS-PCR+ O1:K41 strain); lane 11, strain VPHY216 (a GS-PCR+ O4:K12 strain); lane 12, strain VPHY141 (a GS-PCR− O4:K68 strain); lane 13, strain VPHY135 (a GS-PCR− O1:KUT strain); lane 14, strain VPHY262 (a GS-PCR− O1:K41 strain); lane 15, strain VPHY229 (a GS-PCR− O4:K12 strain).

exceptional four GS-PCR+ O1:K25 strains was confirmed by the DNA colony hybridization assay with a polynucleotide probe. These four strains lacked ORF9 but carried ORF10 (results of PCR and the hybridization assays).

The strains representing GS-PCR+ international traveller strains showed the same antibiograms as those of the strains isolated in Hat Yai City.

AP-PCR profiles of the O1:K25 and O1:K41 strains representing GS-PCR+ and GS-PCR− international traveller strains (strain numbers listed in Table 2) and GS-PCR+ Hat Yai City strains were compared (Fig. 3). Since it seemed difficult to cluster the patterns by visual judgement, their relatedness was analysed by constructing the dendrogram from the profiles (Fig. 3). Primers 2 and 4 were used, and the results obtained with the two primers were essentially similar. GS-PCR+ O1:K25 strains from Hat Yai City and international travellers (Fig. 3*a*, *b*, lanes 1–3) and the GS-PCR+ O1:K41 strain from Hat Yai City (Fig. 3*a*, *b*, lane 6) formed a cluster. Of the O1:K41 strains isolated from the international travellers, a GS-PCR+ strain was closely related to this cluster when primer 2 was used (Fig. 3*a*, lane 7), but it was more closely related to two of the GS-PCR− O1:K41 strains isolated from the international travellers when tested with primer 2 (Fig. 3*b*, lanes 7–9). With this exception, GS-PCR− strains of O1:K25 serovar (Fig. 3*a*, *b*; lanes 4 and 5) and of O1:K41 (Fig. 3*a*, *b*; lanes 8–13) were distantly related with GS-PCR+ strains. The GS-PCR− strains were selected to cover a wide range of isolation year (1983–99) whereas the GS-PCR+ strains were isolated during 1998–9 period. Isolation year of the GS-PCR− strains did not correlate with the clustering pattern of the dendrogram and thus isolation year did not influence this analysis.

DISCUSSION

Year-round occurrence of *V. parahaemolyticus* infection in Hat Yai City in 1999 was demonstrated in this study. Although diarrhoea cases due to *V. parahaemolyticus* infections are less than dose due to infections by *Salmonella* and *Shigella* species in Hat Yai City, occurrence of 317 cases in two city hospitals per year is considered very frequent for *V. parahaemolyticus* infection. This tropical city is located between Songkla Lake, a brackish lake connected to Gulf of Thailand, and Andaman Sea. Therefore, fresh seafood is a very popular food in this area. This may

Table 2. *Characteristics of* V. parahaemolyticus *O1:K25, O1:K41, and O4:K12 strains isolated from international travellers arriving in Osaka, Japan between 1982 and 1999*

O:K serovar	No. of strains	Results of GS-PCR†	Presence of gene‡		Presence of phage sequence*						Representative strain§
					ORF8		ORF9		ORF10		
			tdh	*trh*	P	H	P	H	P	H	
1:25	9	+	+	−	+	+	+	+	+	+	KX-V641
	4	+	+	−	−	−	−	−	+	+	KX-V627
	2	−	+	+	−	−	−	−	+w	+	AQ4913
	1	−	−	−	−	−	−	−	−	−	AQ4366
1:41	1	+	+	−	+	+	+	+	+	+	KX-V829
	2	−	−	+	−	−	−	−	−	−	KX-V803
	1	−	+	+	−	−	−	−	+	+	KX-V755
	1	−	+	−	−	−	−	−	+w	+	AQ4750
	1	−	−	−	−	−	−	−	+	+	KX-V326
	1	−	+	−	−	−	+	+	+	+	AQ4592
	1	−	−	+	−	−	−	−	+w	+	AQ3754
4:12	73	−	+	−	−	NT	−	NT	−	NT	
	6	−	+	+	−	NT	+	NT	−	NT	
	2	−	+	−	−	NT	+	NT	−	NT	
	2	−	+	+	−	NT	−	NT	−	NT	
	2	−	−	−	−	NT	−	NT	−	NT	

* Open reading frames in the genome of filamentous phage f237 [19]. Examined by PCR methods (P) and hybridization methods with polynucleotide probes (H). +, present; +w, related sequence present as indicated by weak reaction; −, absent; NT, not tested.

† +, positive; −, negative.

‡ Examined by PCR methods. +, present; −, absent.

§ Strain selected from each group for AP-PCR analysis (Fig. 3).

be an important factor contributing to high incidence of *V. parahaemolyticus* infection.

Analysis of the strains isolated from the patients revealed that the strains with pandemic clone-specific characteristics (GS-PCR+, *tdh*-positive, and *trh*-negative reactions) were responsible for 75·4% of the infections. In addition, all these strains also possessed the ORF8, ORF9, and ORF10 sequences of the phage genome reported from the pandemic O3:K6 strain isolated from an international traveller [19] (Table 1). Furthermore, the representative strains showed nearly identical AP-PCR profiles with those of pandemic strains isolated in other countries previously (Fig. 2). These results support that the strains with pandemic clone-specific characteristics isolated in Hat Yai City are clonal and are indistinguishable from the previously reported pandemic strains. The result obtained in this study that O3:K6 strains (80%) were dominant among the pandemic strains can be a line of evidence to support our hypothesis on the epidemiological association between the clinical and environmental O3:K6 strains in this area. The environmental O3:K6 strain exhibited GS-PCR+, *tdh*-positive, and *trh*-negative reactions and AP-PCR profiles indistinguishable from those of previously isolated pandemic strains [22]. In addition, this strain carried ORF8, ORF9, and ORF10 (M. Nishibuchi, unpublished observation). Agreement of these characteristics with those of the pandemic clone detected among the clinical strains also supports implication of the environmental strain in infection in Hat Yai City. Antibiogram could not serve as a tool in the epidemiological analysis in this study.

The clinical strains exhibiting the above pandemic clone-specific characteristics belonged not only to previously recognized three serovars of pandemic strains but to three other serovars (O1:K25, O1:K41, and O4:K12; Table 1). Complete identity of the 1364-bp *toxRS* sequences of these O1:K25, O1:K41, and O4:K12 strains with those of previously reported pandemic strains supports that the strains belonging to the three newly recognized serovars are very closely related to the pandemic strains so far reported. During the course of our study, O1:K25 strains with pandemic clone-specific characteristics (GS-PCR+, *tdh*+, ORF8+) were also found independently

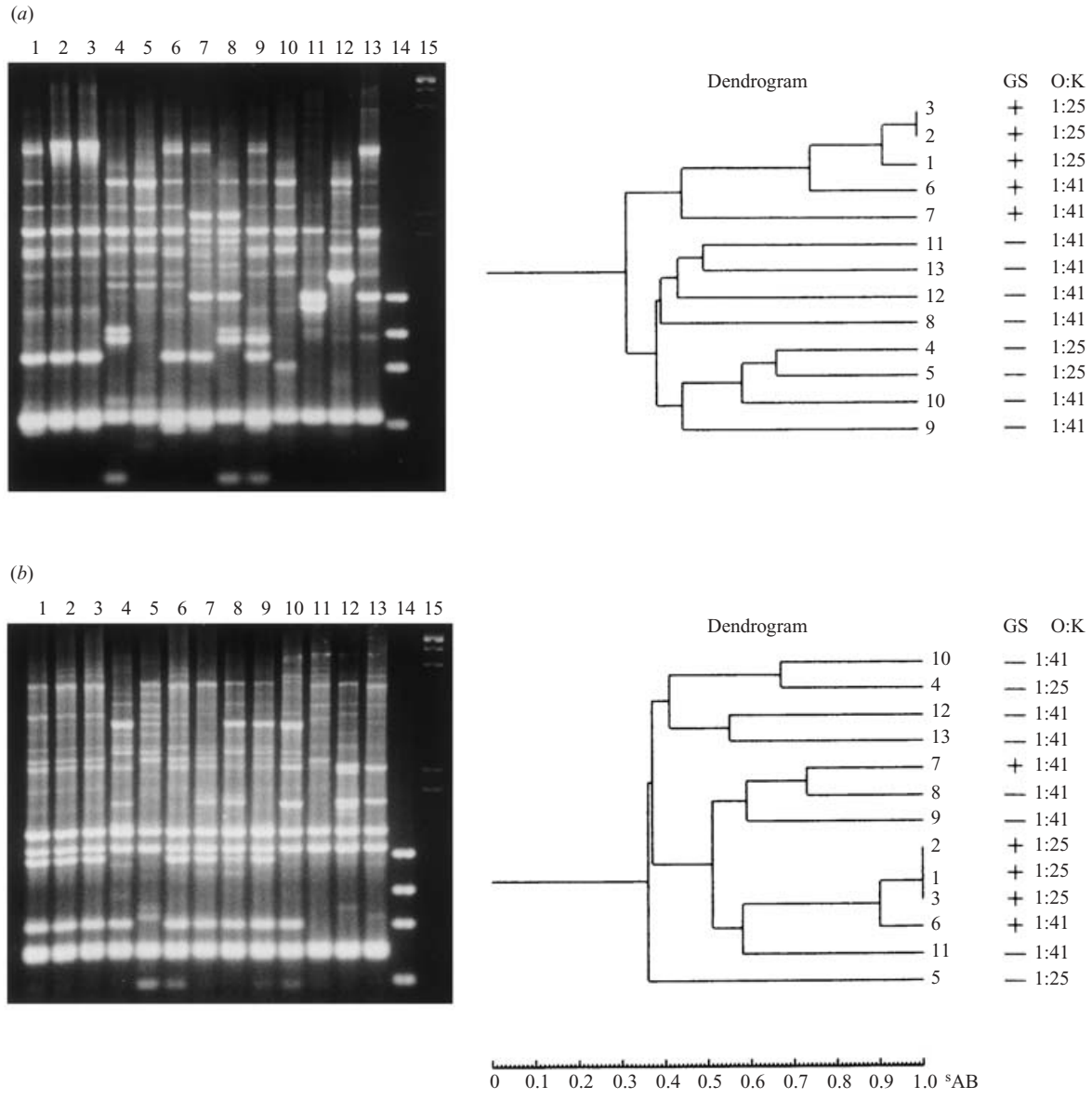

Fig. 3. Results of AP-PCR assay for *V. parahaemolyticus* strains isolated from the international travellers arriving in Osaka, Japan. The result obtained with primers 2 and 4 is presented in panels (*a*) and (*b*), respectively. The AP-PCR profiles are shown on the left. The dendrogram constructed from the AP-PCR profiles is illustrated on the right; the number corresponding to the profile lane, the result of GS-PCR (GS), and O:K serovar (O:K) of the test strain and the scale for the similarity coefficient are indicated. Lanes 1 and 6, control strains isolated in Hat Yai: lane 1, a GS-PCR + O1:K25 strain (VPHY191 [1999]); lane 6, a GS-PCR + O1:K41 strain (VPHY197 [1999]). Lanes 2–5, 7–13, test strains isolated from the international travellers (characteristics of the test strains are shown in Table 2): lanes 2 and 3, GS-PCR + O1:K25 strains (KX-V641 [1998] and KX-V627 [1998], respectively); lanes 4 and 5, GS-PCR − O1:K25 strains (AQ4913 [1994] and AQ4366 [1988], respectively); lane 7, a GS-PCR + O1:K41 strain (KX-V829 [1999]); lanes 8–13, GS-PCR − O1:K41 strains (KX-V803 [1993], KX-V755 [1999], AQ4750 [1992], KX-V326 [1996], AQ4592 [1990], AQ3754 [1983], respectively). The isolation year of each strain is indicated in the brackets. Lanes 14 and 15, molecular size markers: lane 14, phage ϕX174 DNA digested with *Hae*III, 0·60–1·4 kb bands are clearly visible; lane 15, phage λ DNA digested with *Hin*dIII, 2·0–23·1 kb bands are clearly visible.

among clinical strains isolated in Bangladesh [29]. We then sought whether the GS-PCR + strains of O1:K25, O1:K41, and O4:K12 serovars are implicated in pandemic spread of *V. parahaemolyticus* infection. GS-PCR + strains belonging at least to O1:K25 and O1:K41 serovars were found among the strains isolated from international travellers (Table 2). They were isolated between 1998 and 1999 but not

earlier, and the origins of the travellers were Thailand and three other Asian countries. These strains were shown closely related or related to some extent to the GS-PCR + O1:K25 and O1:K41 strains isolated in Hat Yai when compared by AP-PCR profiles (Fig. 3). These results verify that GS-PCR + strains of O1:K25, O1:K41, and possibly O4:K12, serovars have pandemic potential and support our previous hypothesis that the pandemic clone is becoming diversified and more serovariants are emerging [10]. Future surveillance of clinical strains in various countries would confirm the pandemic spread of the strains belonging to the O1:K25, O1:K41, and O4:K12.

Two types of new variants were found among the GS-PCR + strains isolated from international travellers in this study. The first type is an ORF8-negative variant. Other workers claimed that ORF8 is a useful genetic marker for pandemic strains [19, 21]. The perfect correlation between the results of GS-PCR + reaction and ORF8 detection was observed with the strains isolated in Hat Yai City (Table 1). The result supported the proposed utility of ORF8. However, four GS-PCR + O1:K25 strains isolated from international travellers gave negative results in the PCR assays for ORF8 and ORF9 (Table 2). The negative results are not due to mutations in the primer annealing sequence(s) since absence of these ORFs were confirmed by hybridization assays with polynucleotide probes (Table 2). On the other hand, a strain representing this group was shown very closely related to a strain representing the GS-PCR + O1:K25 strains possessing ORF8 and ORF9 by the AP-PCR analysis (Fig. 3*a*, *b*, lanes 2, 3). We therefore concluded that these ORF8- and ORF9-negative strains are the variants of the pandemic clone. The exact reason for the absence of ORF8 and ORF9 and presence of ORF10 in the four O1:K25 strains is not clear. Lysogenic phages can be unstable and deletion of the phage genome may have taken place. However, deletion of the entire phage genome in these strains is unlikely because ORF10 was detected in these strains. Lack of ORF8 in pandemic strains is not unique to O1:K25 strains. GS-PCR + and ORF8-negative strains belonging to O3:K6 and O4:K68 serovars were also found among clinical strains isolated recently in Bangladesh [29]. Therefore, detection of ORF8 in a test strain supports that the strain belongs to the pandemic clone. However, absence of ORF8 does not exclude the possibility that the strain belongs to the pandemic clone.

The second variant is an AP-PCR profile variant. The GS-PCR + O1:K41 strain, KX-V829, had pandemic clone-specific characteristics and possessed ORF8, ORF9, and ORF10 (Table 2). However, comparison of the AP-PCR profiles indicated that this strain (Fig. 3*a*, *b*, lane 7) is closely related to two of GS-PCR − O1:K41 strains isolated from international travellers (Fig. 3*b*, lanes 8, 9) although KX-V829 is not so distantly related to other GS-PCR + strains (Fig. 3*a*, *b*, lanes 1–3, 6). The intermediate AP-PCR profile of KX-V829 may be due to some rearrangement(s) in the genome but exact nature of the rearrangement is unknown.

The GS-PCR assay was originally designed as an easy method to detect pandemic O3:K6 strains [10]. This PCR targets the *toxRS* sequence, an operon well conserved in *Vibrio* species. The variation in the *toxRS* sequence was proven useful for identification of *Vibrio* species at the species level [11–13] and for detection of a particular group of strains at a subspecies level as demonstrated by discovery of serovariants of the pandemic strains by the GS-PCR method [10]. This study also supported the utility of the GS-PCR method by detecting various variants of the pandemic cone including three new serovariants, the ORF8-negative variant, and the AP-PCR profile variant.

In conclusion, this study demonstrated prevalence of infection by the pandemic clone of *V. parahaemolyticus* in Hat Yai City and dominance of O3:K6 strains among the pandemic clone. The results suggest epidemiological significance of the GS-PCR + O3:K6 strain previously detected in this environment. All three serovariants of the pandemic clone and three newly recognized serovars with pandemic clone-specific characteristics detected among the clinical strains suggest a possibility that the pandemic clone is prevalent and that new serovariants may be emerging in this environment. We are currently conducting more intensive study to examine distribution and variation of GS-PCR + strains of *V. parahaemolyticus* in this environment to obtain further support for this hypothesis.

ACKNOWLEDGEMENTS

We are grateful to Yohko Takeda and Chien-Hsien for technical assistance. We thank Kansai Airport Quarantine Station for providing the strains of *V. parahaemolyticus*. This research was supported in part by the funds from the Ohyama Health Foundation and Yuasa International Foundation, by

4th Advanced Scientific Technology Research Fund from Toyota Motor Corporation, by a Grant-in Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan, by the US–Japan Cooperative Medical Science Program, Cholera and Related Diarrhoeal Diseases, Japanese Panel, by the COE programme on 'Making Regions: Proto-Areas, Transformations and New Formations in Asia and Africa', by a Grant-in Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan, by 'Research for the Future' programme from The Japan Society for the Promotion of Science (JSPS-RFTF97L00706), and by a fund from Faculty of Medicine, Prince of Songkla University.

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