MATERIALS AND METHODS

Sample collection and bacteriology. A rectal swab was obtained from each diarrhea patient in Hat Yai hospital, Songkhla Province, Thailand, between 2003 and 2004. Each sample was plated on MacConkey, Salmonella-Shigella and TCBS agars (Difco, Md., U.S.A.). After overnight incubation at 37°C, samples shown growth only on TCBS were selected. Ten non-sucrose fermenting colonies were randomly picked from each plate. The isolates were identified as *V. parahaemolyticus* by standard biochemical tests, and confirmed by PCR targeted to the *toxR* gene (as explained below).

***ToxR* investigation and virulence gene detection.** The test isolate was grown in Luria-Bertani (LB) broth containing 1% NaCl with shaking (160 rpm) at 37°C overnight. One milliliter of the broth culture was centrifuged, and the bacterial cells were washed with sterile saline (0.85% NaCl) and then suspended in it. The cell suspension was boiled for 10 min, and the supernatant was obtained by centrifugation, diluted 10-fold in distilled water, and used as the template for PCR amplification. To investigate the *toxR* gene, PCR using primers T4 and T7 was performed, as described previously (11). To determine the presence of the *tdh* and *trh* genes, the template was prepared as described above. PCR was carried out using the primers D3–D5 and R2–R6, respectively (24).

O:K serotype. The O:K serotype of the test isolate was determined by the slide-agglutination test using anti-O and anti-K antibodies (Denka Seiken, Tokyo, Japan). Briefly, the test isolate was grown in tryptic soy broth containing 3% NaCl at 37 °C for 18 h, and the bacterial cells were suspended in saline (3% NaCl). The bacterial cell suspension was subjected to agglutination with specific anti-K antibodies for the K serotype determination. For the O serotype determination, the bacterial cell suspension

was autoclaved at 121°C for 30 min. Autoclaved bacterial cells were subjected to agglutination with specific anti-O antibodies.

GS-PCR. GS-PCR was carried out using the primers GS-VP1 and GS-VP2 (15). Briefly, the PCR template was prepared in the manner described above. The PCR mixture consisted of 1.5 mM $MgCl_2$, 0.125 mM dNTPs, 0.2 μM of each primer, 0.5 U of *Taq* DNA polymerase in storage buffer A, and 2.5 μl DNA template in a 20-μl volume. Amplification was performed with a single cycle at 96°C for 5 min, followed by 25 cycles of denaturation at 96°C for 1 min, annealing at 45°C for 2 min, and extension at 72°C for 3 min, and a final extension at 72°C for 7 min.

ORF8 detection. The presence of ORF8 was determined by PCR using the primers VP36RF8U and VP36RF8L (13). Briefly, a 20-μl volume contained 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM of each primer, 0.5 U of *Taq* DNA polymerase in storage buffer A, and 2.0 μl of DNA template. The amplification conditions consisted of one cycle at 96°C for 5 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 1 min, and a final cycle at 72°C for 7 min.

AP-PCR. DNA was extracted by a standard phenol-chloroform extraction method (22). AP-PCR was performed using primer 2 (5′-GTTTCGCTCC-3′) and primer 4 (5′-AAGAGCCCGT-3′) as described previously (15). Briefly, amplification was performed in a 30-μl mixture composed of 0.33 mM dNTPs (TaKaRa Biochemicals, Tokyo), 25 ng template DNA, 2.5 U *Ex Taq* (TaKaRa), 0.83 pmol primer, and 1× *Ex Taq* Buffer (TaKaRa). The PCR was performed in a thermal cycler (Program Temp Control System PC-808, Astec Co., Japan). The thermocycle was started with a cycle at 95°C for 4 min.

This was followed by 45 cycles of denaturation at 95°C for 1 min, annealing at 36°C for 1 min, and extension at 72°C for 2 min, in which a transition time of 5 min was set between the denaturation and annealing, annealing and extension, and extension and denaturation steps. The thermocycle finished with one cycle at 72°C for 7 min. The amplification products were analyzed by electrophoresis in a 1.5% agarose gel.

Pulsed-field gel electrophoresis (PFGE). *V. parahaemolyticus* was grown in LB broth supplemented with 1% NaCl at 37°C overnight with continuous shaking (160 rpm). A 1-ml sample of culture was centrifuged and resuspended in 150 µl SE buffer (75 mM NaCl and 25 mM EDTA (pH 8.0)). An agarose plug was prepared by mixing equal volumes of bacterial suspension with melted agarose (2% low-melting agarose in a buffer consisting of 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 0.1 mM EDTA), and was transferred to a disposable plug mold (Bio-Rad, Laboratories, Hercules, CA). After solidification, lysis was achieved with 950 µl lysis solution (containing 50 mM Tris-HCl (pH 8.0), 50 mM EDTA, 1% *N*-lauryl sarcosine, and 1 mg proteinase K per ml), and the DNA was cleaved by the *Not*I restriction enzyme (TOYOBO Co. Ltd., Osaka, Japan) at 37°C overnight. The lambda DNA ladder marker (Bio-Rad) was incubated at 50 °C for 10 min before loading into the gel. The digested DNA fragments and DNA markers were separated in 1% Pulse-Field Certified agarose (Bio-Rad Laboratories) using 0.5× TBE buffer on a CHEF-DRIII system (Bio-Rad). Electrophoresis was performed at 6 V/cm with a field angle of 120° at 14°C. The pulse times were 1–18 s for 36 h. After electrophoresis in 1% PFGE agarose gel, the gel was stained with ethidium bromide and DNA was visualized with a UV transilluminator.

Southern blot hybridization. DNA probes specific to the *tdh*, *trh*1, and *trh*2 genes were excised from recombinant plasmids (12, 23), purified using agarose gel electrophoresis and a DNA extraction kit (QIAGEN, Germany), and then labeled with digoxigenin (Roche Diagnostics, Germany) according to the manufacturer's specifications. *Hin*dIII-digested total bacterial DNA was subjected to electrophoresis in 1% agarose gel and transferred to a nylon membrane (Boreheinger Manheim, Germany). The hybridization was carried out under high-stringency conditions (at 37°C for the *tdh* probe and at 30°C for the *trh*1 and *trh*2 probes). The hybridized probes were detected using a DNA detection kit (Roche Diagnostics, Germany) according to the manufacturer's specifications.

Stability of the serotype and genotype of isolates *in vivo* and *in vitro*. To examine whether changes in the serotype or genotype of *V. parahamolyticus* isolates from patients could occur *in vivo*, three isolates from patient group 16, PSU1681, PSU1690 , and PSU1683, and three additional new isolates with varying toxin gene profile or serotype, PSU1958, PSU2056, and PSU2490, were examined before and after passage through a rabbit. Detailed characteristics of the test isolates are given in Table 2. Each isolate was grown in LB agar, the bacterial cells were washed twice and suspended in 1% NaCl at 10^4, 10^9 or 10^{12} cfu/ml. Three ml of bacterial suspension was mixed with 50 g of rabbit food grain. Individual New Zealand White rabbits approximately 3 months old were fasted for 24 h and then fed with 50 ml of 5% sodium bicarbonate solution to neutralize stomach acid and then allowed to feed on contaminated food grain. Rabbits were challenged with increasing doses of *V. parahaemolyticus* once a week for 3 weeks. Feces were collected 24 h before feeding and every 6 h for 4 days after feeding. Collected feces were enriched in APW and plated on TCBS agar. After incubation at 37°C for 24 h,

sucrose non-fermenting isolates were identified to *V. parahaemolyticus* as described above and their serotype and toxin gene profile (presence or absence of the *tdh* and *trh* genes) were determined as described above.

To investigate whether *in vitro* sub-culture techniques could influence the serotype or genotype of the isolates, the three isolates from patient group 16 (PSU1681, PSU1690, and PSU1683) and one of the new isolates (PSU 2056) were continuously sub-cultured using TCBS agar plate. Inoculated isolate was grown on this medium at 37°C for 24 h and transferred to the new TCBS agar plate for 50 passages. The serotype and the toxin gene profile were determined for five colonies taken from each passage.