DISCUSSION

Only a small portion of the *V. parahaemolyticus* isolates in the environment are thought to be virulent and to carry virulence genes (*tdh* or *trh*), whereas most clinical isolates are virulent (12, 17, 23). We have accumulated data to support this notion; only 16 virulent isolates were obtained from 13 of the 302 seafood samples in our survey carried out between the years 2000 and 2002 (28). In addition, we have confirmed that a single seafood sample can be contaminated by different strains of *V. parahaemolyticus*; for example, we isolated *V. parahaemolyticus* belonging to five serotypes (O3:K6, O4:KUT, O11:KUT, O rough:KUT, and O2:KUT) from one oriental hard clam sample (V. Vuddhakul, unpublished observations). In this study, we found that 597 of 629 isolates obtained from 63 patients possessed either *tdh* or *trh* or both virulence genes suggesting they were virulent isolates and able to proliferate in human hosts. This was the basis for the assumption that isolates in clinical specimens are virulent and represent a homogeneous population.

Recent observation that some clinical isolates lack the *tdh* and *trh* genes (18, 27) prompted us to examine whether the isolates in a single patient specimen were homogeneous or if mixed infection could explain the occurrence of clinical *tdh*$^-$ *trh*$^-$ isolates. Here we showed that up to 33% of the patient specimens contained heterogeneous populations of *V. parahaemolyticus*. The isolates obtained from the patients in groups 18 and 24 were a mixture of strains containing or lacking one or both of the virulence genes *tdh* and *trh* (Table 1). This result indicates that *V. parahaemolyticus* infections may be mixed and supports the possibility of accidental isolation of an avirulent isolate, rather than a virulent isolate, from a patient specimen.

However, 30 isolates from the three patient specimens in patient group 5 carried neither the *tdh* nor the *trh* gene when examined by PCR, and their absence was confirmed

by Southern blot hybridization (data not shown). No known enteric pathogen was isolated from these patients. We therefore cannot rule out the possibility that these isolated have unknown virulence mechanism other than the *tdh* and *trh* genes. However, it is possible that this could be explained by deletion of the *tdh* or *trh* gene during infection. The isolates in this group are under investigated to clarify its pathogenicity in patients.

Our observations also suggest that *in vivo* changes in the genotype of *V. parahaemolyticus* may contribute to strain heterogeneity in clinical specimens. In patients group 9, one and nine isolates from two patients lacked ORF8 although other isolates from the same patients carried ORF8 and DNA fingerprints of all isolates were indistinguishable. ORF8 is contained in a lysogenic filamentous phage. This phage may be unstable and may have been lost during infection in these patients. Similarly, the possibility that the *tdh* gene might be deleted during infection was raised. All 10 isolates from patient group 10 had identical characteristics, including DNA fingerprints, except for the possession of the *tdh* gene. The *tdh* gene is located in a transposon-like structure, which contains an insertion sequence-like element named ISV (26). Therefore, this genetic structure could mediate *tdh* deletion. We have detailed molecular genetic evidence to support the theory that an insertion sequence-mediated *tdh* deletion at least in the isolate in patient group 10 (M. Kamruzzaman, unpublished data). We suspect the *tdh* deletion took place *in vivo* rather than *in vitro* after isolation because the toxin gene genotypes of the isolates have been stable during maintenance of the culture.

In addition, the distribution of serotypes and genotypes within the isolates from patient groups 11, 12 and 16 suggest the possibility of *in vivo* serotype change. In patient group 16 (one patient) three O3:K6, GS-PCR-positive isolates and two O4:K8, GS-PCR-positive isolates showed identical DNA fingerprints, including PFGE profiles. In contrast,

five O4:K8, GS-PCR-negative isolates from this patient group showed different DNA fingerprints from the O4:K8, GS-PCR-positive isolates (Fig. 1 and 2). It is possible that this patient was infected with a single GS-PCR-positive strain, and that some host factors affected the genes expressing the O and K antigens located at the cell surface. Patient groups 11 and 12 consisted of one and nine isolates that differed slightly in their serotypes: O1:KUT and O1:K25 in patient group 11, and OR:K56 and O3:K56 in patient group 12. The O1:KUT and OR:K56 serotypes might have been derived from the O1:K25 and O3:K56 serotypes, respectively. However, the possibility of infection by strains with different serotypes in a single patient cannot be ruled out. We therefore examined whether serotype change or loss of the *tdh* and *trh* genes in *V. parahaemolyticus* can occur *in vitro* and *in vivo*. In the *in vitro* experiment using TCBS medium, no serotype change or loss of *tdh* or *trh* was detected in isolates from patient group 16 and other recent isolates, suggesting that the genotype and serotype are stable under routine culture conditions. However, we did observe serotype change, KUT to K15, in one of test isolates that induced *V. parahaemolyticus*-positive stool in a rabbit model. Considerable proportion of environmental isolates of *V. parahaemolyticus* belongs to KUT serotype (Vuddhakul, unpublished data). The K antigen is the outermost antigen of *V. parahaemolyticus*. The *in vivo* conversion of the O11:KUT isolate to O11:K15 might be an adaptive change to avoid host immunity as different K antigens have been reported to confer different levels of immune protection (6, 20). It is important to note that rabbits are probably less susceptible to *V. parahaemolyticus* infections than humans. Because, as much as 3×10^{12} cfu of test isolate, which is 10^6 times higher than human infective dose, incorporated into rabbit food caused only softened stools but no diarrhea symptoms in this study. No change in O antigen or deletion of *tdh* or *trh* was observed after passage

through the rabbit model; however our sample size may not have been large enough to detect such events. Study on *in vivo* serotype change merits further study.

The ORF8 of a filamentous phage, f237, has been reported to be associated with O3:K6 and other pandemic serotypes (9, 16, 21). However, Bhuiyan et al. (2) and Okura et al. (19) failed to detect ORF8 in some pandemic strains (GS-PCR-positive strains). We found, in the current study, that up to 38.4% of GS-PCR-positive isolates were ORF8 negative. Therefore, ORF8 might not be an appropriate genetic marker for pandemic strains. As discussed above, possible instability of the phage can explain absence of ORF8 in these GS-PCR-positive strains.

In total, 70% of all *V. parahaemolyticus* isolates were GS-PCR-positive, and thus were considered as pandemic strains. These belonged mostly to O3:K6, followed by O1:K25, O4:K68 and O1:KUT serotypes. This result was consistent with the findings of our survey carried out in the Hat Yai hospital in 2000–2004 (V. Vuddhakul, unpublished data) and indicates that this trend has remained unchanged for at least five years in the study area.

In conclusion, the current study showed that *V. parahaemolyticus* isolates obtained from 33% of the patient specimens were not homogeneous and that this was due, in part, to mixed infection by two or more different strains. Our observations of the distribution of genotypes and serotypes in Hat Yai hospital patients also suggests that *in vivo* virulence gene deletion, and *in vivo* serotype conversion may play a role in generating heterogeneity after infection. This study did not rule out the possibility that *V. parahaemolyticus* lacking both virulence markers (*tdh*$^-$, *trh*$^-$) may have other unknown virulence mechanism responsible for diarrhea in patients.