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Genotypic Analyses of *Vibrio parahaemolyticus* and Development of a Pandemic Group-Specific Multiplex PCR Assay

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A total of 54 *Vibrio parahaemolyticus* strains including pandemic O3:K6 strains and newly emerged O4:K68, O1:K25, O1:K26, and O1:K untypeable strains (collectively referred to as the "pandemic group") were examined for their pulsed-field gel electrophoresis (PFGE) and arbitrarily primed PCR (AP-PCR) profiles and for the presence or absence of genetic marker DNA sequences, *toxRS*/*new* or *orf8*, that had been reported elsewhere to be specific for the pandemic group. Both PFGE and AP-PCR analyses indicated that all strains of the pandemic group formed a distinct genotypic cluster, suggesting that they originated from the same clone. In addition to the pandemic group, four O3:K6 strains that did not possess the thermostable direct hemolysin (*tdh*) gene also belonged to this cluster and possessed the *toxRS*/*new* sequence. However, three O3:K6 strains that clearly belonged to the pandemic group by PFGE and AP-PCR did not possess the *orf8* sequence. The evidence suggests that neither the *toxRS*/*new* nor the *orf8* sequence is a reliable gene marker for definite identification of the pandemic group. We therefore developed a novel multiplex PCR assay specific for the pandemic group. The assay successfully distinguished pandemic group strains from other *V. parahaemolyticus* strains by yielding two distinct PCR products for *tdh* (263 bp) and the *toxRS*/*new* sequence (651 bp).

Vibrio parahaemolyticus causes one of the major forms of seafood-borne gastroenteritis, often associated with the consumption of raw or undercooked seafood (4). Past epidemiological studies (7, 8, 14) revealed a strong association between the thermostable direct hemolysin (TDH) and another hemolysin termed TDH-related hemolysin (TRH), which are produced by members of *V. parahaemolyticus*, and its pathogenicity. Both hemolysins are thus considered major virulence factors of *V. parahaemolyticus*. The structural genes for the hemolysins, *tdh* and *trh*, respectively, are encoded chromosomally; and PCR-based methods for the detection of the genes have been successfully developed (16, 19). *V. parahaemolyticus* can be classified into 13 O serotypes and 71 K serotypes (9). Although various serovars of the bacterium can cause infections, O3:K6 has been recognized as the predominant serovar responsible for most outbreaks worldwide since 1996 (5, 13, 17).

Past molecular studies based on pulsed-field gel electrophoresis (PFGE) (1, 20) and arbitrarily primed PCR (AP-PCR) (13, 17) revealed that those pandemic strains and other recently emerged serovars such as O4:K68 and O1:K untypeable (O1:KUT) showed almost identical fragment patterns, suggesting that these strains are clonally related and form what is referred to as the "pandemic group." Furthermore, Matsumoto et al. (13) reported that the members of the pandemic group exhibit a unique sequence within the *toxRS* operon which encodes transmembrane proteins in the regulation of virulence-associated genes conserved in the genus *Vibrio*. However, we have recently shown that not only the pandemic group but also several PFGE-untypeable TDH-nonproducing O3:K6 strains were positive for the *toxRS* sequence (18). Meanwhile, Nasu et al. (15) isolated filamentous phage possessing a unique open reading frame, *orf8*, from a pandemic strain. Moreover, Iida et al. (10) also found *orf8* in another recently emerging serovar (i.e., O4:K68), claiming that *orf8* is a useful genetic marker for the pandemic group. Nevertheless, Bhuiyan et al. (3) reported that they did not detect *orf8* in several O3:K6 strains clinically isolated between 1998 and 2000. These findings suggest that neither the *toxRS* nor the *orf8* sequence is specific enough to distinguish the pandemic group. A more reliable genetic maker is therefore sought. Here we describe the molecular profiles of the pandemic group through PFGE and AP-PCR and reevaluate the use of the genetic markers described above. On the basis of the results obtained, we have developed a multiplex PCR-based assay for the successful identification of pandemic group strains.

MATERIALS AND METHODS

Bacterial strains. A total of 54 strains of *V. parahaemolyticus* with known serological identities that had been isolated from various sources were used in the present study and are listed in Table 1. These included 34 strains of O3:K6 consisting of 17 strains isolated before 1996 and 17 strains isolated after 1996, 6 strains of O4:K68 isolated after 1998, 3 strains belonging to O1:KUT, 3 strains belonging to O1:K25, and another 8 strains belonging to diverse serotypes, as listed in Table 1. The strains were maintained on heart infusion agar (Difco Laboratories, Detroit, Mich.) containing NaCl (final concentration, 2%) until use.

PFGE. PFGE typing of strains was performed with genomic DNAs digested with the restriction enzyme *Not*I, as described elsewhere (12), with minor modifications. Briefly, bacterial cells on 2% NaCl heart infusion agar (Difco) were directly embedded in low-melting-temperature agarose (FMC BioProducts, Rockland, Maine). The DNAs in each plug were then digested with 30 U of *Not*I (Takara Shuzo, Tokyo, Japan) at 37°C for 7.5 h. PFGE was performed with a 1%

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TABLE 1. *V. parahaemolyticus* isolates used in this study

Group and strain no.	Serotype	Yr of isolation	Country of isolation	Source
O3:K6, isolated since 1996				
KE10495	O3:K6	1996	Japan	Human
KE10457	O3:K6	1998	Japan	Human
KE10472	O3:K6	1998	Japan	Human
KE10481	O3:K6	1998	Japan	Human
KE10484	O3:K6	1998	Japan	Human
KE10524	O3:K6	1998	Japan	Seawater
KE10527	O3:K6	1998	Japan	Food
KE10531	O3:K6	1998	Japan	Human
NIID 956–98	O3:K6	1998	USA	Human
NIID K7	O3:K6	1998	Japan	Human
AN-2416	O3:K6	1998	Bangladesh	Human
AN-7410	O3:K6	1998	Bangladesh	Human
AN-8373	O3:K6	1998	Bangladesh	Human
NIID 59–99	O3:K6	1999	Thailand	Human
AO-97	O3:K6	1999	Bangladesh	Human
AP-9251	O3:K6	2000	Bangladesh	Human
AP-14861	O3:K6	2000	Bangladesh	Human
O3:K6, isolated before 1996				
KE9967	O3:K6	1981	Japan	Human
KE9971	O3:K6	1981	Japan	Food
KE9984	O3:K6	1981	Japan	Human
KE10461	O3:K6	1982	Japan	Seawater
KE10491	O3:K6	1983	Japan	Human
KE10492	O3:K6	1984	Japan	Human
KE10465	O3:K6	1985	Japan	Human
KE10462	O3:K6	1986	Japan	Food
KE10463	O3:K6	1987	Japan	Food
KE10464	O3:K6	1988	Japan	Food
KE10443	O3:K6	1995	Japan	Human
KE10466	O3:K6	1995	Japan	Human
TVP 1919	O3:K6	Before 1996	Japan	Human
TVP 1908	O3:K6	Before 1996	Japan	Human
TVP 1841	O3:K6	Before 1996	Japan	Human
TVP 1499	O3:K6	Before 1996	Japan	Human
TVP 1894	O3:K6	Before 1996	Japan	Human
Other serovars				
AN-2189	O4:K68	1998	Bangladesh	Human
AN-11127	O4:K68	1998	Bangladesh	Human
AN-14142	O4:K68	1998	Bangladesh	Human
KE10545	O4:K68	1999	Indonesia	Human
NIID 181-99	O4:K68	1999	Thailand	Human
NIID 242-200	O4:K68	2000	Korea	Human
AN-16000	O1:KUT	1998	Bangladesh	Human
AP-11243-2	O1:KUT	2000	Bangladesh	Human
AP-32241	O1:KUT	2000	Bangladesh	Human
AO-24491	O1:K25	1999	Bangladesh	Human
AP-18000	O1:K25	2000	Bangladesh	Human
AP-18296	O1:K25	2000	Bangladesh	Human
AP-11243-1	O1:K26	2000	Bangladesh	Human
KE10471	O4:K6	1997	Japan	Human
KE10460	O3:K56	1998	Japan	Human
KE10538	O4:K8	1999	Thailand	Human
KE10540	O3:K46	1999	Thailand	Human
KE10541	O8:K41	1999	Thailand	Human
KE10542	O3:K48	1999	Thailand	Human
KE10579	O1:K1	2000	Japan	Seawater

agarose gel (L03; Takara) on a CHEF DRII apparatus (Bio-Rad Laboratories, Richmond, Calif.) in 0.5× TBE (Tris-borate-EDTA) buffer at 14°C and 200 V. Electrophoresis was performed for 18 h at 6 V/cm with a 2- to 40-s linear ramp time. After PFGE, the gels were stained with ethidium bromide (1 μg/ml) and were photographed under UV transillumination.

DNA preparation. For subsequent PCR-based assays, the whole genomic DNAs of the strains were prepared in Tris-EDTA buffer (TE; pH 8.0) essentially as described elsewhere (2). The purity and the amount of DNA in each preparation were estimated colorimetrically, and the DNAs were stored at 4°C until use.

AP-PCR. AP-PCR was performed with the genomic DNAs essentially by the method described by Okuda et al. (17). An oligonucleotide primer, primer 2 (5′-GTTTCGCTCC-3′), provided with the Ready-To-Go RAPD analysis kit (Amersham Biosciences Corp., Piscataway, N.J.), is used with this method. The PCR mixture was heated at 95°C for 4 min prior to 45 cycles of PCR amplification in a DNA thermal cycler (GeneAmp PCR System 2700; Applied Biosystems, Foster City, Calif.); one PCR cycle consisted of denaturation at 95°C for 1 min, primer annealing at 36°C for 1 min, and extension at 72°C for 2 min; after the last cycle, the PCR mixtures were incubated at 72°C for 7 min. The PCR products were electrophoresed in a 2.0% agarose gel and were visualized by UV illumination for specifically amplified fragments after ethidium bromide staining.

Similarities among PFGE and AP-PCR patterns. The PFGE and AP-PCR patterns were converted to PICT files and entered into the GelComparII program (Applied Maths, Kortrijk, Belgium) to generate a dendrogram based on the Dice coefficient (6) by using the unweighted pair group method with 1% position tolerance.

***tdh* and *trh* assays.** The presence of *tdh* and *trh* in the strains was determined by PCR with a set of primers for *tdh*, 5′-GGTACTAAATGGCTGACATC-3′ (sense) and 5′-CCACTACCACTCTCATA-TGC-3′ (antisense), and another set of primers for *trh*, 5′-GGCTCAAAATGGTTAAGCG-3′ (sense) and 5′-CATT TCCG-CTCTCATATGC-3 (antisense), by the protocols established by Tada et al. (19).

***toxRS*-targeted PCR.** PCR was performed as described by Matsumoto et al. (13) with the genomic DNAs of the strains in order to detect the *toxRS* sequence of the new O3:K6 clone (*toxRS*/*new*) and that of the old O3:K6 clone (*toxRS*/*old*) with primers 5′-TAATGAGGTAGAAACA-3′ (primer GS-V1) (13) and 5′-AC GTAACGGGCCTACA-3′ (primer GS-V2) (13) and primers 5′-TAATGAGG TAGAAACG-3′ and 5′-ACGTAACGGGCCTACG-3′, respectively.

As reported previously (18), four *tdh*-negative O3:K6 strains produced the PCR amplicon specific for *toxRS*/*new*. It was thus necessary to confirm whether the strains indeed possessed the unique DNA sequence. We therefore designed three additional sets of primers to amplify the sequences containing other group-specific bases described by Matsumoto et al. (13) (*toxRS*/*new* 1, 2, and 3; Fig. 1). The three primer sets used the sequence 5′-ACTCGTTACCAGTGGAAGT A-3′ (primer toxRS/newF; positions 881 to 900 in the work of Lin et al. [11]; GenBank accession no. L11929) as a sense primer and the sequences 5′-AATT CGGCGGCTTTGTTCA-3′ (primer toxRS/new1R; positions 1481 to 1463 in the work of Lin et al. [11]; GenBank accession no. L11929), 5′-ATGTAATCGCC ATTCGGT-3′ (primer toxRS/new2R; positions 1261 to 1244 in the work of Lin et al. [11]; GenBank accession no. L11929), and 5′-CGTTCGACTCCACATTC ACA-3′ (primer toxRS/new3R′; positions 1233 to 1214 in the work of Lin et al. [11]; GenBank accession no. L11929) as the three antisense primers, respectively. PCR amplification was then performed in a DNA thermal cycler (GeneAmp PCR System 2700; Applied Biosystems) with the genomic DNAs of all strains by the methodology described by Matsumoto et al. (13), except that the annealing temperature was 47°C instead of 45°C. The PCR products were electrophoresed in a 1.5% agarose gel, and after ethidium bromide staining, the specifically amplified fragments were visualized by UV illumination.

***orf8*- and other *orf*-targeted PCR.** A PCR amplification (10) which is designed to amplify a partial DNA sequence of *orf8* (upper *orf8* in Fig. 2) was performed with the genomic DNAs by using a set of primers, 5′-GTTCGCATACAGTTG AGG-3′ (primer ORF8A) and 5′-AAGTACAGCAGGAGTGAG-3′ (primer ORF8B). In the present study, three *tdh*-positive O3:K6 strains whose PFGE fragment patterns belonged to that of the pandemic clone did not produce any amplicon by the *orf8*-targeted PCR. In order to determine whether the strains had lost the phage encoding *orf8* entirely, we performed PCRs with five additional sets of primers that were designed to amplify different parts of *orf8* or other *orf* genes of the lysogenized phage genome (Fig. 2). The primer sets that were designed by referring to the work of Nasu et al. (15) (GenBank accession no. AP000581) were as follows: 5′-CGTCGTTAACCAGTATGGCAA-3′ (primer ORF3 + 4/F) and 5′-TTAGCTTGACCACCGGATACC-3′ (primer ORF3 + 4/R) for partial amplification of the sequence including open reading frames (ORFs) 3 and 4; 5′-ACCCATCATTCCACCGGATA-3′ (primer ORF5/F) and 5′-CACCAAGCCCTTTTAAATCG-3′ (primer ORF5/R) for partial amplification of ORF 5; 5′-TGCTCGAAGAATATGGCGT-3′ (primer ORF6 + 7/F) and 5′-AAACCT-GCATTGACCGAGAA-3′ (primer ORF6 + 7/R) for partial amplification of the sequence including ORFs 6 and 7; 5′-GGGACTTTAAAGAA ACAACGA-3′ (primer ORF8C) and 5′-TGCTTCTTCTAGCGATAATCC-3′ (primer ORF8D) for partial amplification of sequence of ORF8 (primer lower ORF8); and 5′-TATCCCATTCTTTGACCGTCC-3′ (primer ORF9 + 10/F) and 5′-AAAGCAAAAACGCACGAAGC-3′ (primer ORF9 + 10/R) for partial

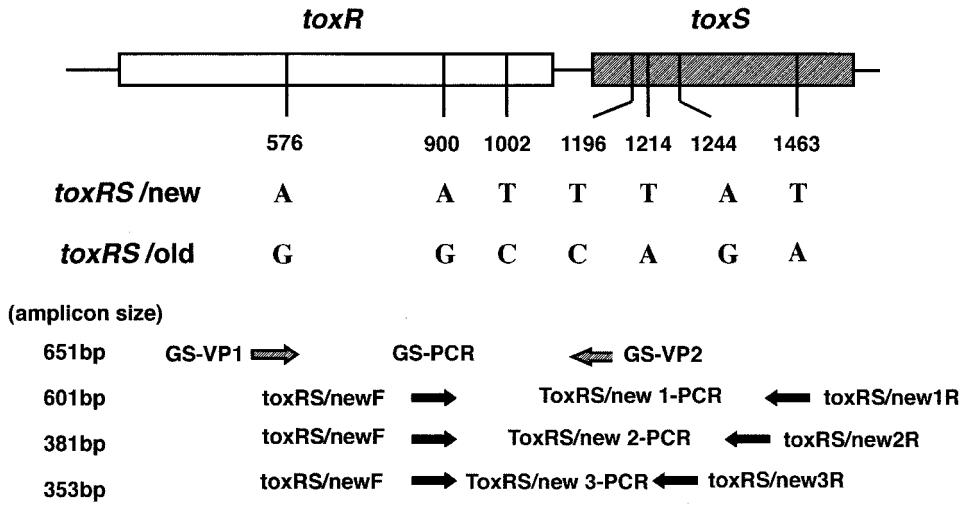

FIG. 1. Target positions of the PCR primers used to amplify the *toxRS/new* and the *toxRS/old* sequences with essential base differences. Numerals indicate the base positions that correspond to those in the *toxRS* sequence of strain AQ3815 reported by Lin et al. (11) (GenBank accession no. L11929). The figure was drawn with reference to Fig. 1 of Matsumoto et al. (13).

amplification of the sequence including *orf9* and *orf10*. PCR amplification was then performed in a DNA thermal cycler (GeneAmp PCR System 2700; Applied Biosystems) with the genomic DNAs of all strains by the methodology described by Iida et al. (10), with slight modifications, in which the annealing temperature, the extension time, and the PCR cycles were set at 60°C, 30 s, and 25 cycles, respectively.

Pandemic group-specific multiplex PCR with primer sets targeting *tdh* and *toxRS/new*. As will be shown below, strains giving positive PCR results for both *tdh* and *toxRS/new* were found to belong to the pandemic group. In this context, we developed a novel multiplex PCR targeting both gene markers. The oligonucleotide primers used in the multiplex PCR were 5′-TGACTGTGAACATT AATGA-3′ (sense primer) and 5′-CGATTCTTTGTTGGATATAC-3′(ant isense primer), which are specific for positions 451 to 469 and 713 to 694 in *tdh*, respectively (position numbers are according to Honda et al. [8]; GenBank accession no. D90238), and which yield a 263-bp fragment, and 5′-TAATGAG GTAGAAACG-3′ (sense primer) and 5′-ACGTAACGGGCCTACA-3′(ant isense primer), which are specific for positions 561 to 576 and 1211 to 1196 in *toxRS/new*, respectively (position numbers are according to Lin et al. [11]; GenBank accession no. L11929), and which yield a 651-bp fragment. PCR amplification was performed in a total volume of 20 μl. Two microliters of each genomic DNA preparation (1 ng of DNA/μl of TE) was added to the PCR master mixture, which consisted of 2 μl of 10× PCR buffer (Mg^{2+} free; Promega Corporation, Madison, Wis.), 2.4 μl of 25 mM $MgCl_2$ (final concentration, 3.0 mM), 0.25 μl of a deoxynucleoside triphosphate mixture (0.125 mM each deoxynucleoside triphosphate), 0.125 μl of each primer (0.125 μM each primer), and 0.125 μl (0.625 U) of *Taq* DNA polymerase (Promega), with the remaining volume consisting of distilled water. A GeneAmp PCR System 2700 thermal cycler (Applied Biosystems) was used for PCR amplification, which consisted of an initial denaturation at 94°C for 3 min; 25 cycles of denaturation at 94°C for 30 s, annealing at 45°C for 30 s, and extension at 72°C for 60 s; and a final extension at 72°C for 5 min. Five microliters of the PCR products was electrophoresed on 2% agarose gels, stained with ethidium bromide (0.25 μg/ml), and photographed under UV light.

RESULTS

Genotypes determined by PFGE. A total of 33 PFGE patterns were observed with the strains examined (Fig. 3). Software analysis of the PFGE profiles revealed the presence of nine distinct genotypes (genotypes A, B, C, D, E, F, G, H, and I) at the 70% similarity level (Fig. 3). PFGE type A could be further subdivided into two clusters at the 75% similarity level. One cluster consisted of 14 patterns (patterns A1 to A14) for the *tdh*-positive O3:K6, O4:K68, O1:K25, O1:K26, and O1:KUT strains isolated since 1996; and the other cluster consisted of 4 patterns (patterns A15 to A18) for the *tdh*-negative O3:K6 strains isolated in the 1980s (Fig. 3 and Table 2). The strains with the patterns assigned to type B that were indistinguishable from the strains with type A patterns at the 65% similarity level included the *tdh*-negative O3:K6 strains isolated in the 1980s and the *trh*-positive O3:K6 strains (Fig. 3 and Table 2).

Genotypes determined by AP-PCR. A total of 14 AP-PCR patterns were observed among the strains examined (Fig. 4). Software analysis of the profiles distinguished six distinct genotypes (genotypes a, b, c, d, e, and f at the 70% similarity level) (Fig. 4). The AP-PCR patterns designated type a con-

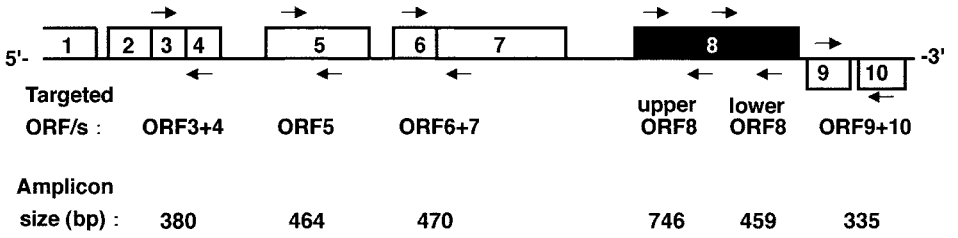

FIG. 2. Target positions of the PCR primers used to amplify the ORF genes specific for the pandemic group. The numerals in the blocks denote ORF numbers. See the text for the base positions of the primers. The figure was drawn with reference to the ORFs of pO3K6 in Fig. 2 of Nasu et al. (15).

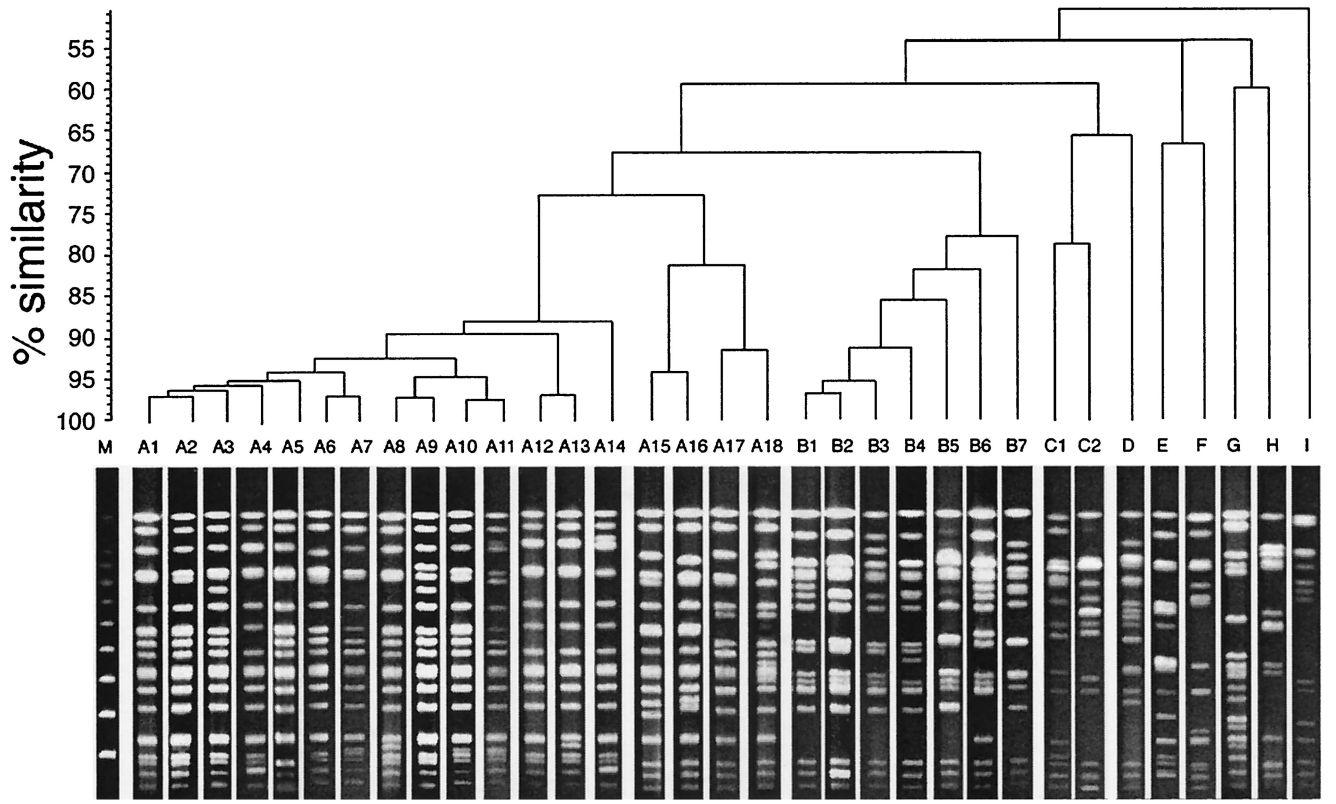

FIG. 3. Representative PFGE patterns for *V. parahaemolyticus* genomic DNAs digested with *Not*I and dengrogram illustrating the clustering of patterns by percent similarity (shown at the left of the dendrogram). Lane M, molecular size marker (bacteriophage lambda ladder PFG Marker; New England BioLabs, Beverly, Mass.).

sisted of four patterns (patterns a1, a2, a3, and a4) for the *tdh*-positive O3:K6, O4:K68, O1:K25, O1:K26, and O1:KUT strains isolated since 1996 and the four *tdh*-negative O3:K6 strains isolated in the 1980s (Fig. 4 and Table 2), all of which belonged to PFGE type A, as described above.

Prevalence of conventional gene markers specific to the pandemic group. The presence or absence of *toxRS*/*new*, *toxRS*/*old*, and *orf8* in the strains determined by the PCR-based assays is shown in Table 2. The *toxRS*/*new* sequence was detected in all strains belonging to PFGE type A or AP-PCR type a, while the rest of the strains were found to possess the *toxRS*/*old* sequence. The *toxRS*/*new*-positive strains included the four *tdh*-negative O3:K6 strains (strains KE10462, KE10464, KE10465, and KE10491) isolated in the 1980s. Subsequent PCR-based analyses with primer sets targeting different sites specific for the *toxRS*/*new* sequence revealed that those *tdh*-negative O3:K6 strains possessed the whole sequence (Fig. 5). The *orf8* gene was detected in all O3:K6 strains belonging to PFGE type A or AP-PCR type a except for the three *tdh*- and *toxRS*/*new*-positive strains (strains AN-2416, AN-8373, and AP-9251) that had been isolated from clinical cases in Bangladesh between 1998 and 2000 and the four *tdh*-negative O3:K6 strains (strains KE10462, KE10464, KE10465, and KE10491). These *orf8*-negative strains were further assayed by the PCR methods targeting other ORF genes and were found to be devoid of all ORF sequences (data not shown).

Evaluation of multiplex PCR assay. We subsequently analyzed the strains by the multiplex PCR assay targeting both *tdh* and *toxRS*/*new*. All PFGE type A (or AP-PCR type a) strains except the four *toxRS*/*new*-positive and *tdh*-negative O3:K6 strains (strains KE10462, KE10464, KE10465, and KE10491) gave the *toxRS*/*new*-specific amplicon of 651 bp as well as the *tdh*-specific amplicon of 263 bp, while other strains failed to produce either or both amplicons (Fig. 6 and Table 2).

DISCUSSION

Our PFGE analysis of *V. parahaemolyticus* strains revealed that the *tdh*-positive strains of O3:K6, O4:K68, O1:K25, O1:K26, and O1:KUT isolated since 1996 (collectively referred to as the "pandemic group") were all within a distinct genotypic cluster that included 14 PFGE profiles (A1 to A14). It is noteworthy that many Bangladeshi and Japanese O3:K6 strains presented an identical PFGE profile (profile A1), implying some epidemiological linkage between these two countries. The results of our AP-PCR analysis were also consistent with these findings; the pandemic strains formed a homogeneous cluster distinct from any of the other strains. This evidence supports the view presented by other workers (1, 13, 17, 20) that the pandemic group might have originated from the same clone. However, both analyses indicated that the four *tdh*-negative O3:K6 strains isolated well before 1996 were included in the pandemic strain cluster (>75% similarity level), although their PFGE profiles (profile A15 to A18) were slightly distant from those of the pandemic strains. Furthermore, our PCR test indicated that the strains possessed *toxRS*/

TABLE 2. Genotypic characteristics of *V. parahaemolyticus* isolates

Group and strain no.	Serotype	Genotype determined by:		Results of PCR targeting the following gene(s)[c]:					
		PFGE[a]	AP-PCR[b]	*tdh*	*trh*	*toxRS/new*	*toxRS/old*	*orf8*	*tdh* and *toxRS/new*[d]
O3:K6, isolated since 1996									
KE10495	O3:K6	A2	a1	+	−	+	−	+	+
KE10457	O3:K6	A1	a1	+	−	+	−	+	+
KE10472	O3:K6	A1	a1	+	−	+	−	+	+
KE10481	O3:K6	A1	a1	+	−	+	−	+	+
KE10484	O3:K6	A8	a2	+	−	+	−	+	+
KE10524	O3:K6	A9	a1	+	−	+	−	+	+
KE10527	O3:K6	A3	a1	+	−	+	−	+	+
KE10531	O3:K6	A1	a1	+	−	+	−	+	+
NIID 956-98	O3:K6	A5	a1	+	−	+	−	+	+
NIID K7	O3:K6	A1	a1	+	−	+	−	+	+
AN-2416	O3:K6	A1	a1	+	−	+	−	−	+
AN-7410	O3:K6	A1	a1	+	−	+	−	+	+
AN-8373	O3:K6	A6	a1	+	−	+	−	−	+
NIID 59-99	O3:K6	A10	a1	+	−	+	−	+	+
AO-97	O3:K6	A1	a1	+	−	+	−	+	+
AP-9251	O3:K6	A6	a3	+	−	+	−	−	+
AP-14861	O3:K6	A1	a1	+	−	+	−	+	+
O3:K6, isolated before 1996									
KE9967	O3:K6	B7	d3	+	−	−	+	−	−
KE9971	O3:K6	B7	d3	+	−	−	+	−	−
KE9984	O3:K6	B1	c1	−	+	−	+	−	−
KE10461	O3:K6	G	f	−	−	−	+	−	−
KE10491	O3:K6	A18	a4	−	−	+	−	−	−
KE10492	O3:K6	B6	c1	−	+	−	+	−	−
KE10465	O3:K6	A16	a4	−	−	+	−	−	−
KE10462	O3:K6	A17	a4	−	−	+	−	−	−
KE10463	O3:K6	B5	c1	−	−	−	+	−	−
KE10464	O3:K6	A15	a4	−	−	+	−	−	−
KE10443	O3:K6	B1	c1	−	+	−	+	−	−
KE10466	O3:K6	B2	c1	−	+	−	+	−	−
TVP 1919	O3:K6	B1	c1	−	+	−	+	−	−
TVP 1908	O3:K6	B1	c1	−	+	−	+	−	−
TVP 1841	O3:K6	B4	c1	−	+	−	+	−	−
TVP 1499	O3:K6	B3	c1	−	+	−	+	−	−
TVP 1894	O3:K6	B3	c1	−	+	−	+	−	−
Other serovars									
AN-2189	O4:K68	A7	a1	+	−	+	−	+	+
AN-11127	O4:K68	A7	a1	+	−	+	−	+	+
AN-14142	O4:K68	A7	a1	+	−	+	−	+	+
KE10545	O4:K68	A7	a1	+	−	+	−	+	+
NIID 181-99	O4:K68	A11	a1	+	−	+	−	+	+
NIID 242-200	O4:K68	A7	a1	+	−	+	−	+	+
AN-16000	O1:KUT	A12	a1	+	−	+	−	+	+
AP-11243-2	O1:KUT	A12	a1	+	−	+	−	+	+
AP-32241	O1:KUT	A13	a1	+	−	+	−	+	+
AO-24491	O1:K25	A4	a1	+	−	+	−	+	+
AP-18000	O1:K25	A4	a1	+	−	+	−	+	+
AP-18296	O1:K25	A14	a1	+	−	+	−	+	+
AP-11243-1	O1:K26	A12	a1	+	−	+	−	+	+
KE10471	O4:K6	E	e	+	−	−	+	−	−
KE10460	O3:K56	I	c3	−	−	−	+	−	−
KE10538	O4:K8	H	c2	+	−	−	+	−	−
KE10540	O3:K46	C1	d1	+	+	−	+	−	−
KE10541	O8:K41	D	d4	+	−	−	+	−	−
KE10542	O3:K48	C2	d2	−	−	−	+	−	−
KE10579	O1:K1	F	b	+	+	−	+	−	−

[a] The genotype corresponds to that shown in Fig. 3.
[b] The genotype corresponds to that shown in Fig. 4.
[c] +, presence of gene sequence; −, absence of gene sequence.
[d] +, amplicons for both *tdh* and *toxRS/new* are produced.

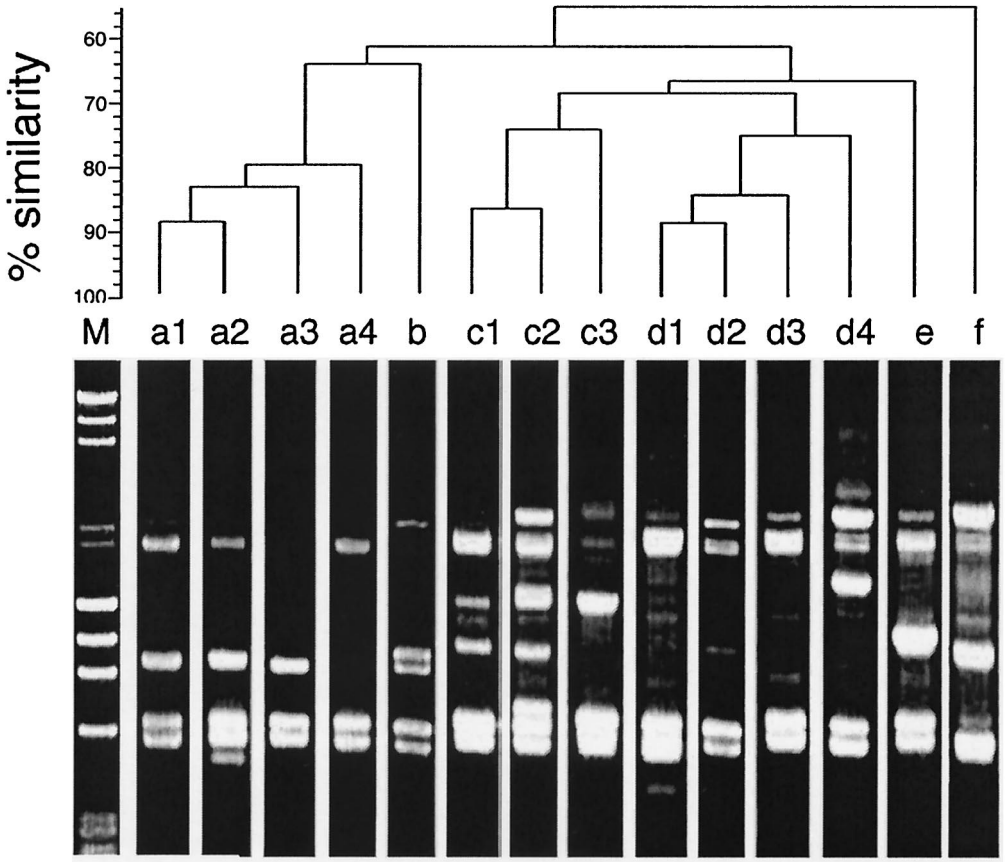

FIG. 4. Representative AP-PCR patterns for *V. parahaemolyticus* genomic DNAs and dendrogram illustrating the clustering of patterns by percent similarity (shown at the left of the dengrogram). Lane M, molecular size marker (a mixture of phage λ DNA digested with *Hind*III and phage ϕX174 DNA digested with *Hae*III).

new. These findings suggest that the presence of the *toxRS*/*new* sequence is not a newly emerged genetic profile in members of the species *V. parahaemolyticus*. The pandemic group might have stemmed from those nonpathogenic strains with *toxRS*/*new* after acquisition of the *tdh* gene, although this hypothesis is highly speculative. Meanwhile, the PCR assays targeting *orf8* and other ORFs that were reportedly encoded by the genome of a filamentous bacteriophage specifically lysogenized in the pandemic strains (15) failed to produce any amplicon from several strains of the pandemic group, indicating that they were

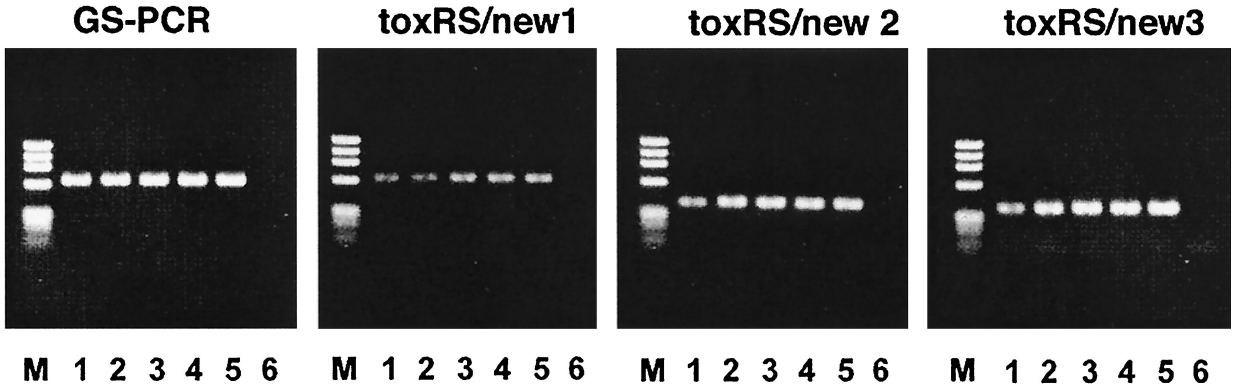

FIG. 5. Agarose gel electrophoresis showing the results of four different PCR amplifications (with the GS, toxRS/new1, toxRS/new2, and toxR/new3 sets of primers) targeting *toxRS*/*new*. Lanes M, molecular size markers (phage ϕX174 DNA digested with *Hae*III); lanes 1, NIID 956-98 (O3:K6, isolated after 1996) as a positive control; lanes 2, KE10491 (O3:K6, isolated before 1996); lanes 3, KE10465 (O3:K6, isolated before 1996); lanes 4, KE10462 (O3:K6, isolated before 1996); lanes 5, KE10464 (O3:K6, isolated before 1996); lanes 6, KE 9967 (O3:K6, isolated before 1996) as a negative control. See Fig. 1 for the primer positions and the amplicon size for each PCR.

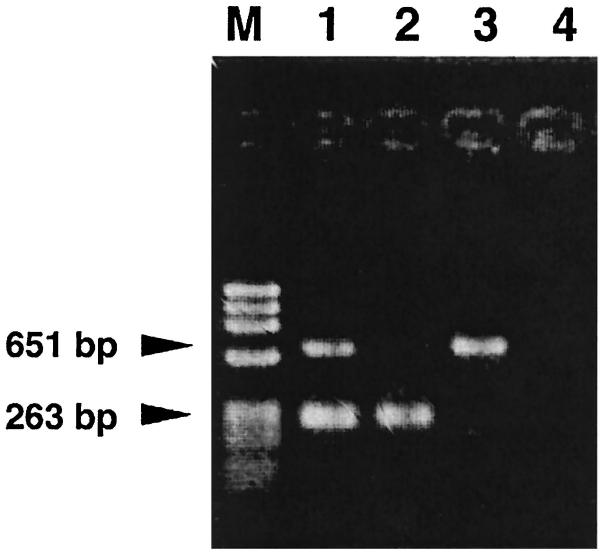

FIG. 6. Agarose gel electrophoresis showing the representative results of multiplex PCR amplification targeting *tdh* and *toxRS*/*new*. The PCR amplicon sizes for *tdh* and *toxRS*/*new* are 263 and 651 bp, respectively. Lane M, molecular size marker (phage ϕX174 DNA digested with *Hae*III); lane 1, NIID 956-98 (O3:K6, isolated after 1996; *tdh* positive, *toxRS*/*new* positive); lane 2, KE9967 (O3:K6, isolated before 1996; *tdh* positive, *toxRS*/*new* negative); lane 3, KE10462 (O3:K6, isolated before 1996; *tdh* negative, *toxRS*/*new* positive); lane 4, KE10460 (O3:K56; *tdh* negative, *toxRS*/*new* negative).

not lysogenized by the phage. Whether the strains had been accidentally cured of the phage during laboratory processing has yet to be determined.

On the basis of the results of our investigation, it can be seen that neither *toxRS*/*new* nor *orf8* is a reliable genetic marker for PCR-based identification of the pandemic strains; detection of *toxRS*/*new* is necessary but not always sufficient for the identification of the pandemic strains, while detection of *orf8* is sufficient but not always necessary for the identification of pandemic strains. This in turn suggests that a strain possessing both *tdh* and *toxRS*/*new* can be considered a pandemic strain. On this basis, we have developed a novel PCR-based assay for the successful identification of pandemic strains. The assay uses a multiplex PCR designed to amplify either *toxRS*/*new* or *orf8* or both *toxRS*/*new* and *orf8* simultaneously, in which only pandemic strains including *orf8*-negative strains produce two specific fragments. Although the assay needs to be evaluated further for its reliability with more strains of a much wider range of serologies or sources, it can be a useful diagnostic or epidemiological tool for investigating outbreaks of food poisoning caused by *V. parahaemolyticus*, with specific reference to the pandemic group.

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