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Genotyping of Pandemic *Vibrio parahaemolyticus* O3:K6 Still Open to Question

Vibrio parahaemolyticus is one of the major seafood-borne gastroenteritis-causing bacteria, frequently associated with consumption of raw or inappropriately cooked seafood. Thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) are considered major virulence factors for the organism (7). *V. parahaemolyticus* can be classified into 13 O serotypes and 71 K serotypes (3). 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). The pandemic strains and other recently emerged serovars such as O4:K68 and O1:K untypeable showed almost identical pulsed-field gel electrophoresis (PFGE) patterns (1), suggesting that these strains are clonally related. Matsumoto et al. (5) reported that the pandemic strains exhibit a unique sequence within the *toxRS* operon, which encodes transmembrane proteins in the regulation of virulence-associated genes conserved in the genus *Vibrio*. On the other hand, Nasu et al. (6) isolated filamentous phage possessing a unique open reading frame, ORF8, from one of the pandemic strains, and Iida et al. (4) claimed that ORF8 was a useful genetic marker for identifying strains. It was thus necessary to evaluate the use of the *toxRS* sequence or ORF8 as a reliable genetic marker for the identification.

A total of 24 strains of *V. parahaemolyticus* that had been isolated from various sources with known serological identities were used in the present study and are listed in Table 1. These include 21 strains of O3:K6, consisting of 12 strains isolated before 1996 and 9 strains isolated since1996, and 3 strains of O4:K68 isolated since 1999. PFGE typing was performed on genomic DNAs of the O3:K6 and O4:K68 strains digested with the restriction enzyme *Sfi*I, following a method described previously (1). Of 21 strains of O3:K6 subjected to the typing, 9 strains isolated since 1996 were classified as type A, 2 strains isolated in 1981 were classified as type B, and 5 strains isolated between 1981 and 1996 were classified as type C (Table 1). The 5 strains isolated between 1982 and 1988 showed PFGE fragment patterns different from any of the above PFGE types (Table 1).

The presence of TDH gene (*tdh*) and TRH gene (*trh*) was determined by PCR with a set of primers, 5′-GGTACTAAATG GCTGACATC-3′ and 5′-CCACTACCACTCTCATA-TGC-3′, and another set of primers, 5′-GGCTCAAAATGGTTAAGC G-3′ and 5′-CATTTCCG-CTCTCATATGC-3, respectively, fol-

TABLE 1. *V. parahaemolyticus* O3:K6 and O4:K68 strains used and their genotypic characteristics

Strain	O:K serovar	Yr of isolation	Country of isolation	Source	PFGE genotype[a]	PCR result for[b]:				
						tdh	*trh*	*toxRS/new*	*toxRS/old*	*ORF8*
KE10495	O3:K6	1996	Japan	Human	A	+	−	+	−	+
KE10457	O3:K6	1998	Japan	Human	A	+	−	+	−	+
KE10481	O3:K6	1998	Japan	Human	A	+	−	+	−	+
KE10484	O3:K6	1998	Japan	Human	A	+	−	+	−	+
KE10524	O3:K6	1998	Japan	Sea water	A	+	−	+	−	+
KE10527	O3:K6	1998	Japan	Food	A	+	−	+	−	+
KE10531	O3:K6	1998	Japan	Human	A	+	−	+	−	+
NIID965–98	O3:K6	1998	United States	Human	A	+	−	+	−	+
NIID 59–99	O3:K6	1999	Thailand	Human	A	+	−	+	−	+
KE10545	O4:K68	1999	Indonesia	Human	A	+	−	+	−	+
NIID181–99	O4:K68	1999	Thailand	Human	A	+	−	+	−	+
NIID 242–2000	O4:K68	2000	Korea	Human	A	+	−	+	−	+
KE9967	O3:K6	1981	Japan	Human	B	+	−	−	+	−
KE9971	O3:K6	1981	Japan	Food	B	+	−	−	+	−
KE9984	O3:K6	1981	Japan	Human	C	−	+	−	+	−
KE10492	O3:K6	1984	Japan	Human	C	−	+	−	+	−
KE10443	O3:K6	1995	Japan	Human	C	−	+	−	+	−
KE10463	O3:K6	1987	Japan	Food	C	−	−	−	+	−
KE10466	O3:K6	1996	Japan	Human	C	−	+	−	+	−
KE10461	O3:K6	1982	Japan	Environmental	UT[c]	−	−	−	+	−
KE10491	O3:K6	1983	Japan	Human	UT	−	−	+	−	−
KE10465	O3:K6	1985	Japan	Human	UT	−	−	+	−	−
KE10462	O3:K6	1986	Japan	Food	UT	−	−	+	−	−
KE10464	O3:K6	1988	Japan	Food	UT	−	−	+	−	−

[a] According to the PFGE typing of *Sfi*I digests described by Arakawa et al. (1).
[b] +, presence of gene; −, absence of gene.
[c] UT, untypeable.

lowing the protocols established by Tada et al. (7). All strains of PFGE types A and B were positive for *tdh* but negative for *trh*, whereas all strains of type C were negative for *tdh*, with four of them positive for *trh* (Table 1). The O3:K6 strains with the untypeable PFGE patterns were negative for both genes (Table 1).

PCRs using a method (5) designed to specifically detect the *toxRS* sequence of the new O3:K6 clone (*toxRS*/*new*) and that of the old O3:K6 clone (*toxRS*/*old*) were performed on the strains with primer set 5′-TAATGAGGTAGAAACA-3′ and 5′-ACGTAACGGGCCTACA-3′ and primer set 5′-TAATGAGGTAGAAACG-3′ and 5′-ACGTAACGGGCC-TACG-3′, respectively. All strains belonging to PFGE type A are found to possess *toxRS*/*new*, whereas all strains of type B and C strains possessed *toxRS*/*old* (Table 1). Interestingly, four strains of the PFGE untypeable O3:K6 strains were positive for the *toxRS*/*new* sequence (Table 1), suggesting that the sequence was not specific to the pandemic PFGE type (type A).

We also performed a PCR amplification (4) which was designed to amplify a partial DNA sequence of ORF8 in the genomic DNA, using primer set 5′-GTTCGCATACAGTTGAGG-3′ and 5′-AAGTACAGCAGGAGTGAG-3′. ORF8 was detected in all strains belonging to the PFGE type A but not in the rest of the strains tested (Table 1). The results suggest that ORF8 rather than *toxRS*/*new* is a more reliable genetic marker for identification of the pandemic strains. However, Iida et al. (4) pointed out that one of the O3:K6 strains belonging to the pandemic PFGE type was negative for ORF 8. More recently, Bhuiyan et al. (2) reported that eight of the O3:K6 clinical strains isolated between 1998 and 2000 were negative for ORF8, claiming that ORF8 was a poor genetic marker for the pandemic genotype. Nevertheless, the claim was made without any reference to the PFGE types of the strains used. Genotyping of the pandemic O3:K6 strains is thus still an open question. Further comprehensive investigation with a larger collection of the strains from various sources is necessary to draw any conclusion on this matter.

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