[Review]

Characteristics of Pandemic \textit{Vibrio parahaemolyticus} O3:K6 strains

Hin-chung Wong*

\textit{Department of Microbiology, Soochow University, Taipei, Taiwan}

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*To whom correspondence and reprint requests should be addressed. Mailing address: Department of Microbiology, Soochow University, Taipei, Taiwan 111, Republic of China. Tel: 8862-8819471 Ext. 6852; Fax 8862-28831193; E-mail: wonghc@mail.scu.edu.tw

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ABSTRACT

*Vibrio parahaemolyticus* is an important foodborne pathogen in many Asian countries, but no dominant serovars were involved in food poisonings until the appearance of the new O3:K6 strains in India in 1996. The pandemic spread of these new O3:K6 strains has since occurred, involving other Asian countries including Taiwan. The new O3:K6 strains are genetically homogenous as determined by several molecular typing methods, and are very unlike other clinical strains. Notably, no significant differences in toxin productivity, and susceptibility to drug and environmental stress were found in these new O3:K6 strains compared to other strains. Changes involving several bases in the *toxRS* and *tdh* genes occurred in these new O3:K6 strains. Moreover, filamentous phage was found in these new O3:K6 strains. Specific identification methods were developed that targeted the specific *toxRS* sequence and the ORF8 elements of the filamentous phage. Meanwhile, positive reactions were found in some other serovars. These new O3:K6 strains contained the pandemic genotype, but so too did many other serovars, such as the O4:K68 and O1:KUT strains isolated in many countries, suggesting the spreading of these pandemic genotypes.

**Key Words:** *Vibrio parahaemolyticus*, O3:K6, pandemic, characteristics
**INTRODUCTION**

*Vibrio parahaemolyticus* is one of the most common food-borne pathogens in Taiwan, Japan and other countries with a high consumption of seafood. In Taiwan, this pathogen typically accounts for 35-63% of annual bacterial food-poisoning outbreaks (Chiou et al, 1991; Pan et al, 1997). Clinical manifestations include diarrhea, abdominal cramps, nausea, vomiting, headache, fever, and chills, with incubation periods ranging from 4 to 96 hours (Takeda, 1983).

*V. parahaemolyticus* is a gram-negative halophilic bacterium, frequently associated with marine organisms or existing freely in seawater. Notably, *V. parahaemolyticus* is seldom isolated in seawater with a temperature of below 13-15°C, (Kaneko and Colwell, 1975). Most clinical strains of *V. parahaemolyticus* produce a major virulence factor, the thermostable direct hemolysin (TDH), and are designated as Kanagawa phenomenon positive (KP+) strains, which display β-hemolysis on Wagatsuma agar. Another virulence factor, the TDH-related hemolysin (TRH) is typically associated with the KP-strains or with urease positive strains of *V. parahaemolyticus* (Kelly and Stroh, 1989). Interestingly, KP- strains are also involved in some food-poisoning outbreaks (Honda et al, 1988) and are sporadically involved in wound infections (Johnson et al, 1984).

TDH, comprising 189 amino acid residues, also displays enterotoxigenic activity, as shown by animal tissue culture (Raimondi et al, 2000) and by molecular manipulation (Nishibuchi et al, 1992). The *tdh* genes encoding TDH are generally present in the 1.3 and 2.7 kb *HindIII*
restricted chromosome fragments in the pathogenic strains (Nishibuchi and Kaper, 1990). Highly homologous tdh sequences have also been found in other vibrios, such as *V. cholerae* (Honda et al, 1986; Yoh et al, 1985), *V. mimicus* (Terai et al, 1990; Uchimura et al, 1993; Yoshida et al, 1991) and *V. hollisae* (Yamasaki et al, 1991; Yoh et al, 1986). The presence of insertion sequence-like elements flanking the tdh gene could explain the spread of this gene among *Vibrio* species (Terai et al, 1991).

Outbreaks of *V. parahaemolyticus* poisoning in America are generally attributed to the consumption of contaminated seafood, particularly raw seafood, such as raw oysters (Ellison et al, 2001). *V. parahaemolyticus* strains isolated from seafood are genetically highly heterogenous (Wong et al, 1999), and the percentages of KP+ strains are extremely low (Cook et al, 2002; Wong et al, 1992). In the United States, 345 sporadic *V. parahaemolyticus* infections were reported between 1988 and 1997: 59% were from gastroenteritis, 34% were from wound infections, 5% were from septicemia, and 2% were from other exposures. Forty-five percent of patients suffering from these infections were hospitalized, and 88% of cases of acute gastroenteritis reported having eaten raw oysters in the week before their illness (Daniels et al, 2000).

Isolates of *V. parahaemolyticus* can be differentiated by serotyping. Thirteen O groups and 71 K types have been identified by commercial antisera (Iguchi et al, 1995). Outbreaks generally involve highly variable serovars. The serovars most frequently clinically isolated from 1992-1995 in Taiwan were O5:K15 (18.5%), O4:K8 (16.2%), O3:K29 (12.5%), O1:K56 (8.3%), O2:K3 (6.5%) and O4:K12 (6.0%) (Wong et al, 2000a). Notably, the K types identified for clinical strains from 1983-1993 in
Taiwan also varied significantly (Wang et al, 1996).

Usually the O3:K6 strains accounted for less than one percent of food poisoning outbreaks in Taiwan and elsewhere before 1996 (Chiou et al, 2000). New strains belonging to the O3:K6 serovar first appeared in February 1996 in Calcutta, India, and later came to account for 50 to 80% of the strains isolated from clinical specimens from February to August 1996 in India (Okuda et al, 1997). Subsequently, the new O3:K6 strains have been recognized as the first pandemic strains of *V. parahaemolyticus*, and are now account for a high percentage of food-borne poisoning outbreaks in many Asian nations (Arakawa et al, 1999; Matsumoto et al, 2000). In Taiwan, the prevalence of *V. parahaemolyticus* rapidly increased from 1996 to 1999, and O3:K6 strains accounted for 55.5, 85.4, 68.2, and 71.6% of *V. parahaemolyticus* outbreaks in Taiwan in 1996, 1997, 1998 and 1999, respectively (Chiou et al, 2000). Moreover, the first isolation of a new O3:K6 strain in Taiwan occurred in June 1996, as determined by molecular typing, showing the high spread rate of this pandemic strain (unpublished data).

Food poisoning outbreaks attributed to the new O3:K6 strain have also occurred in the United states, where they were associated especially with the consumption of oysters (Anonymous, 1998a; Anonymous, 1998b; Anonymous, 1999; Khan et al, 2002).

As the first pandemic strains of *V. parahaemolyticus*, the characterization of these new O3:K6 strains is an important issue and is discussed below.
V. parahaemolyticus O3:K6 can be isolated from clinical specimens and food using traditional enrichment and selective plating procedures, and can be identified by biochemical traits and serotyping with commercial serotyping antisera (Denka Seiken, Tokyo, Japan). Application of an immunomagnetic enrichment method selective for V. parahaemolyticus serovar K6 successfully isolated a pandemic O3:K6 strain from fresh shellfish in southern Thailand (Vuddhakul et al, 2000). Notably, a similar immunomagnetic enrichment method has been applied to isolate new O3:K6 strains from Asari samples in Japan (Hara-Kudo et al, 2001).

A new polymerase chain reaction (PCR) method that targeted two of the base positions of the toxRS sequence unique to the new O3:K6 strains has been developed for rapidly identifying these pandemic strains (Matsumoto et al, 2000). However, several old O3:K6 strains reacted positively to this PCR method, suggesting that the sequence is not absolutely specific to the pandemic strains (Osawa et al, 2002b). Notably, an unique open reading frame, ORF8, of a specific filamentous phage can also be used as an identification marker (Nasu et al, 2000). However, some of the newly isolated O3:K6 strains are negative for ORF8 (Bhuiyan et al, 2002).

When outbreak isolates were analyzed for the enterobacterial repetitive intergenic consensus sequences, the new O3:K6 strains displayed a specific 850-bp DNA fragment with no homology with any known Vibrio spp. gene sequences. A PCR method based on this unique sequence can
be used to specifically identify O3:K6 *V. parahaemolyticus* isolates under 6 h (Khan et al, 2002).

The new O3:K6 strains can also be differentiated from others using molecular typing methods (Gendel et al, 2001; Okuda et al, 1997; Wong et al, 2000b). Specifically, automated ribotyping with a Qualicon Riboprinter was used to determine the clinical isolates of *V. parahaemolyticus* O3:K6 recovered from U.S. in 1998, and the patterns produced using the restriction enzymes *Eco*RI and *Pst*I suggest that the new O3:K6 strains were responsible for the outbreak in the Northeastern United States (Gendel et al, 2001). Nevertheless, some strains belonging to other serovars have been found to have geneotypes similar to these pandemic O3:K6 strains (Matsumoto et al, 2000; Osawa et al, 2002b), limiting the usefulness of these typing methods in differentiating the new O3:K6 of *V. parahaemolyticus*.

**GENETIC VARIATION**

The O3:K6 strains isolated from India and other Asian countries since 1996 are genetically similar. Arbitrarily primed polymerase chain reaction suggests that all of the strains imported to Japan belong to a single clone (Okuda et al, 1997). The Indian strains were also examined by ribotyping and pulsed-field gel electrophoresis (PFGE), revealing five ribotypes among the O3:K6 strains examined, of which ribotype R4 was the major type. PFGE following *Not*I digestion revealed several subtypes, indicating genomic reassortment among these strains (Bag et al, 1999; Yeung et al, 2002).
In our laboratory, a large number of recently isolated clinical O3:K6 strains from India, Japan, Korea, and Taiwan, as well as old O3:K6 strains, were examined by PFGE following SfiI restriction. The O3:K6 strains were grouped into two genetically unrelated groups, namely the old and new O3:K6 groups, respectively. The old group comprised O3:K6 strains isolated before 1996, and was separated into six patterns (A1, A2, A3, A8, B2 and R) in the novel PFGE typing scheme. Patterns A8 and B2 were isolated in Taiwan, while others were isolated from Hong, Kong, the Maldives, Singapore and Thailand. The recently isolated O3:K6 strains all belong to the new O3:K6 group (group I), which comprised eight closely related patterns, of which I1 (81%) and I5 (13%) were the most frequent. Pattern I1 was the most prevalent among strains from Japan, Korea, and Taiwan. The results presented in this work confirmed that the recently isolated O3:K6 strains of *V. parahaemolyticus* are genetically similar, and are distinct from the old O3:K6 strains (Wong et al, 2000b). Subsequently, such group I extends to comprise 13 patterns (I1 to I13), with only a few strains belonging to patterns other than I1 and I5 (unpublished data).

Sequence analysis of the toxRS (Matsumoto et al, 2000) and tdh genes (Yeung et al, 2002) also revealed the close genetic relationship of these new O3:K6 strains.

**VIRULENCE ANALYSIS**

The suckling mouse model was used to compare the virulence of the
new O3:K6 strains and other serovars, revealing no significant difference (unpublished data). Interestingly, some in vitro assays indicated a statistical difference between the new O3:K6 strains and other strains. In vitro adherence to HeLa cells revealed that O3:K6 isolates displayed statistically higher levels of adherence and cytotoxicity to host cells than did non-O3:K6 isolates (Yeung et al, 2002). Furthermore, higher cytotoxicity was also found in the new O3:K6 and closely related strains than those non-O3:K6 isolates (Yeung et al, 2002). However, the non-O3:K6 strains examined in (Yeung et al, 2002) included clinical KP+ and food originated KP- strains, and most of the KP- strains should be non-virulent. Consequently, such in vitro toxicity data may have limited applicability in characterizing these new O3:K6 strains.

Levels of TDH production in these strains did not differ significantly from other KP+ strains (Okuda et al, 1997). Our investigation compared levels of TDH production in 25 new O3:K6 strains isolated in Korea and Taiwan to the levels in 12 other tdh-gene-positive strains isolated before 1996. The comparison confirmed that O3:K6 strains recently isolated in Taiwan and Korea did not produce significantly more TDH than the strains isolated before 1996 (Wong et al, 2000b).

In most new O3:K6 and other strains TDH production was enhanced by the presence of bile and taurocholic acid in the growth medium, but this enhancement was not specific to the pandemic strains (Osawa et al, 2002a).

The pilus of a new O3:K6 strains was purified, and comprised an 18 kDa pilin protein which differed antigenically from the previously reported pilins. Nevertheless, all these pilins shared a high degree of
homology in their N-terminal amino acid sequences. Notably, the pilus from this new O3:K6 strain was shown to help adherence to rabbit intestine (Nakasone et al, 2000).

CHARACTERIZATION OF THE tdh GENE

The presence of the tdh and related genes in these new O3:K6 strains has been examined. Okuda et al. noted that the new O3:K6 strains isolated in India and Japan were tdh positive and trh1/trh2 negative (Okuda et al, 1997). Furthermore, in an earlier study we also verified the presence of the tdh gene in all the new O3:K6 strains (Wong et al, 2000b). Determining the nucleotide sequence of the tdh gene new O3:K6 strains has revealed considerable high similarity. Moreover, the predicted amino acid sequence of the TDH protein products were identical, except that the Gly109 in the O3:K6 group was replaced by Asp109 in the non-O3:K6 group. Three nucleotide bases in the tdh gene differed between the new O3:K6 group and the non-O3:K6 group (Yeung et al, 2002). A new O3:K6 strain, strain no 1114, isolated in Taiwan was tdh positive while no TDH protein was detected. This phenomenon may result from mutation in the promoter of the tdh gene (Okuda and Nishibuchi, 1998).

The toxR gene regulates the expression of many virulence factors and resistance to environmental stress in Vibrio species (Lee et al, 2000; Provenzano et al, 2000). The toxR gene is a global regulatory gene conserved in Vibrio species and assisted by the toxS gene located immediately downstream. Notably, the toxRS sequences of the
representative strains of the new O3:K6 clone differ from those of the old O3:K6 strains at least at seven base positions within a 1,346-bp region (Matsumoto et al, 2000).

**SUSCEPTIBILITY TO ANTIBIOTICS**

Resistance to antibiotics is usually a transferable factor and is important to the growth and spread of pathogenic bacteria. However, the KP+ and KP- strains of *V. parahaemolyticus* generally display no difference in sensitivity to antibiotics (Anand et al, 1981). We investigated the antibiotic susceptibility of O3:K6 strains from several Asian countries and compared it with that of other serovars. Most of these strains were resistant to ampicillin and vancomycin, and 56.4, 48.7 and 69.2% of these strains were resistant to cephalothin, erythromycin, and rifampin, respectively. The new O3:K6 strains did not differ significantly from other strains in susceptibility to most of the antibiotics assayed, as shown by Okuda et al. (Okuda et al, 1997). Meanwhile, our work found that a higher percentage of the O3:K6 strains were resistant to rifampin and streptomycin compared to other serovars (Wong et al, 2000b). The new O3:K6 strains displayed higher levels of resistance to ampicillin, cephalothin, rifampin and streptomycin compared to the *V. parahaemolyticus* strains isolated from foodborne poisoning outbreaks in Taiwan during 1992-1995 (Wong et al, 2000a).

The patterns of antibiotic susceptibility of the strains examined in our work were grouped into 39 different antibiograms (unpublished data).
Unlike the PFGE analysis, the distribution of different antibiograms was not associated with particular serovar or PFGE patterns.

**SUSCEPTIBILITY TO ENVIRONMENTAL STRESSES**

*V. parahaemolyticus* is a vulnerable to environmental stresses and is generally rapidly inactivated at 48 and 55 °C, with salinity below 0.5% or pH 4.0 (Beuchat, 1975). Therefore, strains with high resistance to environmental stress may survive and spread better in environmental substrate. We have examined new O3:K6 strains from Taiwan, Korea, and Japan for their susceptibility to different environmental stresses and have compared them to other reference strains. No strain was identified as being particularly stress-resistant. Grouping the strains according to their location of isolation and comparing them revealed some significant differences. For example, the O3:K6 strains recently isolated in Japan displayed significantly lower low temperature survival and higher resistance to mild acid and low salinity treatments than those from other locations. Furthermore, comparing all of the recent O3:K6 strains with other strains revealed that the recent O3:K6 strains exhibited no specific trait that enhanced their survival (Wong et al, 2000b). Finally, susceptibility to acetic, citric and hydrochloric acids were examined, revealing no differences between serovar O3:K6 and the other serovars in resistance to these acids (Hasegawa et al, 2002).

In our previous study (Wong et al, 2000b) stationary cultures of different strains were compared. Resistance of bacterial cells to
environmental stresses is generally acquired during the stationary phase, and cells may behave differently during other growth phases. Comparing the exponential phase cultures of different strains revealed several new O3:K6 strains that were more resistant to pH 3.0 inactivation than other serovars (unpublished data).

**PRESENCE OF FILAMENTOUS PHAGE**

The presence of polyhedral and filamentous phages in *V. parahaemolyticus* was demonstrated before the spread of these pandemic strains (Koga and Kawata, 1991; Taniguchi et al, 1984). The sudden appearance of pandemic O3:K6 strains may be the result of genetic transmission. Notably, filamentous phage is suspected to be vehicle of genetic transfer in *V. parahaemolyticus* (Chang et al, 1998). A filamentous phage, Vf33, specific to *V. parahaemolyticus*, was reported early in 1984, but only the K38 strain was sensitive to this phage (Taniguchi et al, 1984). Moreover, a filamentous phage, lvpf5, was isolated from a new O3:K6 strain in Laos and characterized. The host range of this phage was not restricted to serotype O3:K6, but 7 of 99 *V. parahaemolyticus* strains with various serovars were also susceptible to this phage. Finally, the amino-terminal amino acid sequence of the coat protein was identified as AEGGAADPFEAIDLLGVATL (Nakasone et al, 1999).

Nucleotides homologous to the VF33 DNA were detected in many numerous pandemic and non-pandemic strains of *V. parahaemolyticus*
using Southern Blot analysis (Chang et al, 2000). Furthermore, plasmid-like replication form of this filamentous phage was found in all 24 O3:K6 strains examined in Japan. Among the open reading frames (ORF) found in this filamentous phage genome, the sequence of ORF8 was not significantly correlated with any of the proteins in databases. Using colony hybridization, ORF8 was detected only in O3:K6 strains isolated since 1996, and not in O3:K6 strains isolated before 1996 or clinical \textit{V. parahaemolyticus} strains of other serovars (Iida et al, 2001; Nasu et al, 2000). Furthermore, a deleted form of this phage was isolated in O4:K68 strain, which genetically resembles the pandemic O3:K6 strains (Chan et al, 2002). Recent finding suggested that this phage may not present in all of the new O3:K6 strains (Bhuiyan et al, 2002).

The function of the filamentous phage is unknown, but it is suspected to encode the adherence factor (Nasu et al, 2000).

**EXISTENCE OF OTHER PANDEMIC STRAINS**

Strains with serovars other than O3:K6 but which were genetically very similar to the pandemic strains have been isolated from many regions. The \textit{tdh}+ and \textit{trh}- strains that belonged to the O4:K68 and O1:KUT serovars showed positive reaction to the group specific PCR detection method, which targeted specific tox\textit{RS} sequences of the new O3:K6 strain. Presence of such specific tox\textit{RS} sequences in these strains suggested that they may have diverged from the new O3:K6 clone owing to alteration of the O:K antigens (Matsumoto et al, 2000). Clinical strains
of O1:K25, O1:KUT, and O4:K68 serovars isolated in Dhaka from 1998 to 2000 also displayed positive results using this group specific PCR method (Bhuiyan et al, 2002; Nakasone et al, 2000). Finally, these strains also displayed ORF8 homologous element (Bhuiyan et al, 2002).

During 1998-1999, eight strains of serovars O1:K25, O1:KUT, O6:K18 and O4:K68, originating from India, Singapore, Thailand and Taiwan, were typed by PFGE and found to belong to the pandemic I group and also reacted positively to the group specific PCR method (Unpublished data). Also determined by AP-PCR, ribotyping and PFGE, the O4:K68 and O3:K6 strains from India and Thailand formed a cluster with 78-91% similarity, thus indicating a close genetic relationship between two different serovars isolated within a similar time-frame but from widely separated geographical regions (Chowdhury et al, 2000a).

The above investigations have demonstrated the spread of the pandemic O3:K6 genotype to other serovars. Characterization based on ribotyping and PFGE, the O4:K68 and O1:KUT strains most likely originate from the pandemic O3:K6 clone (Chowdhury et al, 2000b). The production of TDH and suckling mouse lethality had been determined for these special strains and does not differ significantly from the new O3:K6 strains. Furthermore, no discernable increase in the incidence of these special strains in food poisoning outbreaks in Taiwan has been noted (unpublished data). Additional epidemiological data is required before claiming that these special serovars represent another pandemic strain in *V. parahaemolyticus*. 
CONCLUSIONS

New O3:K6 strains of *V. parahaemolyticus* are the first pandemic strains of this pathogen, and have spread rapidly throughout many Asian countries. These pandemic strains have been characterized in terms of phenotype, molecular nature and virulence, and have been compared with other serovars. However, the origin of these new O3:K6 strains and the reason for the rapid spread remains uncertain.

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