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Detecting and Molecular Typing of *Vibrio parahaemolyticus*

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ABSTRACT

Vibrio parahaemolyticus is a prevalent food-borne pathogen in many Asian countries and has been spreading rapidly following the appearance of pandemic strains. This article reviews conventional and molecular methods for culturing, identifying, enumerating and rapidly detecting *V. parahaemolyticus* and its virulence factors, such as thermostable direct hemolysin (TDH), TDH-related hemolysin and thermolabile hemolysin. It also discusses various molecular typing methods, namely, pulsed-field gel electrophoresis, ribotyping, random amplified polymorphic DNA and three other polymerase chain reaction methods based on specific conserved nucleotide sequences, such as ribosomal gene spacer, repetitive extragenic palindromic and enterobacterial repetitive intergenic consensus sequences, for use in epidemiological investigations of this pathogen.

Key words: *Vibrio parahaemolyticus*, detection, molecular typing, pulsed-field gel electrophoresis, PCR

INTRODUCTION

Vibrio parahaemolyticus was first isolated following food poisoning outbreaks in Japan in the early 1950s and is now one of the most prevalent food-borne pathogens in many Asian countries where seafood is often consumed [1,2].

V. parahaemolyticus is a gram-negative halophilic bacterium, frequently associated with marine organisms or existing freely in seawater. When the temperature of seawater is below 13~15°C, *V. parahaemolyticus* is rarely isolated[3] and probably exists in a viable but non-culturable state[4].

Most clinical strains of *V. parahaemolyticus* generate a major virulence factor, thermostable direct hemolysin (TDH), and are designated as Kanagawa phenomenon positive (KP+) strains and exhibit β-hemolysis on Wagatsuma agar. Another virulence factor, TDH-related hemolysin (TRH), is typically associated with KP- strains or with urease positive strains of *V. parahaemolyticus*[5]. KP- strains have also been involved in some food-poisoning outbreaks[6] and sporadically in wound infections[7]. *V. parahaemolyticus* strains isolated from seafood are genetically highly heterogeneous[8] and KP+ strains occur in extremely low proportions in seafood[9,10].

Outbreaks of *V. parahaemolyticus* poisoning are usually attributed to the consumption of contaminated seafood, especially raw seafood, such as raw oyster in the United States[11]. Clinical manifestations have included diarrhea, abdominal cramps, nausea, vomiting, headache, fever and chills, with incubation periods from 4 to 96 hours[12].

Serotyping can differentiate isolates of *V. parahaemolyticus*. Thirteen O groups and 71 K types have been identified by these commercial antisera[13]. Normally, a wide variety of serovars are involved in outbreaks. The most frequent serovars clinically isolated during 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%)[14]. Wang et al. also documented the presence of highly variable serovars obtained from clinical specimens[15]. However, a predominant serovar O3:K6 appeared after 1996. New strains belonging to the O3:K6 serovar appeared for the first time in February 1996 in Calcutta, India and later accounted for 50 to 80% of the strains isolated from clinical specimens from February to August 1996 in India[16]. Since then, the new O3:K6 strains have been considered to be the first pandemic strains of *V. parahaemolyticus*, and are involved in a high proportion of food-borne poisoning outbreaks in several Asian countries[17-19]. Food poisoning outbreaks attributable to new O3:K6 infections also occurred in the United states and were associated with the consumption of oysters[20-23].

The rapid spread of *V. parahaemolyticus* strains over recent years is such that this pathogen may become an important etiologic agent in several countries. Detection methods and molecular typing of *V. parahaemolyticus* are thus of great concern and are discussed as follows.

ENRICHMENT AND SELECTIVE PLATING

Samples are transported in the Cary-Blair medium, or directly enriched in alkaline peptone water (APW) or alkaline peptone salt broth (APS) incubated for 16~18 hr at 35~37°C to detect *V. parahaemolyticus*[24]. All kinds of diluents and enrichment media are supplemented with 3% NaCl. The growth of *V. parahaemolyticus* in glucose salt teepol broth (GST) or APW at 37°C is markedly faster than that of marine and fecal flora[25]. Selective plating is usually conducted using thiosulfate-citrate-bile salt-sucrose (TCBS) agar incubated for 18 hr at 35°C. Typical colonies

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on TCBS agar are round, 2~3 mm in diameter, with green or blue centers[24]. Isolates are identified as *V. parahaemolyticus* if they meet the following conditions[24]. They show positive reactions for oxidase, gelatinase, lysine and ornithine decarboxylases; they demonstrate indole production, motility, nitrate reduction, and sensitivity to 2,4-diamino-6,7-diisopropylpteridine phosphate; they produce acid from D-mannose and grow in 3 to 8% NaCl at 42°C; they show a negative reaction for arginine dihydrolase; they are negative for the Voges-Proskauer reaction; they do not grow in 0 and 10% NaCl, and they do not produce acid from L-arabinose, m-inositol or sucrose.

Bhathena and Doctor found that a two-time serial enrichment technique, using 8% salt-alkaline broth and salt-polymyxin broth (pH 8) for incubation periods of 8 hours and 18 hours, respectively, at 37°C led to the recovery of maximum numbers of *V. parahaemolyticus* from prawns[26].

Hara-Kudo et al. developed a new enrichment procedure and selective agar medium for detecting *V. parahaemolyticus* in seafood. The samples were cultured in non-selective salt trypticase soy broth, then moved to selective salt polymyxin broth (SPB), and streaked on a new chromogenic agar medium CHROMagar *Vibrio* (CV) agar medium (CHROMagar Microbiology, Paris, France). The *V. parahaemolyticus* colonies developed a purple color on this medium[27].

V. parahaemolyticus cells are often injured by food-processing; such injured cells may not be fully recovered by plating on selective media. Therefore, enrichment should involve special media to ensure the optimum recovery of both injured and healthy cells. Horie arabinose ethyl violet broth (HAE, pH 9.0) was recommended for recovering *V. parahaemolyticus* from refrigerated and frozen oyster homogenates[28]. Each liter of HAE broth contained 5 g of peptone, 3 g of beef extract, 30 g of NaCl, 0.03 g of bromothymol blue, 0.001 g of ethyl violet and 5 g of arabinose. Supplementing with 20 mM to 100 mM magnesium salt or ferric ions significantly increased the number of *V. parahaemolyticus* cells recovered from these oyster homogenates[29]. In another experiment, Beuchat found that the GST broth and the tryptic soy broth that contained 7% NaCl (TSBS) were lethal to cold or heat-injured *V. parahaemolyticus*, whereas HAE broth and water blue-alizarin yellow broth (WBAY) were not lethal to stressed cells. The 3% NaCl in 0.1 M potassium phosphate (pH 7.0) diluent was the most appropriate solution for protecting cold- and heat-injured cells against inactivation[28]. While enumerating *V. parahaemolyticus* from refrigerated or frozen seafood using the Most Probable Number (MPN) method, APW was better than SPB due to higher recovery rate and lower detection limits[30].

Tomoyasu developed a method that involved immunomagnetic separation to isolate the specified K serovar of *V. parahaemolyticus* from a mixture of a large number of bacteria with other K serovars[31]. By this method, cells of mixed serovars were mixed with commercially available antiserum against K antigens, pelleted and resuspended in phosphate-buffered saline (PBS). Cells of the special K type were then bound to anti-rabbit immunoglobulin G, coated on superparamagnetic polystyrene beads and harvested using a magnetic particle concentrator. Such immunomagnetic enrichment was used to isolate a pandemic *V. parahaemolyticus* O3:K6 strain from fresh shellfish not implicated in a clinical case in southern Thailand[32] and from Asari samples in Japan[33].

ENUMERATION

Pathogenic bacteria in food are generally enumerated by dilution plating on a selective agar medium or by using the MPN method with selective enrichment broths. The Food and Drug Administration describes three-tube MPN method, using APW or APS broth, in the Bacteriological Analytical Manual, for enumerating *V. parahaemolyticus*[24]. However, the method is time-consuming and the growth of bacteria in tubes should be verified by streaking these bacteria on the TCBS plate, so that typical colonies can be identified by biochemical traits[24]. Such conventional identification procedures can be replaced with the use of nonradioactive probes to shorten the identification process[34].

Karunasagar et al. also noted that the recovery of *V. parahaemolyticus* inoculated into fish homogenates by the MPN technique using salt broth, SPB, GST broth and nutrient broth with eosin yellow (NBYE) was meager, while direct plating on TCBS agar yielded greater recovery. During the period of incubation of homogenates in broth, other flora may have overgrown *V. parahaemolyticus* isolates[35]. Direct plating on TCBS agar medium seems to be a good method for enumerating *V. parahaemolyticus*; however, it normally recovers up to two orders of magnitude fewer cells than the original number[35].

V. parahaemolyticus can be enumerated by a membrane filtration procedure[36]. According to such method, the membrane filter was placed on the surface of an mVP agar plate and incubated for 18-20 hours at 41°C. The mVP agar comprised, per 100 mL, 1 g of polypeptone peptone, 0.7 g of yeast extract, 1.0 g of D-galactose, 3.0 g of NaCl, 2.5 mg of bromthymol blue, 0.15 g of $CuSO_4$ and 1.5 g of agar. The agar medium was prepared by boiling and cooling to 50~55°C, before adding 0.15 g of sodium cholate per 100 mL of medium and adjusting the acidity of the medium to pH 8.6. The background microbial growth on this medium was reduced using sodium cholate and copper sulfate, a high pH, 3% NaCl, and an elevated incubation temperature. A series of in situ tests, including galactose, sucrose and oxidase tests were recommended to prevent the need to pick colonies to be identified. Hence, *V. parahaemolyticus* could thus be enumerated within 30 hours. DePaola et al. also stated that the membrane filtration method using a hydrophobic grid and a 4~5 hour repair step on nonselective agar at 35°C was superior to the MPN method for enumerating *V. parahaemolyticus*[37].

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RAPID DETECTION

Conventional methods are usually time-consuming and take several days to implement. Many methods have been developed for the rapid detection of *V. parahaemolyticus* in food. Trypsin-like activity was reported to be high only in *V. parahaemolyticus*, independently of its Kanagawa response, particularly when the bacteria were enriched for 6 hours in a selective arabinose-glucuronate (AG) medium, which was comprised of 0.5% arabinose, 0.25% glucuronate, 0.1% polypeptone, 0.1% ammonium sulfate, 0.1% yeast extract, 2% NaCl, and 2 μg of polymyxin B sulfate per ml at pH 8.5[38]. Bacterial cells in the AG medium were pelleted and suspended in a buffer that contained the fluorogenic substrate benzoyl-L-argininine-7-aminomethylcoumarin. *V. parahaemolyticus* was measured by determining the fluorescent intensity with a fluorospectrometer[38]. The AG medium was noted to be better than APW for enhancing the trypsin-like activity of *V. parahaemolyticus* in seafood samples[39]. In another study, when a large number of bacterial strains of various *Vibrio* species were examined, all the *V. alginolyticus* and *V. parahaemolyticus* exhibited strong intracellular trypsin-like activity[39]. Consequently, such fluorogenic assay was unusable for directly detecting *V. parahaemolyticus*. However, this method could be used as a simple and rapid method for identifying *V. parahaemolyticus* strains, which had previously been differentiated from *V. alginolyticus* cultures using a selective agar medium like TCBS agar.

Chen and Chang developed another immunofluorescence microscopy method to target two specific outer membrane proteins (34 and 34 kDa)[40]. All *V. parahaemolyticus* tested produced strong fluorescence under the fluorescence microscope, while only six of the other 63 bacteria generated weak to moderate fluorescence[40]. Based on these species-specific proteins, a rapid latex agglutination method has been developed to identify *V. parahaemolyticus* species[41].

Species-specific genetic markers can be used to identify *V. parahaemolyticus*. A 0.76-Kb *Hin*dIII DNA fragment with unknown function was cloned from the chromosomal DNA of a *V. parahaemolyticus* strain, designated as the pR72H fragment, and used as a species-specific marker[42,43]. The *gyrB* gene that encodes the B subunit protein of DNA gyrase (topoisomerase type II) was also used as a species-specific marker and a polymerase chain reaction (PCR) protocol based on this gene sequence has been developed[44].

DETECTION OF TOXIN AND TOXIN GENES

Most environmental or food isolates of *V. parahaemolyticus* are not virulent strains, so the presence of virulent factors in these strains, such as toxin or toxin genes, must be identified before such isolates are claimed to be pathogenic. The presence of TDH is primarily determined by observing clear beta-hemolysis around the colony, using a special blood agar plate, the Wagatsuma agar plate, supplemented with fresh human or rabbit red blood cells[24]. However, two other hemolysins, TRH and thermolabile hemolysin (TLH), with sequential homology with TDH but exhibiting no hemolysis on Wagatsuma agar, also exist in some *V. parahaemolyticus* strains[6,45].

The presence of TDH can be detected using enzyme-linked immunosorbent assays (ELISA)[46] or a commercial latex agglutination kit. A modified Elek test and an immuno-halo test were developed to detect the TDH[47]. Briefly, the modified Elek test was performed by adding anti-TDH serum to a well cut in agar medium near a colony on the medium, and the immuno-halo test was carried out by inoculating bacteria onto agar medium that contains anti-TDH serum or anti-TDH immunoglobulin G. The sensitivity of the modified Elek test was reported to be similar to that of KP on Wagatsuma medium, and the results of both reactions were consistent. The immuno-halo test was slightly less sensitive than the modified Elek test[47]. Bromthymol blue-teepol agar and modified arabinose-ammonium sulfate-cholate agar were modified to perform the modified Elek test and an immuno-halo test[48].

Nucleic acid probes can be used to verify the presence of these toxin genes in *V. parahaemolyticus* strains. Radioactive or non-radioactive labeled polynucleotides as well as oligonucleotide probes have been developed. The detection target elements include the TDH gene (*tdh*)[49-53], the TRH gene (*trh*)[51,53] and the TLH gene (*tlh*)[45].

Toxin gene elements in *V. parahaemolyticus* can be detected by colony hybridization[52,54] or dot blot hybridization[51] using appropriate nucleic acid probes. The target sequences can also be detected by PCR[44,53,55,56]. Multiplex PCR has been developed to detect *tdh*, *trh* and *tlh* simultaneously[57].

IDENTIFICATION OF SPECIFIC STRAINS

Since the pandemic *V. parahaemolyticus* O3:K6 strains appeared, several methods have been developed to identify these strains rapidly. A PCR method that targets two of the base positions of the *toxRS* sequence is unique to the new O3:K6 strains[17]. However, several old O3:K6 strains also reacted positively to this PCR method, implying that the sequence was not specific to the pandemic strains[58]. A unique open reading frame, ORF8, of a specific filamentous phage can also be used as a marker to identify the new O3:K6 strains[59]. However, some of the new O3:K6 strains are negative for ORF8[60].

When outbreak isolates were analyzed for the presence of enterobacterial repetitive intergenic consensus sequences, the new O3:K6 strains revealed a specific 850-bp DNA fragment which was not homologous with any known *Vibrio* spp. gene sequences. A PCR method based on this unique sequence can be used to identify O3:K6 *V. parahaemolyticus* isolates specifically under 6 hours[23].

Molecular typing can also differentiate the new O3:K6 strains from other strains[16,61,62]. Automated ribotyping with a Qualicon Riboprinter was employed to identify the clinical O3:K6 isolates of *V. parahaemolyticus* in the U.S. in 1998. The patterns produced using the restriction enzymes *Eco*RI and *Pst*I imply that the new O3:K6 strains caused the outbreak in the Northeastern United States[62]. Nevertheless, some strains of other serovars have been identified to have genotypes similar to those of these pandemic O3:K6 strains[17,58], limiting the application of these typing methods in differentiating the new O3:K6 of *V. parahaemolyticus*.

MOLECULAR TYPING

The subspecies typing for epidemiological investigation or controlling the spread of pathogenic bacteria is always required. The first pandemic O3:K6 strains of *V. parahaemolyticus* have spread very rapidly in Asia and other continents[61]. The increasing prevalence of *V. parahaemolyticus* demands an effective subspecies typing scheme to determine the origin and divergence of strains[19]. *V. parahaemolyticus* can be differentiated by serotyping with commercially available antisera (Denka Seiken, Tokyo, Japan). Generally, serotyping cannot distinguish all isolates that originate from various regions or sources, because of the limited availability of antisera. Several molecular typing approaches have been developed and are effective for typing *V. parahaemolyticus* strains.

A pulsed-field gel electrophoresis (PFGE) method was developed in our laboratory for the molecular typing of this pathogen[63]. In this method, genomic DNA was digested with *Sfi*I, and the fragments were resolved on 1% agarose with a contour-clamped homogeneous electric field apparatus set to 190 V and a pulse time of 3 to 80 s[63]. Later, the method has been used to type highly genetically diverse *Vibrio parahaemolyticus* strains in seafood imported from Asian countries[8] and strains obtained in a nosocomial outbreak in five wards of a hospital[64]. A total of 371 isolates of *V. parahaemolyticus* collected from patients involved in foodborne illness outbreaks in Taiwan from 1992 to 1995 were analyzed using this PFGE method. Fifty seven patterns were distinguished, and A, B, C, E and H were the major domestic types altogether representing 76% of the isolates, while the pandemic O3:K6 strains (PFGE type I) were genetically distant from the main domestic types[14].

A large number of old O3:K6 strains collected before 1996 and new O3:K6 strains from India, Japan, Korea and Taiwan, were studied using this PFGE method. All the O3:K6 strains were separated into two genetically unrelated groups, the old O3:K6 group and the new O3:K6 group. The old group included O3:K6 strains isolated before 1996, and was further divided into six patterns (A1, A2, A3, A8, B2 and R in the PFGE typing scheme. Patterns A8 and B2 were isolated in Taiwan; others were from Hong, Kong, the Maldives, Singapore and Thailand. The recently isolated O3:K6 strains were all in the new O3:K6 group (group I), which included eight closely related patterns, of which I1 (81%) and I5 (13%) were the most common patterns. Pattern I1 was the dominant one for strains from Japan, Korea and Taiwan[61]. Later, group I was extended to consist of 13 patterns (I1 to I13), all of which, except I1 and I5, consisted of only a few strains (unpublished data).

PFGE after *Apa*I digestion was also used to characterize an outbreak of *V. parahaemolyticus* on Canada's west coast in 1997. Digestion by *Apa*I and *Not*I was noted to yield a similar characterization[65]. PFGE following *Not*I digestion was also used to type new O3:K6 strains[66,67].

A ribotyping method was also developed in the author's laboratory. Genomic DNA was digested with 28 restriction enzymes, separated and hybridized with digoxigenin-labeled cDNA probes derived from the *Escherichia coli* 16S and 23S rRNA genes to develop this ribotyping method. Genomic DNA digested with *Bst*XI, *Dra*I, *Hin*dIII, *Sca*I and *Sph*I generated 10 to 17 visualized hybridization bands in a reference strain. *Hin*dIII was used to conduct further investigation. A total of 121 isolates obtained from outbreaks in Taiwan during 1992 and 1994 were characterized by this ribotyping method and grouped into 30 different ribotypes. The discriminatory ability of this ribotyping method, as determined by Simpson's index of diversity, was 0.93, very close to that of the previously reported PFGE method[68]. Ribotyping (*Bgl*I restriction enzyme digestion) was also used to group 28 new O3:K6 strains into five patterns[66].

PFGE and ribotyping are reliable molecultar typing methods. Nevertheless, these two methods usually take several days to complete. Molecular typing using PCR techniques can be completed in several hours. A novel, random amplified polymorphic DNA (RAPD) method was also developed for the molecular typing of this pathogen. From a set of a hundred primers, the 10-mer primer, 5'-CAG GCG CAC A-3', was selected to generate polymorphic amplification profiles of the genomic DNA at an annealing temperature of 38°C. A total of 308 clinical and environmental domestic isolates of *V. parahaemolyticus* were analyzed by this RAPD method. A total of 41 polymorphic RAPD patterns were recognized, and these patterns were arbitrarily grouped into 16 types (A to P)[69]. The RAPD is also known as the arbitrarily primed PCR method (AP-PCR) and two primers, 5'-GGT GCG GGA A-3' and 5'-GTT TCG CTC C-3', have been used to type urease-positive strains of *V. parahaemolyticus*[70] and the new O3:K6 strains[16]. The discriminatory ability of these RAPD methods was less than that of the PFGE and the ribotyping methods[61].

The RAPD method is often complicated by variations in band intensity and a lack of reproducibility of some minor bands. Three rapid PCR methods for typing this pathogen, using primers designed according to the conserved ribosomal gene spacer sequence (RS-PCR), repetitive extragenic palindromic sequence (REP-PCR) and

the enterobacterial repetitive intergenic consensus sequence (ERIC-PCR), were further developed to avoid the use of random primers[71]. These methods apparently differentiated *V. parahaemolyticus* strains from interspecific *E. coli*, V. cholerae and *V. vulnificus*. The abilities of these PCR methods were compared to those of PFGE, ribotyping and RAPD methods. The results showed that the effectiveness of these PCR methods closely approached or even exceeded that of the PFGE and ribotyping methods. REP-PCR is favored over ERIC-PCR, because of the greater reproducibility of its fingerprints. While RS-PCR is a practical method, it has fewer amplification bands and patterns than the other methods[71].

The feasibility of these molecular typing methods depends on the use of various enzymes, primers and experimental conditions. Each method may have special applications on certain occasions. Marshall compared the use of ERIC-PCR, restriction fragment length polymorphisms of flagellum (*fla* locus) and PFGE in typing a *V. parahaemolyticus* outbreak. He concluded that ERIC-PCR and ribotyping were the preferred methods. PFGE (follow *Apa*I digestion) discriminated effectively but suffered from a high incidence of DNA degradation[65]. In the author's laboratory, only around 7% of the *V. parahaemolyticus* strains suffered from DNA degradation and were not typeable by PFGE, following *Sfi*I digestion. The reliable PFGE or ribotyping method is recommended for used as the routine molecular typing method, while the PCR method may be used to conduct rapid analysis. In the author's laboratory, many clinical strains were collected from many Asian countries and from the United States, and analyzed by PFGE. A detailed subspecies typing scheme will be established for general reference.

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