# Molecular Typing of Vibrio parahaemolyticus

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**Abstract**

Vibrio parahaemolyticus is a Gram-negative, halophilic bacterium and a causative agent of seafood-borne gastroenteritis in human worldwide. Genotyping is a method used for characterizing the epidemiological spreads of pathogenic bacteria. The aim of this study is to compare the molecular typing techniques including arbitrarily primed polymerase chain reaction (AP-PCR) and variable-number tandem repeat (VNTR) methods for classifying V. parahaemolyticus clinical isolates. The results revealed 4 AP-PCR patterns, whereas two VNTR loci showed 4 and 6 types of V. parahaemolyticus strains, respectively. This suggested that the VNTR method has more discrimination power than the AP-PCR method. However, the PCR product sizes from the VNTR method are sometimes difficult to classify. Therefore, the consensus size, the copy number of tandem repeat and the suitable PCR condition should be concerned.

**Keywords: Vibrio parahaemolyticus / AP-PCR / VNTR**

## Introduction

V. parahaemolyticus is a Gram-negative halophilic bacterium in the Vibrionaceae family (Letchumanan et al., 2014; Su and Liu, 2007). The infection of V. parahaemolyticus is mainly occurred via the fecal-oral route and the predominant cause of infection is ingestion of bacteria in raw or undercooked seafood resulting in gastroenteritis. However, this organism can cause other illness such as wound infection and septicemia (Xiao et al., 2011; Nair et al., 2007). The basis method to classify the strains of V. parahaemolyticus is serotyping. Based on the antigenic properties of the somatic (O) and capsular (K) antigens, the serotyping is done using the commercial antisera developed from strains of clinical isolates (Xiao et al., 2011; Nair et al., 2007). However, many strains of V. parahaemolyticus are still untypable, therefore the serotyping method is not suitable for classifying the epidemiological spreads of V. parahaemolyticus (Xiao et al., 2011). Many molecular typing methods have been developed to dissect genetic variabilities and epidemiological spread of V. parahaemolyticus including random amplified polymorphic DNA analysis (RAPD) or arbitrarily primed polymerase chain reaction (AP-PCR), pulsed field gel electrophoresis (PFGE), multilocus sequence typing (MLST), variable-number tandem repeat (VNTR) (Xiao et al., 2011). The aim of this study is to compare the molecular typing techniques including AP-PCR and VNTR methods for classifying V. parahaemolyticus clinical isolates.

**Objective**

To compare the molecular typing techniques including arbitrarily primed polymerase chain reaction (AP-PCR) and variable-number tandem repeat (VNTR) methods for classifying V. parahaemolyticus clinical isolates.

**Scope of study**

To investigate the genetic variabilities of V. parahaemolyticus isolated from 22 clinical specimens.

**Literature Reviews**

1. Vibrio parahaemolyticus

1.1 General information

V. parahaemolyticus is a Gram-negative halophilic bacterium in the Vibrionaceae family (Letchumanan et al., 2014; Su and Liu, 2007). This bacterium is motile using a single polar flagellum which can attach to inert and animate surfaces such as fish, shell-fish, zooplankton or any suspended matter underwater (Letchumanan, 2014). The sources of V. parahaemolyticus are marine environments and it can usually be found in various kinds of seafood including clam, crab, scallop, oyster, shrimp, lobster, crawfish, codfish, sardine and mackerel. The distribution in marine environments of this bacterium is related to the water temperatures (Su and Liu, 2007). It has been shown that V. parahaemolyticus is likely to be isolated from oysters in the spring and the summer than in the winter. This organism is rarely found in sea water until water temperature arises to 15ºC or higher (Su and Liu, 2007).

V. parahaemolyticus was discovered as a human pathogen by Tsunesaburo Fujino in 1950. This bacterium was identified as a causative agent of food-borne disease in Japan when 272 individuals became ill with 20 deaths after the consumption of shirasu, the semidried juvenile sardines (Letchumanan, et al., 2014; Nair et al., 2007). The infection of V. parahaemolyticus is mainly occurred via the fecal-oral route and the predominant cause of infection is ingestion of bacteria in raw or undercooked seafood resulting in gastroenteritis. However, this organism can cause other illness such as wound infection and septicemia (Xiao et al., 2011; Nair et al., 2007).

1.2 Virulence factors

V. parahaemolyticus possesses many virulence factors required for its survival in the environment and pathogenesis. Different strains of this bacterium contain different number of virulence factors leading to the different degree of pathogenicity underwater (Letchumanan et al., 2014).

1.2.1 Flagella and capsule

V. parahaemolyticus has two types of flagella with different functions, swimming and swarming. The presence of flagella and the ability to produce capsule facilitate the bacterium to survive in the environment and to colonize a human host (Letchumanan et al., 2014).

1.2.2 Toxins

Some strains of V. parahaemolyticus produces the thermostable direct hemolysin (TDH) and the TDH-related hemolysin (TRH) enzymes. TDH is an enzyme that can lyse red blood cell on Wagatsuma agar. This hemolytic activity is known as the Kanagawa Phenomenon (KP) (Letchumanan et al., 2014; Su and Liu, 2007). The TDH and TRH enzymes are associated with hemolysis and cytotoxicity in the host cell of V. parahaemolyticus (Letchumanan et al., 2014). Usually, most of the clinical strains of V. parahaemolyticus isolated from patients with gastroenteritis are differentiated from the environmental strains based on the production of TDH enzymes. The TDH production and KP are related to the clinical strains but not environmental strains. Nevertheless, molecular epidemiology studies reported that V. parahaemolyticus strains isolated from outbreak patients were KP negative but produced TRH enzyme (Letchumanan et al., 2014; Su and Liu, 2007).

1.2.3 Type III secretion systems

Type III secretion systems (T3SSs) are the needle-like structure used by bacteria to inject bacterial effector proteins directly into eukaryotic cells without contacting with the extracellular environments (Letchumanan et al., 2014). A secretion apparatus is composed of a basal body, a needle and a translocon pore that is inserted into the eukaryotic cell membrane. The targets of effector proteins of T3SSs are usually the innate immune signaling, actin cytoskeleton and autophagy (Letchumanan et al., 2014). In V. parahaemolyticus, two types of T3SSs are identified, T3SS1 and T3SS2. T3SS1 of V. parahaemolyticus is found in all environmental and clinical strains. This system contains the number of genes, its gene identity and is induced by the increasing temperature or decreasing calcium concentration similar to Yersinia T3SS (Letchumanan et al., 2014). T3SS1 is responsible for a series of events including autophagy, membrane blebbing, cell rounding, and cell lysis during tissue cell infection.

V. parahaemolyticus T3SS2 is located on a pathogenicity island on chromosome 2, closely associated with pathogenicity island VPAI-7 and flanked by two tdh genes (Letchumanan et al., 2014). This system is found in clinical isolates and is related to pandemic strains of V. parahaemolyticus. T3SS2 system is different from T3SS1 but has high homology to the Hrp1 system of Pseudomonas syringae. The protein effectors of T3SS2 cause cytotoxicity of colon epithelial and enterotoxicity within the host in a rabbit ileal loop model (Letchumanan et al., 2014).

1.3 Pathogenesis

Pathogenesis of V. parahaemolyticus is related to its virulence factor. V. parahaemolyticus infection leads to gastroenteritis characterized by diarrhea with abdominal cramps, headache, vomiting, nausea, and low fever (Letchumanan, et al., 2014; Nair et al., 2007). Diarrhea caused by V. parahaemolyticus is sometimes bloody, with reddish watery stool described as “meat wash”. However, this bloody stool is different from that seen in dysentery of Shigella species or amebiasis (Nair et al., 2007). The incubation period of V. parahaemolyticus infection ranges between 4 to 96 h and the self-limiting illness, moderate severity and last an average of 3 days are found in immunocompetent patients (Nair et al., 2007).

2. Epidemiology of V. parahaemolyticus

The food borne bacteria that cause human illness worldwide are mostly transmitted by seafood. In Asian, V. parahaemolyticus was responsible for many food poisoning cases in Japan, Taiwan, China, Bangladesh, Laos, Hong Kong and Indonesia (Letchumanan et al., 2014). In addition, the pathogenic and antimicrobial resistant strains of V. parahaemolyticus were also isolated from white leg shrimp, black leg shrimp and cockles in Thailand and Malaysia. In India, V. parahaemolyticus has been isolated in both environmental and clinical samples. The prevalent strains among the isolates were multidrug resistant (Letchumanan et al., 2014). In Europe, this bacterium has been isolated from the North Sea, the Mediterranean Sea, the Baltic Sea and the Black Sea. In United States, V. parahaemolyticus has been isolated throughout the U.S. coastal region related to the consumption of raw shellfish or uncooked seafood (Letchumanan et al., 2014).

3. Molecular typing methods for V. parahaemolyticus

The basis method to classify the strains of V. parahaemolyticus is serotyping. Based on the antigenic properties of the somatic (O) and capsular (K) antigens, the serotyping is done using the commercial antisera developed from strains of clinical isolates (Xiao et al., 2011; Nair et al., 2007). This method is able to differentiate V. parahaemolyticus into 13 O groups and 71 K types. Since 1996, the pandemic strains that cause the worldwide outbreak of V. parahaemolyticus-induced gastroenteritis and diarrhea are O3:K6 serovar and its derivatives including O4:K68, O1:K25, O1:KUT, and O6:K18. These strains are genetically conserved to constitute a clonal complex. Therefore, the serotyping method seems not suitable for characterizing the epidemiological spreads of V. parahaemolyticus (Xiao et al., 2011). Many molecular typing methods have been developed to dissect genetic variabilities and epidemiological spread of V. parahaemolyticus including random amplified polymorphic DNA analysis (RAPD) or arbitrarily primed polymerase chain reaction (AP-PCR), pulsed field gel electrophoresis (PFGE), multilocus sequence typing (MLST), variable-number tandem repeat (VNTR) (Xiao et al., 2011).

3.1 Random amplified polymorphic DNA analysis (RAPD)

A RAPD or AP-PCR is one of the simplest methods used to demonstrate DNA variation by constructing the unique fingerprints of individual strains (Small et al., 2014). This technique requires little genomic data from the organism of interest. However, the data from this method is sometimes difficult to analyze (Small et al., 2014).

3.2 Variable-number tandem repeat (VNTR)

A VNTR method is used to demonstrate the multiple short sequence repeats in the bacterial genome. These multiple sequence repeats are named “tandem repeats” and is usually variable in their repeat number among individual genomes (Xiao et al., 2011). VNTRs have high resolution and reproducibility to discriminate genotypically diverse collections of environmental and clinical V. parahaemolyticus strains and have been used as markers for discrimination between bacterial strains (Xiao et al., 2011). Nevertheless, unlike the RAPD method, this method requires the genomic data from the organism of interest and the bioinformatics program to find the appropriate tandem repeats.

**Materials and Methods**

1. Bacterial strains

V. parahaemolyticus strains isolated from 22 clinical specimens were grown in LB agar containing 2% NaCl at 37ºC. The bacteria were confirmed using standard biochemical test and the growth of TCBS agar. Genomic DNA of all bacteria was extracted using GF-1 Bacterial DNA Extraction Kit (Vivantis). The quality of DNA was determined by 1% agarose gel electrophoresis.

2. AP-PCR

AP-PCR was performed using AP-primer4 (5-AAGAGCCCGT-3) as previously described (Bhoopong et al., 2007). A volume of 20 µl PCR mixture contained 1X PCR buffer, 1.5 mM MgCl2, 2.5 µM AP-primer4, 0.5 units Taq DNA polymerase and 25-100 ng of template DNA. The PCR condition included initial denaturation at 94 ºC for 5 min followed by 35 cycles of 94 ºC for 1 min, 35 ºC for 1 min and 72 ºC for 2 min, and final extension step of 72 ºC for 5 min. The PCR products were analyzed by 1.2% agarose gel electrophoresis with ethidium bromide staining.

3. VNTR

The tandem repeats of V. parahaemolyticus were obtained from whole genome sequence of V. parahaemolyticus RIMD 2210633 (accession PRJNA360) using Tandem Repeats Finder program (http://tandem.bu.edu/trf/trf.basic.submit.html). The tandem repeats for VNTR were selected and primers specific for each loci were designed based on 5'and 3' flanking regions of each loci using Primer3 program (http://bioinfo.ut.ee/primer3-0.4.0/). The selected VNTR loci were amplified from each strain by PCR with the specific primer pairs and the PCR products were analyzed by 1.5% agarose gel electrophoresis with ethidium bromide staining.

**Results**

AP-PCR

There are 4 different AP-PCR patterns from 22 clinical isolates denoted AP-A, AP-B, AP-C and AP-D. Eight isolates are classified into AP-A, whereas one isolate in AP-B, five isolates in AP-C and eight isolates in AP-D (Figure1).

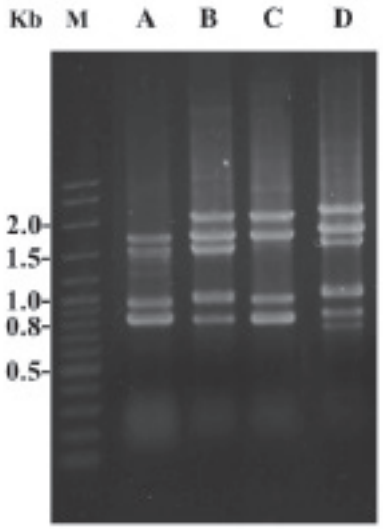


**Figure 1.** AP-PCR patterns of V. parahaemolyticus using primer AP-primer4 and V. parahaemolyticus chromosomal DNA was used as template. Lane A, B, C and D represent AP-PCR pattern AP-A, AP-B, AP-C and AP-D, respectively. M represents 100 bp DNA ladder.

VNTR

Two VNTR loci were selected and characterized. The first locus is located in indices 1341472-1341653 with consensus size 8, 22.8 copy numbers and percent matches 100. The specific primer pairs were designed including C2L3-F (5'-AACGTGCCCACTACGAATGT-3') and C2L3-R (5'-CACGGCAATAAGCAGTAAGC-3') with product size 320-420 bp. The second VNTR locus is in indices 3077908-3078076 with consensus size 6, 28.2 copy numbers and percent matches 100. Primers specific for this loci were designed including C1L2-F (5'-TGCTCGACCTCGCTAAAT-3') and C1L2-R (5'-CGTGATTCGGTGCTGTCTTA-3') with product size 800-1,050 bp. The PCR condition included initial denaturation at 94 ºC for 5 min followed by 30 cycles of 94 ºC for 1 min, 53 ºC for 30 s and 72 ºC for 1 min, and final extension step of 72 ºC for 5 min. The PCR products were analyzed by 1.2% agarose gel electrophoresis with ethidium bromide staining.

The amplified products from the first locus revealed variable sizes from 320-420 bp. Based on the product sizes, 17 clinical isolates of V. parahaemolyticus were classified into 4 types (Figure 2) including VT1 (320 bp), VT2 (390 bp), VT3 (400 bp) and VT4 (420 bp). No amplification was obtained with 5 isolates. The product sizes from the second locus were also variable and 22 isolates of V. parahaemolyticus were classified into 6 types (Figure 3) including VN1 (850 bp), VN 2 (1,050 and 500 bp), VN3 (800 bp), VN4 (900 and 500 bp), VN5 (870 bp) and VN6 (890 bp).

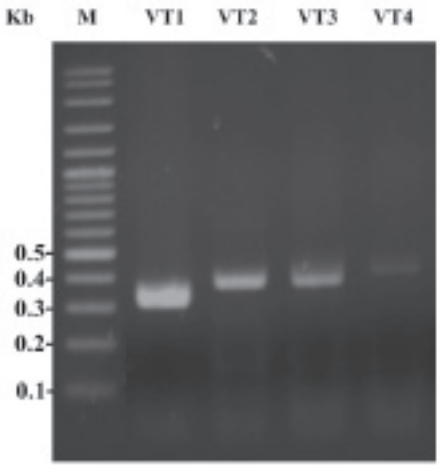


**Figure 2.** VNTR patterns of V. parahaemolyticus using primers C2L3-F and C2L3-R and V. parahaemolyticus chromosomal DNA was used as template. Four patterns from the first VNTR locus are composed of VT1, VT2, VT3 and VT4. M represents 100 bp DNA ladder.

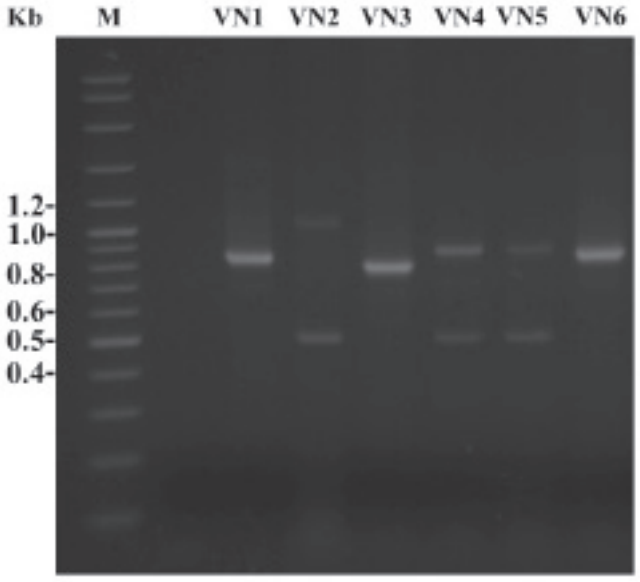


**Figure 3.** VNTR patterns of V. parahaemolyticus using primers C1L2-F and C1L2-R and V. parahaemolyticus chromosomal DNA was used as template. Six patterns from the second VNTR locus are composed of VN1, VN2, VN3, VN4, VN5 and VN6. M represents 100 bp DNA ladder.

**Discussions**

V. parahaemolyticus is a human pathogen that can be found worldwide. In United States, this bacterium is a leading cause of seafood-borne gastroenteritis and most of infection is the result from consumption of raw or mishandled seafood (Nordstrom et al., 2007). In Thailand, the emergence of

pandemic strains is also found and the strains isolated from white leg shrimp and black leg shrimp at inland ponds are antimicrobial resistance (Letchumanan et al., 2014). Therefore, a surveillance program for V. parahaemolyticus has been established in the South of Thailand (Bhoopong et al., 2007).

The molecular typing methods are more reliable than the serotyping method for characterizing the epidemiological spreads of V. parahaemolyticus (Xiao et al., 2011). Moreover, these genotypic methods can be used to demonstrate molecular diversity of this organism (Ansaruzzaman et al., 2008). Various molecular typing methods have been developed and applied for dissecting genetic variation and epidemiological spread of this bacterium (Letchumanan et al., 2014). The results from this study demonstrated that the VNTR method has more power to dissect the genetic variability of V. parahaemolyticus than the AP-PCR method. In the AP-PCR method, this method is easier and less expensive comparing to the VNTR method. However, this method is less specific and has lower reproducible ability than the VNTR method. In the VNTR method, this method is more complicate but more accurate than the AP-PCR method. This method has been used as a marker to discriminate between bacterial strains because it offers high resolution and reproducibility for discriminating genotypically diverse collections of environmental and clinical isolates of V. parahaemolyticus (Xiao et al., 2011). In this study, primers specific for the second loci of the VNTR method gave two amplicons for VN2 and VN4 (Figure 3) suggesting that the primers might be able to bind to anywhere else in the genomic DNA of V. parahaemolyticus. Although one amplicon of VN2 and VN4 is equal (500 bp), however the other amplicon can be used to discriminate between these types. In addition, the size of some amplicons between different types of VNTR is difficult to differentiate by agarose gel electrophoresis. This indicated that although the VNTR method is more accurate and more reproducible than the AP-PCR method, but the selections of the copy number and the consensus size of the tandem repeat should be concerned.

**Reference**


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