**Detection and microbiological and molecular characterization of *Vibrio parahaemolyticus* in the clam *Phacoides* (*Lucina*) *pectinatus* (Gmelin, 1791) and the oyster *Crassostrea rhizophorae* (Guilding, 1828) from the southwest coast of Puerto Rico**

By

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# ABSTRACT

Bivalves such as clams and oysters are filtering mollusks that inhabit aquatic environments. These organisms are relevant in the food industry for the potential presence of pathogenic bacteria, such as *Vibrio parahaemolyticus*, that these mollusks can accumulate during their filter feeding. Recently, the Centers for Disease Control and Prevention (CDC) estimated a higher incidence (7,880 cases/year) of *Vibrio* infections, where approximately 2,800 cases were associated with *V. parahaemolyticus*. The Food and Drug Administration (FDA) recognized this bacterium as a leading cause of human gastroenteritis associated with seafood consumption. However, the outbreaks caused by seafood contaminated with this pathogen have been also described in countries like United States, Spain, Japan, Taiwan and Brazil. Because the foodborne illness distribution, the PulseNet International network was created in order to track foodborne infections worldwide. In Puerto Rico, there are no laws or regulatory agencies that assess quality bivalve for sale. Therefore, the Island does not have statistics on foodborne disease incidence caused by consumption of raw bivalves. This research sought the detection of *V. parahaemolyticus* in both raw bivalves consumed in Puerto Rico, established the potential of pathogenicity of the isolates, and sought the molecular typing of the *V. parahaemolyticus* isolated strains using Pulse Field Gel Electrophoresis (PFGE). This study used standardized tests, including molecular protocols established by the FDA in the Bacteriological Analytical Manual (BAM), for the detection of *V. parahaemolyticus* strains in oyster and clam samples from the southwest coast. Finally, molecular analysis involved the use of Multiplex PCR to detect the presence of *V. parahaemolyticus* pathogenic associated genes and the use of a PFGE technique followed the standardized protocol established by the PulseNet International Network to molecularly subtyping of *V. parahaemolyticus*. To confirm the isolated strains as part of genus *Vibrio*, a PCR was performed to amplify part of the 16S rDNA and the PCR products were sequenced at McLab facilities. After 4 seasonal samplings, 58 strains of presumptive *V. parahaemolyticus* were isolated, where 36% were able to grow at 3%, 6% and 8% of NaCl; while, 64% of isolates were able to grow also at 10% of NaCl. Based on species-specific marker amplicon, and the absence of pathogenic genes signal, 90% of the isolates in the study are *V. parahaemolyticus* and none of them are potentially pathogenic. The PFGE analysis with *NotI* and *SfiI* allowed the discrimination of 52 isolates into 21 different strains. These strains were grouped into 18 types with >65% patterns similarity. Both,

*NotI* and *SfiI* restriction patterns, revealed a similar discriminatory power. *In-silico* analysis of the bacteria isolated confirmed all isolates as *Vibrio spp*. In this study, do not detect the presence of pathogenicity markers (*tdh, trh*) in *V. parahaemolyticus* isolated from mollusk samples from the southwest coast of Puerto Rico. A comparison with isolates from patients with diarrhea associated to bivalve consumption will provide more information on potential foodborne disease associated to raw shellfish consumption from these waters. All restriction patterns were novel in comparison with the restriction patterns of the strains in the PulseNet USA *V. parahaemolyticus* database. This molecular study serves as a baseline to continue developing food safety studies of bivalves in the Island. Also, demonstrated not only the diversity of *V. parahaemolyticus* in the southwest coast of Puerto Rico, but the uniqueness and how they contrast to the isolates from outbreaks in USA.

# RESUMEN

Los bivalvos, como las ostras y las almejas, son moluscos filtradores que habitan ambientes acuáticos. Estos organismos son relevantes en la industria de alimentos por la posible presencia de bacterias patogénicas, como es *Vibrio parahaemolyticus*, que estos moluscos pueden acumular durante su alimentación por filtración. Recientemente, el Centro de Control y Prevención de Enfermedades (CDC, por sus siglas en inglés) estimó una alta incidencia de infecciones causadas por *Vibrio* (7,800 casos/año), donde aproximadamente 2,800 casos se asociaron con *V. parahaemolyticus*. La Administración de Drogas y Alimentos (FDA, por sus siglas en inglés) reconoce esta bacteria como la causa líder de gastroenteritis humana asociada al consumo de mariscos. Sin embargo, brotes causados por maricos contaminados con este patógeno han sido descritos en países como Estados Unidos, España, Japón, Taiwán y Brasil. Debido a la distribución de las enfermedades causadas por alimentos, se creó la red de PulseNet Internacional para rastrear las infecciones trasmitidas por alimentos en todo el mundo. En Puerto Rico, no existen leyes ni agencias reguladoras que evalúen la calidad de los bivalvos para la venta. Por lo tanto, la Isla no tiene estadísticas sobre la incidencia de enfermedades transmitidas por alimentos causadas por el consumo de bivalvos crudos. Esta investigación buscó la detección de *V. parahaemolyticus* en ambos bivalvos crudos consumidos en Puerto Rico, estableció el potencial de patogenicidad de los aislados y buscó la tipificación molecular de las cepas aisladas de *V. parahaemolyticus* utilizando la electroforesis en gel de campo pulsado (PFGE, por sus siglas en inglés). Este estudio utilizó pruebas estandarizadas, incluyendo protocolos moleculares establecidos por la FDA en el Manual Analítico Bacteriológico (BAM, por sus siglas en inglés), para la detección de cepas de *V. parahaemolyticus* en muestras de ostras y almejas de la costa suroeste. Finalmente, el análisis molecular implicó el uso de la PCR Múltiple para detectar la presencia de genes asociados a patogenicidad en *V. parahaemolyticus* y el uso de la técnica de electroforesis de campo pulsado siguiendo el protocolo estandarizado establecido por la Red Internacional PulseNet para la subtipificación molecular de *V. parahaemolyticus*. Para confirmar las cepas aisladas como parte del género *Vibrio*, se realizó una PCR para amplificar parte del 16S rADN y el producto de PCR se secuenció en las instalaciones McLab. Después de 4 muestreos estacionales, se asilaron 58 presuntas cepas de *V. parahaemolyticus*, donde 36% fue capaz de crecer a 3%, 6% y 8% de NaCl; mientras que el 64% de las aislados fue capaz de crecer también al 10% de NaCl. Basado en el

amplicón del marcador especie-especifico, y la ausencia de los genes asociados a patogenicidad, el 90% de los aislados en el estudio son *V. parahaemolyticus* y ninguno de ellos es potencialmente patógeno. El análisis de PFGE con *NotI* y *SfiI* permitió la discriminación de 52 aislamientos en 21 cepas diferentes. Estas cepas se agruparon en 18 tipos con una similitud de más del 65%. Ambos, los patrones de restricción de *NotI* y de *SfiI*, mostraron un poder discriminatorio similar. En el análisis *in-silico* de las bacterias aisladas, todos los aislados fueron confirmados como *Vibrio spp*. En este estudio no se detectó la presencia de los marcadores de patogenicidad (*tdh, trh*) en los aislados de *V. parahaemolyticus* de las muestras de moluscos de la costa suroeste de Puerto Rico. Una comparación con aislados de pacientes con diarrea asociada al consumo de bivalvos proporcionará más información sobre la posible enfermedad transmitidas por los alimentos contaminados asociada al consumo crudo de mariscos de estas aguas. Todos los patrones de restricción resultaron nuevos en comparación con los patrones de restricción de las cepas en la base de datos de *V. parahaemolyticus* de PulseNet USA. Este estudio molecular sirve como una base de referencia para continuar desarrollando estudios de seguridad alimentaria de bivalvos en la Isla. Además, demostró no sólo la diversidad de *V. parahaemolyticus* en la costa suroeste de Puerto Rico, sino la singularidad y la forma en que contrastan con los aislados en brotes en Estados Unidos.

# DEDICATION

I would like to dedicate this work to my family and my colony because they always believed in me and supported me through this journey. To my parents, Carlos and Eneida, because they taught me how valuable are the sacrifices to make this dream come true. To my brother Carlos and my sister Neishlen, because they gave me courage and all your unconditional love. And last but never least, to my colony, Jesús M. Rivera Hernández, because you always understand me, even when I was illogical; also, thank you for doing me company every night in the laboratory.

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## Introduction and Literature Review

## 1.1 Introduction

Seafood has become a healthy food choice for people of all ages, because it provides a lower fat source of high-quality protein, a good source of omega -3 fatty acids and is a supplement rich in vitamins and minerals. Nutritionists and leading health organizations recommend eating 227 or more grams of seafood per week as part of a healthy diet (*Dietary Guidelines for Americans*, 2010). However, along with these nutritional benefits come the potential risks of seafood as a vehicle of transmission of foodborne bacteria that can cause illness. Pathogens such as *Vibrio parahaemolyticus* and *Vibrio vulnificus* may cause foodborne outbreaks throughout the world with seafood as the vehicle of transmission. For example, a large outbreak linked to consumption of raw oysters was reported in the United States and Canada in 1997 (209 infection cases in Oregon, Washington, California and British Columbia), in 1998 (43 cases in Washington and 416 in Texas) and in 2006 (177 cases in Washington and British Columbia). Other countries such as Japan, India, China and Taiwan have also reported outbreaks caused by consumption of contaminated seafood (shirasu, sardines, and shrimp, among others). These pathogens can be present in a variety of fish and shellfish, although oysters and clams are the most common food associated with *Vibrio* infections in most countries. The Food and Drug Administration (FDA) in the United States and other government regulatory agencies throughout the world, recognized *Vibrio parahaemolyticus* as the leading cause of human gastroenteritis associated with seafood consumption and other seafood-associated illness.

Bivalves are of particular concern for the presence of pathogenic bacteria such as *Vibrio spp.*, because bacteria accumulate during their filter feeding. Filtered *Vibrio* concentrates in the gut of molluscan shellfish, such as oysters, clams, and mussels, where they adhere and multiply. The filtrating activity of bivalves is influenced by concentration of phytoplankton, quality and size of food particles and size of the bivalves (Khalil, 1996). Other physical parameters like temperature, salinity and flow of water also affect the filtration rate (MacDonalds and Thompson, 1985).

In Puerto Rico, the consumption of bivalves is a very common cultural activity on the southwest coast of the Island, especially in the city of Cabo Rojo. Street vendors in the area have stands selling fresh clams and oysters which are visited by thousands of people every year. These

products are sold raw to consumers, which could represent a health risk to the population. The most common bivalve species consumed in that area are *Crassostrea rhizophorae* (oyster) and *Phacoides pentinatus (Lucina pectinata)* (clam). Because the consumption of these bivalves is very popular, it is important to determine the possibility of microbial contamination to protect public health.

The "mangrove oyster", *Crassostrea rhizophorae*, is always found on the roots (rhizophores) of the red mangrove tree (*Rhizophora mangle*), present in bays and estuaries throughout the Caribbean region. The mangrove tree characterizes a complex coastal ecosystem distributed in the tropical and subtropical zones in the same range of latitudes near the equator. The oyster *Crassostrea rhizophorae* has an optimal filtration rate in conditions of 25‰ of salinity and a temperature of 28ºC (Madrigal-Castro *et al*., 1985). The clam *Phacoides pectinatus* is a large tropical *Lucinidae* that is found on sulfide-rich mud environments, specifically in the high stress environment of shallow water mangrove swamps. It lives in symbiosis with chemoautotrophic bacteria and is characterized by abundant tissue hemoglobin in its deep-red gills. This bivalve clam is one of the most resistant species to environmental changes and ranges from the Caribbean region to Brazil.

Some other factors that affect the incidence and distribution of *Vibrio* spp. in the environment are temperature and salt concentration. In the Caribbean Sea, specifically in Boquerón, PR, water has warm temperatures fluctuating between 25-28ºC, thus providing optimal environment for their prevalence. De Paola *et al*. (1990) found that the densities of *V. parahaemolyticus* in seawater could increase when water temperature increased to around 25°C.

*Vibrio parahaemolyticus* is a Gram-negative halophilic bacterium indigenous to estuarine, marine and coastal environments. It is commonly found in a free-swimming state, with its motility conferred by a single polar flagellum, and is commensally associated with various species of shellfish. Not all strain of *V. parahaemolyticus* are considered pathogenic; potentially virulent strains are primarily distinguished by the presence of two hemolysins that play a significant role in precipitating the disease. The Centers for Disease Control and Prevention and the U.S. Food and Drug Administration estimated an annual rate of 4,500 U.S. infections per year and recently the outbreaks have been increasing in size and frequency (Iwamoto *et al*., 2010). The pathogenesis of *Vibrio parahaemolyticus* strains involves a different virulent factors, such as adhesins,

thermostable direct hemolysin (TDH) and the TDH- related hemolysin (TRH) as well as two type of secretion systems, that allow the bacteria invade, survive and replicate in host cells (Broberg *et al*., 2011).

Due to the elevated prevalence of *Vibrio* infections in the USA, two national surveillance systems were developed: Cholera and Other *Vibrio* Illness Surveillance (COVIS) and The Foodborne Diseases Active Surveillance Network (FoodNet). In Puerto Rico, there are no laws or regulatory agencies that assess the quality of bivalves for sale. Therefore, the Island does not have statistics on the incidence of foodborne diseases caused by consumption of raw bivalves (R. Cuevas, Bacteriology Supervisor from the Public Health Laboratory of Puerto Rico, personal communication, July, 14, 2015).

The main focus of this research was the detection and the molecular characterization of pathogenic *Vibrio parahaemolyticus* strains that might be present in raw bivalves consumed in Puerto Rico using microbiological and molecular approaches, such as multiplexed polymerase chain reaction and pulsed field gel electrophoresis. Also, this research aimed to develop awareness among local fishermen and the general community of the public health problems associated to eating raw shellfish.

## 1.2 Literature Review

### 1.2.1 Physiology and general features of the target organism

*Vibrio parahaemolyticus* is a motile Gram-negative marine, facultative anaerobic rod, with the morphology of a single rigid curve. It is widespread along estuarine, marine and coastal environments throughout the world, being found in waters, sediments, suspended particles, plankton, fish and shellfish (Joseph *et al.*, 1982). This bacterium can grow and survive in diverse ranges of temperature (5-43ºC), pH (4.8-11), and NaCl concentrations (0.5-10%) (Covert and Woodburn, 1972; Jackson, 1974; Zhu *et al.*, 1992).

Depending on the environmental conditions, this organism can produce a capsule and two different types of flagella with distinct functions. A polar flagellum is made of six different

flagellin proteins and is sheathed, which may aid in attachment and is also used for swimming. Another peritrichous or lateral flagella, are non-sheated and allow the bacterium to swarm over solid or semi-solid substrates (McCarter, 1999). The polar flagellum is produced continuously, whereas production of the lateral flagella is induced when growing on surfaces (Belas *et al*., 1986; McCarter *et al*., 1988).

Furthermore, at the physiological level, *V. parahaemolyticus* exhibits some additional virulence traits such as hemolysins, urease production and systems of secretion.

#### 1.2.1.1 Hemolysins

To date, the most distinctive traits of pathogenic *V. parahaemolyticus* strains are the thermostable direct hemolysin (TDH) encoded by the *tdh* gene and the TDH- related hemolysin (TRH) encoded by the *trh* gene. All strains have the gene encoding for the thermolabile hemolysin (thl) that is considered a signature molecular marker and is not related to virulence (Fujino *et al.*, 1969; Miwatani *et al.*, 1972). The purified TDH is heat-stable (up to 100ºC for 10 mins), is cytolytic against cultured mammalian cells, and lyses erythrocytes of various animal species (Douet *et al.*, 1992; Matsuda *et al.*, 2010).

Virulent strains usually exhibit β- hemolytic activity on Wagatsuma Blood agar (Wagatsuma, 1968; Honda and Iida., 1993). This activity has been termed the Kanagawa phenomenon (KP). Thermostable direct hemolysin damages the erythrocyte membrane by acting as a pore-forming toxin. This hemolysis occurs in three steps: binding to the erythrocyte membrane, formation of a trans-membrane pore, and disruption of the cell membrane, where the fairly large pore size allows both water and ions to flow through the membrane. An alterations in the ion flux in the membrane of the intestinal cells, are also the mechanism of diarrhea during the infection (Honda *et al.*, 1992; Shimohata *et al*., 2010). Some clinical isolates which are KP- negative and lack the *tdh* gene have been shown to produce a second hemolysin, TRH. In cultured human colonic epithelial cells, TRH increases Cl- secretion, followed by increased calcium concentrations (Takahashi *et al.*, 2000). In contrast to TDH, the activity of TRH is labile to heat treatment at 60ºC for 10 mins. Pathogenic *V. parahaemolyticus* produces either the TDH, the TRH, or both, while few isolates from the environment have the genes that codify for these properties (Nishibuchi and Kaper, 1995). Few studies have reported the presence of *tdh* and *trh* genes in *V. parahaemolyticus* strains of

environmental isolation; only 6% of the samples analyzed from the coasts of the USA, Europe, and Asia, contained these genes (DePaola *et al.*, 2003; Nair *et al.*, 2007). However, in some regions of Mexico and the United States, the proportion of environmental strains with pathogenic genes *(tdh* and/or *trh*) increased from 48 to 52% between 2004 and 2010, respectively (Newton *et al.*, 2012).

#### 1.2.1.2 Urease (Uh) production

Urease production in *Vibrio parahaemolyticus* is variable, but it is correlated with the pathogenesis. Usually, the majority of clinical and environmental isolates found are urease negative. The first report of urease production in *V. parahaemolyticus* was established by Abbott et al. (1989). This study reported that *V. parahaemolyticus* urease positive had become the predominant biotype in California outbreaks. Osawa *et al*. (1996) isolated a total of 132 clinical strains of *V. parahaemolyticus* from patients in Japan and suspected food items of foodborne cases. This study identified a total of 106 strains that carried a *tdh* gene, but less than 6% were positive to urease, whereas all trh-carrying strains were positive to urease. These results suggest that urease hydrolysis may be a marker for *trh* carrying strains. Nevertheless, Nakaguchi *et al*. (2003) demonstrated that the urease gene has no influence on the regulation of *tdh* and *trh* expresions.

#### 1.2.1.3 Type III secretion system

Another virulent factor of *V. parahaemolyticus* is a new type III secretion system (T3SS), found by Makino *et al.* (2003) when comparing sequences associated with the *V. parahaemolyticus* genome to those of *V. cholera*. The T3SS was identified on the genome of *V. parahaemolyticus*, but not in *V. cholerae*. Type 3 secretion systems are a needle-like protein machinery used to inject bacterial effectors directly into the membrane and cytoplasm of eukaryotic cells without encountering the extracellular environment (Cornelis, 2006; Caburlotto *et al*., 2010). Ono *et al.* (2006) showed that a clinical strain of *V. parahaemolyticus* (RIMD2210633) contained 2 sets of the gene clusters (T3SS1 and T3SS2) that encode for the T3SS. Both clusters are conserved and widespread in both clinical and environmental strains of *V. parahaemolyticus*. The T3SS1 effectors help *V. parahaemolyticus* evade the host immune response, inducing autophagy followed by cell rounding and lysis (Higa *et al.*, 2013). However, *V. parahaemolyticus* uses a T3SS2

mediated mechanism for invasion, intracellular replication and lysis of infected cells (Zhang *et al.*, 2012), and has been found only in Kanagawa phenomenon positive strains.

#### 1.2.1.4 Type VI secretion system

The most recent virulent factor identified in *V. parahaemolyticus* is the type VI secretion system (T6SS1 and T6SS2), detected when a comparison was performed between pandemic and non-pandemic strains (Boyd *et al.*, 2008; Izutsu *et al.*, 2008). The type VI secretion system is a widespread protein secretion apparatus used by Gram-negative bacteria to deliver toxic effector proteins into adjacent bacterial or host cells. *V. parahaemolyticus* T6SS was used as a virulence marker to differentiate pathogenic strains. T6SS1 is predominantly found in clinical isolates, whereas T6SS2 is found in all isolates tested to date (Salomon *et al*., 2013). The function of T6SS1 has not been demonstrated, but recent studies suggest that T6SS2 plays a role in the adhesion and export of effectors by inducing enterocytotoxicity (Park *et al*., 2004; Yu *et al.*, 2012). Salmon *et al*. (2015) identified that at least four antibacterial effectors are secreted by T6SSI and three by T6SS2.

#### 1.2.1.5 The O3:K6 pandemic clone

Epidemiological studies have indicated that specific clones of certain serotypes, notably O3:K6 having enhanced virulence, have become endemically established in certain global locales. Strains of O3:K6 serovar were found for the first time in Calcutta, India, during ongoing surveillance, causing an increase in patients with *V. parahaemolyticus* infections. The O3:K6 serotype exhibited the specific genetic markers *tdh*, *toxRS/New* and *orf8*. The *orf8* gene is believed to encode an adherence protein that increases the ability of *V. parahaemolyticus* to attach to host intestinal cells or to the surface of marine plankton (Nasu *et al.*, 2000). Furthermore, the *toxRS* operon of the pandemic strains encodes for transmembrane proteins involved in the regulation of virulence associated genes (Chowdhury *et al.*, 2000; Okura *et al.*, 2003). Another characteristic of the O3:K6 pandemic clone is the absence of the *trh* gene and the lack of urease production.

### 1.2.2 Clinical symptoms due to infections by *V. parahaemolyticus*

Since its discovery in 1950, *V. parahaemolyticus* has become a leading cause of seafood-derived food poisoning throughout the world. This organism causes three important syndromes of

clinical illness, which are: gastroenteritis, wound infections, and septicemia. The most common syndrome is gastroenteritis, and its clinical symptoms include: diarrhea with abdominal cramps, nausea, vomiting, cefalea, low-grade fever and chills. Gastrointestinal infections due to *V. parahemolyticus* are usually mild with duration of 2-3 days. The mean incubation period for infection is 15 hours in a range of 4 to 96 hours after consumption of contaminated food (Yeung and Boor, 2004; Nair *et al.*, 2007; Yeung *et al.*, 2002). A more severe and debilitating dysenteric form of gastrointestinal infection with bloody stools and marked leukocytosis is due particularly to strains of the serotype O3:K6 (Bolen *et al*., 1974; Hughes *et al.*, 1978; Daniels *et al.*, 2000).

Although gastroenteritis may be self-limited, the infection can cause septicemia that is life-threatening in the case of preexisting medical conditions. Septicemia occurs when the bacterium enters the bloodstream and is disseminated throughout the body. This can result in hypovolemic shock, multisystem organ failure and death (Su *et al.*, 2007). Extraintestinal infections due to *V. parahaemolyticus* can also be associated with wound infections at the extremities. Wound infections are sometimes limited to cellulitis, but may progress to necrotizing fasciitis. Sanyal and Sen (1974) concluded that the infectious dose of *V. parahaemolyticus* in other to trigger the symptoms of gastroenteritis is ~$10^5$ CFU (colony forming units). However, recent studies have suggested that unusually virulent strains may be infectious at lower cell numbers (DePaola *et al.*, 2000).

### 1.2.3 Use of molecular techniques for detection of *V. parahaemolyticus*

#### 1.2.3.1 Polymerase Chain Reaction and Multiplex PCR

The genetic composition of *Vibrio* species is extremely variable thus the genes present in the environmental isolates can be used to distinguish this genus from other bacterial groups. From all the molecular techniques used for the identification and detection of *V. parahaemolyticus*, Polymerase Chain Reaction (PCR) has become the most popular. This method is an efficient and cost-effective way to copy or amplify small segments of DNA. Since 1992, Tada *et al.* (1992) established PCR protocols for the specific detection of the *tdh* and *trh* genes of *V. parahaemolyticus*. Lee et al. (1995) developed a species-specific PCR assays to distinguish *V.*

*parahaemolyticus* from *V. alginolyticus* using a pR72H DNA region that is present in *V. parahaemolyticus* but absent in *V. alginolyticus*.

Another molecular technique used for the detection of *V. parahaemolyticus* is the Multiplex Polymerase Chain Reaction (multiplex PCR). This technique is a variant of PCR in which two or more target sequences can be amplified using more than one pair of primers. In the field of infectious diseases, multiplex PCR is a valuable tool for identification of virus and bacteria (Markoulatos *et al.*, 2002). A number of multiplex PCR assays have been developed for detection of pathogenic *Vibrio*. Brasher *et al*. (1998) designed a double multiplex PCR assay enabling the simultaneous detection in shellfish of *V. vulnificus*, *V. cholera* and *V. parahaemolyticus* based on amplification of regions corresponding to gene targets *vvhA, ctx* and *tlh*, respectively. The sensitivity of detection for each species was $<10^1$- $10^2$ CFU/g, when these investigators applied the assay to artificially inoculated oyster homogenates. Bej *et al*. (1999) developed a multiplex PCR assay for pathogenic and non-pathogenic strains of *V. parahaemolyticus* based on the amplification of a 450bp sequence of the species specific marker (*tlh* gene), a 269bp sequence of the *tdh* gene, and a 500bp sequence of the *trh* gene. All isolates in this study showed the *tlh* amplicons, but only 60 isolates showed the *tdh* gene and 43 the *trh* gene. This research found that the presence of the *tdh* gene, in some cases, was not related with the Kanagawa phenomenon.

Recently, Neogi *et al*. (2010) developed a multiplex PCR for the simultaneous detection of the most clinically important *Vibrio* species: *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus*. This method was validated with 488 *Vibrio* strain associated with human diseases, and in each case showed 100% of specificity and sensitivity. Another multiplex PCR was developed by No *et al.* (2011) using *hns*, *tdh* and *trh* primers. However only the sensitivity of *hns* for detection of particular strains was examined in their study. Tofazzal *et al*. (2013) developed a multiplex PCR using primer sets for a species-specific marker, *groEL*, and two virulence markers, *tdh* and *trh*. This investigation is useful for the differentiation of *V. parahaemolyticus* strains.

#### 1.2.3.2 Molecular typing of *V. parahaemolyticus* using Pulsed Field Gel Electrophoresis

Several molecular methods have been applied to *V. parahaemolyticus* strain typing. Molecular studies based on Pulsed-Field Gel Electrophoresis (PFGE) and arbitrarily primed PCR (APPCR)

have indicated that the pandemic strains (*Vibrio parahaemolyticus* O3:K6) exhibit almost identical fragment patterns. Pulsed-Field Gel Electrophoresis (PFGE) is a molecular technique used to separate large restriction fragments of chromosomal DNA by alternating the direction of the electric field during the electrophoresis process. Standardization in comparison and interpretation of profile is an integral part of the analysis of gels from PFGE (Fakruddin *et al.*, 2013). The value of the database will depend on the reproducibility of the experimental data stored and on the quality of the documentation. PulseNet International (http://www.pulsenetinternational.org/) has developed and implemented highly standardized PFGE protocols for different microorganisms associated with food-borne diseases. In the standard PFGE protocol for *V. parahaemolyticus* and *V. cholera*, two restriction enzymes, *Sfi*I and *Not*I, are used to generate appropriate number of DNA fragments for analysis. The combination of both restriction enzymes increases the discriminatory power of the method. Kam *et al.* (2008) recommend the use of *Not*I as the primary enzyme, while *Sfi*I can be used when further differentiation is needed (PulseNet, 2013).

PFGE generates information on genetic diversity among strains that are not provided by other molecular techniques such as GS-PCR and *orf8*-PCR. Much of the PFGE typing has been done by Wong *et al*. (1996, 2000, 2002, and 2007). In these research works, Wong *et al*. examined more than 532 *V. parahaemolyticus* strains from clinical samples obtained in Taiwan and 14 other countries. The PFGE procedure was applied to classify the strains, resulting in the detection of 115 differentiated PFGE patterns (fingerprints) that were grouped based of band similarity into more than 13 types. Each type had a dissimilarity value of less than 15%. In other studies, Wong *et al*. (1999) examinated 315 *V. parahaemolyticus* isolates from contaminated seafood. Results revealed 96 patterns and 22 types. Studies by Yeung *et al.* (2002) and Wong *et al*. (2000) confirmed the ability of Restriction Fragment Length Polymorphism (RFLP) PFGE to differentiate between pandemic and non-pandemic isolates. Martínez *et al*. (2004) conducted a characterization and comparison of pathogenic *V. parahemolyticus* isolates from different countries (Spain, Asia, and North America) using a PFGE. This analysis clustered the European isolates into two related PFGE types, and these strains were widely differentiated from the isolates of Asia and North America.

### 1.2.4 Prevalence of *Vibrio parahaemolyticus*

More than 80 serotypes of *Vibrio parahaemolyticus* have been described worldwide, based on antigenic properties of the somatic (O) and capsular (K) antigens. The presence and distribution

of *V. parahaemolyticus* is influenced by several environmental factors including water temperature, salt and oxygen concentrations, interaction with plankton, presence of sediments, organic matter in suspension and marine biota (Cabrera *et al.*, 2004).

In the American territories, only five countries have reported the presence of the pandemic *V. parahaemolyticus* clone O3:K6 and its serovariants in clinical and environmental samples. Figure 1.1 (Velázquez-Román *et al*., 2014) shows the countries where pandemic and non- pandemic strains were observed (Cabanillas *et al*., 2006; Iwamoto *et al*., 2010).

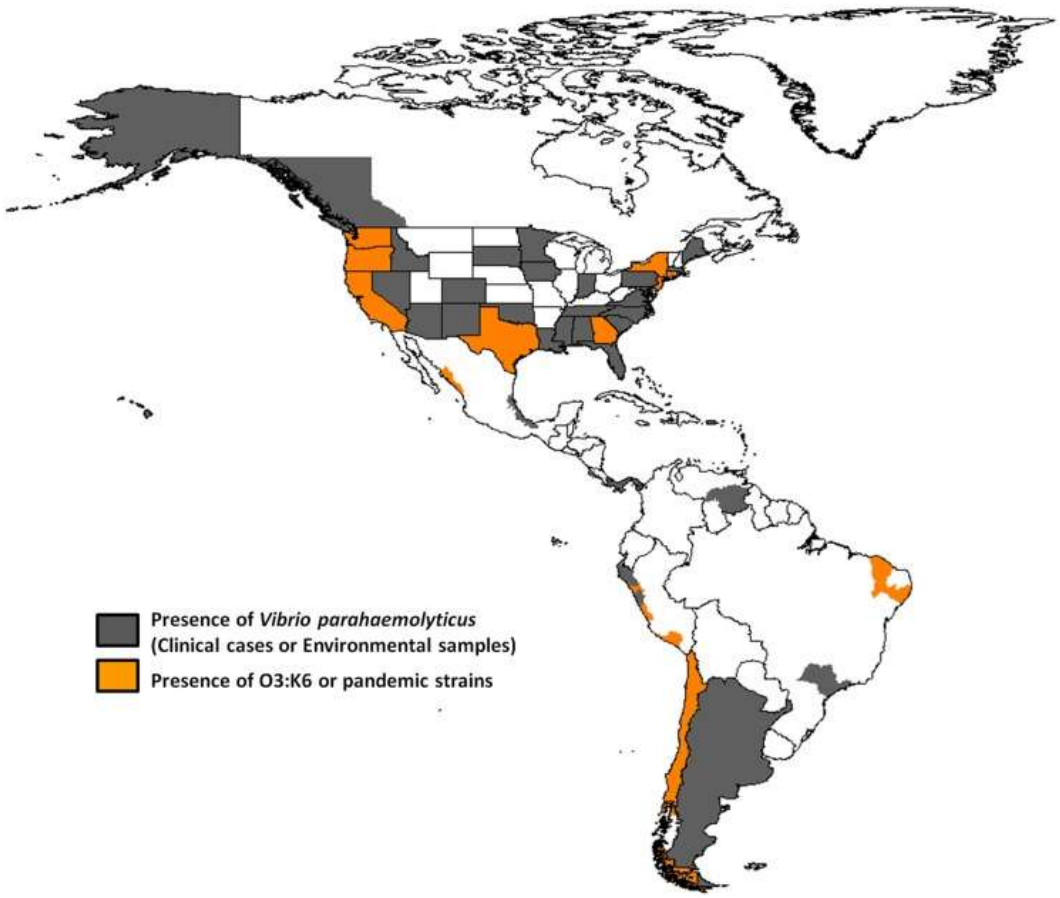


**Figure 1.1** Presence of *V. parahaemolyticus* throughout America. The map shows the presence of *Vibrio parahaemolyticus* and the dissemination of pandemic O3:K6 clone throughout America (Velázquez-Román *et al*., 2014)

Since 1971 sporadic *V. parahaemolyticus* outbreaks have been reported throughout the U.S. coastal regions. Over 700 cases in four outbreaks were linked by the Centers for Disease Control

and Prevention (CDC) to consumption of raw oysters in the Gulf Coast, Pacific Northwest, and Atlantic Northeast regions between the years 1997 and 1998. CDC, in May 2015, reported a significantly higher incidence of *Vibrio* infections with an increase of 100% in comparison with 2006-2008 incidences (see Figure1.2). However, the recent report of the CDC's Foodborne Diseases Active Surveillance Network (FoodNet) in April 2017 reported not significant increase incidence of *Vibrio spp* in comparison with the previous 3-year average (Marder *et al.*, 2017).

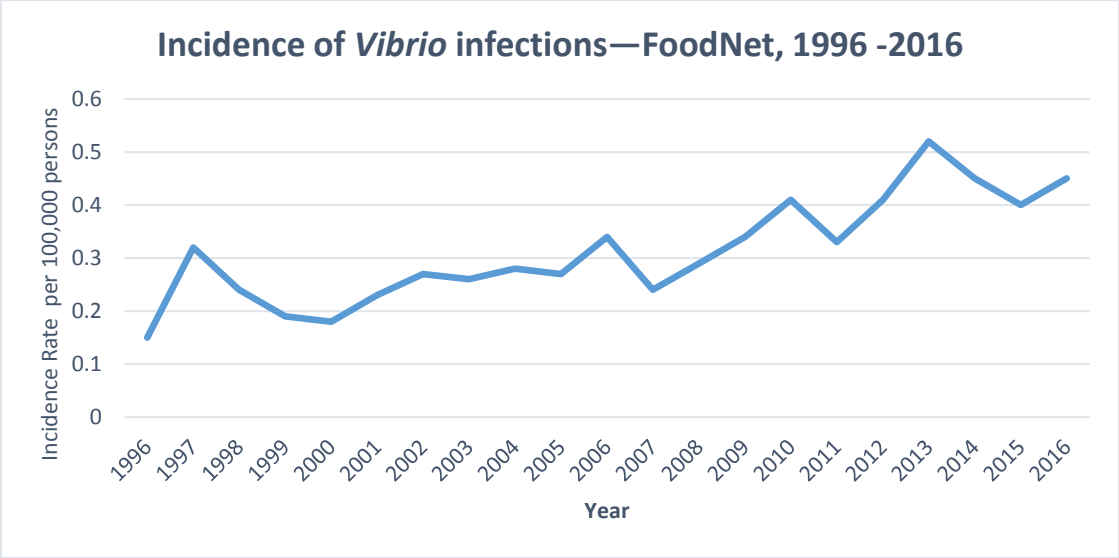


**Figure 1.2**. Incidence Rates of *Vibrio* infections 1996 through 2016, by year. The position of the line indicates the relative incidence rate (cases per 100,000 persons) (FoodNet, Crim *et al*., 2015; Marder *et al*., 2017).

#### 1.2.4.1 *Vibrio parahaemolyticus* in Puerto Rico

There have been a few researches about *V. parahaemolyticus* in Puerto Rico. However, since the decade of 1980, several researchers in Puerto Rico have tried to establish public health importance of *V. parahemolyticus*. Ducklow *et al.* (1980) conducted a research using V. parahaemolyticus as a model of pathogen and biological control of schistosome vector snail, *Biomphalaria glabrata*, in Puerto Rico. Rivera *et al.* (1988) collected environmental samples from estuaries, mangroves and beaches along the east coast of Puerto Rico focusing on the presence of *V. vulnificus* and *V. parahaemolitycus*, including the detection and identification of strains, while

their research did not follow the Bacteriological Analytical Manual (BAM) method. The samples were filtered throught a 0.45µm membrane filters incubated in Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar. To determine pathogenicity, mice were injected an inoculum of *V. vulnificus,* while the virulence of *V. parahaemolyticus* was determined by the Kanagawa test. These studies demonstrated a positive correlation between high levels of fecal coliforms and the density of *V. parahaemolyticus* in the water column using a one factor analysis of variance (ANOVA). These researches are the first scientific projects published in a peer review journal about species of *Vibrio* in Puerto Rico.

Toro Martinez (1989) performed a study about the effects of two methods for microbial purification samples from bivalves, specifically in *Phacoides pectinatus* (*Lucina pectinata*). This investigation compared the depuration and relocation methods of bivalve purification. Bacteriological analyses of the samples for the presence of *Salmonella* and *Vibrio* were performed according to the procedures described in the BAM. Although *Vibrio* were not detected in this study, it was determined that the depuration process is more effective than relocation as a mean of reducing the microbial load in samples from bivalves.

Pérez Gónzalez (1992) performed a research regarding the relationship between the Vibrionaceae and the process of depuration in the oyster *Crassostrea rhizophorae*. The methodology of this research was based on oysters being subjected to a depuration process for five days, following microbial monitoring. This research concluded that *Vibrio parahaemolyticus* could be isolated five days after depuration, indicating that this species be more resistant to this process than *V. vulnificus* which could only be isolated until three days after starting the process. Fontánez Barris (2005) evaluated the microbiological profiles of *Phacoides pectinatus* and *Crassostrea rhizophorae*. Density of *Vibrio* in the clams and oysters were found to range between $< 0.48$ to 5.01 $\log_{10}$ cfu/g.

# CHAPTER 2

## Detection and Identification of *Vibrio parahaemolyticus* strains associated with the clam *Phacoides pectinatus* and the oyster *Crassostrea rhizophorae*

## 2.1 Introduction

Vibriosis is a leading cause of seafood-borne illnesses in the United States and typically is associated with the consumption of raw or partially cooked bivalves, such as clams and oysters. *Vibrio parahaemolyticus* causes a seafood- associated bacterial gastroenteritis that has increased globally during the last decade. Some strains of this halophilic bacterium are strictly environmental with non-pathogenic forms. However, certain strains are pathogenic to humans and could cause gastroenteritis, septicemia and less commonly extra intestinal infection such as wound infections (Wang *et al*., 2015). As a joint effort of the CDC and the FDA, standard tests and procedures were established to reduce the annual incidence of cases. For the isolation and detection of *Vibrio parahaemolyticus*, the enrichment on alkaline peptone water, the selection of *Vibrio* on Thiosulfate Citrate Bile Salts Sucrose (TCBS) and differentiation of species based on growth in different concentrations of NaCl were performed as standardized test. *Vibrio parahaemolyticus* is the species with most isolates in cases of infection associated to the genus *Vibrio* in the United States (CDC, 2016).

## 2.2 Methodology

The detection and identification of presumptive *Vibrio parahaemolyticus* strains were performed in 6 stages, which included: sample preparation, enrichment, selective isolation, purification, biochemical tests and, finally, determination of the level of pathogenicity. The standard procedures for the detection of pathogenic *Vibrio spp.* were followed those described in the FDA Bacteriological Analytical Manual (BAM) with some modifications.

### 2.2.1 Protocols and Strains

All the manipulations in the laboratory were performed in a Bacteriological Safety Level 2 Hood using aseptic techniques. *Vibrio parahaemolyticus* O4:K12 strain 48057 (NR-21990) ATCC® was used as positive control strain in all procedures performed in the laboratory. This strain was supplied by the Biodefense and Emerging Infections Research Resources Repository (BEI Resources) from the National Institute of Allergy and Infectious Diseases (NIAID).

### 2.2.2 Sample preparation

Samples were collected from the roots of red mangrove trees and the seafloor in Boquerón, Cabo Rojo, PR (18°01'39.8"N 67°11'00.2"W). Samples were collected every 3 months throughout a year (specifically in March, June, September, and December) to characterize the effects of water temperature changes on the extant *Vibrio* populations. Each sample consisted of two dozen of clams and two dozens of oysters. These were transported to the laboratory in a Whirl-Pak® bag without ice at 28ºC and processed within 3 hours of their collection. The salinity and temperature data were obtained from the National Oceanic and Atmospheric Administration (NOAA) National Weather Services (http://graphical.weather.gov/sectors/puertoricoLoop.php#tabs), NASA's OceanColor Web (http://oceancolor.gsfc.nasa.gov/cms/) and Caribbean Coastal Ocean Observing Systems (http://www.caricoos.org/drupal/).

### 2.2.3 Enrichment, isolation and enumeration of *Vibrio parahaemolyticus*

For the enrichment of the samples, the FDA has recommended Alkaline Peptone Water [(APW; 10.0g Peptone (Sigma-Aldrich®) and 10.0g NaCl (Sigma-Aldrich®) in 1000mL of distilled water)] for all *Vibrio* species because it has a pH level between 8.5 - 9, and more than the minimum concentration of NaCl required (0.5% NaCl). *Vibrio* species grow best under alkaline conditions. Each sample of twelve shellfish, clams or oysters, was pooled and blended in a stomacher (Tekmar Stomacher Lab Blender 400) for 90 seconds. For each sample, 50 grams were mixed in 450 mL of Phosphate Buffered Saline (PBS), and serially diluted from $10^{-1}$ to $10^{-7}$. Three replicates (1mL) for each serial dilutions were transferred into 10mL of APW and incubated overnight at 35 ± 2ºC. After incubation, a 3 mm loopfull from the top 1cm of the APW tubes that showed turbidity was streaked on Thiosulfate Citrate Bile Salts Sucrose (TCBS, Criterion™) Agar, and subsequently incubated at 35 ± 2ºC overnight. TCBS is a highly selective differential diagnostic medium with sucrose/bromothymol blue, where *Vibrio parahaemolyticus* colonies would be typically 2-3 mm in diameter, round, opaque, and green or bluish.

Presumptive (green) *V. parahaemolyticus* colonies obtained from TCBS agar were transferred to Tryptone Broth (Teknova®) in different concentrations of NaCl: $T_1N_0$, $T_1N_3$, $T_1N_6$, $T_1N_8$, $T_1N_{10}$ (where $T_n$ is the % of tryptone and $N_n$ is the % of NaCl). Tryptone Broth consisted of 10.0g tryptone and 0, 10, 30, 60, 80 or 100g NaCl in 1000mL of distilled water. All isolates were

tested for the presence of urease in 3% NaCl using urease broth (Difco®) at 35 ± 2ºC, and subsequently propagated in Tryptic Soy Agar (TSA, Difco®) or Tryptone Agar plates supplemented with 3% of NaCl and incubated overnight at 35 ± 2ºC. The TSA cultures were used as inocula for the morphological and other physiological tests.

### 2.2.4 Kanagawa phenomenon

For the differentiation of *tdh* and non-*tdh* producing strains, the test for Kanagawa phenomenon was performed. *Vibrio parahaemolyticus* isolates were inoculated on 5% rabbot red blood cells Wagatsuma medium (Himedia®) incubated at 37ºC for 24 hours, and screened for the presence of a hemolytic zone around colonies. Isolates producing a clear hemolytic zone (β-hemolysis) were identified as Kanagawa phenomenon positive, those without hemolytic zone were identified as Kanagawa phenomenon negative.

### 2.2.5 Biofilm production

Biofilm production in *V. parahaemolyticus* isolated strains was detected using a Microbial Biotechnology and Bioprospecting Laboratory modified protocol version of the Microtiter Dish Formation Assay of George A O'Toole (2011). The isolated strains were inoculated on $T_1N_3$ and incubated at 37ºC for 24 hours. After incubation, 2µL of the bacterial growth was inoculated in a well of a ELISA plate with 150µL of $T_1N_3$. This procedure was performed in triplicate with each isolate. Then, the ELISA plate was covered and incubated at 37ºC for 24 hours. For biofilm staining, the medium was discarded and the plate was washed twice with water. Then, 200µL of 0.1% crystal violet was added to each inoculated microtiter well and incubated for 15min. The plate was rinsed 4 times with water and let dry for 1hr. The biofilm production was evaluated by scoring cell aggregates 4- times in the bottom or walls of the wells.

## 2.3 Results

### 2.3.1 Sampling site and physical-chemical parameters using NOAA Software

The physical-chemical parameters for the four sampling are documented in the Table 2.1. The figure 2.1 illustrates the location of the Boquerón Bay in Cabo Rojo, PR, where clams and oyster samples were collected.

**Table 2.1** Temperature and Salinity at Boquerón Bay in different seasons.

| Sampling | Sample Collection Date | Temperature (ºC) | Salinity (psu) |
|---|---|---|---|
| **First** | 12/3/2014 | 28 | 34.5 |
| **Second** | 3/28/2015 | 27 | 35 |
| **Third** | 7/4/2015 | 28 | 35 |
| **Fourth** | 10/2/2015 | 30 | 34.5 |


**Figure 2.1** Geographical location of bivalves sampling site (htttp://maps.google.com/). Yellow star in the map represents the sampling site and the red star represents the capital of Puerto Rico.

### 2.3.2 Identification and Isolation of *Vibrio parahaemolyticus* using standardized methods described by the BAM.

After four seasonal sampling, a total of 58 presumptive *Vibrio parahaemolyticus* strains were isolated6 20 from clams and 38 from oysters. *Vibrio parahaemolyticus* colonies shows a typically 2-3mm in diameter, round, opaque and green color. The Gram stain shows a Gram-negative rod. Figure 2.2 illustrates a representation of presumptive *V. parahaemolyticus* isolated and Gram staining. Differentiation of species based on growth at different NaCl concentrations were performed, and all isolates were able to grow at 3%, 6% and 8% of NaCl (Figure 2.3A), while 64% of the isolates were capable of growing also at 10% of NaCl (Figure 2.3B). Only one strain was positive for the production of urease as show in the Figure 2.4.

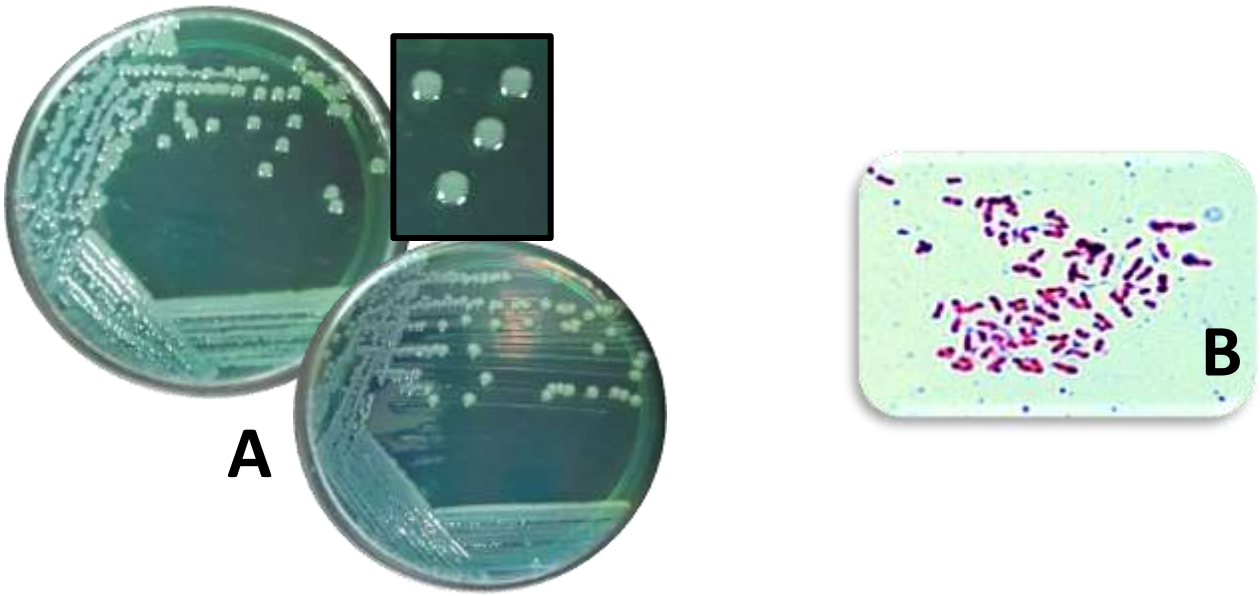


**Figure 2.2** Colonial growth of *Vibrio parahaemolyticus* isolated strains on TCBS (A), and Gram stain (B).

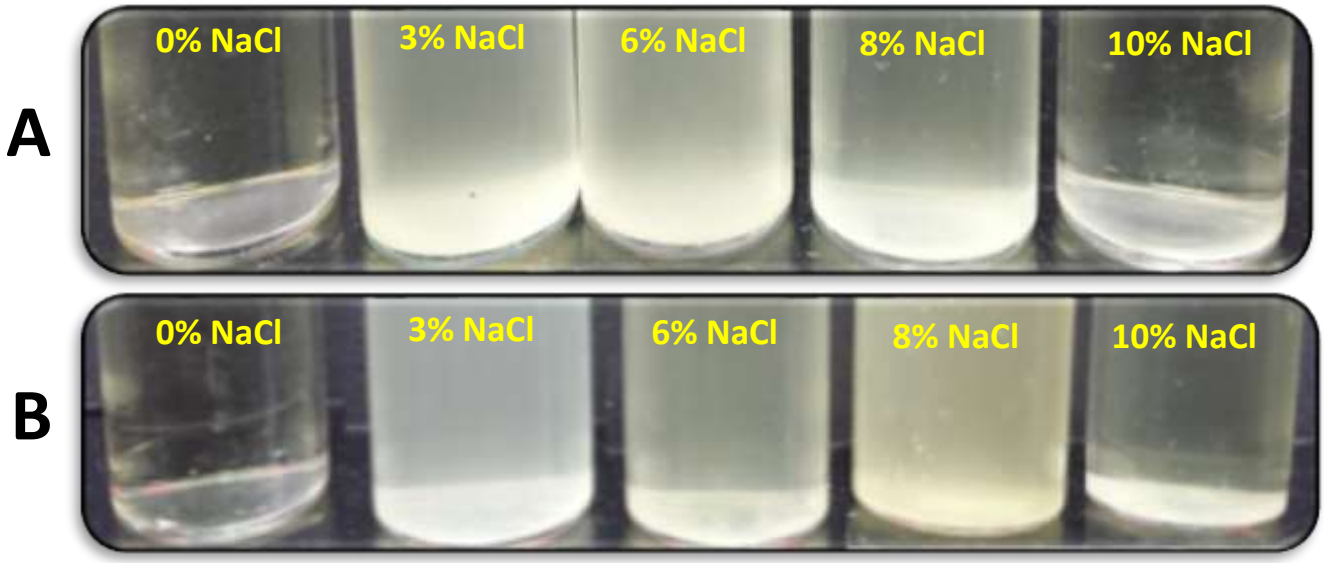


**Figure 2.3** Growth of *Vibrio parahaemolyticus* isolated strains in tryptone broth at different NaCl concentrations. All 58 isolates grew on 3%, 6% and 8% of NaCl (Figure A), but only 37 isolates were able to grow also at 10% of NaCl (Figure B).

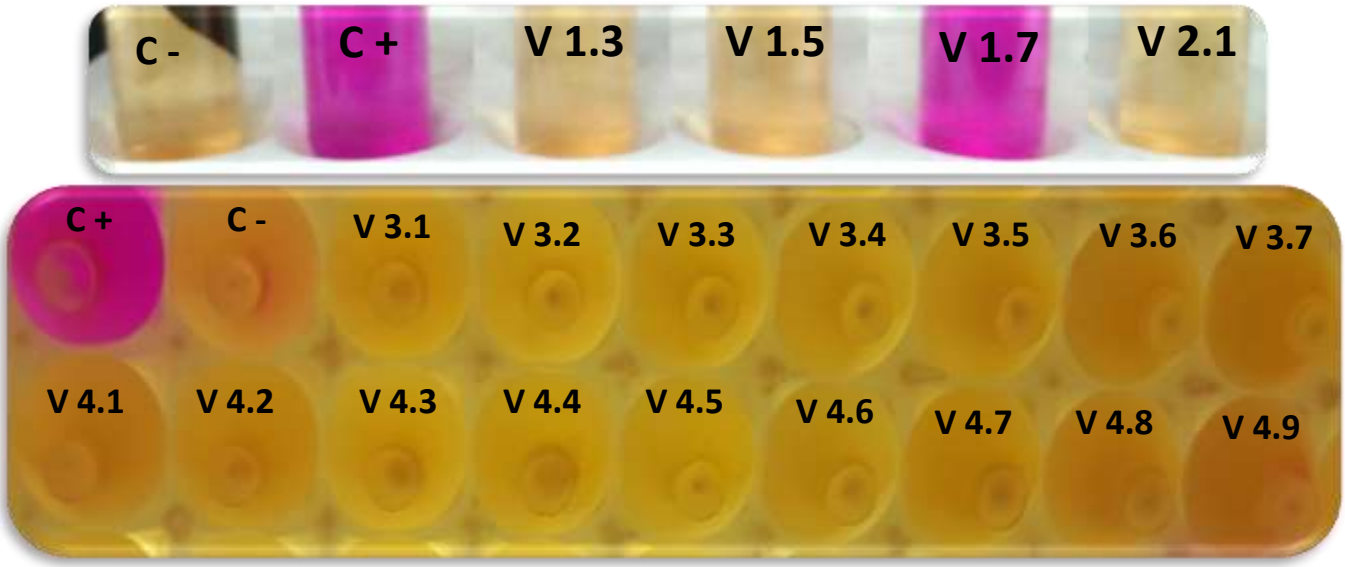


**Figure 2.4** Urease tests. The label of each isolates indicates V as *V. parahaemolyticus* and the unique number in the database.

### 2.3.3 Kanagawa phenomenon

For the preparation of the Wagatsuma agar a rabbit blood was used within 6 hours of drawing. The characteristics of beta-hemolysis were observed in only 11 presumptive hemolytic isolates as show in Figure 2.5, where 3 were from clams and 8 from oysters.

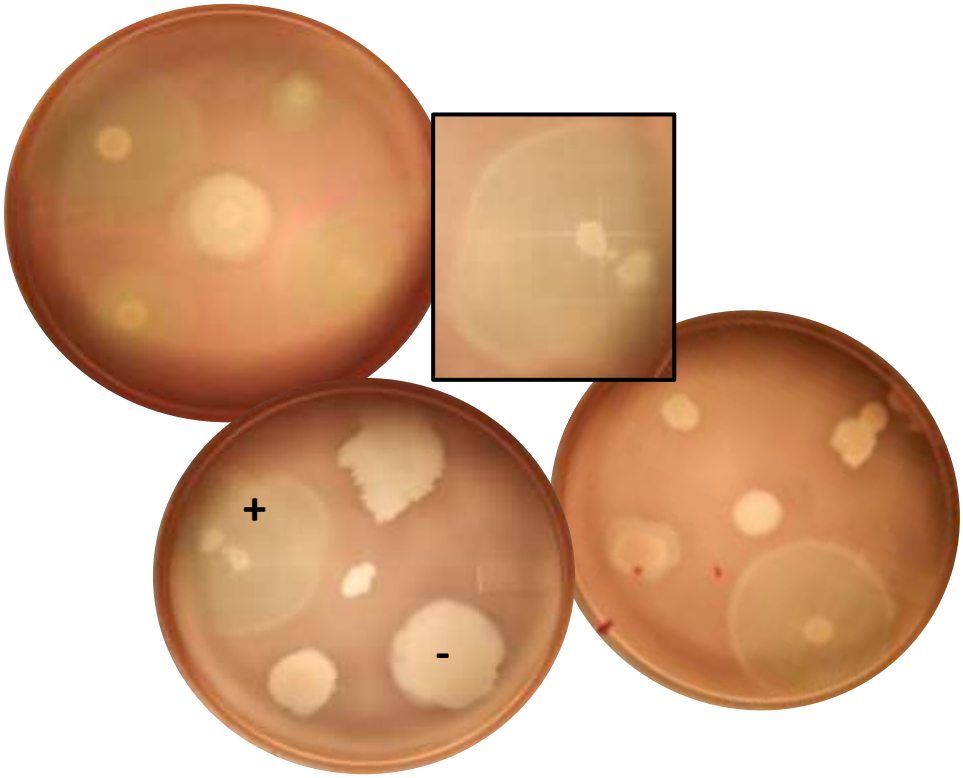


**Figure 2.5** Presumptive hemolytic analysis of growth on Wagatsuma agar by the *Vibrio parahaemolyticus* isolated strains. Positive symbol indicates hemolytic activity and negative symbol a negative result.

### 2.3.4 Biofilm Test

Biofilm production was observed in 6 (1 from clams and 5 from oysters) of 58 isolates by staining with 0.1% crystal violet.

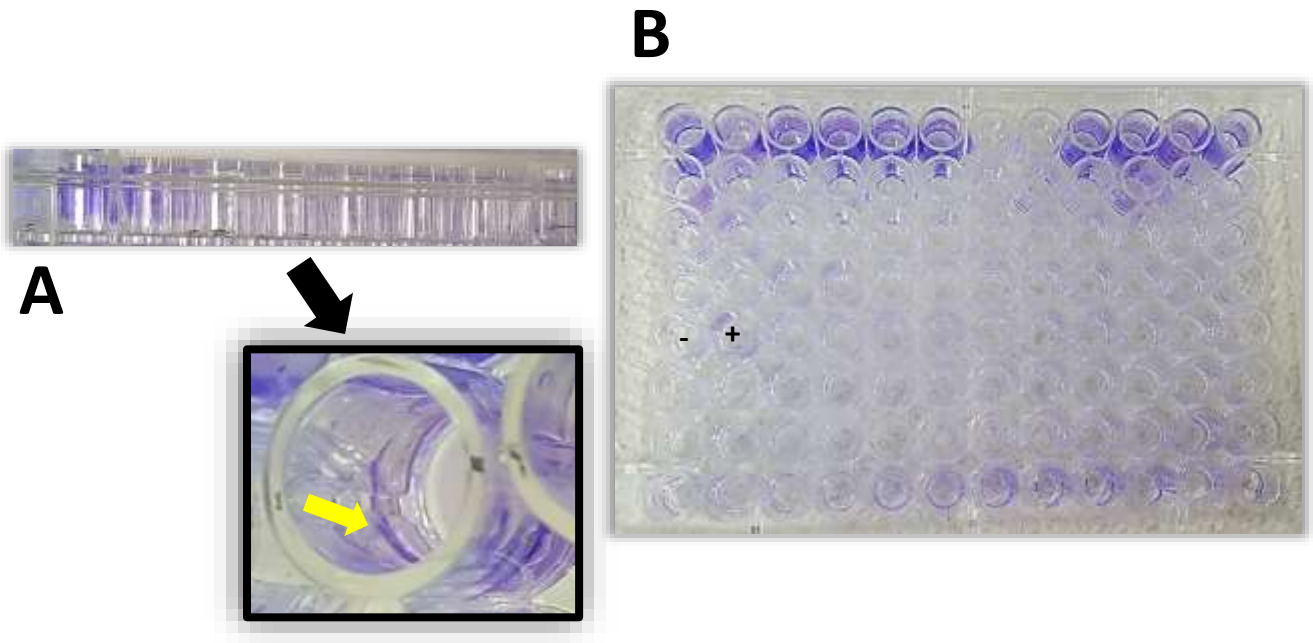


**Figure 2.6** Staining of the biofilm produced by *V. parahaemolyticus* isolated strains. Panel A: Biofilm stain of *V. parahaemolyticus* isolates. The yellow arrow indicates the biofilm production by a *V. parahaemolyticus* isolated strain. Panel B: + = positive control ; - = negative control (*E.coli*).

## 2.4 Discussion of Results

This stage of the study consisted of the detection and identification of *V. parahaemolyticus* in bivalves (clams and oysters) from the southwest coast of Puerto Rico using standard tests and procedures. Among environmental factors, the seawater temperature and salinity are the most important factors that govern the temporal and spatial distribution of *V. parahaemolyticus* (Martinez-Urtaza *et al*., 2008; Zimmerman *et al*., 2007; Sobrinho *et al*., 2010). Previous studies have proven that this species was detected in seawater when water temperature is $\geq$ 15ºC. A study of *V. parahaemolyticus* occurrence in oyster-growing environments at Oregon (USA) found a positive correlation between *V. parahaemolyticus* in seawater and higher water temperature (Duan and Su, 2005). However, in another study conducted in Spain, the salinity was the primary factor influencing the distribution of *V. parahaemolyticus* and the seawater temperature has a secondary effect. Only the temperature could modulate the levels of *V. parahaemolyticus* when the combined effects of low salinity and warm temperature are present (Martinez-Urtaza *et al*., 2008). In Puerto Rico, the seawater temperature fluctuates between 26 to 29ºC (https://www.nodc.noaa.gov); an optimal range for the proliferation of this bacterium. In this study is probably that the *V. parahaemolyticus* populations are exposed to the influence of temperature over the entire year. The sampling site has this characteristic as documented on the Table 2.1.

*Vibrio parahaemolyticus* is a halophilic foodborne pathogen that has been commonly isolated from seafood, such as oysters, scallops, octopus, shrimp, clams, crabs, sardines and others organisms (Kaysner *et al*., 1990; Wong, 2000; Su and Liu, 2007). In this study a total of 58 presumptive *V. parahaemolyticus* was detected in oyster and clams from the southwest coast of Puerto Rico using the standard procedures set forth by the FDA Bacteriological Analytical Manual for the isolation and identification of *Vibrio spp*. All isolates presented the microscopic characteristic of the species: Gram negative rods (Figure 2.2B). The BAM methods are recommended for official analysis but there are not representative of the latest technology or optimal methodology for detection. The US FDA manual recommends the use of TCBS as selective-differential medium to isolate *V. parahaemolyticus*. In TCBS, all presumptive *V. parahaemolyticus* strains present green or green- blue colonies of 2 to 3mm of diameter, as this bacterium does not ferment the sucrose present in the selective medium. The inability of *V. parahaemolyticus* isolates to ferment sucrose is a key differential characteristic, but, TCBS

medium may be insuffient to differentiate among other *Vibrio* species that do not ferment sucrose. Although, other alternatives have been developed to address this problem; for example, CHROMagar™ *Vibrio*. This medium contains colorimetric substrate for β-galactosidase to differentiate *V. parahaemolyticus* from other closely related bacteria, such as *V. vulnificus* and *V. mimicus*. Also, in this study was observed the presence of yellow colonies in larger numbers on TCBS agar from both bivalves. These yellow colonies can be other presumptive vibrios with the ability to ferment sucrose, such as *Vibrio cholerae*, *V. alginolyticus*, *V. fluvialis*, and *V. metschnikovii*.

The total number of isolates obtained from clams and oyster differs between bivalves in 47%; being the oyster, the bivalve with more isolated strains. This difference may be caused by the ecosystem that each bivalve inhabits. The oyster *Crassostrea rhizophorae* is always associated with the mangrove roots. Various studies suggest that the nitrogen-fixing *Vibrio spp*. might be involved in an unknown potential interaction with the mangrove and *Vibrio* species are the most abundant bacteria detected in mangrove rhizosphere sample (Criminger *et al*., 2007; Gomes *et al*., 2010; Rameshkumar *et al*., 2009). Also, it is possible that the filtration rate of oysters, under sampling conditions, was closer to optimal when compared to clams, which would contribute with consequent accumulation of bacteria.

To confirm the presumptive green colonies as *V. parahaemolyticus*, the salt-tolerance test as used to differentiate between sucrose non-fermenting *Vibrio*. In this study, a total of 21 (36%) presumptive *V. parahaemolyticus* grew on 3%, 6% and 8% of NaCl in 1% tryptone broth; meanwhile, 37 (64%) were also able to grow in 10% of NaCl. Due to the strict halophilic nature of *V. parahaemolyticus*, this pathogen requires a minimum of 0.5% NaCl for growth, but it can grow in media containing up to 10.5% NaCl (Naughton *et al.*, 2009; Ongagna and Boyd, 2013). The optimal growth of this bacterium occurs at 3% NaCl. *Vibrio parahaemolyticus* and other proteo-bacteria can respond to osmotic stress with a short-term response resulting in the accumulation of potassium (K) or in long-term strategy with the synthesis and/or accumulation of compatible solutes that can be amassed in high concentrations without disturbing vital cellular functions (da Costa *et al*., 1998). The biosynthesis systems of ectoine and glycine betaine are contained in the *V. parahaemolyticus* genome and these substances act as compatible solutes (Ongagna and Boyd, 2013).

The virulence of *V. parahaemolyticus* has been associated with the Kanagawa phenomenon (KP), which involves the production of beta-hemolysis on Wagatsuma agar. In the past, the KP was an indicator for the identification of pathogenic strains of *V. parahaemolyticus*,because have the strong correlation with the presence of *tdh* gene (Zhang and Austin, 2005; Ono *et al*., 2006). Now, it is recommended to use DNA probe methods to determine potential virulence of *V. parahaemolyticus*. A total of 11 presumptive *Vibrio parahaemolyticus* strains were identified as potential pathogenic ones using Wagatsuma Agar. These presumptive pathogenic strains may encode another pathogenic gene that has not been considered in this study, for example T3SS2α. The T3SS2α codes for the type III secretion systems, which contribute to pathogenicity (Noriea *et al*., 2010). Another virulence trait that is associated with *V. parahaemolyticus* pathogenic strains is the ability to produce urease. Urease production is highly correlated with the presence of *trh* gene, but a poor evidence exists on the function of urea in this bacterium (Suthienkul *et al*., 1995). In the present study, only one *V. parahaemolyticus* strain resulted positive for the urea production.

Some strains of *V. parahaemolyticus* can form biofilms on seafood (Rajkowski *et al*., 2009). Biofilms are assemblies of microorganisms on or in biotic or abiotic surfaces, characterized by interactions between different populations. *Vibrio parahaemolyticus* can produce distinct types of adherence factors that allowed the bacterium to adhere to the surface and initiate the biofilm formation (Donlan, 2002). Han *et al*. (2016) suggested that low temperatures (4-10ºC) may decrease biofilm formation, while the increment of temperatures ranging from 15-37ºC enhances biofilm formation, virulence, and quorum sensing of *V. parahaemolyticus* on seafood. In the present study, only 6 isolated strains were able to form biofilm. One of these strains also is able to produce a beta- hemolysis in Wagatsuma agar and other also resulted positive for urea production.

# CHAPTER 3

## Molecular Characterization of *Vibrio parahaemolyticus* strains isolated from the clam *Phacoides pectinatus* and the oyster *Crassostrea rhizophorae*

## 3.1 Introduction

In the past, the traditional culture- dependent methods were the gold standard for the identification of the foodborne pathogens. However, in the last 25 years, molecular biology has developed techniques which are more efficient at rapidly confirming the identification of pathogens as a result of the demand for rapid results. Molecular techniques, such as PCR, Multiplex-PCR, RFLP, RAPD, PFGE and NGS, make possible the detection of low numbers of pathogens in a time much shorter than conventional methods (Lauri and Mariani, 2009). DNA-based detection methods can be specific and able to detect or differentiate at the species level. In more specific cases, nucleic acid- based detection methods can target specific serotypes or genotypes based on identify virulence genes or other marker genes. These molecular methods have replaced or supplemented culture detection for most bacterial foodborne pathogens (Ceuppens *et al*., 2014; Smith *et al*., 2000).

For the identification and characterization of *Vibrio parahaemolyticus*, both the cultivable and molecular methods supplement each other and are described in the Bacteriological Analytical Manual (BAM) of the FDA. A molecular method for identification of virulent *V. parahaemolyticus* strain is the multiplex-PCR for the amplification of the pathogenic genes, *tdh* and *trh* genes, associated to clinical isolates from patients with diarrhea related to infection with *V. parahemolyticus*. Another molecular typing technique is PFGE, which was used to evaluate the genetic diversity among strains.

Advances in molecular biology are rapidly evolving, nowadays, research and surveillance of foodborne outbreak are moving to the use of whole genome sequencing as a routine tool.

## 3.2 Methodology

### 3.2.1 Genomic DNA Extraction

For the genomic DNA extraction, 1,500µl of an overnight grown culture of each purified *V. parahaemolyticus* was collected by centrifugation at maximum speed and the cell pellets were resuspended in 200µl of Lysis buffer (40mM Tris pH 7.8, 20mM Sodium-acetate, 1.0mM EDTA and 1% SDS) combined with 66µl of the 5M NaCl, followed by vigorous mixing by pipetting. The lysed cells were centrifuged for 10 minutes at 13,000RCF. The supernatant was transferred to a sterile microtube of 1.5ml. Subsequently, one volume of chloroform was added to clean the DNA

(removing lipids and proteins). The aqueous phase was transferred to a sterile 1.5ml microtube. The DNA in the solution was precipitated by adding two volumes of cold absolute ethanol for 30mins at -20ºC. After centrifugation (5min/13,000rpm) and washing the DNA pelleted with 70% ethanol, the resulting DNA pellet was air dried, resuspended in 50µl of 1X TE buffer, and stored at -20ºC.

### 3.2.2 Multiplex PCR identification of *Vibrio parahaemolyticus*:

Isolated *Vibrio parahaemolyticus* was confirmed by a Multiplex PCR method to detect the presence of the *tlh*, *tdh*, and *trh* genes (Bej *et al.*, 1999). The master mix consisted of 1µM of each of the primers for *tlh*, *tdh*, *trh*, 200µM of each of the dNTP's, 2.5 units of Taq DNA polymerase, 5µL of a 10x PCR reaction buffer (10x buffer consisted of 500mM Tris•Cl, 500mM KCl and 25mM MgCl) and the appropriate volume of deionized sterile water. Another alternative for the PCR reaction is the use of Go Taq® Green Master Mix (Promega Corporation). This master mix is a premixed (ready to use) solution containing derived Taq DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentration for amplification of DNA templates. The final volume of PCR (50µl) consisted of 1µl of purified genomic DNA with a concentration between 50-100ng/µL and the master mix. The multiplex PCR primer sets are shown in Table 1.

All multiplex PCR reactions were carried out in a DNA thermal cycler (T-100™ Thermal cycle, Bio-Rad) using the following temperature-cycling parameters: initial denaturation at 94°C for 3 min followed by 25 cycles of amplification; each cycle consisted of denaturation at 94°C for 1 min, primer annealing at 53.2°C for 1 min, and primer extension at 72°C for 2 min. A final extension step was carried out kept at 72°C for 3 min to allow the extension of the incompletely synthesized DNA (BAM, 2004). The size of the PCR product was confirmed by agarose gel electrophoresis (2% agarose) using a voltage of 70V for 100 min. The gels were stained with ethidium bromide (1µg/ml) for 5 min, destained in distilled water for 15 min and were photographed with a UV photo documentation system (Gel Doc™ $XR^+$ System, Bio Rad®). A PCR reaction containing DNA from *V. parahaemolyticus* O4:K12 strain 48057 as template was included as a positive control.

To differentiate between amplicon size of *tlh* gene and *trh* gene, each amplification was carried out in a single PCR reaction following the same temperature-cycling parameters of the multiplex-PCR reactions.

**Table 3.1** Primer sets used for multiplex PCR assays. From Bet *et al*. (1990).

| Primer ID | Direction | Sequence |
|---|---|---|
| ***tlh* gene** | F | L-TL (5'- AAA GCG GAT TAT GCA GAA GCA CTG- 3') |
| | R | R-TL (5'- GCT ACT TTC TAG CAT TTT CTC TGC – 3') |
| ***trh* gene** | F | TRH-L (5'- TTG GCT TCG ATA TTT TCA GTA TCT -3') |
| | R | TRH-R (5'- CAT AAC AAA CAT ATG CCC ATT TCC G -3') |
| ***tdh* gene** | F | TDH-L (5'- GTA AAG GTC TCT GAC TTT TGG AC-3') |
| | R | TDH-R (5'- TGG AAT AGA ACC TTC ATC TTC ACC-3') |

F= forward; R= reverse

### 3.2.3 Polymerase Chain Reaction (PCR) identification of *Vibrio parahaemolyticus*

Polymerase Chain Reaction was performed with the genomic DNA of the isolates as template in order to partially amplify of the 16S rDNA. This procedure was done to confirm the isolates affiliation to the genus *Vibrio*. The PCR reactions were prepared using the Green Tag Master Mix (Promega) and consisted of Taq DNA Polymerase 2X, Taq Reaction Buffer, 400µM of each dNTP, 3mM $MgCl_2$ and 1pmol bacterial universal primers oligos 27-F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492-R (5'-GGTTACCTTGTTAGGACTT-3')(Weisburg *et al*., 1991). The following cycling parameters were used: initial denaturalization at 95ºC for 3 minutes; followed by 30 cycles of denaturalization at 95ºC for 1 second, annealing at 52ºC for 30 seconds, extension at 72ºC for 1 minute and a final extension at 72ºC for 10 minutes. All amplifications were confirmed by gel electrophoresis (1% agarose, 80V in Tris-EDTA IX buffer). The PCR products were sequenced at McLab facilities (California, USA). The sequence

reads were searched againt GenBank (nucleotide collection) using the BLASTn program (Zhang *et al*., 2000). The phylogenetic analysis was performed using the MEGA6 software (http://www.megasoftware.net/). The distance model used was p-distance and the bootstrap test of phylogeny was calculated for 2000 replicates. The generation of the consensus tree was performed by the Neighbor-Joining method.

### 3.2.4 Molecular typing of *V. parahaemolyticus* using Pulsed Field Gel Electrophoresis (PFGE)

The Standard Operating Procedure PNL06 for PulseNet PFGE was used in this study to validate inter- laboratory comparability of the generated results. For the band pattern comparison of gel images, *Salmonella* serotype Braenderup (H9812) ATCC® BAA-664™ digested with *Xba*I was used as a universal standard strain and marker.

All strains positive for the presence of *tlh*, *tdh* and/or *trh* genes were analyzed using the PFGE molecular method. An overnight culture of the isolates in 3% NaCl TSA was embedded in agarose plugs, lysed and digested with *Sfi*I (50ºC) and *Not*I (37ºC) (New England BioLabs) restriction enzymes during 4 hours. The agarose plugs were prepared with 1.0% SeaKem Gold agarose in Tris-EDTA (TE) buffer (10mM Tris: 1mM EDTA, pH 8.0). Cell suspension from the agar plate was performed in Cell Suspension Buffer (100mM Tris: 100mM EDTA, pH 8.0) and the optical density was adjusted spectrophotometrically to 0.9 (610 nm wavelength). Cells were embedded in 20µL of Proteinase K (20 mg/ml stock) combined with 400µL melted 1% agarose in a labeled 1.5ml sterile microtube and mixed gently by pipetting. Immediately, the mixture was dispensed in plug molds and allowed to solidify at room temperature for 10-15mins.

The cells in agarose plugs were transferred to a 15mL polypropylene screw- cap tube and lysed by adding 5mL of a Proteinase K (0.1mg/ml)/ Cell Lysis Buffer (50mM Tris: 50mM EDTA, pH 8.0 + 1% Sarcosyl) and incubated in an orbital shaker water bath at 54-55ºC for 2 hours with constant and vigorous agitation (150-175 rpm). After lysis, the plugs were washed 3 times for 15min with sterile distilled water at 54-55ºC in an orbital shaker water bath. Final washing step of the agarose plugs was performed using TE Buffer in the same conditions of the previous washes.

After cell lysis, the digestion of the agarose – embedded DNA was performed in order to generate a genomic profile for each strain. The digestion was incubated for 4 hours at the appropriate temperature (*Sfi*I at 50ºC and *Not*I at 37ºC). Then, the restriction enzyme was removed and substituted with 0.5X TBE, followed by a final incubation for 5mins at room temperature.

For the generation of a genomic digestion profile, agarose plugs containing the digested DNA were processed for 18 hours in a CHEF Mapper® XA Pulsed Field Electrophoresis System, using default parameters and the Autoalgorithm mode in for molecular weight to 78kb – 396kb. The autoalgorithm requires that for DNA < 2.5mb, 0.5X TBE at 14ºC, in a 1.0% agarose gel must be used. After the electrophoresis, the agarose gel was removed and stained with ethidium bromide (1µg/ml) for 20mins, destained in distilled water for 90mins (the water was changed every 20mins) and photographed with a UV photo documentation system.

The analysis (comparison and interpretation) of gels from PFGE was performed using BioNumerics™ software in collaboration with the Centers of Diseases Control and Prevention (CDC), Atlanta, GA.

### 3.3 Results

#### 3.3.1 Genomic DNA Extraction

Total genomic DNA of all pure cultures of *V. parahaemolyticus* isolated strains was purified following a chemical extraction. A high molecular weight DNA (> 20Kbp) was obtained as illustrated in the Figure 3.1.

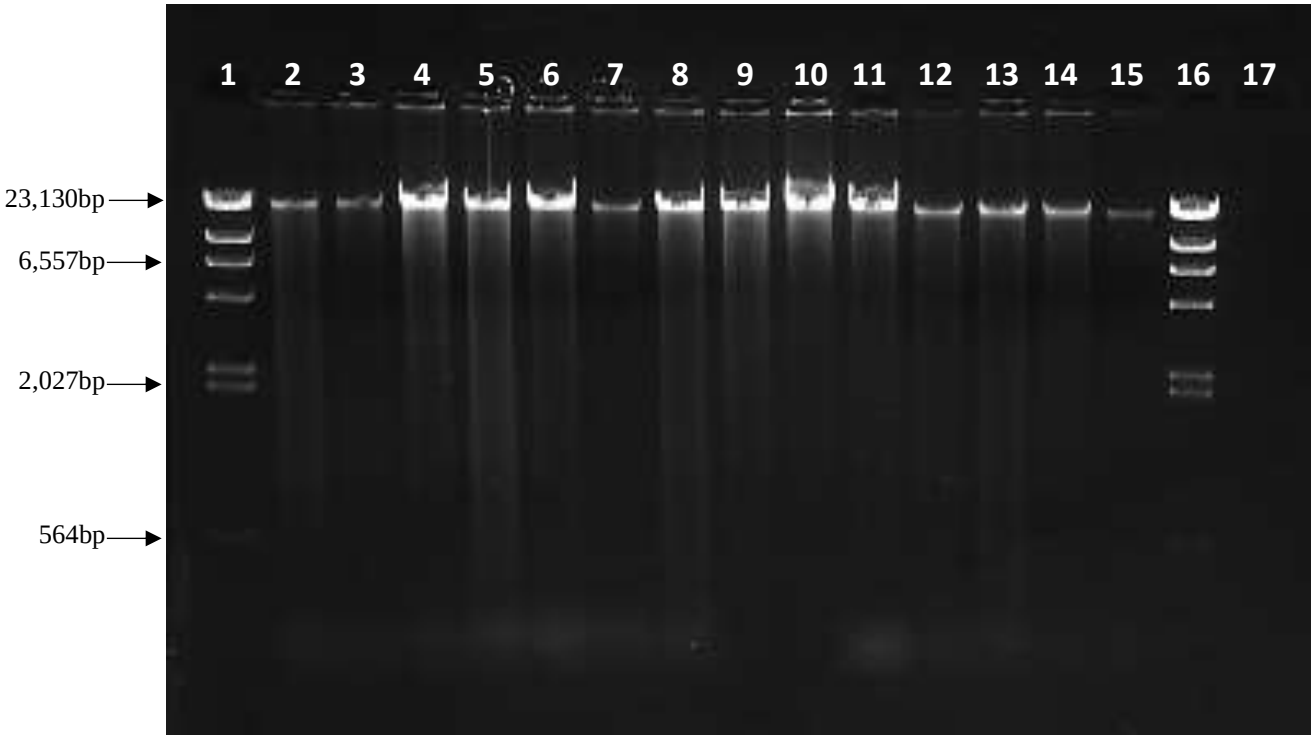


**Figure 3.1** Agarose gel electrophoresis of genomic DNA from *Vibrio parahaemolyticus* strains recovered from oysters and clams samples. Electrophoresis agarose gel run of isolated strains, 1.0% agarose gel run during 65mins at 80V. Lambda/*Hind* III marker in lanes 1 and 16; lanes 2 to 15 contain presumptive *Vibrio parahaemolyticus* strains isolated from oysters and clams.

### 3.3.2 Multiplex PCR identification of *Vibrio parahaemolyticus*

A total of 52 (90%) presumptive *V. parahaemolyticus* isolates presented the 450bp amplicon representative of the species-specific marker, *tlh* gene, confirming the amplified strain as a *Vibrio parahaemolyticus*. As illustrates in Figure 3.2 and 3.5, several isolates that were negative for the presence of the *tlh* gene. However, all of the strains lack the pathogenicity associated markers, *tdh* (270bp) and *trh* (500bp) genes (Figure 3.3 and 3.5). The amplification of all three genes, *tlh*, *tdh* and *trh* only was show in the positive controls.

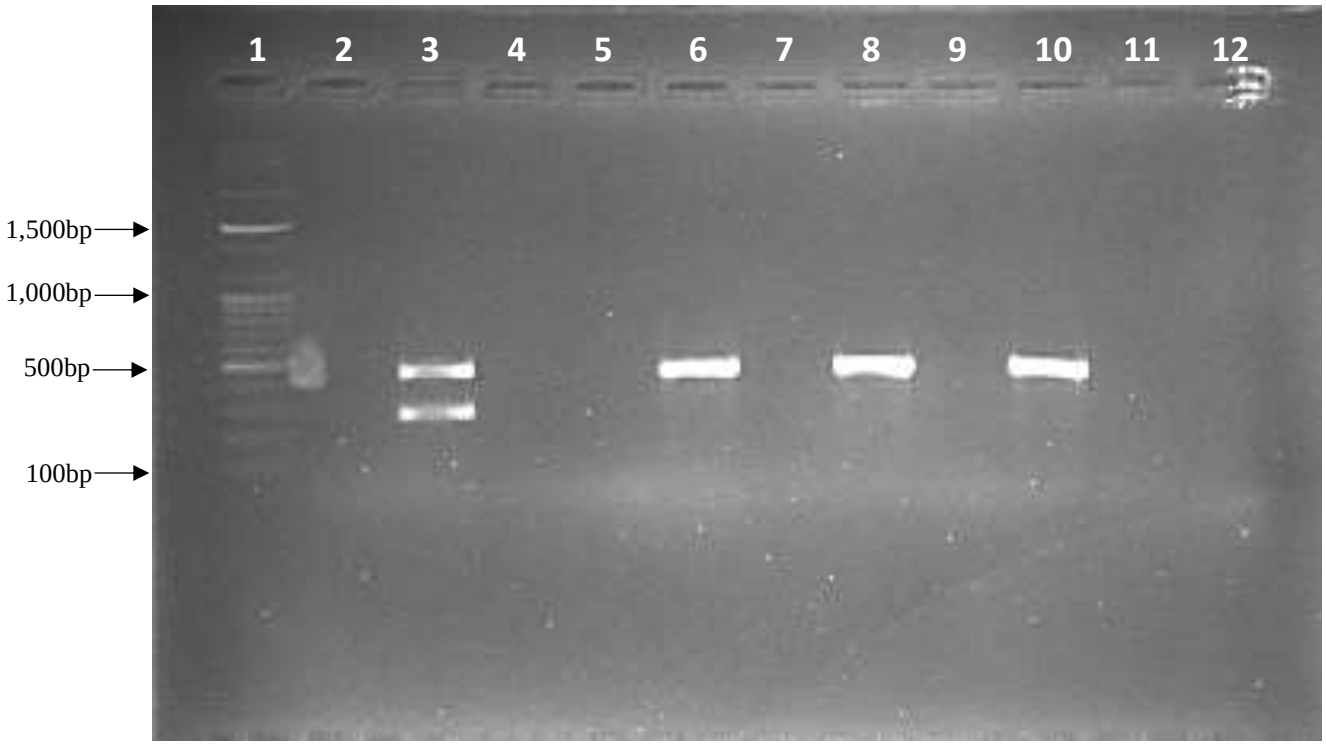


**Figure 3.2** Agarose gel electrophoresis of Multiplex PCR products detected in *Vibrio parahaemolyticus* isolates recovered from oysters and clams samples. Agarose gel electrophoresis run of the isolated strains, 2.0% agarose gel run during 2 hours at 70V. 100bp plus marker, lane 2 negative control, lane 3 *Vibrio parahaemolyticus* O4:K12 48057 as positive control, lanes 4 to 12 contain presumptive *Vibrio parahaemolyticus* strains isolated from oysters and clams.

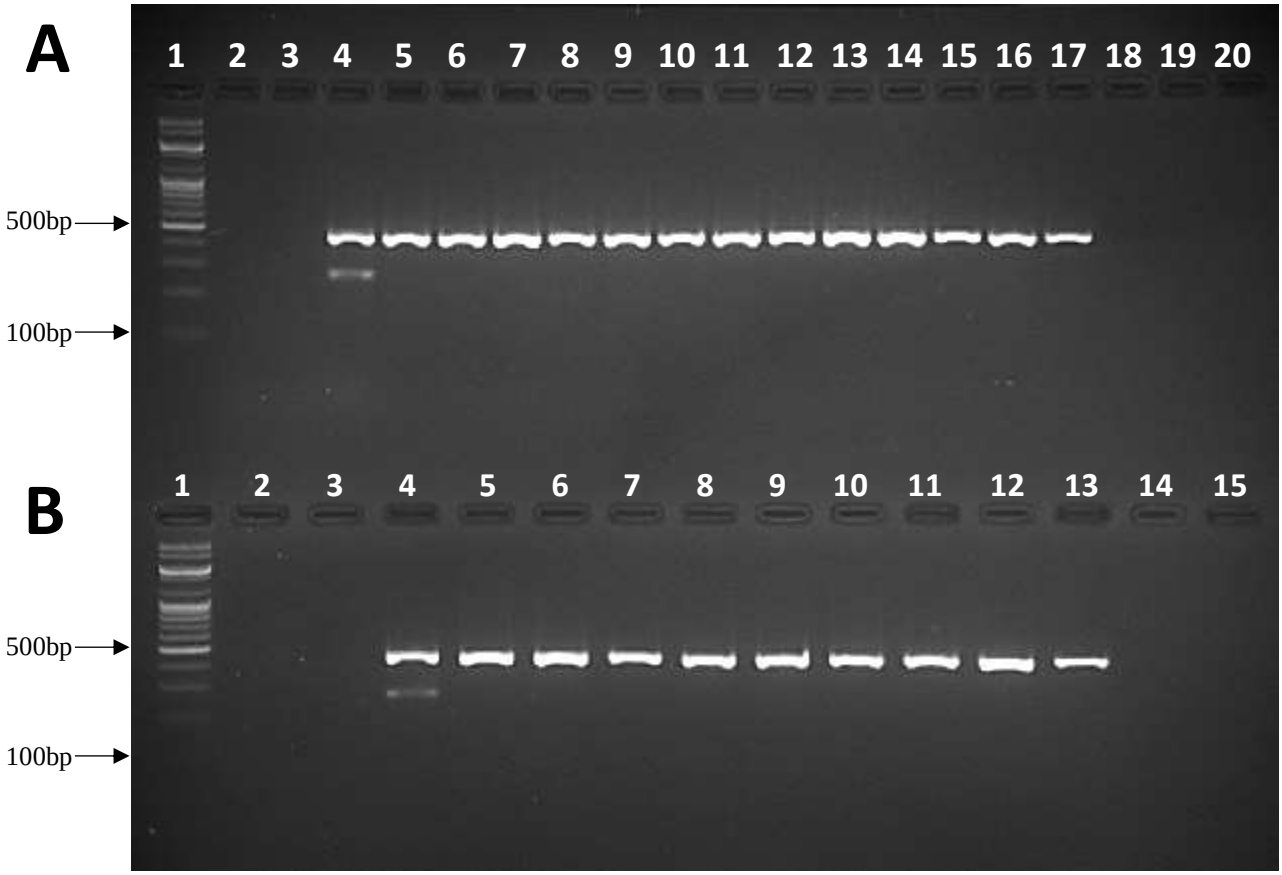


**Figure 3.3** Agarose gel electrophoresis of Multiplex PCR products that lack pathogenicity associated markers. Agarose gel electrophoresis run of isolated strains, 2.0% agarose gel run during 2 hours at 70V. Lane 1: 100bp Plus marker, lane 2 contains PCR mix without DNA as negative control, lane 2 *Vibrio fisheri* DNA as negative control, lane 4 *Vibrio parahaemolyticus* O4:K12 48057 as positive control, and lanes 5 to 17 contain presumptive *Vibrio parahaemolyticus* strains isolated from oysters and clams (Panel A). Lane 1: 100bp Plus marker, lane 2 contain PCR mix without DNA as negative control, lane 2 *Vibrio fisheri* DNA as negative control, lane 4 *Vibrio parahaemolyticus* O4:K12 48057 as positive control, and lanes 5 to 13 contain presumptive *Vibrio parahaemolyticus* strains isolated from oysters and clams (Panel B).

As illustrated in Figure 3.4, the *V. parahaemolyticus* O4:K12 48057 strain used as a positive control exhibited the presence of all three genes, *tlh*, *tdh* and *trh*. This strain was isolated in from a patient with clinical disease in Washington, USA.

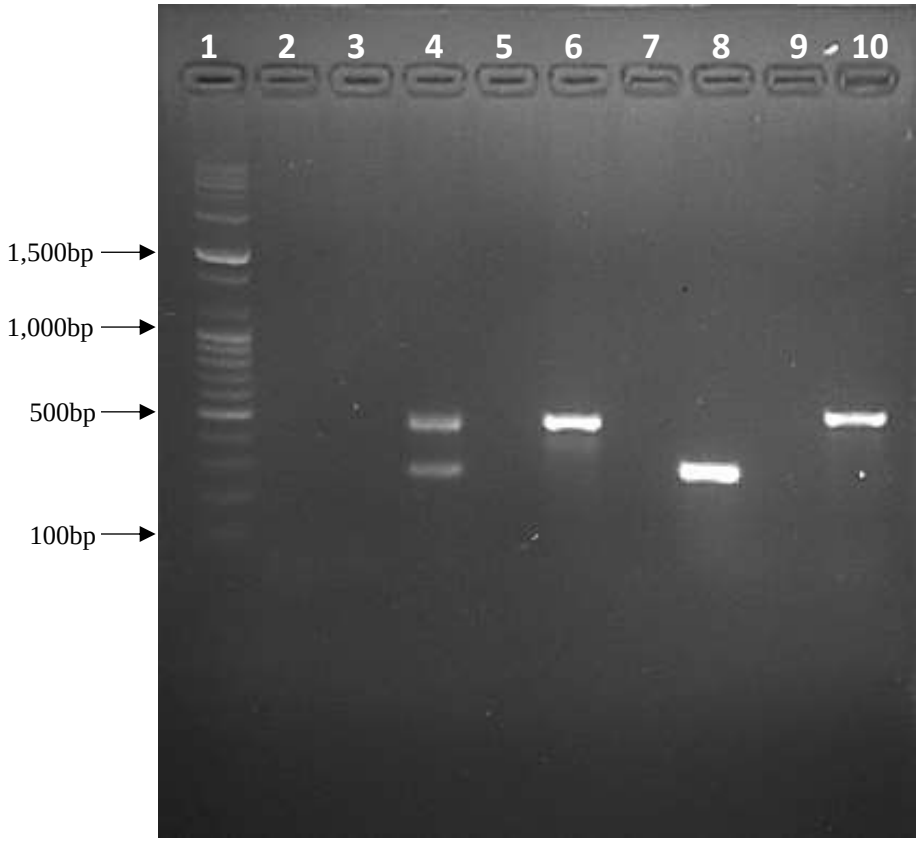


**Figure 3.4** Agarose gel electrophoresis of Multiplex PCR products and PCR products from *Vibrio parahaemolyticus* O4:K12 48057; strain used as a positive control of pathogenicity associated markers. Agarose gel electrophoresis run of isolated strains, 2.0% agarose gel run during 2 hours at 70V. Lane 1: 100bp Plus marker, lanes 2,5,7, and 9 contain PCR mix without DNA as negative control, lane 2 *Vibrio fisheri* DNA as negative control, lane *Vibrio parahaemolyticus* O4:K12 48057 as positive control of multiplex PCR, and lanes 6, 8 and 10 contain *Vibrio parahaemolyticus* O4:K12 48057 as positive control for the amplification of *tlh*, *tdh* and *trh* genes, respectively.

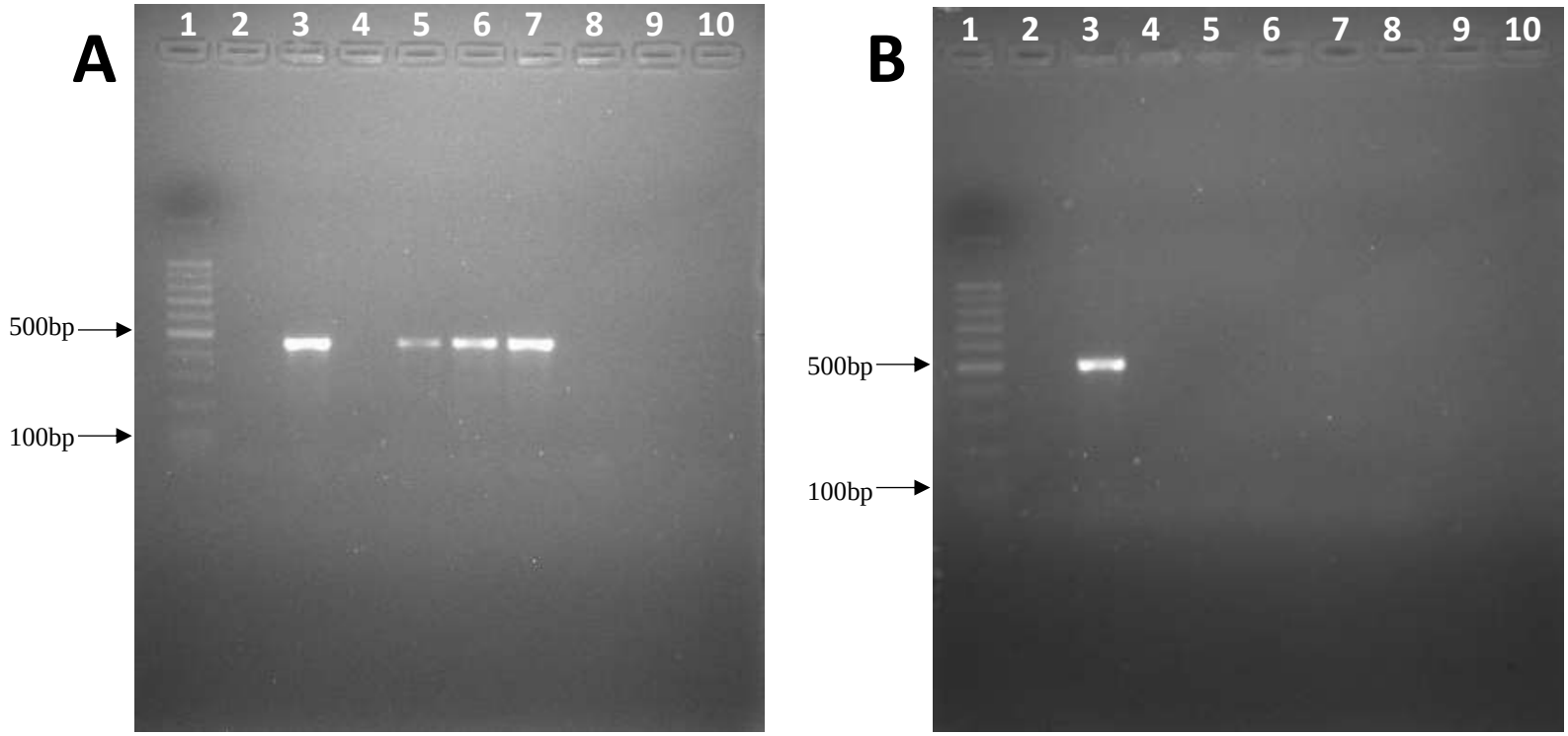


**Figure 3.5** *tlh* and *trh* genes amplification of the presumptive *V. parahaemolyticus* isolated. Lane 1: 100bp Plus marker, lane 2 contains PCR mix without DNA as negative control, lane 3 *Vibrio parahaemolyticus* O4:K12 48057 as positive control, and lanes 4 to 8 contain presumptive *Vibrio parahaemolyticus* strains isolated from oysters and clams (*tlh* gene: Panel A; *trh* gene: Panel B).

### 3.3.3 Polymerase Chain Reaction (PCR) identification of *Vibrio parahaemolyticus*

Amplification of the 16S rDNA was done by PCR with positive amplification of a DNA fragment of approximately 1500bps (Figure 3.6). Sequencing of the 16S rDNA fragment was performed by McLab (California, USA). *In-silico* analysis of the bacteria isolated suggested that all isolates belong to the genus *Vibrio* as show in the Table 3.1. The phylogenetic tree constructed using the 16S rRNA gene from the *V. parahaemolyticus* isolates displayed divergence and splitted the isolates in four different subgroups (Figure 3.7).

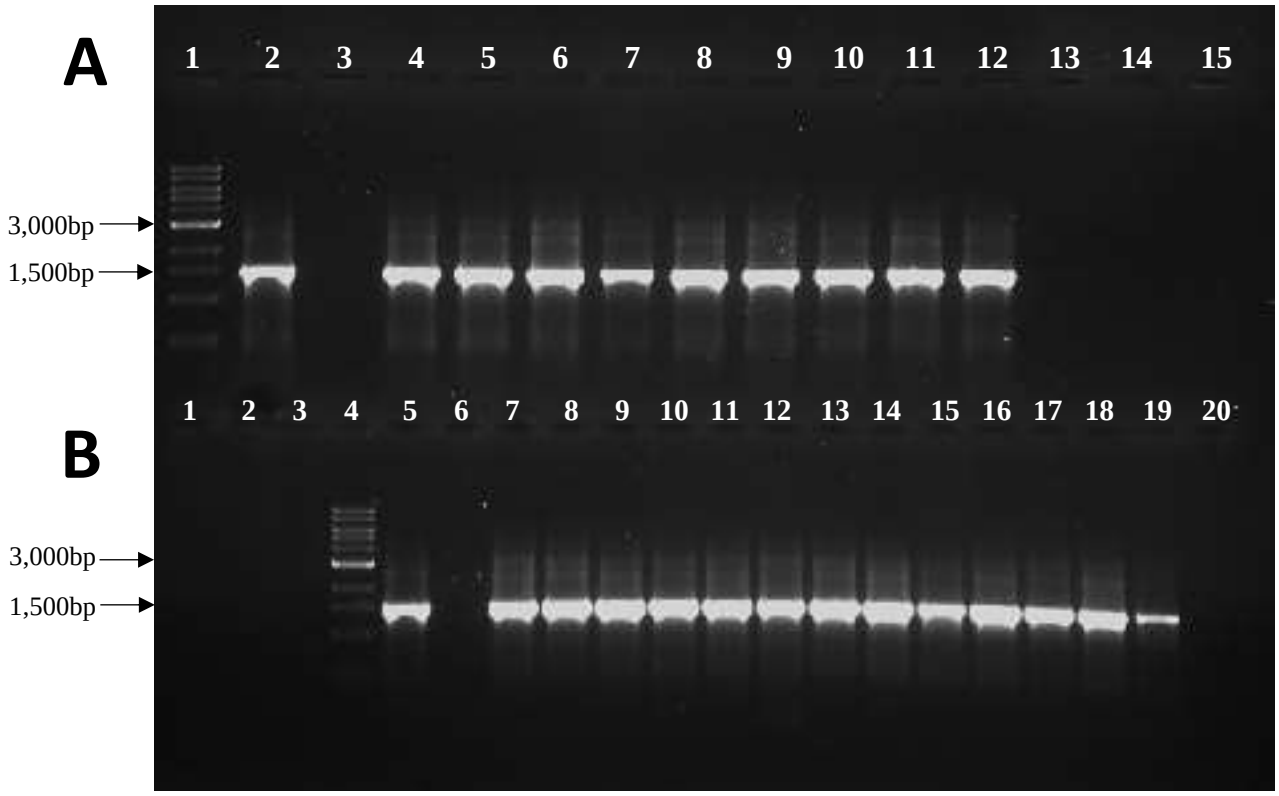


**Figure 3.6** 16S rDNA gene amplification of the presumptive *V. parahaemolyticus* isolated. The molecular marker used was 1Kb ladder (lanes1A, 4B). The positive control (lines 2A, 5b) was a PCR using a *Vibrio parahaemolyticus* O4:K12 48057 genomic DNA. The negative control (lanes 3A, 6B) was the PCR mixture without DNA. Lanes 4 to 12 (Panel A) and lanes 7 to 19 (Panel B) contain presumptive *Vibrio parahaemolyticus* strains isolated from oysters and clams.

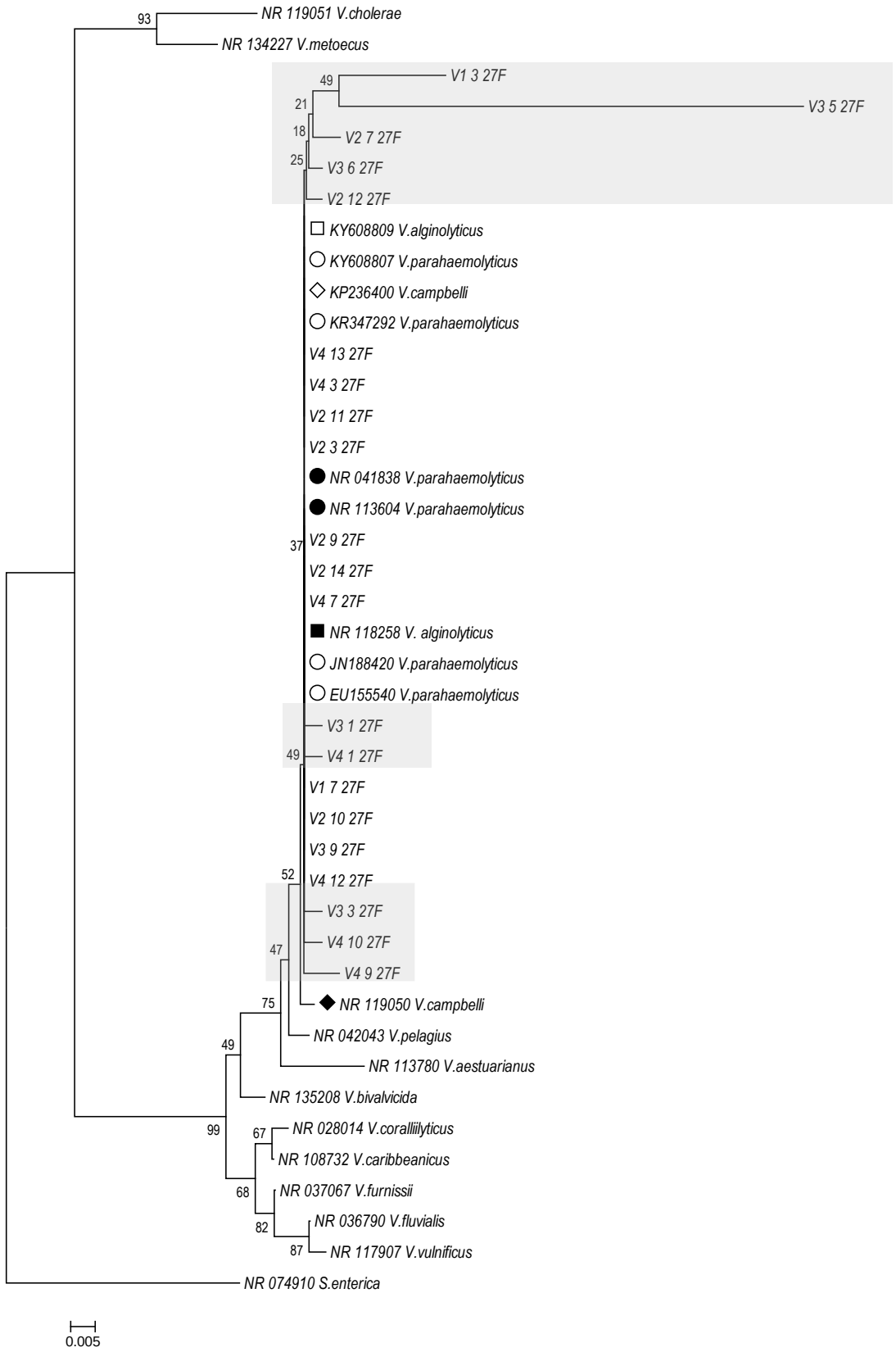


**Figure 3.7** Phylogenetic relationships of *Vibrio parahaemolyticus* isolates**.** The evolutionary history was inferred using the Neighbor-Joining method and the p-distance model. The analysis involved 42 nucleotide sequences. Black and white labels depict the positions of phylotypes representative of type and no-type strains, respectively. All positions in the alignment containing gaps and missing data ("N") were eliminated. There were a total of 273 positions in the final dataset. The analyses were carried out in MEGA6.

**Table 3.2** Results from i*n-silico* analysis of partially sequenced 16S rRNA gene.

| Isolates ID | Suggested Candidates (BLAST) | Query Cover | E-value | Max. Identity | Accession Num. |
|---|---|---|---|---|---|
| **VP 1.3** | *Vibrio alginolyticus* Xmb011 | 93% | 1e-178 | 94% | KT986141.1 |
| | *Vibrio parahaemolyticus* strain FORC 023 | 100% | 2e-176 | 93% | CP012950.1 |
| | *Vibrio* sp. I9 | 100% | 2e-176 | 93% | KR108389.1 |
| **VP 1.7** | *Vibrio parahaemolyticus* strain 1682 ch2 | 99% | 0.0 | 94% | CP019060.1 |
| | *Vibrio parahaemolyticus* strain 1682 ch1 | 99% | 0.0 | 94% | CP019059.1 |
| | *Vibrio parahaemolyticus* strain FORC 018 | 99% | 0.0 | 94% | CP013827.1 |
| **VP 2.3** | *Vibrio sp.* BBT27 | 98% | 0.0 | 92% | FJ981850.1 |
| | *Vibrio sp*. BBT71 | 98% | 0.0 | 91% | FJ981891.1 |
| | *Vibrio parahaemolyticus* strain An 3 | 98% | 0.0 | 91% | FJ386958.1 |
| **VP 2.7** | *Vibrio alginolyticus* strain CH11 | 96% | 4e-178 | 96% | KC210814.1 |
| | *Vibrio sp.* strain HEP1B2 | 95% | 1e-177 | 96% | KY608808.1 |
| | *Vibrio parahaemolyticus* strain BF11 | 95% | 1e-177 | 96% | KU955359.1 |
| **VP 2.9** | *Vibrio parahaemolyticus* strain 1682 ch2 | 99% | 0.0 | 98% | CP019060.1 |
| | *Vibrio parahaemolyticus* strain 1682 ch1 | 99% | 0.0 | 98% | CP019059.1 |
| | *Vibrio parahaemolyticus* strain FORC 018 | 99% | 0.0 | 98% | CP013827.1 |
| **VP 2.10** | *Vibrio parahaemolyticus* isolate VPJR3 | 97% | 0.0 | 92% | DQ991215.1 |
| | *Vibrio parahaemolyticus* strain CHB-5 | 97% | 0.0 | 92% | KR347274.1 |
| | *Vibrio campbellii* strain 0284 | 97% | 0.0 | 92% | KP236400.1 |
| **VP 2.11** | *Vibrio harveyi* strain Lmb041 | 100% | 0.0 | 99% | KT986106.1 |
| | *Vibrio azureus* strain CAIM 1457 | 100% | 0.0 | 99% | JN603238.1 |
| | *Vibrio parahaemolyticus* strain CHB-35 | 99% | 0.0 | 99% | KR347292.1 |
| **VP 2.12** | *Vibrio parahaemolyticus* strain FORC 006 | 99% | 0.0 | 95% | CP009765.1 |
| | Uncultured *Vibrio sp.* clone L.j-7 | 99% | 0.0 | 95% | HM031439.1 |
| | *Vibrio parahaemolyticus* strain J-C1-5 | 99% | 0.0 | 95% | EU652247.1 |
| **VP 2.14** | *Vibrio sp.* strain HEP1B2 | 100% | 0.0 | 99% | KY608808.1 |
| | Vibrio campbelli strain 0284 | 100% | 0.0 | 99% | KP236400.1 |
| | *Vibrio parahaemolyticus* strain HEP1B1 | 100% | 0.0 | 99% | KY608807.1 |
| **VP 3.1** | *Vibrio parahaemolyticus* strain CICC21617 | 99% | 9e-165 | 98% | KJ643938.1 |
| | *Vibrio sp.* CON-A4-1 | 97% | 9e-165 | 98% | EF100859.1 |
| | *Vibrio alginolyticus* strain HEP1B3 | 98% | 1e-163 | 98% | KY608809.1 |
| **VP 3.3** | *Vibrio parahaemolyticus* strain 1682 ch2 | 99% | 0.0 | 99% | CP019060.1 |
| | *Vibrio parahaemolyticus* strain 1682 ch1 | 99% | 0.0 | 99% | CP019059.1 |
| | *Vibrio parahaemolyticus* strain FORC 018 | 99% | 0.0 | 99% | CP013827.1 |
| **VP 3.5** | *Vibrio sp*. Oct07-TCBS-7BB-5 | 81% | 3e-140 | 82% | GQ215077.1 |
| | *Vibrio alginolyticus* strain ZDS-6 | 82% | 1e-129 | 81% | JN188406.1 |
| | *Vibrio parahaemolyticus* strain VPMP34 | 82% | 1e-128 | 81% | JQ663904.1 |
| **VP 3.6** | *Vibrio alginolyticus* strain Xmb006 | 99% | 0.0 | 92% | KT986136.1 |
| | *Vibrio parahaemolyticus* strain S9-891 | 99% | 0.0 | 92% | KC520577.1 |
| | *Vibrionaceae* bacterium | 99% | 0.0 | 92% | FJ178086.1 |
| **VP 3.9** | *Vibrio parahaemolyticus* strain VP01 | 100% | 0.0 | 98% | JN188420.1 |
| | *Vibrio parahaemolyticus* strain MCCB 373 | 100% | 0.0 | 98% | KT982480.1 |
| | *Vibrio parahaemolyticus* strain mee 16 | 100% | 0.0 | 98% | KY565419.1 |

**Table 3.2 (Cont.)** Results from i*n-silico* analysis of partially sequenced 16S rRNA gene.

| Isolates ID | Suggested Candidates (BLAST) | Query Cover | E-Value | Max. Identity | Accession Num. |
|---|---|---|---|---|---|
| **VP 4.1** | *Vibrio sp*. S1194 | 100% | 0.0 | 99% | KM273120.1 |
| | *Vibrio campbelli* strain 0284 | 100% | 0.0 | 99% | KP236400.1 |
| | *Vibrio sp.* M-137-16 | 100% | 0.0 | 99% | KF746900.1 |
| **VP 4.3** | Uncultured *Vibrio* sp. clone C1A01 | 99% | 0.0 | 96% | KP016653.1 |
| | *Vibrio parahaemolyticus* strain NSTH20 | 99% | 0.0 | 97% | KF886631.1 |
| | *Vibrio parahaemolyticus* strain NSTH21 | 99% | 0.0 | 96% | KF886632.1 |
| **VP 4.7** | *Vibrio parahaemolyticus* strain FORC 006 | 98% | 0.0 | 97% | CP009765.1 |
| | *Vibrio parahaemolyticus* strain J-C1-5 | 98% | 0.0 | 97% | EU652247.1 |
| | *Vibrio sp*. KYJ 962 | 98% | 0.0 | 97% | AY542526.1 |
| **VP 4.9** | *Vibrio sp*. S2-s3 | 99% | 0.0 | 99% | JX468071.1 |
| | *Vibrio sp.* S3-m2 | 99% | 0.0 | 99% | JX468072.1 |
| | *Vibrio parahaemolyticus* isolate Vp481 | 99% | 0.0 | 99% | EU155540.1 |
| **VP 4.10** | *Vibrio parahaemolyticus* strain 1682 ch1 | 99% | 0.0 | 97% | CP019059.1 |
| | *Vibrio parahaemolyticus* strain FORC 023 | 99% | 0.0 | 97% | CP012950.1 |
| | *Vibrio parahaemolyticus* strain CHN25 ch1 | 99% | 0.0 | 97% | CP010883.1 |
| **VP 4.12** | *Vibrio parahaemolyticus* strain NSP1 | 100% | 0.0 | 99% | JN188415.1 |
| | *Vibrio harveyi* Lmb041 | 100% | 0.0 | 99% | KT986106.1 |
| | *Vibrio campbelli* strain 0284 | 100% | 0.0 | 99% | KP236400.1 |
| **VP 4.13** | *Vibrio sp.* strain MS977 | 100% | 0.0 | 98% | KY473999.1 |
| | *Vibrio alginolyticus* strain APM TUR D11 | 100% | 0.0 | 98% | KX685339.1 |
| | *Vibrio parahaemolyticus* strain FORC 023 | 100% | 0.0 | 98% | CP012950.1 |

### 3.3.4 Molecular typing using a Pulsed Field Gel Electrophoresis (PFGE)

PFGE analysis was performed by the PulseNet International team at Centers of Diseases Control and Prevention (CDC), Atlanta, GA, using a BioNumerics Software. The analysis of *NotI* and *SfiI* allowed differentiation of 52 isolates into 21 restriction patterns, as show in Figure 3.7. These strains were grouped in 18 types with >65% patterns similarity (Figure 3.8, 3.9). Both, *NotI* and *SfiI* restriction patterns, revealed a similar discriminatory power. All restriction patterns were novel in comparison with the restriction patterns of the strains in the PulseNet USA *V. parahaemolyticus* database. All *SfiI* restriction patterns exhibited a conserved band with an approximate size of 650 – 700 kilobase pairs (kbp) and other band with approximate size of 30 kbp. In the case of *NotI* restriction patterns, all isolates had the same preserved band at approximate size of 650 – 700 kbp. Furthermore, the patterns exhibited two additional conserved bands with a size of 40 kbp and 140 kbp, respectively.

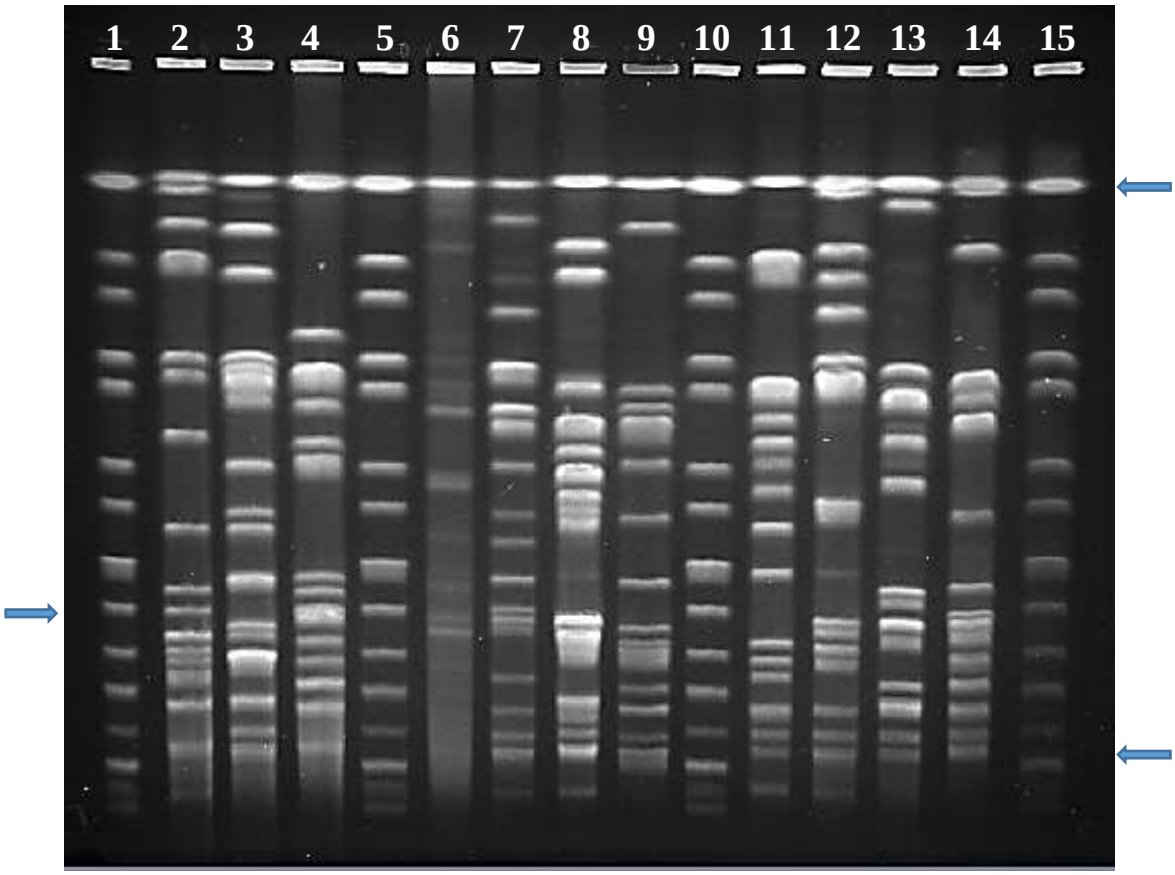


**Figure 3.8** Representative results of Pulsed Field Gel Electrophoresis of *V. parahaemolyticus* isolates digested with *SfiI*. The conditions for PFGE were as follows: 1% SeaKem Gold agarose gel, 0.5X TBE buffer, 6V, pulse time of 10 to 35 s, and a run time of 18 h. Genomic DNA from *Salmonella* serotype Braenderup reference standard (H9812) restricted with *XbaI* was used as a molecular weight marker (lanes 1, 5, 10 and 15). Lanes 2 to 4, 6 to 9, and 11 to 14 contain the genomic DNA of *V. parahaemolyticus* isolates digested with *SfiI*. The blue arrow indicates the conserved bands between isolated strains.

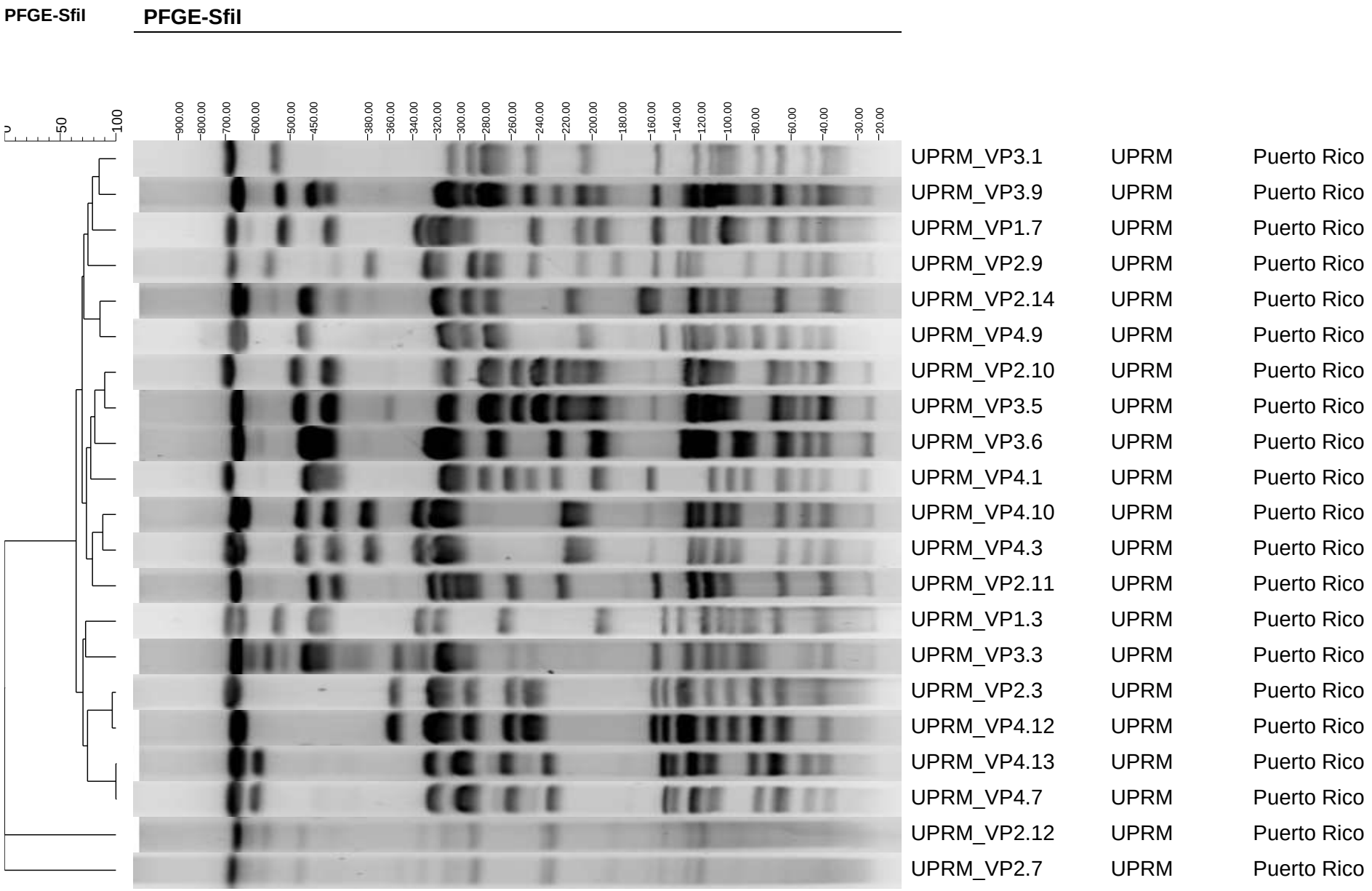


**Figure 3.9** PFGE dendrogram generated by BioNumerics software, showing the relationship of fingerprinting (*SfiI* PFGE) for 21 *V. parahaemolyticus* isolates. The numbers of the top at the figure indicate molecular sizes of kilobase pairs (kbp).

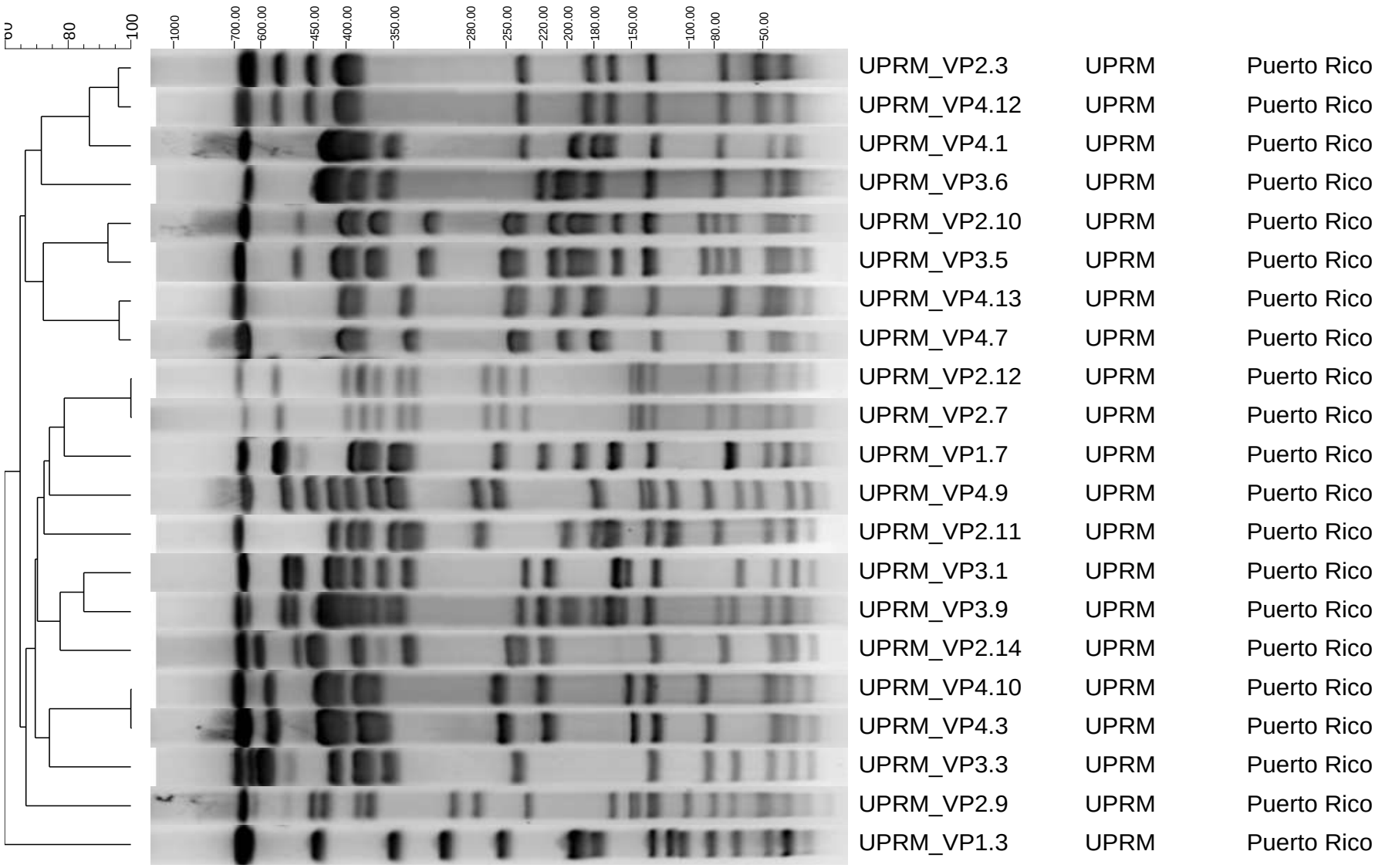


**Figure 3.10** PFGE dendrogram generated by BioNumerics software, showing the relationship of fingerprinting (*NotI* PFGE) for 21 *V. parahaemolyticus* isolates. The numbers at the top of the figure indicate molecular sizes of kilobase pairs (kbp).

### 3.4 Discussion of results

The main goal of this study consisted of the molecular characterization of the *V. parahaemolyticus* strains detected in clams and oyster recovered from the southwest coast of Puerto Rico. The study combined both genotypic characterization and molecular typing to gain a better understanding of the diversity of *V. parahaemolyticus* strains in Puerto Rico. *Vibrio parahaemolyticus* is a halophilic bacterium inhabiting marine and estuarine environments, that can be isolated in two form: pathogenic and non-pathogenic. In the environment, some related *Vibrio* species can exchange mobile genetic material, such as the genes associated to virulence (*tdh*, *trh*, T3SS genes, and T6SS genes), resulting in the change of the genome that can turn a non-pathogenic strain into pathogenic or viceversa (Gennari *et al*., 2012). The presence of pandemic genomic regions in non-pandemic strains provides evidence for horizontal gene transfer and evolution of *V. parahaemolyticus* (Ceccarelli *et al*., 2013). All *Vibrio* spp. have two chromosomes; in the case of *Vibrio parahaemolyticus*, the chromosomes are approximately 3.2-3.3Mb and 1.9Mb. The genomic DNA of the *Vibrio parahaemolyticus* strains isolated in this study possess a high molecular weight DNA (> 20Kbp); it coincides with what is described in the literature (Figure 3.1). The first sequenced and annotated genome is a pandemic *V. parahaemolyticus*, O3:K6 strain RimD221063, that was isolated in Japan from a patient with traveler's diarrhea (Nasu *et al*., 2000). It has been used as the reference sequence in pathogenesis analysis of numerous *V. parahemolyticus* strains (Makino *et al*., 2003).

Biochemical identification of *V. parahaemolyticus* is progressively being substituted by DNA- based detection techniques. The need for detection and differentiation of virulent strains, that do not exhibit noticeable phenotypic characteristics of pathogenesis, has turned molecular assays like PCR and Multiplex-PCR as alternative methods of ease use, high efficient and low cost. In the case of *V. parahaemolyticus*, diverse multiplex-PCR protocols targeting the *toxR*, *tlh*, *tdh*, *trh*, *groEL*, and *fla* genes have been developed to detect the total and pathogenic strains from clinical and environmental samples (Rosec *et al*., 2009; Izumiya *et al*., 2011; Wang *et al*., 2011; Hossain *et al*., 2013). In this study, the molecular biological analysis for the identification of *Vibrio parahaemolyticus* strain was performed using a multiplex- PCR developed for Bej *et al*. (1999), that is recommended by the BAM. This multiplex- PCR is based on the amplification of a 450bp sequence of *tlh*, 269bp sequence of *tdh*, and 500bp sequence of TRH. From a total of 58

presumptive *Vibrio parahaemolyticus* isolated strains, some 90% (52) strain showed a species-specific marker amplicon (*tlh* gene) and were confirmed as *Vibrio parahaemolyticus* (Figure 3.2). The *tlh* gene encodes the thermolabile hemolysin, a phospholipase A2, and is widely recommended as a species- specific marker (Bej *et al*., 1999; Zhang and Austin, 2005). It also lyses human erythrocytes and may play a role in human infection (Broberg *et al.*, 2011). Other studies do not recommend the use of *tlh* gene as a species specific marker, because it can generate false positive amplifications in other *Vibrio* species, reducing the accuracy and specificity of the detection method. Wang *et al*. (2007) reported that *tlh* gene is widespread in vibrios, including *V. aglinolyticus, V. harveyi, V. vulnificus, V. natriegens*, and others. An alternative solution to this problem is the use of *toxR* gene to diferenciate a *V. parahaemolyticus* strain at the species level (Kim *et al*., 1999; Vimala *et al*., 2010; Payder *et al*., 2013; Suffredini *et al*., 2014). The *toxR* gene is present in pathogenic and non-pathogenic strains of *V. parahaemolyticus* and it stimulates the expression of *tdh* (Sujeewa *et al*., 2009).

The most distinctive virulence factors associated with pathogenic strains of *V. parahaemolyticus* are two principal genes that codify for the thermostable direct hemolysin (TDH) and TDH- related hemolysin (TRH) (Fujino *et al.*, 1969; Miwatani *et al.*, 1972). Previous studies reported two enzymatic activities associated with pathogenesis. First a hemolytic activity, TDH binds to the membrane of host cells, and forms a pore in the surface. Second a cytotoxicity activity, THD forms a channel in the cell membrane, which induces an increase of extracellular $Ca^{2+}$ concentration and $Cl^{-}$ secretion. The cell expansion results in a high osmotic pressure, eventually ending in cell death (Matsuda *et al*., 2010). TRH has the same enzymatic activities that TDH (Takahashi et al., 2000; Ceccarelli *et al*., 2013). Also, all strains isolated in this study lacked the pathogenicity marker signal related to the isolates from patients with diarrhea associated to bivalve consumption (Figure 3.3). These results do not reflect that there is no a potential public health risk. Recently, Jones *et al*. (2012) reported clinical isolates that lacked *tdh, trh*, and *T3SS2* genes, indicating that the *tdh* or *trh* genes are not necessarily predictive of pathogenic potential. The same results are observed in four *V. parahaemolyticus* strains isolated from acute gastroenteritis cases caused by consumption of mussels in Italy. These strains present cytotoxic and adhesive activities, but lacked the typical pathogenicity genes (Ottaviani *et al*., 2012).

For the confirmation of isolated strains as member of the genus of *Vibrio*, amplification and sequencing of the 16S rDNA were performed. All strains PCR products showed the amplicon of approximately 1500bp (Figure 3.6). The *Vibrio* genus contains closely related bacterial species that differs less than 1% in 16S rRNA gene sequence (Dorsch *et al*., 1992; Ruimy *et al*., 1994). The analysis on the 16S rRNA gene is useful to allocate species to different branches of the family *Vibrionaceae*, but is largely inadequate for the discrimination of closely related species. *In-silico* analysis of the 16S rDNA partial sequences of the isolated strains (Table 3.2) suggest as possible candidate species the following: *V. parahaemolyticus*, *V. alginolytuicus*, *V. harveyi* and *V. campbelli*. It is difficult to differentiate these species using 16S rRNA and 23 rRNA genes as the species specific targets due to their high sequence similarity (Croci *et al*., 2007; Haldar *et al*., 2010). Also, a disadvantage of these genes is that they cannot be used to distinguish *Aeromonas* and *Vibrio* species (Teh *et al*., 2010). *Aeromonas* species are relevant in the identification of *Vibrio* because they can grow in TCBS agar and represent false- positives.

Molecular characterization should be included to establish an accurate relationship between isolates from diverse geographical regions. Pulsed-field gel electrophoresis is used to evaluate the genetic diversity among *V. parahaemolyticus* strains and detect the relatedness between environmental and food isolates with reference isolates of pathogenic significance (Ellingsen *et al*., 2008; Rodríguez-Castro *et al*., 2010; Wagley *et al*., 2009). The diversity of *Vibrio parahaemolyticus* strains between regions has been studied in different countries, such as Japan, Europa, Australia and the United States (Suffredini *et al*., 2011; Martínez-Urtaza *et al.*, 2004). The PFGE is the most common molecular typing method using in the food safety; it is based on restriction digest of the genomic DNA using enzymes with few cut sites in the genome. PFGE has a highly discriminatory index between 0.900- 0.9998 (Chen *et al*., 2012; Lüdeke *et al*., 2014; Fuenzalida *et al*., 2007). This method is the only subtyping technique that has been standardized and allowed comparison of data between PulseNet certified Laboratoties (Person *et al*., 2007; Kam *et al*., 2008). In this investigation, the PFGE was performed followed the PulseNet International standardized protocol with some modifications in the number of cells in the suspension. The restriction digest of the 52 *V. parahemolyticus* isolated strains allowed the differentiation of 21 different strains with DNA fragments profiles ranging between approximately 28 to 700kb (Figure 3.7). The PFGE analysis with *SfiI* (the primary enzyme) grouped the isolates into 18 types with >65% patterns similarity (Figure 3.8). Moreover, the strains analysis with *NotI* (the secondary

enzyme) were grouped into 16 types with >65% patterns similarity (Figure 3.9). Same patterns were present in strains isolated from both bivalves (clam and oyster); also, similar patterns were presented in different seasons of the year (spring and fall). All 21 restriction patterns were novel in comparison with the restriction patterns of the strains in the PulseNet USA *V. parahaemolyticus* database. These results suggest that the *V. parahaemolyticus* diversity in Puerto Rico are not necessarily related to the USA isolated strains. This diversity can be the product of horizontal transfer of mobile genetic elements with other members of the marine microbial communities; it may lead to the emergence of new strains with expanded ecological persistence, infectivity and dispersion. A previous study on the genetic variation of pathogenic *V. parahemolyticus* in Peru, Gavilan *et al*. (2013) concluded that horizontal genic transfer and homologous recombination as major events shaping the structure and diversity of pathogenic strains. The presence of genomic regions characteristic of the pandemic clone, such as T3SS, T6SS and mannose-sensitive hemagglutinin (MSHA) pilus in other non-pandemic strains may represent early evidence of genetic transfer from the introduced population to the local communities. Another possible reason for vast diversity of *V. parahaemolyticus* isolated in this study is the diversity of marine organisms that inhabit the aquatic environment of the sampling site. Some fish species that can be found in the Cabo Rojo area are: *Megalops atlanticus* (tarpon); *Lutjanus spp*. (lane snapper, mutton snapper), *Centropomus undecimalis* (snook), *Albula vulpes* (bone fish), *Elops saurus* (ladyfish), *Lachnolaimus maximus* (hogfish), *Trachinotus goodei* (palometa), *Archosargus rhomboidalis* (sea bream), *Caranx crysos* (blue runner) and others (D. Matos and L. Rivera, personal communication, November 15, 2016). Cabrera-García *et al*. (2014) reported *V. parahemolyticus* strains associated to fish products herein from the Gulf of Mexico.

The phylogenetic tree (Figure 3.7) shows the approximate division of the *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, and *Vibrio campbelli*; these results are consistent with the *V. metoecus* phylogenic generated based on a concatenated dataset of six partial gene sequences: *mdh*, *adk*, *gyrB*, *recA*, *pgi* and *rpoB* (Kirchberger *et al*., 2014). According to the Neighbor-Joining phylogenetic tree, the isolates in this study clustered with type and no-type strains of *Vibrio parahaemolyticus* and its closest relatives. This analysis shows the diversity of the isolates in based of the sequence of 16S rRNA gene, fourth subgroups can be distinguished and each one have a different divergent degree. The clustering of the isolates of *V. parahaemolyticus* was different in both molecular method, 16S rRNA gene sequencing and PFGE. Some isolates, such as Vp 2.7 and

Vp 2.12, displayed greater divergence in comparison with the other isolates in the study, though exhibited the same PFGE pattern and a distance relation in based of 16S rRNA gene. In some cases, similar 16S rRNA gene genotypes may exhibit different PFGE patterns due to genomic rearrangement or genomic plasticity in bacteria (Nakatsu *et al*., 1998; Mavingui *et al*., 2002). DNA replication, repair and homologous recombination can induce genome instability and chromosomal rearrangement. Bacteria utilize genome instability to increase their gene diversity and control gene expression (Darmon *et al*., 2014).

In summary, the results of the study presented confirmed the presence of *V. parahaemolyticus* in two bivalve species consumed in Puerto Rico. Furthermore, the data show that some of the characteristic associated with pathogenicity were exhibited, suggesting that some strain have pathogenic potential. In Puerto Rico, the lack of regulations for the handling and consumption of bivalves generate unknowing among people. The findings of this study allow to raise awareness about the species' pathogenic potential. More studies such as this should be carried out on the Island to validate the presence of *V. parahaemolyticus* and be able to establish safe food handling regulations. Some techniques for the reduction of the microbial load that can be established are depuration and relocation. In addition, the collection must be restricted to uncontaminated water.

# CHAPTER 4

## Educational activities in order to create awareness about the pathogenicity of *Vibrio parahaemolyticus* and the potential public health problem associated to shellfish

### 4.1 Disseminating food safety and *Vibrio parahaemolyticus* to the community

In a joint effort with Sea Grant and the Department of Natural and Environment Resources (DRNE) of Puerto Rico, an educational talk and an informative brochure were developed with the purpose of informing, making awareness and educating the community about the pathogenicity of *Vibrio parahaemolyticus*. This initiative came to support the mission of the Sea Grant program (http://seagrantpr.org/es/) on research, education and public service. Specifically, the mission was providing the community information generated by research about marine food safety and experience to help solve the problems of handling that communities face every day. In Puerto Rico, the lack of bivalves management regulations and the limited information about the prevention of *V. parahaemolyticus* in seafood among bivalve's sellers and fishermen led the development of the educational outreach activities.

Among the medically important *Vibrio* species, *V. parahaemolyticus* is recognized as an important seafood-borne pathogen. However, this issue does not have the particular concerns in the seafood industry in the Island. For these reasons, the topics that were discussed in the activities include: the general characteristic of *Vibrio parahaemolyticus*, the relationship between the bacterium and the shellfish, the risks of eating raw shellfish, the safety food handling and the prevention of *V. parahaemolyticus* in seafood (relocation and depuration). In addition, the research findings related to public health of the study titled "Detection and molecular characterization of *Vibrio parahaemolyticus* in the clam *Phacoides (Lucina) pectinatus* and the oyster *Crassostrea rhizophorae* from the southwest coast of Puerto Rico" , the same as present in this document, was disseminated to the community in trough informative talks. The educational outreach activities were developed in colloquial language based on the educational levels of the audience.

During a PR Commercial Fisheries Project (PEPCO) meeting of the Caribbean Fishery Management Council, the educational talk presented in this chapter was shared and discussed with the fishermen of the southwest coast of Puerto Rico, which work near the sampling site of my research. A group of twenty fishermen and other general public, including DNER employees, between 25 and 70 years of age, constituted the audience. The purpose of this discussion, entitled "Let's be careful with the handling and consumption of oysters and clams…", was to develop

awareness among local fishermen and the community on the potential public health problem associated with eating raw shellfish and how they can help to prevent.


**Figure 4.1** Educational talk with the fishermen. The meeting was held in the Dockey at Mayaguez, PR.

### 4.2 "Let's be careful with the handling and consumption of oysters and clams…"

The presentation, entitled: "Let's be careful with the handling and consumption of oysters and clams…", contained a total of 19 slides, where some of the topics added included: general information about filter-feeding bivalves (oysters, clams) and *V. parahaemolyticus*, and the strategies to identify and prevent *V. parahaemolyticus*. After a presentation, a Question and Answers session of 10 minutes took place. The purpose of this session was to clarify any questions about the topics and know the fishermen's opinion about the information presented.


Seamos cuidadosos con el manejo y consumo de ostras (ostiones) y almejas...
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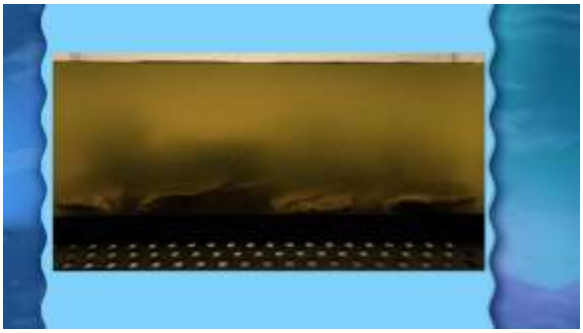
Sea Grant


¿Qué sabemos de las ostras y almejas?
1. ¿Quién las recolecta?
2. ¿Quién las distribuye y vende?
3. ¿Quién la consume?


¿Qué es una ostra, una almeja?
1. Marinos
2. Bivalvos
3. Filtradores
4. Comestibles


Las ostras y las almejas viven en ambientes distintos.


Las ostras y las almejas filtran agua para alimentarse.




Las ostras y las almejas pueden filtrar microorganismos.
¿Son buenos o malos?


Vibrio parahaemolyticus
Bacteria halofílica (necesita sal para vivir)
Habita (vive) en ambiente marinos
Playa
Mares
Oceanos
Puede encontrarse en dos formas:
No patogénica (inofensiva)
Patogénica (causa enfermedad)

## *Vibrio parahaemolyticus* patogénica

- Gastroenteritis
- Infecciones en la piel
- Septicemia (infección en la sangre)

- Causa líder de gastroenteritis por consumo de mariscos crudos a nivel mundial.
  - Diarrea
  - Dolor abdominal
  - Nausea
  - Vómitos
  - Fiebre

## *Las ostras y las almejas pueden filtrar microorganismos.*

## *Seguridad alimentaria ("food safety") en ostras y almejas.*

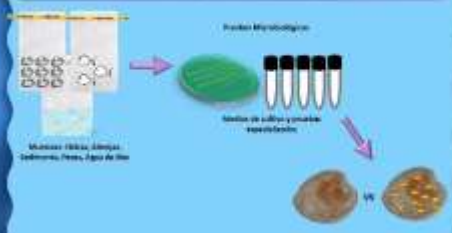
1. Crudas:
   a. Salsa picante
   b. Limón
   c. Temperatura
      i. Baja temperatura
      ii. Abuso temperatura
2. Cocinarlas

## *Seguridad alimentaria ("food safety") en ostras y almejas.*

VS

***¿Cómo sabemos si el alimento es seguro o no?***

## ¿Cómo sabemos si en las almejas y ostras hay *Vibrio parahaemolyticus*?



## ¿Cómo podemos reducir la presencia de *Vibrio parahaemolyticus* ?

**Cocinarlos**

**Mantenerlos a baja temperatura (fríos)**

## ¿Cómo podemos reducir la presencia de *Vibrio parahaemolyticus* ?

**Proceso de relocalización**

**Proceso de depuración**

## *¿Hay Vibrio parahaemolyticus* en ostras y almejas en Puerto Rico?

## En resumen....

- Las ostras y almejas son organismos filtradores que pueden acumular microorganismos.
  - Vibrio parahaemolyticus
- Existes varias técnicas para el manejo seguro de estos alimentos; entre ellas:
  - Cocinarlo adecuadamente
  - Depuración
  - Reloncalización

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# CHAPTER 5
## Conclusions, Recommendations and Literature Cited

## 5.1 Summary/Conclusions

- This study generated the first molecular characterization of *V. parahaemolyticus* strains isolated from the southwest coast of Puerto Rico.
- Based on the data, the clam *Phacoides pectinatus* and the oyster *Crassostrea rhizophorae* harbor *V. parahaemolyticus* strains with a pathogenic potential, because its presented virulent characteristics as hemolytic activity, biofilm production and urease production. More *V. parahaemolyticus* strains were isolated in oyster, and these isolates exhibited higher diversity than in clams.
- All *V. parahaemolyticus* strains isolated from this study presented some similar microscopic and phenotypic characteristics: Gram-negative rods with green colonies of 2-3mm in TCBS.
- Species – specific gene of *tlh* was found in all *V. parahaemolyticus* strains isolated from both types of bivalves.
- Pathogenicity markers associated to clinical strain (*tdh* and *trh*) in *V. parahaemolyticus* were not found in the isolates from mollusk.
- All PFGE patterns generated from the *V. parahaemolyticus* isolated strains (21) are novel for the PulseNet USA database; thus suggesting that the bivalves of the southwest Puerto Rico has a distinctive diversity.

## 5.2 Recommendations

- Driving similar studies in other areas of Puerto Rico where selling bivalves to the community. These studies will provide more data of the *V. parahaemolyticus* diversity in the Island and give more evidence for the potential public health problem by eating raw shellfish.
- Compare the PFGE patterns of *V. parahemolyticus* isolates with other *V. parhaemolyticus* database, for example PulseNet Latin America and Caribbean, to establish relatedness between diverse environmental and geographical regions.
- Continue with the characterization of the *V. parahaemolyticu* isolated strain; this included the lipopolysaccharide (O) and capsular (K) serotype, the numbers of plasmids, and the resistance of antibiotics.
- Confirm the presence of the *V. parahemolyticus* specie- specific gene of *toxR*.
- Subject all *V. parahaemolyticus* isolated strains to a whole genome sequencing to recognize the pathogenicity island and genes associated to virulence. This technique is the tools use by PulseNet International to foodborne investigations and surveillance.

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