ECOLOGY, VIRULENCE AND DETECTION OF PATHOGENIC AND PANDEMIC *VIBRIO PARAHAEMOLYTICUS*

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ECOLOGY, VIRULENCE AND DETECTION OF PATHOGENIC AND PANDEMIC *VIBRIO PARAHAEMOLYTICUS*

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Harvesting bivalves. Photo by Iddya Karunasagar

Vibrio parahaemolyticus is a gram negative, halophilic bacterium that occurs in the coastal and estuarine environments worldwide and is implicated in several cases of seafood-born gastroenteritis around the globe.

However, not all strains of *V. parahaemolyticus* are pathogenic. Clinical isolates of *V. parahaemolyticus* most often produce either the thermostable direct haemolysin (TDH) or TDH-related haemolysin (TRH) encoded by *tdh* and *trh* genes, respectively. A pandemic clone of O3:K6 which was first detected in Kolkata (India), has been responsible for many outbreaks in Asia

and the USA. With the emergence of pandemic clone of *V. parahaemolyticus*, this organism has assumed significance.

Although most of the *V. parahaemolyticus* outbreaks are invariably related to seafood consumption, pathogenic strains are rarely isolated from seafood. Virulent strains producing TDH or TRH and the pandemic clone, which is responsible for most of the outbreaks (that have occurred after 1996) have been rarely isolated from seafood and other environmental samples. This could be due to the occurrence of pathogenic strains in the estuarine environment at a lower level compared to non-pathogenic strains. Another reason can be that the pathogenic stains are more sensitive to dystropic conditions in the aquatic environment and rapidly become non-culturable. Similarity in growth kinetics between virulent and non-virulent strains also made the isolation of virulent strains from the aquatic environment difficult.

Several studies were done to determine the factors responsible for an increased virulence and persistance of pandemic clone. However, none of those studies were conclusive. Several researchers have proposed various genetic markers for specific detection of pandemic clone of *V. parahaemolyticus*. But many of those genetic markers were found to be unreliable. Recently, seven genomic islands (VPaI-1 to VPaI-7) unique to pandemic clone were identified.

This Research Topic is dedicated to improve our current understanding of ecology, pathogenesis and detection of pathogenic and pandemic clone of *V. parahaemolyticus*, and will also strive to identify areas of future development.

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EDITORIAL
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Editorial: Ecology, Virulence, and Detection of Pathogenic and Pandemic *Vibrio parahaemolyticus*

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Keywords: ***Vibrio parahaemolyticus*, pathogen, marine, seafood, virulence, ecology, pandemic, detection**

The Editorial on the Research topic

Ecology, Virulence, and Detection of Pathogenic and Pandemic *Vibrio parahaemolyticus*

Vibrio parahaemolyticus is a very versatile halophilic organism that can adapt to a wide variety of environments and can cause infections in both humans and aquatic animals. This versatility in terms of host and habitat is attributable to the ability to acquire genes that improve fitness of the organism in different situations. Several genomic islands have been described in this organism. Human pathogenic strains are characterized by the presence of pathogenicity islands that encode certain specific Type Three Secretion Systems (TTSS) and hemolysins that are not present in most environmental strains (Chen et al., 2011). Recently characterized shrimp pathogenic *V. parahaemolyticus* strains have plasmid borne virulence genes (Sirikharin et al., 2015). *V. parahaemolyticus* is associated with zooplankton like copepods in off-shore waters and same genotype has been found over large areas (Martinez-Urtaza et al., 2012). The global spread of pandemic *V. parahaemolyticus* has been attributed to the El Nino phenomenon characterized by the arrival of equatorial warm waters to South American coast in a sequence of invasive waves lasting about 6 months in 1997 (Martinez-Urtaza et al., 2008). Studies conducted using molecular techniques such as realtime PCR and multilocus sequence typing have helped detecting pathogenic *V. parahaemolyticus* in environmental samples and in understanding their global spread.

Thus, this organism has attracted attention of both seafood safety managers as well as aquatic animal health professionals. Being an autochthonous aquatic organism, *V. parahaemolyticus* has global distribution, occurring wherever environmental conditions are favorable. *V. parahaemolyticus* is a model organism for the "one health" concept, which recognizes that human health is connected to the health of animals and the environment. In order to better manage both public health and aquatic animal health, we need a better understanding of the factors effecting the ecology of this organism, the virulence factors present in human and animal pathogenic strains.

The papers in this research topic cover the three major aspects of pathogenic and pandemic *V. parahaemolyticus*: ecology, virulence, and detection. Lopez-Joven et al. discuss the prevalence of pathogenic and non-pathogenic strains in association with molluscs while Zavala-Norzagaray et al. describe *Vibrio* spp. associated with sea turtles in Mexico. Host colonization depends on the ability of the organism to acquire difficult to get nutrients such as iron. León-Sicairos et al. describe strategies of *V. parahaemolyticus* to obtain iron. Improvements in the detection methods of pathogenic strains has been presented by Escalante-Maldonado et al. Genetic characterization of clinical and environmental strains has enabled Xu et al. to understand the emergence of indigenous and non-indegenous pathogen lineages. Genomic and molecular typing studies provide insights into the environmental reservoirs and genetic diversity of pathogenic and pandemic strains as described by Hazen et al., de Jesús Hernández-Díaz et al., Lüdeke et al., and Haendiges et al.

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Function of genes involved in Type IV secretion system of *V. parahemolyticus* has been investigated by Yu et al. and conditions leading to loss of plasmid in this organism has been described by Letchumanan et al. Raghunath presented insights into the role of virulence genes involved in human infections. Thus, the articles presented in this research topic contribute to a better understanding of the ecology, virulence, and detection of this important aquatic organism that impacts for both public health and aquaculture.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct, and intellectual contribution to the work, and approved it for publication.

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Roles of thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) in *Vibrio parahaemolyticus*

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Vibrio parahaemolyticus is the leading cause of seafood borne bacterial gastroenteritis in the world, often associated with the consumption of raw or undercooked seafood. However, not all strains of *V. parahaemolyticus* are pathogenic. The thermostable direct hemolysin (TDH) or TDH-related hemolysin (TRH) encoded by *tdh* and *trh* genes, respectively, are considered major virulence factors in *V. parahaemolyticus*. However, about 10% of clinical strains do not contain *tdh* and/or *trh*. Environmental isolates of *V. parahaemolyticus* lacking *tdh* and/or *trh* are also highly cytotoxic to human gastrointestinal cells. Even in the absence of these hemolysins, *V. parahaemolyticus* remains pathogenic indicating other virulence factors exist. This mini review aims at discussing the possible roles of *tdh* and *trh* genes in clinical and environmental isolates of *V. parahaemolyticus*.

Keywords: *V. parahaemolyticus, tdh, trh,* virulence factors

INTRODUCTION

Vibrio parahaemolyticus is a Gram-negative, halophilic bacterium that occurs in estuarine environments worldwide (Su and Liu, 2007; Nelapati et al., 2012; Ceccarelli et al., 2013; Zhang and Orth, 2013). It is the leading cause of seafood borne bacterial gastroenteritis in the world, often associated with the consumption of raw or undercooked seafood. However, not all strains of *V. parahaemolyticus* are pathogenic. Although mechanism by which *V. parahaemolyticus* causes enteric disease is not fully understood, clinical isolates most often produce either the thermostable direct hemolysin (TDH) or TDH-related hemolysin (TRH) encoded by *tdh* and *trh* genes, respectively (Zhang and Austin, 2005). TDH and TRH are considered major virulence factors in *V. parahaemolyticus* (Ceccarelli et al., 2013).

CORRELATION OF *tdh* AND *trh* WITH THE VIRULENCE OF *V. parahaemolyticus*

Thermostable direct hemolysin exerts a variety of biological activities such as hemolytic activity, cytotoxicity, cardiotoxicity, and enterotoxicity. TDH is a pore-forming toxin, forms pores of ~2 nm in diameter on erythrocyte membrane (Matsuda et al., 2010). The fairly large pore size allows both water and ions to flow through the membrane (Honda et al., 1992). These alterations in ion flux in the intestine is responsible for the diarrhea observed during infection. TRH is a heat labile toxin and immunologically similar to TDH (Honda et al., 1988). Both genes, *trh* and *tdh* share approximately 70% homology (Kishishita et al., 1992). Similar to TDH, TRH also activates cl^- channels resulting in altered ion flux (Takahashi et al., 2000). Although TDH and TRH correlate with pathogenic strains, they do not fully account for *V. parahaemolyticus* pathogenicity (Lynch et al., 2005). Several studies have reported that some of the clinical strains do not contain *tdh* and/or *trh* (Jones et al., 2012; Li et al., 2014; Pazhani et al., 2014). Even in the absence of these hemolysins, *V. parahaemolyticus* remains pathogenic indicating other virulence factors exist (Jones et al., 2012). Mahoney et al. (2010) reported that environmental isolates of *V. parahaemolyticus* lacking *tdh* and/or *trh* produced putative virulence factors like extracellular proteases, biofilm, siderophore, and highly cytotoxic to human gastrointestinal cells. Park et al. (2004a) reported that deletion of both copies of *tdh* did not affect the cytotoxicity to HeLa cells and enterotoxicity assayed by the rabbit ileal loop test was lowered by *tdh* deletion, but the mutant still showed partial fluid accumulation in rabbit intestine. Ming et al. (1994) reported that *trh* deletion resulted in partial but apparent fluid accumulation in ligated rabbit small intestine. These results clearly indicate that cytotoxicity and enterotoxicity of pathogenic *V. parahaemolyticus* are not explained by TDH and TRH alone and suggest that an unkown virulence factor (s) could be responsible for pathogenicity.

TYPE III SECRETION SYSTEMS

The type III secretion system (T3SS) of *V. parahaemolyticus* has been suggested as an important virulence factor is (Shimohata and Takahashi, 2010; Broberg et al., 2011). Two non-redundant T3SSs are reported from many *V. parahaemolyticus* strains (Park et al., 2004b; Broberg et al., 2011). Many studies suggest that T3SS1 is responsible for cytotoxicity, mouse lethality, and possibly induction of autophagy (Park et al., 2004b; Burdette et al., 2009; Hiyoshi et al., 2010). T3SS2 appears to be responsible for enterotoxicity and may play a role in the environmental fitness of strains (Park et al., 2004b; Hiyoshi et al., 2010; Matz et al., 2011). All *V. parahaemolyticus* isolates possess T3SS1 (Park et al., 2004b; Noriea et al., 2010). While T3SS2 is commonly associated with *V. parahaemolyticus*

carrying *tdh* and/or *trh*. Two distinct lineages of T3SS2 have been described, showing correlations of *tdh* with T3SS2α and *trh* with T3SS2β (Park et al., 2004b; Noriea et al., 2010). However, recently T3SS2β has been detected in *tdh*- and *trh*-negative environmental strains of *V. parahaemolyticus* (Paranjpye et al., 2012). In another study, authors screened 77 clinical isolates of *V. parahaemolyticus*, which were submitted to the Centers for Disease Control and Prevention (CDC) in 2007 from wound infections or food-borne illness and reported that 21 of 77 (27%) clinical *V. parahaemolyticus* strains were negative for *tdh*, *trh*, and T3SS2 (Jones et al., 2012). The results of these studies raise some concerns about the reliability of the *tdh*, *trh*, and T3SS2 genes as predictors of overall strain virulence.

TYPE VI SECRETION SYSTEMS

Comparison between pandemic and non-pandemic strains of *V. parahaemolyticus* led to identification of type VI secretion systems, T6SS1 (VP1386–VP1420) and T6SS2 (VPA1030–VPA1043), located on chromosome 1 and 2 of *V. parahaemolyticus* RIMD 2210633, respectively, (Boyd et al., 2008; Izutsu et al., 2008). The role of T6SS2 is under investigation, preliminary data suggested that the T6SS2 is not involved in cytotoxicity, helps in adhesion to host cells (Yu et al., 2012). Since T6SS2 and T3SS2 systems co-exist, it is proposed that both systems might cooperate during an infection process in host. T6SS2 initiates the first step of infection by adhering to host cells and T3SS2 exports effector molecules by inducing enterocytotoxicity (Yu et al., 2012). Role of T6SS1 has not yet been demonstrated. Recently, researchers suggested the role of T6SSs in environmental fitness of *V. parahaemolyticus*. Salomon et al. (2013) reported that T6SS1 is most active under warm marine-like conditions, while T6SS2 is active under low salt conditions. T6SS was used as a virulence marker to differentiate *V. parahaemolyticus* strains. Chao et al. (2010) reported that most pandemic strains isolated in China had the complete set of T6SS genes, whereas the majority of non-pathogenic strains had a partial set of T6SS genes.

POSSIBLE ROLES OF *tdh* AND *trh* IN CLINICAL AND ENVIRONMENTAL ISOLATES OF *V. parahaemolyticus*

First outbreak of foodborne gastroenteritis due to *V. parahaemolyticus* was reported in the year 1951 in Osaka, where people frequently consume raw or uncooked seafood (Fujino et al., 1953). Since then *V. parahaemolyticus* has reported from many food poisoning cases in Japan (Su and Liu, 2007; Hara-Kudo et al., 2012), in Taiwan (Yu et al., 2013), in China (Li et al., 2014), Bangladesh (Bhuiyan et al., 2002), HongKong, and Indonesia (Matsumoto et al., 2000). In India, a recent study, reported that 178 *V. parahaemolyticus* strains were isolated from 13,607 diarrheal patients admitted in Infectious Diseases Hospital, Kolkata since 2001–2012 (Pazhani et al., 2014). Kanungo et al. (2012) have reported *V. parahaemolyticus* diarrheal cases from the urban slums of Kolkata, India.

The majority of clinical cases of *V. parahaemolyticus* have been associated with *V. parahaemolyticus* strains carrying *tdh* and/or *trh* (Kanungo et al., 2012; Li et al., 2014; Pazhani et al., 2014). However, pathogenic strains including the pandemic clone have been rarely isolated from seafood and other environmental samples. This could be due to the occurrence of pathogenic strains in the estuarine environment at a lower level compared to non-pathogenic strains or that the pathogenic stains are more sensitive to dystrophic conditions in the aquatic environment and rapidly become non-culturable (Pace and Chai, 1989; Alam et al., 2002). We thought that host factors like bile or its component bile acids might trigger release from dormancy and increase virulence in *V. parahaemolyticus* strains. This could be the reason for selection of pathogenic strains in human gastrointestinal system inspite of low prevalence in aquatic environment. With this in view, a new enrichment broth containing bile salt, sodium taurocholate (ST broth) has been formulated and the efficiency of new enrichment broth in detecting and recovering pathogenic *V. parahaemolyticus* from seafood was compared with the traditional APW. Results of the study suggested that the ST broth is superior to APW for detection and isolation of pathogenic *V. parahaemolyticus* from seafood.

Previous studies suggested that expression of *tdh* gene is upregulated under conditions simulating those in the human intestine (Gotoh et al., 2010; Broberg et al., 2011). Gotoh et al. (2010) are reported that TDH and T3SS2 proteins were detected in much higher concentrations when bacteria were cultured at 37 and 42°C, which corresponds to the temperature of the intestine, than at lower temperatures. In the same study, they also identified bile as a potent stimulator of the production of TDH and T3SS2 proteins (Gotoh et al., 2010). Pathogenic strains may produce TDH and/or TRH more abundantly in hostile environment and these toxins might be helpful to acquire nutrients from host cells through their cytotoxic activities. This could be the reason for selection of pathogenic strains in human intestine compared to non-pathogenic strains. Within the human host, whether TDH and TRH play any other roles other than cytotoxicity and enterotoxicity of *V. parahaemolyticus* need to be studied. Bacterial pathogens frequently use environmental cues to discriminate between host and non-host environments. In response to these environmental cues, bacteria regulate their virulence gene expression for more efficient utilization of bacterial resources and facilitate colonization, leading to infection. *V. parahaemolyticus*, upon reaching a human host from environment, should expose to number of environmental cues such as temperature, pH, osmolarity, oxygen levels, carbon sources, and concentration of various ions and compounds. In response to these environmental cues, *V. parahaemolyticus* carrying *tdh* and/or *trh* might tightly coordinate their virulence associated genes expression. Whereas, *V. parahaemolyticus* strains lacking *tdh* and/or *trh* may not be able to regulate their virulence associated genes expression and not able to establish colonization and infection in the human host. Mahoney et al. (2010) reported that in clinical strains carrying *tdh* and/or *trh*, the expression of virulence associated traits including hemolysin, protease, motility, biofilm formation, and cytotoxicity correlated with increased temperature from 28 to 37°C. In contrast, the environmental isolates did not induce their virulence associated traits in response to a temperature of 37°C.

The occurence of *tdh* and/or *trh* genes among environmental *V. parahaemolyticus* isolates is typically 1–10%, but this depend

on location, sample source and detection method. For example, we detected *tdh* and *trh* genes in 20.7 and 41.4% of the seafood samples, respectively, from southwest coast of India by PCR after 18 h enrichment in ST broth. In the same study, we isolated *tdh* and *trh*-carrying *V. parahaemolyticus* isolates from 19 to 44.8% of seafood samples, respectively, by colony hybridization following enrichment using ST broth (Raghunath et al., 2009). Kaysner et al. (1990) reported that between 49 and 78% of the sediment, water or oyster samples from Willapa Bay (WA, USA) contained *trh*-bearing *V. parahaemolyticus*. Alam et al. (2002) reported that *tdh* and *trh* genes were positive in 55 and 20% of environmental (water and sediment) samples, respectively, by MPN-PCR technique. But no *tdh* and/or *trh*-carrying strains were isolated by the conventional MPN-culture procedure. The *tdh* and *trh* genes are also present in non- *V. parahaemolyticus* vibrionaceae species such as *V. mimicus*, *V. cholerae* non-O1/non-O139, *V. hollisae*, *V. diaboilcus*, *V. alginolyticus*, and non-*vibrio* species such as *Aeromonas veronii* (Nishibuchi and Kaper, 1995; Gonzalez-Escalona et al., 2006; Raghunath et al., 2010; Shinoda, 2011; Klein et al., 2014). Such high frequency of these hemolysin genes in environmental strains of *V. parahaemolyticus* and occurance of these genes in the environmental strains of other *Vibrio* species indicate other potential roles of these hemolysins in the environment. Aquatic environment such as estuaries contain limited amount of nutrients. These hemolysins might be used to acquire nutrients through damage to cells of estuarine organisms. Matz et al. (2011) performed co-culture experiment of *V. parahaemolyticus* with a nanoflagellate *Cafeteria roenbergensis* and reported that the *tdh* gene is required for the persistence of *V. parahaemolyticus*.

CONCLUSION

In conclusion, the reason for the selection of *V. parahaemolyticus* strains carrying *tdh* and/or *trh* in the human host and the role of these hemolysins in coordinating virulence associated gene expression in response to the environmental cues to facilitate colonization and infection needs to addressed. The reason for the high frequency of *tdh* and *trh* genes in environmental strains of *V. parahaemolyticus* is not clear. When compared to *tdh* gene, the detection rate of *trh* gene in clinical strains is very less but relatively more in environmental strains. Pathogenic potential of these environmental strains of *V. parahaemolyticus* carrying *trh* gene needs to be studied. Future studies should also focus on the role of TDH and TRH in the environmental strains of *V. parahaemolyticus* for contribution of environmental fitness.

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Characterization of *Vibrio parahaemolyticus* clinical strains from Maryland (2012–2013) and comparisons to a locally and globally diverse *V. parahaemolyticus* strains by whole-genome sequence analysis

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Vibrio parahaemolyticus is the leading cause of foodborne illnesses in the US associated with the consumption of raw shellfish. Previous population studies of *V. parahaemolyticus* have used Multi-Locus Sequence Typing (MLST) or Pulsed Field Gel Electrophoresis (PFGE). Whole genome sequencing (WGS) provides a much higher level of resolution, but has been used to characterize only a few United States (US) clinical isolates. Here we report the WGS characterization of 34 genomes of *V. parahaemolyticus* strains that were isolated from clinical cases in the state of Maryland (MD) during 2 years (2012–2013). These 2 years saw an increase of *V. parahaemolyticus* cases compared to previous years. Among these MD isolates, 28% were negative for *tdh* and *trh*, 8% were *tdh* positive only, 11% were *trh* positive only, and 53% contained both genes. We compared this set of *V. parahaemolyticus* genomes to those of a collection of 17 archival strains from the US (10 previously sequenced strains and 7 from NCBI, collected between 1988 and 2004) and 15 international strains, isolated from geographically-diverse environmental and clinical sources (collected between 1980 and 2010). A WGS phylogenetic analysis of these strains revealed the regional outbreak strains from MD are highly diverse and yet genetically distinct from the international strains. Some MD strains caused outbreaks 2 years in a row, indicating a local source of contamination (e.g., ST631). Advances in WGS will enable this type of analysis to become routine, providing an excellent tool for improved surveillance. Databases built with phylogenetic data will help pinpoint sources of contamination in future outbreaks and contribute to faster outbreak control.

Keywords: NGS, WGS, *Vibrio parahaemolyticus*, clinical, phylogenetic analysis, phylogeny, SNPs

INTRODUCTION

Vibrio parahaemolyticus is a natural inhabitant of temperate and tropical coastal waters and is the leading cause of seafood-borne gastroenteritis in the United States (US) (Scallan et al., 2011). Cases of illness are usually associated with eating raw or undercooked seafood. Strains of *V. parahaemolyticus* carrying genes for thermostable direct hemolysin (*tdh*) and/or thermostable direct hemolysin-related hemolysin (*trh*) are considered pathogenic (Turner et al., 2013) and typically represent <1% of environmental *V. parahaemolyticus* strains (DePaola et al., 2000). However, this frequency may depend on the location, sample sources, and detection methods (Kaysner et al., 1990; Alam et al., 2002; Cook et al., 2002; Hervio-Heath et al., 2002; Martinez-Urtaza et al., 2008). During the last two decades *V. parahaemolyticus* infections and outbreaks have increased throughout the world. Most of these new cases belong to a pandemic clonal complex, known as CC3, first identified in in February of 1996 in India (Martinez-Urtaza et al., 2005; Nair et al., 2007; Gonzalez-Escalona et al., 2008; Haendiges et al., 2014).

The emergence of CC3 caused public health concerns about the potential worldwide spread of virulent *V. parahaemolyticus*, which previously had been restricted to particular regions. Other clonal complexes of *V. parahaemolyticus*, specifically CC36 and CC34, have been observed among coastal US strains (Gonzalez-Escalona et al., 2008), and strains of Sequence Type (ST) 36, the ancestral type of CC36, have also been detected on the western coast of Canada (Banerjee et al., 2014). Although infections in the US are typically caused by strains from CC36, which is endemic to the West Coast (Abbott et al., 1989; Gonzalez-Escalona et al., 2008), a Maryland outbreak in August 2012 (Haendiges et al., 2014) was caused by strains belonging to CC3.

Foodborne illnesses due to *V. parahaemolyticus* are uncommonly reported in the US and in the state of Maryland. Forty-six cases of *V. parahaemolyticus* gastroenteritis associated illnesses were reported between 2012 and 2013. From those cases, 34

strains were isolated. During the summer of 2012 a multistate outbreak associated with harvested shellfish was reported on the East Coast of the US (Newton et al., 2014), caused by *V. parahaemolyticus* strains belonging to CC36 (Martinez-Urtaza et al., 2013), subsequently shown to be ST36. That outbreak affected 28 persons in nine states (Newton et al., 2014), and the isolated strains were of the O4:K12 or O4:K (unknown) serotypes. Usually strains from this serotype/ST isolated in the US are from the West Coast (Gonzalez-Escalona et al., 2008; Newton et al., 2014). Another outbreak, involving 104 cases, occurred in the summer of 2013 affecting people in 13 US states and were caused by the same ST36 (Newton et al., 2014). An outbreak in Spain, during the summer of 2012, was attributed to strains that were ST36 as well (Martinez-Urtaza et al., 2013), associated with cooked seafood that had been cooled with untreated local seawater (Martinez-Urtaza et al., 2013); contrarily to the infections in the US which are often linked to the consumption of contaminated oysters (Newton et al., 2014).

The numbers of *V. parahaemolyticus* infections appears to have increased approximately 4 times in US in the last decade (Mead et al., 1999; Scallan et al., 2011). Scallan et al. (2011) reported that the domestically acquired foodborne average for *V. parahaemolyticus* infections annually were around 35,000 in the period 2000–2008, while Mead et al. (1999) reported around 8000 infections annually in the period 1992–1997. The increasing number of infections could have detrimental impacts on public health and economic growth, particularly in regions where seafood harvest and consumption are important. However, active surveillance depends upon having effective ways to identify and monitor the nature or identity of the *V. parahaemolyticus* strains causing outbreaks.

Various typing methods have been used to distinguish bacterial isolates for epidemiological investigations (Foxman et al., 2005). Pulsed Field Gel Electrophoresis (PFGE) has been a favored method for genotyping *V. parahaemolyticus* isolates (Marshall et al., 1999) and it is considered the "gold standard" for outbreak investigations (Parsons et al., 2007; Wagley et al., 2008; Dauros et al., 2011; Banerjee et al., 2014; Haendiges et al., 2014; Ma et al., 2014; Pazhani et al., 2014). While useful for short-term epidemiology, PFGE does not provide details of the genetic relationships among isolates (e.g., evolutionary relationships) (Foxman et al., 2005). Another common method for characterizing *V. parahaemolyticus* isolates is Multilocus Sequence Typing (MLST). This method is based on direct sequence analysis of housekeeping genes, making MLST better for long term evolutionary studies (Gonzalez-Escalona et al., 2008; Harth et al., 2009; Yan et al., 2011; Gavilan et al., 2013; Martinez-Urtaza et al., 2013; Turner et al., 2013). A public database was established to archive *V. parahaemolyticus* sequences (http://pubmlst.org/vparahaemolyticus) (Gonzalez-Escalona et al., 2008).

In the last 5 years, scientists have begun using genomic techniques to analyze historical collections of pathogens and outbreak isolates, providing new insights for outbreak investigations. Whole genome sequencing (WGS) and WGS-SNP data analyses allow us to better understand population dynamics and mechanisms contributing to increased virulence among foodborne bacterial pathogens, including outbreaks of *Salmonella* Montevideo in 2010 (Bakker et al., 2011; Allard et al., 2012), *Vibrio cholerae* in Haiti in 2010 (Chin et al., 2011), *E. coli* O104:H4 (in Germany in 2011, Rasko et al., 2011) and *Salmonella* Enteritidis in the US in 2010 (Allard et al., 2013). In the current project, we utilize a similar approach to explore the diversity and relationships among *V. parahaemolyticus* isolates causing outbreaks in Maryland.

Specifically, in order to better understand potential changes in *V. parahaemolyticus* populations in the state of Maryland and to investigate whether the spikes in cases during the summers of 2012 and 2013 were related to this recently introduced East Coast clone, ST36, we used WGS to compare genomes from those outbreaks with those of the other *V. parahaemolyticus* strains causing illnesses in recent years (2012–2013). We began by sequencing the 34 strains from MD, then 10 additional historical *V. parahaemolyticus* strains from different sources (clinical and environmental) from the East and West Coasts of US were also sequenced and used for phylogenetic comparative analysis. Additional available genomes from global *V. parahaemolyticus* strains were also used in the phylogenetic comparative analysis.

MATERIALS AND METHODS

BACTERIAL STRAINS AND MEDIA

The *V. parahaemolyticus* isolates sequenced for this project are listed, along with their assigned CFSAN numbers, in **Tables 1**, **2**. **Table 1** lists the 34 clinical isolates from Maryland, and **Table 2** lists the national and international *V. parahaemolyticus* isolates used for comparison. All isolates were retrieved from storage (−80°C freezer), transferred to Luria-Bertani (LB) medium with 3% NaCl and incubated at 250 rpm at 37°C.

DNA EXTRACTION AND QUANTIFICATION

Genomic DNA from each isolate was isolated from overnight cultures using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA). The quality of the DNA was checked using a NanoDrop 1000 (Thermo Scientific, Rockford, IL) and the concentration was determined using a Qubit double-stranded DNA HS assay kit and a Qubit 2.0 fluorometer (Life Technologies, Grand Island, NY), according to each manufacturer's instructions.

WHOLE GENOME SEQUENCING, CONTIG ASSEMBLY, AND ANNOTATION

The genomes of the historical isolates were sequenced using 200 bp reads chemistry and the 36 MD outbreak isolates were sequenced using 300 bp reads chemistry, using an Ion Torrent (Thermo Scientific) sequencer, according to manufacturer's instructions, at approximately 20X coverage (Haendiges et al., 2014). Genomic sequence contigs were *de novo* assembled using CLC Genomics Workbench (QIAGEN). These draft genomes were annotated using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP, http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html) (Klimke et al., 2009).

IN SILICO MLST PHYLOGENETIC ANALYSIS

The initial analysis and identification of the isolates was performed using an *in silico V. parahaemolyticus* MLST approach, based on information available at the *V. parahaemolyticus* MLST website (http://pubmlst.org/vparahaemolyticus). Seven

Table 1 | Clinical *V. parahaemolyticus* isolates sequenced in this study and source information.

Isolate name	Accession no(s) WGS	CFSAN number	STs	County	State	Collection date	Source	*tdh, trh* presence[a]	References
VP1	JNSM01000000	CFSAN007429	631	Anne Arundel	MD	6/15/2012	stool	2,1	Haendiges et al., 2014
VP8	JNSN01000000	CFSAN007430	631	Cecil	MD	7/12/2012	stool	2,1	Haendiges et al., 2014
VP9	JNSO01000000	CFSAN007431	631	Queen Anne's	MD	7/17/2012	stool	2,1	Haendiges et al., 2014
VP31	JNSP01000000	CFSAN007432	631	Harford	MD	6/16/2013	stool	2,1	Haendiges et al., 2014
VP35	JNSQ01000000	CFSAN007433	631	Baltimore City	MD	7/11/2013	stool	2,1	Haendiges et al., 2014
VP41	JNSR01000000	CFSAN007434	631	Unknown	MD	7/17/2013	stool	2,1	Haendiges et al., 2014
VP44	JNSS01000000	CFSAN007435	631	Unknown	MD	8/3/2013	stool	2,1	Haendiges et al., 2014
VP45	JNST01000000	CFSAN007436	631	Anne Arundel	MD	8/9/2013	stool	2,1	Haendiges et al., 2014
VP2	JNSU01000000	CFSAN007437	651	Montgomery	MD	5/31/2012	stool	0,0	Haendiges et al., 2014
VP4	JNSW01000000	CFSAN007439	653	Out of State-DE	MD	6/17/2012	stool	0,2	Haendiges et al., 2014
VP34	JNSX01000000	CFSAN007440	653	Prince George's	MD	7/11/2013	stool	0,2	Haendiges et al., 2014
VP7	JNSZ01000000	CFSAN007442	113	Charles	MD	7/10/2012	stool	2,1	Haendiges et al., 2014
VP10	JNTC01000000	CFSAN007445	43	Montgomery	MD	6/12/2012	stool	2,1	Haendiges et al., 2014
VP16	JNTG01000000	CFSAN007449	3	Anne Arundel	MD	8/21/2012	stool	1 and 2, 0	Haendiges et al., 2014
VP17	JNTH01000000	CFSAN007450	3	Anne Arundel	MD	8/22/2012	stool	1 and 2, 0	Haendiges et al., 2014
VP18	JNTI01000000	CFSAN007451	3	Anne Arundel	MD	8/24/2012	stool	1 and 2, 0	Haendiges et al., 2014
VP39	JNTL00000000	CFSAN007455	896	Wicomico	MD	7/19/2013	stool	0,0	Haendiges et al., 2014
VP12	JNTM00000000	CFSAN006129	36	Montgomery	MD	8/3/2012	stool	2,1	Haendiges et al., 2014
VP32	JNTN00000000	CFSAN006131	36	Anne Arundel	MD	6/30/2013	stool	2,1	Haendiges et al., 2014
VP33	JNTO01000000	CFSAN006132	36	Howard	MD	6/17/2013	stool	2,1	Haendiges et al., 2014
VP36	JNTP01000000	CFSAN006133	36	Baltimore County	MD	7/5/2013	stool	2,1	Haendiges et al., 2014
VP38	JNTQ00000000	CFSAN006134	36	Baltimore City	MD	7/16/2013	stool	2,1	Haendiges et al., 2014
VP40	JNTR00000000	CFSAN006135	36	Baltimore County	MD	7/21/2013	stool	2,1	Haendiges et al., 2014
VP42	JNTS00000000	CFSAN007460	36	Baltimore City	MD	8/7/2013	stool	2,1	Haendiges et al., 2014
VP43	JNTT00000000	CFSAN007461	36	Talbot	MD	7/31/2013	stool	2,1	Haendiges et al., 2014
VP30	JNTV00000000	CFSAN006130	36	Pringe George's	MD	6/2/2013	stool	2,1	Haendiges et al., 2014
VP46	JNTU00000000	CFSAN007462	36	Pringe George's	MD	8/27/2013	stool	2,1	Haendiges et al., 2014
VP5*	JNSY01000000	CFSAN007441	113	Calvert	MD	6/5/2012	wound	0,0	Haendiges et al., 2014
VP13*	JNTD01000000	CFSAN007446	678	Charles	MD	8/5/2012	wound	0,0	Haendiges et al., 2014
VP6*	JNTB01000000	CFSAN007444	677	Baltimore City	MD	6/25/2012	wound	0,0	Haendiges et al., 2014
VP15*	JNTF01000000	CFSAN007448	679	Out of State-PA	MD	8/3/2012	wound	0,0	Haendiges et al., 2014
VP3*	JNSV01000000	CFSAN007438	652	Anne Arundel	MD	6/8/2012	wound	0,0	Haendiges et al., 2014
VP11*	JNTA01000000	CFSAN007443	113	Somerset	MD	7/23/2012	ear	0,0	Haendiges et al., 2014
VP14*	JNTE01000000	CFSAN007447	162	Wicomico	MD	8/10/2012	ear	0,0	Haendiges et al., 2014

The isolates were organized by their isolation source.

[a] *type of tdh or trh gene present in that strain by in silico reference mapping using CLC Genomics workbench. Example: 2,1 means tdh2 and trh1 types, respectively, and 0 means not present.*

**Strains isolated from wound or ear.*

loci (*dnaE, gyrB, recA, dtdS, pntA, pyrC,* and *tnaA*), described previously for *V. parahaemolyticus* (Gonzalez-Escalona et al., 2008), were used for MLST analysis. The same *V. parahaemolyticus* MLST database was also used to assign numbers for alleles and STs.

ASSIGNMENT TO CLONAL COMPLEXES

The program goeBURST v1.2.1 was used to identify CCs among the isolates (http://goeburst.phyloviz.net) (Francisco et al., 2009). To qualify as members of the same CC, isolates must share at least 5 of the 7 alleles used in the *V. parahaemolyticus* MLST scheme (Feil et al., 2004). Those STs that differ by two alleles are considered double locus variants (DLV), and those which differ at only a single locus are single locus variants (SLV).

PHYLOGENOMIC ANALYSIS AND TARGETED SNP ANALYSIS

We performed two phylogenetic analyses using the software kSNP v2 (Gardner and Hall, 2013) (kmer length = 31). The first one included a broad SNP analysis of all the genomes included in the analysis, including local, national and international isolates, and was performed to provide an evolutionary context for all the *V. parahaemolyticus* isolates used for the analysis. The second was conducted to provide a more detailed phylogenetic relationship among the isolates found in each individual cluster and were targeted for more refined SNP analyses. Our goal was to recover variations unique to each respective lineage. To do this, we created a matrix that included only core SNPs (i.e., SNPs that were present in all genomes) for each lineage, using the reference-free SNP- finding program, kSNP v2 (Gardner and Hall, 2013) (kmer

Table 2 | Locally and globally diverse *V. parahaemolyticus* isolates genomes used for phylogenomic analyses available at the National Center for Biotechnology Information.

Isolate name	Accession no(s) WGS	CFSAN number	STs	State	Country	Isolation year	Source	References
029-1(b)	JNTW00000000	CFSAN001611	36	OR	USA	1997	E	Haendiges et al., 2014
48057	JNTX00000000	CFSAN001612	36	WA	USA	1990	C	Haendiges et al., 2014
K1198	JNTY01000000	CFSAN001614	59	AK	USA	2004	E	Haendiges et al., 2014
10292	JNTZ00000000	CFSAN001617	50	WA	USA	1997	C	Haendiges et al., 2014
48291	JNUA00000000	CFSAN001618	36	WA	USA	1990	C	Haendiges et al., 2014
F11-3A	JNUB00000000	CFSAN001619	36	WA	USA	1988	E	Haendiges et al., 2014
NY-3483	JNUC00000000	CFSAN001620	36	NY	USA	1998	E	Haendiges et al., 2014
K1203	JNUD00000000	CFSAN001173	59	AK	USA	2004	E	Haendiges et al., 2014
98-513-F52	JNUE00000000	CFSAN001160	34	LA	USA	1998	E	Haendiges et al., 2014
10290	JNUF00000000	CFSAN001613	36	WA	USA	1997	C	Haendiges et al., 2014
10329	NZ_AFBW01000000	N/A	36	WA	USA	1998	C	Gonzalez-Escalona et al., 2011
RIMD 2210633	NC_004603, NC_004605	N/A	3	Osaka	Japan	1996	C	Makino et al., 2003
BB22OP	CP003973.1, CP003972.1	N/A	88	?	Bangladesh	1980	E	Jensen et al., 2013
AN-5034	ACFO00000000	N/A	3	?	Bangladesh	1998	?	Chen et al., 2011
AQ3810	AAWQ00000000	N/A	87	?	Singapore	1983	C	Unpublished
AQ4037	ACFN00000000	N/A	96	?	Maldives	1985	?	Chen et al., 2011
K5030	ACKB00000000	N/A	3	?	?	2005	?	Chen et al., 2011
PCV08-7	AOCL00000000	N/A	808	Selangor	Malaysia	2008	E	Tiruvayipati et al., 2013
Peru-466	ACFM00000000	N/A	3	?	Peru	1996	?	Chen et al., 2011
SNUVpS-1	AMRZ00000000	N/A	917	?	Korea	2009	E	Jun et al., 2013
V110	AQPJ00000000	N/A	809	?	China	2010	E	Liu and Chen, 2013
3259	AVOL01000000	N/A	479	?	USA	2007	C	Unpublished
949	AVPV01000000	N/A	3	?	USA	2006	C	Unpublished
NIHCB0603	AVOM00000000	N/A	3	?	Bangladesh	2006	C	Unpublished
NIHCB0757	AVPX01000000	N/A	65	?	Bangladesh	2006	C	Unpublished
VP-NY4	AVON01000000	N/A	3	?	India	1997	C	Unpublished
VP2007-095	NZ_AVOI01000000	N/A	631	FL	USA	2007	C	Unpublished
VP232	NZ_AVOJ01000000	N/A	3	?	India	1998	C	Unpublished
VP250	NZ_AVOK01000000	N/A	3	?	India	1998	C	Unpublished
VPCR-2010	NZ_AVPW01000000	N/A	308	?	USA	2010	E	Unpublished
12310	AYXP00000000	N/A	36	WA	USA	2006	C	Unpublished
3256	AZGS00000000	N/A	36	WA	USA	2007	C	Unpublished

N/A, not applicable; ?, unknown.

length = 31). Maximum-likelihood phylogenies for each core SNP matrix were then constructed within the kSNP, labeled with the number of unique SNPs present in every descendant of that node. Draft assemblies were used to detect SNPs using the program, kSNP. Filtering was applied to eliminate detectable horizontal gene transfer such as mobile elements and paralogs, but we were not able to filter for genome recombination. Although important, reconstructing the reticulate evolutionary history of *V. parahaemolyticus* was outside the scope of this research effort.

Using the kSNP approach offers several advantages. First, the kSNP approach to gathering reference-free SNPs for downstream phylogenetic analysis has been effective for microbial-scale phylogenomic analyses (Sahl et al., 2013; Timme et al., 2013), Second, these kSNP analyses are not as vulnerable to assembly errors as other methods because the putative SNPs are extracted from kmers (in our present analysis, one SNP per 25 mer), effectively eliminating any influence of assembly bias. Third, the algorithm behind SNP identification is especially conservative about common pyrosequencing errors, such as the homopolymeric runs commonly observed in data from 454 and Ion Torrent sequencing procedures: if an insertion or deletion is present in a region, the resulting 25-mer will no longer match the other homologous kmer regions, thus that SNP will be missing in that taxon. Additionally, kSNP omits kmers where all SNPs are in the center of a homopolymer repeat (Gardner and Slezak, 2010). These features make our approach conservative and prevent repeat regions from becoming a factor in our analyses: any k-mers comprising such regions will fail to be unique, and therefore will not be used.

NUCLEOTIDE SEQUENCE ACCESSION NUMBERS

The draft genome sequences for all 46 *V. parahaemolyticus* strains in our analyses are available in GenBank under the accession numbers listed in **Tables 1**, **2**.

RESULTS AND DISCUSSION

MARYLAND *V. PARAHAEMOLYTICUS* OUTBREAK STRAINS AND *IN SILICO* MLST

Our MLST analyses provide a broad look at the diversity of *V. parahaemolyticus* clinical samples collected during 2010–2013 in the state of Maryland. These 34 *V. parahaemolyticus* strains isolated from patients—stool (80%), ear (6%), and wound (14%) were sequenced using Ion Torrent. A quarter of these strains (25%) came from an outbreak in the summer of 2013. All the reported cases occurred during the warmer months between June and August (**Table 1**).

In silico MLST (Gonzalez-Escalona et al., 2008) identified 13 different STs, showing that the sporadic outbreaks in Maryland were caused by highly diverse *V. parahaemolyticus* strains. Seven STs were novel (STs: 651, 652, 653, 677, 678, 679, and 896) and represented strains that have only been found (so far) in Maryland (**Table 1**). The most prevalent STs were ST36 (28%), ST631 (22%), ST3 (8%), ST113 (8%), and ST653 (6%). The rest of the STs were represented by a single strain.

Some of the identified STs were isolated from more than one location, more than one person, and in different years (**Table 1** and **Figure 1**); most of the illnesses associated with these isolates occurred in MD coastal counties (**Figure 1**). Interestingly, most of the cases on 2013 (88%) were caused by ST631 (31%) and ST36 (56%). This suggests these two STs are either more prevalent and/or have replaced the endemic *V. parahaemolyticus* populations in this region. Additional surveys of pathogenic *V. parahaemolyticus* in MD could confirm or clarify our theories.

Our eBURST analysis (goeBURST) showed that the MD isolates do not form any clonal complexes and were not related to each other (**Figure 2**). Therefore, the outbreaks during this period in MD were caused by *V. parahaemolyticus* belonging to different populations. However, some findings suggested we had reached the limits of the MLST method: the ST36 observed in MD could not be distinguished from ST36 from the West Coast by this method. It is at this point we must turn to the higher discriminatory power of WGS analyses.

PHYLOGENOMIC ANALYSIS

Using 7 *V. parahaemolyticus* genomes available from NCBI (www.ncbi.nlm.nih.gov/genome/?term=vibrioparahaemolyticus) and the 10 genomes from historical US *V. parahaemolyticus* strains (**Table 2**), we determined the phylogenetic relationships among the MD strains using a whole-genome SNP analysis (**Figure 3**). As can be seen from **Figure 3**, *V. parahaemolyticus* is a highly diverse microorganism, documented by the high number of SNPs defining each branch, and a low-resolution tree is obtained when using this entire genome database. Nevertheless, a similar clustering to the one achieved by MLST analysis can still be observed for the Maryland *V. parahaemolyticus* strains, where the same STs were also grouping at the genome level. Five clusters of related strains were identified, cluster I (ST36), cluster II (ST653), cluster III (ST113), cluster IV (ST631), and cluster V (ST3).

Besides Maryland ST36 strains (which appear in blue text on the **Figure 3**), Cluster I also contained strains that were isolated on the West Coast (1988–2004) (represented by the red text in **Figure 3**) and are part of CC36 (Gonzalez-Escalona et al., 2008). In this broad phylogeny, little differentiation can be observed among these ST36 strains. The same can be observed for most other clusters. But this broad phylogeny does allow us to confirm

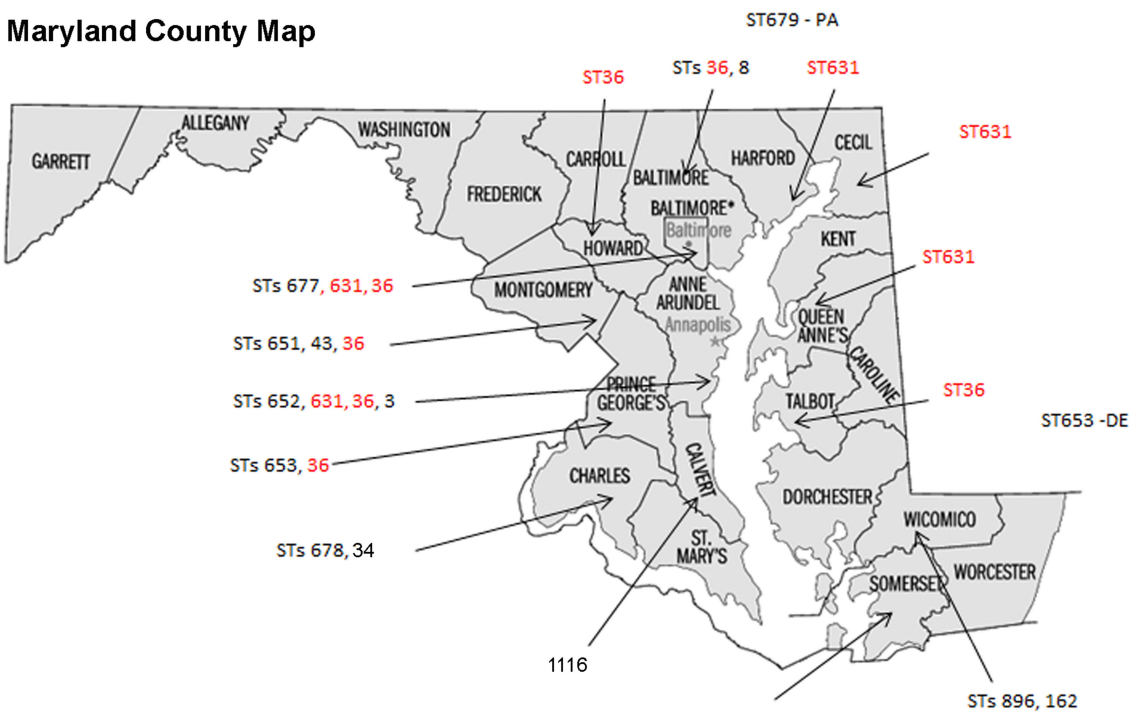

FIGURE 1 | Sequence types (STs) isolation locations on the state of Maryland, USA. STs found in more than one location are in red fonts.

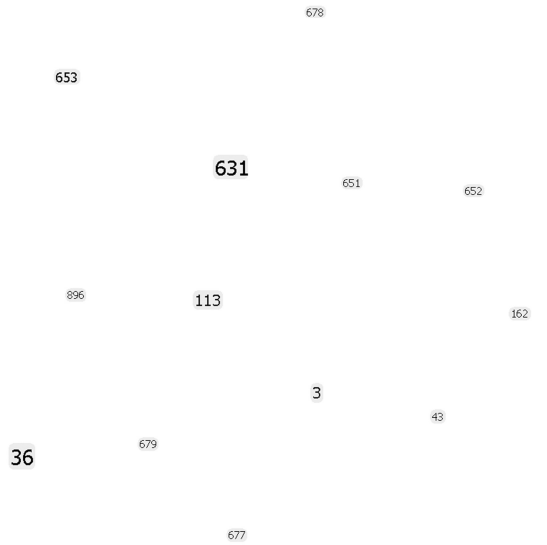

FIGURE 2 | goeBURST population snapshot using the *in silico* MLST analysis of the strains sequenced in this study indicating the absence of any clonal complex and the relative frequency of each ST. Font sizes varied according to the frequency of the ST.

the diversity of populations causing the outbreaks in MD during that 2 year period.

Given these patterns, we then used a cluster-based analysis that enabled us to identify relationships among strains that would not have been found by examining the entire dataset (**Figure 4**). Each individual cluster (when more than three strains) was analyzed separately. This type of analysis helped us to clearly distinguish strains in ST36 from MD (2012–2013) from those originating on the West Coast (1988–1998). These groups differed by at least 150 SNPs (**Figure 4A**). Interestingly, MD strains were similar to ST36, also from the West Coast, of US but from a later time point: 2006–2007 (strains 12310 and 3256). This clearly indicates that ST36 strains have evolved in the last decade and the new ST36 strains from the West Coast are more similar to the ST36 from MD (2012–2013) than the historical ST36 from the West Coast. Also, it seems that the outbreak in 2013 in MD of ST36 was also composed by strains from at least three different subgroups within lineage ST36. One subgroup contained the strain responsible for the cases in 2012 and most 2013 cases (VP43, 12, 42, 33, 36, 40, and 38), and the other two subgroups were composed of one (VP32) and two strains (VP30 and VP46). These differences in subgroups within lineage ST36 could have arisen from adaptations to surviving in different oysters/habitats and/or to the introduction of new variants.

This is why WGS-SNP analyses provide clearer insights than earlier methods: many of these strains were unable to be distinguished from one another by MLST and PFGE analysis. The general understanding of *V. parahaemolyticus* evolution suggests that the typical mechanism is recombination, with a recombination ratio estimated at 2.5:1 and 8.8:1 by allele and site, respectively (Gonzalez-Escalona et al., 2008). Example of this are observed among strains belonging to CC345, where all known variants (SLV) of the ancestral ST type of the CC (ST345) have arisen by recombination, mainly in the *recA* allele (González-Escalona et al., in press).

Cluster II and Cluster III were composed by two and three strains each, respectively; no other genomes have been reported for those STs, therefore we could not use a targeted SNP analysis to further analyze those strains. Nevertheless, these clustered with other US strains (**Figure 3**) indicating that they are probably strains endemic to the US. Strains belonging to ST113 were isolated from wound, ear, and stool in MD, suggesting that Clusters II and III are composed of local strains. ST113 was first described for a *V. parahaemolyticus* strain isolated from oysters in Louisiana state in 2007 (Johnson et al., 2009).

The second most numerous ST observed among the MD outbreak strains was Cluster IV (ST631): these strains were highly similar, suggesting that they came from the same region 2 years in a row. In our analyses, the Cluster IV MD strains also clustered with a strain isolated in FL in 2007 (**Figure 4B**). If we fit this data together with the fact that ST631 had initially been reported through a strain isolated in Prince Edward Island (Canada) in 2009 (Banerjee et al., 2014), we have reason to believe that ST631 is a regional ST, endemic to the East Coast.

Finally, Cluster V (ST3), included the MD 3 strains recovered from an outbreak that caused six illnesses during the summer of 2012 (Haendiges et al., 2014). These strains had previously been characterized by Haendiges et al. (2014), using a different phylogenetic tool (BIGSdb genome comparator tool). Due to homopolymer errors (inherent to Ion Torrent sequencing), no further resolution could be observed among strains belonging to Cluster V, so we could not determine whether the strains causing the outbreak were the same or not. The other analyses performed in our study were not impacted by such errors, since the miscalls were pruned from the analysis and only true high quality SNPs were included. With this targeted SNP based analysis, these strains were undistinguishable and can be clearly separated from other ST3 strain which genome were available at NCBI by at least 57 unique SNPs and can conclusively be called as being undistinguishable and probably the same strain and coming from the same source (**Figure 4C**). Therefore, this targeted SNP analysis can be used for outbreak detection caused by a specific *V. parahaemolyticus* strain.

PRESENCE AND ABSENCE OF *tdh* AND *trh* GENES AND THEIR RELATION TO ETIOLOGY

There are five different subtypes of *tdh* genes that differ by sequence (*tdh1* through *tdh5*); these subtypes share 96–98% identity (Nishibuchi and Kaper, 1990). In the case of *trh* genes there are two recognized subtypes (*trh*1 and 2), which share 84% identity (Kishishita et al., 1992). Most of the clinical MD *V. parahaemolyticus* analyzed carried *tdh* and/or *trh* genes (72%) (**Table 1**). Ten isolates (28%) were negative for *tdh* and *trh*, three (8%) were only *tdh* positive, four (11%) were only *trh* positive, and the remaining 19 (53%) isolates contained both genes.

It was unusual to find that 28% of these clinical *V. parahaemolyticus* did not carry *tdh* or *trh*, since these strains are usually considered non-pathogenic (Nishibuchi and Kaper, 1995; Blackstone et al., 2003; DePaola et al., 2003). There have been

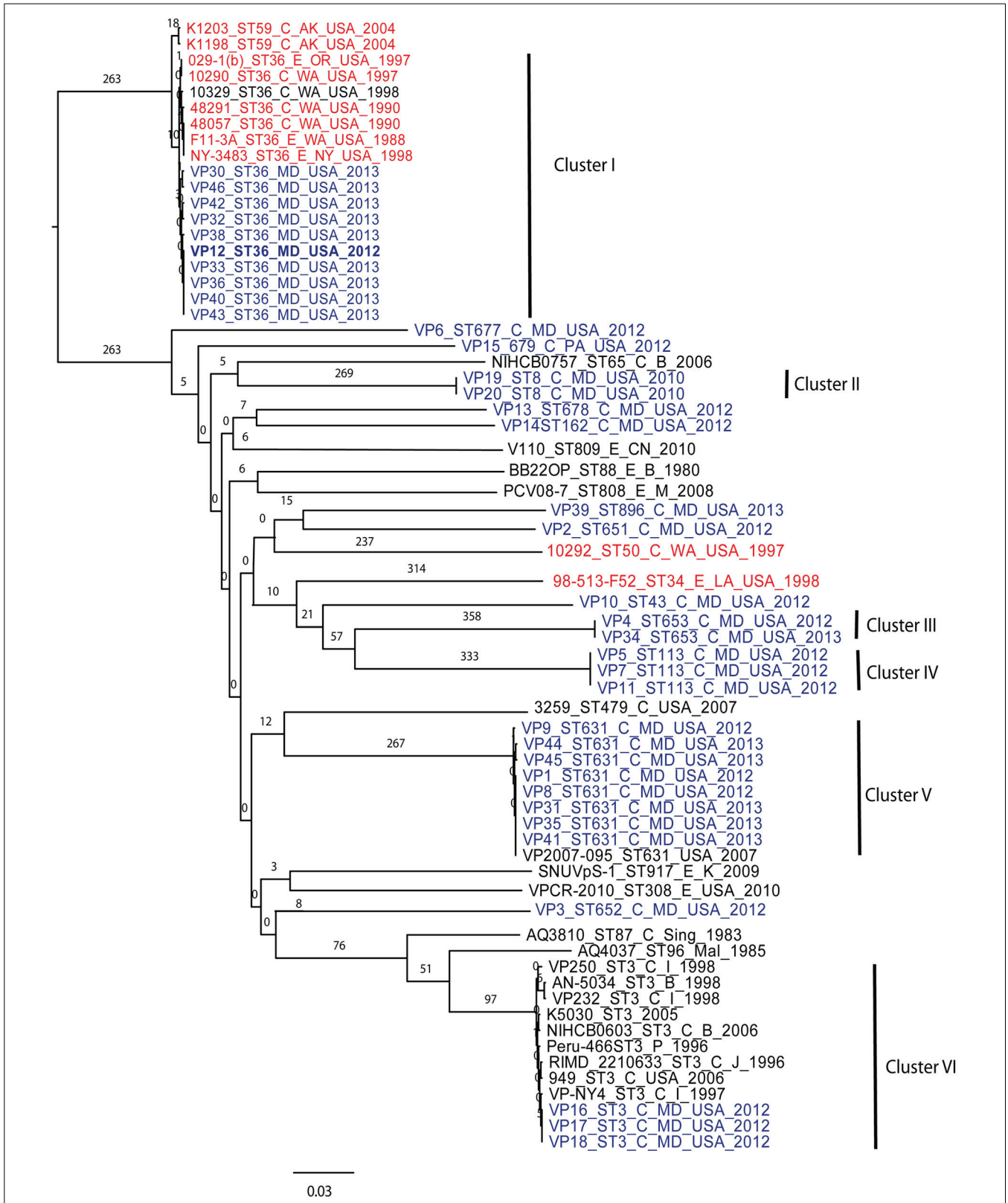

FIGURE 3 | Phylogenetic clustering analysis of the outbreak strains of *V. parahaemolyticus*, Maryland, 2012–2013, by whole-genome SNP analysis. A k-SNP (Timme et al., 2013) analysis was performed. The maximum-likelihood tree shows *V. parahaemolyticus* MD outbreak isolates (in blue fonts), historical US strains sequenced in this study (in red fonts) and other related but unassociated *V. parahaemolyticus* strains (retrieved from NCBI, black fonts). Maximum-likelihood phylogeny was constructed from a

(Continued)

FIGURE 3 | Continued
46,963 SNP matrix. Samples are annotated as follows: strain name, sequence type (ST), source (C, clinical; E, environmental), country of isolation (B, Bangladesh; CN, China; M, Malaysia; Mal, Maldives; USA, United States of America; Sing, Singapore; I, India, P, Peru; and K, Korea), and year when the samples were collected. All gene alignments of the SNPs observed in the entire dataset as well as in each individual cluster are available on request from the authors.

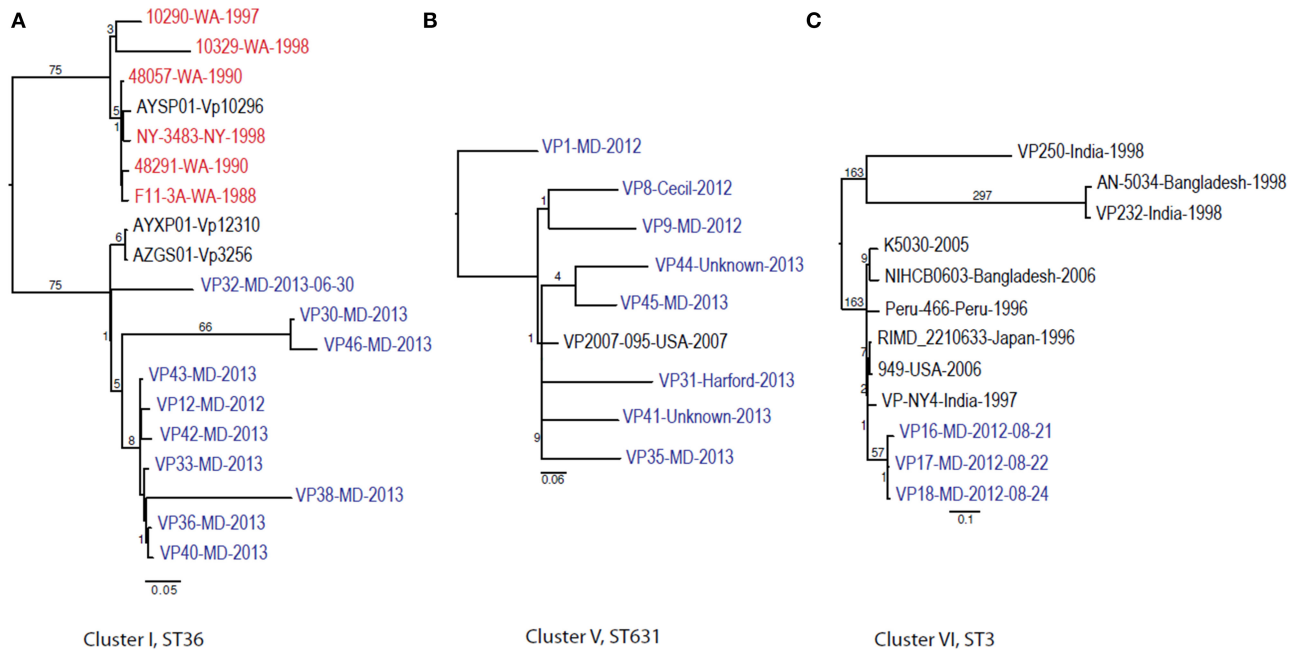

FIGURE 4 | Maximum-likelihood trees for each individual *V. parahaemolyticus* outbreak cluster identified in Figure 3 and other related (if any) but unassociated *V. parahaemolyticus* strains (retrieved from NCBI). Maximum-likelihood phylogeny was constructed from a 347, 931, and 88 core SNP matrix for clusters I, V, and VI, respectively. **(A)** Cluster I, **(B)** cluster V, and **(C)** cluster VI. Samples are annotated as in **Figure 3**. All gene alignments of the SNPs observed in the entire dataset as well as in each individual cluster are available on request from the authors.

some recent reports of *V. parahaemolyticus* without either of these two pathogenicity markers causing sporadic cases of illness (Jones et al., 2012; Banerjee et al., 2014) and having these 10 genomes may help researchers discover what makes these strains pathogenic. Jones et al. (2012) reported that 27% of their clinical isolates collected across US from July 2006 to November 2007 were *tdh/trh* negative while Banerjee et al. (2014) reported the same genotype for 4% of the clinical *V. parahaemolyticus* collected in Canada during 2000–2009. Furthermore, the majority of the *tdh/trh* negative strains (70%) were isolated from either wound or ear infections (**Table 1**). This further indicates that these two markers may not be necessary for human illness to occur (Broberg et al., 2011), although it is possible that these patients had some kind of immune deficiencies that could make them more prone to infections (chronic liver disease), as observed for *V. vulnificus* (Kim et al., 2014). The actual percentage of *tdh/trh* negative clinical strains is currently hard to estimate and might depend on several factors including sample size, *V. parahaemolyticus* endemic populations, type of infection, as well as errors caused by sporadic outbreaks that often go unnoticed or underreported (Scallan et al., 2011).

Most of the *tdh* and/or *trh* positive strains carried *tdh*2 and *trh*1 combination. Most clinical US strains carry both *tdh/trh* (Okuda et al., 1997a; Jones et al., 2012; Paranjpye et al., 2012; Turner et al., 2013) while pandemic strains carried *tdh*1 and *tdh*2 (Okuda et al., 1997b; Matsumoto et al., 2000; Makino et al., 2003; Gonzalez-Escalona et al., 2005). Among the MD *V. parahaemolyticus* strains, only three were both *tdh*1 and two positive and these were identified as belonging to the pandemic clonal complex and were ST3 (Haendiges et al., 2014). Only two MD strains carried exclusively *trh*2. A study of environmental *V. parahaemolyticus* in Norway also identified strains containing only the *trh* gene that were also *trh*2, however those strains belonged different STs than the one observed in this study (Ellingsen et al., 2013). The availability of these genomes would help in future analysis in determining which other genes besides *tdh* and *trh* are necessary for pathogenesis in this marine bacterium.

DISTRIBUTION OF T3SS AND OTHER GENOMIC REGIONS IN MD *V. PARAHAEMOLYTICUS* CLINICAL STRAINS

We performed an *in silico* analysis of each strain's genome using CLC Genome Workbench software (QIAGEN) for 24 known regions described for pandemic strains, including both T3SS, T3SS1 and 2α (Boyd et al., 2008) and well as for T3SS2β (Park et al., 2000), containing the *ure* genes and *trh* gene and described previously for strains carrying both *tdh* and *trh*

genes (Supplementary Table 1). All strains carried NK, T3SS-1, Osmotolerance (chromosome I), Gametolysin, Osmotolerance (chromosome II), CPS, Type I secretion, Type I pilus, Multidrug efflux, and Ferric uptake. Most strains lacked all the pathogenicity islands described for the pandemic strain RIMD2210633 (VPaI1-7). Four other MD strains carried either VpaI1 (VP10) or VpaI2 (VP2, and VP14), although carrying a sequence different from that of the pandemic strain. Of the nine MD strains lacking *tdh* or *trh* gene, six carried a T6SS different from the pandemic, for the presence/absent of the other elements or PIs were very similar to the strains carrying *tdh*, *trh*, or *tdh/trh* strains. Seven of these strains were isolated from wound or ear infections. Since *tdh/trh* and T3SS2s were described as needed for producing infections at the intestinal level [because *tdh/trh* causes intestinal fluid secretion as well as cytotoxicity in a variety of cell types (Raimondi et al., 2000) while TTSS2 has a role in enterotoxicity (Park et al., 2004)], then these strains could have caused infections by a rather opportunistic way than by an actual infection as observed for gastroenteritis. These strains still possess TSS1 which is known for being involved in the cytotoxicity (Park et al., 2004). A more detailed study could reveal additional elements we did were not able to assess in this study; such analyses will certainly shed more light on the infection mechanisms of these unusual *V. parahaemolyticus* strains.

CONCLUSIONS

Our study used WGS to provide a high resolution investigation of genetic diversity and relationships of clinical *V. parahaemolyticus* strains isolated in the state of Maryland during 2012–2013 period in comparison to those isolated from other geographical locations. Having an archive of *V. parahaemolyticus* genomes allowed us to perform a phylogenetic analysis of strains and revealed that strains causing outbreaks in MD were highly diverse, genetically distinct, and clearly different from other regional and global strains. Despite the geographic breadth of our strains, we are aware that our study captured only a fraction of the actual number of *V. parahaemolyticus* strains causing illness in the state of Maryland, due in part to the sporadic nature of outbreaks, the low number of strains recovered per outbreak, and the fact that many outbreaks are never investigated (Scallan et al., 2011).

We suggest using a similar approach during future outbreaks to analyze the identity of suspect *V. parahaemolyticus* strains and their relationship to strains causing previous outbreaks. Our WGS analysis of *V. parahaemolyticus* strains highlights the need for a genome database for *V. parahaemolyticus* to improve traceback and responses to outbreaks. We have created a new BioProject (PRJNA245882) at NCBI, spearheaded by FDA-CFSAN and the state of Maryland's DHMH, in which we are going to continually deposit new available genomes of *V. parahaemolyticus* causing outbreaks that would improve detection of new outbreak strains, track the emergence of new clonal strains in geographical regions where these strains are not endemic, and will be used in source tracking. We suggest and encourage the scientific community to deposit further *V. parahaemolyticus* genomes from their respective countries in centralize databases, such as NCBI, in order to improve source tracking.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2015.00125/abstract

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VgrG2 of type VI secretion system 2 of *Vibrio parahaemolyticus* induces autophagy in macrophages

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Type VI secretion system (T6SS) is a macromolecular transenvelope machine encoded within the genomes of several proteobacteria species. *Vibrio parahaemolyticus* contains two putative T6SS systems, VpT6SS1 and VpT6SS2, both contributing to adherence to Caco-2 and/or HeLa cells. However, it remains unknown if these systems are involved in cellular responses. In order to exclude the effects of other virulence factors known to induce cytotoxicity or autophagy, a triple deletion mutant dTTT (with deletion of *tdh*, and T3SS1 and T3SS2 structural protein genes) was used as the parent strain to construct deletion mutants of T6SS genes. The mutant dTTT-Δ*icmF2*, but not dTTT-Δ*icmF1*, reduced autophagic response upon 4 h of infection of the macrophage. Further attempt was made to search for the possible effector proteins that might be responsible for direct induction of autophagy by deletion of the genes encoding Hcp2 and VgrG2, two putative translocons of T6SS2 of *V. parahaemolyticus*. Deletion of either *hcp2* or *vgrG2* did reduce the autophagic response. However, increased LC3-II lipidation was seen only in the macrophage cells transfected with pVgrG2, but not with pHcp2. Chloroquinine treatment increased accumulation of LC3-II, suggesting that VgrG2 enhanced autophagic flux. The fact that *vgrG2* deletion led to reduced level of intracellular cAMP suggests a possible role of cAMP signaling in autophagic responses to the bacterium. We conclude that VgrG2 of *V. parahaemolyticus* induces autophagy in macrophages.

Keywords: *Vibrio parahaemolyticus*, type VI secretion system, secretion system, effector proteins, autophagy

INTRODUCTION

Vibrio parahaemolyticus is one of the leading causes of human foodborne gastroenteritis in China (about one-third of the foodborne outbreaks reported between 1991 and 2001) due to consumption of raw or under-cooked seafood (Liu et al., 2004). Early studies have linked gastroenteritis to the presence of thermostable direct hemolysin (TDH), TDH-related hemolysin (TRH) and two sets of type III secretion systems (T3SS1 and T3SS2), which are able to induce general cytotoxicity or enterotoxicity to host cells (Kaper et al., 1984; Park et al., 2004; Yeung and Boor, 2004; Okada et al., 2009).

Type VI secretion system is a macromolecular transenvelope machine encoded within the genomes of several proteobacterial species (Mougous et al., 2006; Pukatzki et al., 2007; Bingle et al., 2008; Bernard et al., 2010). The system contains 13–20 proteins [Intracellular multiplication Factor (IcmF)-associated homologous proteins, IAHP] coded by the gene cluster (Boyer et al., 2009). Deletion of *icmF* associated proteins did not affect expression of the translocon proteins but prevents their translocation (Pukatzki et al., 2006; Suarez et al., 2008). The T6SSs of *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, and *Vibrio anguillarum* were found to participate in pathogenicity: adhesion to epithelial cells, cytotoxicity, resistance to phagocytosis, tolerance to stress sensing, and replication inside the host cells (Mougous et al., 2006; Pukatzki et al., 2006; Zheng and Leung, 2007; Suarez et al., 2008; Weber et al., 2009; Jani and Cotter, 2010). Of the two sets of putative T6SS in *V. parahaemolyticus* (VpT6SS), we found that VpT6SS1 is present in majority of clinical isolates (90.9%), but less in environmental or food isolates (25.0%) while VpT6SS2 exists in all isolates, and both systems contribute different aspects of adherence to Caco-2 and/or HeLa cells (Yu et al., 2012).

Autophagy acts as an intracellular surveillance system to monitor and trap invading pathogens and influence both the innate and adaptive immune responses (Burdette et al., 2009b; Deretic and Levine, 2009). For most intracellular bacteria, host cells use autophagy to prevent cytoplasmic replication or invasion of intracellular pathogens by engulfing the pathogens in autophagic vesicles and targeting them to lysosomes (Levine and Deretic, 2007). In extracellular bacteria like *Vibrio* spp, secreted proteins are involved in autophagy (Gutierrez et al., 2007). With *V. parahaemolyticus*, VP1680 (VopQ) and VP1659 (two secretion proteins of T3SS1) are found to induce autophagy accompanied with cytotoxicity, disruption of actin structure, and cell death (Burdette et al., 2009a; Zhou et al., 2010). By examining LC3 lipidation and EGFP-LC3 punctation in macrophages infected with *V. parahaemolyticus*, we provide the first evidence that the VgrG2, a translocon of VpT6SS2, induces autophagy.

MATERIALS AND METHODS

BACTERIAL STRAINS AND PLASMIDS

The bacterial strains and plasmids used in this study are listed in **Table 1**. *V. parahaemolyticus* strain HZ is a clinical isolate

Table 1 | Bacterial strains and plasmids used in this study.

Plasmids or strains	Description	Reference or source
Plasmids		
pMD18T	A clone vector, Amp^r	Takara
pYAK1	A suicide vector with ori R6K *sacB*; Cm^r	Park et al. (2004)
pET-30a	PBR322 origin, pT7, *his*-tag	Novagen
pcDNA3.1		Invitrogen
pcDNA-*egfp*		Zhu et al. (2012)
pcDNA-*egfp-lc3b*		Zhu et al. (2012)
pHcp2	pcDNA-*egfp* fused with *hcp2* of VpT6SS2	This study
pVgrG2	pcDNA-*egfp* fused with *vgrG2* of VpT6SS2	This study
Escherichia coli		
CC118λpir	Λpir lysogen of CC118 Δ(*ara-leu*) *araD*Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1*	Yu et al. (2012)
DH5α	F^- φ80*lacZ*Δ*M15* Δ*(lacZYA-argF)* U169 *deoR recA1 endA1 hsdR17 phoA supE44* λ^- *thi-1 gyrA96 relA1*	Invitrogen
BL21		Novagen
Vibrio parahaemolyticus		
HZ	Wild type (WT), clinical strain, Cm^s	Yu et al. (2012)
dTTT	Strain HZ with in-frame deletion of *tdh*, *vcrD1*, and *vcrD2*	Yu et al. (2012)
Δ*icmF1*	Strain dTTT with in-frame deletion of *icmF1*	Yu et al. (2012)
Δ*icmF2*	Strain dTTT with in-frame deletion of *icmF2*	Yu et al. (2012)
Δ*icmF1/icmF2*	Strain dTTT with in-frame deletion of *icmF1* and *icmF2*	Yu et al. (2012)
Δ*hcp2*	Strain dTTT with in-frame deletion of *hcp2*	Yu et al. (2012)
Δ*vgrG2*	Strain dTTT with in-frame deletion of *vgrG2*	This study

from the Zhejiang Provincial Center for Disease Control and Prevention, Zhejiang, China. *Escherichia coli* strains DH5α, BL21, and CC118λpir were used for general manipulation of plasmids, prokaryotic expression of proteins, and mobilization of plasmids into *V. parahaemolyticus*, respectively. The bacterial strains were grown at 37°C in Luria-Bertani (LB) broth (*E. coli*) or LB broth supplemented with 3% NaCl (*V. parahaemolyticus*). LB agar supplemented with 3% NaCl, 10 μg/ml chloramphenicol, and 25 μg/ml polymyxin was used for screening mutant strains. The culture media were supplemented, where appropriate, with the following antibiotics: chloramphenicol (Cm, 10 μg/ml), ampicillin (Amp, 100 μg/ml), and kanamycin (Kan, 50 μg/ml).

PLASMID CONSTRUCTION

The mammalian expression vector pcDNA-*egfp* and pcDNA-*egfp-lc3b* were constructed from pcDNA3.1 (Invitrogen) in our laboratory (Zhu et al., 2012). To construct pHcp2 and pVgrG2 in pcDNA3.1 background for expression of these proteins fused with GFP (**Table 1**), *egfp* was PCR-amplified from pcDNA-*egfp* by primers GFP-F/R, and genes *hcp2* and *vgrG2* were from *V. parahemolyticus* strain HZ amplified by primers *hcp2*-F/R and *vgrG2*-F/R, respectively. The *egfp-hcp2* and *egfp-vgrG2* fusion fragments were obtained by overlap PCR using primers GFP-F/*hcp2*-R and GFP-F/*vgrG2*-R, respectively, and cloned into the multiple cloning site of pcDNA3.1. The above primers are listed in **Table 2**. All constructs were confirmed by DNA sequencing.

HOMOLOGOUS RECOMBINATION

In-frame gene deletion of *vgrG2* was generated by *sacB*-based allelic exchange as described previously (Park et al., 2004; Yu et al., 2012). Briefly, PCR amplification was performed to generate the upstream and downstream fragments of the *vgrG2* gene (using respective primer pair vgrG2-A/B and vgrG2-C/D, **Table 2**). Overlap PCR was performed to construct a fragment with deletion of the *vgrG2* gene using the primer pair vgrG2-A/D. The fragment was cloned into pMD18T vector (Takara) and then subcloned into the suicide vector pYAK1 that contains the *sacB* gene conferring sensitivity to sucrose. The recombinant plasmid was introduced into *E. coli* CC118λpir and then mated with *V. parahaemolyticus* dTTT (strain HZ with in-frame deletion of *tdh*, *vcrD1*, and *vcrD2*)

Table 2 | Primers used in this study.

Primers	Sequence (5′–3′)	Reference
EGFP-F	TAGGATTCGCCACCATGGTGAGCAAGGGCGA	Zhu et al. (2012)
EGFP-R	TCCTCCGCTTCCTCCCTTGTACAGCTCGTCCAT	
hcp2-F	GGAGGAAGCGGAGGAATGCAGTCTAATAC	This study
hcp2-R	GAACTCGAGTTACATTTGTTGACCT	
vgrG2-F	GGAGGAAGCGGAGGAATGAAAAAAGCAAGTC	This study
vgrG2-R	GGCCTCGAGTTAATTCAAAGAGATT	
hcp2-KF	CACGGATCCATGCAGTCTAATAC	This study
hcp2-KR	GAACTCGAGTTACATTTGTTGACCT	
vgrG2-KF	AAAGGATCCATGAAAAAAGCAAGTC	This study
vgrG2-KR	GGCCTCGAGTTAATTCAAAGAGATT	
vgrG2-A	AAAGGATCCTTGTACTTGGATGACCACC	This study
vgrG2-B	GTATCCAGAGGGAACTTAGAATGGGTAAAC	
vgrG2-C	GTTCCCTCTGGATACTTATATTTCCTTTTGAA	
vgrG2-D	ATTGCATGCAAGCGACAGCGGA	
vgrG2-E	CTAACTTGCACTTCCTCATCGTC	
vgrG2-F	CTTCAAGATCGTTCGTCTCC	
sacB-F	ACGGCACTGTCGCAAACTAT	Yu et al. (2012)
sacB-R	TTCCGTCACCGTCAAAGAT	

as the parent strain. The resulting mutant strains were screened using selective LB agar as specified above.

PREPARATION OF PROTEIN SAMPLES FROM BACTERIAL SUPERNATANTS AND PELLETS

Secreted proteins from the parent and mutant *V. parahaemolyticus* strains were prepared from the supernatant samples of cultures grown for 16 h at 28°C in LB broth. The samples were passed through a 0.2 μm pore-size syringe filter and precipitated by adding trichloroacetic acid to a final concentration of 10% (vol/vol). The proteins were collected by centrifugation at 15,000 *g* for 30 min at 4°C. The precipitates were solubilized in 40 μl 0.1M NaOH, and 10 μl of 5x SDS-PAGE loading buffer was added prior to SDS-PAGE with 10% polyacrylamide. For separation of T6SS proteins associated with the bacterial cells, *V. parahaemolyticus* cultures were pelleted by centrifugation, and the pellets were resuspended in 10 mM phosphate buffered saline pH 7.2 (PBS, 100 mg wet weight pellet per ml). A volume of 160 μl was then mixed with 10 μl of 5X SDS-PAGE loading buffer, and the mixtures were heat-treated for 5 min in a boiling water-bath to release proteins from the bacterial cells before SDS-PAGE.

CELLS CULTURE, BACTERIAL INFECTION, AND VECTOR TRANSFECTION

Murine RAW264.7 macrophage cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% new-born calf serum, L-glutamine (1%), penicillin G (100 U/ml), and streptomycin (100 μg/ml).

The macrophage cells were infected with mid-log phase cultures (3–4 h) of *V. parahaemolyticus* wild-type strain (WT) HZ, and single or multiple deletion mutants at multiplicity of infection (MOI) of 10 at 37°C and 5% CO_2. Infection was allowed to proceed for 0.5 h and the supernatant was then removed. The cell monolayers were rinsed with sterile PBS to remove unattached bacteria and then incubated in the presence of fresh medium for 2 or 4 h at 37°C and 5% CO_2 for cytotoxicity assay or SDS-PAGE and immunoblotting as described below. Rapamycin treated (0.5 μM, Merck) or mock-infected cells were included as controls (here and in other relevant experiments).

The cells stably expressing GFP-LC3B on the coverslips were infected with the strain dTTT or dTTT-Δ*icmF2* at MOI of 10. The remaining procedure is the same as above. At the end of 4 h incubation, the cell monolayers were subjected for confocal microscopic imaging.

The macrophage cells were transfected with pcDNA-*egfp*, pHcp2, or pVgrG2 using lipofectamine 2000 (Invitrogen) for transient expression of the target proteins. The transfection medium was replaced 6 h post-transfection by complete medium containing G418 (0.5 mg/ml), and the cells were incubated for 24 h at 37°C and 5% CO_2 for analysis of LC3 lipidation by immunoblotting.

For examination of the effects of 3-methyladenine (3-MA) and chloroquinine (CQ) on autophagic response, the RAW264.7 cells were first treated with these agents (10 mM 3-MA, and 5 mM CQ) for 1 h before they were transfected with vectors pHcp2, pVgrG2, and pcDNA-*gfp* as described above. The cell pellets were then collected for SDS-PAGE/Western blotting for LC3-II and β-actin.

ANALYSIS OF cAMP IN MACROPHAGES INFECTED WITH *Vibrio parahaemolyticus* STRAINS

For measurement of cAMP within macrophages infected with the bacterial strains, the RAW264.7 cell monolayers were infected with the parent and mutant strains (triple deletion mutant dTTT with additional deletion of *hcp2* or *vgrG2*, namely dTTT-Δ*hcp2* or dTTT-Δ*vgrG2*; about 10 MOI). After 4 h of incubation at 37°C and 5% CO_2, the cell monolayers were washed twice with PBS and lysed with deionized H_2O for 10 min followed by repeated pipetting and vortex-mixing in Eppendorf tubes. The cell lysates were subjected to centrifugation as above and the supernatant samples were collected for analysis of intracellular cAMP using the ELISA-based cAMP assay kit (detection range from 0.5 to 6 nM, lot No HL30028, Shanghai Haling Biol. Technol. Co., Ltd., China) according to the manufacturer's instruction.

SDS-PAGE AND IMMUNOBLOTTING

After infection or transfection for indicated times, all cell samples were lysed for 10 min in ice cold lysis buffer [50 mM Tris-HCl pH 8.0, 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% NP-40 with complete protease inhibitor cocktail (Roche)]. Cell debris was pelleted by centrifugation and clear supernatants transferred to new tubes. Protein concentration was measured by BCA protein assay kit (MultiSciences, Hangzhou, China). Protein samples were boiled for 5 min in the presence of 5x SDS-PAGE loading buffer.

Proteins on the gels were electro-transferred onto an Immobilon-P membrane (Millipore). The membranes were blocked with 5% skim milk in tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.6) containing 0.05% Tween 20 and probed for 1 h with rabbit anti-Hcp2 or rabbit anti-VgrG2 polyclonal antibodies (Yu et al., 2012), rabbit anti-LC3 polyclonal IgG (Sigma–Aldrich) or anti-β-actin monoclonal IgG (MultiSciences, Hangzhou, China) for 3 h at room temperature. The blots were then probed with goat anti-rabbit or anti-mouse horse-radish peroxidase-labeled antibodies (KPL), and developed by the Super-Signala West Pico Chemiluminescent Substrate (Pierce) according to the manufacturer's instruction.

CONFOCAL MICROSCOPY

Cells infected as above were washed with PBS, fixed and permeabilized with 80% cold acetone in PBS at −20°C for 20 min, and washed again with PBS. Cells were then counterstained with 4′,6-diamidino-2-phenylindole (DAPI) nucleic acid stain (Invitrogen). Fluorescence was observed under a laser scanning confocal microscope (Leica TCS SP5, Munich, Germany). The average number of EGFP-LC3 punctae per cell from at least 60 cells per sample was counted (Mizushima et al., 2010).

CYTOTOXICITY ASSAY

The culture supernatants from infected macrophage cells as above were collected for lactate dehydrogenase activity (LDH) using CytoTox 96 non-radioactive cytotoxicity assay (Promega).

STATISTICAL ANALYSIS

All data, where appropriate, are mean ± SD from three independent experiments and analyzed by Student's *t*-test.

RESULTS

T6SS2 INDUCES AUTOPHAGY IN MACROPHAGE CELLS

Our preliminary data indicate that there was no apparent autophagic response of the macrophage cells in 2-h infection with *V. parahaemolyticus*, either the WT strain or its triple deletion mutant strain dTTT void of *tdh*, *vcrD1,* and *vcrD2* with or without additional deletion of *icmF1* and *icmF2* (structural genes of VpT6SS1 and VpT6SS2, respectively; data now shown). When infection continued up to 4 h, T3SS1 was found to be involved in autophagy shown as reduced LC3-II transformation in the macrophage cells infected with Δ*vcrD1* deletion mutant (**Figure 1A**), as previously reported (Burdette et al., 2009a; Zhou et al., 2010). In cells infected with the WT strain or its mutants Δ*tdh* and Δ*vcrD1*, significant cytotoxic effect at 4 h was seen possibly due to T3SS1. To exclude the confounding effects of cytotoxicity, the triple deletion mutant (dTTT, in-frame deletion of *tdh*, *vcrD1,* and *vcrD2*) was used as the parent strain to examine the possible role of VpT6SS in autophagy after further deletion of both *icmF1* and *icmF2*.

Initial experiments with the multiple deletion strain did show reduced LC3 lipidation upon further deletion of both *icmF1 and icmF2* [dTTT-Δ*icmF1/2*, as compared with its parent strain dTTT or rapamycin positive control (**Figure 1A**)]. To explore which VpT6SS system triggered autophagy of macrophages, further deletion of *icmF1* or *icmF2* was attempted from the triple mutant strain dTTT. **Figures 1B,C** shows that LC3 lipidation was significantly reduced in dTTT-Δ*icmF1/2* or dTTT-Δ*icmF2*, but not dTTT-Δ*icmF1* ($P < 0.01$, as compared with its parent strain dTTT; **Figure 1C**), indicating that VpT6SS2 might be involved in the autophagic response. TDH and/or T3SS, but not VpT6SS contribute to cytotoxicity as revealed by lactate dehydrogenase release (**Figure 1D**). To further confirm whether VpT6SS2 was indeed related to autophagy and induce the formation of autophagosomes, the EGFP-LC3-expressing macrophage cells were infected with *V. parahaemolyticus* dTTT and its mutant dTTT-Δ*icmF2*. **Figure 2** shows that the mutant dTTT-Δ*icmF2* exhibited significantly reduced number of cells containing punctae of EGFP-LC3 (i.e., autophagosome-like vesicles), as compared with its parent strain dTTT ($P < 0.01$).

VgrG2 OF VpT6SS2 IS THE EFFECTOR PROTEIN DEVOTING TO AUTOPHAGY

A number of Gram-negative bacteria use T6SS to infect eukaryotic cells by its effectors, translocon, or secretion proteins (Cascales, 2008; Pukatzki et al., 2009). We paid attention to Hcp2 and VgrG2, two known translocon family proteins of VpT6SS2 (Yu et al., 2012). Deletion of either *hcp2* or *vgrG2* decreased the ratio of LC3-II to β-actin in cells infected with the mutant strains in comparison with its parent strain dTTT (**Figure 3**, $P < 0.05$), indicating that Hcp2, VgrG2, or even other secretion proteins translocated by them might contribute to autophagy in the macrophage cells.

To further examine if Hcp2 or VgrG2 of VpT6SS devotes to autophagy, plasmids pHcp2, and pVgrG2 were made based on pcDNA-*egfp* (Zhu et al., 2012) to express EGFP-Hcp2 and EGFP-VgrG2 in macrophage cells after transfection for 24 h. We found that VgrG2, but not Hcp2, increased the conversion from LC3-I to LC3-II with significant elevation of LC3-II/β-actin ratio ($P < 0.05$) in comparison with the mock pcDNA-*egfp* (**Figures 4A,B**).

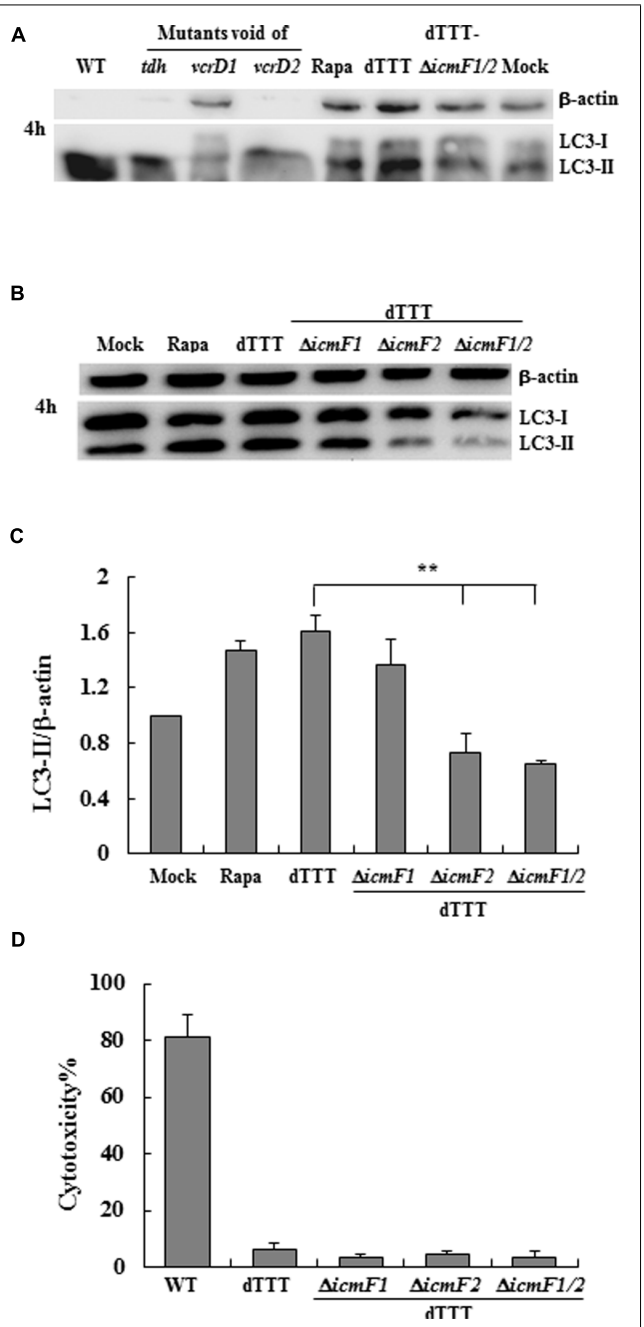

FIGURE 1 | *Vibrio parahaemolyticus* T6SS2 induces autophagy of macrophage cells without causing apparent cytotoxicity. (A,B) Autophagy of macrophage cells was measured by LC3-II accumulation after infection for 4 h with the wild-type strain (WT), its triple deletion mutant dTTT or dTTT with further deletion of T6SS1 and T6SS2 structural genes. Rapamycin-treated (Rapa) and mock-infected (Mock) cells were used as positive and negative controls. **(C)** LC3-II to β-actin ratio (mean ± SD) of three independent experiments as shown in **(B**; $^{**}P < 0.01$). **(D)** Cytotoxicity to macrophages infected as above was measured by LDH release.

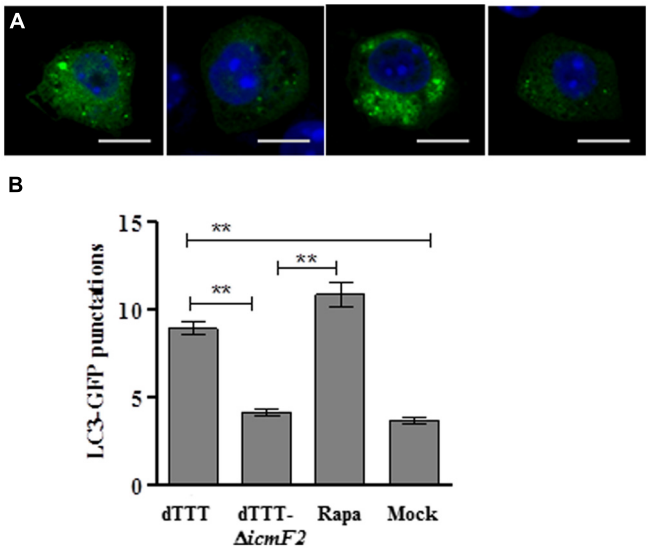

FIGURE 2 | *Vibrio parahaemolyticus* T6SS2 induces autophagosome formation in macrophages. (A) Formation of autophagosome vesicles shown as green punctae in EGFP-LC3 expressing macrophage cells infected with strain dTTT and its *icmF2* deletion mutant. Rapamycin-treated (Rapa) and mock-infected (Mock) cells were used as positive and negative controls (scale bar: 10 μm). **(B)** Mean ± SD of EGFP-LC3-II punctae per cell from 60 cells of three independent experiments as represented in (**A**; $^{**}P < 0.01$).

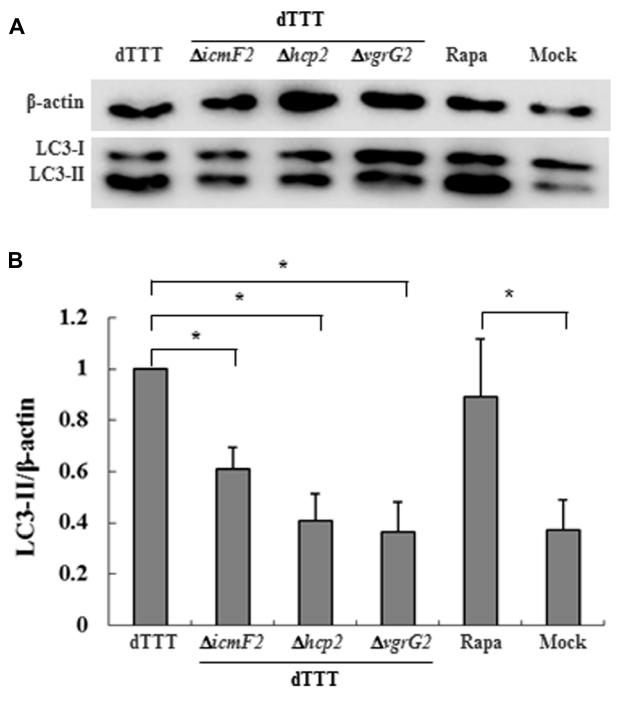

FIGURE 3 | *Vibrio parahaemolyticus* T6SS2 translocons might be involved in autophagic response of macrophages. (A) LC3-II accumulation in cells infected for 4 h with strain dTTT and its mutants. Rapamycin-treated (Rapa) and mock-infected (Mock) cells were used as positive and negative controls. **(B)** Ratios of LC3-II to β-actin were calculated from '**A**' and reported as mean ± SD of three independent experiments with the dTTT ratio normalized to 1.0 ($^{*}P < 0.05$).

RAW264.7 cells were pretreated with P13-kinase inhibitors 3-MA and lysosome-phagosome fusion inhibitors chloroquinine. 3-MA is competent in its ability to inhibit induction of autophagy by rapamycin, a TOR kinase inhibitor, and well-characterized inducer of autophagy (Burdette et al., 2009a). The VgrG2-induced autophagy appears to be dependent of PI3-kinase activation because treatment with 3-MA repressed VgrG2-induced LC3-II accumulation (**Figures 4C,D**). Chloroquinine is a inhibitor of lysosome–phagosome fusion, which blocks degradation of LC3-II (Menzies et al., 2012). Chloroquinine treatment increased LC3-II lipidation in VgrG2-expressing cells, suggesting that VgrG2 enhanced the autophagic flux. These results indicate that VgrG2 of *V. parahaemolyticus* induces autophagy by targeting the initial events of autophagic signaling.

Besides, Cyclic AMP is known to activate AMPK which might contribute to autophagy due to repression of mTOR (Shaw, 2009). **Figure 5** indicates that the ΔvgrG2 mutant infected cells showed similar level of intracellular cAMP to the mock-infected cells, but had significantly lower level of intracellular cAMP than its parent strain, suggesting that *V. parahaemolyticus* vgrG2 might activate the cAMP signaling pathway to induce the autophagic response.

DISCUSSION

Bacterial T6SS is a newly found secretion system which could be involved in pathogenesis and environment adaptation (Schwarz et al., 2010). Our previous study reveals that both T6SS1 and T6SS2 of *V. parahaemolyticus* contribute to adherence to mammalian cells (Yu et al., 2012). Here, we further show that T6SS2 of *V. parahaemolyticus* is involved in autophagic response of macrophages RAW264.7.

Vibrio parahaemolyticus contains several cytotoxic factors such as TDH and effector proteins of T3SS1 and T3SS2 which disrupt mammalian cell structure and lead to release of cellular contents (Burdette et al., 2008, 2009a; Zhou et al., 2010). In this study, we found the absence of β-actin bands and altered motility patterns of LC3 in cells infected with the WT, Δ*tdh* and Δ*vcrD2* strains, but not in the Δ*vcrD1* mutant. This might indicate that T3SS1 contributed to loss of β-actin as a result of acute autophagy accompanied with cell wall disruption and release of cellular contents, as shown in previous studies (Burdette et al., 2009a,b; Zhou et al., 2010). Recent studies show that two T3SS1 effectors, VopQ and Vp1659, contribute to autophagy in eukaryotic cells (Burdette et al., 2009a; Zhou et al., 2010). In order to examine autophagy induction by T6SS independent of other factors and to avoid interference by cytotoxic effects, we constructed T6SS deletion mutants based on the triple deletion mutant void of *tdh*, *vcrD1*, and *vcrD2* (strain dTTT) as the parent strain. It is clear that the triple deletion mutant dTTT and the mutant strains having further deletions of T6SS genes only showed marginal cytotoxicity as shown by LDH release.

Initially, we found that the multiple deletion mutant dTTT-Δ*icmF2*, but not dTTT-Δ*icmF1*, reduced autophagic response upon 4 h of infection of the macrophage, suggesting involvement of the putative T6SS2. Since *icmF1* or *icmF2* is the inner membrane protein of T6SS (Cascales, 2008), we attempted to search for

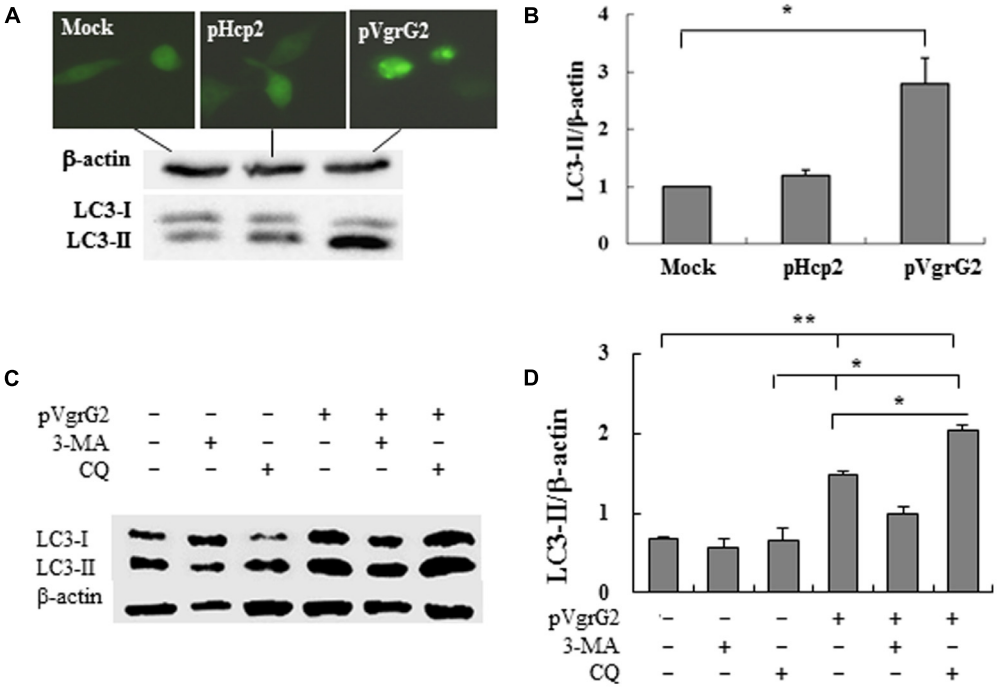

FIGURE 4 | *Vibrio parahaemolyticus* T6SS2 VgrG2 is the effector protein inducing autophagy of macrophages. (A) Hcp2 and VgrG2 protein were expressed in macrophage cells as the EGFP fusion proteins. LC3-II accumulation was measured by immunoblotting. **(B)** Ratios of LC3-II to β-actin of '**A**' were calculated and reported as mean ± SD of three independent experiments with the ratio of Mock cells (transfected with the control vector pcDNA-*egfp*) normalized to 1.0. **(C)** Macrophage cells were pretreated for 1 h with 3-methyl adenine (3-MA) or chloroquinine (CQ) and then transfected with pVgrG2 or pcDNA-*egfp* for analysis of LC3-II by Western blotting. **(D)** Ratios of LC3-II to β-actin were calculated and reported as mean ± SEM of three independent experiments. $*P < 0.05$; $**P < 0.01$.

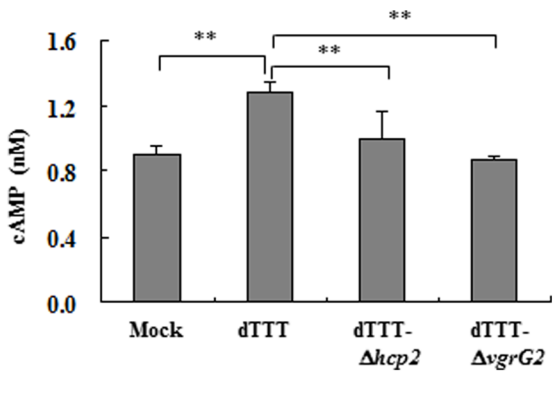

FIGURE 5 | Intracellular cAMP of macrophages infected with *V. parahaemolyticus* vgrG2 deletion mutant was significantly reduced as compared with its parent triple deletion strain dTTT. The confluent RAW264.7 cell monolayers were infected with bacterial strains, each in triplicate wells, for 4 h. The culture supernatants were then removed, and the cell monolayers washed and lysed for cAMP determination. Each datum point represents mean ± SD of triplicate wells ($**P < 0.01$).

the possible effector proteins that could be responsible for direct induction of autophagy by further deletion of the genes encoding Hcp2 and VgrG2, two putative translocons of T6SS2 seen in several bacterial species (Cascales, 2008) and also in T6SS2 of *V. parahaemolyticus* according to our previous study (Yu et al., 2012). Deletion of either *hcp2* or *vgrG2* did lessen the autophagic response as shown by decreased ratio of LC3-II to β-actin. However, increased LC3-II lipidation was seen only in the macrophage cells transfected with pVgrG2, but not with pHcp2. Therefore, we conclude that VgrG2, but not Hcp2, is the autophagy inducer of *V. parahaemolyticus*. Hcp might form as the tubular structure of VpT6SS for effector proteins including VgrG to translocate across the bacterial cell wall (Cascales and Cambillau, 2012). Deletion of *hcp2* could apparently prevent VgrG2 translocation, leading to decreased LC3-II.

The fact that chloroquinine treatment caused accumulation of LC3-II indicates that VgrG2 could probably act on the upstream autophagic pathways. We also provide evidence that the cAMP signaling might be involved in autophagy induction by VgrG2 of *V. parahaemolyticus* since *vgrG2* deletion led to reduced level of intracellular cAMP as compared with its parent strain. Different pathogens might explore distinct mechanisms in autophagic responses. *Staphylococcus aureus* was reported to enhance non-canonical autophagic response by its α-hemolysin in a PI3K/Beclin1-independent way, which is inhibitable by treating the cells with a permeable dibutyryl cAMP (a cAMP analog; Mestre and Colombo, 2012). They found that such inhibition was related to recruitment of Epac (Rap guanine nucleotide exchange factor/exchange protein activated by cAMP) and Rap2b via calpain activation. However, Chen et al. (2013) show that cAMP was necessary for resveratrol-induced PRKA-AMPK-SIRT1

activation of autophagy in human umbilical vein endothelial cells treated with this naturally occurring phytoalexin compound). Therefore, the possible mechanisms of cAMP signaling in *V. parahaemolyticus* VgrG2 induced autophagy require further investigation.

In conclusion, *V. parahaemolyticus* is clearly equipped with two arms of autophagy inducers, T3SS1 and T6SS2. However, T6SS2 induces autophagy without involving cytotoxic effect as seen in T3SS1.

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A pandemic *Vibrio parahaemolyticus* O3:K6 clone causing most associated diarrhea cases in the Pacific Northwest coast of Mexico

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Between September and October of 2004, more than 1230 cases of gastroenteritis due to pandemic O3:K6 strains of *Vibrio parahaemolyticus* (*V. parahaemolyticus*) were reported in the relatively small geographical area of Southern Sinaloa, a state located in Northwest Mexico. Since then, *V. parahaemolyticus*-associated gastroenteritis cases have gradually increased in prevalence spreading from south to north. The present study conducted an epidemiological surveillance of *V. parahaemolyticus* strains in both environmental and clinical samples along the Pacific coast of Sinaloa from 2011 to 2013. The genetic relatedness, serotype dominance and antibiotic resistance of isolates were investigated. A total of 46 strains were isolated from environmental samples (e.g., sediment, seawater and shrimp), whereas 249 strains were obtained from stools of patients with gastroenteritis. Nine different O serogroups and 16 serovars were identified. Serovars O3:K6 and O6:K46 were identified in both environmental and clinical strains. Whereas most environmental isolates carried the *tdh* gene (71.74%, 33/46), only three (6.52%) belonged to pandemic clones (O3:K6, O3:KUT and OUT:KUT). In contrast, 81.1% (202/249) of clinical isolates belonged to pandemic serotypes, with O3:K6 (*tdh*, *toxRS/new*, and/or *orf8*) representing the predominant serovar (97%, 196/202). This prevalence of pathogenic (*tdh* and/or *trh* positive) and O3:K6 pandemic *V. parahaemolyticus* isolates in this study were similar to those found from 2004 to 2010. As investigated by REP-PCR, genetic lineages of selected O3:K6 strains isolated in this study and some isolated earlier were nearly identical. Antimicrobial susceptibility testing showed that most strains (93.8%) were resistant to ampicillin but sensitive to *chloramphenicol* (98.8%). Multidrug resistance significantly increased from 8.6% (2004–2010) to 22.93% (2011–2013; $p < 0.05$). Our data indicate that pandemic O3:K6 clone has endemically established in the Pacific Coast of Mexico.

Keywords: serologic, isolation, *Vibrio parahaemolyticus*, biosurveillance, public health

Introduction

Vibrio parahaemolyticus is a Gram stain-negative bacterium autochthonous of marine and estuarine environments worldwide (Kaneko and Colwell, 1973, 1978; Joseph et al., 1982). While the majority of environmental strains are innocuous members of the marine microbiota, small subpopulations are opportunistic pathogens of humans (Johnson et al., 2008). Potentially virulent strains are commonly differentiated from likely avirulent strains by the presence of the thermostable direct (*tdh*) and/or *tdh*-related (*trh*) hemolysin genes (Shirai et al., 1990; Bej et al., 1999). Acute gastroenteritis is the most common manifestation of illness and often associated with the consumption of raw or undercooked oysters, which can bioaccumulate the bacterium through filter-feeding (Daniels et al., 2000; Su and Liu, 2007; Iwamoto et al., 2010).

Previously, *V. parahaemolyticus* infections have been typically sporadic cases attributed to multiple serotypes, with at least 13 O serogroups and 71 K serotypes detected (Ishibashi et al., 2000). There was not a clear association between *V. parahaemolyticus*-mediated infection and serovars until 1996. Serogroup O3:K6 was first isolated in 1996 from diarrhea patients in Kolkata, India (Okuda et al., 1997) and subsequently worldwide. Since then an increasing incidence of gastroenteritis caused by the serogroup O3:K6 has been reported in many countries, including Africa (Ansaruzzaman et al., 2005), Europe (Martinez-Urtaza et al., 2004, 2005), and Latin America (Gonzalez-Escalona et al., 2005). Serotype O3:K6 strain was then identified as a dominant pandemic clone from clinical cases of *V. parahaemolyticus*-induced diarrhea reported globally (Okuda et al., 1997; Chowdhury et al., 2000).

Up to now, a wide variety of O3:K6 clonal derivatives, including O4:K68, O1:K25, O1:K26, and O1:KUT, have been recognized as the predominant group responsible for most outbreaks since 1996 (Okuda et al., 1997; Matsumoto et al., 2000; Okura et al., 2004;Ansaruzzaman et al., 2005; Hayat Mahmud et al., 2006). Pandemic strains typically belong to serotype O3:K6 and encodes a unique *orf8* gene (Nasu et al., 2000). It has been hypothesized that *orf8* encodes for an adherence protein that increases the ability of *V. parahaemolyticus* to adhere to host intestinal cells or the surfaces of marine plankton (Nasu et al., 2000; Yeung et al., 2002). Several studies reported that the *tox*RS operon of pandemic strains contains a unique sequence, thereby referred as *tox*RS/new, encoding transmembrane proteins involved in the regulation of virulence associated genes (Chowdhury et al., 2000; Okura et al., 2003, 2005). In general, an isolate possessing both *tdh* and *toxRS*/new can be considered as a pandemic strain (Okura et al., 2003). Another known virulence gene, *trh*, is not specific to pandemic strains and it is rarely present in environmental strains compared to clinical ones (DePaola et al., 2000; Parvathi et al., 2006).

In Mexico, the first outbreak of gastroenteritis caused by the pandemic strain of *V. parahaemolyticus* O3:K6 was reported in 2004 (Cabanillas-Beltran et al., 2006). More than 1230 cases of infection with *V. parahaemolyticus* were associated to consumption of contaminated seafood in a relatively small geographic area in southern Sinaloa (Cabanillas-Beltran et al., 2006). The incidence of *V. parahaemolyticus* infections in Mexico was unknown until 2004 when the O3:K6 pandemic strain with the *tdh* virulence gene was detected in this region. In subsequent years, recurrent sporadic cases has been detected in both South and North areas with the pandemic strain O3:K6 causing >79% of reported cases between 2004 and 2010 (Velazquez-Roman et al., 2012).

In an effort to understand the prevalence and dissemination of *V. parahaemolyticus* strains (toxigenic and pandemic O3:K6) we have characterized since 2004 strains of *V. parahaemolyticus* isolated from both clinical cases and environmental samples obtained from South and North areas of the Sinaloa state (Velazquez-Roman et al., 2012). The present report describes a more extensive investigation that evaluated the prevalence of *V. parahaemolyticus* strains in clinical and environmental samples collected from 2011 through 2013 from along all Sinaloa state. Our studies characterized the isolates by serotyping, investigated their antimicrobial susceptibility or non-susceptibility and assessed the presence of toxigenic and pandemic genetic markers. We also investigated the genetic relationships of strains isolated between 2004 and 2010 to those investigated in this study and isolated in 2011–2013. Our results indicate the persistence in the environment and clinical cases of the O3:K6 pandemic strain in Northwest Mexico from 2004 to 2013 To our knowledge, this is the first report describing that the pandemic O3:K6 clone has endemically established in the Pacific Coast of Mexico and causes most *V. parahaemolyticus* attributable diarrhea cases.

Materials and Methods

Area of Study, Collection of Environmental Samples, and Stool Samples

This study was performed in the state of Sinaloa, which is located in Northwest Mexico. Sinaloa has over 650 km of coastline, with most of it (∼75%) facing the Sea of Cortez and the rest (∼25%) bordering the Pacific Ocean. Sample collection was performed in eleven sites from the southern to the northern region in Sinaloa, during the years of 2011, 2012, and 2013. Regions sampled include were leading shrimp producers in Sinaloa are located; clinical cases were also detected near these regions. A total of 1,895 environmental samples (shrimp $N = 204$, sediment $N = 9$, and seawater $N = 1{,}682$) were collected (**Figure 1**). Stool specimens or rectal swabs ($N = 10{,}521$) were collected in Cary-Blair transport medium from persons with clinical gastroenteritis who had eaten seafood and requested attention in public-sector health care agencies during the period 2011–2013 (**Figure 1**). Written informed consent was obtained from patients or their families. Procedures for collection of stool samples were approved by the ethics committee of the Faculty of Medicine-UAS and the Sinaloa State Public Health Laboratory.

Bacteriological Analyses

Samples were processed following procedures found in the Bacteriological Analytical Manual of the Food and Drug Administration (Kaysner and De Paola, 2004) and as described by Canizalez-Roman et al. (2011). Briefly, 50 g of shrimp, or

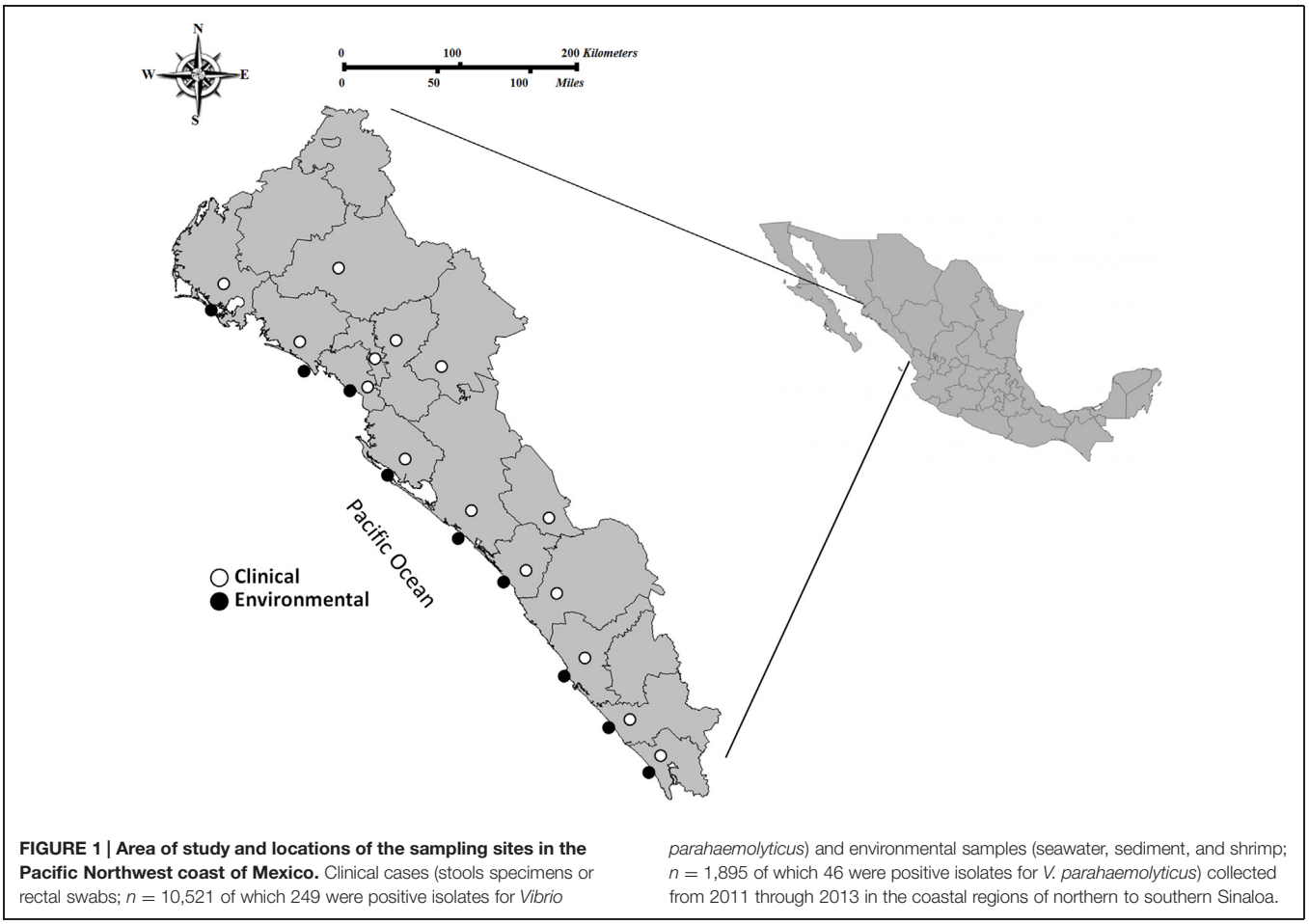

FIGURE 1 | Area of study and locations of the sampling sites in the Pacific Northwest coast of Mexico. Clinical cases (stools specimens or rectal swabs; $n = 10{,}521$ of which 249 were positive isolates for *Vibrio parahaemolyticus*) and environmental samples (seawater, sediment, and shrimp; $n = 1{,}895$ of which 46 were positive isolates for *V. parahaemolyticus*) collected from 2011 through 2013 in the coastal regions of northern to southern Sinaloa.

sediment samples, or 50 mL of seawater, were homogenized with 450 mL of sterile alkaline peptone water (APW; pH 8.6) in a Stomacher 400 circulator. The APW homogenate was incubated at 37°C for 6–8 h. Stool samples, or rectal swabs, were placed in Cary-Blair transport medium and transported at room temperature (RT) to the laboratory within 2 h and immediately processed. These specimens were also enriched in APW (pH 8.6) for 6–8 h at 37°C. After incubation, the enrichment broths were streaked onto thiosulfate-citrate-bile salts-sucrose (TCBS) agar plates and/or CHROMagar Vibrio (CV) medium (CHROMagar, Paris, France) and incubated at 37°C for 18–24 h. At least three typical colonies of *V. parahaemolyticus* were isolated from each plate and subjected to identification by biochemical test and polymerase chain reaction (PCR) as mentioned below. After identification of *V. parahaemolyticus* a single colony from each sample was used to continue the analysis (serotyping and virulence genes).

Extraction and Purification of Chromosomal DNA

Chromosomal DNA was extracted using the Wizard genomic DNA purification kit (Promega Corp.) according to the manufacturer's instructions. Briefly, strains were inoculated in LB broth containing 3% NaCl an incubated overnight at 37°C. This culture (3 mL) was pelleted by centrifugation at 16,000 × *g* for 5 min. Cells were lysed at 80°C in nucleic lysis solution. RNase solution was added to the cell lysate, followed by incubation at 37°C for 1 h and cooling at RT. Protein precipitation solution was added to the RNase-treated cell lysate and vortexed vigorously. DNA was precipitated by adding 0.6 volumes of isopropanol at RT and then washed with 70% ethanol; air dried, and solubilized using DNA rehydration solution. Our DNA preparations were stored at −20 or −80°C until use.

PCR Assays

Polymerase chain reaction amplification was performed in 25 μL reactions consisting of 1X GoTaq green master mix (Promega), primers targeting either the *tl* gene (Bej et al., 1999), pR72H plasmid (Lee et al., 1995; Robert-Pillot et al., 2002), *tdh* or *trh* genes (Bej et al., 1999), and 0.5 μL of purified genomic DNA template, with the remaining volume consisting of molecular biology grade water. PCR was routinely carried out in a Thermal Cycler C1000 (Bio-Rad Laboratories, Hercules, CA, USA) under the following cycling conditions: an initial period of DNA denaturation at 94°C for 3 min, followed by 35 cycles of 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C, and a final extension of 5 min at 72°C. PCR assays to amplify the *toxRS/new* and *orf8* genes (pandemic markers) were performed using specific primers previously reported

to detect *toxRS/new* (Matsumoto et al., 2000) sequence unique to the pandemic clone of *V. parahaemo*lyticus and the *orf8* (Myers et al., 2003) sequence of phage f237, respectively. PCR conditions for these assays were the following: for the *tox*RS/*new* gene, initial denaturation at 94°C for 3 min, followed by 25 cycles of 30 s at 94°C, 30 s at 45°C, and 1 min at 72°C, with a final extension of 5 min at 72°C, and for the *orf8* gene, denaturation at 94°C for 3 min, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, with a final extension of 5 min at 72°C (Matsumoto et al., 2000; Myers et al., 2003). Ten microliter aliquots of each amplification product were separated by electrophoresis in 2% agarose gels. Ethidium bromide staining (0.5 mg/mL) allowed for the visualization of DNA fragments with a digital imaging system (Gel Doc EZ imager, Bio-Rad, Hercules, CA, USA). The sizes of the PCR fragments were compared against a 50-bp DNA ladder (Promega DNA step ladder).

Serotyping

Serotyping of *V. parahaemolyticus* isolates was done by using a commercially available *V. parahaemolyticus* antiserum test kit (Denka Seiken, Tokyo, Japan) with O1–O11 antisera and 71 K antisera according to the manufacturer's instructions. Briefly, strains were grown overnight at 37°C on LB agar containing 3% NaCl. A pool of colonies was suspended in 1 mL of saline and then split in two 500 μL aliquots. For serotyping, an aliquot was heated up to 121°C for 1 h for O serotyping; if the serotype could not be obtained, the bacterial lysate was heated for an additional hour and then used for O serotyping. The second aliquot was used for serotyping based on the K antigen.

REP-PCR

More than 50% ($n = 150$) of strains isolated between 2004–2010 and 2011–2013 were selected for repetitive extragenic palindromic PCR (REP-PCR) analysis. Of these only nine strains are shown in the results section. Strains from the 2004 to 2010 period were obtained from a previous study (Velazquez-Roman et al., 2012) and from the MEMC (Medical and Enviromental Microorganism Collection, School of Medicine, Autonomous University of Sinaloa, Culiacan, Sinaloa, México). Seven strains belonged to pandemic O3:K6 strains, one strain sharing serogroup and pandemic characteristics O3:KUT isolated from shrimp sample, and a clinical isolate belonging to serotype O1:K20. Reactions were performed with primers REP-1D, 5-NNN RCG YCG NCA TCM GGC-3, and REP-2D, 5-RCG YCT TAT CMG GCC TAC-3, where M is A or C, R is A or G, Y is C or T, and N is any nucleotide (Wong and Lin, 2001; Maluping et al., 2005). PCR was routinely carried out in a Thermal Cycler C1000 (Bio-Rad Laboratories, Hercules, CA, USA) under the following cycling conditions: an initial period of DNA denaturation at 95°C for 7 min, followed by 35 cycles of 0.30 min at 94°C, 1 min at 45°C, and 8 min at 72°C, and a final extension of 10 min at 72°C (Wong and Lin, 2001; Maluping et al., 2005). PCR products were resolved by gel electrophoresis (1.5% agarose) buffered in Tris acetate EDTA (TAE) at 80 V for 2 h, stained with ethidium bromide (0.5 mg/mL) allowed for the visualization of DNA fragments with a digital imaging system (Bio-Rad Gel Doc EZ Imager, Wayne, PA, USA).

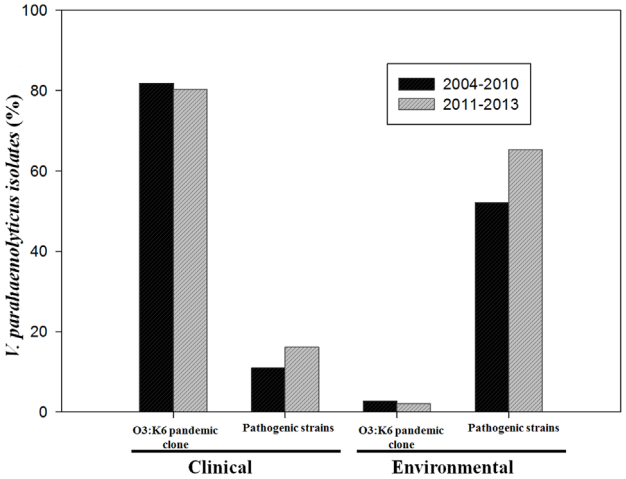

FIGURE 2 | Prevalence of pandemic *Vibrio parahaemolyticus* O3:K6 and pathogenic strains isolated from environmental and clinical samples that were collected in periods 2004–2010 or 2011–2013.

Antibiotic Susceptibility Testing

To evaluate antimicrobial-susceptibility of *V. parahaemolyticus* strains, 65 clinical and 87 enviromental isolates from 2004 to 2010 (from MEMC, a previous study (Velazquez-Roman et al., 2012), and 77 clinical and 32 enviromental isolates from 2011 to 2013, were tested by a standard disk diffusion method on Mueller-Hinton II agar (CLSI, 2011). The antibiotic sensi-disk (BD BBL, Sensi-Disc, Becton, Dickinson and Company, USA) used were the following: ampicillin (10 μg), tetracycline (30 μg), trimethoprim–sulfamethoxazole (1.25 μg/23.75 μg), chloramphenicol (30 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), ceftazidime (30 μg), gentamicin (10 μg), and cefotaxime (30 μg). In the absence of Clinical and Laboratory Standards Institute (CLSI) definitive standards for interpreting *V. parahaemolyticus* susceptibility to antibiotics, zone diameters were determined and recorded as sensitive, intermediate, or resistant according to established standards for *V. cholerae* and *Enterobacteriaceae*. The following *V. parahaemolyticus* strains were used as a control organism: ATCC 17802, (tdh^-) and multidrug resistant strain 727 (Leon-Sicairos et al., 2009).

Statistical Analysis

All statistical analysis was performed using SPSS v.20.0 (IBM Corp., Armonk, NY, USA). We carried out Chi-square to evaluate significance.

Results

Isolation of *V. parahaemolyticus* from Environmental and Stool Samples

From 2011 to 2013, a total of 1,895 environmental samples were analyzed for the presence of *V. parahaemolyticus* strains; these samples included 204 shrimp, 1682 seawater and nine sediment

samples (**Figure 1**). Overall, *V. parahaemolyticus* strains were isolated from 2.4% ($N = 46$) of samples. Of these 46 strains, 38 (82.6%) were obtained from shrimp samples, 5 (10.9%) from sediment, and 3 (6.5%) from seawater. In clinical samples taken during the same period, *V. parahaemolyticus* strains were isolated in 249 (2.4%) out of 10,521 stool specimens or rectal swabs collected from persons with gastroenteritis who had eaten seafood. The presence of *V. parahaemolyticus* in both, environmental samples and in cases of diarrhea by this bacterium were detected from southern to northern Sinaloa state (**Figure 1**).

Virulence Genes, Serotypes and Pandemic Characteristics of *V. parahaemolyticus* Isolates

Based on the presence or absence of virulence genes, we classified the isolates into three groups: pandemic (*tdh*$^+$, *toxRS/new*$^+$ and/or *orf8*$^+$), pathogenic (*tdh*$^+$ and/or *trh*$^+$), and non-pathogenic strains (*tdh*$^-$ and *trh*$^-$). Among environmental *V. parahaemolyticus* strains, three strains (6.5%) were identified as pandemic isolates. One of these strains belonged to serotype O3:K6 and carried the *tdh*, t*oxRS/new*, and *orf8* genes (isolated from shrimp), whereas two pandemic O3:KUT strains carried the *tdh*, *toxRS/new* and/or *orf8* genes (isolated from sediment and shrimp). A total of 65.2% (30/46) of environmental isolates carried the virulence *tdh* gene and therefore are considered as pathogenic strains. We did not detect isolates encoding the *trh* gene (**Table 1**). Approximately 28% (13/46) of environmental isolates were non-pathogenic. The most prevalent serovars were O3:KUT (23.9%, 11/46) and O5:K30 (10.8%, 5/46). Non-typeable strains represented the 39.13% (18/46; **Table 1**). Regarding *V. parahaemolyticus* strains isolated from diarrhea cases, 81.1% (202/249) of these isolates were identified as pandemic serotypes (**Table 1**). Of these, 97% (196/202) belonged to serovar O3:K6, carrying the *tdh*, *toxRS*/new and/or *orf8* genes. One isolate belonged to serovar O3:K29 (*tdh*$^+$ and *toxRS/new*$^+$), and one isolate belonged to serotype OUT:KUT (*tdh*$^+$, *toxRS/new*$^+$, and *orf8*$^+$). A total of 16.1% (40/249) of these clinical isolates were pathogenic strains (*tdh*$^+$ and/or *trh*$^+$) including several serotypes (e.g., O1:KUT, O4:K12, O4:K29, O4:K55, O6:K18, O10:KUT, OUT:KUT, OUT:K53, O1:K56, O3:KUT, O4:KUT, O8:K21). Only few clinical isolates, 2.8% (7/249), were classified within the non-pathogenic group. Unlike serovars detected in environmental isolates, pandemic serotype O3:K6 (80.3%, 200/249) was the most prevalent among those isolated from clinical samples (**Table 1**). Serotypes O1:KUT, O2:KUT, O3:KUT, O3:K6, O6:K46, and OUT:KUT were isolated from both environmental samples and stool samples.

Prevalence of O3:K6 Pandemic Clone and Pathogenic Strains between 2004–2010 and 2011–2013

The pandemic clone O3:K6 serotype was the most prevalent strain isolated from gastroenteritis cases in both periods 2004–2010 (81.8%) and 2011–2013 (80.3%). Among environmental strains the prevalence of serotype O3:K6 was also similar, 2.7 or 2.1%, for those isolated in 2004–2010, or 2011–2013, respectively (**Figure 2**). The percentage of clinical pathogenic strains isolated during the period 2011–2013 (16.1%) was slightly higher than that obtained during the period 2004–2010 (11%) but statistical analysis revealed no significant difference ($p > 0.05$). In the case of environmental pathogenic strains the prevalence increased from 52% in 2004–2010 to 65.3% in our period of analysis 2011–2013 (**Figure 2**). Similarly, no statistically significant difference was detected ($p > 0.05$). This indicates that the incidence of *V. parahaemolyticus* infection by the pandemic strains (O3:K6) in this region of Mexico had remained constant since 2004. It is noteworthy that between 2004 and 2013, O3:K6 strains were isolated from clinical samples in high proportions (80.3–81.8%) whereas pathogenic strains were detected in low proportions (11–16.1%). Conversely, in environmental samples the pandemic clone O3:K6 was detected in low proportions (2.1–2.7%) and pathogenic strains were detected in high proportions (52–65.3%).

REP-PCR Typing of Clinical and Environmental *V. parahaemolyticus* Strains

To investigate whether infections due to pandemic isolates were caused by genetic related clones throughout the years, DNA fingerprints of 150 strains obtained during 2004–2013 were examined using REP-PCR, but only seven randomly selected O3:K6, one O3:KUT and one O1:K20 isolates are shown in the **Figure 3**. Our REP-PCR studies revealed 11 discernible products (i.e., PCR bands) ranging in size from 400 to 3,000 bp. Several REP-PCR products with molecular size of 600, 750, and 1500 bp were common to most strains, while products of 400, 800, 1000, and 3000 bp were present in all *V. parahaemolyticus* strains (**Figure 3**). One REP-PCR banding pattern was obtained for O3:K6 (*tdh*$^+$, *toxRS/new*$^+$, *orf8*$^+$, and *trh*$^-$) strains; these seven isolates (**Figure 3**, lanes 1–5, 7, 9), yielded an identical banding pattern to that observed for the control strains (**Figure 3**, lane 10). A second banding pattern comprised one isolate, O3:KUT (*tdh*$^+$, *toxRS/new*$^+$, *orf8*$^+$ and *trh*$^-$), (**Figure 3**, lane 6) and a third REP-PCR banding pattern was obtained with one isolate, O1:K20 (*tdh*$^-$, *toxRS/new*$^-$, *orf8*$^-$, and *trh*$^-$; **Figure 3**, lane 8). Similar REP-PCR banding pattern were observed when REP-PCR was repeatedly performed, at least three times, demonstrating the reproducibility of our data. Except for the O3:KUT, lane 6 and O1:K20, lane 8 which displayed non-identical REP-PCR profiles, isolates from any year with the same serotype mostly produced identical REP-PCR profiles.

Antibiotic Resistance Profiles of *V. parahaemolyticus* Strains

Of the environmental and clinical strains tested, a significant increase in cefotaxime resistance was observed from 2004–2010 to 2011–2013 ($p < 0.05$) and most isolates were resistant to ampicillin (**Table 2**). However, among clinical strains a significantly ampicillin-resistance decreased was observed from 2004–2010 to 2011–2013 ($p < 0.05$). Low resistance was determined for gentamicin, nalidixic acid, sulfamethoxazole-trimethoprim, ceftazidime, chloramphenicol, and tetracycline from 2004–2010 to 2011–2013 (**Table 2**). Clinical and environmental isolates were all susceptible to ciprofloxacin or chloramphenicol, respectively.

TABLE 1 | Serovar and virulence attributes of 295 *Vibrio parahaemolyticus* strains isolated between 2011 and 2013.

O serogroup and serovar	Total no. of isolates	Presence of each virulence gene				No. of clinical isolates (from feces)	No. of environmental isolated from:		
		tdh	*trh*	*toxRS/new*	*Orf8*		Sh	Sw	S
O1									
O1:KUT	2	+	–-	–	–	1	1		
	1	–	–	–	–		1		
	1	+	+	–	–	1			
O1:K20	1	–	–	–	–	1			
O1:K56	1	+	+	–	–	1			
O2									
O2:KUT	2	+	–	–	–		2		
	1	–	–	–	–	1			
O3									
O3:KUT	2	–	–	–	–	1	1		
	6	+	–	–	+		5		1
	2	+	–	–	–			1	1
	1	+	–	+	–				1
	1	+	–	+	+		1		
	1	+	+	–	–	1			
O3:K6	192	+	–	+	+	191	1		
	5	+	–	+	–	5			
	21	+	–	–	+	21			
	4	+	+	+	+	4			
O3:K29	1	+	–	+	–	1			
O3:K30	1	–	–	–	–	1			
O3:K33	1	+	–	–	+		1		
	2	–	–	–	–		2		
O3:K54	1	–	–	–	–	1			
O4									
O4:KUT	2	+	+	–	–	2			
O4:K4	1	+	–	–	–				1
O4:K12	1	+	–	–	–	1			
O4:K29	1	+	–	–	–	1			
O4:K55	1	+	–	–	–	1			
O4:K63	1	–	–	–	–	1			
O5									
O5:K30	2	–	–	–	–		2		
	1	+	–	–	+		1		
	2	+	–	–	–		2		
O6									
O6:K18	1	+	+	–	–	1			
	1	+	–	–	–	1			
O6:K46	2	–	–	–	–	1	1		
O8									
O8:K21	1	+	+	–	–	1			
O10									
O10:KUT	2	+	–	–	–	2			
O11									
O11:KUT	1	–	–	–	–		1		
OUT									
OUT:KUT	14	+	–	–	–	2	11	1	
	1	–	+	–	–	1			
	1	+	–	+	+	1			
	1	+	–	–	+		1		

(Continued)

TABLE 1 | Continued

O serogroup and serovar	Total no. of isolates	Presence of each virulence gene				No. of clinical isolates (from feces)	No. of environmental isolated from:		
		tdh	*trh*	*toxRS/new*	*Orf8*		Sh	Sw	S
	1	+	+	−	−	1			
	5	−	−	−	−		3	1	1
OUT:K6	1	+	−	−	−		1		
OUT:K53	1	+	−	−	−	1			
							38	3	5
TOTAL	**295**					**Total clinical: 249**	**Total environmental: 46**		

Sh, Shrimp; Sw, Seawater; S, Sediment.

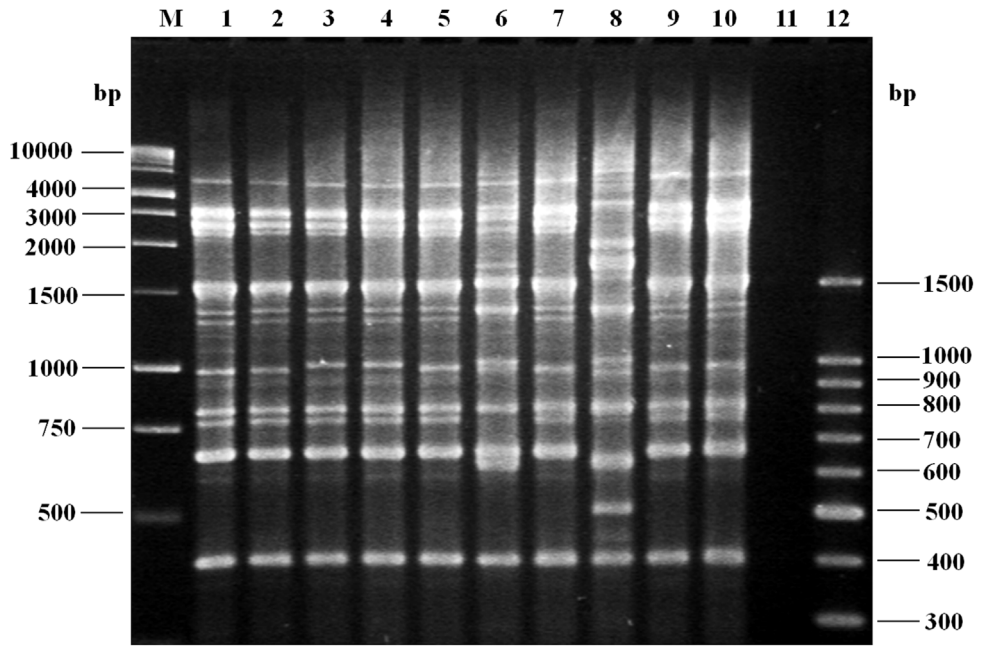

FIGURE 3 | Agarose gel (1.5%) electrophoresis showing the results of polymerase chain reaction (PCR) amplification of representative fingerprint patterns for the REP-PCR of *Vibrio parahaemolyticus* strains of serotype O3:K6 (*tdh*$^+$, *toxRS/new*$^+$, *orf8*$^+$, and *trh*$^-$), isolated from 2004 to 2013. Lane M, 1 kb molecular size markers; lane 1, clinical isolate (2004, O3:K6); lane 2, clinical isolate (2006, O3:K6); lane 3, clinical isolate (2008, O3:K6); lane 4, clinical isolate (2010, O3:K6); lane 5, clinical isolate (2011, O3:K6); lane 6, shrimp isolate (2011, O3:KUT, *tdh*$^+$, *toxRS/new*$^+$, *orf8*$^+$, and *trh*$^-$); lane 7, clinical isolate (2012, O3:K6); lane 8, clinical isolate (2012, O1:K20, *tdh*$^-$, *toxRS/new*$^-$, *orf8*$^-$, and *trh*$^-$); lane 9, clinical isolate (2013, O3:K6); lane 10, control strain *V. parahaemolyticus* RIMD 2210633 (*tdh*$^+$, *toxRS/new*$^+$, *orf8*$^+$, and *trh*$^-$); lane 11, negative control (no DNA template added to the rep-PCR reaction); lane 12, molecular size markers.

Regarding overall antibiotic resistance, most environmental (>78%) and clinical strains (>70%) were non-susceptible to at least one antibiotic (**Table 3**). This high prevalence of resistance decreased in strains isolated in 2011–2013 in comparison to previous period, 2004–2010, with an expected increase on the prevalence of strains resistant to two or more antibiotics (multidrug-resistant; **Table 3**). The increased prevalence, however, of strains with resistance to two antibiotics was only statistically significant among clinical isolates ($p < 0.05$). Importantly, strains with resistance to 5 or 7 antibiotics were detected among clinical strains isolated in the period 2011–2013 (**Table 3**).

Discussion

In Mexico, the first outbreak of gastroenteritis caused by pandemic *V. parahaemolyticus* strain O3:K6 was reported in a relatively small geographical area of the Southern part of the Sinaloa State (Velazquez-Roman et al., 2012). Since its arrival back in 2004, Sinaloa has experienced recurrent sporadic cases of gastroenteritis caused by *V. parahaemolyticus* strains which have gradually spread from south to north from 2004 to 2010 (Velazquez-Roman et al., 2012). The present study conducted an epidemiological surveillance of *V. parahaemolyticus* strains in both environmental and clinical samples along the Pacific coast

TABLE 2 | Distribution of antibiotic resistance among clinical and environmental *Vibrio parahaemolyticus* strains isolated from 2004–2010 and 2011–2013 periods.

Antimicrobial agents	Sample type			
	Environmental		Clinical	
	2004–2010 *n* = 87 (%)	2011–2013 *n* = 32 (%)	2004–2010 *n* = 65 (%)	2011–2013 *n* = 77 (%)
Aminoglycoside				
Gentamicin	2 (2.3%)	1 (3.1%)	0	3 (3.9%)
Quinolones and fluoroquinolones				
Ciprofloxacin	1 (1.1%)	0	0	0
Nalidixic acid	1 (1.1%)	0	1 (1.5%)	4 (5.2%)
Sulfonamides and potentiated sulfonamides				
Sulfamethoxazole-trimethoprim	2 (2.3%)	0	0	2 (2.6%)
Tetracyclines				
Tetracycline	1 (1.1%)	0	1 (1.5%)	1 (1.3%)
Beta lactams				
Ampicillin	78 (89.7%)	32 (100%)	65 (100%)∗	70 (90.1%)∗
Cephalosporins				
Ceftazidime	3 (3.4%)	0	0	4 (5.2%)
Cefotaxime	3 (3.4%)∗	7 (21.9%)∗	4 (6.1%)∗	14 (18.2%)∗
Phenicols				
Chloramphenicol	0	0	0	3 (3.9%)

∗*Significant at 0.05 level.*

TABLE 3 | Drug resistance and multidrug-resistance among clinical and environmental *Vibrio parahaemolyticus* strains isolated from 2004–2010 and 2011–2013 periods.

Number of drugs resistant to:	Sample type			
	Environmental		Clinical	
	2004–2010 *n* = 87 (%)	2011–2013 *n* = 32 (%)	2004–2010 *n* = 65 (%)	2011–2013 *n* = 77 (%)
0	7 (8.1%)	0	0	5 (6.5%)*
1	72 (82.8%)	25 (78.1%)	60 (92.3%)*	54 (70.1%)*
2	6 (6.9%)	6 (18.8%)	4 (6.2%)*	13 (16.9%)*
3	1 (1.1%)	1 (3.1%)	1 (1.5%)	3 (3.9%)
4	1 (1.1%)	0	0	0
5	0	0	0	1 (1.3%)
6	0	0	0	0
7	0	0	0	1 (1.3%)
≥8	0	0	0	0

∗*Significant at 0.05 level.*

of Sinaloa from 2011 to 2013. We demonstrate that the pandemic clone O3:K6 (encoding the *tdh* and *tox*RS/*new* genes and with or without *orf8*) still remains the most prevalent serotype isolated from cases of *V. parahaemolyticus*-induced diarrhea cases. The pandemic clone has endemically established in the Pacific Coast of Mexico. Furthermore, most strains were resistant to ampicillin and resistance to multiple first-line antibiotics significantly increased from 2004–2010 to 2011–2013. These observations represent, to the best of our knowledge, the first report demonstrating 10 years of persistence of the pandemic clone O3:K6 in the Mexico's pacific coast.

As in our previous study where most strains isolated from 2004 to 2010 belonged to the O3:K6 serotypes, most strains isolated from 2011 to 2013 were serotype O3:K6 (Velazquez-Roman et al., 2012). The prevalence of O3:K6 pandemic and pathogenic strains isolated from environmental and clinical samples was not significantly different from that detected in 2004–2010. This indicates that (1) the incidence of *V. parahaemolyticus* infection by the pandemic strains remains similar and (2) that the pandemic clone is a permanent resident of the environment in this region of Mexico. We hypothesize that the presence of pandemic strains in the environment is at least partially due to shedding in the feces of patients with gastroenteritis.

Regional persistence of O3:K6 pandemic strains have been reported in different geographic areas. For example, O3:K6 was the predominant serovar in studies conducted in Peru in 2007

(Gil et al., 2007), China (2007–2012; Li et al., 2014), and other Asian countries (Arakawa et al., 1999; Chowdhury et al., 2000; Wong et al., 2000) as well as in Chile when investigated from 2004 to 2009 (Gonzalez-Escalona et al., 2005; Cabello et al., 2007; Fuenzalida et al., 2007; Garcia et al., 2009). A follow up study in Chile conducted in by Harth et al. (2009) made an interesting observation of serotype replacement. The authors reported a decrease in outbreaks caused by O3:K6 but an increase of cases caused by pandemic isolates belonging to serotype O3:K59. In contrast, no change in the prevalence of the O3:K6 pandemic clone was observed in Northwest Mexico from 2004 to 2013. Our studies also identified new serovariants (O3:K29 and OUT:KUT) emerging with virulence attributes (*tdh* positive, *tox*RS/*new* positive and/or *orf8* positive) of pandemic strains. The emergence of new serovariants warrants further investigation since clones can potentially produce outbreaks along the northern Mexican coastline of the Pacific Ocean and spread to South Mexico or head North to US and Canada.

Results showed that the serovars of *V. parahaemolyticus* in environmental and clinical isolates were abundant and variable. We identified 10 novel serovars (from 2011 to 2013) in the area that were not isolated in our previous investigation conducted during 2004–2010. These newly identified serovars were isolated from diarrhea cases, $N = 7$, (O1:K20, O3:K30, O3:K54, O4:K29, O4:K55, O6:K18, and OUT:K53) and three from environmental samples (O4:K4, O5:K30, and OUT:K6). Data from the present study are in accordance with other reports in which *V. parahaemolyticus* environmental strains show a high serological variability (Nair et al., 2007; Chao et al., 2009; Garcia et al., 2009).

Previous studies have demonstrated that up to 90% of clinical strains encode the *tdh* and/or *trh* gene (Okuda et al., 1997; Chao et al., 2009; Garcia et al., 2009; Velazquez-Roman et al., 2012; Li et al., 2014; Pazhani et al., 2014), whereas their presence in environmental isolates is rare (Shirai et al., 1990; DePaola et al., 2000; Yeung and Boor, 2004; Nair et al., 2007; Chao et al., 2009; Velazquez-Roman et al., 2012). More recently, however, an increased proportions (48–52%) of strains encoding virulence genes (i.e., *tdh* and/or *trh*) have been detected in environmental isolates obtained in Mexico and the US. (Paranjpye et al., 2012; Velazquez-Roman et al., 2012; Gutierrez West et al., 2013). Accordingly, our studies detected high prevalence of the *tdh* gene (encoding for the TDH hemolysin) as it was carried by 58.6% of all environmental strains isolated from 2004 to 2013. Besides this demonstrated high serodiversity in the environment of *V. parahaemolyticus* strains with pathogenic potential (i.e., non-O3:K6 strains encoding the *tdh* gene), the pandemic strain O3:K6 caused >81% of reported cases of gastroenteritis, attributable to *V. parahaemolyticus* between 2004 and 2013 in the Pacific Northwest coast of Mexico. The detection of *tdh* gene in environmental isolates suggests that *tdh* alone is not an adequate marker for potentially virulent *V. parahaemolyticus* strains (Paranjpye et al., 2012).

As expected, our studies found a high serodiversity of *V. parahaemolyticus* in the environment, including isolates obtained from shrimp, sediment, and seawater. It is worth to mention that in 2013 we observed a high mortality of cultured *Penaeus vannamei* in shrimp farms located in northern Mexico including the states of Nayarit, Sinaloa and Sonora. Mortality was due to acute hepatopancreatic necrosis disease (AHPND), which has also been referred to as early mortality syndrome (EMS), and the pathogen associated with EMS was *V. parahaemolyticus* (Gomez-Gil et al., 2014; Gomez-Jimenez et al., 2014; Nunan et al., 2014). Additional studies should provide a link between pathogenic traits of *V. parahaemolyticus* strains and/or serotype or serovars, if any, associated to this syndrome in shrimps.

Rep-PCR genomic fingerprinting is known to have a greater resolving power than serotyping (Maluping et al., 2005). Our rep-PCR studies intended to demonstrate genetic similarities, or differences, between pandemic strains isolated from the environment with those isolated from human cases of gastroenteritis. Whereas molecular divergence was noticed on the banding profile obtained from O1:K20 and O3:KUT strains, we obtained a similar rep-PCR profile in all O3:K6 pandemic isolates utilized which indicates that O3:K6 strains circulating in the environment have the same clonal origin than those infected patients and therefore a source of infection and transmission.

Another important contribution in our work was the investigation of susceptibility, or not, of the isolated *V. parahaemolyticus* strains to first-line antibiotics utilized in the region. In agree with our genetic evidences (i.e., rep-PCR) indicating genetic relationships of the isolated strains, our results revealed similar resistance patterns in both clinical and environmental isolates. Most *V. parahaemolyticus* isolates were resistant to ampicillin which was not a surprise as non-susceptibility to ampicillin is very common in *V. parahaemolyticus* strains isolated from enviromental and clinical samples (Okuda et al., 1997; Wong et al., 2000; Roque et al., 2001; Sun et al., 2013), suggesting that these drugs have a negligible role in the treatment of *V. parahaemolyticus*. In contrast, most isolates were sensitive to tetracycline, trimethoprim–sulfamethoxazole, chloramphenicol, nalidixic acid, ciprofloxacin, ceftazidime and gentamicin, which can be used as an alternative antibiotic therapy. Resistance to cefotaxime increased from 4.6% in 2004–2010 to 19.3% in 2011–2013. A similar prevalence of resistance to cefotaxime (20%) has also been reported in strains isolated in Italy from shellfish and clinical samples (Ottaviani et al., 2013). While the percentage of isolates expressing resistance to the newer generation of cephalosporins was relatively low, these antibiotics are considered to be some of the best defenses against the severe infections that these organisms can elicit, so even a small percentage of resistant isolates could be cause for concern. Therefore, all isolates must be tested for antimicrobial susceptibility to monitor resistance patterns of each antibiotic. In Mexico and others countries, patients suffering *V. parahaemolyticus* disease are treated with empiric antibiotic therapy which generates more resistance to first line antibiotics. Unlike other bacterial infections, little to nothing is reported about antibiotic resistance of *V. parahaemolyticus* in Mexico, and perhaps other Latin American countries, as clinical laboratories do not routinely test susceptibility to different classes of antimicrobial agents. Furthermore, until 2004 where we reported the first outbreak of gastroenteritis caused by *V. parahaemolyticus* in Mexico, there had not been other outbreaks published in the scientific literature (Velazquez-Roman et al., 2012, 2014). Prior to our studies, only

few environmental strains had been isolated from water and fish in Mexico (Cabrera-Garcia et al., 2004).

To the best of our knowledge, our findings represent the first investigation in Mexico about the prevalence, pathogenic potential, and antimicrobial susceptibility over a 10-years period of continue surveillance of *V. parahaemolyticus* (pathogenic and pandemic O3:K6 clone) in both clinical specimens and environmental samples. Most gastroenteritis cases attributable to *V. parahaemolyticus* strains are caused by the same pandemic clone which warrants extended surveillance in the region and across the country. Continued monitoring of *V. parahaemolyticus* strains and their susceptibility to antibiotics seem to be necessary to unsure the best treatment, and prognosis, to patients with *V. parahaemolyticus* diseases in the area. This information should also be relevant to health authorities in the case of a local or multistate foodborne outbreak of *V. parahaemolyticus* gastroenteritis.

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Insights into the environmental reservoir of pathogenic *Vibrio parahaemolyticus* using comparative genomics

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Vibrio parahaemolyticus is an aquatic halophilic bacterium that occupies estuarine and coastal marine environments, and is a leading cause of seafood-borne food poisoning cases. To investigate the environmental reservoir and potential gene flow that occurs among *V. parahaemolyticus* isolates, the virulence-associated gene content and genome diversity of a collection of 133 *V. parahaemolyticus* isolates were analyzed. Phylogenetic analysis of housekeeping genes, and pulsed-field gel electrophoresis, demonstrated that there is genetic similarity among *V. parahaemolyticus* clinical and environmental isolates. Whole-genome sequencing and comparative analysis of six representative *V. parahaemolyticus* isolates was used to identify genes that are unique to the clinical and environmental isolates examined. Comparative genomics demonstrated an O3:K6 environmental isolate, AF91, which was cultured from sediment collected in Florida in 2006, has significant genomic similarity to the post-1995 O3:K6 isolates. However, AF91 lacks the majority of the virulence-associated genes and genomic islands associated with these highly virulent post-1995 O3:K6 genomes. These findings demonstrate that although they do not contain most of the known virulence-associated regions, some *V. parahaemolyticus* environmental isolates exhibit significant genetic similarity to clinical isolates. This highlights the dynamic nature of the *V. parahaemolyticus* genome allowing them to transition between aquatic and host-pathogen states.

Keywords: genomics, *Vibrio parahaemolyticus*, environment, phylogenomics, O3:K6

Introduction

Vibrio parahaemolyticus is halophilic aquatic bacterium that is ubiquitous in coastal marine and estuarine environments. The majority of isolates derived from environmental sources, that is water and sediments, are believed to be non-pathogenic (Depaola et al., 1990; Nair et al., 2007); however,

some *V. parahaemolyticus* isolates are capable of causing human illness, and are primarily associated with food-borne derived gastroenteritis and diarrhea. *V. parahaemolyticus* infection can also be associated with wound infections and sepsis (CDC, 2007, 2008; Tena et al., 2010). In 1996, an increase in diarrheal illness in India associated with *V. parahaemolyticus* infections were attributed to the emergence of a novel genetic variant in 1995 that had the O3:K6 serotype (Okuda et al., 1997). This novel disease-associated O3:K6 clone rapidly disseminated worldwide and is considered to be pandemic (Vuddhakul et al., 2000; Myers et al., 2003; Quilici et al., 2005; Ottaviani et al., 2008). Previously, isolates belonging to the post-1995 O3:K6 clone were identified with the serotypes O1:Kuk, O1:K25, and O4:K68, indicating the O3:K6 clone has undergone serogroup conversion in the years since the original clonal expansion (Nair et al., 2007; Chen et al., 2011).The disease-associated *V. parahaemolyticus* clinical isolates usually carry one or both of the thermostable direct hemolysins (*tdh* and *trh*) (Kaper et al., 1984; Nishibuchi et al., 1992; Nishibuchi and Kaper, 1995; Makino et al., 2003; Nair et al., 2007). In addition to the hemolysins, two type III secretion systems (T3SS) have been demonstrated to secrete effectors that induce cytotoxicity or enterotoxicity (Park et al., 2004; Lynch et al., 2005; Ono et al., 2006; Caburlotto et al., 2010; Broberg et al., 2011; Ham and Orth, 2012; Zhang and Orth, 2013). A previous study revealed a second version of T3SS2, T3SS2β, which was identified in clinical isolates that also possess the *trh* gene (Okada et al., 2009). Although the hemolysins and type III secretion have been identified as a major components of the *V. parahaemolyticus* virulence mechanism (Park et al., 2004; Burdette et al., 2008; Caburlotto et al., 2010; Ham and Orth, 2012; Zhang and Orth, 2013), disease-associated isolates have been identified that do not encode the thermostable direct hemolysins (Yu et al., 2006; Bhoopong et al., 2007; Meador et al., 2007), suggesting there may be additional, as yet, uncharacterized genes contributing to *V. parahaemolyticus* virulence mechanisms.

The genetic diversity of *V. parahaemolyticus* has been investigated using numerous molecular methods, including the identification of known virulence genes (Meador et al., 2007; Noriea et al., 2010; Jones et al., 2012), multi-locus sequence typing (MLST) (Chowdhury et al., 2004; González-Escalona et al., 2008; Gavilan et al., 2013; Turner et al., 2013), phylogenetic analysis of housekeeping genes (Thompson et al., 2005), microarray (Han et al., 2008), and pulsed-field gel electrophoresis (PFGE) (Parsons et al., 2007; Ludeke et al., 2014). MLST was used to identify two new clonal complexes in addition to a clonal complex of the post-1995 O3:K6 isolates (González-Escalona et al., 2008). The second clonal complex consisted of O4:K12 and O12:K12 isolates from the Pacific coast of the United States, and the third clonal complex was comprised primarily of isolates from oysters in the Gulf of Mexico (González-Escalona et al., 2008).

Genome sequencing and comparative analysis of the post-1995 *V. parahaemolyticus* O3:K6 isolate RIMD2210633 (Makino et al., 2003) revealed seven genomic islands, including four that are characteristic of post-1995 O3:K6 isolates (Hurley et al., 2006; Boyd et al., 2008; Chen et al., 2011). Genomic subtraction demonstrated that an 80-kb pathogenicity island (VpPAI) encoding T3SS2 was associated with the post-1995 O3:K6 pandemic isolates (Okura et al., 2005). The sequencing of additional *V. parahaemolyticus* genomes has confirmed that the emergence of the post-1995 O3:K6 pandemic isolates coincided with the acquisition of genomic islands as these regions were mostly absent from the genomes of pre-1995 O3:K6 isolates (Makino et al., 2003; Boyd et al., 2008; Chen et al., 2011).

The purpose of this study was to investigate the genetic diversity of *V. parahaemolyticus* isolates from human clinical (stool, blood, wound specimens, or unknown sample types) or environmental (sediment, water, oysters) sources using multiple molecular methods including a PCR assay of known *V. parahaemolyticus* virulence-associated genes, phylogenetic analysis of housekeeping genes, PFGE, and whole-genome sequencing. Investigation of the genomic diversity of two clinical isolates and four environmental isolates by whole-genome sequencing and comparative analysis identified genes that are shared or exclusive to the clinical or environmental isolate genomes sequenced. These methods highlight the genetic similarity among clinical and environmental isolates, and the different combinations of virulence-associated genes demonstrate the dynamic nature of the *V. parahaemolyticus* genome.

Materials and Methods

Bacterial Isolates and Media

V. parahaemolyticus clinical isolates included in this study were provided by the Centers for Disease Control and Prevention (Atlanta, GA). The *V. parahaemolyticus* environmental isolates were cultured from sediment, water, and oysters of Skidaway Island, GA, and Apalachicola Bay, FL in September 2006, and Skidaway Island, GA in September 2007 (Hazen et al., 2009). Additional environmental isolates were obtained from the rhizosphere sediment of a salt marsh in North Inlet, NC (Bagwell et al., 1998). The environmental isolates were cultured by plating environmental samples on thiosulfate citrate bile salts sucrose (TCBS) agar (Difco) and incubating them overnight at 30°C. Water samples were directly plated onto TCBS, while sediment, and oysters were homogenized with sterile water then plated onto TCBS agar. Presumptive *V. parahaemolyticus* colonies that were green on TCBS were confirmed by PCR by screening for the thermolabile hemolysin (*tl*) as previously developed (Bej et al., 1999), which is characteristic of *V. parahaemolyticus* (Meador et al., 2007). The culture collection strain, ATCC 17802, was used as a reference isolate for molecular characterizations of *V. parahaemolyticus.*

Serotyping

Serotypes were determined using *V. parahaemolyticus* Seiken typing antisera (Denka Seiken, Tokyo, Japan).

PCR Assay of Virulence-Associated Genes

The known *V. parahaemolyticus* virulence-associated genes were detected by PCR assay for all *V. parahaemolyticus* clinical and environmental isolates examined in this study using primers listed in Supplemental Table 5. All isolates that were positive for *tl* as described above were then PCR screened for previously-characterized virulence-associated genes. The thermostable direct hemolysins *tdh* and *trh* were detected as

described (Bej et al., 1999; Meador et al., 2007). In addition, the ORF8 gene of the pandemic phage f237 was detected using primers that were previously developed (Myers et al., 2003). The presence of the T3SS1 and T3SS2 were determined by PCR assay for two effectors and one gene involved in translocation from each T3SS. The T3SS1 effectors *vp1680* and *vp1686* and the structural gene *vp1670* was identified by PCR assay using previously developed (Vora et al., 2005; Meador et al., 2007) primers, and additional primers made in this study that are listed in Supplemental Table 5. The presence of T3SS2α and T3SS2β was determined by PCR assay for the effectors *vpa1346* and *vpa1362,* and the export protein-encoding gene *vpa1354* using primers listed in Supplemental Table 5.

Phylogenetic Analysis of Housekeeping Genes

The genetic similarity was investigated for 116 *V. parahaemolyticus* clinical and environmental isolates examined in this study by phylogenetic analysis of a concatenation of four housekeeping genes (*recA, gyrB, pyrC, dtdS*) using previously developed primers (González-Escalona et al., 2008). The genes were PCR amplified using NEB Phusion high-fidelity polymerase (NEB; Ipswich, MA) and purified by separation on a 0.7% Seakem LE agarose gel (Lonza; Allendale, NJ). The target amplicon was excised from the gel and the DNA was recovered using the Sigma GenElute gel extraction kit (Sigma Aldrich; St. Louis MO). Sequencing was performed with M13 primers at the Georgia Tech Genome Center on an ABI 3130 Genetic Analyzer (Applied Biosystems) using BigDye Terminator chemistry (Applied Biosystems). Sequences were assembled in BioEdit (v. 7.0.4.1) (Hall, 1999) and aligned using MEGA5 (Tamura et al., 2011), and all sequences for a particular gene were trimmed to the same length. The partial sequences of each housekeeping gene analyzed were concatenated in the same order for each *V. parahaemolyticus* isolate, generating a single representative sequence. A maximum-likelihood phylogeny with 100 bootstrap replicates was generated using RAxML v7.2.8 (Stamatakis, 2006) and visualized using FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

The genetic similarity was further investigated for 52 of the clinical and environmental isolates by analysis of three additional genes (*dnaE, tnaA, pntA*) for a total of seven genes. These three additional genes were PCR amplified and described above using previously developed primers (González-Escalona et al., 2008). Phylogenetic analysis of all seven (*recA, gyrB, dnaE, pyrC, dtdS, tnaA, pntA*) of the conserved genes for this subset of clinical and environmental isolates was performed as described above.

PFGE

Pulsed-field gel electrophoresis of 44 *V. parahaemolyticus* clinical and environmental isolates was performed according to the *V. parahaemolyticus* PulseNet USA standardized protocol (Parsons et al., 2007). Restriction endonuclease profiles were generated using the enzymes *Sfi*I and *Not*I (Roche, Mannheim, Germany). Restricted plugs were run on a CHEF Mapper™ electrophoresis system (Bio-Rad Laboratories, Hercules, CA). *Salmonella Braenderup* H9812 restricted with 50 U of *Xba*I (Roche, Mannheim, Germany) was used as a control strain for gel normalization. PFGE patterns were analyzed with BioNumerics v. 5.1 (Applied-Maths, Kortrijk, Belgium) and dendrograms were generated using the Dice coefficient and unweighted pair group method with arithmetic averages (UPGMA) with a band position tolerance and optimization of 1.5% for cluster analysis.

Genome Sequencing and Assembly

Following the molecular characterization of the *V. parahaemolyticus* clinical and environmental isolates, we generated high-quality draft genome sequences of two clinical isolates (K1275, K1461) and four environmental isolates (AF91, SG176, J-C2-34, 22702) (**Table 2**). The clinical isolates analyzed have unique combinations of the known virulence-associated genes compared to the epidemic post-1995 O3:K6 isolates (Supplemental Table 1). The environmental isolates analyzed by genome sequencing were obtained from samples of three different states (NC, GA, FL) (Supplemental Table 1). The *V. parahaemolyticus* isolates analyzed by whole-genome sequencing were grown overnight in Luria Bertani (Difco) at 37°C with shaking (225 rpm). Genomic DNA was isolated from the overnight cultures using the Sigma GenElute genomic kit (Sigma Aldrich; St. Louis MO). The genome sequences of *V. parahaemolyticus* isolates K1461, K1275, SG176, J-C2-34, and AF91 were generated using the Roche 454-Titanium sequencing platform at the Centers for Disease Control and Prevention. The 454 reads were assembled into high-quality draft genomes at the Institute for Genome Sciences, using the Mira assembler (Chevreux et al., 1999), and the assemblies were filtered to contain contigs ≥500 bp.

The genome sequence of *V. parahaemolyticus* 22702 was generated using paired-end libraries with 300 bp inserts on the Illumina HiSeq2000 at the Institute for Genome Sciences, Genome Resource Center. The Illumina reads generated for 22702 were assembled into a high-quality draft genome using the Velvet assembly program (Zerbino and Birney, 2008) with kmer values determined using VelvetOptimiser v2.1.4 (http://bioinformatics.net.au/software.velvetoptimiser.shtml), and the assembly was filtered to contain contigs ≥500 bp.

Information regarding the genome assembly size, number of contigs, and the GenBank accession numbers for each of the genomes sequenced in this study are listed in **Table 2**.

Comparative Genomics

Phylogenomic analysis of the *V. parahaemolyticus* genomes sequenced in this study compared to previously sequenced *V. parahaemolyticus* genomes available in the public domain, was performed as previously described (Sahl et al., 2011). The genomes were aligned using Mugsy (Angiuoli and Salzberg, 2011), and the aligned regions were concatenated then used to construct a maximum-likelihood phylogeny with 100 bootstrap values using RAxML v7.2.8 (Stamatakis, 2006), and visualized using FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

BLAST score ratio (BSR) analysis was performed as previously described (Rasko et al., 2005) and used to identify the presence of virulence-associated genes in each of the genomes analyzed (**Table 2**). Briefly, the predicted amino acid sequences of virulence-associated genes and genomic regions (Hurley et al., 2006; Chen et al., 2011; Salomon et al., 2013) were compared

using TBLASTN (Gertz et al., 2006) to all the *V. parahaemolyticus* genomes analyzed in this study. The protein-encoding genes that were considered present with significant similarity had BSR values ≥0.8.

Genetic similarity of chromosomes I and II of the O3:K6 isolate RIMD2210633 to genes in each of the genomes sequenced in this study was determined using BLASTN (Altschul et al., 1990) BSR analysis as previously described (Rasko et al., 2005). A circular display of the BLASTN BSR values was generated using Circos 0.65 (Krzywinski et al., 2009).

Large-scale BSR analysis (Hazen et al., 2013; Sahl et al., 2013, 2014) was used to identify the shared and unique features present in the *six* genomes sequenced in this study compared with eight previously sequenced genomes listed in **Table 2**. Each protein-encoding gene was considered present with high identity (BSR value ≥0.8), present but with sequence divergence (BSR value ≥0.4, ≤0.8), or absent (BSR value <0.4). A representative sequence of each predicted protein-encoding gene is included in Supplemental Data Set 1. The predicted function of protein-encoding genes was identified using the RAST annotation server (Overbeek et al., 2014).

Plasmid and Phage Analyses

The number of extrachromosomal elements (plasmids and prophage) was determined for a subset of the isolates using a modified acid phenol extraction method (Kieser, 1984; Sobecky et al., 1997). PCR amplification for sequencing was performed using NEB Phusion high-fidelity polymerase reaction mix with GC buffer and reaction and cycle conditions as recommended by the manufacturer (NEB; Ipswich, MA). Primers used to PCR amplify *rstA* are listed in Supplemental Table 5. The M13 sequence at the 5′ end of each *rstA* primer was used for sequencing. Sequencing was performed as described for the housekeeping genes. The sequences were aligned using MEGA5 (Tamura et al., 2011), and a maximum-likelihood phylogeny using the Kimura 2-parameter model (Kumar et al., 2004) and 1,000 bootstrap replications was constructed using MEGA5 (Tamura et al., 2011). Bootstrap values ≥50 are shown.

Nucleotide Sequence Accession Numbers

All individual gene sequences generated in this study are deposited in GenBank under the accession numbers FJ847518-FJ847829. The genome sequences are deposited in GenBank under the accession numbers JMMO00000000, JMMP00000000, JMMQ00000000, JMMR00000000, JMMS00000000, and JMMT00000000.

Results and Discussion

Identification of Virulence-Associated Genes in a Collection of Clinical and Environmental *V. Parahaemolyticus*

As a measure of the virulence potential of *V. parahaemolyticus* clinical and environmental isolates analyzed, we detected common markers of virulence including: the ORF8 gene of the filamentous vibriophage, the hemolysins (*tdh* and *trh*), the type III secretion systems of chromosome I (T3SS1), and chromosome II (T3SS2α and T3SS2β) (**Table 1**). These genes have been previously identified in association with illness-associated *V. parahaemolyticus,* and the hemolysins and T3SS2 were characterized for their role in pathogenesis (Kaper et al., 1984; Nishibuchi and Kaper, 1995; Nasu et al., 2000; Park et al., 2004; Lynch et al., 2005; Ono et al., 2006; Nair et al., 2007; Broberg et al., 2011; Ham and Orth, 2012; Zhang and Orth, 2013). The ORF8 gene encoded by the filamentous vibriophage f237, which has previously been linked to the post-1995 O3:K6 pandemic clinical isolates (Nasu et al., 2000), was identified in 32% of the clinical isolates, and none of the environmental isolates in this study (**Table 1**). The ORF8 gene was identified in all post-1995 O3:K6 isolates analyzed, and only 13% of the non-O3:K6 clinical isolates (**Table 1**; Supplemental Table 1). The T3SS1 genes and *tl* were detected among all *V. parahaemolyticus* isolates, which is consistent with previous reports that these genes are universal among *V. parahaemolyticus* isolates (Vora et al., 2005) (**Table 1**, Supplemental Table 1). The T3SS2α genes were present in 93% (13/14) of the O3:K6 clinical isolates and 100% (4/4) of the O4:K8 clinical isolates, but only 25% (12/49) of the clinical isolates

TABLE 1 | Identification of *V. parahaemolyticus* virulence-associated genes in a collection of *V. parahaemolyticus* clinical and environmental isolates using PCR assays.

Source	Serotype	No. isolates	No. of isolates with virulence genes (%)					
			ORF8	*tdh*	*trh*	T3SS1	T3SS2α	T3SS2β
Clinical	O3:K6	14	14 (100)	13 (93)	0 (0)	14 (100)	13 (93)	0 (0)
	O4:K8	4	0 (0)	4 (100)	0 (0)	4 (100)	4 (100)	0 (0)
	O4:K12	10	1 (10)	10 (100)	9 (90)	10 (100)	0 (0)	9 (90)
	Other serotypes	48	9 (19)	29 (60)	14 (29)	48 (100)	12 (25)	22 (46)
Total clinical	All serotypes	76	25 (32)	57 (74)	23 (30)	77 (100)	29 (38)	31 (40)
Environmental	O3:K6	1	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
	Other serotypes	56	0 (0)	0 (0)	2 (3)	56 (98)	0 (0)	2 (3)
Total environmental	All serotypes	57	0 (0)	0 (0)	2 (3)	57 (100)	0 (0)	2 (3)

that had other serotypes (**Table 1**). T3SS2β was detected in 90% (9/10) of the O4:K12 isolates, and 46% (22/48) of the clinical isolates with other serotypes (**Table 1**). Included in this study were 10 clinical isolates with the O4:K12 serotype, and all but one (K4358) of these isolates were *tdh*+/*trh* + /T3SS2β+ (**Table 1**, Supplemental Table 1). Similar virulence-associated gene content (*tdh*+/*trh* + /T3SS2β+) was identified in clinical isolates of six other serotypes (O4:K53, O4:K63, O1:K56, O8:K21, O6:K18, O11:Kuk) (Supplemental Table 1). While T3SS2α was not identified in any of the environmental isolates, including the O3:K6 environmental isolate AF91, T3SS2β was present in two environmental isolates that were *trh*+ (**Table 1**, Supplemental Table 1). *V. parahaemolyticus* environmental isolates that possess T3SS2 have been demonstrated to adhere to eukaryotic cells and disrupt membrane tight junctions (Caburlotto et al., 2010). This study demonstrated that *V. parahaemolyticus* isolates residing in the environment that possessed some of the known virulence factors also had the potential to cause disease.

There were also clinical isolates that had an atypical combination of virulence genes, or were missing most of the known virulence-associated genes. Several clinical isolates contained only *tdh* (K0071, F5828, K4358, K4279), or both *tdh* and *trh* genes (K5067), but lacked detectable T3SS2 genes (**Table 2**). In addition, the clinical isolates K4763, K3528, and K4305 contained *tdh* and T3SS2β genes, which is unusual as the *tdh* gene is typically associated with T3SS2α (Sugiyama et al., 2008). It is possible these isolates may have contained both *tdh* and *trh*, similar to O4:K12, and they may have lost *trh* during infection or during laboratory passage. None of the isolates analyzed contained the *trh* gene and also the T3SS2α genes. Three clinical isolates (F8950, F8937, K4377) contained T3SS2α but lacked *tdh* and *trh*, and five clinical isolates (K0456, K4237, K4638, K5323G, K5330) contained T3SS2β but lacked *tdh* and *trh* (Supplemental Table 1). The presence of T3SS2 genes and the absence of hemolysins in clinical isolates has been previously described (Meador et al., 2007). There were 12 clinical isolates (K1275, K0851, K0850, F9974, F6658, F8132, F7979, F6179, K4434, F8190, K4981, K1000) that did not encode *tdh, trh*, or the T3SS2 genes (Supplemental Table 1). These isolates were obtained from blood (K1275), wound infections (F8132, K4434), or unknown clinical sample types (F6658, F6179, F8190, K4981, K1000). Although the *V. parahaemolyticus* clinical isolates that lacked the hemolysin and/or T3SS2 genes were obtained from clinical specimens, they may have been co-occurring in the host with other *V. parahaemolyticus* isolates that did encode the hemolysins or T3SS2 genes and were the primary cause of illness. A previous study demonstrated that multiple *V. parahaemolyticus* isolates were present in disease-associated samples; however, some of these isolates lacked the hemolysin genes (Bhoopong et al., 2007). Another possible explanation is that these isolates may have contained the hemolysin genes and T3SS2 genes and may have lost them following passage through a host or during passage in the laboratory. This was previously observed for the enteropathogenic *Escherichia coli* isolate E2348/69, which exhibited loss of the EPEC virulence plasmid in a subset of culturable isolates following passage through adults in a clinical trial (Levine et al., 1985). Also, it may be possible that some of these *V. parahaemolyticus* isolates have as yet uncharacterized virulence factors. Further research is necessary to determine whether these *V. parahaemolyticus* clinical isolates are capable of causing disease without the hemolysin genes and/or T3SS2 genes. These findings highlight the many combinations of virulence-associated genes in *V. parahaemolyticus* clinical isolates, demonstrating the dynamic nature of the virulence repertoires of *V. parahaemolyticus* isolates.

Molecular Analysis of the Genetic Similarity of Clinical and Environmental *V. parahaemolyticus*

Phylogenetic analysis of housekeeping genes was used to investigate the genetic similarity of *V. parahaemolyticus* clinical and environmental isolates representing diverse serotypes, isolation sources, and date of isolation (**Figure 1**, Supplemental Table 1). This approach has previously been used to investigate the evolutionary relationships of isolates within a single *Vibrio* species (Chowdhury et al., 2004; Boyd et al., 2008; González-Escalona et al., 2008; Turner et al., 2013), and among isolates belonging to multiple *Vibrio* species (Thompson et al., 2005, 2007, 2008; Sawabe et al., 2007, 2013; Lin et al., 2010). A phylogeny analyzing the genetic relatedness of 52 *V. parahaemolyticus* isolates (42 clinical, 10 environmental) was constructed using the partial nucleotide sequences of seven housekeeping genes (**Figure 1**). The phylogeny contained three distinct clades (colored boxes), which were primarily comprised of isolates with the O4:K12, O3:K6, and O4:K8 serotypes (**Figure 1**). Notably, all but two of the isolates (AF91 and BB22OP) that formed these three clades were derived from clinical sources (**Figure 1**). The other 18 isolates analyzed that were outside of these three clades included a mixture of clinical and environmental isolates that have diverse serotypes, and these isolates exhibited considerable phylogenetic diversity (**Figure 1**). Furthermore, this demonstrated that clinical isolates with serotypes other than O3:K6, O4:K12, and O4:K8 had genetic similarity to the environmental isolates analyzed in this study (**Figure 1**).

To further investigate the genetic diversity observed for the clinical and environmental isolates that had serotypes other than O3:K6, O4:K12, and O4:K8, we analyzed partial sequences of two housekeeping genes of chromosome I (*recA* and *gyrB*), and two housekeeping genes of chromosome II (*pyrC* and *dtdS*) in a larger collection of 116 *V. parahaemolyticus* clinical and environmental isolates (Supplemental Figure 1). In a phylogenetic analysis of the concatenation of all four genes, there were four clinical isolates that formed a sub-clade with a long branch. To investigate whether the long branch of this sub-clade resulted from sequence divergence within a particular analyzed gene we generated individual phylogenies for each gene. Three of these genes (*gyrB, pyrC,* and *dtdS*) had similar topologies to the concatenated phylogeny, while the *recA* phylogeny demonstrated there was additional sequence divergence within *recA* for the four clinical isolates with the longer branch. Therefore, we analyzed the diversity of these four housekeeping genes by constructing a phylogeny for three of the genes (Supplemental Figure 1A), compared with a separate phylogeny of only *recA* sequences (Supplemental Figure 1B). Overall, phylogenetic analysis of the three genes (*gyrB, pyrC, dtdS*) indicated the clinical and environmental isolates analyzed have extensive

TABLE 2 | Genome characteristics and *in silico* detection of virulence-associated genes in the sequenced *V. parahaemolyticus* genomes.

								Hemolysin		T3SS2		Post-1995 O3:K6 isolate RIMD2210633 genomic islands[b]							Other virulence-associated regions			
Strain Id	Serotype	Year	Isolate Source	Sample type[a]	Accession No.	No. Contigs	Genome Size (Mb)	*tdh*	*trh*	T3SS2α (VPal-7)	T3SS2β	VPal-1 (VP0380-VP0403)	VPal-2 (VP0635-VP0643)	VPal-3 (VP1071-VP1094)	VPal-4 (VP2131-VP2144)	VPal-5 (VP2900-VP2910)	VPal-6 (VPA1253-VPA1270)	VPal-7 (VPA1312-VPA1398)	f237 (VP1549-VP1562)	T6SS1 (VP1386-VP1414)	T6SS2 (VPA1025-VPA1046)	Superintegron (VP1787-VP1865)
Post-1995 O3:K6 Clinical Isolates																						
RIMD2210633	O3:K6	1996	Thailand (Japan airport)	clinical (NK)	NC_004603, NC_004605	2	5.15	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
K5030	O3:K6	2005	India	clinical (NK)	ACKB00000000	164	5.03	+	-	+	-	+	+	[c]+	+	+	+	+	+	+	+	+
AN5034[d]	O4:K68	1998	Bangladesh	clinical (NK)	ACFO00000000	54	5.20	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
Peru-466	O3:K6	1996	Peru	clinical (NK)	ACFM00000000	149	5.04	+	-	+	-	+	+	[c]+	+	+	+	+	+	+	+	+
Pre-1995 O3:K6 Clinical Isolates																						
AQ4037	O3:K6	1985	Maldive Islands	clinical (NK)	ACFN00000000	164	4.94	-	+	-	+	-	-	-	-	-	-	-	-	+	+	-
AQ3810	O3:K6	1983	Singapore	clinical (NK)	AAWQ00000000	1037	5.77	+	-	+/-	-	-	-	+/-	-	-	-	+/-	-	-	+	-
10329	O4:K12	1998	Washington, USA	clinical (NK)	AFBW00000000	33	5.09	+	+	-	+	-	-	-	-	-	-	-	-	+/-	+	-
K1275	O3:K54	2004	Texas, USA	clinical (blood)	JMMP00000000	63	5.11	-	-	-	-	-	+/-	-	-	-	-	-	-	+/-	[c]+	-
K1461	O4:K12	2004	Massachusetts, USA	clinical (stool)	JMMO00000000	65	5.17	+	+	-	+	-	-	-	-	-	-	-	-	+/-	[c]+	-
Environmental Isolates																						
AF91	O3:K6	2006	Florida, USA	environmental (sediment)	JMMS00000000	111	5.17	-	-	-	-	-	-	+/-	-	-	-	-	-	+/-	[c]+	+/-
BB22OP	O4:K8	1980s	Bangladesh	environmental (NK)	CP003972, CP003973	2	5.10	+	-	+/-	-	-	+/-	-	-	-	-	+/-	-	+/-	+	-
22702	O5:Kuk	1998	Georgia, USA	environmental (sediment)	JMMT00000000	43	4.95	-	-	-	-	-	-	-	-	-	-	-	+/-	-	+	-
J-C2-34	O5:K19	1998	North Carolina, USA	environmental (sediment)	JMMR00000000	91	5.15	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
SG176	O5:Kuk	2006	Georgia, USA	environmental (water)	JMMQ00000000	48	4.95	-	-	-	-	-	-	-	-	-	-	-	+/-	-	[c]+	-

[a] *NK, not known;*

[b] *A + indicates a gene or all genes within a region were detected with BSR values ≥0.8, a +/− indicates that at least half of the genes were detected with BSR values ≥0.8;*

[c] *All genes were detected with BSR values ≥0.8, except one gene that was <0.8;*

[d] *This isolate was described as having seroconverted from O3:K6 to O4:K68 (Chen et al., 2011).*

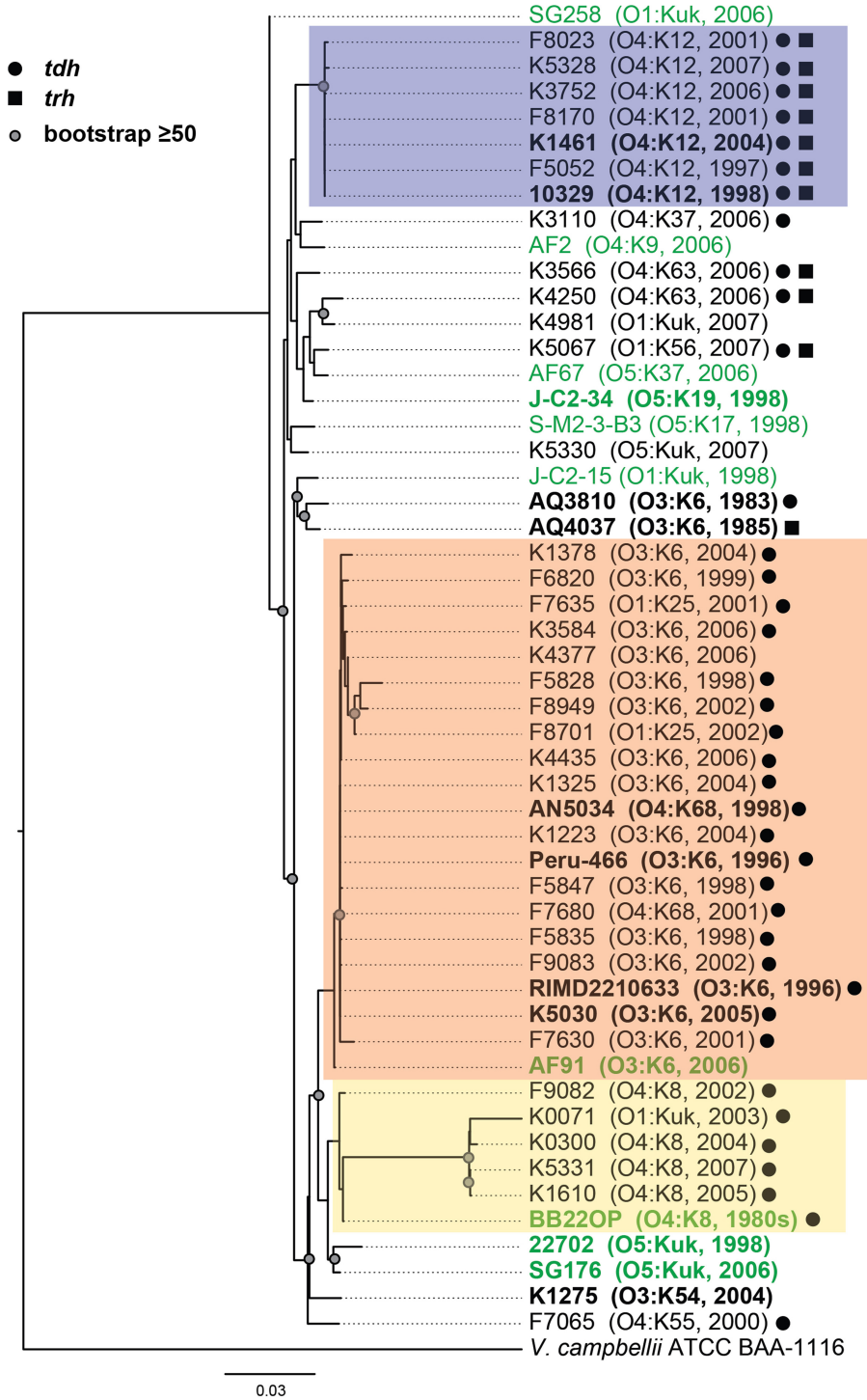

FIGURE 1 | Maximum-likelihood phylogeny of *V. parahaemolyticus* clinical and environmental isolates analyzed in this study compared to *V. parahaemolyticus* isolates that have been previously characterized by complete or draft genome sequencing and are available in the public domain. The nucleotide sequences of seven conserved genes (*recA, gyrB, pyrC, dtdS, tnaA, dnaE,* and *pntA*) were concatenated for each *V. parahaemolyticus* isolate, and *V. campbellii* ATCC BAA-1116 was included as an outgroup. The phylogeny was constructed using RAxML (Stamatakis, 2006) with 100 bootstrap replications, and visualized using FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/). Only bootstrap values ≥50 are shown. The scale bar represents 0.03 nucleotide substitutions per site. The genomes that were sequenced in this study or in previous studies that are available in the public domain are indicated in bold and are listed in **Table 2**. The post-1995 *V. parahaemolyticus* O3:K6 isolates are indicated by an orange box, the O4:K12 isolates are indicated by a purple box, and the O4:K8 isolates are indicated in yellow. The *V. parahaemolyticus* isolates obtained from environmental sources are indicated in green, while the isolates from clinical sources are indicated in black. The presence of the virulence-associated thermostable direct hemolysins, *tdh* and *trh*, in each of the genomes is indicated by symbols.

genetic diversity (Supplemental Figure 1). Previous studies have demonstrated there is considerable genetic diversity of *V. parahaemolyticus* isolates from around the world (Chowdhury et al., 2004; González-Escalona et al., 2008).

The genetic relatedness of *V. parahaemolyticus* clinical and environmental isolates was also investigated using PFGE, which is a cost-effective method for routine identification of disease-associated bacteria, including *V. parahaemolyticus* (Parsons et al., 2007). PFGE was performed on a total of 37 clinical isolates and 7 environmental isolates (Supplemental Figure 2). Analysis of the *Sfi*I and *Not*I patterns of these isolates demonstrated the presence of three main clades corresponding to those identified based on phylogenetic analysis of the housekeeping genes and the phylogenomic analysis (**Figure 1**, Supplemental Figure 1). A notable exception is that of the O3:K6 environmental isolate, AF91, which was not present in the O3:K6 clade by PFGE analysis as it was in the housekeeping gene phylogenies (**Figure 1**, Supplemental Figure 1). Similar to the housekeeping gene phylogenies, PFGE also demonstrated there is considerable genetic diversity among the clinical and environmental isolates analyzed that had serotypes other than those of the three main clades (O3:K6, O4:K12, and O4:K8) (Supplemental Figure 2). The PFGE pattern of the *V. parahaemolyticus* environmental isolate AF91 was different from the O3:K6 clinical isolates and the other environmental isolates examined. The *NotI* pattern of AF91 was similar to that of other *V. parahaemolyticus* isolates; however, the *Sfi*I pattern had multiple large bands that ranged from approximately 485- to 693-kb. In addition, the *Sfi*I pattern of this strain was missing four or more small bands that were present in the *Sfi*I patterns of the other *V. parahaemolyticus* isolates. The presence of the larger bands in the *Sfi*I pattern of AF91 suggested the absence of several *Sfi*I restriction sites that may correlate with the absence of the genomic islands of the post-1995 O3:K6 isolates (Hurley et al., 2006).

Comparative Genomics of Clinical and Environmental *V. parahaemolyticus*

To investigate whether there are shared or exclusive genome features of *V. parahaemolyticus* clinical and environmental isolates, we generated high-quality draft genome sequences of six *V. parahaemolyticus* isolates (K1461, K1275, SG176, J-C2-34, AF91, and 22702) that had diverse isolation sources, serotypes, and virulence factor content (Supplemental Table 1). Phylogenomic analysis of the six *V. parahaemolyticus* genomes sequenced in this study compared to previously sequenced *V. parahaemolyticus* genomes (**Table 2**) demonstrated there is considerable genomic diversity among isolates from clinical and environmental sources (**Figure 2**). Three of the environmental isolate genomes (22702, SG176, and J-C2-34) grouped together in the whole-genome phylogeny, while the other two environmental isolate genomes (AF91, BB22OP (Jensen et al., 2013) were within a larger group that contained the clinical isolate genomes (**Figure 2**). The phylogenomic analysis further confirmed that the O3:K6 environmental isolate, AF91, was more related to the post-1995 O3:K6 genomes than to the pre-1995 O3:K6 isolate genomes that have been previously sequenced (**Figure 2**).

In silico identification of the known *V. parahaemolyticus* virulence-associated genes and genomic islands (Hurley et al., 2006; Boyd et al., 2008) in the clinical and environmental genomes sequenced demonstrated that these regions were primarily identified in the post-1995 O3:K6 genomes (**Table 2**). However, some but not all of the genes in a few of these regions (VPaI-2, VPaI-3, and VPaI-7 encoding T3SS2α) were identified in some of the other clinical or the environmental isolate genomes (**Table 2**). This finding is similar to previous studies that demonstrated the T6SS gene cluster of chromosome I (T6SS1) is more frequently associated with *V. parahaemolyticus* clinical isolates than environmental isolates (Yu et al., 2012). The genes of T6SS1 were identified in nearly all the clinical isolate genomes, except the pre-1995 O3:K6 isolate AQ3810, and they were not identified in the genomes of the environmental isolates except for AF91 and BB22OP, which encode genes with similarity to those of T6SS1 (**Table 2**). However, AF91 and BB22OP have the serotypes O3:K6 and O4:K8, respectively, which are serotypes that have been linked to cases of human illness (Okuda et al., 1997; Matsumoto et al., 2000; Chowdhury et al., 2013; Ma et al., 2014). Further investigation is necessary to determine whether these environmental isolates may be more likely to cause disease than other environmental isolates that do not possess the T6SS1 genes.

Comparison of the *V. parahaemolyticus* genome content was analyzed using large-scale BLAST score ratio (LS-BSR) (Sahl et al., 2014) and further demonstrated the extent of the overall genome similarity among the clinical and environmental isolate genomes analyzed (**Table 3**). There were a total of 7782 genes identified in the 14 genomes analyzed in this study, 3494 of these genes were present with significant similarity (LS-BSR value $\geq$0.8) in all of the genomes analyzed (**Table 3**). Of the total genes identified there were 755 that were present in one or more of the clinical isolate genomes with significant similarity (LS-BSR value $\geq$0.8) that were not identified (LS-BSR value $<$0.4) in any of the environmental genomes sequenced (**Table 3**, Supplemental Table 2). Among these were genes encoding T3SS proteins, which likely belong to T3SS2 since these genes were not identified in any of the environmental isolates sequenced (**Table 2**). Also included among these genes were a multidrug resistance efflux pump and a putative RTX toxin (Supplemental Table 2). There were a similar number of genes (838) that were identified in one or more of the environmental isolate genomes that were not identified in any of the clinical isolate genomes (**Table 3**, Supplemental Table 2).

There were no genes identified in all of the clinical isolate genomes that were not present in one or more of the environmental isolate genomes, or vice versa (**Table 3**). This is likely due to significant genetic similarities between clinical and environmental isolate genomes such as the O3:K6 environmental isolate AF91 and the O3:K6 clinical isolates (**Figures 1**, **2**). The inability to identify genes that are exclusive to all clinical isolate genomes also can likely be attributed to the inclusion of the environmental isolate BB22OP, which encodes known virulence-associated genes such as *tdh* (Jensen et al., 2013). However, upon exclusion of the AF91 and BB22OP genomes from the analysis, there were 26 genes that were highly-conserved (LS-BSR values $\geq$0.8) in all

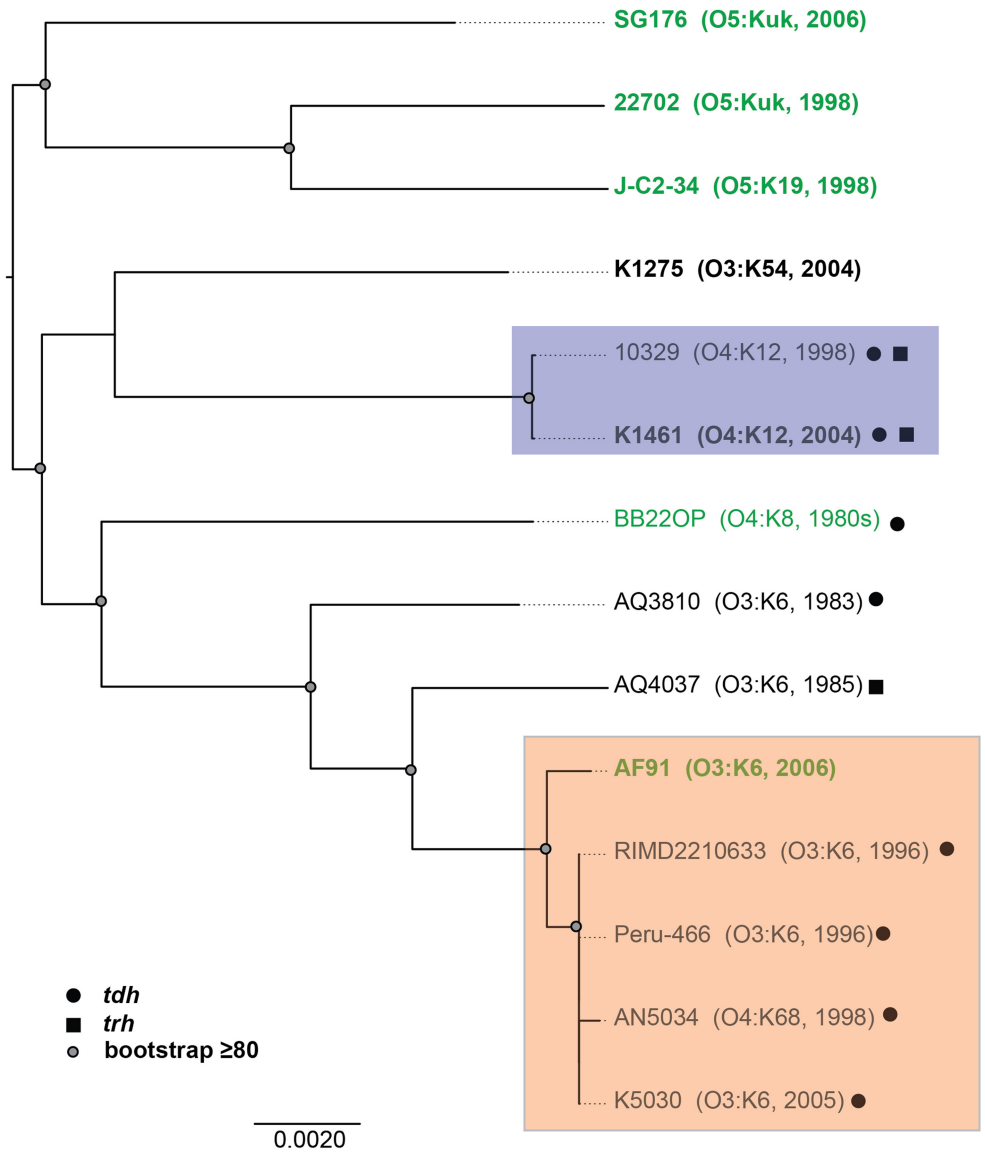

FIGURE 2 | Phylogenomic analysis of the six *V. parahaemolyticus* clinical and environmental isolates sequenced in this study (indicated in bold) compared to eight *V. parahaemolyticus* genomes that have been previously sequenced (Makino et al., 2003; Boyd et al., 2008; Chen et al., 2011; Gonzalez-Escalona et al., 2011; Jensen et al., 2013) and are available in the public domain. The genomes were aligned using Mugsy (Angiuoli and Salzberg, 2011), and an approximately 4.0 Mb region of each genome that aligned was concatenated to generate a single sequence for each isolate as previously described (Sahl et al., 2011). A maximum-likelihood phylogeny with 100 bootstrap replicates was constructed using RAxML (Stamatakis, 2006) and visualized using FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/). Bootstrap values ≥80 are indicated by a circle. The genomes of *V. parahaemolyticus* isolates from environmental sources are indicated in green, while the isolates from clinical sources are indicated in black. The post-1995 O3:K6 isolates are identified by the orange box, and the O4:K12 isolates are indicated by a purple box. The presence of the virulence-associated thermostable direct hemolysins, *tdh* and *trh*, in each of the genomes is indicated by symbols.

clinical isolate genomes that were divergent (LS-BSR values <0.8, ≥0.4) or absent (LS-BSR values < 0.4) from the three remaining environmental isolate genomes (22702, J-C2-34, SG176), and 20 that were highly-conserved (LS-BSR values ≥0.8) in the three environmental isolate genomes that were divergent (LS-BSR values <0.8, ≥0.4) or absent (LS-BSR values <0.4) from the clinical isolate genomes (**Table 3**). The small number of genes that were universal to clinical or environmental isolates could also be a result of the genetic diversity or misclassification of the clinical and environmental isolates (**Figures 1**, **2**).

The number of genes that were exclusive (LS-BSR values ≥0.8, and <0.4 in all other genomes) to the six *V. parahaemolyticus* genomes sequenced in this study ranged from 20 to 173 (Supplemental Table 3). The fewest number of exclusive genes (20) was identified in the genome of the O4:K12 isolate K1461, which can be attributed to the significant genomic similarity of this isolate

TABLE 3 | LS-BSR analysis of the genomic similarity of select *V. parahaemolyticus* clinical and environmental isolates.

Genomes	No. of genomes	No. of gene clusters	
		All	≥ 1[c]
All genomes analyzed	14	3473	7782
All clinical isolate genomes	9	0 (26)[b]	755 (407)[d]
All environmental isolate genomes	5	0 (20)[b]	838 (230)[d]
Clinical Isolate Genomes			
Post-1995 O3:K6 (including AF91)[a]	5	17	423
Post-1995 O3:K6 (not including AF91)[a]	4	78	198
Pre-1995 O3:K6	2	26	391
O4:K12	2	169	244

[a] *This comparison group includes an O4:K68 isolate that is a seroconversion from O3:K6, and the environmental isolate AF91 where indicated in parentheses.*

[b] *Genes are highly conserved (LS-BSR ≥0.8) in all genomes of the group and divergent (LS-BSR <0.8, ≥0.4) or absent (LS-BSR < 0.4) in the other genomes. The number of gene clusters in parentheses is conserved in all genomes when BB22OP and AF91 are not included.*

[c] *Genes that are highly conserved (LS-BSR ≥ 0.8) in one or more of the genomes of the group and divergent or absent (LS-BSR < 0.8) in the other genomes.*

[d] *The number of gene clusters that are highly-conserved (LS-BSR ≥0.8) in one or more genomes of this group, and absent (LS-BSR <0.4) from the other genomes. The number in parentheses is the number of gene clusters that are highly-conserved (LS-BSR ≥0.8) in one or more genomes of this group, but are divergent (LS-BSR <0.8, ≥0.4) in one or more of the other genomes.*

to the previously sequenced genome of the O4:K12 isolate 10329 (Gonzalez-Escalona et al., 2011) (**Figure 2**). The genome of the *V. parahaemolyticus* clinical isolate that was *tdh*−/*trh*−/T3SS2−, K1275, encoded 150 genes that were exclusive to this isolate (Supplemental Table 3). Among these unique genes were many hypothetical proteins and other genes that lacked similarity to any previously characterized genes, which suggests there is extensive genomic diversity that has yet to be characterized from *V. parahaemolyticus* isolates (Supplemental Table 3).

Many of the genes identified as exclusive to a particular genome were hypothetical or were similar to genes of mobile genetic elements including plasmids and phage (Supplemental Table 3), highlighting the contribution of mobile elements such as plasmids and phage to the diversification of *V. parahaemolyticus*. In addition to being present in the post-1995 O3:K6 genomes, the protein-encoding genes of the filamentous vibriophage f237 of RIMD2210633 were identified in the genomes of two environmental isolate genomes (22702, SG176) (**Table 2**). Sequence analysis of partial nucleotide sequences of the vibriophage replication protein-encoding gene, *rstA* obtained from *V. parahaemolyticus* clinical and environmental isolates revealed these sequences had 95–100% nucleotide identity to the *rstA* of the filamentous phage f237 (Nasu et al., 2000). Phylogenetic analysis of the partial nucleotide sequences of *rstA,* demonstrated there is no discernible pattern of genetic similarity of the filamentous vibriophage based on serotype, isolation source, or geographical location, with the exception of the O4:K12 isolates and the post-1995 O3:K6 isolates (Supplemental Figure 3).

In addition to the identification of genetic similarity of filamentous vibriophage from clinical and environmental isolates, there was an approximately 90-kb phage-like element identified in the genome sequences of the O4:K12 clinical isolate K1461, and the environmental isolate and J-C2-34. Analysis of the plasmid content of K1461 and J-C2-34 using a modified acid-phenol extraction method (Kieser, 1984; Sobecky et al., 1997) demonstrated that both of these isolates contain a single large extrachromosomal element that is approximately 90-kb, which is likely the prophage identified in the genomes of these isolates. Sequence characterization of these prophage demonstrated they encode numerous phage-like genes with a conserved organization that exhibited 80–100% nucleotide identity to each other (Supplemental Figure 4). This finding provides additional evidence of the horizontal transfer of similar prophage-like elements among *V. parahaemolyticus* clinical and environmental isolates. These phage-like elements also exhibited divergent similarity (BSR values ≥0.4, <0.8) to genes encoded by a previously sequenced plasmid, p0908, from *V. fluvialis* (Hazen et al., 2007) and the bacteriophage P1 (Lobocka et al., 2004), suggesting they belong to a phage family that has relatives in other enteric bacteria.

Genomic Similarity of the O3:K6 Environmental Isolate, AF91, to Pre- and Post-1995 O3:K6 Isolates

Phylogenomic analysis demonstrated that the 2006 O3:K6 environmental isolate, AF91, exhibited greater genomic similarity to the post-1995 O3:K6 isolate genomes (Makino et al., 2003; Chen et al., 2011) than to the two pre-1995 O3:K6 isolate genomes (Boyd et al., 2008; Chen et al., 2011) (**Figure 2**). A PCR-based assay and *in silico* analysis of the AF91 genome demonstrated that AF91 does not encode the hemolysins or T3SS2 that are typically found in *V. parahaemolyticus* clinical isolates (**Table 2**, Supplemental Table 1). The AF91 genome was also missing most of the genomic islands of the post-1995 O3:K6 isolate RIMD2210633 (**Table 2**, **Figure 3**). However, AF91 did contain genes with similarity to those encoded by the genomic island VPaI-3 (**Table 2**), which was previously identified in post-1995 O3:K6 isolates and related isolates (AN5034) that have undergone seroconversion (Boyd et al., 2008; Chen et al., 2011) (**Table 2**). AF91 also encoded genes with significant similarity to genes of T6SS1 (**Table 2**, **Figure 3**), which has primarily been identified in *V. parahaemolyticus* clinical isolates (Yu et al., 2012). Their findings demonstrated that the T6SS1 genes exhibited bacteriolytic activity against other bacteria when grown in conditions similar to the marine environment, suggesting T6SS1 provides a fitness advantage that allows the disease-associated isolates to be competitive and persist in marine environments (Salomon et al., 2013). T6SS1 may have contributed to the emergence and spread of the post-1995 O3:K6 isolates (Okuda et al., 1997; Matsumoto et al., 2000).

Comparative analysis of the O3:K6 genomes using LS-BSR demonstrated there were only 17 gene clusters identified in all post-1995 O3:K6 isolates, including the O3:K6 environmental isolate AF91 (**Table 3**) that were divergent (LS-BSR value <0.8, ≥0.4) or absent (LS-BSR value <0.4) from the other genomes. However, there were 78 gene clusters present with significant similarity in all of the post-1995 O3:K6 isolate genomes when AF91 was not included (**Table 3**). This finding

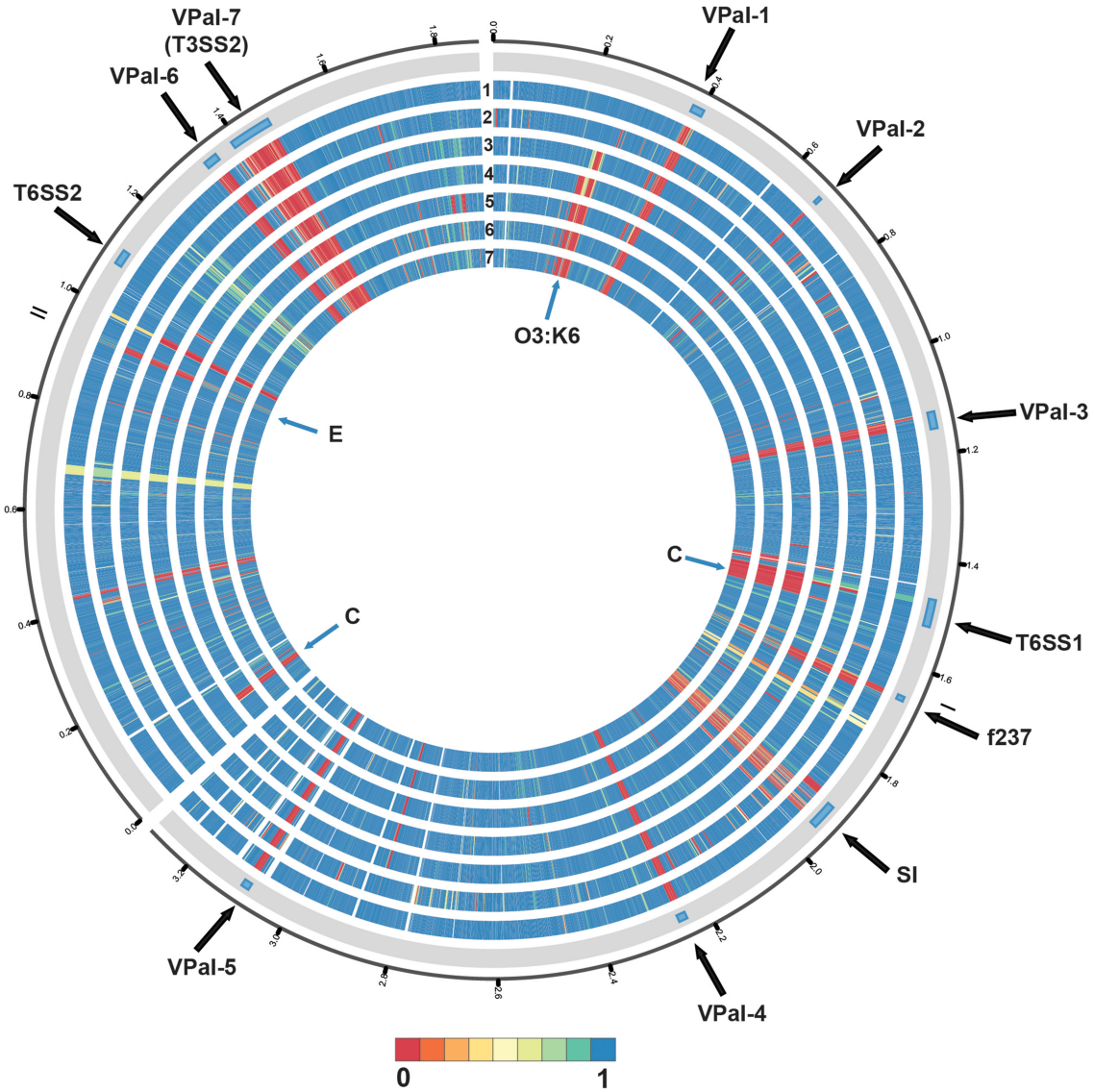

FIGURE 3 | Circular display of the BLASTN BSR values demonstrating the genetic similarity of each gene of RIMD2210633 in the genome of the pre-1995 O3:K6 isolate AQ4037 (Chen et al., 2011), and the genomes sequenced in this study. The previously described putative pathogenicity islands (Hurley et al., 2006) and other regions of interest (Chen et al., 2011) in the genome of RIMD2210633 (Makino et al., 2003) are indicated in the outermost track. The genomes in tracks labeled 1–7 are as follows: (1) AF91, (2) AQ4037, (3) K1275, (4) K1461, (5) SG176, (6) J-C2-34, and (7) 22702. The location of the O3:K6 O-antigen lipopolysaccharide region is indicated by "O3:K6." An "E" designates the location of RIMD2210633 genes that are present with greater similarity in the environmental isolate genomes than in the clinical isolate genomes. A "C" designates the location of RIMD2201633 genes that are present in the clinical isolate genomes analyzed and not in the environmental isolate genomes, with the exception of AF91. The circular display of the BSR values was generated using Circos (Krzywinski et al., 2009). Blue indicates genes were present with significant similarity, yellow indicates the genes were present but divergent, and red indicates genes were absent.

demonstrates that although AF91 has the O3:K6 serotype and was isolated a decade after the emergence and spread of the O3:K6 pandemic clone (Okuda et al., 1997; Vuddhakul et al., 2000; Myers et al., 2003; Quilici et al., 2005; Ottaviani et al., 2008), the genome of AF91 exhibits genetic differences compared to other post-1995 O3:K6 isolate genomes. Not surprisingly, many of these genes were encoded within the genomic regions that were previously described as unique to the post-1995 O3:K6 genomes (Hurley et al., 2006; Chen et al., 2011) (Supplemental Table 4). In addition to the 78 genes that are divergent or missing (LS-BSR value <0.8) from the AF91 genome compared to other post-1995 O3:K6 genomes, there were 173 genes that were identified by LS-BSR as being unique to the AF91 genome compared to the other genomes analyzed (**Table 3**). The genes that were unique to AF91 included integrases and transposases, putative transcriptional regulators, a GGDEF domain-containing protein,

and many genes encoding proteins with unknown functions (Supplemental Table 3).

This study describes an environmental O3:K6 isolate that exhibits significant genetic similarity to the post-1995 O3:K6 isolates, yet does not encode most of the known virulence-associated genes of these isolates. Due to the genomic similarity of this O3:K6 environmental isolate to the post-1995 O3:K6 isolates, this isolate may have originally carried the missing genomic islands, and after entering the environment may have begun to transition to an environmental niche by losing the virulence-associated genomic regions. For example, genomic islands of the uropathogenic *Escherichia coli* isolate 536, a strain isolated from a urinary tract infection, were demonstrated to be unstable (Middendorf et al., 2004; Soto et al., 2006). Interestingly, two of the *E. coli* 536 genomic islands, including one island that encoded a hemolysin, were lost in response to altered environmental conditions such as lower temperature and higher cell density (Middendorf et al., 2004). Further experiments would be necessary to determine whether AF91 would have a fitness advantage over other *V. parahaemolyticus* environmental isolates, and whether the absence of the other post-1995 O3:K6 genomic islands and virulence-associated regions gives it an additional advantage for surviving in the environment. Another possibility is that AF91 may represent an intermediate isolate that was involved in the emergence of the post-1995 O3:K6 isolates, and AF91 may have persisted without acquiring the post-1995 O3:K6 genomic islands due to a fitness advantage for surviving in the environment. Further experiments are needed to determine if AF91 would have a similar pathogenic potential as other post-1995 O3:K6 isolates, following the acquisition of the post-1995 O3:K6 genomic islands and other virulence-associated regions.

Previous research investigating the disease-causing potential of *V. parahaemolyticus* in the environment has typically examined the presence of prevalent clinical serotypes and known virulence-associated genes. However, we have demonstrated that investigating the genetic diversity of environmental isolates that do not carry the known virulence-associated genes can yield insight into the emergence of human disease-associated *V. parahaemolyticus*. Sequencing and characterization of *V. parahaemolyticus* AF91, an O3:K6 environmental isolate, demonstrated that environmental isolates that do not carry the known virulence-associated genes can have significant genetic similarity to disease-associated *V. parahaemolyticus* clinical isolates, including the pandemic post-1995 O3:K6 isolates. Additional genome sequencing of *V. parahaemolyticus* clinical and environmental isolates that have diverse serotypes and unique combinations of known virulence-associated genes and genomic regions would yield further insight into the ability of *V. parahaemolyticus* isolates to transition from an environmental niche and to emerge as pathogens.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2015.00204/abstract

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Genetic characterization of clinical and environmental *Vibrio parahaemolyticus* from the Northeast USA reveals emerging resident and non-indigenous pathogen lineages

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Gastric infections caused by the environmentally transmitted pathogen, *Vibrio parahaemolyticus,* have increased over the last two decades, including in many parts of the United States (US). However, until recently, infections linked to shellfish from the cool northeastern US waters were rare. Cases have risen in the Northeast, consistent with changes in local *V. parahaemolyticus* populations toward greater abundance or a shift in constituent pathogens. We examined 94 clinical isolates from a period of increasing disease in the region and compared them to 200 environmental counterparts to identify resident and non-indigenous lineages and to gain insight into the emergence of pathogenic types. Genotyping and multi-locus sequence analysis (MLSA) of clinical isolates collected from 2010 to 2013 in Massachusetts, New Hampshire, and Maine revealed their polyphyletic nature. Although 80% of the clinical isolates harbored the *trh* hemolysin either alone or with *tdh*, and were urease positive, 14% harbored neither hemolysin exposing a limitation for these traits in pathogen detection. Resident sequence type (ST) 631 strains caused seven infections, and show a relatively recent history of recombination with other clinical and environmental lineages present in the region. ST34 and ST674 strains were each linked to a single infection and these strain types were also identified from the environment as isolates harboring hemolysin genes. Forty-two ST36 isolates were identified from the clinical collection, consistent with reports that this strain type caused a rise in regional infections starting in 2012. Whole-genome phylogenies that included three ST36 outbreak isolates traced to at least two local sources demonstrated that the US Atlantic coastal population of this strain type was indeed derived from the Pacific population. This study lays the foundation for understanding dynamics within natural populations associated with emergence and invasion of pathogenic strain types in the region.

Keywords: disease ecology, emergent pathogen, MLSA, Vibriosis, population structure, pathogen evolution, hemolysin

Introduction

Rare pathogenic variants of *Vibrio parahaemolyticus*, a ubiquitous yet typically harmless estuarine bacterium, can cause human gastric infections most often from the consumption of raw or improperly handled seafood, and wound infections from recreational aquatic activities (Daniels et al., 2000; Scallan et al., 2011). Infections typically occur seasonally during warmer months when total populations of these bacteria rise, and with them, the risk of exposure to an infectious dose of pathogens increases (Parveen et al., 2008). Even so, temperature and total abundance do not fully explain infection trends as some infections occur when water temperatures and abundance of total *V. parahaemolyticus* are low (Zimmerman et al., 2007; Johnson et al., 2012; Jones et al., 2012). Furthermore, recurrent infections and outbreaks have also occurred in cooler waters of the Pacific Northwest (PNW) for decades where pathogens re-emerge each year from among diverse residential populations (Altekruse et al., 2000; Johnson et al., 2010; Paranjpye et al., 2012; Turner et al., 2013; Banerjee et al., 2014). A better understanding of conditions that promote emergence and relative abundance of pathogens is necessary to develop appropriate strategies for disease prevention.

Comparatively fewer and sporadic infections have been associated with shellfish harvested from the cooler waters of the Northeastern US. One notable exception was a large multi-state outbreak in 1998, which included oysters harvested from Long Island Sound bordered by New York (NY) and Connecticut (CT) (**Figure 1**) (CDC, 1999) That outbreak was attributed to a non-indigenous strain from the pandemic clonal complex which are typically serotype O3:K6 and sequence type (ST) 3 and that spread globally from Southeast Asia (CDC, 1999; Depaola et al., 2000; Nair et al., 2007; García et al., 2009; Harth et al., 2009; Martinez-Urtaza et al., 2010; Alikhan et al., 2011). The ensuing low disease incidence in the Northeastern US implied that the regional environmental conditions did not sustain either invasive or endemic pathogenic populations (Mahoney et al., 2010; Ellis et al., 2012). In the last several years, however, reported cases have been increasing in the Northeast, with outbreaks in NY in 2012, and in NY, CT, and Massachusetts (MA) in 2013 (Newton et al., 2014) (**Figure 1**). This abrupt increase in cases coincided with both warmer than usual ocean temperatures in the region (**Figure 1**) and the probable Atlantic ecological invasion of a lineage of ST36 strains, which are indigenous to the cooler waters of the PNW (Martinez-Urtaza et al., 2013; Newton et al., 2014). Thus, the emergent disease in the Northeast appears to be unlike other situations under study in the US. Characterizing clinical strains from the region and relating them to native non-pathogens during this period of increased disease incidence could provide insight into how changes in the bacterial population have led to increased disease.

A significant obstacle for the study of emergent pathogenic strains of *V. parahaemolyticus* is our lack of understanding of factors that define virulence and that could be used to detect pathogens within mostly non-pathogenic populations. Few of the diagnostic markers most commonly used to define pathogens are actually implicated in disease, including two hemolysin genes (*tdh* and *trh*) and a horizontally acquired type-three secretion system (T3SS2) (Honda and Iida, 1993; Hiyoshi et al., 2010). Although hemolysins are sufficient for inducing some disease symptoms, they are not necessary for disease in either mice or humans, indicating they are not the only virulence determinant (Nishibuchi et al., 1992; Xu et al., 1994; García et al., 2009; Thongjun et al., 2013; Banerjee et al., 2014). Perhaps more concerning, the abundance of hemolysin-containing strains in the environment often does not correlate with increased incidence of disease, calling into question the ability of these markers to sufficiently predict risk evaluation (Johnson et al., 2012; Jones et al., 2012).

In the absence of a definitive diagnostic marker of virulence, identification of related strains or lineages of pathogens that are known to cause infections would aid in the study of seasonal population dynamics associated with emergence of these pathogenic types. The many commonly applied strain typing tools, including serotyping, rep-PCR, pulse field electrophoresis (PFGE), intergenic spacer region (ISR-1), and restriction fragment length polymorphisms can group similar strains but have some limitations for resolving evolutionary relationships, especially when used individually (Chowdhury et al., 2000, 2004; Nair et al., 2007; Jones et al., 2012; Banerjee et al., 2014; Lüdeke et al., 2014). Multi-locus sequence analysis (MLSA) of conserved housekeeping genes can better define relatedness and recombination among strains (Depaola et al., 2003; Jolley et al., 2004; Thompson et al., 2005; Sawabe et al., 2007; González-Escalona et al., 2008; Ellis et al., 2012; Paranjpye et al., 2012; Martinez-Urtaza et al., 2013; Turner et al., 2013; Banerjee et al., 2014). But the analysis of pathogens alone, in the absence of related non-pathogenic relatives, will not provide a complete picture of evolution of virulence.

Here we combine MLSA, genotyping, and phylogenetic analysis to relate clinical strains with environmental isolates from northern New England. The strains analyzed include 94 clinical isolates from reported cases in three northern New England US states and more than 200 environmental isolates identified from the region since 2007 (Mahoney et al., 2010; Ellis et al., 2012). The study demonstrated that disease-causing strains are genetically diverse and polyphyletic. Some infections were caused by pathogens that are resident, but the strains that caused a steep rise in infections in 2013 are derived from the PNW ST36 population as previously suggested (Martinez-Urtaza et al., 2013; Newton et al., 2014). This study lays the foundation for detailed analysis of conditions that have promoted emergence and invasion of pathogenic types in the region.

Materials and Methods

Bacterial Strains, Strain Designations, and Culture Conditions

Ninety-four *V. parahaemolyticus* clinical isolates, defined as isolated from a clinical (patient) source (e.g., wounds or stool), from 2010 to 2013 were provided by cooperating public health laboratories in MA, New Hampshire (NH), and Maine (ME) (**Table 1**). For the purposes of this study and in the absence

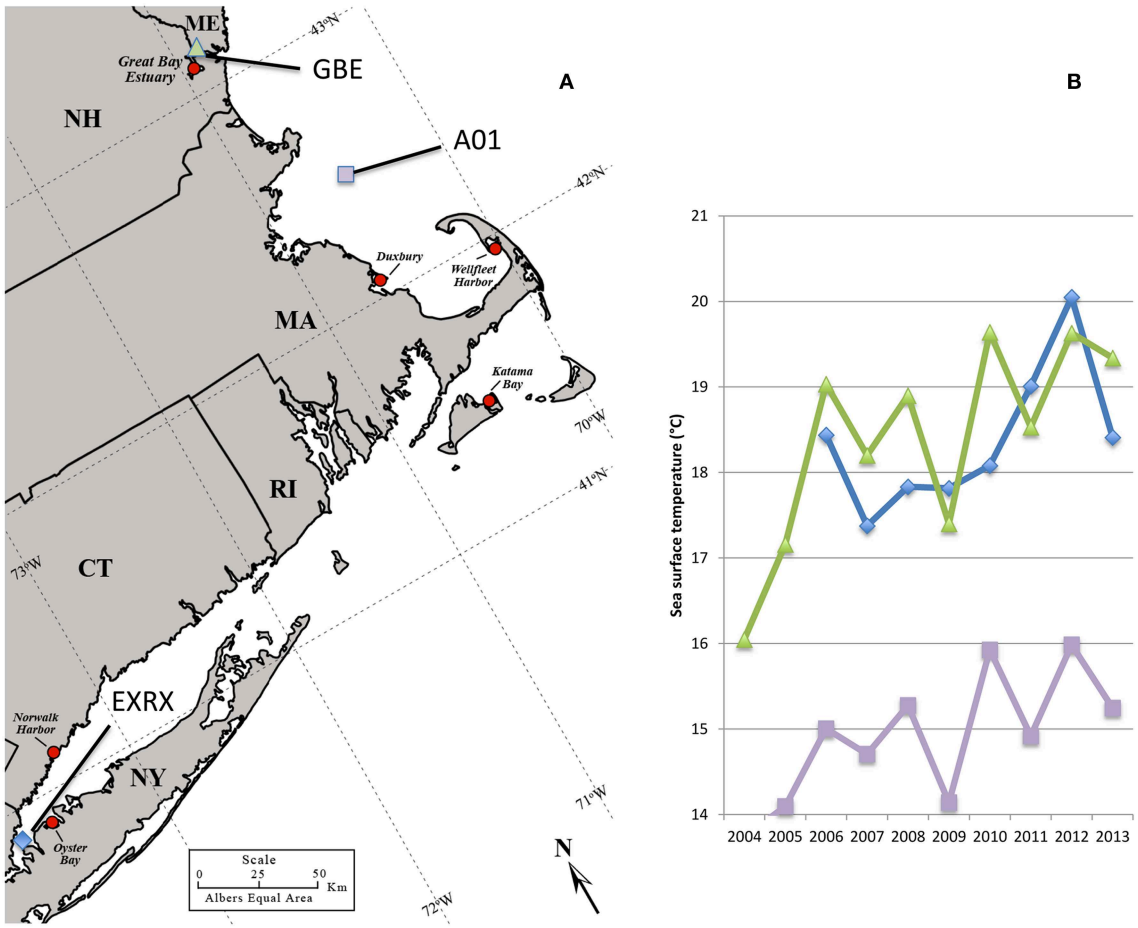

FIGURE 1 | Regional oyster production areas and ocean temperatures. Regional oyster harvest and production areas all identified with red circle, some of which have been linked to cases or outbreaks of *V. parahaemolyticus* including: Oyster Bay NY in 1998, 2012, and 2013; Norwalk Harbor CT in 1998, and 2013; Katama Bay MA in 2013; and Duxbury MA in 2013. No closures had occurred at the Great Bay Estuary site in NH or Wellfleet Harbor in MA as of 2013 **(A)**. Ocean buoys used for temperature measurements identified by blue diamond, purple square, and green triangle symbols **(A,B)**. Increasing average monthly sea surface water temperatures during June 2004–2013 at offshore buoys in Long Island Sound (EXRX), the Gulf of Maine (A01), and the Great Bay Estuary (GBE) **(B)**. Map was adapted from the US Department of Agriculture Natural Resource Conservation Service.

of contradictory data, all these clinical isolates are considered pathogenic, regardless of genotype. Additional environmental strains from the Great Bay Estuary bordered by NH and ME prior to 2011 were also included for comparisons (Supplementary Table 1) (Mahoney et al., 2010; Ellis et al., 2012). A few previously unreported strains collected from the Great Bay Estuary in 2010–2013, and two environmental isolates from oysters harvested in CT that were recalled due to an outbreak were also used in this study (Supplementary Table 1). For the purpose of this study, we define environmental strains that lack hemolysin genes, and that are not phylogenetically related to strains from clinical sources as non-pathogenic. Environmental isolates that harbor either or both hemolysin genes, or are related to isolates from clinical sources (e.g., shared phylogeny or identical ST) are defined as potentially pathogenic. Finally, although not yet identified locally, isolates from the environment that are genetically indistinguishable from a clinical isolate by genotype and ST, and deemed clonal based on whole genome phylogeny are considered pathogenic. Several pathogenic *V. parahaemolyticus* isolates acquired previously included ST36 strain F11-3A (González-Escalona et al., 2008), pandemic strain MDOH-04-5M732 (Davis et al., 2007) and pre-pandemic strain BB22OP LM5132 (Mccarter, 1998), which were used as controls. Strains were grown in heart infusion (HI) medium with 3% NaCl (Fluka, Buchs, Switzerland) at 28°C (for environmental strains) or 37°C (for clinical strains) for routine culturing. Strains from environmental sources are enriched in APW, isolated on CHROMAgar Vibrio (CHROMagar, Paris, France) as purple colonies and cultured on T-soy agar as previously described (Schuster et al., 2011).

Genotypic Analysis

Genotyping (**Table 2**, Supplementary Table 1) was performed by PCR amplification of template genomic DNA isolated from

TABLE 1 | Sources and reporting states for clinical isolates available for this study.

Type of infection	Year	Strains by reporting state[1]	Potential exposure source traced to location[2]
Gastric	2010	MAVP-E[a]	MA[3]
		MAVP-A, MAVP-T, NHVP-1	Unknown
	2011	MAVP-H[a], MAVP-M[a], MAVP-Q[a]	MA[3]
		MAVP-R[a]	MA
		MAVP-B, MAVP-C, MAVP-D[a], MAVP-J, MAVP-K,MAVP-L, MAVP-N, MAVP-O[a], MAVP-U[a]	Unknown
	2012	NHVP-2[a]	Unknown
	2013	MAVP-56[b], MAVP-57[b]	Canada
		MAVP-7[b]	Canada, MA, or ME
		MAVP-55	Canada, MA, or other than North America
		MAVP-11[b], MAVP-14[b], MAVP-29[b]	Canada, MA, or WA
		MAVP-18[b], MAVP-19[b], MAVP-23[b], MAVP-31[b], MAVP-38[b]	CT
		MAVP-32[b], MAVP-52[b]	CT or MA
		MAVP-44[b]	Canada, CT, or MA
		MAVP-40[b]	CT or VA
		MAVP-6[b], MAVP-9[s], MAVP-17[b], MAVP-20[b], MAVP-26[b], MAVP-33[f], MAVP-34[b], MAVP-36[b], MAVP-37[b], MAVP-45[b], MAVP-46[r], MAVP-48[b], MAVP-54[b], MAVP-59[b], MEVP-4[b]	MA
		MAVP-8[b], MAVP-24[b]	MA, or ME
		MAVP-2[b]	MA, VA, or WA
		MEVP-2[e,d,i,l], MEVP-6[c]	ME
		MAVP-3[p]	Other than North America
		MAVP-1[b], NHVP-3[b]	VA
		MAVP-5[f], MAVP-12[b], MAVP-16[c,g], MAVP-22[c],MAVP-25[j], MAVP-27[b], MAVP-28, MAVP-30[b], MAVP-35[f], MAVP-39, MAVP-41[b], MAVP-43[b], MAVP-49, MAVP-50[b], MAVP-51, MAVP-55[b], NHVP-4[c], MEVP-1[c,k], MEVP-3[b,c,m,n,o], MEVP-5[c,i,q]	Unknown
Wound	2011	MAVP-F, MAVP-G, MAVP-I, MAVP-X	MA
	2013	MAVP-13	MA
Unknown	2010	MAVP-P	Unknown
	2011	MAVP-S, MAVP-V, MAVP-Y	
	2012	MAVP-W	
	2013	MAVP-4, MAVP-10, MAVP-15, MAVP-21, MAVP-42, MAVP-47, MAVP-53, MAVP-58	

[1] *Strains are coded by reporting state in the reference strain name and blinded by random assignment of letters (for MA isolates prior to 2013) or numbers (for all others); MAVP for MA, NHVP for NH, MEVP for ME. Potential exposure source(s) for each isolate is identified when reported as follows: [a]Oysters; [b]Raw oysters; [c]Lobsters; [d]Striped bass; [e]Cooked lobster; [f]Raw clams; [g]Fried clams; [h]Cooked clams; [i]Clams; [j]Quahogs; [k]Haddock; [l]Sea scallops; [m]Crab; [n]Shrimp; [o]Crawfish; [p]Sushi; [q]Fish chowder; [r]Handled bait; [s]Swallowed seawater while swimming.*

[2] *Location where the V. parahaemolyticus contaminated food was harvested or where water exposure occurred. For wound infections, exposure presumed in reporting state.*

[3] *Inferred that oysters potentially harvested from MA sources for these isolated cases reported from Cape Cod locations.*

cultures grown in HI medium for 6 h using either Master Taq (5 PRIME, MD, US) or AccuStart PCR Supermix (Quanta, MD, US). DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, WI, USA), using columns and manufacturer-provided recipes (Epochlifescience Inc., TX, US), or by cetyltrimethylammonium bromide protein precipitation and organic extraction (Ausubel et al., 1990). The presence of a species-specific gene (*tlh*), both hemolysin genes (*tdh* and *trh*) and the pandemic marker ORF8 was determined using published primers and cycling parameters (Panicker et al., 2004) (**Table 2**, Supplementary Table 1). The presence of additional virulence-associated genes including a homolog of *Escherichia coli* cytotoxic necrotizing factor *vopC* as well as several additional genes located within pathogenicity islands of strain MDOH-04-5M732 including T3SS2 genes *vscC2* and effector *vopP*, was assessed using published primers and cycling parameters (Caburlotto et al., 2009). For each reaction, the presence of an amplicon of the correct size was determined following electrophoresis in 0.7% (for large amplicons) or 1.2% (for small amplicons) Sea Kem LE agarose (Lonza Group Ltd., NH, US) in 1× TAE buffer with 1× GelRed (Phenix Research Products, NC, US) for amplicon visualization. The size of each amplicon was determined by comparison to 1 Kb-plus ladder (Invitrogen Inc., NY, US) and also compared to amplicons from control strains including F11-3A

TABLE 2 | Distribution of genotypes* among Northeastern US clinical isolates.

	tdh[a]	*trh*[b]	ORF8[c]	*vscC2*[d]	*vopP*[e]	*vopC*[f]
REFERENCE STRAINS BY SEROTYPE						
O3:K6[G]	+	−	+	+	+	+
O4:K12[H]	+	+	−	−	−	−
NUMBER OF NEW ENGLAND ISOLATES						
2	+	−	+	+	+	+
2	+	−	−	+	+	+
2	+	−	−	−	−	−
3	−	+	−	+	−	−
1	−	+	−	−	−	+
4	−	+	−	−	−	−
1	+	+	−	+	−	−
66	+	+	−	−	−	−
2	−	−	−	+	−	−
11	−	−	−	−	−	−

*Presence (+) or absence (−) of gene as determined by PCR.
[a]Thermostable direct hemolysin.
[b]Thermostable-related hemolysin; [c]O3:K6 Pandemic marker.
[d]Putative type III secretion system EscC protein. Chromosome II T3SS-pathogenic V. parahaemolyticus.
[e]Putative type III secretion effector YopP protein. Chromosome II T3SS-pathogenic V. parahaemolyticus.
[f]Homolog of E. coli cytotoxic necrotizing factor. Gene located on a pathogenicity island of V. parahaemolyticus.
[G]MDOH-04-5M732.
[H]F11-3A.

TABLE 3 | Correlation of urease activity with the presence of *trh*.

Hemolysin genotyp		% of urease positive (# of strains tested)
tdh	*trh*	
FOR CLINICAL ISOLATES		
+	+	100% (67)
+	−	25% (8)
−	+	100% (8)
−	−	33% (11)
FOR ENVIRONMENTAL ISOLATES		
+	+	100% (7)
+	−	0% (1)
−	+	100% (2)
−	−	0% (10)

and MDOH-O4-5M732. Presence of the correct size amplicon or gene (for sequenced strains) is denoted by (+), whereas absence of amplicon is denoted by (−).

Urease Activity

Urease activity (**Table 3**, Supplementary Table 1) was determined on strains first grown in HI medium for 6 h at 28°C or 37°C, and then inoculated in triplicate as 10 μl samples onto 200 μl modified Christensen's urea agar, containing 2% NaCl, 0.1% peptone, 0.1% dextrose, 0.2% KH_2PO_4, 2% urea, 0.12% phenol red, and 2% agar in the wells of a 96-well plate. The plates were sealed with Breathe-Easy membrane (USA scientific Inc., FL, US) and incubated with ventilation at 37°C overnight. A positive reaction is observed as a change in color from yellow to pink. Wells without bacterial inoculum remained yellow. The association of the presence of a hemolysin gene (*tdh* or *trh*) and urease activity in isolates was determined using a two-tailed Fisher's exact test (Preacher and Briggs, 2001).

Genome Sequencing and Assembly

The *V. parahaemolyticus* ST631 strain MAVP-E, and four representative ST36 isolates including MAVP-26, MAVP-36, MAVP-45, all traced to shellfish harvest areas in MA, and MAVP-V, from an unknown source, were sequenced using an Illumina-HiSeq2500 device at the Hubbard Center for Genome Studies at the University of New Hampshire. Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, WI, USA) as recommended by some sequencing centers or by a cetyltrimethylammonium bromide and organic extraction method (Ausubel et al., 1990) that provides both higher quality and quantity of DNA but requires more technical skill. The DNA quality was assessed visually by electrophoresis. Sequencing libraries were generated from 1 μg of genomic DNA as determined using the Qubit 2.0 fluorimeter (LifeTech, CA, US). DNA was sheared on the Covaris M220 Ultasonicator to a mean size of 500 bp. Libraries were generated using the TruSeq Kit and targeted size selection of 500 bp was completed using the optional gel-extraction method in the TruSeq protocol (Illumina). MAVP-E was sequenced by a high output mode run, 101 bp paired-end whereas MAVP-26 and MAVP-36 were sequenced using a rapid output mode run, 150 bp paired-end with 152-fold coverage for MAVP-E, 249-fold coverage for MAVP-26, 238-fold coverage for MAVP-36, 355-fold coverage for MAVP-45, 847-fold coverage for MAVP-V, 84-fold coverage for MAVP-M, and 167-fold coverage for CT4287. Raw sequences were processed and *de novo* assemblies performed using the A5 pipeline (Tritt et al., 2012).

Multi-locus Sequence Analysis and Phylogenies, and Analysis of Population Structure

Phylogenetic analysis was performed from concatenated sequences derived by PCR amplification of multiple housekeeping loci. The amplicons were generated using Master Taq (5 PRIME, MD US), and sequenced by the Sanger method at the UNH Hubbard Center for Genome Studies or by Functional Biosciences (WI, US). For inferring multi-locus phylogeny, we used either seven loci (See Supplementary Figure 1) from two schemes as previously described (Ellis et al., 2012) including three loci adopted to determine the relationships broadly among *Vibrio* spp. (Sawabe et al., 2007) (*gyrB, pryH, and recA*) and four loci adopted to closely examine within species relationships (González-Escalona et al., 2008) (*dnaE, dtdS, pntA,* and *tnaA*); because these four are the only sequenced loci that overlap with those from strains in the public database (www.pubmlst.org), the phylogenetic relationships of a larger collection of isolates in this study with those of a global distribution were inferred using only four loci (*dnaE, dtdS, pntA,* and *tnaA*) (**Figure 2**). The

Clade with Identified ST	*tdh*	*trh*	ORF8	*vscC2*	*vopP*	*vopC*	color
36	+	+	-	-	-	-	
322	+	-	-	+	-	-	
773	+	-	-	-	-	-	
631	-	-	-	+	-	-	
	+	+	-	-	-	-	
3	+	-	+	+	+	+	
674	+	+	-	-	-	-	
	-	-	-	-	-	-	
34	+	+	-	-	+	+	
1123	+	+	-	-	-	-	
1126	-	-	-	+	-	-	
1125	-	-	-	-	-	-	
1127	-	-	-	-	-	-	
	+	+	-	-	-	-	
	+	-	-	-	-	-	
	-	+	-	-	-	-	

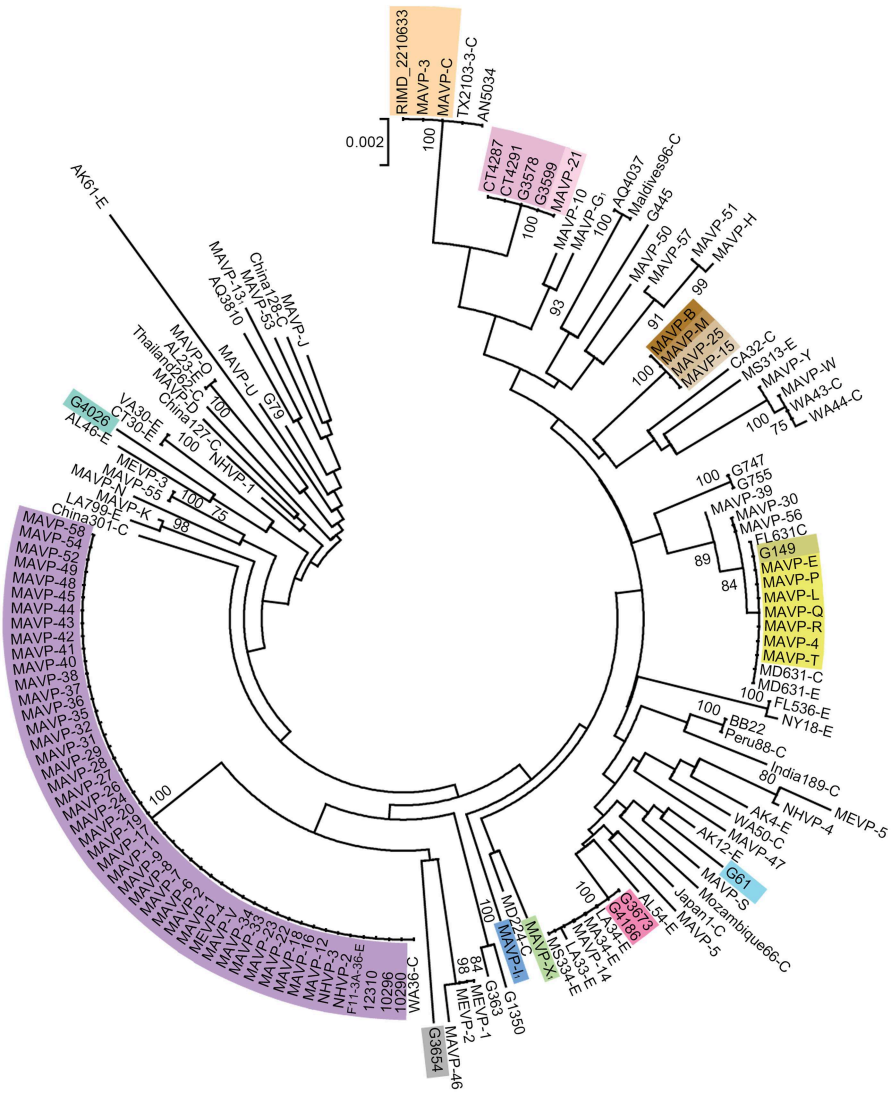

FIGURE 2 | Population structure of Northeast US clinical, related environmental, and some unique worldwide isolates of *V. parahaemolyticus*. A consensus neighbor-joining tree was constructed from four concatenated housekeeping gene loci including *dnaE, dtdS, pntA,* and *tnaA* sequences by using a Jukes-Cantor model. For ease in distinguishing pertinent information associated with worldwide strains from the MLST database, a single representative strain from several sequence

(Continued)

FIGURE 2 | Continued
types was identified from among the available strains, and the representative strain identified by geographic location (USA by state, international by country name), sequence type number, and as clinical (C) or environmental (E). Environmental strains from the region included isolates from the Great Bay Estuary (all with prefix G) or Connecticut (CT). For seven strains whose draft or complete genomes are publicly available, the loci were recovered from the available assemblies. Among related strains where the probable sequence type of the strains was determined, unique genotypes are indicated by color provided in the key and overlaid upon the tree. The bar indicates 0.2% divergences, and branches with less than 70% bootstrap support are unlabeled. Several clinical strains, for which one or more housekeeping loci were not successfully amplified and sequenced were excluded from the analysis (MAVP-A, MAVP-F, MAVP-59, MEVP-6). [1]Isolates were from wound infections.

primer sequences (Supplementary Table 2) and corresponding cycling parameters were used exactly as in published protocols (Sawabe et al., 2007; González-Escalona et al., 2008; Jolley, 2010). For phylogenies inferred from all seven loci, each forward and reverse raw sequence for 25 clinical isolates from 2010 to 2012 was assembled, and the contiguous sequences were then aligned and trimmed to match the length of corresponding sequence of 192 Great Bay Estuary environmental isolates (Ellis et al., 2012), only two of which harbor hemolysin genes. An additional eight isolates collected during 2013 were also included in some analysis. The sequences for individual isolates were then concatenated in alphabetic order. For phylogenies inferred from four loci (*dnaE, dtdS, pntA,* and *tnaA)*, each raw sequence was assembled, aligned, and trimmed to match the exact corresponding amplicon sequence from the public database. Neighbor-joining trees for concatenated sequence of either four loci (1868 bp) or seven loci (2988 bp) were constructed by Mega 5.0 software (Tamura et al., 2013) using Jukes-Cantor model. The statistical support was assessed by 1000 bootstrap re-assemblies.

Comparisons with the published MLST database (http://pubmlst.org) to identify STs were performed on 12 clinical and 16 environmental isolates for which the sequencing of three additional loci (*gyrB*, *pyrC,* and *recA*) were completed as described (González-Escalona et al., 2008). Raw sequences were assembled, aligned, and trimmed as described above. Allele numbers and ST numbers were determined by matching the public database. The STs of sequenced strains were determined from raw short read sequences using the short read sequence typing (SRST2) pipeline (Inouye et al., 2012).

The extent of recombination and mutation within the population was visualized and analyzed by several approaches. The contribution of recombination to phylogeny was evaluated visually using SplitsTree v4 neighbor net analysis of four loci, and the Phi test module was applied for determining statistical support (Huson and Bryant, 2006). The standardized index of association (I_A^S) was determined from a non-redundant allele database for the collection of 90 clinical and 16 environmental strains using the LIAN 3.5 linkage analysis program (Haubold and Hudson, 2000). This statistic describes the linkage disequilibrium in a multilocus data set where a low rate of recombination relative to mutation is indicative of linkage disequilibrium ($I_A > 1$). The null hypothesis that variation of the observed data (V_D) does not differ from that predicted for a population in equilibrium (i.e., experiencing a high rate of recombination relative to mutation) (V_e) was tested by a non-parametric Monte Carlo simulation, with the 5% critical value to determine significant linkage (*L*). ClonalFrame 1.1 was used to determine the relative influence of recombination compared to mutation (r/m) to nucleotide variation (Didelot and Falush, 2007).

Reconstruction of Whole Genome Phylogenies

Representative strains within the species *V. parahaemolyticus* were selected from among the 25 NCBI genome groups (defined as such by ∼90% genome identity) from NCBI genomes phylogeny (http://www.ncbi.nlm.nih.gov/genome/691) that had accompanying information on geographic isolation, year, and sample source (environmental or clinical including wound, stool, and ear). The raw sequences from MAVP-E, MAVP-26, MAVP-36, MAVP-45, MAVP-V, MAVP-M, CT4287, (see **Table 1** and Supplementary Table 1 for a description of these isolates) were processed and *de novo* assembled using the A5 pipeline (Tritt et al., 2012). The assembled contigs of all isolates were analyzed using REALPHY v. 1.09 (Bertels et al., 2014). Sequences were analyzed in three separate alignments, each with a unique reference strain including 10290 (GCA_000454205.1), BB22OP(NC_019955.1, NC_019971.1), and RIMD 2210633 (NC_004605.1, NC_004603.1), for phylogenies across a broad distribution of strains, and 10290, 10329 (NZ_AFBW01000001.1 - 33.1), and 10296 (GCA_000500105.1) for analysis of strains within the ST36 clonal complex clade. From these alignments multiple alignment positions were extracted and then merged into a single alignment. Neighbor-joining phylogenies were reconstructed using the maximum likelihood method in PhyML, with a GTR substitution matrix and a gamma-distributed rate heterogeneity model (Guindon et al., 2010). Phylogenies were visualized as trees using FigTree 1.4.2 (Rambaut, 2012). The branch length reflects nucleotide changes per by total number of nucleotides in the sequence.

Environmental Data and Analysis

Sea surface water temperature (SST) data were acquired from US Integrated Ocean Observing System buoys in Long Island Sound and the Gulf of Maine (http://www.neracoos.org/datatools/climatologies) and from the NOAA National Estuarine Research Reserve System Wide Monitoring Program (SWMP) buoys in the Great Bay Estuary (http://cdmo.baruch.sc.edu/get/export.cfm). Monthly average SST data from 2004 to 2013 were compiled from representative buoys for the areas of interest. In Long Island Sound, the EXRX buoy is in close proximity to Norwalk Harbor, CT and Oyster Bay, NY, the AO1 buoy is representative of the southwestern end of the Gulf of Maine, and the Great Bay SWMP buoy is representative of the Great Bay estuary.

Nucleotide Sequences

MLST loci for strains, MAVP-I, MAVP-M, MAVP-E, MAVP-26, MAVP-36, G61, G3654, G4026, G3673, and CT4287 are available at www.pubmlst.org, and Illumina short read genomic sequences for bioproject PRJNA263814 including MAVP-26 (SAMN03107383), MAVP-36 (SAMN03107385), MAVP-E (SAMN03107386), MAVP-V (SAMN03177809), MAVP-M (SAMN03177808), CT4287 (SAMN03177811), and MAVP-45 (SAMN03177810) are available at NCBI.

Results

Genetic Diversity Among Clinical Isolates and Distribution of *tdh, trh*, and Urease Activity

In light of the recent rise of *V. parahaemolyticus* infections in the northeastern US, clinical isolates from infections reported in MA, NH, and ME were obtained to identify pathogenic strain types, and determine whether they belong to resident or invasive lineages (See **Table 1**). A total of 94 clinical *V. parahaemolyticus* strains from infections reported from 2010 through 2013 were compared to each other and 200 environmental isolates from the region collected since 2007 (Mahoney et al., 2010; Ellis et al., 2012) (**Table 1**). Even though clinical isolates were not archived from every infection, and information was incomplete, this collection provides an extensive survey of regional infections (**Table 1**). Prior to 2013, consumption of local shellfish was implicated in only five, isolated infections (**Table 1**). However, by 2013 many clinical isolates ($n = 37$) were traced to at least one local source (**Table 1** and see Newton et al., 2014). Gastric infections most often were attributed to contaminated oysters ($n = 49$), some of which were either definitively or potentially locally harvested ($n = 27$). In 2013, two infections were traced to recreationally harvested seafood in ME, including oysters, cooked fish, and lobster (**Table 1**). No illnesses were definitively traced to seafood from NH, although it is important to note that at the time of these collections commercial harvesting of oysters from this state was limited. Remarkably, two gastric infections were apparently induced by handling of raw product and casual ingestion of water while swimming, consistent with the presence of highly infective strains with a low infectious dose in near shore waters. Relatively fewer isolates ($n = 5$) were recovered from wound infections (**Table 1**).

The distribution of genotypes among clinical isolates indicated that infections were caused by a variety of strains, and although no specific gene or combination of virulence-associated genes defines pathogens reported in the region, certain genotypic profiles were more abundant than others (**Table 2**, and Supplementary Table 1). Very few isolates contained only the *tdh* gene ($n = 6$). Two isogenic, *tdh*-containing isolates, MAVP-C and MAVP-3, were identified as the pandemic O3:K6-type (**Table 2**, and Supplementary Table 1). Most clinical isolates from the Northeast US (80%) harbored the *trh* gene either alone or in combination with *tdh* (**Table 2**, Supplementary Table 1). In fact, strains harboring both *tdh* and *trh* were highly prevalent among clinical isolates from reported cases in this region even prior to 2013 (**Table 1**, and Supplementary Table 1). Yet several clinical isolates ($n = 13$), only three of which were wound isolates, harbored neither hemolysin.

Because urease activity may be useful for easily identifying some pathogenic types due to the proximity of the urease locus only ∼7-Kb from the *trh* gene in the same pathogenicity island of some characterized strains (Park et al., 2000), we investigated whether urease activity correlated with the presence of hemolysin genes and clinical status among northern Northeast isolates (**Tables 2, 3**). Urease activity significantly correlated with the presence of *trh* (100% of isolates) either in the absence of *tdh* in clinical strains ($p = 0.012$) or in combination with the *tdh* gene for both clinical ($p < 0.0001$) and environmental ($p < 0.0001$) strains. Urease activity did not correlate significantly with *tdh* alone ($p = 1.0$). A few environmental isolates of unknown virulence were also urease positive, all of which harbor *trh* (**Table 3**).

Phylogenetic Relationships Among Clinical and Environmental Isolates and Identification of Resident Clades

Neighbor-joining phylogenies identified shared lineages of environmental strains and pathogens isolated in the region (Supplementary Figure 1, **Figure 2**). Related environmental and clinical isolates were first identified by visual inspection of a neighbor-joining phylogeny based on seven genes sequenced from clinical strains isolated prior to 2013 (Supplementary Figure 1). This tree revealed that only eight out of 192 characterized environmental isolates were closely related to clinical isolates as inferred by bootstrap values greater than or equal to 70. The relationships of 90 clinical isolates, and 16 environmental (eight strains isolated prior to 2010, and eight previously unreported hemolysin-encoding strains isolated in 2013) to strains collected worldwide were then examined more broadly by MLSA using four loci shared with the public database (**Figure 2**). This analysis demonstrated that regional clinical isolates are polyphyletic and include some strains of known STs (**Figure 2**, Supplementary Table 3). Strains within clades are not always clonal when assessed using virulence-associated genotypes (**Table 2**, Supplementary Table 1, **Figure 2**).

Even though definitive traceback data was incomplete prior to 2013, the reporting data (see **Table 1**) did facilitate identification of the isolates most likely from local sources, and those from outside the region. Both isolates harboring ORF8 grouped with the globally distributed pandemic ST3 strains, but these were not from local sources. The ST631 clade grouped isolates from multiple years that were traced to oysters consumed at various Cape Cod MA locations where oysters are farmed and imported oysters are not typically marketed and thus they were identified as putatively local, and a single isolate was traced to a local source (**Table 1**, **Figure 2**). A single environmental ST631 isolate (G149) that based on whole genome phylogeny is a close relative to these strains (Xu, unpublished data) was also isolated in NH in 2007. In agreement with the persistence of this strain type, ST631 are not strict clones (Supplementary Table 1, **Figure 2**). MAVP-14, and two environmental isolates from NH harboring both *tdh* and *trh* grouped with several ST34 environmental strains from the Gulf of Mexico, specifically Louisiana (LA) (González-Escalona

et al., 2008) (Supplementary Table 3). MAVP-21 was most closely related to four tdh^+/trh^+ ST674 environmental isolates, two of which were recovered from oysters harvested from CT and two from the Piscataqua River of the GBE (**Figure 1**). Finally, even though genetically diverse strains identified as a new ST, ST1127, caused four infections, three were from unknown sources and no environmental isolates of this ST have been identified, thus it is not clear whether they are residents.

We evaluated the MLSA data by several statistical tests to determine the relative contributions of mutation and recombination to population structure. The influence of recombination on clonal structure was supported by the Phi test in SplitsTree v.4 ($P < 0.001$), and was also indicated by the reticulate structure of the Neighbor Net analysis (**Figure 3**). However, the LIAN test of recombination indicated the population is in linkage disequilibrium ($P < 0.01$; $L = 0.1009$), with a standardized index of association of $I_A^S = 0.2228$, which is also visible in the Neighbor Net analysis as long branch lengths emerging from a central network of recombination. Approximately one recombination event for every three mutations is predicted (r/m = 0.337707; 95% credibility region = 0.145408 – 0.571994), which indicates an effect of both recombination and mutation upon population structure.

Occurrence of Non-Indigenous ST36 Clinical Isolates in the Northeast US

Most clinical isolates from 2013 ($n = 42$) were closely related to each other and to ST36 clonal complex strains from the PNW (**Figure 2**) (Newton et al., 2014). The ecological invasion of the ST36-clade strain in the Atlantic from the Pacific was suggested when these genotypes were associated with infections reported in NY in 2012 (Martinez-Urtaza et al., 2013). All isolates from the northern New England region in this ST36-clade shared the same virulence associated genotype; however, MAVP-V of unknown source and that was isolated in 2011, is distinct from the others from the Northeast in that it is missing a phage-encoding island that is also missing in closely related strain 12310 from Washington state (Haendiges et al., 2014) (**Table 1**, Supplementary Table 1, **Figure 2**, and Supplementary Figure 1). Remarkably, the ST36-clade isolates were not only traced to oysters harvested south of Cape Cod proximal to Long Island Sound and Oyster Bay NY, but also from north of Cape Cod in the Gulf of Maine

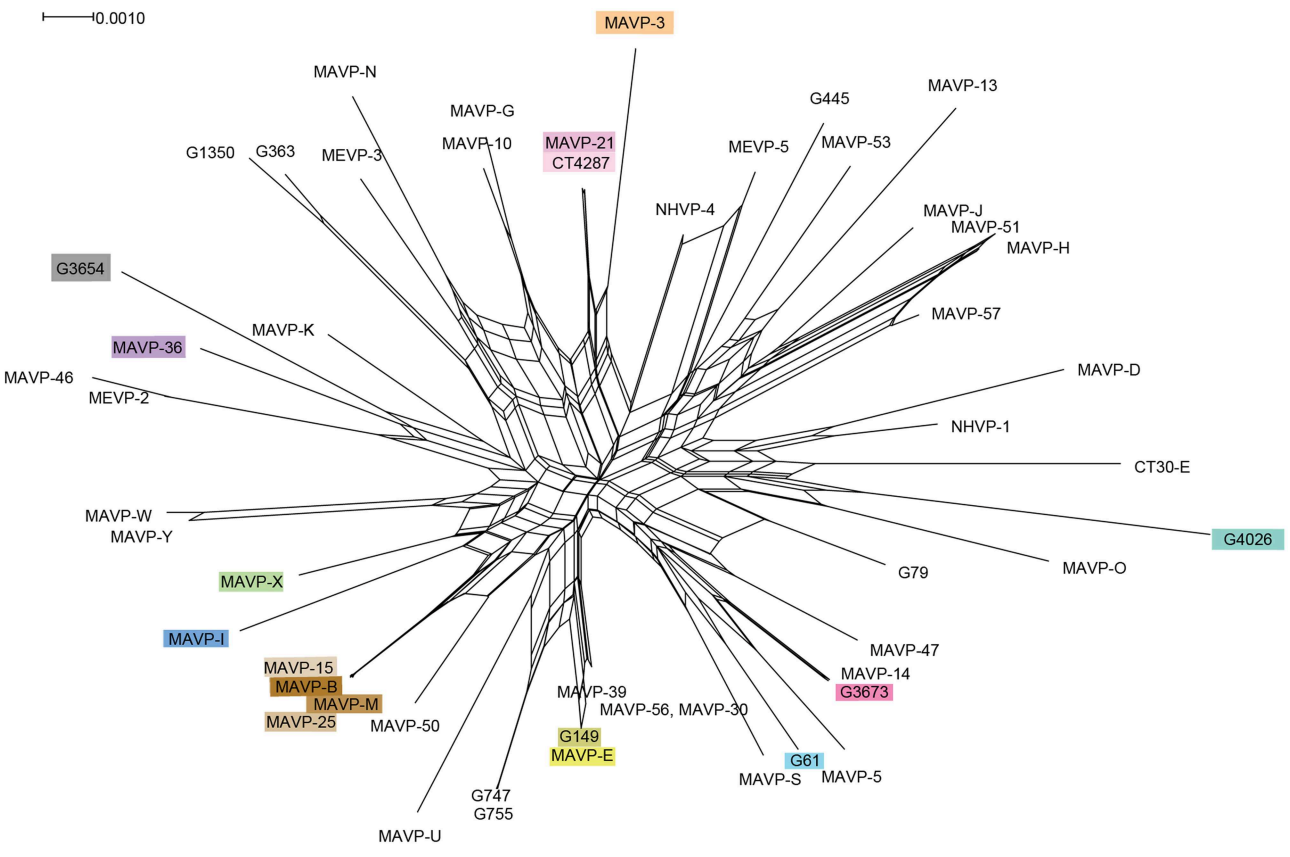

FIGURE 3 | Recombination network revealed by splits decomposition analysis of genotypes of Northeast regional *V. parahaemolyticus* isolates. The Splits Tree analysis is based on four concatenated gene loci including *dnaE, dtdS, pntA,* and *tnaA* (1868 bp) from 54 unique regional clinical and related environmental isolates. Where multiple strains were identified as sharing the same allelic profile or ST, one representative strain per unique genotype was selected for inclusion in analysis. For ease in identifying resident and invasive clades, strains are designated by colors that correspond exactly to color scheme in **Figure 2**.

(See **Figure 1**). This geographical distance suggests that the New England ST36-clade strains both spread northward and grew sufficiently to increase infections (Newton et al., 2014). MAVP-26, MAVP-36, and MAVP-45 (traced to at least two, and potentially three shellfish harvest areas in MA), and MAVP-V (from an unknown source) were subsequently confirmed to be ST36 by whole genome sequencing.

Whole Genome Phylogeny of *V. parahaemolyticus* and Relationships Between ST36 Atlantic and Pacific Populations

From the collection of available draft genomes of strains of known identity (i.e., clinical and environmental source) and geographic location of isolation, we examined phylogenetic relationships among isolates of *V. parahaemolyticus*. Although the selected strains likely underrepresent the diversity and distribution of pathogen types, they nevertheless provide insight into the evolution of different lineages. Distinctive pathogen lineages grouped within three major nodes, one of which included the pandemic RIMD 2210633, BB22OP, and northern New England resident ST631 clades, a second of which grouped the ST36 clade and other clinical and environmental strains from both the Atlantic and Pacific, and a third that grouped fewer and more distantly-related representative strains from the Atlantic and Gulf of Mexico (**Figure 4**). This broad phylogenetic relationship illustrates that although infectious strains of *V. parahaemolyticus* are polyphyletic, they may yet belong to genetic clusters that can be diagnostically and epidemiologically informative.

To test this hypothesis, the genomes and relationships of related ST36-clade strains were compared to gain insight into the patterns of microevolution within this clade (**Figure 5**). As expected from a dynamic of recent ecological invasion in the Atlantic, most strains within this clade isolated prior to 2011 were from the PNW, where this strain is indigenous.

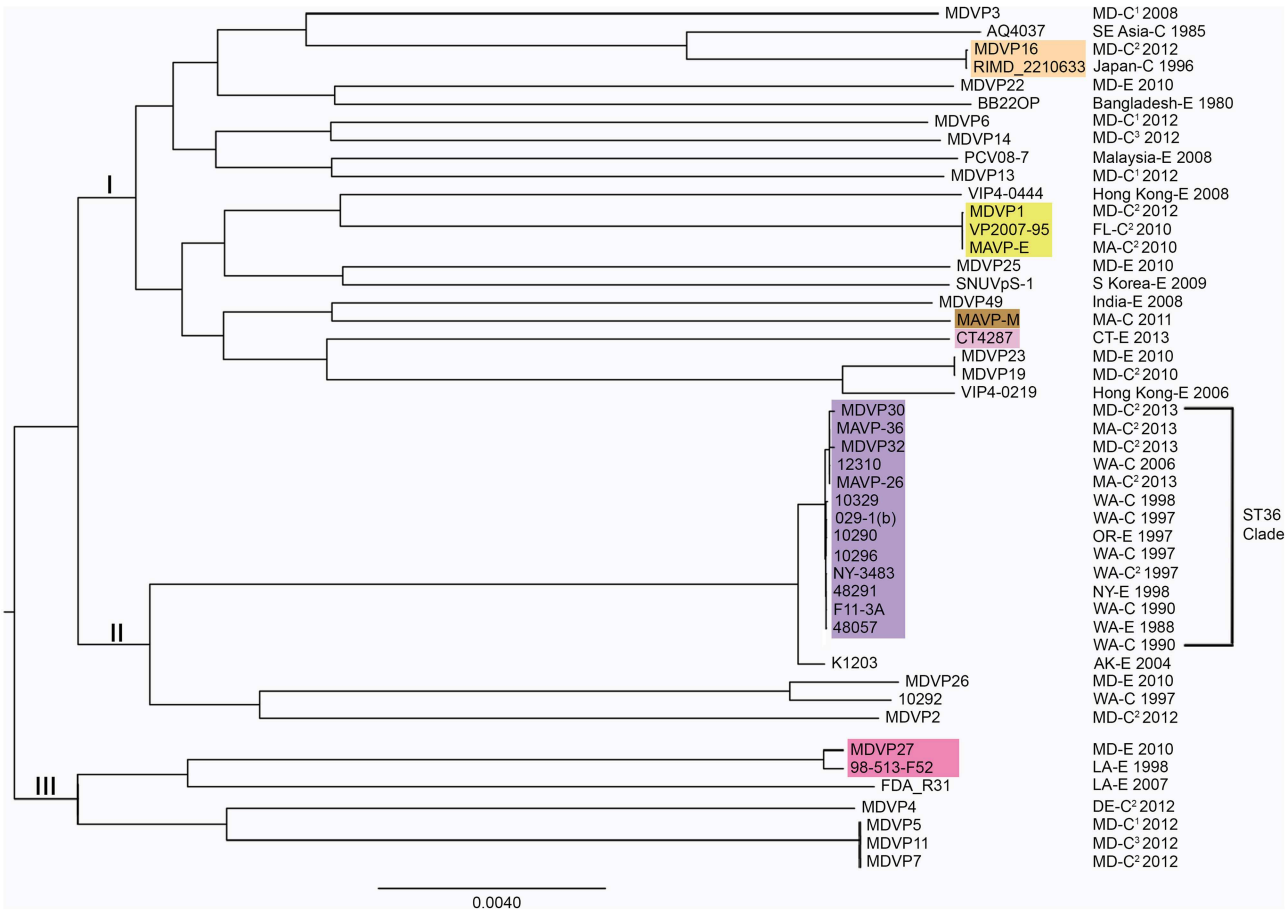

FIGURE 4 | Phylogenies of pathogenic lineages of *V. parahaemolyticus*. Multiple genome reference-sequence alignment based phylogeny were reconstructed using REALPHY v1.09 to evaluate the evolution and relatedness of pathogenic lineages, where related pathogens grouped within three major nodes (I, II, and III). Maximum likelihood phylogenies of strains of broad phylogenetic distribution were reconstructed based on merged reference strains 10329, BB22OP, and RIMD 2210633, where the merged alignment represents 63% coverage of sites of the largest reference genome (Vp10329). The branch length reflects nucleotide changes per by total number of nucleotides in the sequence. Representative strains are identified by geographic location (USA by state, international by country name), as clinical (C) or environmental (E) and year isolated. For ease in identifying strains or sequenced types identified in the Northeast, select strains are designated by colors that correspond exactly to color scheme in **Figure 2**. [1]Isolates were from wound infections. [2]Isolates were from gastric infections. [3]Isolates were from ear infections.

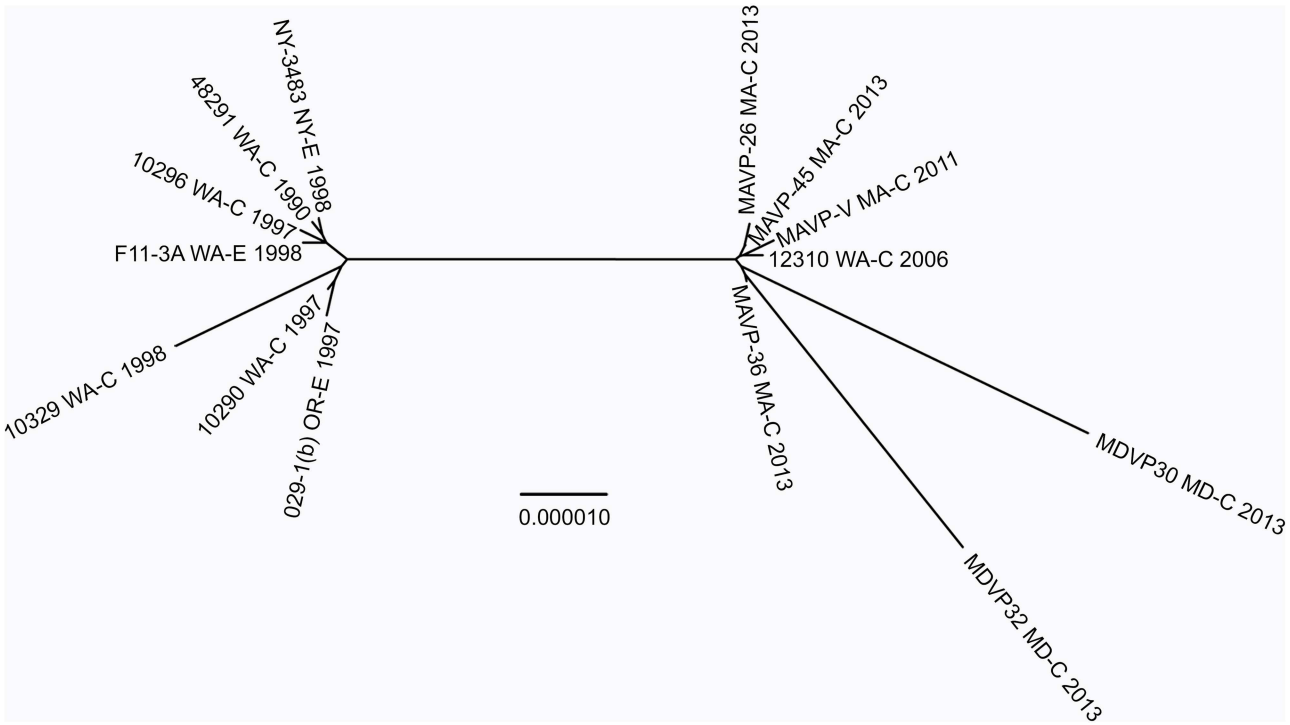

FIGURE 5 | Phylogenetic relationship between Atlantic and Pacific ST36 populations. Multiple genome reference-sequence alignment based phylogeny were reconstructed using REALPHY v1.09 to evaluate the evolution and relatedness of ST36 pathogenic lineages. Maximum likelihood phylogenies within the ST36 clade were reconstructed based on merged reference strains 10329, 10290, and 10296 where the merged alignment represents 92% coverage of sites of the largest reference genome (Vp10329). The branch length reflects nucleotide changes per by total number of nucleotides in the sequence. Representative strains are identified by geographic location [USA by state, as clinical (C) or environmental (E) and year isolated].

One exception was a single environmental isolate from NY in 1998 NY-3483. NY-3483 grouped with these historic PNW ST36 strains. More recent clinical isolates from 2013 traced to Atlantic sources were closely related to and share a common ancestor with strain 12310, a Washington State (WA) isolate from 2006. This topology strongly supports that the Atlantic ST36 strains are non-indigenous, and were derived from the PNW ST36 population.

Discussion

V. parahaemolyticus has become the most common bacterial infection acquired from seafood in the US, with an estimated 35,000 cases each year (Scallan et al., 2011). Even with protective measures in place in the Gulf States, and the PNW, reported cases continue to increase nationwide (Crim et al., 2014). But disease burden in the northeastern US had until recently remained relatively low, even with these national trends. Over the last several years, assumptions that pathogens could not thrive in the cooler waters of the Northeast have given way as several unprecedented outbreaks occurred across multiple states leading to the implementation of protective measures in both CT and MA. The water temperatures in the Long Island Sound and the Gulf of Maine were at or near 11-year maxima from October 2011 through September 2012, and again in July 2013 (Dawicki, 2013; NERACOOS, 2014) (**Figure 1**). The first outbreaks coincided with these unusually mild conditions that have also had profound effects on the Gulf of Maine ecosystem (Mills et al., 2013). Likewise, many previous US outbreaks and the spread of the pandemic strain type on the Pacific US coast have also been linked to warmer ocean temperatures (Mclaughlin et al., 2005; Martinez-Urtaza et al., 2008), a predicted effect of global climate change, indicating that these recent outbreaks in the Northeast US may herald a continuing trend of increased disease in this region, and for others in more northern latitudes (Baker-Austin et al., 2013).

The increase in recent infections in the Northeast was associated with both resident and non-indigenous *V. parahaemolyticus* lineages. The establishment of the ST36 strain, first reported in the region during an outbreak in Oyster Bay NY in 2012 explains the abrupt increase in cases traced to several different locations of northern New England in 2013, including one location in CT proximal to Oyster Bay NY, and two geographically distant locations in MA, to the south and north of Cape Cod (Martinez-Urtaza et al., 2013; Newton et al., 2014) (See Results Section – Occurrence of Non-Indigenous ST36 Clinical Isolates in the Northeast US, **Figure 1**, and **Table 1**). However, even before these unprecedented multi-state outbreaks, cases were on the rise in the region from potentially emergent residential

lineages including ST631, ST34, and ST674 (See **Table 1**, Supplementary Figure 1, and **Figure 2**). Genetically diverse ST631 strains were responsible for seven isolated infections between 2010 and 2013 (**Figures 1**, **2**, **Table 1**, Supplementary Figure 1). Previously identified pathogenic ST631 isolates have been traced to Florida (FL) (Noriea Iii et al., 2010), and Maryland (MD) (Haendiges et al., 2014) indicating a fairly wide Atlantic US-coastal distribution of this ST (Supplementary Table 3). The identification of a single environmental ST631 isolate from NH (G149) lacking *tdh* and *trh*, that was closely related to the clinical isolates based on whole genome maximum likelihood phylogeny (Xu and Whistler, unpublished) indicates the clade is resident rather than transient in New England (Ellis et al., 2012). A single clinical isolate potentially from a local oyster and two environmental isolates were identified as ST34 (See **Table 1**, **Figure 2**). Although environmental ST34 isolates of unknown virulence are broadly distributed from the Pacific and Gulf of Mexico waters, two previously identified ST34 isolates are from clinical sources, indicating the potential for virulence among this clade (Supplementary Table 3) (González-Escalona et al., 2008). Finally, within the ST674 lineage, a single clinical isolate from an unknown source and four environmental isolates from CT and NH oysters were identified (**Table 1**, **Figure 2**). These are the first ever reported strains of this ST and their presence in more than one location in New England suggests these related strains are resident and potentially endemic. Further analysis of the clinical and environmental strains from these resident lineages may provide insight into evolution and recent emergence of pathogenic types in the region.

The recent appearance of ST36-clade strains in the Atlantic is apparently not the first invasion: one isolate, NY-3483, was identified in NY in 1998, when one of the largest multi-state outbreaks of *V. parahaemolyticus* in the US occurred in NY and CT (CDC, 1999). That outbreak was attributed to the invasive pandemic O3:K6 strain type, not the ST36 strain type. This coincidence could indicate that certain conditions promoted the ecological invasion of non-resident pathogenic strain types, but neither strain was apparently able to establish residency. The whole genome phylogeny of these ST36 strains indicates that current ST36 strains causing infections in the Northeast are not derived from NY-3483 (**Figure 5**). Rather, the current ecologically invasive population shares a common ancestor with a quite distinctive 2006 isolate from the PNW (**Figure 5**). Curiously, the serologically unique ST36 strains responsible for several outbreaks in the PNW in 2006 (both WA and Canada) did not persist in that region (Banerjee et al., 2014). Furthermore, current trends in the PNW suggest that infections by ST36 strains have declined locally for as yet unknown reasons (http://www.cdc.gov/vibrio/investigations/). In the Atlantic, the non-indigenous ST36 lineage has persisted for several years, and spread northward into the Gulf of Maine (**Figure 1**). This strain recurrence suggests that yet-undefined environmental factors, perhaps in combination with a particular genetic predisposition, allowed it to compete with resident *V. parahaemolyticus* strains. Furthermore, two unique MD ST36 isolates chosen for this analysis for comparison (Haendiges et al., 2014) also share a common ancestor with the MA isolates (and more specifically MAVP-36), suggesting the clinical populations in the Northern and Mid-Atlantic could undergo admixture. However, the greater genetic distance of the MD isolates from other strains could reflect subsequent evolution following an earlier introduction or could indicate more rapid reproduction for this subpopulation as would be anticipated in the warmer ocean waters compared to the Northeast (**Figure 5**).

The analysis of the contributions of mutation and recombination indicates a significant effect of both processes upon population structure (**Figure 3**) (Vos and Didelot, 2009). The reticulate nature of the SplitsTree phylogeny is consistent with multiple evolving subpopulations of pathogens undergoing recombination, but not frequently enough to break up the distinct population structure of the major lineages (**Figure 3**) (Huson and Bryant, 2006). This data and its interpretation may appear to contrast with those inferred from other *Vibrio* sp. for which mutation is lower, not higher than recombination (Schuster et al., 2011; Turner et al., 2013); however, the subpopulations under scrutiny are often more closely related, and sometimes exclude environmental counterparts. When isolates that were not traced specifically to Northeast sources were excluded from this analysis, the population structure of regional isolates agreed with other studies that indicate substantial recombination among *V. parahaemolyticus* (González-Escalona et al., 2008; Ellis et al., 2012) (**Figure 3**). As one example, the SplitsTree analysis indicates a striking and more recent recombination history among ST631 and related non-ST631 environmental and clinical strains that could have influenced the evolution and potentially the emergence of this and new pathogenic types in the region (**Figure 3**). In contrast, the Northeast ST36 population has evidently evolved primarily through mutation, but this recent population expansion likely has provided insufficient time for the effects of recombination to be apparent. Even so, several resident strain lineages from local sources, including G3654, MAVP-46, and MEVP-2, were identified as potentially having a past recombinatorial history with the ST36 lineage.

Pathogenic strains from the Northeast, even those that are resident, are genotypically diverse (**Table 2**, Supplementary Table 1, **Figure 2**), making the development of an optimal detection strategy for all pathogens extremely challenging. The majority of infections in the Northeast were caused by *trh*-containing, urease positive strains (**Tables 2**, **3**, Supplementary Table 1), and both attributes are used for assignment of environmental isolates as pathogens since *trh*-containing strains have increased among clinical isolates in North America in recent years (**Tables 2, 3**) (Jones et al., 2012; Martinez-Urtaza et al., 2013; Banerjee et al., 2014). Utility of urease activity as a surrogate for *trh* detection in Northeast *V. parahaemolyticus* populations is well supported by our analysis (**Table 3**). Yet the reliability of *trh* as a virulence marker for environmental surveillance is not thoroughly validated because the pathogenic potential of environmental isolates with this trait from any coastal population is still untested. Directed experimentation must determine whether environmental isolates that harbor hemolysins are in fact virulent and, conversely, that those lacking these genes are not virulent. However, virulence studies with environmental isolates are uncommon and reveal potential shortcomings in virulence models currently in use (Caburlotto et al., 2009; Mahoney et al., 2010). For instance,

Mahoney et al. examined the cytotoxicity, a commonly-used proxy for virulence, of a variety of known pathogenic strains compared to environmental isolates lacking any of the classic virulence determinants used in surveillance, including *tdh*, *trh*, and T3SS2 (Mahoney et al., 2010). Surprisingly, many environmental isolates that would otherwise be identified as non-pathogenic are more cytotoxic to human CaCo-2 cells than most clinical strains, indicating relative cytotoxicity as a measurement for virulence may only be useful for studies comparing known pathogens, and not for environmental isolates (Mahoney et al., 2010). Thus, the determination of virulence potential and assignment of an environmental isolate as a pathogen is not an easy task given the limitations of current models for disease and infection. Ultimately, a better understanding of the prevalence of any potential diagnostic trait among non-pathogens in the environment is necessary.

The current state of knowledge supports multiple points of view on the utility of hemolysins as a diagnostic trait, even with their strong correlation with clinical isolation (Nishibuchi et al., 1992; Honda and Iida, 1993; Xu et al., 1994; García et al., 2009; Hiyoshi et al., 2010; Thongjun et al., 2013; Banerjee et al., 2014). It is concerning that 14% of clinical isolates, 11% if counting those from gastric infections only, harbored neither hemolysin gene (**Table 2**), and therefore, these strains would evade detection as human pathogens in any monitoring program relying upon only these markers. A similar prevalence of clinical strains lacking these virulence markers has been observed in other regions of North America (Jones et al., 2012; Banerjee et al., 2014). One explanation for the lack of *tdh* or *trh* in these stool isolates evokes the possibility of misidentification of non-pathogens consumed along with pathogens in an oyster or other food, since standard practices limit analysis to only one isolated colony in confirmed laboratory tests. However, we see this as improbable given that such isolates would need to both colonize and proliferate in order to achieve a high enough population to allow detection at this relatively high frequency. Furthermore, the detection of the same strain repeatedly during an outbreak would be unlikely if other, non-pathogenic *V. parahaemolyticus* were so abundant during infection. Even though published reports support that hemolysins contribute to virulence and are sufficient for some symptoms (Nishibuchi et al., 1992), other studies demonstrate virulence is unaffected by the deletion of these genes (Xu et al., 1994; Park et al., 2000). Thus this relationship is unresolved and supports the view that non-hemolysin producers isolated from humans may be pathogenic. More thorough analyses of the diversity of isolates from single infections are necessary to address this limitation in our knowledge of the defining characteristics of pathogens. If non-hemolysin producers are pathogenic, analysis of their genomic attributes may provide useful insight into fundamental attributes that promote their virulence. Even in the absence of a definitive (or even a few) virulence marker(s) harbored by all pathogenic *V. parahaemolyticus* strains, there is promise for monitoring particular lineages of concern (see **Figure 2**), by the use of markers or pathogenicity islands identified by whole genome comparisons. Assays informed by genomics comparisons could be tailored to different regions, but would need to include new strain diagnostic loci as populations continue to evolve and are influenced by invasive and introduced strains. Ultimately, the above limitations suggest that a combined trait or multi-locus-based assessment that includes hemolysins may be necessary for monitoring how changing population structure correlates with increased disease incidence, and for assessing public health risk.

Conclusions

Environmental conditions that create a warmer and longer season conducive for rapid growth do not entirely explain the current trend of increasing numbers of *V. parahaemolyticus* infections from local northern New England sources. This study suggests that changes in the bacterial populations underlie enhanced disease risk in the region. Yet, other factors may also contribute to increases in the reported number of cases. First, commercial shellfish harvesting has risen steeply in both MA and CT in recent years, with overall increased summer harvesting and consumption of product from the area (MADMF, 2013). But this increase does not explain the rise in cases in ME from recreationally harvested shellfish. Second, Vibriosis is now a reportable disease in all New England states, and an increased awareness of the pathogen by the public and health practitioners may contribute to the rise in reported cases. Regardless, the status of *V. parahaemolyticus* as an emergent pathogen of significant concern in the region is justified by projections of changing climate that may be conducive to *V. parahaemolyticus* growth. This concern is amplified by the finding that a non-indigenous pathogen invaded the Atlantic during a period with warmer than usual ocean temperature and has now likely established populations in the Northeast (Newton et al., 2014). Since *Vibrio* species are known to undergo recombination readily (Turner et al., 2013), and recombination influenced the population structure of these isolates (**Figure 3**) it is not yet clear how the now likely-established population of the Atlantic ST36 strain could further shape the Northeast resident pathogen population. Our findings lay a foundation for future research aimed at understanding the interplay between pathogen genotype and environment that leads to disease emergence.

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Supplementary Material

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Improvement of the quantitation method for the *tdh*$^+$ *Vibrio parahaemolyticus* in molluscan shellfish based on most-probable-number, immunomagnetic separation, and loop-mediated isothermal amplification

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Vibrio parahaemolyticus is a marine microorganism that can cause seafood-borne gastroenteritis in humans. The infection can be spread and has become a pandemic through the international trade of contaminated seafood. Strains carrying the *tdh* gene encoding the thermostable direct hemolysin (TDH) and/or the *trh* gene encoding the TDH-related hemolysin (TRH) are considered to be pathogenic with the former gene being the most frequently found in clinical strains. However, their distribution frequency in environmental isolates is below 1%. Thus, very sensitive methods are required for detection and quantitation of *tdh*$^+$ strains in seafood. We previously reported a method to detect and quantify *tdh*$^+$ *V. parahaemolyticus* in seafood. This method consists of three components: the most-probable-number (MPN), the immunomagnetic separation (IMS) targeting all established K antigens, and the loop-mediated isothermal amplification (LAMP) targeting the *tdh* gene. However, this method faces regional issues in tropical zones of the world. Technicians have difficulties in securing dependable reagents in high-temperature climates where we found MPN underestimation in samples having *tdh*$^+$ strains as well as other microorganisms present at high concentrations. In the present study, we solved the underestimation problem associated with the salt polymyxin broth enrichment for the MPN component and with the immunomagnetic bead-target association for the IMS component. We also improved the supply and maintenance of the dependable reagents by introducing a dried reagent system to the LAMP component. The modified method is specific, sensitive, quick and easy and applicable regardless of the concentrations of *tdh*$^+$ *V. parahaemolyticus*. Therefore, we conclude this modified method is useful in world tropical, sub-tropical, and temperate zones.

Keywords: *Vibrio parahaemolyticus*, most-probable-number, immunomagnetic separation, loop-mediated isothermal amplification, K antigen

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Introduction

Vibrio parahaemolyticus inhabits estuarine and marine environments (Joseph et al., 1982). This bacterium thrives in high-temperature environments and thus it is prevalent in tropical areas year around and is found at lower concentrations only in summer in temperate regions.

Vibrio parahaemolyticus is the major cause of seafood-borne infections in the world (Raghunath, 2015). This bacterium can cause gastroenteritis in humans only when it propagates in the harvested seafood to the number exceeding the infectious dose when consumed by humans without proper cooking (Okuda et al., 1997a). A large number of *V. parahaemolyticus* cells distributed in the eutrophic coastal environments may accumulate in the digestive tract of molluscan bivalves because they filter-feed (Nishibuchi and Kaper, 1995; Manas et al., 2014). Therefore, molluscan shellfish accumulates *V. parahaemolyticus* at high concentrations and very frequently cause infection. However, not all strains are considered pathogenic. Only those possessing and expressing the gene (*tdh*) encoding the thermostable direct hemolysin (TDH) and/or the gene (*trh*) encoding the TDH-related hemolysin (TRH) are considered pathogenic (Nishibuchi and Kaper, 1985). Most clinical isolates of *V. parahaemolyticus* carry the *tdh* and *trh* genes, either alone or in combination; however, distribution of these genes in environmental isolates is usually low (1–2%; Nishibuchi and Kaper, 1995; Yamazaki et al., 2010) although some workers reported extremely frequent detection (up to 48%; Rodriguez-Castro et al., 2010; Gutierrez West Casandra et al., 2013).

The concentration of pathogenic *V. parahaemolyticus* can exceed a detectable level all the year around in the tropical zone but only exists at lower levels in the summer season in temperate zones. To not under-report pathogenic *V. parahaemolyticus*, sensitive methods have been devised for shellfish examination. These include the use of alkaline peptone water (APW) and salt polymyxin broth (SPB) as selective media in an enrichment procedure (Hara-Kudo et al., 2003); the immunomagnetic separation (IMS) technique targeting the K6 antigen shown to be useful in selective isolation of the O3:K6 pandemic strain of *V. parahaemolyticus* (Okuda et al., 1997b; Vuddhakul et al., 2000); and loop-mediated isothermal amplification (LAMP) reported to be more sensitive than conventional PCR (Yamazaki et al., 2010). Based on these reports, our group recently developed an most-probable-number (MPN) procedure for enumeration of *tdh*$^+$ *V. parahaemolyticus* in shellfish samples where a PickPen-assisted IMS technique (hereinafter abbreviated simply as IMS) targeting as many as 69 established K antigens and a LAMP assay targeting the *tdh* gene were incorporated (Tanaka et al., 2014; hereinafter referred to as MPN-IMS-LAMP).

Experiments in southern Thailand show the MPN-IMS-LAMP performed well in general in detection and quantitation of *tdh*$^+$ *V. parahaemolyticus* in shellfish products (Tanaka et al., 2014). However, there was a problem of underestimating MPN values because the study was conducted in a tropical environment where the total microbial population including target and non-target organisms is generally large; and, in such an environment, it is difficult to properly detect *tdh*$^+$ *V. parahaemolyticus* (Vuddhakul et al., 2006). Overgrowth of non-target organism(s) and a failure in the IMS and the SPB enrichment are most likely responsible for the underestimation problem.

PickPen, an eight-channel intrasolution magnetic particle separation device enables a straight forward 96-well plate-based IMS procedure was successfully applied to increase the sensitivity and specificity of *Escherichia coli* O157:H7 detection in food (Nou et al., 2006). The IMS consists of two steps: the incubation of immunomagnetic beads (IMBs) with enriched culture containing the target bacterium and others (hereinafter referred to as IMB Incubation); and the washing step using the PickPen (hereinafter referred to as PickPen Operation) to remove non-targeted microbial population. The *E. coli* O157:H7 study demonstrated that constant shaking during the IMB Incubation could increase the efficiency of IMS (Nou et al., 2006). Our previous method employed incubation with intermittent mixing but not constant shaking (Tanaka et al., 2014). We also noticed loss of IMB during PickPen Operation suggesting improvement of this step in the IMS component. In this study we adopted the IMB Incubation with constant shaking and examined whether increased PickPen Operation time could further improve the efficiency of IMS.

Loop-mediated isothermal amplification allows one-step detection of gene amplification at a single temperature (Notomi et al., 2000) and it has been reported that LAMP is more simple and sensitive than the currently popular conventional PCR methods targeting the *tdh* and *trh* genes (Yamazaki et al., 2010). The conventional liquid LAMP reagent is practically inconvenient because refrigerated environment is recommended during storage, transportation, and operation; whereas the dried form does not require a refrigerated environment and therefore it can be used to detect pathogenic microorganisms even in tropical countries (Boehme et al., 2007; Mitarai et al., 2011). If the dried LAMP reagent is applicable to detection of the pathogenic genes of *V. parahaemolyticus*, it would be very useful for examination of seafood in various parts of the world.

In the present study, we attempted to improve the previously reported MPN-IMS-LAMP for quantitation of *tdh*$^+$ *V. parahaemolyticus*. We evaluated the improvement by applying a series of modifications in the three components of the protocol: MPN, IMS, and LAMP. First, we evaluated two important factors (IMB incubation and PickPen Operation time) in IMS and we determined the benefits of the dried LAMP reagents for seafood analysis. Then, we compared the MPN values at three steps (after APW incubation, after IMS application, and after SPB enrichment). Finally, based on the results we present a new recommended MPN-IMS-LAMP method where the high sensitivity, MPN accuracy, and shellfish analysis applicability was determined.

Materials and Methods

Preparation of IMB

Immunomagnetic bead was prepared as previously described (Tanaka et al., 2014). In brief, magnetic beads were coated with antibodies partially purified with ammonium sulfate

precipitation from polyvalent K antisera groups I–IX and monovalent anti-K70 to -K75 antibodies using commercially available *V. parahaemolyticus* K antisera from Denka Seiken Co., Ltd., Tokyo, Japan.

Enrichment Procedures for Shellfish Samples

The shellfish sample was shucked and homogenized in a plastic bag. A three-tubes MPN dilution series was prepared as described in the U.S. Food and Drug Administration's Bacteriological Analytical Manual (DePaola and Kaysner, 2004) with slight modifications (shown schematically in **Figure 1**).Briefly, a 25 g portion of the homogenate was added to 225 ml of APW (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). For determination of the MPN, the shellfish homogenate was diluted 10-fold as follows. Ten-ml of shellfish homogenate in APW was transferred to an empty tube, and subsequent 10-fold dilutions were prepared by transferring 1 ml aliquot to the tube containing 9 ml of APW in triplicate. The tubes were incubated at 37°C for 6 h. The modified IMS was applied as described below (IMS subsection). The 1 ml suspension in PBS resulting from IMS was used for DNA template preparation followed by LAMP assay using dried reagent as described below. Finally, MPN values were determined.

Immunomagnetic Separation

Immunomagnetic separation was performed as previously described (Nou et al., 2006; Tanaka et al., 2014) with modifications. Briefly, 1-ml aliquots of each culture were transferred to a well in a 96-well (2-ml capacity) titer plate. The cultures were mixed with 25 μl of the IMB were incubated on a shaker (140 rpm) at room temperature for 30 min. The subsequent bead washing and bead suspension steps were performed without changing the tips. The beads were captured with PickPen (BioControl Systems, Bellevue, WA, USA) by gently stirring the cultures with an up-and-down motion for 3 min. The captured beads were then washed twice by releasing into and recapturing from 1 ml of phosphate buffered saline (SIGMA-Aldrich Co., St. Louis, MO, USA) and were finally suspended in 1 ml of the same buffer.

DNA Template Preparation

A 1-ml aliquot of a test culture was centrifuged at 15,000 rpm for 1 min, and the supernatant was discarded. The pellet was washed and suspended in 1 ml 0.85% NaCl solution, heated at 100°C for 10 min, and immediately cooled on ice for 5 min. After centrifugation at 15,000 rpm for 5 min, the supernatant was transferred to a new tube and was stored at −20°C until used.

LAMP Assay Using Dried Reagent

The microtubes containing the dried reagent were taken from a commercially available kit for detection of Influenza virus (Loopamp Type A Influenza detection kit, Eiken Chemical Co., Ltd., Tokyo, Japan). The microtubes were transported, stored, and rehydrated at room temperature. Five μl of DNA template solution, 1.3 μl of *tdh* LAMP primer set (Yamazaki et al., 2010) and 23.7 μl of distilled water were added to make a final volume of 30 μl per reaction were added to the microtube. The cap was tightly secured and the microtube was inverted for 3 min in order to rehydrate the reagent which is located in the cap of the tube. The tube was heated at 65°C for 1 h (reaction) and at 80°C for 5 min (enzyme inactivation). Results of the reaction was judged using the visual system by the color change from brown to green in the reaction solution.

Effect of PickPen Operation Time

Vibrio parahaemolyticus O3:K6 strain VP81 originally isolated from a fecal sample of a patient with diarrhea in India (Matsumoto et al., 2000) was used in this study. The strain was grown in 5 ml Luria broth (SIGMA-Aldrich Co.) at 37°C with shaking at 160 rpm for 18 h. Serial 10-fold dilutions of the culture were made in sterile saline. Based on our preliminary experiment (data not shown), to best evaluate the effect of the PickPen Operation time, a concentration of 10^3 CFU/ml was used for this experiment. Six sets of 25 μl of IMB mixed with the diluted culture were prepared and incubated for 30 min with shaking as explained above. The IMB in each set was then washed with different PickPen Operation times (1, 2, 3, 4, 5, or 6 min). After the IMB capture, the remaining supernatant as well as the diluted culture without IMS treatment were plated onto thiosulfate citrate bile salt sucrose agar (TCBS, Eiken Chemical Co.) and incubated at 37°C for 24 h and colonies were counted. The capture efficiency (CE) was calculated using the following equation: CE (%) = (Co-Ca)/Co x 100%, where Co is the total CFU/ml present in the sample, and Ca is the CFU/ml not bound to IMB (Zeng et al., 2014).

Comparison of Liquid and Dried LAMP Reagent

To compare the performance between liquid reagent (DNA amplification kit, Eiken Chemical Co.) and the dried reagent (contained in the microtubes provided in the Loopamp Type A Influenza detection kit), the DNA templates prepared from 14 *V. parahaemolyticus* reference strains were used. LAMP reaction with liquid reagent was performed according to the manufacture's instruction. LAMP reaction with dried reagent is described above. The LAMP primer sets for the detection of *tdh*, *trh*1 and *trh*2 genes were used to conduct four LAMP assays targeting the *tdh*, *trh*1, *trh*2 and *tdh* plus *trh*2 genes as previously reported (Yamazaki et al., 2010). We judged the results using a turbidimetric system 1 h after the beginning of the reaction using the Loopamp EXIA LA-320A (Eiken Chemical Co.). Results were judged using the visual system after 1 h by a change in the color of the reaction solution. Finally, to assess the utility of the dried LAMP reagent in tropical countries, we tested its stability at high-temperatures. The reagents were exposed to temperatures of 30, 40, 50, and 60°C for 15 days; and after, a *tdh* LAMP assay using a standard *tdh*$^+$ *V. parahaemolyticus* strain was performed (Yamazaki et al., 2010).

Shellfish Samples

Twenty eight samples of shellfish harvested in Thailand consisting of 12 bloody clams (*Anadara granosa*), 12 hard clams (*Meretrix lusoria*), and four green mussels (*Perna viridis*) were purchased at a local morning market in Hat Yai, Thailand, during

April and May 2014. The shellfish samples were transported to the Prince of Songkla University, Hat Yai, Thailand at room temperature (∼30°C) and were processed within 1 h of purchase. Sixteen samples of shellfish harvested in Japan consisting of 12 Japanese littlenecks (*Ruditapes philippinarum*) and four hard clams were obtained at Osaka Municipal Wholesale Market, Osaka, Japan during August and September 2014. These shellfish samples were transported to the laboratory at Kyoto University at room temperature (∼25°C) and processed within 2 h of purchase.

Modified Protocol for Comparative Experiments

A minor modification was employed in the experiments in southern Thailand for comparison. Samples were processed as described above after up to 6 h incubation in APW at 37°C (**Figure 1**, Step 1). After IMS application (**Figure 1**, Step 2), 500 μl of 1-ml bead suspension was inoculated into 4 ml of SPB (Nissui Pharmaceutical Co., Ltd.) and was incubated at 37°C for 18 h (**Figure 1**, Step 3). One-milliliter aliquots from each culture tube at Steps 1 and 3 and 500 μl of the bead suspension at Step 2 were used for DNA template preparation. LAMP using the liquid reagent was employed in these experiments.

Sensitivity of the Recommended Protocol

Two-hundred-fifty ml of the Japanese littleneck sample suspension in APW was prepared by inoculation of *tdh*$^+$ *V. parahaemolyticus* VP81 in the mid log phase at known concentrations (0.1, 1.0, 10.0 CFU/ml). These VP81 suspensions were examined for MPN of *tdh*$^+$ *V. parahaemolyticus* using the protocol recommended in this study.

Statistical Analysis

All experiments were performed in triplicate. The means and standard deviations of all collected data were calculated for every triplicate group. A student's *t*-test was used for statistical analysis between two groups. A p-value ≤ 0.05 was considered statistically significant.

Results

The Strategy Used to Improve the Previous Quantitation Method

To improve the MPN-IMS-LAMP previously reported (Tanaka et al., 2014), we evaluated the components of the protocol in two stages to propose the recommended procedure for quantitation of *tdh*$^+$ *V. parahaemolyticus* and evaluated the recommended procedure as schematically shown in **Figure 2**.

Stage I: Laboratory Evaluation of the Techniques

IMS: PickPen Operation Time

As explained in the introduction, we noticed loss of IMB during PickPen Operation suggesting improvement of this step. Therefore, we evaluated whether increasing the

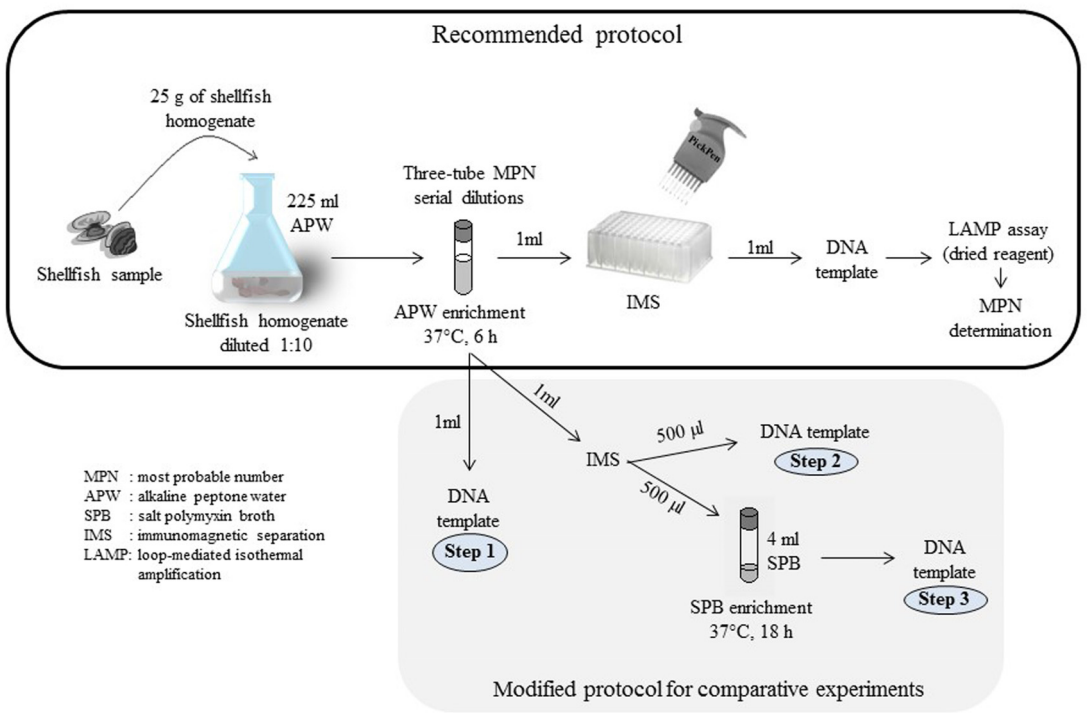

FIGURE 1 | MPN-IMS-LAMP protocol for quantitation of *tdh*$^+$ *V. parahaemolyticus* recommended in this study and the scheme of the comparative experiments used in this study.

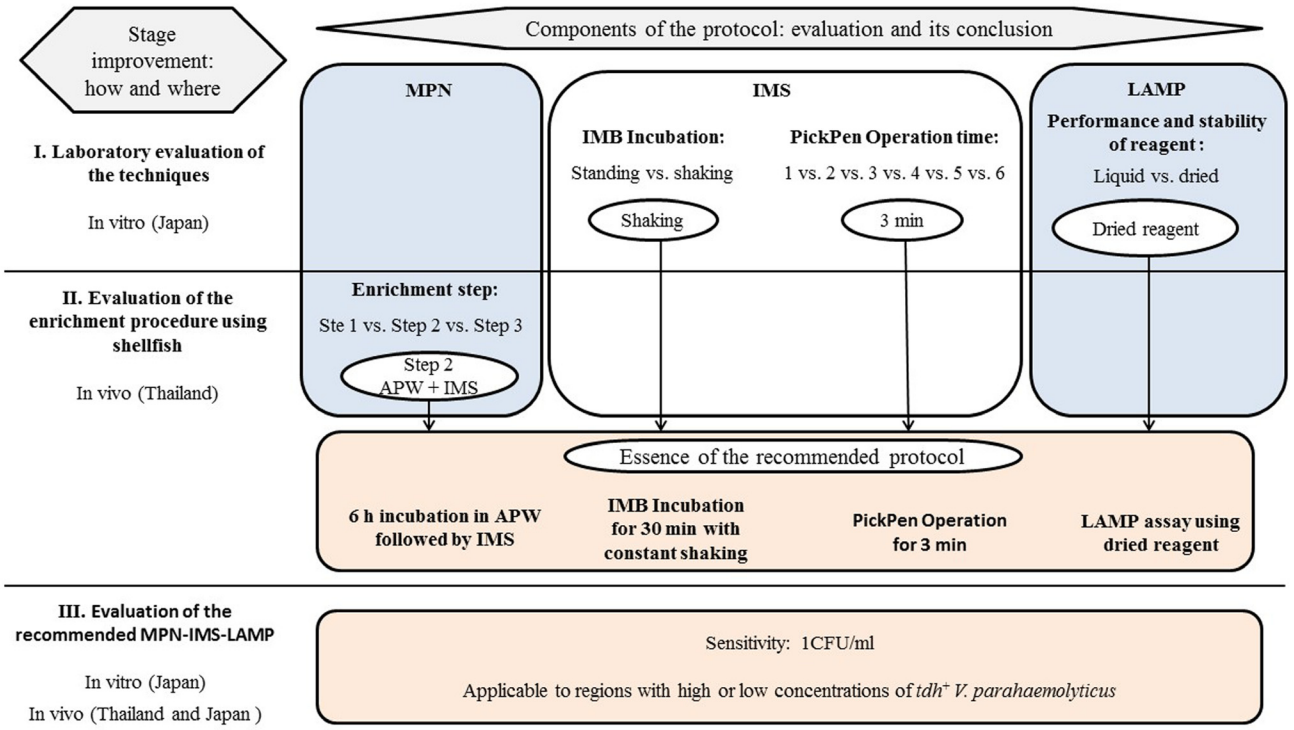

FIGURE 2 | Schematic representation of the strategy used to improve the method for quantitation of *tdh*+ *Vibrio parahaemolyticus.*

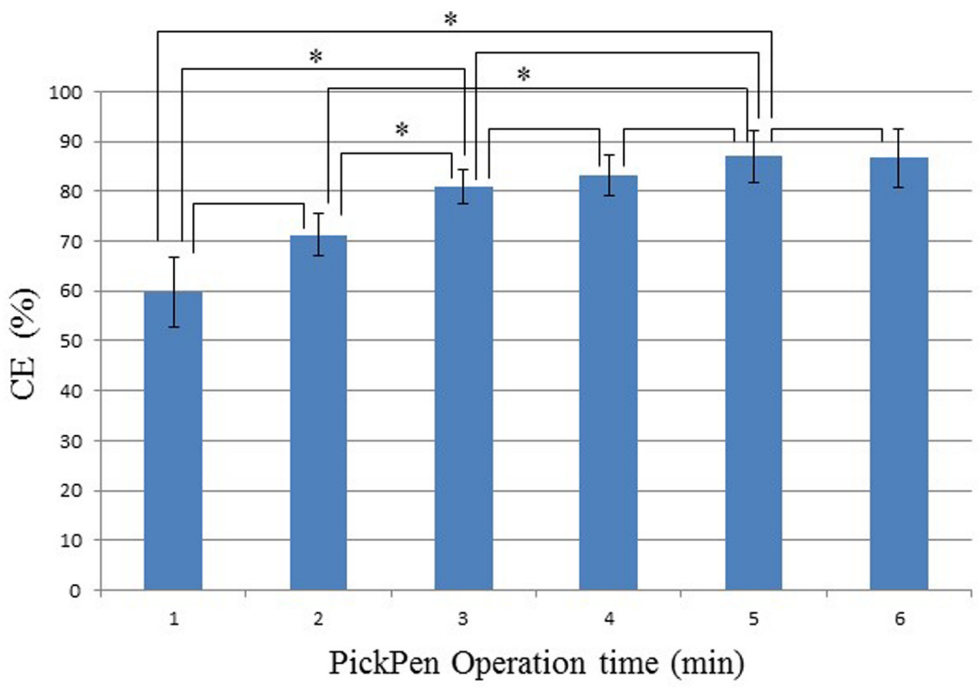

FIGURE 3 | Effect of PickPen Operation time on the capture efficiency (CE) of *V. parahaemolyticus*. CE (%) = (Co-Ca)/Co x 100%, where Co is the total CFU/ml present in the sample, and Ca is the CFU/ml not bound to IMB (Zeng et al., 2014). The pairs of the samples varying in PickPen Operation time that were compared for the difference in CE (%) using the Student's *t*-test are indicated; *pairs showing a significant difference ($p = 0.05$).

time during the washing step of IMB helped to minimize the loss of IMB and thus improve the CE of IMB. We used the student's *t*-test to compare the different PickPen Operation times (1–6 min) and to evaluate the significance of increasing time (**Figure 3**). The CE value gradually increased with increase in the PickPen Operation time until it reached 5 min; in particular, the significant increase in CE was apparent after an PickPen Operation time of 3 min.

LAMP: Dried Reagent vs. Liquid Reagent

To evaluate whether easy-to-use dried reagent can replace the standard liquid reagent we compared the two methods using 14 reference strains. The method using the liquid reagent or using dried reagent were equally sensitive and specific when the results were judged using the turbidimetric system (**Table 1**). In addition, when the product of the reaction was judged by eye, the results using the dried reagent were clearer, due to the color change of the rehydrated reagent from brown to green, than that using the liquid reagent (**Figure 4A**). Furthermore, it was more difficult to judge by eye the results of *trh* (*trh*1 and *trh*2) detection than *tdh* detection using the liquid reagent. Conversely, judgment of color by eye of the results for all these genes was equally easy when the dried reagent was used (data not shown). Further, the positive results in the *tdh* LAMP assays obtained after the dried LAMP reagent was exposed to different temperatures (30, 40, 50, and 60°C), showed the dried reagent was equally stable at all of these temperatures during the examination period (15 days).

Stage II: Evaluation of the Enrichment Procedure Using Shellfish

Change in the Enrichment Step for MPN Determination

We explored the possibility of finding a factor affecting the protocol in the enrichment step. We compared the MPN values after each of the three treatments (**Figure 1**, Steps 1–3) in the new protocol including the above modifications. Twenty-eight shellfish samples purchased in southern Thailand were examined for the MPN of *tdh*$^+$ *V. parahaemolyticus* as described above (**Table 2**). The data clearly shows *tdh*$^+$ *V. parahaemolyticus* is prevalent and the concentration is very high in these samples, with the log MPN/10 g value ranging from 1.0 to 5.4. Hard clams gave higher log MPN than the other two shellfish. Regardless of the kind of the shellfish, the values in Step 3 were lower than those of the other two steps. When the MPN values of Steps 1 and 2 are compared, three samples (**Table 2**, sample no. 8–10) were higher in Step 1, two samples (**Table 2**, sample no. 1 and 2) were higher for Step 2 and 23 samples were the same. The average MPN values for all samples between Steps 1 and 2 did not differ significantly.

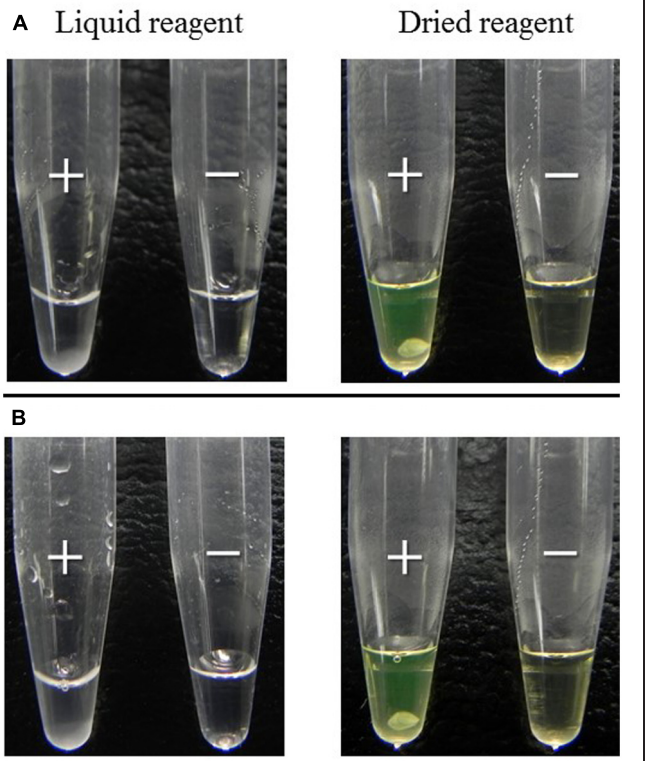

FIGURE 4 | Comparison of the LAMP results between liquid and dried reagents. DNA template obtained using the boiling method from: **(A)** the pure culture of control strains examined for LAMP component at Stage I (**Figure 2**); **(B)** retail Thai shellfish samples examined at Stage III **(Figure 2)** by the recommended MPN-IMS-LAMP protocol **(Figure 1)**. Positive (+) and negative (−) LAMP reaction directly judged by eye.

TABLE 1 | Comparison of liquid reagent and dried reagent for detection of the *tdh*, *trh*1 and *trh*2 genes in *Vibrio parahaemolyticus* using the turbidimetric system.

Reference strains tested		No. of positive strains detected by the LAMP method targeting the gene(s)* and LAMP reagent in liquid (L) or dried (D) form							
Genotype	No. of strains	*tdh*		*trh*1		*trh*2		*tdh*+*trh*2	
		L	D	L	D	L	D	L	D
tdh$^+$	11	11	11	0	0	0	0	11	11
*trh*1$^+$	1	0	0	1	1	0	0	0	0
*trh*2$^+$	2	0	0	0	0	2	2	2	2

*Yamazaki et al. (2010).

Stage III: Evaluation of the Recommended MPN-IMS-LAMP

Sensitivity and MPN Accuracy Determination

Shellfish homogenates artificially contaminated with a reference strain (*tdh*$^+$) at known concentrations were examined by the recommended protocol. Positive tubes in the three-tubes MPN format were 0-0-0, 3-0-0, and 3-3-0, for the homogenates contaminated at 0.1, 1.0 and 10.0 CFU/ml, respectively. The sensitivity of the recommended MPN-IMS-LAMP was therefore determined to be 1 CFU/ml. Based on the 3-tubes MPN format results, the MPN values obtained were <0.03 (range: <0.005–0.09) MPN/ml, 0.23 (range: 0.04–1.2) MPN/ml, and 2.4 (range: 0.36–13.0) MPN/ml, respectively.

Examination of Retail Shellfish in Thailand and Japan

Twenty-eight Thai samples were examined using liquid LAMP reagent in Stage II. Three (**Table 2**, sample designations 4, 21, and 25) of the 28 samples representing each shellfish group were also examined using dried LAMP reagent as in the new recommended protocol. The MPN values obtained at this stage were exactly the same, indicating the dried reagent had the same sensitivity as the liquid reagent as judged by the turbidimetric system. However, we confirmed that judgment by eye is easier when dried reagent is applied (**Figure 4B**).

Sixteen Japanese shellfish samples were also examined by the recommended new protocol (**Table 3**). The *tdh* gene was not detected in 14 of the 16 samples, with the MPN values being below the detection limit (<3 MPN/10 g). Two of the samples were positive for the *tdh* gene but the MPN values (15 and 23 MPN/10 g) were much lower than those obtained with Thai samples.

Discussion

Our international research group attempted to develop an easy and sensitive method for quantitative detection of *tdh*$^+$ *V. parahaemolyticus* in molluscan shellfish that can be applied in any

TABLE 2 | Comparison of the MPN values of *tdh*$^+$ *V. parahaemolyticus* obtained at the three Steps of the modified protocol*.

			log MPN/10 g		
Kind of shellfish	**Sample designation**	**Date of examination**	**Step 1**	**Step 2**	**Step 3**
Bloody clam	1	26-April	1.6	2.4†	1.6
(*Anadara granosa*)	2	22-April	2.0	2.6†	1.0
	3	25-April	2.4	2.4	1.4
	4	5-May	3.0	3.0	2.4
	5	6-May	3.0	3.0	2.6
	6	8-May	3.0	3.0	2.0
	7	12-May	3.4	3.4	2.6
	8	24-April	3.6†	3.0	2.0
	9	6-May	3.6†	3.4	2.6
	10	9-May	3.6†	3.4	2.6
	11	11-May	3.6	3.6	2.6
	12	8-May	3.6	3.6	3.4
		Average	3.03	3.07	2.23
Hard clam	13	23-April	2.4	2.4	2.0
(*Meretrix lusoria*)	14	28-April	3.4	3.4	3.0
	15	29-April	3.6	3.6	2.6
	16	25-April	3.6	3.6	3.0
	17	1-May	3.6	3.6	2.6
	18	5-May	4.0	4.0	3.0
	19	12-May	4.0	4.0	3.0
	20	7-May	4.6	4.6	3.6
	21	9-May	5.2	5.2	4.0
	22	6-May	5.2	5.2	4.4
	23	13-May	5.4	5.4	3.4
	24	24-April	5.4	5.4	3.4
		Average	4.2	4.2	3.2
Green mussel	25	22-April	2.4	2.4	2.0
(*Perna viridis*)	26	30-April	3.0	3.0	2.0
	27	13-May	4.0	4.0	2.9
	28	1-May	5.0	5.0	4.0
		Average	3.6	3.6	2.7
		Total average	**3.61**	**3.62**	**2.7**

Explained in **Figure 2. † MPN/10 g that differs between Steps 1 and 2.*

TABLE 3 | Examination of the Japanese shellfish for the MPN of *tdh*$^+$ *V. parahaemolyticus*.

Kind of shellfish	No. of samples	MPN/10 g	Origin and date of examination
Japanese littleneck (*Ruditapes philippinarum*)	11	<3.0	Shizuoka: 8-August (1), 4-September (1), 23-September (2)
			Kumamoto: 8-August (1), 4-September (2), 23-September (1)
			Chiba: 8-August (1), 4-September (1), 23-September (1)
	1	15.0	Kumamoto: 8-August
Hard clam (*Meretrix lusoria*)	3	<3.0	Ehime: 8-August (2), 4-September (1)
	1	23.0	Ehime: 23-September

The numbers in the parentheses indicate the numbers of the samples examined.

part of the world; being practical and feasible even in resources-limited and/or tropical countries. We previously reported an MPN-IMS-LAMP method that was shown to be more sensitive than the MPN-PCR-based method (Hara-Kudo et al., 2003; Jones et al., 2012; Tanaka et al., 2014). However, a problem of under-estimation of the MPN values for some samples in a tropical environment was found as well as the technical practical inconvenience of the liquid LAMP reagent. In the current study, we improved the previously reported method using a series of technical modifications in the MPN and IMS components (**Figure 2**). In addition, we replaced the liquid reagent by the dried reagent in the LAMP component. As a result, much of the technical problems were solved (**Tables 2** and **3**; **Figure 4**).

While our study was in progress, a LAMP (targeting the *tlh* gene) and IMS (using nanoparticules targeting flagella) detection method for *V. parahaemolyticus* was reported (Zeng et al., 2014). However, CODEX 2011 recommends quantitative detection of pathogenic rather than total *V. parahaemolyticus* for risk assessment of *V. parahaemolyticus* in seafood (Food and Agriculture Organization World Health Organization of the United Nations [FAO/WHO], 2011). Along this line, our IMS method screens for clinically important *V. parahaemolyticus* by targeting all established K antigens and our LAMP method targets the *tdh* gene. Though, primer sets for *trh*1 and *trh*2 are available, variation in the *trh* gene sequence is widely observed in environmental strains is of major concern (Kishishita et al., 1992). A new *trh* LAMP primer set to overcome this issue is currently under development (Escalante-Maldonado and Nishibuchi, unpublished data).

The IMS component is an essential part of our protocol, therefore we needed to improve the efficiency of the IMS performance. *In vitro* experiments in Stage I confirmed that IMB incubation with constant shaking (Nou et al., 2006) for 30 min (Tanaka et al., 2014) enhances IMB-target association (data not shown). **Figure 3** shows that increasing the PickPen Operation time up to 3 min (1–3 and 2–3 min) increased CE value significantly. Increase in operation time after 3 min showed no significance difference in CE value where further extension of PickPen Operation time increases the workload. Taken together, we judged 3 min is the most effective PickPen Operation time. *In vivo* experiments conducted in Thailand employing the new protocol confirmed that these two modifications are a valuable contribution to the IMS performance (**Table 2**). The MPN values obtained after IMS application in this study are higher than the under-estimated MPN values reported in our previous study where similar shellfish samples were examined (Tanaka et al., 2014).

Another very valuable contribution to our recommended protocol is the application of the dried LAMP reagent. We utilized the dried reagent included in the commercially available kit for detection of two other pathogens. Results of the comparative experiments in Stage I indicate that both dried and liquid reagents are equally sensitive and specific for the LAMP assays for *V. parahaemolyticus* regardless of the primer set (**Table 1**). Evaluation of the stability of the dried reagent at different temperatures (30–60°C) confirmed the dried reagent is very stable even at high-temperatures during the examination period, corroborating its utility even in tropical coastal areas of the world. Likewise, the dried reagent was proven useful because its transportation and storage do not necessarily require refrigerated or frozen conditions as confirmed during experiments in southern Thailand in Stage III.

Prevalence of infection by *tdh*$^+$ *V. parahaemolyticus* was reported previously in southern Thailand. This prevalence is due to consumption of under-cooked molluscan shellfish, which is very common in southern Thailand (Laohaprertthisan et al., 2003). A risk assessment study conducted in southern Thailand reported low concentration of *tdh*$^+$ *V. parahaemolyticus* in the bloody clam sold in the evening market (Yamamoto et al., 2008). However, pre-incubation of shellfish samples prior to examination may allow growth of *tdh*$^+$ *V. parahaemolyticus* to a detectable level (Hara-Kudo et al., 2003; Yamamoto et al., 2008). Our previous study indicated that molluscan shellfish kept overnight and sold at the morning market contained relatively high levels of *tdh*$^+$ *V. parahaemolyticus* (Tanaka et al., 2014). Under this condition, the under-estimation problem of the MPN values was raised. The current study examining similar shellfish samples solved the under-estimation problem. Comparative experiments in Stage II showed the problem is due at least in-part to the addition of the SPB enrichment step (**Figure 1**, Step 3). Among the MPN values observed at the three different steps, those of Step 3 were lower for all seafood samples examined (**Table 2**). SPB enrichment probably supported preferentially the growth of competing bacterial population rather than that of *tdh*$^+$ *V. parahaemolyticus*.

The method yielding the highest MPN values is presumed to be most sensitive. We therefore compared the values obtained at Steps 1 and 2. The averages of the MPN values for these two steps were indistinguishable (**Table 2**). Difference in MPN values between Step 1 and Step 2 were observed only with five of 12 bloody clam samples. Two samples require special attention. They showed lower MPN values at Step 1 which were close to the detection limit. Application of IMS could avoid

under-estimation of these values and assure the detection of the target.

Conclusion

Modifications in the three components of the protocol (**Figure 2**) were critical to improve the MPN-IMS-LAMP. The under-estimation problem was solved by modifying the IMS and excluding the SPB from the enrichment step. Introduction of the dried LAMP reagent made the method quicker, easier and allows its use at high environmental temperatures. We therefore recommend the modified MPN-IMS-LAMP for detection and quantitation of *tdh*$^+$ *V. parahaemolyticus* as a universal method useful in tropical, subtropical, and temperate zones of the world.

Author Contributions

Conceived and designed the experiments: OE-M and MN. Performed the experiments and analyzed data: OE-M and AK. Technical assistance: WY, VV, and YN. Interpretated and wrote the paper: OE-M and MN.

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Examination of clinical and environmental *Vibrio parahaemolyticus* isolates by multi-locus sequence typing (MLST) and multiple-locus variable-number tandem-repeat analysis (MLVA)

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Vibrio parahaemolyticus is a leading cause of seafood-borne infections in the US. This organism has a high genetic diversity that complicates identification of strain relatedness and epidemiological investigations. However, sequence-based analysis methods are promising tools for these identifications. In this study, Multi-Locus Sequence Typing (MLST) and Multiple-Locus Variable-Number Tandem-Repeat Analysis (MLVA) was performed on 58 *V. parahaemolyticus* isolates (28 of oyster and 30 of clinical origin), to identify differences in phylogeny. The results obtained by both methods were compared to Pulsed-Field Gel Electrophoresis (PFGE) patterns determined in a previous study. Forty-one unique sequence types (STs) were identified by MLST among the 58 isolates. Almost half of the isolates (22) belonged to a new ST and added to the MLST database. A ST could not be generated for 5 (8.6%) isolates, primarily due to an untypable *recA* locus. Analysis with eBURST did not identify any clonal complex among the strains analyzed and revealed 37 singeltons with 4 of them forming 2 groups (1 of them SLV, and the other a DLV). An established MLVA assay, targeting 12 total genes through three separate 4-plex PCRs, was successfully adapted to high resolution melt (HRM) analysis with faster and easier experimental setup; resulting in 58 unique melt curve patterns. HRM-MLVA was capable of differentiating isolates within the same PFGE cluster and having the same ST. Conclusively, combining the three methods PFGE, MLST, and HRM-MLVA, for the phylogenetic analysis of *V. parahaemolyticus* resulted in a high resolution subtyping scheme for *V. parahaemolyticus*. This scheme will be useful as a phylogenetic research tool and as an improved method for outbreak investigations for *V. parahaemolyticus*.

Keywords: ***Vibrio parahaemolyticus*, phylogenetics, MLVA, MLST, HRM**

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Introduction

Vibrio parahaemolyticus can cause acute gastroenteritis associated with consumption of raw or undercooked seafood (Nishibuchi and DePaola, 2005). Presently, this bacterium represents the most common cause for seafood-associated infections in the United States (Iwamoto et al., 2010). *V. parahaemolyticus* has shown a high rate of recombination and mutation which leads to a high genetic diversity (Gonzalez-Escalona et al., 2008). *V. parahaemolyticus* isolates are frequently characterized for their virulence gene profile, serotype, ribotype, and/or Pulsed-Field Gel Electrophoresis (PFGE) pattern for research studies and epidemiological investigations (Broberg et al., 2011; Jones et al., 2012; Paranjpye et al., 2012; Banerjee et al., 2014; Xu et al., 2015). However, there are several phylogenetic and evolutionary methodologies for differentiation of *V. parahaemolyticus,* such as Multi-Locus Sequence Typing (MLST) and Multiple-Locus Variable-Number Tandem-Repeat Analysis (MLVA), which may provide greater discrimination.

For this study, we selected two sequence-based typing methods, MLST and MLVA. MLST is based on sequence diversity of loci which are generally well-conserved, or under purifying selection, as is the case with housekeeping genes. MLST is a frequently used typing method for many organisms and the development of a public database (PubMLST) has simplified sequence analysis and identification of evolutionary relationships within bacterial species (Maiden et al., 1998; Perez-Losada et al., 2013). MLST was selected to examine the current strain collection based on the previous success of the technique for characterizing diverse environmental and clinical *V. parahaemolyticus* isolates (Gonzalez-Escalona et al., 2008) and to add a diverse set of isolates to the MLST database. MLVA has also been used to distinguish isolates with little genetic variation. MLVA uses PCR for amplification of size polymorphisms in several Variable-Number Tandem-Repeat (VNTR) loci (Lindstedt, 2005). The VNTRs are highly polymorphic and are well suited for differentiation of bacterial isolates (Lindstedt, 2005; van Belkum, 2007). MLVA was selected for use in this study as it is able to differentiate between indistinguishable PFGE patterns for *V. parahaemolyticus* (Hayat et al., 1993; Harth-Chu et al., 2009) or identical MLST sequence types (STs) in other organisms (Maiden et al., 1998, 2013). Recently, MLVA was used for epidemiological analysis for discrimination of clinical and environmental *V. parahaemolyticus* isolates with indistinguishable Direct Genome Restriction Enzyme Analysis (DGREA) patterns (Harth-Chu et al., 2009; Garcia et al., 2012). Generally, the PCR products from MLVA are separated by agarose gel or capillary electrophoresis (CE) (Lindstedt et al., 2013). However, differentiation of amplification products using high resolution melt (HRM) analysis has been described for MLVA assays in other organisms (Fortini et al., 2007).

The objective of this study was to evaluate the combined use of MLST and the HRM-MLVA sequence-based methods for discrimination of environmental and clinical *V. parahaemolyticus* isolates previously characterized by other fingerprinting methods, such as PFGE (Ludeke et al., 2014). MLST was selected to identify phylogenetic relationships while MLVA was applied with the hypothesis that it will further discriminate isolates with identical STs. In order to achieve this objective, an HRM-MLVA protocol for rapid and simple characterization of *V. parahaemolyticus* isolates was developed. This is the first study, to the authors' knowledge, to use the combined approach of the subtyping methods MLST, HRM-MLVA, and PFGE for differentiation of *V. parahaemolyticus* isolates.

Material and Methods

Bacterial Strains

For the MLST and MLVA analysis, 58 *V. parahaemolyticus* isolates were selected; among those were 28 environmental (oyster) and 30 clinical isolates. Isolates were selected to represent multiple collection states and serotypes (**Table 1**). Each isolate was inoculated into Luria Bertani broth with 1% NaCl and incubated with shaking overnight at 35°C. Afterwards, 1 mL of the overnight culture was transferred to a 1.5 mL microcentrifuge tube, heated to 100°C for 10 min, and placed in ice for 5 min. The samples were stored at −20°C until used as a PCR template.

Multi-Locus Sequence Typing (MLST)

MLST was performed as described in the protocol for *Vibrio parahaemolyticus* (Gonzalez-Escalona et al., 2008). The PCR products were purified using a PCR purification kit (Qiagen, Valencia, CA) with a total elution volume of 25 μL. The purified samples were sequenced on an ABI 3730 × l sequencer at McLab (South San Francisco, CA). Sequences were analyzed with BioEdit software 7.1.9 (Abbott, Carlsbad, CA). The allelic and sequence type (ST) identification was determined using the MLST database (http://pubmlst.org/vparahaemolyticus). In the cases where whole genome sequence data was available, sequences were *de novo* assembled using CLC Genomics Workbench software 7.0.3 (CLCbio, Germantown, MD) and consensus assemblies submitted to the MLST database for allelic and ST identification.

For identification of clonal complexes, eBURST version 3 was used (http://eburst.mlst.net/). As reported previously, two different STs were considered single-locus variant (SLV) when they differed by a single locus; a double-locus variant (DLV) has two different loci (Gonzalez-Escalona et al., 2008). To be part of a clonal complex, isolates needed to share at least six out of seven alleles.

The minimum evolution tree of the concatenated sequences of the seven loci was built based on the method of Kimura-2-parameter in Mega 6 (Tamura et al., 2013). The ratio between the number of synonymous and non-synonymous substitutions, showing the type of selection at each locus, was calculated using the method of Nei and Gojobori in Mega 6. The hypotheses of neutrality ($d_S = d_N$), purifying selection ($d_S/d_N > 1$), and positive selection ($d_S/d_N < 1$) were tested.

Multiple-Locus Variable-Number Tandem-Repeat Analysis (MLVA)

Three multiplex real-time-PCR assays using HRM curve analysis were performed with the LightCycler® 480 High Resolution

TABLE 1 | Isolates used in this study and their sequence types (ST).

Isolate ID	Source of isolate	Collection state	Serotype	tdh	trh	Allele types							ST
						dnaE	gyrB	recA	dtdS	pntA	pyrC	tnaA	
FDA_R2	Oyster	TX	O3:Kut[a]	–	+	86	300	17	55	12	54	86	**729**[c]
FDA_R5	Oyster	TX	O10:Kut	–	+	214	**329**	30	19	**165**	69	26	**730**
FDA_R10	Oyster	FL	O1:Kut	+	+	142	29	10	7	4	24	20	313
FDA_R12	Oyster	LA	O4:K8	+	+	20	25	15	6	7	11	4	32
FDA_R13	Oyster	LA	O4:K10	–	–	**241**	**330**	205	253	28	22	**188**	**732**
FDA_R16	Oyster	FL	O4:K9	+	+	20	25	15	13	7	11	5	34
FDA_R17	Oyster	FL	O4:Kut	–	–	14	30	49	11	49	11	13	536
FDA_R21	Oyster	TX	O5:Kut	–	+	9	21	15	13	4	10	26	12
FDA_R26	Oyster	NJ	O4:K8	+	+	20	25	15	6	7	11	4	32
FDA_R29	Oyster	FL	O11:Kut	–	–	**235**	22	25	273	164	254	20	**734**
FDA_R30	Oyster	FL	O1:Kut	+	+	17	16	UT	36	15	31	26	–
FDA_R45	Oyster	WA	O5:Kut	–	+	37	14	14	9	14	34	26	61
FDA_R47	Oyster	AL	O4:K8	+	+	20	25	15	6	7	11	4	32
FDA_R51	Oyster	AL	O8:Kut	+	+	60	106	31	72	66	62	65	676
FDA_R52	Oyster	WA	O3:Kut	–	+	4	13	11	38	18	46	23	**735**
FDA_R60	Oyster	ME	O10:Kut	–	+	63	**326**	**231**	13	48	120	24	**736**
FDA_R62	Oyster	ME	O1:Kut	–	+	31	327	UT	157	14	3	20	–
FDA_R74	Oyster	VA	O4:K34	–	–	26	58	53	19	28	9	26	108
FDA_R75	Oyster	VA	O8:Kut	+	+	60	106	31	72	66	62	65	676
FDA_R86	Oyster	FL	O6:Kut	–	–	45	**336**	143	7	171	**255**	36	**737**
FDA_R87	Oyster	FL	O8:K70	+	+	145	177	140	158	4	132	104	320
FDA_R94	Oyster	PEI[b] (Canada)	O3:K5	–	+	47	328	UT	13	2	256	23	–
FDA_R125	Oyster	FL	O11:Kut	+	–	17	**331**	**235**	23	33	137	94	**739**
FDA_R126	Oyster	FL	O4:K42	–	–	36	285	25	250	26	227	26	**740**
FDA_R135	Oyster	SC	O3:Kut	–	–	26	16	41	224	31	32	23	**741**
FDA_R136	Oyster	SC	O1:K20	+	+	31	16	32	36	33	11	19	**775**
FDA_R143	Oyster	FL	O5:Kut	–	–	17	64	137	60	94	11	51	**743**
FDA_R149	Oyster	FL	O1:Kut	+	+	142	29	10	7	4	24	20	313
CDC_K4556_1	Clinical	LA	O1:K25	–	–	31	82	**236**	35	23	26	51	**744**
CDC_K4557	Clinical	LA	O1:K33	–	–	28	4	82	88	63	187	1	**799**
CDC_K4588	Clinical	ME	O5:Kut	–	+	56	16	**237**	8	33	59	20	**746**
CDC_K4857_1	Clinical	HI	O5:K17	–	–	35	43	38	21	31	35	37	79
CDC_K4858	Clinical	HI	O4:K4	–	–	27	84	127	139	54	124	37	283
CDC_K4981	Clinical	OK	O1:Kut	–	–	17	**327**	13	8	172	32	**181**	**748**
CDC_K5009_1	Clinical	MA	O4:K53	+	+	5	71	**238**	162	26	11	107	**749**
CDC_K5010_1	Clinical	MA	O1:Kut	+	–	3	4	19	4	29	4	22	3
CDC_K5058	Clinical	TX	O3:K6	+	–	3	4	19	4	29	4	22	3
CDC_K5067	Clinical	SD	O1:K56	+	+	31	16	13	36	33	11	19	**775**
CDC_K5073	Clinical	MD	O3:K56	+	+	17	57	52	**285**	44	28	36	**750**
CDC_K5125	Clinical	MS	O3:Kut	–	–	195	263	187	75	23	198	**190**	**772**
CDC_K5276	Clinical	NY	O11:Kut	+	+	222	128	21	69	46	236	12	631
CDC_K5278	Clinical	WA	O4:K12	+	+	21	15	1	23	23	21	16	36
CDC_K5282	Clinical	HI	O5:Kut	–	–	19	217	89	175	UT	62	51	–
CDC_K5306	Clinical	GA	O4:K9	+	+	20	25	15	13	7	11	5	34
CDC_K5323_1	Clinical	VA	O5:K17	–	+	83	82	73	83	4	77	58	674
CDC_K5324_1	Clinical	VA	O1:K20	+	+	56	16	32	**286**	14	11	19	**752**
CDC_K5331	Clinical	GA	O4:K8	+	–	11	48	UT	48	26	48	26	–
CDC_K5345_1	Clinical	IA	O4:K12	+	+	21	15	1	23	23	21	16	36

(Continued)

TABLE 1 | Continued

Isolate ID	Source of isolate	Collection state	Serotype	tdh	trh	Allele types							ST
						dnaE	gyrB	recA	dtdS	pntA	pyrC	tnaA	
CDC_K5428	Clinical	NV	O1:Kut	+	+	22	28	17	13	8	19	14	199
CDC_K5433	Clinical	WA	O4:Kut	+	+	21	15	1	23	23	21	16	36
CDC_K5436	Clinical	WA	O4:Kut	+	+	21	15	1	23	23	21	16	36
CDC_K5439	Clinical	WA	O4:K8	+	–	11	48	3	48	26	48	26	189
CDC_K5485	Clinical	NC	O6:K18	–	–	29	5	22	12	20	22	25	50
CDC_K5528	Clinical	GA	O4:K68	+	–	3	4	19	4	29	4	22	3
CDC_K5582	Clinical	GA	O11:Kut	+	+	222	128	21	69	46	236	12	631
CDC_K5618	Clinical	NY	O10:Kut	+	+	223	106	31	221	45	171	165	636
CDC_K5621	Clinical	NY	O1:Kut	–	+	39	9	27	39	3	37	30	65
CDC_K5635	Clinical	MD	O5:K30	–	–	158	131	31	**287**	128	43	**189**	**753**

[a] *ut = untypable.*
[b] *PEI = Prince Edward Island.*
[c] *novel allele type and novel ST in bold.*

Melting Master Kit (Roche, Indianapolis, IN). The primer sequences utilized in this study are listed in **Table 2** and have been previously described (Harth-Chu et al., 2009). These primers were utilized as unmodified oligonucleotides since HRM was used as the detection method rather than CE. Each reaction mixture for the multiplex PCR A and B (Multi A and B) had a final volume of 20 μL and consisted of: 1X master mix solution (Roche), 2 mM $MgCl_2$ (Roche), 0.2 μM each primer (Integrated DNA Technologies; IDT, Coralville, IA), and 5 μL boiled template. For multiplex PCR C (Multi C), reaction concentrations were the same as for Multi A and B, except for primer pair VP2-07; 0.4 μM of each primer was used. The temperature program was as described previously for Multi A and B (Harth-Chu et al., 2009): Initial denaturation at 95°C for 15 min, followed by 20 cycles of a touchdown PCR consisting of denaturation at 94°C for 30 s, annealing starting at 63°C and decreasing 0.2°C per cycle for 1.5 min, and elongation at 72°C for 1 min. A final 10 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 1.5 min, and elongation at 72°C for 1 min was used. The cycling program of Multi C included the same initial denaturation as for Multi A and B; cycling consisted of 30 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 1.5 min, and elongation at 72°C for 1 min. A final annealing step at 60°C for 30 min completed both cycling programs. The melt curve analysis was performed using the Roche High Resolution Melting Kit protocol. The temperature program for the HRM analysis started at 95°C for 1 min with a ramp of 4.4°C per s, followed by 40°C for 1 min with a ramp of 2.2°C per s, 60°C for 1 s with a ramp of 4.4°C per s and a continuous step at 95°C. For the PCR amplification and HRM analysis a LightCycler® 480 (Roche) was used.

Due to overlapping peaks in Multi A and C, the presence of the target loci VNTR5 and VNTR7, VNTR3, and VP2-07 were confirmed using simplex real-time-PCR for each gene with the same PCR reaction conditions and cycling parameters as described for the multiplex, but with omission of the three other primer sets. The melt curves were analyzed with BioNumerics software 6.6 with a customized script (Applied Maths, Austin, TX). This script compares the melting curves of each multiplex PCR, as well as a combination of all curves from the three multiplex PCRs. The combined dendrogram of all three multiplex PCR was built based on the Pearson correlation of average trend curves in BioNumerics as well. This dendrogram was also converted to a rendered rooted tree.

Results

Multi-Locus Sequence Typing (MLST)

From the 58 *V. parahaemolyticus* isolates analyzed, MLST resulted in 41 different STs (**Table 1**). Four (6.9%) and one (1.7%) of the isolates were untypeable for *recA* and *pntA*, respectively. For those strains, no ST could be assigned. Twelve (42.9%) of the oyster and ten (40.0%) of the clinical isolates were a novel ST. The most frequently identified STs were ST36 (13.3%) and ST3 (10.0%) in clinical isolates and ST32 (10.7%), ST313 (7.1%), and ST676 (7.1%) in oyster isolates.

All loci showed ratios of synonymous and non-synonymous substitutions (d_N/d_S) below 1 and therefore under purifying selection, as expected for housekeeping genes. eBURST analysis divided the 53 isolates for which a ST could be identified into 37 singletons and two groups: one SLV and one DLV (data not shown). No clonal complexes could be identified; demonstrating that none of the STs identified in this study share more than six alleles and, therefore, belong to different *V. parahaemolyticus* lineages.

A minimum evolution tree was constructed using the concatenated sequences of each allele (**Figure 1**). The isolates grouped into two main clusters, or lineages (I and II), with each lineage containing ST of clinical and oyster isolates. Isolates with the same ST generally had the same serotype; ST631 isolates possessed serotype O11:Kut, ST676 were serotype O8:Kut, ST36 were serotype O4:K12 or O4:Kut, and ST313 were serotype O1:Kut. However, the three ST3 isolates had all different serotypes.

TABLE 2 | Primer sequences including their amplification product length and melting temperature for MLVA multiplex PCR.

Multiplex-PCR	Locus	Primer	5′-3′ Sequence	Product length [bp]	Melting temperature Tm [°C]	References
Multi A	VP1-11	VP1-11 F	CTGCCTGGAGAATTGGCTTA	854	95	8
		VP1-11 R	TGAGCCTGAAGCTGAAAACA			
	VP2-03	VP2-03 F	CATAAACGAGCGACACGAGA	168	57	8
		VP2-03 R	GCGCAAAAATTCATTGTGATT			
	VPTR5	VPTR5 F	GCTGGATTGCTGCGAGTAAGA	204	82	22
		VPTR5 R	AACTCAAGGGCTGCTTCGG			
	VPTR7	VPTR7-1F	TATCTACAAAGGTGGCGGAGAT	200	80	8
		VPTR7-1R	AAGGTGTTACTTGTTCCAGACG			
Multi B	VP1-17	VP1-17 F	TCAACACGAGCTTGATCACC	206	69	8
		VP1-17 R	GAAATCCGGAGTACCTGCAA			
	VP1-10	VP1-10 F	CGTCTTGCTCGTGAACGTAA	955	94	8
		VP1-10 R	TCATTAAGTCAGGCGTGCTG			
	VPTR1	VPTR1 F	TAACAACGCAAGCTTGCAACG	253	54	22
		VPTR1 R	TCATTCTCGCCACATAACTCAGC			
	VPTR8	VPTR8 F	ACATCGGCAATGAGCAGTTG	301	89	22
		VPTR8 R	AAGAGGTTGCTGAGCAAGCG			
Multi C	VP2-07	VP2-07 F	TGATTTTGAAGCAGCGAAGA	296	98, smaller peak at 74	8
		VP2-07 R	TTTGTGACTGCTGTCCTTGC			
	VPTR3	VPTR3 F	CGCCAGTAATTCGACTCATGC	331	77	22
		VPTR3 R	AAGACTGTTCCCGTCGCTGA			
	VPTR4	VPTR4 F	AAACGTCTCGACATCTGGATCA	227	85	22
		VPTR4 R	TGTTTGGCTATGTAACCGCTCA			
	VPTR6	VPTR6 F	TGTCGATGGTGTTCTGTTCCA	316	107, smaller peak 97, 72	22
		VPTR6 R	CTTGACTTGCTCGCTCAGGAG			

Multiple-Locus Variable-Number Tandem-Repeat Analysis (MLVA)

The 58 *V. parahaemolyticus* isolates from this study were further analyzed by MLVA with HRM analysis. Three multiplex PCRs covering twelve different loci were used. Each multiplex PCR generated reproducible melting curve profiles of select isolates (data not shown). **Table 3** shows the percentage of isolates from which each target sequence in the MLVA scheme was amplified. From the Multi A, VP2-03, VPTR7, or VP1-11 was not amplified in 10% and 14.3%, 46.6% and 39.3%, or 3.3% and 3.6% of clinical and oyster isolates, respectively; VPTR5 was amplified from all isolates. In Multi B, VP1-10 or VPTR8 was not present in 96.7% and 17.9%, 23.3% and 17.9% of clinical and oyster isolates, respectively; VPTR1 and VP1-17 was amplified in all isolates with the exception of 6.7% of clinical isolates not amplifying VPTR1. From Multi C, VPRT6 was not amplified in 83.3% and 85.7% of clinical and oyster isolates, respectively. All isolates amplified VPTR4, VPTR3, and VP2-07, with the exception of 3.6% of oyster isolates for VPTR4.

Using the trend curves of each individual multiplex PCR, dendrograms were constructed (data not shown). Each multiplex PCR generally clustered the isolates based on their serotypes and ST. The individual multiplex PCR dendrograms demonstrated the ability of MLVA to differentiate between the same ST (data not shown).

Comparison of MLST, MLVA, and PFGE

Based on the hypothesis MLVA can differentiate isolates with the same ST and PFGE pattern, these isolates' MLVA patterns were compared to the MLST data, as well as previously published PFGE results (Ludeke et al., 2014). To compare these methods, dendrograms were built of the combined melting curves from the three MLVA multiplex PCRs and correlated to the PFGE cluster and ST of each isolate. MLVA allowed further differentiation of isolates with identical STs and PFGE clusters (**Figure 2**). Specifically, the isolates with ST3 and ST36 share the same PFGE cluster, but were distinguishable by MLVA melting curve profiles (**Figure 3**). The dendrogram with only ST3 and ST36 isolates showed ST-specific clusters, but separation within those clusters based on the combined melting curves of MLVA.

Discussion

This study analyzed 58 *V. parahaemolyticus* isolates by MLST and a newly-developed HRM-MLVA assay to investigate the

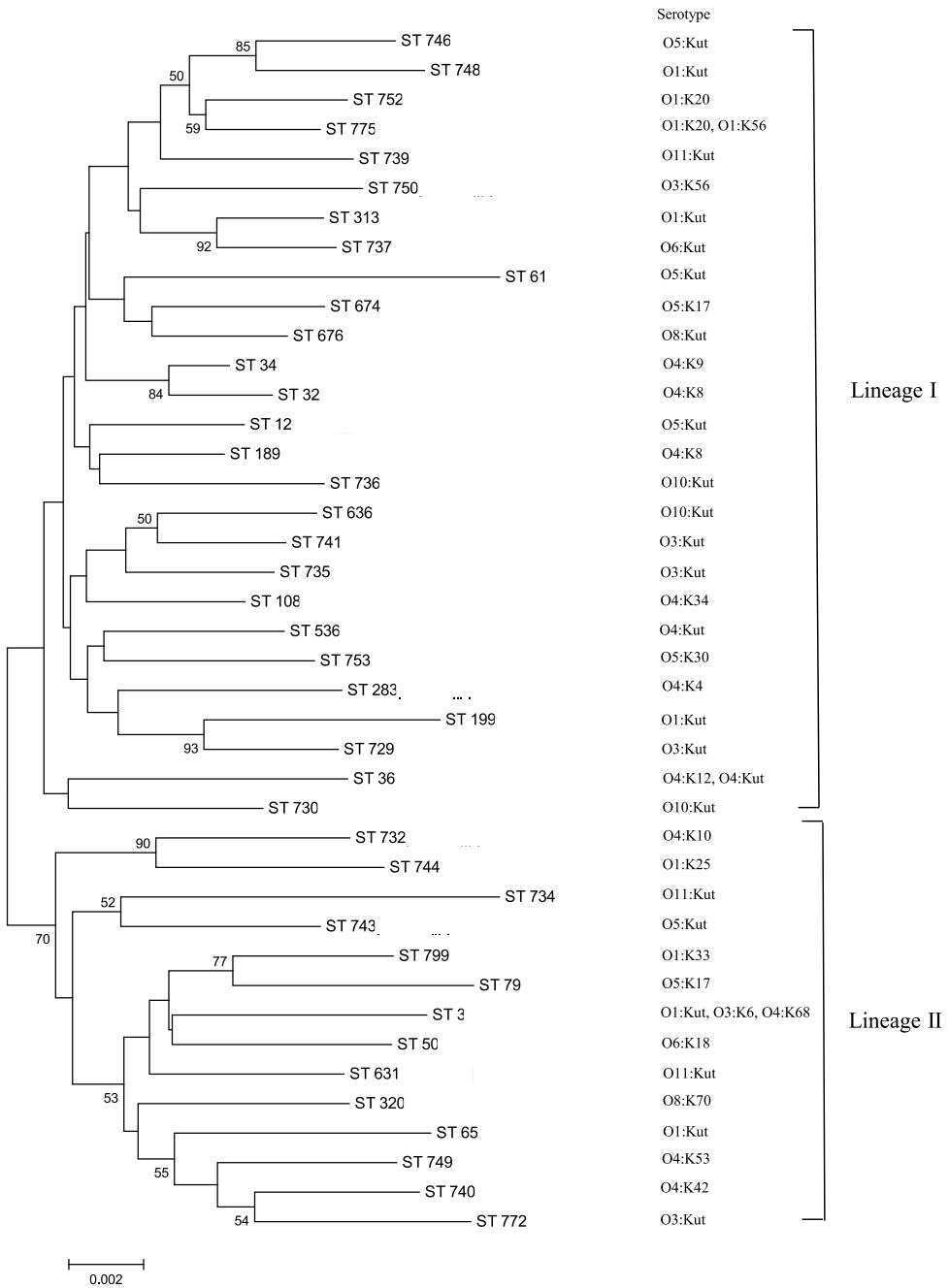


FIGURE 1 | MLST minimum evolution tree of the 58 *V. parahaemolyticus* isolates. The tree was built with Mega six software using concatenated sequences. The scale represents the evolutionary distance and the branches show bootstrap values above 50%.

relatedness of the isolates. The seven gene MLST protocol reported in a previous study was employed in this study (Gonzalez-Escalona et al., 2008). A different MLST method using ten housekeeping genes was described (Yan et al., 2011). Both methods report the same level of discrimination; however, the seven gene protocol was selected due to the availability of a public repository for the data (http://pubmlst.org/vparahaemolyticus), which allows comparisons to be made with isolates analyzed by other researchers. With 22 novel STs, this study substantially contributed to the diversity in the MLST database. Four of our isolates were untypeable for *recA*. In a recently published study, a *V. parahaemolyticus* strain contained a *recA* gene that was fragmented by a 30 kb DNA insertion (Gonzalez-Escalona et al., 2015). It is possible a similar insertion exists in the *recA* gene of

TABLE 3 | Presence of individual MLVA genes in clinical and oyster isolates.

	Multi A				Multi B				Multi C			
	VP2-03	**VPTR7**	**VP1-11**	**VPTR5**	**VP1-10**	**VPTR1**	**VPTR8**	**VP1-17**	**VPTR4**	**VPTR3**	**VPTR6**	**VP2-07**
Clinical isolates ($n = 30$)	27	16	29	30	1	28	23	30	30	30	5	30
Percentage (%)	*90.0*	*53.3*	*96.7*	*100.0*	*3.3*	*93.3*	*76.7*	*100.0*	*100.0*	*100.0*	*16.7*	*100.0*
Oyster isolates ($n = 28$)	24	17	27	28	23	28	23	28	27	28	4	28
Percentage (%)	*85.7*	*60.7*	*96.4*	*100.0*	*82.1*	*100.0*	*82.1*	*100.0*	*96.4*	*100.0*	*14.3*	*100.0*

some of the strains in the current study, but further analysis is needed to confirm.

ST3, ST32, and ST36 were the STs that occurred most often in our isolates, as well as in the public database. In our study, the fourth most frequent ST observed was ST676, which is one of the novel STs reported here. Two of these STs (ST3 and ST36) have been reported as part of clonal complexes CC3 and CC36, respectively (Gonzalez-Escalona et al., 2008), and correlated with outbreaks in multiple countries, including the US and Chile (Fuenzalida et al., 2006; Martinez-Urtaza et al., 2013). ST3 was identified as the ancestral ST of CC3 with ST27, ST42, and ST51 as SLVs. None of other STs from CC3 were identified in this study. A previous study using MLST on a set of clinical and environmental isolates from the Pacific Northwest Region of the United States showed that some environmental isolates were of ST3, suggesting a higher potential for virulence than other environmental isolates (Turner et al., 2013). In this study, three clinical isolates were ST3 and no direct relationship to environmental or other clinical clades were observed.

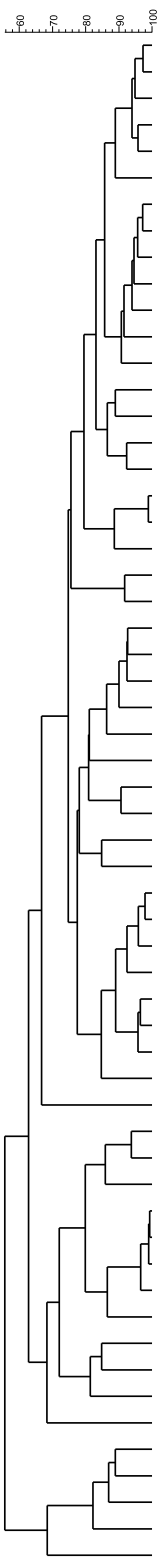
We developed an HRM-MLVA method, based on an existing MLVA method that uses CE, for subtyping of *V. parahaemolyticus* and to differentiate between similar PFGE patterns or STs. The CE method provides the actual number of tandem repeats while HRM does not. However, the HRM analysis still recognizes allelic variants and is able to distinguish between otherwise indistinguishable strains. For example, the ST3, ST32, and ST36 strains in this study also shared common PFGE profiles, but each isolate produced a unique HRM curve combination. This resolving power of HRM-MLVA is similar to previous reports of CE-MLVA, where Chilean isolates which shared a DGREA pattern and were ST3 could be differentiated by CE-MLVA (Gonzalez-Escalona et al., 2008; Harth-Chu et al., 2009). These data demonstrate that the HRM-MLVA method developed in this study provides similar discrimination as previously reported for the CE-MLVA method and is suitable examination of *V. parahaemolyticus* isolates. Additionally, the use of HRM-MLVA on the LightCycler®480 saves the step of electrophoretic detection, thus minimizing the potential for cross contamination by PCR amplicons during that additional handling step.

The loci amplified in this study by MLVA are coding proteins such as a putative hemolysin (VPTR4) and putative collagenase (VPTR3) (Kimura et al., 2008). Most of these genes, could be amplified from the current strain selection with the exception of VPTR7 (Multi A), VP1-10 (Multi B), and VPTR6 (Multi C). Nearly all clinical isolates failed to amplify the VP1-10 locus and approximately half failed to amplify the locus VPTR7. A failure to amplify VPTR7 from some shellfish isolates has been reported previously (Harth-Chu et al., 2009). Fewer than 20% of isolates amplified VPTR6 in this study. Additionally, a previous study found VPTR6 to be one of the few loci with high genetic diversity (Harth-Chu et al., 2009). Together, these data suggest that these two loci might not be suitable targets for future MLVA studies, especially for environmental isolate screening. Nonetheless, the HRM-MLVA method successfully discriminated between otherwise indistinguishable *V. parahaemolyticus* isolates.

Previous studies have employed multiple methods for characterization and subtyping of *V. parahaemolyticus* isolates from various sources. For example, DePaola et al. used a combination of serotyping and ribotyping to identify types more highly associated with clinical isolates (DePaola et al., 2003). Turner et al. utilized REP-PCR, as fingerprint-based subtyping method, followed by MLST to identify region-specific clades of *V. parahaemolyticus* (Turner et al., 2013). Banerjee et al. employed PFGE, MLST, serotyping, and ribotyping to examine clinical *V. parahaemolyticus* isolates and provided combinatorial analysis to determine relatedness (Banerjee et al., 2014). However, none of these previous studies utilized the combination of two highly discriminatory, sequence-based methods as does the current study.

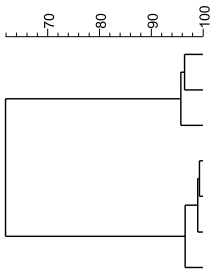
This combined method approach described here using PFGE, MLST, and MLVA has not been previously reported for discrimination of *V. parahaemolyticus* isolates, but is similar to approaches used for other organisms: for example, the Centers for Disease Control and Prevention has used a combination of PFGE followed by MLVA for epidemiological investigations of STEC O157 to discriminate between closely related isolates (Hyytia-Trees et al., 2006). Also, a combination of MLST and MLVA has been used as an epidemiological tool for distinguishing between clones of *Listeria monocytogenes* (Chenal-Francisque et al., 2013).

This study used a combined method approach to increase the discrimination of *V. parahaemolyticus* isolates. MLST was able to determine the identity and the phylogenetic relatedness of the isolates in this collection. As hypothesized, the developed HRM-MLVA method further refined the relationship of isolates by being able to distinguish between isolates with indistinguishable PFGE groupings or STs. Our data demonstrates



Key	Serotype	ST	PFGE pattern
FDA_R26	O4:K8	32	21
FDA_R47	O4:K8	32	21
FDA_R12	O4:K8	32	21
FDA_R10	O1:Kut	313	5
FDA_R86	O6:Kut	737	69
FDA_R143	O5:Kut	743	34
CDC_K4858	O4:K4	283	46
FDA_R136	O1:K20	775	55
FDA_R2	O3:Kut	729	1
CDC_K5067	O1:K56	775	56
CDC_K5073	O3:K56	750	47
CDC_K4588	O5:Kut	746	70
CDC_K5324-1	O1:K20	752	57
FDA_R125	O11:Kut	739	31
FDA_R29	O11:Kut	734	64
FDA_R16	O4:K9	34	22
FDA_R17	O4:Kut	536	53
CDC_K5276	O11:Kut	631	13
CDC_K5582	O11:Kut	631	13
CDC_K5618	O10:Kut	636	52
FDA_R126	O4:K42	740	16
FDA_R5	O10:Kut	730	39
FDA_R135	O3:Kut	741	38
FDA_R62	O1:Kut	-	26
FDA_R149	O1:Kut	313	6
FDA_R30	O1:Kut	-	59
FDA_R74	O4:K34	108	2
FDA_R45	O5:Kut	61	73
CDC_K4981	O1:Kut	748	33
CDC_K5621	O1:Kut	65	43
CDC_K4857-1	O5:K17	79	45
FDA_R13	O4:K10	732	60
CDC_K5331	O4:K8	-	61
CDC_K5439	O4:K8	189	62
FDA_R75	O8:Kut	676	20
FDA_R51	O8:Kut	676	20
CDC_K5010-1	O1:Kut	3	78
CDC_K5528	O4:K68	3	77
CDC_K5058	O3:K6	3	77
CDC_K5125	O3:Kut	772	37
CDC_K5306	O4:K9	34	22
CDC_K5282	O5:Kut	-	14
FDA_R60	O10:Kut	736	42
CDC_K5428	O1:Kut	199	3
CDC_K5278	O4:K12	36	8
CDC_K5345-1	O4:K12	36	8
CDC_K5433	O4:Kut	36	8
CDC_K5436	O4:Kut	36	8
FDA_R94	O3:K5	-	15
CDC_K4557	O1:K33	799	30
CDC_K5009-1	O4:K53	749	10
FDA_R52	O3:Kut	735	74
FDA_R87	O8:K70	320	72
CDC_K5323-1	O5:K17	674	23
FDA_R21	O5:Kut	12	9
CDC_K4556-1	O1:K25	744	58
CDC_K5635	O5:K30	753	7
CDC_K5485	O6:K18	50	4

FIGURE 2 | Combined dendrogram of MLVA melting curves of the three multiplex PCRs built with BioNumerics software version 6.6. using Pearson correlation and the unweighted pair group method using arithmetic averages (UPGMA). Isolates originated from oysters starting with "FDA," isolates from clinical origin labeled with "CDC." The PFGE pattern designations are as previously reported (Ludeke et al., 2014).



Key	Serotype	ST	PFGE pattern
CDC_K5010-1	O1:Kut	3	78
CDC_K5528	O4:K68	3	77
CDC_K5058	O3:K6	3	77
CDC_K5278	O4:K12	36	8
CDC_K5345-1	O4:K12	36	8
CDC_K5433	O4:Kut	36	8
CDC_K5436	O4:Kut	36	8

FIGURE 3 | Dendrogram of MLVA melting curves of the three multiplex PCRs for the isolates carrying ST3 and ST36 built with BioNumerics software version 6.6. using Pearson correlation and the unweighted pair group method using arithmetic averages (UPGMA). The PFGE cluster designations are as previously reported (Ludeke et al., 2014).

that a combination of PFGE, MLST, and HRM-MLVA would be the most suitable approach for outbreak and evolutionary investigations of *V. parahaemolyticus*, due to the high resolution provided. In instances where further discrimination is needed, and if available, next generation sequence data could be used to determine relatedness or to generate subtyping results *in silico*.

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Isolation, characterization, and antibiotic resistance of *Vibrio* spp. in sea turtles from Northwestern Mexico

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The aerobic oral and cloacal bacterial microbiota and their antimicrobial resistance were characterized for 64 apparently healthy sea turtles captured at their foraging grounds in Ojo de Liebre Lagoon (OLL), Baja California Sur (BCS), Mexico (Pacific Ocean) and the lagoon system of Navachiste (LSN) and Marine Area of Influence (MAI), Guasave, Sinaloa (Gulf of California). A total of 34 black turtles (*Chelonia mydas agassizii*) were sampled in OLL and eight black turtles and 22 olive ridley turtles (*Lepidochelys olivacea*) were sampled in LSN and MAI, respectively from January to December 2012. We isolated 13 different species of Gram-negative bacteria. The most frequently isolated bacteria were *Vibrio alginolyticus* in 39/64 (60%), *V. parahaemolyticus* in 17/64 (26%), and *V. cholerae* in 6/64 (9%). However, *V. cholerae* was isolated only from turtles captured from the Gulf of California (MAI). Among *V. parahaemolyticus* strains, six O serogroups and eight serovars were identified from which 5/17 (29.4%) belonged to the pathogenic strains (*tdh*$^+$ gene) and 2/17 (11.7%) had the pandemic clone (*tdh*$^+$ and *toxRS/new*$^+$). Among *V. cholerae* strains, all were identified as non-O1/non-O139, and in 4/6 (66%) the accessory cholera enterotoxin gene (*ace*) was identified but without virulence gene *zot*, *ctxA*, *and ctxB*. Of the isolated *V. parahaemolyticus, V. cholerae*, and *V. alginolyticus* strains, 94.1, 33.4, and 100% demonstrated resistance to at least one commonly prescribed antibiotic (primarily to ampicillin), respectively. In conclusion, the presence of several potential (toxigenic) human pathogens in sea turtles may represent transmission of environmental microbes and a high-risk of food-borne disease. Therefore, based on the fact that it is illegal and unhealthy, we discourage the consumption of sea turtle meat or eggs in northwestern Mexico.

Keywords: antibiotic resistance, bacterial diversity, *Chelonia mydas agassizii*, *Lepidochelys olivacea*, Mexico, sea turtles, *Vibrio spp.*

Introduction

Sea turtles are air-breathing, marine reptiles of the order *Testudines*. The advances in their medical management, the studies on causes of morbidity and mortality during stranding events, and the efforts to conserve them, have increased in recent years. Despite these efforts, six of the seven species of sea turtles are classified as threatened or endangered by IUCN Red List of Threatened Species (IUCN, 2014). Sea turtles can be considered excellent sentinel species of marine ecosystem health due to their ecological and physiological characteristics, including long life spans, long period of time to reach sexual maturity, and high site fidelity to near-coastal feeding habitats (Aguirre and Lutz, 2004). Sea turtles appear to be highly susceptible to biological and chemical insults despite their robust appearance (Lutcavage et al., 1997). As with other marine vertebrate species, sea turtles are threatened by increasing anthropogenic activities including fisheries bycatch; illegal traffic of meat, eggs, and their parts; coastal development; various forms of plastic; global environmental change; and, environmental pollution (Aguirre and Lutz, 2004).

The presence of many contaminants in Northwestern Mexico is related to agricultural runoff. For example, methoxychlor, endrin, and heptachlor levels in the Navachiste-Macapule lagoon system suggested that these compounds were continuously applied although their use is forbidden (Montes et al., 2012). Several recent studies evaluating heavy metals and their potential impact on sea turtles in Northwestern Mexico have been reported (Frías-Espericueta et al., 2006; Ley-Quinonez et al., 2011; Ley-Quiñónez et al., 2013; Zavala-Norzagaray et al., 2014). The region encompasses the northern nesting and feeding distribution for black (*Chelonia mydas agassizii*) and olive ridley (*Lepidochelys olivacea*) turtles (Ley-Quiñónez et al., 2013; Aguilar-Gonzalez et al., 2014; Zavala-Norzagaray et al., 2014). The effects of heavy metals and other environmental contaminants in sea turtles have been previously documented as one of the potential synergic etiologies of marine turtle fibropapillomatosis (Aguirre et al., 1994, 2006; Lutcavage et al., 1997; Aguirre and Lutz, 2004). In addition, contaminant loads can increase the incidence of other diseases and could affect various functional processes (Camacho et al., 2013) representing a serious threat to dwindling sea turtle populations (Garcia-Fernandez et al., 2009).

Although there is no food safety microbiology for the consumption of sea turtle meat or eggs, this illegal practice is common in countries with coastal areas worldwide. The health effects of humans consuming sea turtles infected with zoonotic pathogens have been reported (Aguirre et al., 2006). For example, *Vibrio mimicus* in Costa Rica (Campos et al., 1996), *V. cholerae* in China (Lu et al., 2006), and *Salmonella chester* in Australia (O'grady and Krause, 1999) were associated to human disease by consumption of sea turtle meat and/or eggs. Therefore, it is important to effectively communicate accurate information regarding the potential human health hazards associated with the consumption of sea turtles and their eggs in areas where this practice is common (Aguirre et al., 2006).

Many bacteria have been identified as the cause of diseases in marine turtles kept in captivity (Chuen-Im et al., 2010; Arena et al., 2014). In addition, many of these bacteria may be pathogenic to humans (Warwick et al., 2013). Other bacteria including *Aeromonas hydrophila*, *V. alginolyticus*, *Pseudomonas fluorescens*, *Flavobacterium* spp., and *Bacillus* spp. are common bacterial microbiota in sea turtles from Hawaii and Australia and are associated with other diseases such as ulcerative stomatitis, obstructive rhinitis-pneumonia complex and fibropapillomatosis (Glazebrook and Campbell, 1990; Glazebrook et al., 1993; Aguirre et al., 1994). Among these bacteria, *Vibrio spp.* are commonly found, naturally, in aquatic environments and can cause infections to humans (Chowdhury et al., 1989; West, 1989; Chakraborty et al., 1997). Particularly, *V. parahaemolyticus* infections have increased globally; they are usually associated with eating raw or undercooked sea products (Nair et al., 2007; Velazquez-Roman et al., 2012, 2014; Hernández-Díaz et al., 2015). To the best of our knowledge, there are no reports of the presence of the bacterial diversity linked to disease in sea turtles in Mexico.

The aim of this study was to identify, characterize, and determine antibiotic resistance of potentially pathogenic bacteria isolated from oral and cloacal swabs from black turtles and olive ridley turtles in northwestern Mexico.

Materials and Methods

Study Site

During January to December 2012, sea turtle surveys were conducted in selected feeding grounds in the states of Baja California Sur (BCS) and Sinaloa (SIN). Ojo de Liebre Lagoon (OLL) (27.7500° N, 114.2500° W) is located on the Pacific coast near the border between BCS and Baja California and it is part of the El Vizcaino Biosphere Reserve. Eelgrass (*Zostera marina*) and several species of benthic macroalgae are abundant in the lagoon (Lopez-Castro et al., 2010). The highly productive waters of BCS have been revered for decades and recognized for centuries for the abundance and diversity of charismatic megafauna they attract (Lopez-Castro et al., 2010; Micheli et al., 2012). SIN has 16 coastal lagoons with mostly surrounded by irrigation districts where commercial fisheries and aquaculture farms represent important economic activities (Hernández-Cornejo et al., 2005; Gonzalez-Farias et al., 2006; Aguilar-Gonzalez et al., 2014). The Navachiste-Macapule lagoon (25.4–25.7° N and 108.85–108.55° W) is a complex coastal system with an approximate area of 24,000 ha. It is located in the municipality of Guasave, in the southeast coast of the Gulf of California (Montes et al., 2012). This lagoon has a great ecological and economic importance, as it supports a variety of oyster (*Crassostrea virginica*), clam (*Protothaca staminea*), mullet (*Mugil cephalus*), mojarra (*Gerres cinereus*), puffer (*Arthron hispidus*), snapper (*Pagrus auratus*), jewfish (*Argyrosomus hololepidotus*), and snook (*Centropomus undecimalis*) fisheries, as well as intensive shrimp (*Penaeus vannamei*) aquaculture activities (Orduna-Rojas and Longoria-Espinoza, 2006; Aguilar-Gonzalez et al., 2014).

Sample Collection

Sea turtles were captured unharmed using fishing nets and snorkel equipment (Ley-Quiñónez et al., 2013) and transported

to a "floating dock" for examination, sampling and determination of morphometric parameters. All turtles were released alive and unharmed. Two nasopharyngeal and cloacal swabs were collected from each turtle for microbiology. The swabs were placed in alkaline peptone water at pH 8.5 (APW) for *Vibrio* spp. and in buffered peptone water pH 7.2 (BPW) for *Enterobacteriacea*; then transported to the School of Medicine laboratory at the Autonomous University of Sinaloa for bacteriological isolation and identification.

Isolation and Identification of Bacterial Strains

For *Vibrio* spp., all the nasopharyngeal and cloacal swabs were placed in APW and streaked onto thiosulfate citrate bile salts sucrose agar (TCBS; Becton-Dickinson, USA), and CHROMagar *Vibrio*, (CHROMagar Paris, France). The plates were incubated overnight at 37°C. From each plate, green and yellow colonies in TCBS or blue and violet colonies in CHROMagar *Vibrio* exhibiting diverse morphology were transferred to TSA-2% NaCl agar for purity. These plates were incubated overnight at 37°C and proceeded with identification using a single isolated colony. Each colony was examined by using the oxidase test and all biochemical tests described in the Bacteriological Analytical Manual of the Food and Drug Administration for *Vibrio* (Kaysner and De Paola, 2004; Canizalez-Roman et al., 2011). At least three typical colonies of *V. parahaemolyticus* and *V. cholerae* were isolated from each plate and subjected to identification by biochemical test and PCR. After identification of *V. parahaemolyticus*, *V. cholera*, and *V. alginolyticus* a single colony from each sample was used to continue the analysis (serotyping, virulence genes or antibiotic susceptibility testing). For *Enterobacteriacea*, specimens were placed in BPW and streaked onto *Salmonella-Shigella*, Hektöen and McConkey agar (Becton-Dickinson, USA). The plates were incubated overnight at 37°C. The presumptive colonies were transferred to TSA agar for purity. These plates were incubated overnight at 37°C and proceeded with identification using a single isolated colony. Each colony was examined by using the biochemical test (*Citrobacter freundii, E. coli, Edwarsiella spp., Aeromonas, Plesiomonas*, *Morganella* or *Proteus*, and *Providencia*) described in the Bacteriological Analytical Manual of the Food and Drug Administration (Andrews and Jacobson, 2013; Andrews et al., 2014; Feng et al., 2014).

PCR Assays

PCR assays were performed in a 25 μL volume consisting of 1X GoTaq green master mix (Promega), primers targeting the *tl* gene, the pR72H plasmid and the *tdh* and *trh* genes, the *toxRS* and *orf8* pandemic marker genes for *Vibrio parahaemolyticus* as previously described (Velazquez-Roman et al., 2012; Hernández-Díaz et al., 2015). *Vibrio cholerae* O1 and O139 were further confirmed for the presence of VC, *rfbO1, O139,* genes, and the *ctxA, ctxB, zot*, and *ace* toxigenic genes (Albert et al., 1997; Sarkar et al., 2002; Di Pinto et al., 2005; Goel et al., 2007) and 0.5 μg of purified genomic DNA template, with the remaining volume consisting of molecular biology grade water. PCR was routinely conducted in a Thermal Cycler C1000 (Bio-Rad Laboratories, Hercules, California). Ten microliter aliquots of each amplification product were separated by electrophoresis in a 2% agarose gel. Ethidium bromide staining (0.5 mg/ml) allowed for the visualization of DNA fragments with a digital imaging system (model E1 logia 100 imaging system; Kodak). The sizes of the PCR fragments were compared against a 50-bp DNA ladder (Promega DNA step ladder). To further identify diarrheagenic *E. coli* strains within our *E. coli* isolates, a protocol of sequential multiplex, duplex and single PCR reactions was used according to a previously published protocol work (Canizalez-Roman et al., 2013).

Serotyping

V. cholerae serotyping was performed by using *V. cholerae* O1–specific polyvalent rabbit antiserum and O139-specific polyvalent rabbit antiserum obtained from the National Institute of Epidemiological Reference (InDRE), Mexico. The microagglutination test was used to determine the serogroup O1, Ogawa and Inaba, and serogroup O139 as described in the Bacteriological Analytical Manual of the Food and Drug Administration for *Vibrio* (Kaysner and De Paola, 2004). Furthermore, serotyping of *V. parahaemolyticus* isolates was performed by using a commercially available *V. parahaemolyticus* antiserum test kit (Denka Seiken, Tokyo, Japan) with O1–O11 antisera and 71 K antisera according to the manufacturer's instructions. Briefly, strains were grown overnight at 37°C on LB agar containing 3% NaCl. A pool of colonies was suspended in 1 mL of saline and then split in two 500 μl aliquots. For serotyping, an aliquot was heated up to 121°C for 1 h for O serotyping; if the serotype could not be obtained, the bacterial lysate was heated for an additional hour and then used for O serotyping. The second aliquot was used for serotyping based on the K antigen.

Antibiotic Susceptibility Testing

All isolates of *V. parahaemolyticus*, *V. cholerae*, and *V. alginolyticus* were tested for antimicrobial susceptibility by a standard disc diffusion method on Mueller–Hinton agar. The protocol was performed as follows: fresh cultures were inoculated into LB broth and incubated until they reached an optical density equal to a MacFarland 0.5 standard. Bacterial cultures were then plated onto Mueller–Hinton agar and, then antibiotic disks (BD BBL, Franklin Lakes, NJ) were placed in a sterile environment. The plates were incubated at 37°C for 18–20 h. The diameters (in millimeters) of clear zones of growth inhibition around each antimicrobial agent disks were measured using a precision digital caliper (Absolute, Mitutoyo, Japan). Each bacterial species was classified as Resistant (R), Intermediately Resistant (I), or Susceptible (S) according to guidelines developed by the Clinical Laboratory Standard Institute (CLSI, 2011). The following antibiotics sensi-disc (BD BBL, Sensi-Disc, Becton, Dickinson and Company, USA), with their concentrations given in parentheses, were tested including ampicillin (10 μg), tetracycline (30 μg), trimethoprim–sulfamethoxazole (1.25 μg/23.75 μg), chloramphenicol (30 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), ceftazidime (30 μg), gentamicin (10 μg), and cefotaxime (30 μg). The following *V. parahaemolyticus* strains were used as a control organism: ATCC

17802, (*tdh*$^{-}$) and multidrug resistant strain 727 (Leon-Sicairos et al., 2009).

Results

Identification of Bacterial Isolates

From January to December 2012, 34 black turtles (*C. mydas agassizii*) were sampled in OLL, and eight black and 22 olive ridley (*L. olivacea*) turtles were captured in LSN and MAI, respectively (**Figure 1**). A total of 82 bacterial isolates (42 from black turtles and 40 from olive ridley turtles) were identified (**Table 1**). According to the geographic site of sampling, 33 (40.3%) and 49 (59.7%) bacterial isolates from BCS and from SIN were detected, respectively (**Table 1**).

Nine different microorganisms were isolated from olive ridley turtles including *Citrobacter freundii*, *E. coli*, *Edwarsiella* spp., *V. alginolyticus*, *V. cholerae*, *V. fluvialis*, *V. furnisii*, *V. parahaemolyticus*, and *Vibrio* spp. Eight different microorganisms were isolated from black turtles in BCS including *Aeromonas* or *Plesiomonas*, *Citrobacter*, *E. coli*, *Morganella*, and *Proteus*, *Providencia*, *V. alginolyticus*, *V. parahaemolyticus*, and *Vibrio* spp. Only three microorganisms were isolated from black turtles including *V. fluviales*, *V. alginolyticus*, and *V. parahaemolyticus* in Sinaloa (**Table 1**).

Overall, the predominant isolates in descending order of frequency were: *V. alginolyticus* (47.5%, 39/82), *V. parahaemolyticus* (20.7%, 17/82), *V. cholerae* (7.3%, 6/82) and other bacteria (25.6%, 21/82) (**Table 1**). *Vibrio alginolyticus* and *V. parahaemolyticus* were isolated in 62% (21/34) and 3% (1/34) from black turtles captured in BCS, respectively. However, in SIN, *V. alginolyticus* were isolated in 75% (6/8) and 55% (12/22), and *V. parahaemolyticus* were isolated in 25% (2/8) and 63% (14/22) from black turtles and olive ridleys, respectively. Interestingly, *V. cholerae* was only isolated in 27% (6/22) of olive ridley turtles (**Table 1**).

Serovars and Detection of Virulence Genes

V. parahaemolyticus was screened for the presence of virulence genes and serovars. There were 6 O serogroups, 4 different K types, and 8 serovars that could be identified in the 17 strains recognized or not by both O and K currently available antisera in *V. parahaemolyticus* isolates, (**Table 2**). A total of 47% (8/17) of

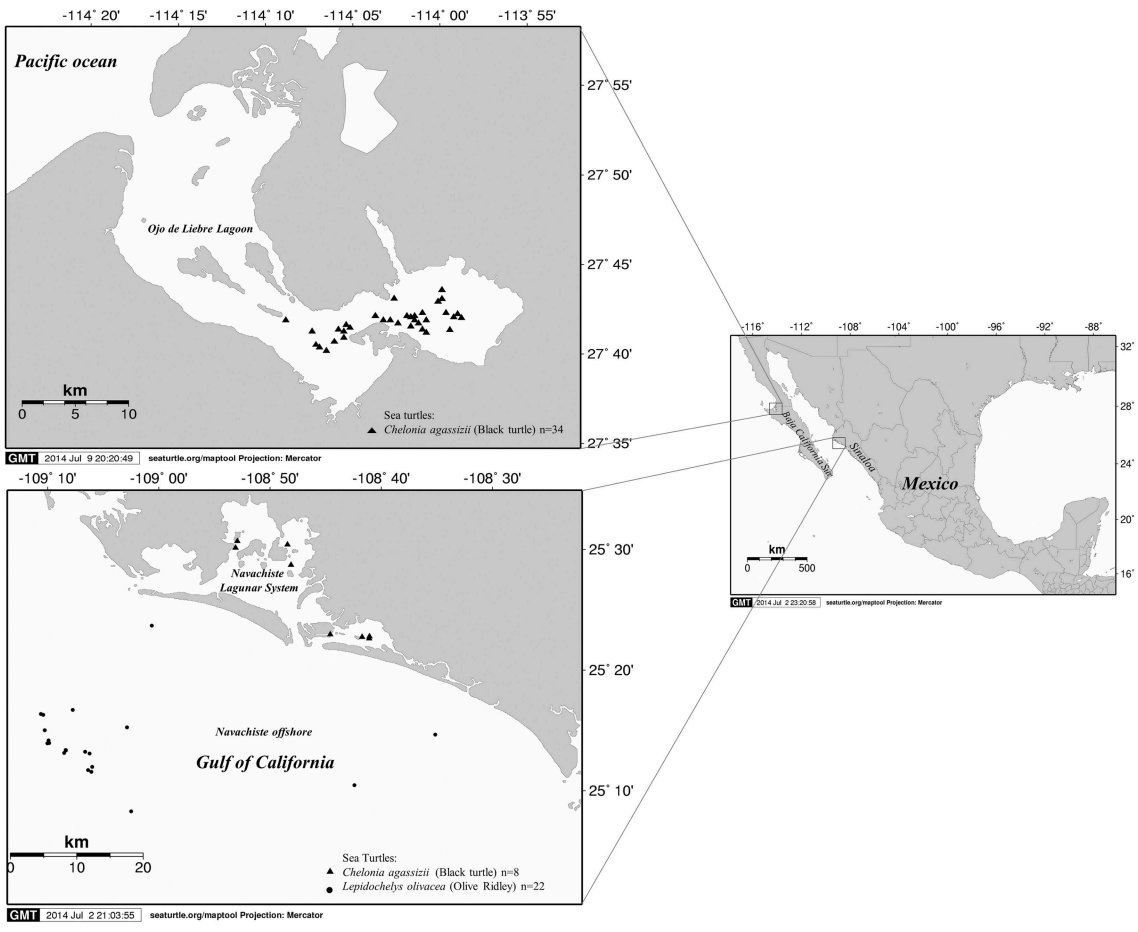

FIGURE 1 | Map of the study area and sites of capture of sea turtles, including in Ojo de Liebre Lagoon in Baja California Sur on the Pacific coast, the lagoon system of Navachiste and its influence zone in the Gulf of California in the state of Sinaloa.

TABLE 1 | Bacterial species isolated from buccal cavity and cloacae of black turtles (*Chelonia mydas agassizii*) and olive ridley turtles (*Lepidochelys olivacea*).

Bacteria	Anatomic isolation site		Sea turtles ($N = 64$)		
	Oral	Cloacal	BCS ($n = 34$)	Sinaloa ($n = 30$)	
			C. mydas agassizii $n = 34$	*C. mydas agassizii* $n = 8$	*L. olivacea* $n = 22$
Aeromonas or Plesiomonas		✓	2 (6%)	–	–
Citrobacter		✓	2 (6%)	–	–
Citrobacter freundii		✓	–	–	2 (9%)
E. coli		✓	1 (3%)	–	2 (9%)
Edwarsiella spp.	✓		–	–	1 (5%)
Morganella or Proteus		✓	2 (6%)	–	–
Providencia		✓	2 (6%)	–	–
Vibrio alginolyticus	✓	✓	21 (62%)	6 (75%)	12 (55%)
Vibrio cholerae	✓	✓	–		6 (27%)
Vibrio fluvialis		✓	–	1 (13%)	1 (5%)
Vibrio furnisii	✓		–	–	1 (5%)
Vibrio parahaemolyticus	✓	✓	1 (3%)	2 (25%)	14 (63%)
Vibrio spp.	✓		2 (6%)	–	1 (5%)

BCS, Baja California Sur.

the strains were not recognized by O antisera, while 82% (14/17) were not recognized by K antisera, and eight of these latter strains did not react to O:K antisera (OUT:KUT). Serogroups O1, O2, O4, O5, and O10 were identified. In BCS, one serotype OUT:KUT was isolated from black turtles. In Sinaloa, serotypes recognized were O1:KUT, O4:K10, O5:KUT, O10:KUT, OUT:KUT, O1:K32, O2:KUT, and O4:K13, isolated from olive ridleys and OUT:KUT, isolated from olive ridley and black turtles (**Table 2**).

Based on the presence or absence of virulence genes, the *V. parahaemolyticus* isolates were classified into three groups: pandemic (tdh^+, $toxRS/new^+$, and/or $orf8^+$), pathogenic (tdh^+ and/or trh^+), and non-pathogenic strains (tdh^- and trh^-). Among *V. parahaemolyticus* strains, two strains (11.7%) were identified as pandemic isolates. One of these strains belonged to serotype OUT:KUT and carried the *tdh*, *trh*, and t*oxRS/new* genes (isolated from black turtles in BCS), whereas one pandemic O4:K10 strain carried the *tdh* and *toxRS/new* genes (isolated from olive ridley turtles in Sinaloa). A total of 29.4% (5/17) of isolates carried the virulence *tdh* gene and therefore were considered pathogenic strains (**Table 2**). Approximately 58.8% (10/17) of *V. parahaemolyticus* isolates were non-pathogenic.

Serological characterization of the six *V. cholerae* strains isolated from olive ridley turtles in Sinaloa, revealed that all strains belonged to the non-O1 and non-O139 serogroup. PCR studies revealed that four of the six isolates of *V. cholerae* non-O1/non-O139 harbored the *ace* gene; however, all strains tested were negative for the *ctxA*, *ctxB*, and *zot* genes (**Table 2**).

Antibiotic Resistance Profiles

Antimicrobial susceptibility testing of *V. parahaemolyticus* isolated shown that all strains were resistant to ampicillin (94.1% resistant and 5.9% intermediate resistance), but >82% of isolates demonstrated susceptibility to tetracycline (88.2%), ceftazidime (82.3%) and chloramphenicol (88.2), and >64% to gentamicin and cefotaxime (**Table 3**). There were no strains resistant to ciprofloxacin, nalidixic acid, and sulfamethoxazole/trimethoprim (SXT) although inter-mediate zone sizes were observed in 23.5, 41.2, and 17.7% of the strains, respectively. Regarding overall antibiotic resistance, most *V. parahaemolyticus* strains (94.1%; 16/17) were non-susceptible to at least one antibiotic and 35.3% (6/17) of strains were resistant to two or more drugs (multidrug-resistant) (**Table 3**).

All strains of *V. cholerae* isolated were susceptible to ciprofloxacin, SXT, tetracycline, and chloramphenicol (**Table 3**). There were no strains resistant to nalidixic acid, ceftazidime, and cefotaxime although inter-mediate zone sizes were observed in 16.7% of the strains for each antibiotic. Low resistance (16.7%) was observed for gentamicin and ampicillin. In relation to antibiotic resistance, only the 33.4% (2/6) of strains were resistant to one antibiotic (**Table 3**).

On the other hand, almost all the *V. alginolyticus* strains (87.2%) exhibited resistance to ampicillin antibiotic, but low resistance was observed for tetracycline (7.7%), chloramphenicol (10.3%), nalidixic acid (5.1%), and to SXT (7.7%) (**Table 3**). There were no strains inter-mediate zone sizes observed to tetracycline and ampicillin. A high proportion of resistance and intermediate resistance were noticed among *V. alginolyticus* strains to gentamicin (82.1%), ciprofloxacin (76.9%), and cefotaxime (74.4%). Regarding overall antibiotic resistance, all *V. alginolyticus* strains were non-susceptible to at least one antibiotic and 69.2% (27/39) of strains were resistant to two or more drugs (multidrug-resistant). The resistance to 3–6 antibiotic was observed in 19 strains representing 48.7% (**Table 3**).

TABLE 2 | Serological and virulence gene characteristics of *Vibrio parahaemolyticus* and *Vibrio cholerae*.

Sea turtle species	*Vibrio parahaemolyticus*						Geographic zone
	Serovar	Total no. of isolates	Presence of each virulence gene				
			tdh	*trh*	*Orf-8*	*toxRS/new*	
C. mydas agassizii	OUT:KUT	1	+	+	−	+	BCS
		2	−	−	−	−	SIN
L. olivacea	O1:KUT	1	−	−	−	−	SIN
	O1:K32	1	−	−	−	−	
	O2:KUT	1	+	−	−	−	
	O4:K10	1	+	−	−	+	
	O4:K13	1	−	−	−	−	
	O5:KUT	1	−	−	−	−	
		1	+	−	−	−	
	O10:KUT	2	+	−	−	−	
	OUT:KUT	4	−	−	−	−	
		1	+	−	−	−	
	Vibrio cholerae						
	Serovar	Total no. of isolates	Presence of each virulence gene				
			ctxA	*ctxB*	*zot*	*ace*	
L. olivacea	non-O1/non-O139	2	−	−	−	−	SIN
		4	−	−	−	+	

+, Presence; −, absence.

Discussion

Sea turtles are long-distance, migratory animals and occupy niches in different marine environments and geographical regions throughout their different life cycle stages, usually ranging from pelagic environments, as hatchlings, to several coastal areas in their juvenile and adult stages. Due to migratory habits, sea turtles are susceptible to threats in both offshore and coastal environments (Bolten, 2003). Five of the world's seven sea turtle species occur along the Pacific coast of Mexico, making this region very important from a biological and socioeconomic point of view (Senko et al., 2009, 2011; Aguilar-Gonzalez et al., 2014). Black turtles that have grown large enough to reside in benthic environments have a nearly exclusive herbivorous diet consisting of selected macroalgae and sea grasses. They are found in the Mexican Pacific during all life history stages and the coastal waters of the eastern Pacific and Gulf of California provide important feeding and developmental habitats (Cliffton and Felger, 1982; Seminoff et al., 2002a,b; Senko et al., 2009, 2011; Aguilar-Gonzalez et al., 2014). Olive ridley turtles have a large range within the tropical and subtropical regions in the Pacific and Indian Oceans as well as the Southern Atlantic Ocean. This species spends most of its time within 15 km of shore, preferring shallow seas for feeding and sunbathing; however, this species is also observed in the open ocean (Eckert et al., 1999).

In Mexico, sea turtles have traditionally been an important resource for many coastal communities for centuries and have been used throughout the region for food, medicine, and decoration especially in BCS and SIN (Senko et al., 2009, 2011; Aguilar-Gonzalez et al., 2014). Coastal communities that consume sea turtles generally utilize the entire animal. While turtle meat is eaten directly (on the grill or stew), internal organs such as kidney and liver are used for soup. Oil is extracted from the fat as a cure for respiratory problems, especially in children, and eggs and blood are drunk raw as a remedy for anemia and asthma, and are valued as an aphrodisiac (Spotila, 2004; Delgado, 2005; Aguirre et al., 2006; Lohmann and Lohmann, 2006; Mancini and Koch, 2009).

The human impacts on the world's oceans have devastated populations, species, and ecosystems at a rapid scale (Aguirre et al., 2002; Aguirre and Lutz, 2004; Aguirre and Tabor, 2008). There are several zoonotic agents spilling over from terrestrial reservoirs to marine species and, on the other hand, zoonotic pathogens spill back to humans and domestic animals with severe consequences to wildlife health. Several bacterial species have been isolated from sea turtles, including *Salmonella*, *Mycobacterium*, *Vibrio*, and *E. coli*, which have been identified as potentially pathogenic to humans (Raidal et al., 1998; O'grady and Krause, 1999; Orós et al., 2005; Lu et al., 2006). In our study, a total of 82 bacterial isolates were identified including *C. freundii, E. coli, Edwarsiella spp., V. alginolyticus, V. cholerae, V. fluvialis, V. furnisii, V. parahaemolyticus,* and *Vibrio spp. Aeromonas* or *Plesiomonas*, *Morganella* or *Proteus*, *and Providencia*. All of these bacteria have been considered to be potentially pathogenic and opportunistic in sick sea turtles (Aguirre and Lutz, 2004; Orós et al., 2004, 2005). Besides, the predominant isolates from sea turtles were, *V. alginolyticus*, *V. parahaemolyticus*, *V. cholerae*, which are considered pathogenic to human health.

TABLE 3 | Antibiotic resistance among *Vibrio parahaemolyticus, Vibrio cholerae, and Vibrio alginolyticus* strains isolated from black turtles and olive ridley turtles.

Class and antimicrobial	*Vibrio parahaemolyticus* No. (%)				*Vibrio cholerae* non-O1/non-O139 No. (%)				*Vibrio alginolyticus* No. (%)			
	n = 17	[a]S	[b]I	[c]R	*n* = 6	[a]S	[b]I	[c]R	*n* = 39	[a]S	[b]I	[c]R
AMINOGLYCOSIDE												
Gentamicin		11 (64.7)	4 (23.5)	2 (11.8)		4 (66.6)	1 (16.7)	1 (16.7)		7 (17.9)	17 (43.6)	15 (38.5)
QUINOLONES AND FLUOROQUINOLONES												
Ciprofloxacin		13 (76.5)	4 (23.5)	0		6 (100)	0	0		9 (23.1)	23 (59)	7 (17.9)
Nalidixic acid		10 (58.8)	7 (41.2)	0		5 (83.3)	1 (16.7)	0		26 (66.7)	11 (28.2)	2 (5.1)
Sulfamethoxazole-trimethoprim		14 (82.3)	3 (17.7)	0		6 (100)	0	0		23 (59)	13 (33.3)	3 (7.7)
TETRACYCLINES												
Tetracycline		15 (88.2)	0	2 (11.8)		6 (100)	0	0		36 (92.3)	0	3 (7.7)
BETA LACTAMS												
Ampicillin		0	1 (5.9)	16 (94.1)		3 (50)	2 (33.3)	1 (16.7)		5 (12.8)	0	34 (87.2)
CEPHALOSPORINS												
Ceftazidime		14 (82.3)	2 (11.8)	1 (5.9)		5 (83.3)	1 (16.7)	0		16 (41)	14 (35.9)	9 (23.1)
Cefotaxime		11 (64.7)	2 (11.8)	4 (23.5)		5 (83.3)	1 (16.7)	0		10 (25.6)	9 (23.1)	20 (51.3)
PHENICOLS												
Chloramphenicol		15 (88.2)	0	2 (11.8)		6 (100)	0	0		31 (79.4)	4 (10.3)	4 (10.3)
NUMBER OF DRUGS RESISTANT TO:												
0	1 (5.9%)				4 (66.6%)				0			
1	10 (58.8%)				2 (33.4%)				12 (30.8%)			
2	4 (23.5%)				0				8 (20.5%)			
3	0				0				12 (30.8%)			
4	1 (5.9%)				0				4 (10.3%)			
5	1 (5.9%)				0				1 (2.5%)			
6	0				0				2 (5.1%)			

[a] *Susceptible,* [b] *Intermediate,* [c] *Resistant. Vibrio parahaemolyticus strains with resistance to 3, 6, 7, 8, and 9 drugs were not detected. Vibrio cholerae no-O1 strains with resistance to 2, 3, 4, 5, 6, 7, 8, and 9 drugs were not detected. Vibrio alginolyticus strains with resistance to 0, 7, 8, and 9 drugs were not detected.*

V. parahaemolyticus, are halophilic, Gram negative bacteria, that naturally inhabit marine and estuarine environments (Gutierrez West et al., 2013; Haley et al., 2014). The pathogen has emerged as a worldwide pandemic causing gastroenteritis related to consumption of sea products in recent years (Nair et al., 2007; Velazquez-Roman et al., 2014). Also, it has been demonstrated that the existence of the *tdh* and/or *trh* gene in a strain is associated with its ability to cause gastroenteritis (Nishibuchi and Kaper, 1995). Interestingly, in SIN, we have reported the presence of these bacteria in environmental samples and invertebrates (seawater, sediment, and shrimp) as well as fecal samples, identifying a high serodiversity and prevalence of pathogenic (tdh^+) and pandemic (O3: K6, tdh^+, and $toxRS/new^+$) strains (Velazquez-Roman et al., 2012; Hernández-Díaz et al., 2015). However, in this study, we identified a novel serovar (04:K13) and the serotype O4:K10 with pandemic features in a different area from our previous investigations conducted during 2004–2013 (Velazquez-Roman et al., 2012; Hernández-Díaz et al., 2015). These novel serovars were isolated from olive ridley turtles, suggesting that this species could contribute to *V. parahaemolyticus* clones migrations in several ecosystems.

Importantly, olive ridley turtles nest from BCS (Lopez-Castro et al., 2004) to Peru (Kelez et al., 2009) with nesting reported from July to March. The scientific communities are coming to the conclusion that ballast discharge, global trade, and climate change represent the major underlying mechanisms for the global spread of pandemic *V. parahaemolyticus*, particularly clone O3:K6 (Velazquez-Roman et al., 2014). This spreading of pandemic *V. parahaemolyticus* is still a speculative question that requires further investigation since the pandemic and pathogenic strains can potentially migrate through sea turtles from Mexico to Peru or others countries including the United States. Due to the wide geographic distribution that sea turtles cover during their life cycle, they can serve as meaningful "sentinels" for overall ecosystem health (Aguirre and Lutz, 2004), and because of this, it is especially important to document and understand any factors that might affect the dissemination of pathogens.

To date, ca. 200 serogroups of *V. cholerae* have been recorded, and two (O1 and O139) have been associated with major cholera epidemics. The other serogroups, referred as non-O1/non-O139, have not been associated with epidemics but rather can cause sporadic diarrhea and occasional outbreaks (Chatterjee et al., 2009). In this study, *V. cholerae* was detected in six (9%) specimens. All isolates were non-O:1/non-O:139 serotypes and negative for *ctxA*, *ctxB*, and *zot* gene, but positive for *ace* gene by PCR. In China, turtles and their breeding environment have been reported major reservoirs of *V. cholerae* and responsible for many cholera outbreaks (Liu et al., 2006; Lu et al., 2006;

Chang et al., 2007). In Zhejiang Province, the incidence of O1 serogroup of *V. cholerae* was found to be high (9%) in turtles and cholera epidemics in this region are associated with consumption of infected turtles (Lu et al., 2006). Identification of the non-O1/non-O139 serogroups of *V. cholerae* carrying virulence genes (*ace* gene) in olive ridley turtles is very important since these new toxigenic strains with epidemic potential may emerge in the future if illegal consumption continues.

V. alginolyticus is pathogenic to a wide range of marine life including fish, mollusks, crustaceans, cnidarians, and sea turtles (Orós et al., 2004, 2005). In our study, *V. alginolyticus* were the predominant isolates from sea turtles and has been associated as an opportunistic pathogen for sea turtles in exudative bronchopneumonia and/or granulomatous pneumonia, traumatic skin lesions, granulomatous nephritis, renal abscesses, and necrotizing and/or granulomatous hepatitis and have been considered important causes of mortality among sea turtles (Orós et al., 2004, 2005). To the best of our knowledge, the present study is the first record of especially of *V. parahaemolyticus, V. cholerae*, and *V. alginolyticus* in black and olive ridley turtles in BCS and SIN, Mexico.

Another important contribution of this study was the investigation of susceptibility, or resistance of the isolated *V. parahaemolyticus*, *V. cholerae*, and *V. alginolyticus* strains to first-line antibiotics utilized in the region. All *V. parahaemolyticus* isolates were resistant to ampicillin, which was not a surprise as non-susceptibility to ampicillin is very common in *V. parahaemolyticus* strains isolated from environmental and clinical samples (Okuda et al., 1997; Wong et al., 2000; Roque et al., 2001; Sun et al., 2013; Letchumanan et al., 2015). We detected 23.5% of resistance to cefotaxime in *V. parahaemolyticus* from sea turtles, a similar prevalence of resistance to cefotaxime (20%) has also been reported in strains isolated in Italy from shellfish and clinical samples (Ottaviani et al., 2013). A number of studies have been reported low (Ceccarelli et al., 2015) and high (Jagadeeshan et al., 2009) prevalence of *V. cholerae* non-O1/non-O139 strains resistant to numerous antibiotics, isolated from environmental samples. In this study, only a few of the *non-O1/non-O139 V. cholerae* isolates from sea turtles were resistant to ampicillin or gentamicin. Most of the isolates were sensitive to all antibiotics tested.

All *V. alginolyticus* strains isolated in this study, were resistant to at least one antibiotic. Several studies reported a wide range of resistance for *V. alginolyticus* (Snoussi et al., 2008; Scarano et al., 2014). Although a high proportion of resistance and intermediate resistance were noticed to gentamicin (82.1%), ciprofloxacin (76.9%), and cefotaxime (74.4%). These results are in accordance with other studies which found high rates of resistance to gentamicin, ciprofloxacin, and cefotaxime (Snoussi et al., 2008; Lajnef et al., 2012).

Despite a federal ban on turtle hunting, consumption and trade in Mexico since 1990 (Gardner and Nichols, 2001; Senko et al., 2009; Aguilar-Gonzalez et al., 2014), sea turtles are captured by bycatch or incidentally mostly in the summer, precisely when consumption presents the greatest potential hazards to human health (Senko et al., 2009; Aguilar-Gonzalez et al., 2014). And when consumed, may have adverse human health effects, such as extreme dehydration, vomiting, diarrhea, and even death, due to the presence of bacteria, parasites, and environmental contaminants found in these animals (Aguirre et al., 2006; Senko et al., 2009; Aguilar-Gonzalez et al., 2014).

In conclusion, although fishermen from northwestern Mexico recognize that sea turtles might be contaminated and infected with potential pathogens, and that eating them could cause health problems (Aguirre et al., 2006; Senko et al., 2009; Aguilar-Gonzalez et al., 2014), they and their families continue consumption. In this study, we found the presence of several potential toxigenic and drug-resistant or multi-drug resistance human pathogens in sea turtles. Therefore, this information is important on possible health risks for humans in hope of behavioral changes that could benefit sea turtle conservation.

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REVIEW
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Strategies of *Vibrio parahaemolyticus* to acquire nutritional iron during host colonization

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Iron is an essential element for the growth and development of virtually all living organisms. As iron acquisition is critical for the pathogenesis, a host defense strategy during infection is to sequester iron to restrict the growth of invading pathogens. To counteract this strategy, bacteria such as *Vibrio parahaemolyticus* have adapted to such an environment by developing mechanisms to obtain iron from human hosts. This review focuses on the multiple strategies employed by *V. parahaemolyticus* to obtain nutritional iron from host sources. In these strategies are included the use of siderophores and xenosiderophores, proteases and iron-protein receptor. The host sources used by *V. parahaemolyticus* are the iron-containing proteins transferrin, hemoglobin, and hemin. The implications of iron acquisition systems in the virulence of *V. parahaemolyticus* are also discussed.

Keywords: *Vibrio parahaemolyticus*, iron, virulence, host iron proteins, mechanism of acquisition

Introduction

Vibrio parahaemolyticus is a halophilic Gram-negative bacterium that naturally inhabits marine and estuarine environments (Gavilan et al., 2013). The more recent distribution of this species has been driven by climatic conditions and the eutrophication of regional waters throughout the world (Baumann et al., 1984). *V. parahaemolyticus* can survive in a wide variety of niches in a free-swimming state, and its motility is conferred by a single polar flagellum. Alternatively, this bacterium can be found in a sessile state, attached to inert or animate surfaces, such as suspended particulates, zooplankton, fish, and shell-fish (McCarter, 1999). Many strains of *V. parahaemolyticus* are strictly environmental, though small subpopulations can be opportunistic pathogens that may cause gastroenteritis, wound infection, and septicemia (Joseph et al., 1982; McCarter, 1999). As with all bacteria, *V. parahaemolyticus* reproduces via binary fission without a systematic exchange of genes with other individuals of the same species, leading to essentially clonal reproduction (Garcia et al., 2013); however, only some of these clones cause diarrhea in humans. Strains of *V. parahaemolyticus* belong to different serogroups and can produce a number of different lipopolysaccharide (O) and capsular (K) antigens, constituting the primary basis for strain classification (Nair et al., 2007; Garcia et al., 2013).

Since its discovery in 1950 by Tsunesaburo Fujino of Osaka University after an outbreak due to the ingestion of contaminated seafood, *V. parahaemolyticus* has been recognized as a leading cause of intestinal infection throughout coastal countries worldwide (Fujino et al., 1953). Following the consumption of raw or undercooked seafood, individuals infected with virulent strains may present clinical symptoms that include diarrhea, vomiting, nausea, abdominal cramping, and low-grade fever. Exposure to *V. parahaemolyticus* can also lead to wound infection and septicemia in certain medical conditions, such as immune deficiency (Su and Liu, 2007).

The infections and outbreaks caused by *V. parahaemolyticus* prior to 1996 were geographically isolated and associated with a diversity of serotypes (Wong et al., 2000; Chowdhury et al., 2004). However, the later increase in outbreaks was linked to the occurrence of gastroenteritis throughout Asia associated with a new and unique clone of serotype O3:K6 that emerged in Kolkata, India, in 1996 (Matsumoto et al., 2000; Ansede-Bermejo et al., 2010; Pazhani et al., 2014). This clone rapidly spread throughout the majority of Southeast Asian countries in the same year (Okuda et al., 1997b; Ansede-Bermejo et al., 2010; Gavilan et al., 2013). This clone, referred to as the pandemic clone, has since spread globally from Southeast Asia to Europe, Africa, and the Americas (Okuda et al., 1997a; Nair et al., 2007; Paranjpye et al., 2012; Yano et al., 2014). In particular, some countries on the American continent have reported cases of gastroenteritis due to the pandemic O3:K6 strain and its serovariants; the pandemic strain was first detected in Peru, subsequently spread to Chile in 1998, to the U. S. in the same year, to Brazil in 2001 and to Mexico in 2004 (Velazquez-Roman et al., 2012, 2013). Based on various molecular methods, these widespread pandemic O3:K6 strains have been found to be genetically closely related and appear to constitute a clone that differs significantly from the non-pandemic O3:K6 strains that were isolated prior to 1996 (Okuda et al., 1997b; Wong et al., 2000; Lan et al., 2009), with several serovariants apparently emerging since 1996 (Nair et al., 2007; Velazquez-Roman et al., 2012, 2013; Al-Othrubi et al., 2014; Pazhani et al., 2014; Letchumanan et al., 2015). The global occurrence of *V. parahaemolyticus* strains emphasizes the importance of understanding their many virulence factors as well as the mechanisms used to acquire nutrients from the environment and the effects on human hosts.

Vibrio parahaemolyticus possesses a wide variety of virulence factors that cause damage such as adhesins, toxins, and secreted effectors involved in attachment, cytotoxicity, and enterotoxicity (Broberg et al., 2011; Zhang and Orth, 2013). The two main factors are a thermostable direct hemolysin (TDH) and a thermostable direct hemolysin-related hemolysin (TRH) encoded by the *tdh* and *trh* genes, respectively (Honda et al., 1982; Nishibuchi et al., 1992; Vongxay et al., 2008). Nonetheless, the isolation of *V. parahaemolyticus* strains lacking functional *tdh* and *trh* genes from human infection cases and the analysis of the genome sequence of *V. parahaemolyticus* strain RIMD2210633 suggest that other virulence factors also play a role in the disease caused by this bacterium (Makino et al., 2003; Okada et al., 2009). The genome of *V. parahaemolyticus* contains two sets of type III secretion system (T3SS) gene clusters that function in the secretion and translocation of virulence factors into eukaryotic cells. These appear on each of the two chromosomes (Makino et al., 2003; Caburlotto et al., 2009). T3SSs utilize a needle-like apparatus to translocate into host cells effectors that target and hijack multiple eukaryotic signaling pathways. Indeed, T3SSs are essential virulence machines used by numerous bacterial pathogens, including *Yersinia, Salmonella, Shigella*, and pathogenic *Escherichia coli* (Makino et al., 2003).

Other systems or mechanisms that play an important role as virulence factors in *Vibrio* and all pathogenic microorganisms are those that confer the ability to acquire nutrients from the environment in which they live. These systems ensure that pathogens successfully reproduce and become established in a host. For example, the capacity to acquire nutrients such as iron (Fe) from a host is an ability obtained by pathogenic microorganisms during evolution. In fact, it has been speculated that the evolutionary pressure for microbes to develop pathogenic characteristics was to access the nutrient resources supplied by animals (Tanabe et al., 2012). The environment inside the colonized host has led to the evolution of new bacterial characteristics to maximize such new nutritional opportunities (Rohmer et al., 2011; Tanabe et al., 2012).

Currently, access to host nutrients is regarded as a fundamental aspect of an infectious disease. During the invasion of the human host, pathogens encounter complex nutritional microenvironments that could change, for example; the increase in inflammatory response due to the infection, local hypoxia in some tissues (Payne, 1993; Cassat and Skaar, 2013). The host can limit microbial access to nutrient supplies as a defense mechanism against the pathogens, however, the pathogens can counteract this by developing metabolic adaptations or improved mechanisms of nutrient acquisition to successfully exploit available host nutrients for their proliferation (Payne, 1993). Recent studies have pointed out an emerging paradigm that has been designated as 'nutritional virulence' (Abu Kwaik and Bumann, 2013). Although this term is applied to the acquisition of amino acids and carbon sources, certain nutritional ions or metals that are essential for cellular growth and other metabolic processes could be considered as part of this paradigm. As one of the most fundamental aspects of infectious diseases is the microbial acquisition of nutrients *in vivo*, which positively impacts in virulence as well as antibiotic resistance (Santic and Abu Kwaik, 2013), we suggest that the process of iron acquisition systems used by pathogenic microorganisms may be considered in the concept of 'nutritional virulence.'

Iron (Fe) is an essential element for almost all cells, including most bacteria because it serves as a cofactor for metabolic processes, such as redox reactions, nucleic acid synthesis, and electron transfer (Tanabe et al., 2012). Iron is the fourth most abundant element on the Earth's crust. In nature, there are two states of iron: (1) ferrous iron (Fe^{2+}), which is toxic because it leads to the production of hazardous reactive oxygen species (ROS), including superoxide, in the presence of oxygen; and (2) ferric iron (Fe^{3+}), which is insoluble under normal physiological conditions. Fe is bound to ligands, primarily proteins, in

iron-dependent organisms, and trace Fe concentrations are necessary for all organisms, ranging between 0.4 and 4 μM in the majority of both eukaryotic and prokaryotic cells (Weinberg, 1974). However, there are bacteria such as some lactobacilli that are iron independent because they utilize manganese (Mn) and other cations as cofactors in their enzymes (Imbert and Blondeau, 1998).

The pathogenic microorganisms that infect mammalian hosts encounter diverse and changing environments. For example; the pH within the human body is usually neutral (7.4), but it can range from 1.0 in the stomach to 8.0 in urine. Also, if they move deeper into host tissues at mucosal surfaces, such as those from the lumen, the multilamellar mucus, and the epithelial cells of the stomach, pathogens confront drastically different or hostile environments. Some mucosal surfaces are well oxygenated, but others possess areas of low oxygen tension, for example the oral cavity, large intestine, female genital tract, abscesses and damaged tissues (Rohmer et al., 2011). The level of free Fe in mammalian bodily fluids is variable ($\sim 10^{-18}$ M) but always far below the concentrations required for optimal bacterial growth (10^{-6} M), causing bacteria to rely on their own strategies or mechanisms for obtaining this metal. In an infected site there are numerous physiologically specialized environments that bacteria might encounter or colonize. For example, within the small intestine, there are variable conditions different from those found between caecum and colon (Rohmer et al., 2011). For all these reasons mentioned above, pathogens move through multiple diverse environments throughout their life cycle, and to accomplish this they require the regulation, coordination, and utilization of multiple bacterial metabolic pathways. Bacteria often use metabolic cues in order to regulate their metabolism and virulence functions to be successful as pathogens (Rohmer et al., 2011). Because they depend upon Fe as a vital cofactor that enables a wide range of key metabolic activities, bacteria must therefore ensure a balanced supply of this essential metal; accordingly, they invest considerable resources into its acquisition and employ elaborate control mechanisms to alleviate both iron-induced toxicity and Fe deficiency (Rangel et al., 2008).

The Fe concentration in coastal waters ranges from 1.3–35.9 nM up to 23.1–573.2 nM (Gledhill and Buck, 2012), a concentration that may be sufficient to support the growth of *V. parahaemolyticus*; however, *V. parahaemolyticus* also infects humans. The human body contains 3.8 g (in men) and 2.3 g (in women) of Fe. 20 mg of Fe is required daily for the production of hemoglobin (Hb) for new erythrocytes in order to preserve Fe homeostasis. Iron absorption from the diet, however, supplies only 1–2 mg daily, and the remaining Fe is derived by recycling Fe from senescent red blood cells (Ganz and Nemeth, 2006). Most bodily Fe is found in heme proteins (Hb, myoglobin, cytochromes, and multiple enzymes), and the second largest Fe pool is found in ferritin (also in hemosiderin). The remaining Fe is found in other proteins, such as iron-sulfur cluster enzymes, Fe-chelating proteins (Tf and Lf), and a pool of accessible Fe ions called the labile Fe pool (LIP), all of them constitutes the iron-containing proteins involved in metabolic pathways from hosts.

Inside the human body the solubility of iron is extremely low, because the Fe exists in insoluble mineral complexes, or under aerobic, aqueous, and neutral pH conditions, that difficult the access of bacteria to this element. Besides, Fe is bound to mammalian high-affinity iron-binding proteins such as Tf, Lf, and Ft and in consequence, many bacteria have developed high-affinity Fe transport systems to acquire Fe from sources in their niches (Rangel et al., 2008; Jin et al., 2009; Tanabe et al., 2012). The Fe sources available in the different environmental niches of *V. parahaemolyticus* are described and discussed in **Figure 1**.

Role of Iron in the Virulence of *Vibrio parahaemolyticus*

Iron regulates virulence factors of *V. parahaemolyticus* and almost all pathogenic bacteria. Inside a host the Fe concentration is very low, so many pathogens uses this (low-iron conditions) for inducing expression of genes involved in the virulence (Litwin and Calderwood, 1993). The presence of ferric Fe in bacterial growth media has been found to increase the adherence intensities of virulent *V. parahaemolyticus* strains to human fetal intestinal (HFI) cells *in vitro* (Hackney et al., 1980). Intraperitoneally injection with *V. parahaemolyticus* in the presence of ferric ammonium citrate in mice increased the bacterial proliferation, thus enhancing the lethality toward infected mice. *V. parahaemolyticus* cultures in low-iron conditions showed better proliferation than iron-rich cultures in response to the addition of supplementary Fe. Also, the production of thermostable direct toxin (TDH) by the hemolytic strains of *V. parahaemolyticus* was higher in iron-limited cultures than in iron-rich cultures, though the production of TDH by both iron-limited and iron-rich cultures was inhibited by the addition of Fe. In conclusion, the enhancement of *V. parahaemolyticus* virulence in the model mice likely occurred through the increase of bacterial proliferation *in vivo* and not the stimulation of TDH production. The *V. parahaemolyticus* precultured under iron-limited conditions may be more adaptable to the *in vivo* environment (Wong and Lee, 1994; Funahashi et al., 2003; Gode-Potratz et al., 2010).

The effect of lysed blood on the virulence of *V. parahaemolyticus* in mice was also investigated, and a factor released by erythrocyte lysis was found to greatly reduce the 50% lethal dose of *V. parahaemolyticus* in mice. Similar effects were observed with ferric ammonium citrate and Mn sulfate. Authors conclude that Fe from the lysed blood is involved in the virulence of *V. parahaemolyticus* (Karunasagar et al., 1984).

In a recent work, Gode-Potratz et al. (2010) demonstrated that metal ions play distinct roles in modulating gene expression and behavior in *V. parahaemolyticus*. In this work, high-calcium and low-iron growth conditions stimulated the induction of swarming and T3SS regulons from *V. parahaemolyticus* (Gode-Potratz et al., 2010). Swarming is a particular adaptation of many bacteria to grow in surfaces. In *V. parahaemolyticus* swarming is done by lateral flagella that enable the bacteria move over and colonize surfaces (Gode-Potratz et al., 2010). The authors concluded that swarming plays a signaling role with global

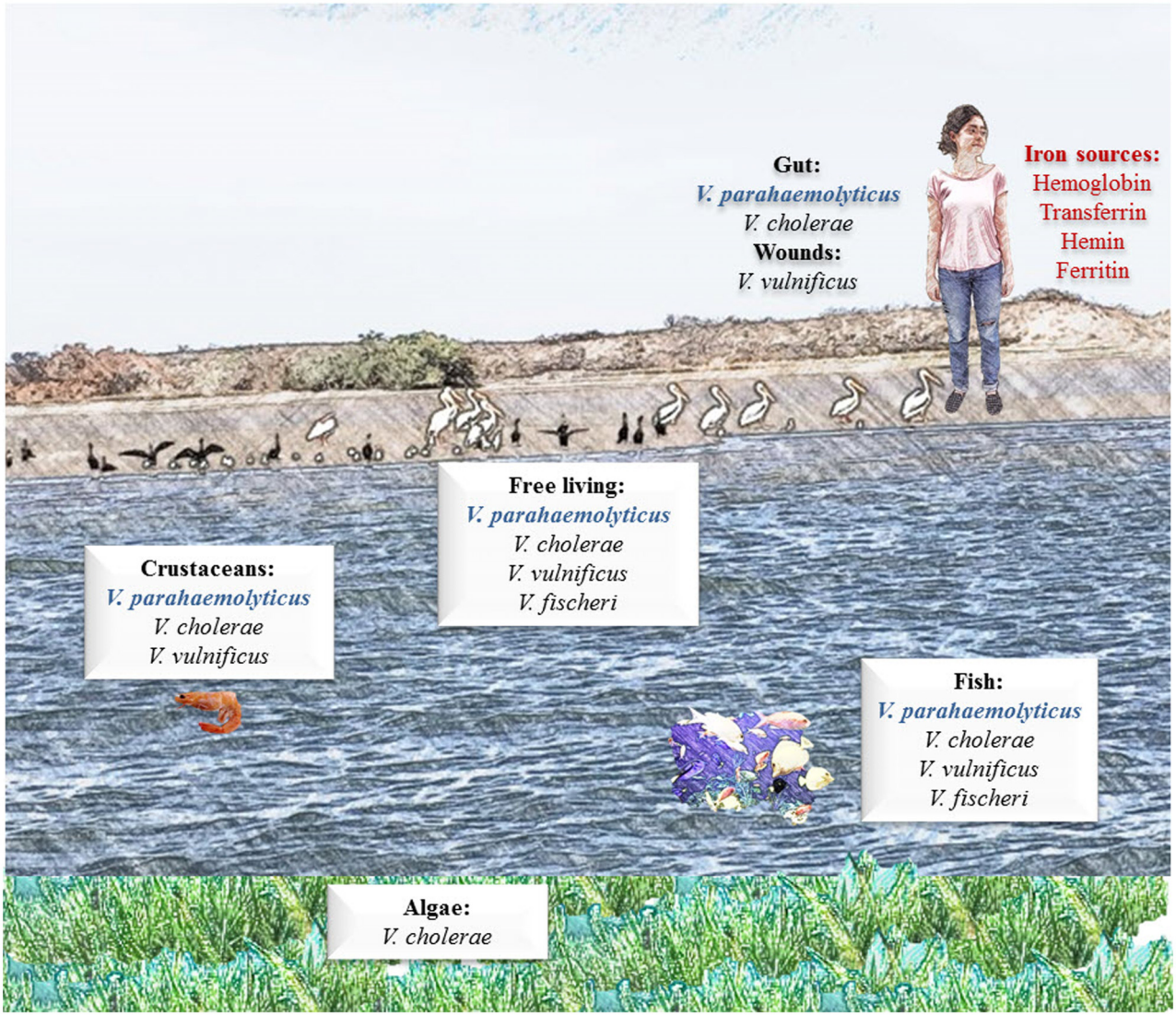

FIGURE 1 | Iron sources available in the environmental niches of *Vibrio parahaemolyticus*. *V. parahaemolyticus* is an obligate halophilic organism, meaning that it requires salt to live. This organism is naturally occurring and found worldwide. It can commonly be found free swimming or attached to underwater surfaces and is found at high concentrations in areas of significant seafood consumption. Humans can acquire *V. parahaemolyticus* infection from infected seafood; once infected, established *V. parahaemolyticus* can acquire iron from different iron-containing proteins, such as hemoglobin (Hb) and transferrin (Tf).

consequences on the regulation of gene sets that are relevant for surface colonization and infection and that stimulation depends on the level of Fe present in the environment (Gode-Potratz et al., 2010).

Iron also regulates virulence factors in other members of the genus *Vibrio*. Based on the description of the virulence-enhancing effect of ferric ammonium citrate on *V. cholerae* by Joo (1975), other work has supported the role and importance of Fe in the virulence of *Vibrio* sp. (Joo, 1975). It has been demonstrated that the iron overload in humans increases the virulence of pathogens *in vivo*. For example in patients with liver diseases and hemochromatosis, iron overload states are common. It is known that these conditions predispose patients to recurrent infections, septicemia, and high mortality due to pathogens such as *V. vulnificus* (Bullen et al., 1991; Barton and Acton, 2009). This was corroborated by injecting mice with *V. vulnificus* and Fe, resulting in a lower 50% lethal dose and in a reduction in the time to death post-infection (Wright et al., 1981). In addition, elevated serum Fe levels were also produced by liver damage due to injections. Because the infections with *V. vulnificus* are acquired through the consumption of contaminated seafood, the role of iron on infections acquired by the oral route was also studied in mice. On the other hand, the role of iron on the growth of *V. vulnificus* in human and rabbit sera with injections of Fe or Tf were also studied. In the results, lethality and growth of *V. vulnificus* were more efficient in rabbit and

human sera; respectively (Wright et al., 1981). Next, an induced peritonitis model was employed in mice to determine whether heme-containing molecules enhance the lethality of infections of *V. vulnificus* (Helms et al., 1984). In this model, the lethality toward mice inoculated intraperitonally with the bacteria and treated with methemoglobin, or hematin but not by myoglobin, was increased compared with those untreated (Helms et al., 1984). These results indicated that *V. vulnificus* has the capacity to produce fatal human infections because of its ability to use human proteins that bind Fe (Helms et al., 1984). Additionally, *V. vulnificus* strains isolated from different sources of Cuddalore coastal waters were tested for their virulence activity based on their LD50 values in mice. The LD50 was in the range of 10^4–10^7 cells in normal mice, but 10^1–10^2 cells in iron-injected mice, thus reinforcing the idea that Fe (acquired from human sources *in vivo*) may play a major role in the pathogenesis of *V. vulnificus* (Jayalakshmi and Venugopalan, 1992). Additionally, the virulence mechanisms of *V. vulnificus* biotype 1 and biotype 2 were studied and compared in mice. Both strains presented several properties in common, including capsule expression, the uptake of various Fe sources, and the production of exoproteins (Wright et al., 1981; Amaro et al., 1994). Taken together, data support the importance of iron in the pathogenesis of *Vibrio* sp. and *V. parahaemoluticus in vivo* and *in vitro*.

Iron regulatory Proteins and Mechanisms in *V. parahaemolyticus*

The study of Fe acquisition systems in *E. coli* led to the discovery of Fur, a DNA binding protein of 16.8 kDa, product of the *fur* (ferric uptake regulation) gene, that represses the transcription of genes involved in Fe uptake systems in iron replete conditions (Litwin and Calderwood, 1993). When the intracellular Fe concentration increases, Fur forms a dimer together with ferrous Fe (Fe^{2+}) and binds to a consensus sequence (Fur-box), which overlaps the promoters of Fur-target genes, resulting in the inhibition of transcription. Although the role of Fur as a repressor is well-documented, emerging evidence demonstrates that Fur can function as an activator (Troxell and Hassan, 2013). Additionally to *E. coli*, Fur has been identified in other Gram-negative and Gram-positive bacteria. An interesting finding was that Fur also participates in functions different to the Fe metabolism for example; defense against oxygen radicals, metabolic pathways, bioluminescence, chemotaxis, swarming and production of toxins, and other virulence factors (Litwin and Calderwood, 1993). We can speculate about the importance of these Fe dependent mechanisms for bacterial virulence inside a host *in vivo*.

Vibrio parahaemolyticus contains a Fur protein that is 81% identical with the Fur protein from *E. coli* and over 90% identical with those of the *Vibrio* sp. (Yamamoto et al., 1997). Funahashi et al. (2002) reported that *V. parahaemo*lyticus *psuA* and *pvuA* genes (which encode the TonB-dependent outer membrane receptors for a putative ferric siderophore and ferric-vibrioferrin), are regulated by Fur (Funahashi et al., 2002). Additionally, a homolog of the *iutA* gene in *V. parahaemolyticus* (which encodes for the receptor of ferric aerobactin) is apparently regulated by Fur (Funahashi et al., 2003). Furthermore, Fur regulates *V. parahaemolyticus peuA* gene (which encodes for an alternative ferric-enterobactin receptor; Tanabe et al., 2014).

Fur has been involved in the regulation of other virulence factors from *Vibrio* sp. Lee et al. (2013) demonstrated that in *V. vulnificus* Fur regulates hemolysin production at the transcriptional (vvhBA operon) and post-translational (by regulating the expression of two VvhA-degrading exoproteases, VvpE, and VvpM) levels (Lee et al., 2013). In contrast, other transcriptional regulators such as AraC-type family members and LysR-type family members, have been shown to activate transcription initiation of genes involved in the synthesis and utilization of siderophores in bacteria (Balado et al., 2009; Tanabe et al., 2012). In *V. cholerae* Fur regulates the expression of a number of genes in response to changes in the level of available iron. Fur usually acts as a repressor, but it has been shown that Fur positively regulates the expression of ompT, which encodes a major outer membrane porin, involved in the virulence of *V. cholerae* (Craig et al., 2011). It has been reported that Fur also represses the synthesis of RyhB, which negatively regulates genes for iron-containing proteins involved in the tricarboxylic acid cycle and respiration as well as genes for motility and chemotaxis (Wyckoff et al., 2007). Mey et al. (2005) reported the effects of iron and Fur on gene expression in *V. cholerae*. According with this work, nearly all of the known iron acquisition genes were repressed by Fur under iron-replete conditions, and also those genes involved in the transport of iron inside of pathogens (Mey et al., 2005). The iron transport systems regulated negatively by Fur in iron-replete conditions were *feo* and *fbp* genes (involved in the transport of ferrous and ferric iron inside cells; respectively). Both were found to be negatively regulated by iron and Fur (Mey et al., 2005). This is consistent with others genes involved in iron acquisition; in high concentrations of this nutrient the genes for iron acquisition systems are repressed.

Iron acquisition Systems Used by Pathogenic Microorganisms

An obligate question is how does *V. parahaemolyticus* acquire iron? This theme is complex; however, it has been well established in other pathogens. To acquire Fe from host sources, microorganisms generally use the iron-acquisition systems described below.

Receptors for Host Iron-Containing Proteins

Transferrins (Tfs) are a family of iron-binding glycoproteins that chelate free ferric Fe in biological fluids (Crichton and Charloteaux-Wauters, 1987). Bacteria such as *Neisseria gonorrhoeae* and *Haemophilus influenzae* are able to use the Fe in these proteins by binding Tf and Lf iron directly through the use of membrane receptors or binding proteins for host iron-glycoproteins (Tbp and Lbp, respectively). These receptors have been reported to be induced in some bacteria in the absence of Fe (Gray-Owen and Schryvers, 1996). Some receptors are specific for one iron-containing protein. On the contrary, other receptors

that recognizes Tf, also can recognize Lf or other iron proteins, these receptors have been found in *Neisseria* (Morgenthau et al., 2013). Tf binding protein (TbpA) and lactoferrin binding protein (LbpA) are receptors that share 25% amino acid identity and a high degree of similarity. These Tf and Lf receptors can be promiscuous; for example, TbpA can recognizes Lf in some bacteria. Moreover, Tbps also exhibit low homology to other transport proteins and siderophore receptors. The homology among the members of this family of transporters suggests that the ancestral meningococcal Tf and Lf receptors may have been a single-unit transporter, similar to siderophore receptors (Perkins-Balding et al., 2004). The best characterized Tfbps and Lfbps to date belong to *Neisseria* species (Schryvers et al., 1998; Schryvers and Stojiljkovic, 1999).

Production and Secretion of Siderophores/Hemophores

Siderophores and hemophores are relatively small (<1 kDa) compounds produced and secreted by some species of bacteria and fungi to acquire Fe. Once produced and secreted from microorganisms, their function is to chelate ferric Fe with very high affinity (formation constant up to 10^{50} M; Wandersman and Delepelaire, 2004; Saha et al., 2013). In fact, these compounds can remove ferric Fe from the host proteins Tf, Lf, and ferritin. Siderophores are produced as common products of microbial metabolism under Fe stress conditions and facilitate the solubilization of ferric Fe and transport Fe via a specific receptor expressed in the cell plasma membrane. In addition, *Bacillus subtilis* and *Mycobacterium smegmatis* that are unable to synthesize siderophores resolve this problem by using the Fe from siderophores derived from other microorganisms (xenosiderophores; Schumann and Mollmann, 2001; Miethke et al., 2013). The capacity of these systems to acquire Fe from various environmental or biological sources is an evident advantage for organisms that may exist in several niches. Hemophores, which are less well studied and only found thus far in Gram-negative bacteria, are employed via the following strategy: they are secreted into the extracellular medium where they scavenge heme from various hemoproteins due to their higher affinity for this compound and then return the heme to hemophore-specific outer membrane receptors (Wandersman and Delepelaire, 2012). These proteins are secreted under conditions of iron depletion (Faraldo-Gomez and Sansom, 2003; Wandersman and Delepelaire, 2012).

Ferrireductases/Proteases

Some microorganisms secrete ferrireductases or produce membrane-associated proteins that reduce the ferric iron in holoTf, holoLf, or ferritin to the more accessible ferrous form. The reduction of ferric iron destabilizes the host iron-containing protein, and the ferrous iron is thus released. It has been reported that some pathogens are able to produce and secrete proteases that cleave host iron-containing proteins, and iron is easily acquired by pathogens in this form, for example, *Entamoeba histoytica* has hemoglobinases (Payne, 1993; Serrano-Luna et al., 1998; Ortiz-Estrada et al., 2012). A scheme for Fe acquisition systems in bacteria is represented in **Figure 2**.

Human Iron Sources and Iron Acquisition Systems in *V. parahaemolyticus*

Vibrio parahaemolyticus and other bacteria from the genus *Vibrio* including *V. cholerae, V. vulnificus*, require Fe for their

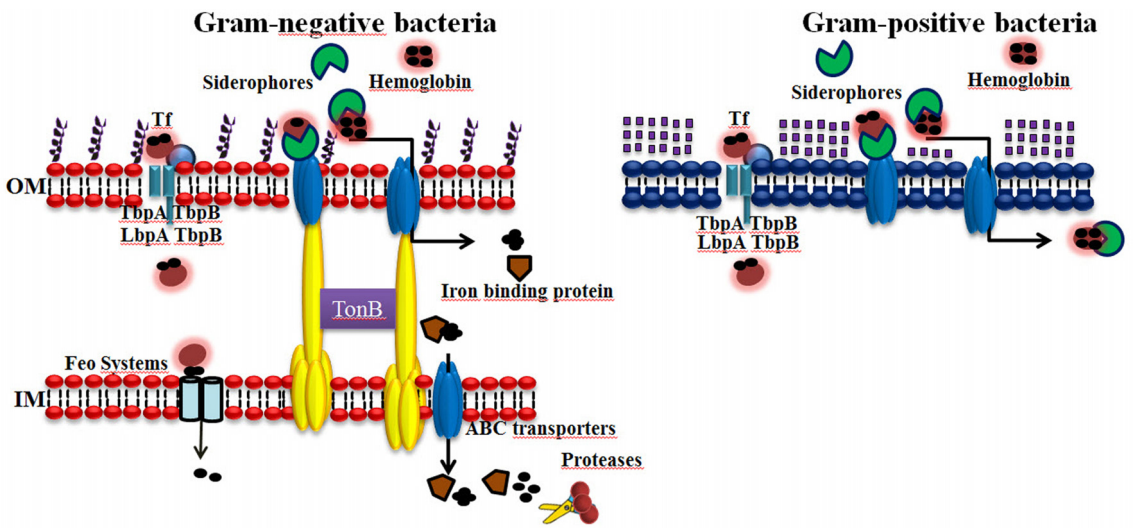

FIGURE 2 | Iron acquisition systems used by bacteria that infect humans. Iron (Fe) is an essential element for virtually all forms of cellular life, including most bacteria, because it serves as a cofactor for several key enzymes required for many metabolic processes. Gram-negative and Gram-positive bacteria can acquire iron using elaborated mechanisms. (1) Receptors for host iron-containing proteins. (2) The production and secretion of siderophores/hemophores. (3) Ferrireductases/Proteases. The abilities of bacterial pathogens to adapt to the environment within a host are essential to their virulence.

growth and have developed systems during evolution to acquire Fe for sustaining metabolism and replication. As mentioned above, the Fe concentration in coastal waters ranges from 1.3–35.9 to 23.1–573.2 nM, which is sufficient for the growth of *V. parahaemolyticus*. In the human host, *V. parahemolyticus* can utilize host sources, such as the iron-proteins Hb, heme, hemin, and Tf (Yamamoto et al., 1994), and possibly others, such as Lf and Ft (**Figure 1**).

Siderophores as a Mechanism of Iron Acquisition Used by *V. parahaemolyticus*

One of the strategies of *V. parahaemolyticus* to obtain the iron from the host iron-proteins is the use of siderophores. Under iron-limited conditions *V. parahaemolyticus* secretes Vibrioferrin to facilitate Fe acquisition. Fe-charged Vibrioferrin is recognized by an outer-membrane receptor composed of the proteins PvuA1 and PvuA2, which also recognizes heme and Hb. This receptor is coupled with the ABC transport system PvuBCDE which is located in the inner membrane and whose function is import the ferric-charged vibrioferrin to the inner membrane (Tanabe et al., 2003, 2011, 2012). *V. parahaemolyticus* also contains the TonB system which consists of three proteins designated TonB1, TonB2, TonB3 (Kuehl and Crosa, 2010). The energy for the transport of ferric-vibrioferrin is provided by the TonB2 system for PvuA1 and both the TonB1 and TonB2 systems for PvuA2 (Tanabe et al., 2011, 2012).

The *V. parahaemolyticus* also is able to utilize siderophores produced by other bacteria, for example, exogenous aerobactin, desferri-ferrichrome, and enterobactin (Funahashi et al., 2003, 2009; Tanabe et al., 2012). We have compared and analyzed *in silico* the mechanism used by *V. parahaemolyticus* with *V. cholerae*; the most studied member of the genus *Vibrio*. *V. cholerae* has multiple strategies for iron acquisition, including the endogenous siderophore vibriobactin and several siderophores that are produced by other microorganisms (Wyckoff et al., 2007). In general the *Vibrio* species, including *V. cholerae* and *V. anguillarum* can use catecholate-type siderophores as their cognate siderophores (Griffiths et al., 1984; Actis et al., 1986; Balado et al., 2009; Tanabe et al., 2012). Furthermore, some *Vibrio* sp. use the xenosiderophore enterobactin (Ent) that is produced mainly by members of the *Enterobacteriaceae* family (Griffiths et al., 1984; Naka and Crosa, 2012; Tanabe et al., 2012). On the other hand, Tan et al. (2014) described the use of the siderophore vulnibactin, essential in Fe uptake from host proteins. The importance of the vulnibactin in *V. vulnificus* pathogenicity was clinically demonstrated (Ceccarelli et al., 2013; Tan et al., 2014).

Once the iron has been acquired, *V. cholerae* has two systems for the transport of free iron: the Feo system, which transports ferrous iron, and the Fbp system, which transports ferric iron (Occhino et al., 1998; Wyckoff et al., 2007). It has been speculated that *V. cholerae* contains one additional high affinity iron transport system. Apparently, iron transport genes are regulated by Fur (Occhino et al., 1998; Wyckoff et al., 2007).

Transferrin Receptors as an Iron Acquisition System Used by *V. parahaemolyticus*

Using a basic local alignment search tool (BLAST) analysis of the sequences in *V. parahaemolyticus,* we identified putative genes that could encode for Tf receptors, but not for Lf receptors. The putative *V. parahaemolyticus* Tf receptor gene (*tbpA*) had 88% of identity with for those reported for *N. meningitidis* (not shown; unpublished data). However, apparently *V. parahaemolyticus* also can use LF as in Fe source (Wong et al., 1996). This probably could be due to the siderophore utilization by this bacterium. Because of Lf is one of the main iron transporters at intestinal level, the *V. parahaemolyticus* capacity of to use Lf as an iron source and the mechanism from iron-Lf acquisition must to be determined. Other members of the genus *Vibrio* have receptors for the use of Tf as iron source. For example, recently; Pajuelo et al. (2015) demonstrated that pVvbt2 from *V. vulnificus*, which causes vibriosis in fish (mainly eels), encodes a host-specific Fe acquisition system that depends on an outer membrane receptor called Vep20. This protein recognizes eel Tf and belongs to a new family of plasmid-encoded fish-specific Tf receptors (Pajuelo et al., 2015). Furthermore, it was found that Vep20 is encoded by an iron-regulated gene that is overexpressed in eel blood during artificially induced vibriosis with *V. vulnificus* both *in vitro* and *in vivo* (Pajuelo et al., 2015). The *Vep20* gene homologs have been identified on the transferable plasmids of two species of fish pathogens with broad host ranges: *V. harveyi* (pVh1) and *Photobacterium damselae* subsp. *damselae* (pPHDD1; Pajuelo et al., 2015). It has been hypothesized that *V. cholerae* contains three proteins that could be Tf receptors. These proteins were shown to be involved in the binding of LF, Hemin, Ft, and Hb (Ascencio et al., 1992).

Binding and Transport of Iron or Iron-Charged compounds in *V. parahaemolyticus*

The expression of two proteolytic proteins of 43 and 90 kDa from *V. parahaemolyticus* were identified. Apparently the protease of 43 kDa is capable of degrading Hb and it has been speculated that this could be one of the strategies of *V. parahaemolyticus* to acquire iron from the human host (Wong and Shyu, 1994). By using the BLAST, we also identified genes that could play a role in Fe acquisition (**Table 1**). Although they have not reported and their biological functions have not been described, these genes likely encode proteins involved in Fe acquisition for *V. parahaemolyticus* in different niches. The probable functions of putative Fe acquisition genes and homologies are described in **Table 1**. Additionally; a schematic view of Fe acquisition systems with putative proteins used by *V. parahaemolyticus* is shown in **Figure 3**.

Apparently, the *V. parahaemolyticus* contain the proteins VctA, VctP, VctG, and VctD (Tanabe et al., 2012). These proteins are involved in the use of the siderophore ferric-enterobactin (Wyckoff et al., 2007, 2009). VctA and IrgA are receptors for

TABLE 1 | Genes related to Iron acquisition systems in *Vibrio cholerae* and putative genes in *Vibrio parahaemolyticus*.

V. cholerae			Homology *V. parahaemolyticus (%)*				
Protein/ Gene	**Function**	**Reference for source**	**Reference for source**	**Accession number**	***Vibrio* sp.**	**Protein identity (%)**	**Gene identity (%)**
VctA*	Outer membrane receptor for enterobactin (VctA) Vct(PGD) participate in the transport of vibriobactin and enterobactin	Wyckoff et al. (2009)	Tanabe et al. (2012)	(CP006005.1)	*V. parahaemolyticus* O1:Kuk	65	67
VctP				(BA000032.2)	*V. parahaemolyticus* RIMD 2210633	56	66
VctG				(CP006005.1)	*V. parahaemolyticus* O1:Kuk	71	66
VctD				(BA000038.2)	*V. parahaemolyticus* RIMD 2210633	75	71
IrgA*	Major membrane receptor for ferric enterobactin	Goldberg et al. (1990)		(CP001805.1)	*Vibrio* sp	69	69
TonB1*	Energy transduction system, provides energy for transport of Enterobactin across the outer membrane	Occhino et al. (1998)	O'Malley et al. (1999)	(CP007005.1)	*V. parahaemolyticus* UCM-V493	51	70
TonB2*	Energy transduction system, provides energy for the transport of vibrioferrin across the outer membrane	Wyckoff et al. (2004)		(CP006007.1)	*V. parahaemolyticus* 13-028/A3	54	72
FhuA*	Transport of ferrichrome across the outer membrane	Rogers et al. (2000)	Funahashi et al. (2009)	(CP003973.1)	*V. parahaemolyticus* BB22OP	64	67
FhuB*				(BA000032.2)	*V. parahaemolyticus* RIMD 2210633	77	70
FhuC*				(AB300920.1)	*V. parahaemolyticus* W-9175	77	65
FhuD*				(AB119276.1)	*V. parahaemolyticus*	62	69
HutA*	Outer membrane receptors for heme and transporters	Mey and Payne (2001)	O'Malley et al. (1999)	(CP003973.1)	*V. parahaemolyticus* BB22OP	68	69
HutR				(CP006007.1)	*V. parahaemolyticus* 13-028/A3	54	67
HutB				(CP003973.1)	*V. parahaemolyticus* BB22OP	68	64
HutC*			O'Malley et al. (1999)	(CP006008.1)	*V. parahaemolyticus* O1:K33	67	70
HutD				(CP006008.1)	*V. parahaemolyticus* O1:K33	68	68
FeoA	Ferrous iron transporter	Cartron et al. (2006)		(CP007004.1)	*V. parahaemolyticus* UCM-V493	73	68
FeoB				(CP006004.1)	*V. parahaemolyticus* O1:Kuk	79	73
FeoC				-	*V. parahaemolyticus*	51	-
FbpA	Ferric iron transporter	Kirby et al. (1998)		(CP006008.1)	*V. parahaemolyticus* O1:K33	81	75
FbpB	Periplasmic ferric iron binding protein			(BA000031.1)	*V. parahaemolyticus*	78	70

**indicate proteins that were studied and reported in V. parahaemoyticus.*
Column 6 contain the V. parahaemolyticus strain with the highest identity.
FeoC was not found in the V. parahaemolyticus strains.

enterobactin in *V. cholerae* and *V. parahaemolyticus* (Goldberg et al., 1990; Tanabe et al., 2012). Also, in *V. parahaemolyticus* were identified the TonB1 and TonB2 system similar to *V. cholerae* (Occhino et al., 1998; Seliger et al., 2001; Wyckoff et al., 2004). The proteins that belong to the TonB systems are involved in the transport of Fe^{3+} in Gram-negative bacteria. The system includes outer membrane receptors, all of which are connected with a complex of proteins located at the inner membrane such as TonB, ExbB, and ExbD. This system also includes a periplasmic binding protein associated with an ABC transporter

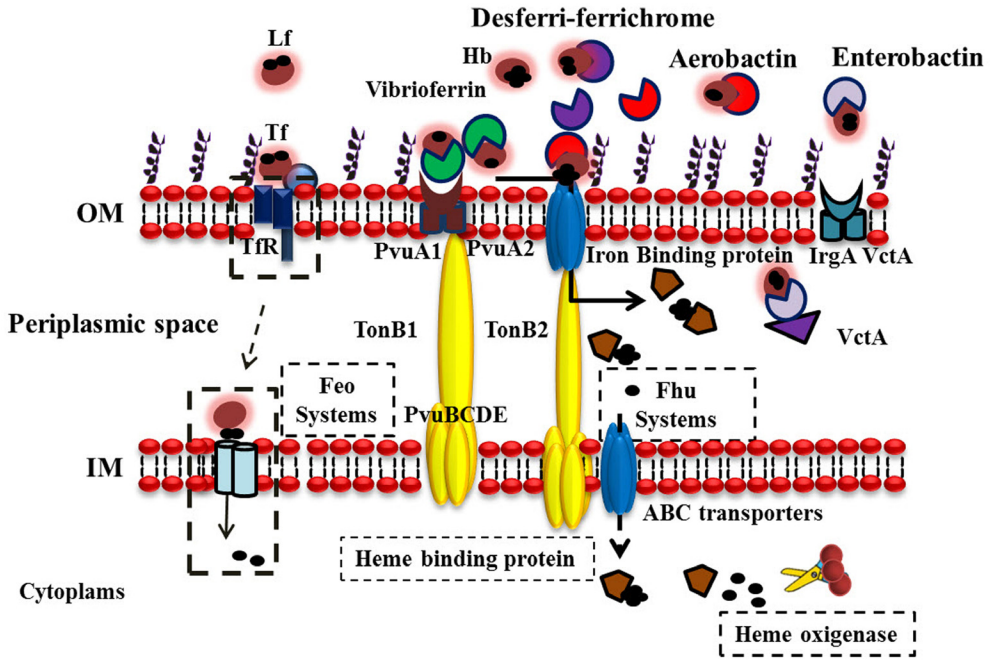

FIGURE 3 | Iron acquisition systems Used by *V. parahaemolyticus* to acquire iron. *V. parahaemolyticus* can be found free swimming or attached to underwater surfaces, seafood products, and humans. The bacterium produces and chelates iron from different sources (depending on the habitat) and transports ferric-charged vibrioferrin into cells via the outer-membrane receptors PvuA1 and PvuA2 and the inner-membrane ABC transport system PvuBCDE. Three sets of TonB systems (TonB1, TonB2, and TonB3) are present in this bacterium, and the energy required for PvuA1 and PvuA2 to transport ferric vibrioferrin is provided by the TonB2 system for PvuA1 and both the TonB1 and TonB2 systems for PvuA2. *V. parahaemolyticus* can also utilize hydroxamate-type xenosiderophores, such as desferri-ferrichrome and aerobactin. Discontinuous lines indicate possible iron sources and iron acquisition mechanisms in *V. parahaemolyticus*.

(Larsen et al., 1996; Koster, 2005). The first step is the binding of the ferric-siderophore to the receptor, it has been speculated that this binding induces a conformational change and then the interaction of the receptor with TonB is enhanced. The proteins ExbB and ExbD provide the energy for the transport of the ferric-siderophore to the inner membrane, in this second step a periplasmic binding protein associated with an ABC transporter delivers the ferric-siderophore into the cytoplasm, in the third step the Fe dissociates from the siderophore (Larsen et al., 1996; Occhino et al., 1998; O'Malley et al., 1999). Little is known about the recycling, storage, and modification of the siderophore. The secretion of *E. coli* enterobactin is mediated by the membrane exporter protein EntS (Furrer et al., 2002; Danese et al., 2004).

In *V. parahaemolyticus* the genes encoding both for the TonB1 and TonB2 systems are located on the small chromosome, and the TonB3 system on the large chromosome (O'Malley et al., 1999; Kuehl and Crosa, 2010; Tanabe et al., 2012). In *V. parahaemolyticus* TonB2 is most active than TonB1 in providing the energy necessary for the transport of ferric-enterobactin via the receptors IrgA and VctA (Tanabe et al., 2012). However, this is different in *V. cholerae*, during the transport of ferric-enterobactin the energy required for IrgA and VctA receptors is provided by the TonB2 system (Seliger et al., 2001; Tanabe et al., 2012). The TonB3 system is not implicated in the transport of iron either *V. parahaemolyticus* or *V. cholerae* (Kuehl and Crosa, 2010; Tanabe et al., 2012), has been reported that the TonB3 system from *V. vulnificus* is induced when the bacterium grows in human serum (Alice and Crosa, 2012). The *V. cholerae* has two TonB systems, which are present on small chromosome (this is different from *V. parahaemolyticus*), and those encoding the TonB2 system are located on the large chromosome. They have unique as well as common functions (Seliger et al., 2001). Both mediate the transport of hemin, vibriobactin, and ferrichrome. However, only TonB1 participates in the use of the siderophore schizokinen, but TonB2 is required for the transport of enterobactin (Seliger et al., 2001).

With respect to FhuA, this protein is the receptor for the siderophores desferri-ferrichrome and aerobactin in *V. parahaemolyticus* (Funahashi et al., 2009). In addition, FhuB, FhuC, and FhuD apparently are involved in the transport of the siderophores, and also are present in *V. cholerae* (Rogers et al., 2000; Funahashi et al., 2009). Regarding the hut genes, it has been reported that HutA is the receptor for the uptake of heme. In addition, HutR has significant homology to HutA as well as to other outer membrane heme receptors (Occhino et al., 1998; Mey and Payne, 2001). In the *V. cholerae* the presence of hutBCD stimulated growth when hemin was the iron source, but these genes were not essential for hemin utilization (Occhino et al., 1998; Mey and Payne, 2001; Wyckoff et al., 2004). Other genes found in the *V. parahaemolyticus* genome were the feo system. The feo system consists of genes

that encoded proteins involved in the transport of ferrous iron (Fe^{2+}), which is expected to be a major iron source in the intestine (Cartron et al., 2006). This Fe^{2+} iron transport system feo is widely distributed among bacterial species such as *V. cholerae*. In this bacterium, the feo operon consists of three genes, feoABC. FeoB is an 83-kDa protein involving in the pore formation for iron transport (Weaver et al., 2013). FeoA and FeoC are all required for iron acquisition; however, their functions have not been described in detail. Apparently, in the genome of *V. parahaemolyticus* there are genes that encode for this Fe^{2+} transport. Moreover, the *V. parahaemolyticus* contains other iron transporters such as FbpA and FbpB. In *Pasteurella haemolytica* the presence of FbpABC family of iron uptake systems has been documented (Kirby et al., 1998). This family of proteins is involved in the utilization and transport of the ferric-xenosiderophore of the bacterium *N. gonorrhoeae,* and is independent of the TonB system (Strange et al., 2011). We speculate that *V. parahaemolyticus* bacteria could have this family of proteins in order to acquire ferric iron from xenosiderophores, in a TonB-independient manner. All of these Fe acquisition systems could be likely involved in the survival of *V. parahaemolyticus* and other *Vibrio* sp. in the different environments that they can colonize, i.e., water, humans, and several other vertebrate hosts (Fouz et al., 1997).

Conclusion

The element Fe is essential for the growth of pathogenic microorganisms, is fundamental and necessary for establishment and replication inside a host, and is required to cause infection. To this end, microbes that live in hostile environments and extracellular spaces of their host must employ different strategies for Fe acquisition to be successful in these niches. It has been postulated that such strategies were acquired during evolution and are involved in the pathogenesis and virulence of bacteria such as *V. parahaemolyticus*. Based on reported findings, this bacterium can utilize the Fe from the proteins Tf, Hb, and hemin by means of siderophores (vibrioferrin, aerobactin, and desferri-ferrichrome) and likely also receptors to acquire Fe from humans during infection of the gut. In low iron, *V. parahaemolyticus* express two proteins of 78 and 83 kDa (now called PvuA2 and PvuA1; respectively), which are the receptors for the siderophore vibrioferrin, and a protease of 43 kDa, which has been hypothesized is involved in one of the strategies of *V. parahaemolyticus* in order to acquire iron from the host. *V. parahaemolyticus* also encodes for LutA, which is the receptor for the siderophore aerobactin. Additionally, according to **Table 1** and other works, this pathogen possesses genes that encode accessory proteins involved in Fe acquisition, transport and synthesis of molecules implicated in Fe acquisition systems.

The iron *per se* has been involved in increase the virulence of *V. parahaemolyticus* and other bacteria. For example, in recent works it have been demonstrated that iron uptake and Quorum sensing (QS) can act together as global regulators of bacterial virulence factors (Wen et al., 2012). QS is a regulatory mechanism used by several bacteria to regulate or modulate the production of extracellular compounds at high cell densities with the aim of establish bacterial biofilms (nowadays, the main medical problem for the control of infectious diseases). Bacterial QS serves as simple indicator of population density, by means of secreting signaling molecules called autoinducers. The link among iron and QS was reported firstly in *Pseudomonas aeuruginos*a (Bollinger et al., 2001). This bacterium in iron-depleted conditions, retarded biofilm formation and increased the twitching motility and expression of QS-related genes, suggesting a link between iron and QS system during biofilm formation (the most important virulence factor of *P. aeruginosa;* Cai et al., 2010), in contrast; *Staphylococcus aureus* in Fe limitation appeared to stimulate biofilm formation (Johnson et al., 2005). These controversial observations can be explained because biofilm formation QS-dependent is nutritionally conditional (Shrout et al., 2006). In other words, in the absence of an acquisition system needed for obtain nutritional iron, or other nutrients such as carbon sources, a bacterium such as *P. aeuruginosa* could establish thin

TABLE 2 | Genes related to quorum sensing in *Vibrio cholerae* and putative genes in *Vibrio parahaemolyticus*.

V. cholerae			*V. parahaemolyticus*			
Protein/ Gen	**Function**	**Reference**	**Accession number**	***Vibrio* sp.**	**Protein identity**	**Gene identity**
luxP	Detects the AI-2 as quorum sensing (QS) signal.	Miller et al. (2002)	CP006007.1	*V. parahaemolyticus* O1:K33	65%	67%
luxQ	Can be autophosphorylated, resulting in the transfer of a phosphate group to LuxO.	Raychaudhuri et al. (2006)	CP006005.1	*V. parahaemolyticus* O1:Kuk	–	64%
cqsA	Acts as an autoinducer to form biofilms.	Zhu and Mekalanos (2003)	BA000032.2	*V. parahaemolyticus* RIMD 2210633	59%	74%
luxO	Activates expression of four sRNAs that destabilize hapR mRNA repressing expression of HapR.	Jobling and Holmes (1997)	CP007004.1	*V. parahaemolyticus* UCM-V493	–	75%
hapR	Master regulator of QS.	Jobling and Holmes (1997)	CP006008.1	*V. parahaemolyticus* O1:K33	72%	75%
aphA	Is a winged-helix transcription factor that controls virulence factor production in the closely related pathogen and QS.	Jobling and Holmes (1997), Skorupski and Taylor (1999)	CP007004.1	*V. parahaemolyticus UCM-V493*		68%

and weak biofilms instead of mature biofilms (Banin et al., 2005; Shrout et al., 2006). At intracellular level the main regulator is Fur. Therefore, Fur regulates genes that are crucial for the iron acquisition needed for the bacteria replication and consequently; in biofilms development (Banin et al., 2005).

Until now, the association between iron and QS on biofilms formed by *V. parahaemolyticus* has not been studied. The results of a BLAST search indicate that some strains of V. *parahaemolyticus* have genes involved in QS and biofilm formation (luxP, luxQ, cqsA, luxO, hapR, aphA), with high identity for those reported in the *V. cholerae* (Jobling and Holmes, 1997; Skorupski and Taylor, 1999; Miller et al., 2002; Zhu and Mekalanos, 2003; Raychaudhuri et al., 2006; **Table 2**). We speculate that iron and QS could be involved in the virulence of *V. parahaemolyticus*. In the *V. vulnificus* the biosynthesis of the siderophore vulnibactin is regulated by Fur and QS (Wen et al., 2012; Kim et al., 2013). Once vulnibactin sequesters the iron needed for the replication of the bacterium, *V. vulnificus* catalizes the enzyme LuxS, which synthetizes the autoinducer (AI-2), involved in the activation of signals for QS and Biofilm formation. At high cell density, *V. vulnificus* enhances the expression of the gene *vvpE*, which encodes for the virulence factor elastase (Kim et al., 2013). It has been observed that mutations in LuxS reduce not only biofilm formation, also reduce virulence factors such as motility, production of proteases and the secretion of the *V. vulnificus* hemolysin, etc. (Wen et al., 2012).

Although the link between Fur and QS is complex, the siderophores production and the coordinated regulation by the two systems (Fur and QS) probably ensures to bacteria maintain an appropriate iron concentration to optimize its survival and propagation within the human host (Wen et al., 2012). It has been shown that blocking the nutritional support and the communication pathways of one's adversaries serves as an effective tactic to disrupt cooperative actions among individuals or groups. Removal of iron as a therapeutic approach has been investigated *in vitro* for several infections, with promising results (Gorska et al., 2014). The generation of analogs that block or alternative signals involved in QS have been developed, in order to disrupt biofilm formation and other virulence factors (LaSarre and Federle, 2013). These strategies could be success in bacteria, because Fe limitation and Fe excess affect QS-dependent biofilm formation, therefore understand how these sophisticated and complex regulatory systems are regulated, is vital to predict bacterial behaviors and possibly then, develop drugs that can interfere with the iron acquisitions systems, or with the response of signal molecules involved in iron acquisition systems or QS (Bollinger et al., 2001; Wen et al., 2012; LaSarre and Federle, 2013).

When the host tries to limit infection by lowering iron, pathogens such as *V. parahaemolyticus* triggered increased expression of virulence factors (that are relevant for surface colonization and infection) in order to cause damage to the host. Based in these results, we conclude that unnecessary or excessive iron administration may be harmful, due the possible multiplication of bacterial growth and increase in their virulence. While it is clear that iron levels are important in infection, it is not an easy task to control their levels in the host. The complete detailed mechanism for Fe acquisition and its role in *V. parahaemolyticus* virulence remains to be determined.

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An insight of traditional plasmid curing in *Vibrio* species

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As the causative agent of foodborne related illness, *Vibrio* species causes a huge impact on the public health and management. *Vibrio* species is often associated with seafood as the latter plays a role as a vehicle to transmit bacterial infections. Hence, antibiotics are used not to promote growth but rather to prevent and treat bacterial infections. The extensive use of antibiotics in the aquaculture industry and environment has led to the emerging of antibiotic resistant strains. This phenomenon has triggered an alarming public health concern due to the increase number of pathogenic *Vibrio* strains that are resistant to clinically used antibiotics and is found in the environment. Antibiotic resistance and the genes location in the strains can be detected through plasmid curing assay. The results derived from plasmid curing assay is fast, cost effective, sufficient in providing insights, and influence the antibiotic management policies in the aquaculture industry. This presentation aims in discussing and providing insights on various curing agents in *Vibrio* species. To our best of knowledge, this is a first review written discussing on plasmid curing in *Vibrio* species.

Keywords: *Vibrio* species, antibiotic, resistance, plasmids, foodborne pathogens

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Introduction

Antibiotic resistant infection has become more challenging to treat with existing antibiotics, leading to infections triggering higher morbidity and mortality, imposing huge costs on our society (Carlet et al., 2011; de Kraker et al., 2011; Finley et al., 2013). This increasing resistance involves many human pathogens including *Vibrio* species. *Vibrio* are Gram-negative bacteria possessing a curved rod shape that naturally inhabits the estuarine and marine environment worldwide (Hazen et al., 2010; Letchumanan et al., 2014; Raghunath, 2015). The presence of this bacterium in the marine environment raises human concern on food safety due to the latter potentially causing disease outbreaks depending on the environmental conditions (Ceccarelli et al., 2013). *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus*, *V. fluvialis*, and *V. alginolyticus* are among the species commonly known to cause human illnesses (Theresa and Kumar, 2014).

The occurrence of multidrug-resistant (MDR) bacteria to clinically used antibiotics is a major health issue and a great challenge to the worldwide drug discovery programs (Alanis, 2005). It is well documented that both clinical and environmental *Vibrio* strains harbors antibiotic resistance traits (Letchumanan et al., 2015; Shrestha et al., 2015; Zavala-Norzagaray et al., 2015). A recent study in Iran has reported multidrug resistance profile towards erythromycin, sulfamethoxazole–trimethoprim and ampicillin in *V. cholerae* isolated from clinical samples (Tabatabaei and Khorashad, 2015). In India, serogroups O1 of *V. cholerae* classical biotype and sub serotype, Ogawa was identified among the *V. cholerae* isolated from clinical strains. All the isolates were

reported to be resistant to ampicillin, nalidixic acid, and cotrimoxazole (Shrestha et al., 2015). Besides *V. cholerae*, *V. parahaemolyticus* have been isolated both from clinical and environmental samples study in India. A clinical study reported 178 *V. parahaemolyticus* strains were isolated from 13,607 diarrheal patients admitted in Infectious Diseases Hospital, Kolkata since 2001–2012 (Pazhani et al., 2014). Reyhanath and Kutty (2014) have reported the detection and isolation of multidrug resistant strains of *V. parahaemolyticus* isolated from a fishing land at South India. In another study, pathogenic and antibiotic resistant *V. parahaemolyticus* strains and other *Vibrio* species strains were isolated from seafood in Cochin. Majority of the strains in this study were resistant to ampicillin and multiple drug resistance was prevalent among the isolates (Sudha et al., 2014). In recent years, environmental *Vibrio* strains have been studied in detail for its potential as a reservoir for the wide spread of antibiotic resistance (Zhang et al., 2012).

Every year, more and more pathogenic *Vibrio* species have been reported to develop higher resistance towards most of the clinically used antibiotics. Drug resistance is an alarming issue worldwide and is spreading rapidly due to overuse, self-medication or the non-therapeutic use of antimicrobials (Slama et al., 2005). Antibiotics and other chemotherapeutic agents are frequently utilized in aquaculture farms as feed additives or immersion baths to achieve either prophylaxis or therapy (Devi et al., 2009; Manjusha and Sarita, 2011; Rico et al., 2012; Cabello et al., 2013). The excessive usage of antibiotics in agriculture and aquaculture environments has caused the development of multidrug resistance in seafood pathogens such as *Vibrio* species (Sudha et al., 2014). Usually the emerging of single or multiple antibiotic resistances are closely associated with various antimicrobial used (Manjusha and Sarita, 2011). Tetracycline, quinolone, oxytetracycline, enrofloxacin, sarafloxacin, and florfenicol are among the antibiotics allowed and used in the aquaculture industry to ensure continuous production of seafood (Roque et al., 2001; Yano et al., 2014).

Vibrio species are usually known to be highly susceptible to most clinically used antibiotics (Mala et al., 2014; Shaw et al., 2014; Letchumanan et al., 2015; Zavala-Norzagaray et al., 2015). Most of the genetic determinants that confer resistance to antibiotics are located on plasmids. Acquired antibiotic resistance in bacteria is generally mediated by extrachromosomal plasmids and transferable to other bacteria in the environment through vertical gene transfer or horizontal gene transfer (Manjusha and Sarita, 2011). Horizontal gene transfer is very important in the evolution and transmission of resistance genes between species and includes the transfer of resistance genes from fecal bacteria to environmental bacteria (Baquero et al., 2008). These extrachromosomal DNA sequences may be responsible for the emergence of resistance to multiple antibiotics (Schelz et al., 2006). In recent years, the presence of antibiotic resistance genes detected in *Vibrio* species have increased and includes β-lactam and penicillin resistance genes *penA* and *blaTEM-1* (Srinivasan et al., 2005; Zhang et al., 2009), chloramphenicol resistance genes *cat*I, *cat*II, *cat*III, *cat*IV, and *floR* (Dang et al., 2007, 2008) and tetracycline resistance genes *tet*A, *tet*B, *tet*C, *tet*D, *tet*E, *tet*G, *tet*H, *tet*J, *tet*Y, *tet*Z (Macauley et al., 2007; Zhang et al., 2009; Kim et al., 2013).

Plasmid-mediated multidrug resistance is one of the most pressing problems in the treatment of infectious diseases. The use of plasmid-curing agents may serve as a possible way to eliminate the plasmid and reduce spreading of antibiotic resistance encoded by antibiotic resistance plasmids (R-plasmids) (Molnar et al., 2003). Plasmid curing occurs naturally through cell division or by treating the cells with any chemical or physical agents (Elias et al., 2013). The inhibition of conjugational transfer of antibiotic resistance plasmid can be used to decrease the spread of antibiotic resistance plasmid in the environment. Inhibition of plasmid replication occurs in various stages and well demonstrated through the "rolling circle" model (replication, partition, and conjugal transfer). This could also be the theoretical basis for the elimination of bacterial virulence in the case of plasmid mediated pathogenicity and antibiotic resistance (Brüssow et al., 2004). The aim of this study is to provide essential insights on the traditional plasmid curing assay in *Vibrio*.

Plasmid Curing in *Vibrio*

Bacterial plasmids are known to harbor genes for resistances to antibiotics and metals; catabolic pathways such as lactose utilization and degradation of hydrocarbons; and biosynthesis of certain antibiotics. Curing of plasmids from bacteria strains is a way to eliminate the bacteria plasmid and determine the antibiotic resistance mediation. There are several methods involving chemical and physical agents that have been developed to eliminate plasmids. Protocols for plasmid curing in *Vibrio* consist of chemical agents such as acridine orange (AO), ethidium bromide (EB), and sodium dodecyl sulphate (SDS), and physical agent (Liu et al., 2012) (**Table 1**). The mechanism of plasmid curing starts from the inhibition of plasmid replication resulted from a single nick, outside of the replication origo of the superhelical structure. The process leads to further relaxation of plasmid DNA, an increase in melting point and circular dichroism. The intercalating agents would then break the superhelical form of plasmid DNA subsequently forming an open circular or linear form plasmid DNA (Spengler et al., 2006). Resistance is usually classified as "chromosomal" when unaffected by plasmid curing and as "plasmidial" when affected.

Intercalating Agents

Intercalating agents such as AO and EB have been successfully used in curing bacterial plasmids. The modes of action of intercalating agents are through preferential inhibition of plasmid replication. Basically, overnight bacteria cultures are inoculated into enrichment broths, Tryptic Soy Broth (TSB), or Luria Bertani Broth (LB). Curing agents at a concentration ranging from 0.1 to 0.5 mg/ml is added to the culture broth. The concentration depends on the organism and curing agent used. The cultures are then incubated overnight at 35 or 37°C under constant agitation. After the treatment, antibiograms assay were again performed to find antibiotic resistance phenotypes.

TABLE 1 | Summary of plasmid curing in *Vibrio* species.

No	Curing agent	[Concentration]	Media	*Vibrio* species	Results		Reference
					Before	After	
1	Sodium dodecyl sulphate	0.2–3% w/v	LBS Broth	*V. parahaemolyticus*	The isolates showed a resistance toward ampicillin, polymixin-B, streptomycin, kanamycin, neomycin, chlorotetracycline, furazolidone.	There was no apparent changes observed when susceptibility was tested against the antibiotics after plasmid curing. It indicates that the resistance to these antibiotics is found to be chromosomal.	Devi et al. (2009)
2	Acridine orange	0.100 mg/ml	Luria Bertani Broth	*V. navarrensis, V. brasiliensis, V. parahaemolyticus, V. xuii, V. coralliilyticus, V. cholerae, V. neptunius, V. alginolyticus, V. diazotrophicus, V. vulnificus B3*	Isolates were resistant to penicillin G, tetracycline, cephalothin, ampicillin, aztreonam, ceftriaxone.	Fourteen isolates with multi-resistance profile was subjected to plasmid curing. Eleven of the isolates became susceptible to the antibiotics they were resistant to after curing and three isolates were still resistant to penicillin G and aztreonam.	Costa et al. (2014a)
3	Acridine orange	0.2 mg/ml	Tryptic Soy Broth	*V. marinus, V. mimicus, V. tubiashii, V. fluvialis, V. hispanicus, V. vulnificus, V. mediterranei, V. metschnikovii, V. alginoyticus, V. harveyi*	Isolates were resistant to ampicillin, cefoxitin, oxytetracycline, nalidixic acid, tetracycline, sulfamethoxazole.	After plasmid curing, two of the isolates became susceptible to all the antibiotics they were resistant to, indicating the resistance was plasmidial. A few isolates were still resistant ampicillin after curing, suggesting it is chromosomal mediated.	Reboucas et al. (2011)
4	Acridine orange	0.05 mg/ml, 0.1 mg/ml, 0.2 mg/ml	Luria Bertani Broth	*Vibrio* species	Isolates were resistant to ampicillin, carbenicillin, cephalothin, gentamicin, netilmicin, nitrofurantoin, oxytetracycline, pefloxacin.	The strains only grew in 0.05 mg/ml AO but it was not enough to cure the strains.	Molina-Aja et al. (2002)
	Ethidium bromide	0.05 mg/ml, 0.1 mg/ml, 0.2 mg/ml	Luria Bertani Broth			All the 21 strains were cured with 0.2mg/ml EB. Six ampicillin resistant strains and one carbenicillin resistant strain became susceptible after plasmid curing	
	Sodium dodecyl sulphate	10%	Luria Bertani Broth			The treatment with 10% SDS did not cure any resistant strains.	
5	Acridine orange	0.05 mg/ml, 0.1 mg/ml, 0.2 mg/ml	Luria Bertani Broth	*V. navarrensis, V. brasiliensis, V. xuii, V. coralliilyticus*	Isolates were resistant to penicillin G, cephalothin, aztreonam, ampicillin, imipenem	All isolate resistance to be chromosomal mediated after plasmid curing.	Costa et al. (2014b)
6	Ethidium bromide	0.2 mg/ml	Tryptic Soy Broth	*V. parahaemolyticus*	Isolates were resistant to ampicillin, amikacin, kanamycin, gentamicin, cefotaxime, ceftazidime	Chloramphenicol and kanamycin resistant strains were subjected to plasmid curing. After plasmid curing, two of its chloramphenicol resistant isolate were plasmidial mediated and six were chromosomal mediated. All the kanamycin resistant strains were still resistant to the antibiotic after plasmid curing.	Letchumanan et al. (2015)

(Continued)

TABLE 1 | Continued

No	Curing agent	[Concentration]	Media	*Vibrio* species	Results		Reference
					Before	After	
7	Ethidium bromide	0.2 mg/ml	Tryptic Soy Broth	*V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*	Isolates were resistant to ampicillin, oxytetracycline, nalidixic acid.	The oxytetracycline resistance phenotype was eliminated thru plasmid curing, suggesting that the resistance to oxytetracycline was related to R-plasmids	Yano et al. (2014)
8	Ethidium bromide	0.05–0.5 mg/ml	Luria Bertani Broth	*V. mimicus*, *V. damsela*, *V. carchariae*, *V. metschnikovii*, *V. proteolyticus*, *V. anguillarum*, *V. mediterranei*, *V. vulnificus*, *V. furnissii*, *V. alginolyticus*, *V. anguillarum*	Isolates were resistant to amoxicillin, ampicillin, amikacin, co-trimoxazole, carbenicillin, cefuroxime, chloramphenicol, ciprofloxacin, chlortetracycline, doxycycline hydrochloride furazolidone, gentamycin, meropenem, nalidixic acid, netilmycin, norfloxacin, neomycin, refampicin, stretomycin, sulfafurazole, trimethoprim, tetracycline.	All of the *Vibrio* strains loss their plasmids when treated with concentration of 0.3 mg/ml EB and demonstrated a change in their resistance pattern. In their study, 79% of the *Vibrio* strains was devoid of plasmid but showed an antibiotic resistance pattern, which indicate resistance via chromosomal.	Manjusha and Sarita (2011)

The effectiveness of a curing agent does vary considerably in ranging of 100- to 1000-fold. This depends on the organisms being treated, curing agent efficiency and efficacy, and the mode of action of respective the curing agent (Carlton and Brown, 1981). Due to these factors, it is essential to use a wide range of curing agent concentration especially when the bacteria are isolated from environmental sources (Trevors, 1986).

Since the 1960s, AO has been used as curing agent and normally involves loss of the whole plasmid (Salisbury et al., 1972; Costa et al., 2014a). In 1970s, Dastidar et al. (1977) reported on the efficiency of AO in eliminating R-plasmids in *V. cholerae* multidrug-resistant strains. Many other studies have demonstrated the usefulness of AO in clinical strains, animal, or environmental isolates (Kamat and Nair, 1992; Barman et al., 2010; Reboucas et al., 2011; Carvalho et al., 2013; Costa et al., 2014a) although the conventional methods for curing plasmids by curing agents may induce mutations in the host chromosomal DNA (Liu et al., 2012).

Reboucas et al. (2011) have used 0.2 mg/ml AO to cure plasmid of *Vibrio* species isolated from marine shrimp. The occurrence of multiple antibiotic resistances was observed in 29% (9/31) of *Vibrio* isolates. Of the total multi-resistant *Vibrio* isolates submitted to plasmid curing, five (55.5%) lost one or more resistance profile while four isolates (44.5%) did not lose their resistance. Out of the five isolates, two isolates became totally susceptible to all the antibiotics they were resistant to, indicating the resistance was plasmidial. Resistance to oxytetracycline was the most frequently lost phenotype after plasmid curing. The results also demonstrated that isolates were still resistant to ampicillin after plasmid curing, suggesting it could be chromosomal mediated (Reboucas et al., 2011).

Similar results were also demonstrated in another study by Costa et al. (2014a). This study subjected fourteen penicillin G, ampicillin and aztreonam resistant isolates to plasmid curing with 0.1 mg/ml of AO. After the plasmid curing assay, 11 of the isolates resistance changed from resistant to susceptible whereas, three other isolates were still resistant to penicillin G and aztreonam. Teo et al. (2000) noted the ampicillin resistant in *Vibrio* with a possible via of mediation by β-lactamase *blaVHW-1* and *blaVHH-1* genes in plasmids. AO has been used in another study by Costa et al. (2014b) and revealed the isolate resistance to be chromosomal mediated after plasmid curing. The loss of phenotype in these studies suggests that AO produce an immediate and complete inhibition of plasmid replication. However, the results may be species dependent and could not be expected with other organisms.

A study was conducted to compare the effectiveness of different plasmid curing agents. Molina-Aja et al. (2002) tested three curing treatments using AO, EB, and SDS. The strains grown in LB was tested with a set of 10% SDS; 0.05, 0.1, and 0.2 mg/ml of AO; 0.05, 0.1, and 0.2 mg/ml of EB. The results stated that the treatment with 10% SDS did not cure any of the study strains. The strains only grew in the lowest concentration of AO (0.05 mg/ml), but it was not enough to cure the stains. All the twenty-one strains were successfully treated

with 0.2 mg/ml EB and seven isolates lost their plasmid. All the treated isolates presented changes in their sensitivity toward the antibiotics (Molina-Aja et al., 2002). The authors suggested that EB was selected as a favorable curing agent in comparative to AO because it is difficult to disposal of AO (Molina-Aja et al., 2002).

Ethidium bromide with a formula molecule $C_{21}H_{20}N_3Br$ is an intercalating agent which resembles a DNA base pair. Due to its unique structure, EB can easily intercalate into DNA strand. Yano et al. (2014) demonstrated the use of EB to eliminate plasmids in antibiotic resistant *Vibrio* species isolated from shrimp cultured in inland ponds in Thailand. Typically the resistant isolates were grown in TSB supplemented with 0.2 mg/ml EB. The oxytetracycline resistance phenotype was eliminated through plasmid curing. The authors suggested that the resistance to oxytetracycline was related to R-plasmids (Yano et al., 2014). Our study also utilized 0.2 mg/ml of EB in curing plasmid of *V. parahaemolyticus* isolates. The study demonstrated chloramphenicol (*catA2*) gene was detected in eight chloramphenicol resistant *V. parahaemolyticus* isolates. Two of the isolates had the gene present in their plasmid whereas another six isolates showed possibility of chromosomal-mediated since the isolates exhibit positive amplification with *catA2* gene and demonstrated phenotypic resistance to chloramphenicol on the disk diffusion test after plasmid curing. In addition, the result of plasmid curing revealed that kanamycin-resistant *V. parahemolyticus* were potentially chromosomal mediated since the isolated exhibit positive amplification with *aphA-3* gene and demonstrated phenotypic resistance after plasmid curing (Letchumanan et al., 2015).

Manjusha and Sarita (2011) performed plasmid curing using 0.05 to 0.5 mg/ml of EB. It was evident from the curing experiment that all of the *Vibrio* strains loss their plasmids when treated with concentration of 0.3 mg/ml EB and demonstrated a change in their resistance pattern. In their study, 79% of the *Vibrio* strains was devoid of plasmid but showed an antibiotic resistance pattern, which indicate chromosomal resistance. The isolates exhibited a chromosomal borne resistance toward amoxicillin, ampicillin, furazolidone, and tetracycline after curing assay. The authors concluded that some of these resistances may be encoded on plasmids in some strains, while the other isolates may be chromosomal mediated.

Sodium Dodecyl Sulfate

Sodium Dodecyl Sulfate is an anionic detergent that is used as a chemical curing agent in *Vibrio* species. Plasmid containing cells are possibly more sensitive to SDS because of plasmid-specified pili on cell surface. The chemical acts in dislodging the indigenous plasmid from its site of attachment. Curing assay was performed using SDS as the agent in a study done in India. The *V. parahaemolyticus* isolates were inoculated into LB with different concentrations of SDS (0.2, 0.4, 0.6, 0.8, 1, 2, and 3% w/v). All the strains were still resistant to the antibiotic after curing. The study suggested that the antibiotic resistance of *V. parahaemolyticus* isolates was chromosomal borne (Devi et al., 2009).

Physical Agents

Based on literature, physical agent such as elevated growth temperature is commonly used in *Vibrio* plasmid curing. The mode of action of elevated growth temperature is through complete or partial deletions of strain's plasmid DNA. Elevated incubation temperature (5–7°C) above the optimal growth temperature can be used as a curing method. Study has demonstrated strains that have an optimal growth temperature of 37°C are incubated at 42°C (Carlton and Brown, 1981). The culture are incubated at the elevated temperature until it reaches late log phase, at which time it is diluted (1:20) and re-incubated at the elevated growth temperature until late log phase growth is reached again. A serial dilution is prepared and plated to obtain single colonies which are individually tested for loss of the plasmid-encoded trait and physical absence of the plasmid using agarose gel electrophoresis (Trevors, 1985).

The elevated growth temperature has been successfully used to cure tetracycline resistant, penicillinase-positive strains of *Staphylococcus aureus* (May et al., 1964). Cultures were grown at 43–44°C to obtain tetracycline-sensitive and penicillinase negative cells. However, these cells did not appear until after several cell generations at the elevated temperature. Elevated incubation temperature (up to 42°C), EB (0.5 mg/ml), 10% SDS, and AO (0.5 mg/ml) were employed to eliminate plasmids from *Vibrio* species isolated from Mai Po Nature Reserve, Hong Kong (Zhang et al., 2012). The study results stated that none of the plasmid curing agents was effective in eliminating the plasmid from host cells. All attempts to cure the plasmids from their hosts were failed, probably due to the relatively high copy number of the plasmids similar to earlier work (Zhang et al., 2007).

Discussion

Vibrio species occur naturally in the aquatic environments and are normal member of the flora occurring in coastal seawater (Manjusha and Sarita, 2012). In recent years, the increasing number of emerging multi-drug resistant bacteria is distressing. The presence of antibiotic resistant genes in the bacterial plasmid have led to transmission and spreading of drug resistance among pathogenic strains. Several studies have shown conclusively that antibiotic resistance is caused by pressures of clinical antibiotics and use of antibiotics in the agricultural.

Literatures have shown bacterial resistance in *Vibrio* strains was both plasmid and chromosomal mediated. The studies have demonstrated a high incidence of antibiotic resistance against ampicillin, chloramphenicol, tetracycline, penicillin G, oxytetracycline, carbenicillin, aztreonam, cefuroxime, streptomycin, rifampicin, and amoxicillin. These are among the clinical antibiotics administrated to prevent diseases in human beings. In addition, high numbers of chromosomal mediated antibiotics resistance toward chloramphenicol and ampicillin was observed frequently in the studies. These two antibiotics along with tetracycline, chlortetracycline, nalidixic acid, gentamycin, sulfafurazole, trimethoprim are among the commonly used antibiotics in aquaculture farms through feeds during culture and hatchery production of seeds (Manjusha and Sarita, 2011).

The extensive use of these antibiotics in the aquatic environments has caused the *Vibrio* species to be resistant and carry the resistant genes either in their plasmid or chromosomal. It is noted that plasmid borne integrons are the main players in being able to acquire, rearrange, and express genes conferring antibiotics resistance (Stokes and Hall, 1989; Manjusha and Sarita, 2012). These intergrons have been found in chromosomes of *Vibrio* species and many other bacteria (Heidelberg et al., 2000; Holmes et al., 2003).

The efficacy of each plasmid curing agent discussed varies depending on the concentration and the organism being cured. Based on the study's results, it could be concluded that, EB and AO may be a better curing agent than SDS. EB was also preferred by many researchers in comparative to AO because the latter is difficult to be disposed (Molina-Aja et al., 2002). All these chemical curing agents are known to be harmful and cause health problems to human beings. Precaution steps should be followed strictly prior in handling with these curing agent during experiments. When compared with chemical curing agents, physical agent such as elevated growth temperature is least favored in *Vibrio* plasmid curing studies due to its low successful rate.

Resistance emerges either passively as an aftereffect of pre-existing innate mechanisms or actively through the acquisition of new hereditary material by mobile genetic elements for example plasmids or transposons (Summers, 2006; Wright, 2007). Traditional plasmid curing assay may be used eliminate the bacteria plasmid and detect the antibiotic resistance mediation. This vital information would be beneficial in the global surveillance management of environmental multidrug resistance. Reducing and improving the use of antibiotics in the aquatic environment can reduce resistance and allow the antibiotic to resurface eventually as an effective therapy (Barbosa and Levy, 2000). The establishment of suitable therapeutic doses of antibiotics may also help reduce potential impacts on the environment and on human health (Nogueira-Lima et al., 2006).

In summary, the paper is to provide insight to readers on the traditional plasmid curing agents its effectiveness in *Vibrio* studies. Nevertheless, a study on *Bacillus anthracis* has addressed the weakness of chemical and physical plasmid curing agents (Liu et al., 2012). The curing agents are said to cause potential mutation in the host chromosome which interferes with the functional analysis of the plasmid. For this reason, the study developed a curing method using plasmid incompatibility to study *Bacillus anthracis* plasmid (Liu et al., 2012). But this concern or approach has not been reported in *Vibrio* studies worldwide. Hence, in view of potential weakness of traditional plasmid curing agents, modern approach to plasmid curing using plasmid incompatibility or next generation sequencing (NGS) could be considered in plasmid curing of *Vibrio* studies. Another alternative approach would be using microarray technology to detect antibiotic resistant genes in bacteria (Perrenten et al., 2005; Law et al., 2015). To date, a few microarray technologies have been developed for identification of antibiotic resistance genes but are either restricted to a class of drug or limited to a certain number of genes only (Perrenten et al., 2005).

Conclusion

To our knowledge, this is the first presentation that discuss on the traditional plasmid curing in *Vibrio* species. In the current era of science technology, traditional plasmid curing may be used to eliminate plasmids and determine antibiotic resistance mediation although there is availability of modern methods such as NGS or diagnostic displacement by specific incompatibility. Next generation sequencing has become easier to be accessed, with high throughput results and helps to locate the resistant gene in the genome. However, when compared with traditional plasmid curing, next generation sequencing involves high cost when sequencing genomes of huge samples. Usually, food safety studies involve huge number of samples thus it would be very costly to sequence all the sample isolates genome by using next generation sequencing platform. Hence, alternative approach using traditional plasmid curing is adapted by researchers. The results derived from plasmid curing assay is fast, cost effective, sufficient in providing knowledge and influence the better antibiotic management policies in the aquaculture industry. The aquaculture industry could adapt the method of switching antibiotics used in the aquatic field from time to time in order to allow withdrawal of antibiotic resistance profile in strains. As the effectiveness of clinical antibiotics has declined, the extensive use of antibiotics in the aquaculture and humans are in distress conditions due to horizontal gene transfer and spread of resistant strains. It is very crucial to deal with this threat posed by overused antibiotics in aquaculture promptly.

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frontiers in Microbiology

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Prevalences of pathogenic and non-pathogenic *Vibrio parahaemolyticus* in mollusks from the Spanish Mediterranean Coast

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Vibrio parahaemolyticus is a well-recognized pathogen of humans. To better understand the ecology of the human-pathogenic variants of this bacterium in the environment, a study on the prevalence in bivalves of pathogenic variants (*tlh*+ and *tdh*+ and/or *trh*+) versus a non-pathogenic one (only *tlh*+ as species marker for *V. parahaemolyticus*), was performed in two bays in Catalonia, Spain. Environmental factors that might affect dynamics of both variants of *V. parahaemolyticus* were taken into account. The results showed that the global prevalence of total *V. parahaemolyticus* found in both bays was 14.2% (207/1459). It was, however, significantly dependent on sampling point, campaign (year) and bivalve species. Pathogenic variants of *V. parahaemolyticus* (*tdh*+ and/or *trh*+) were detected in 3.8% of the samples (56/1459), meaning that the proportion of bivalves who contained *tlh* gene were contaminated by pathogenic *V. parahaemolyticus* strains is 27.1% (56/207). Moreover, the presence of pathogenic *V. parahaemolyticus* (*trh*+) was significantly correlated with water salinity, thus the probability of finding pathogenic *V. parahaemolyticus* decreased 1.45 times with every salinity unit (ppt) increased. Additionally, data showed that *V. parahaemolyticus* could establish close associations with *Ruditapes* spp. (P-*value* < 0.001), which could enhance the transmission of illness to human by pathogenic variants, when clams were eaten raw or slightly cooked. This study provides information on the abundance, ecology and characteristics of total and human-pathogenic *V. parahaemolyticus* variants associated with bivalves cultured in the Spanish Mediterranean Coast.

Keywords: prevalence, human-pathogenic variants, *tdh*, *trh*, *Vibrio parahaemolyticus*, bivalves, salinity, water temperature

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Introduction

Vibrio parahaemolyticus is a bacterium commonly present in marine and estuarine water worldwide (Kaneko and Colwell, 1975).

The virulence of *V. parahaemolyticus*, among other virulence attributes, depends on the presence of a thermostable direct hemolysin (TDH, *tdh* gene) and/or the thermostable direct hemolysin related (TRH, *trh* gene; Takeda, 1982; Shirai et al., 1990; Honda and Iida, 1993; Oliver and Kaper, 2007; Ceccarelli et al., 2013; Raghunath, 2015). Despite that the bacterium can be

found naturally in seafood and taking into account that bivalves are filter feeders and accumulate environmental bacteria in their gills and digestive glands becoming potential vectors for many pathogens (Potasman et al., 2002), pathogenic isolates capable of inducing gastroenteritis in humans are rare in environmental samples (2 to 3%) or even undetectable (Nair et al., 2007; Canizalez-Roman et al., 2011; Velazquez-Roman et al., 2012; Haley et al., 2014). It should be noted that, in recent studies using a new set of primers have shown that higher frequencies of the *tdh* and *trh* genes can be detected in environmental *V. parahaemolyticus* strains than primers described previously (Gutierrez West et al., 2013). Anyway, despite that only, few cases of gastroenteritis by *V. parahaemolyticus* have been reported so far in Europe, there is a growing concern on that non-cholera vibrios could represent an important and increasing clinical threat within the European context (Baker-Austin et al., 2010). Furthermore, the scenario could worsen by climate global change which plays an important role in the dissemination of pathogenic microorganism in the marine environment (Baker-Austin et al., 2013; Huehn et al., 2014; Letchumanan et al., 2014).

This study examines the spatial distribution and temporal changes of the total and pathogenic *V. parahaemolyticus* in the delta of Ebro River, Catalonia, Spain. Catalonia which is the second-most important region of Spain in terms of bivalve production, being Spain the second largest producer in the world and one of the largest consumers of bivalves (APROMAR, 2011; Eurostat, 2012). Farming of bivalves in Catalonia is concentrated in the two bays (Alfacs and Fangar) in the delta of the Ebro River. The average and ranges of water temperature and salinity, along with the moderately alkaline pH of the two bays provided the conditions to support growth of vibrios (Montilla et al., 1994). The risk of potentially pathogenic *Vibrio* spp. in products placed on the market is not addressed by the existing legislative framework related to food safety in the European Union. However, it is recognized, the need for a better knowledge of the prevalence of diarrheal vibrios in seafood products (European Commission, 2001).

This study is one of the few that has focused on the examination of large numbers of oysters, mussels, and clams with the objective to investigate the prevalence, spatial distribution and temporal change of total and pathogenic *V. parahaemolyticus* associated to different aquaculture bivalves in the Ebro delta and its relationship with environmental parameters from the surrounding waters.

Materials and Methods

Sampling Sites and Collection of Samples

Four surveillance campaigns for pathogenic *V. parahaemolyticus* detection in commercial bivalves from the Ebro delta bays (**Figure 1**) were performed from 2006 to 2010 (see Roque et al., 2009 for details).

The sample size was established to achieve a high confidence level (CL), between 95 and 99%, considering that some shellfish are eaten raw or lightly cooked. Thus, the minimum expected prevalence for mussels and oysters was 5 and 10% for clams, where sample availability was limited (**Table 1**). Unfortunately, was not possible to carry out the survey in 2007.

Oysters and mussels samples were collected in each bay, from at least three ropes, at three production units (*fixed* platforms with hanging ropes), while clams were sampled in three different points in one culture area (clam field) in each bay. Mussel and oysters were sampled from six sites of the culture area, three on each bay, at the beginning (N 40°37′112″E0°37′092″-Alfacs; N40°46′723″E0°43′943″-Fangar), middle (N 40°37′125″ E0°38′570″-Alfacs; N40°46′666″E0°45′855″-Fangar), and end (N 40°37′309″E0°39′934″-Alfacs; N40°46′338″E0°44′941″-Fangar) of the culture polygons. Clams were sampled from only one site per bay, in the Alfacs bay from a natural bed of *Ruditapes decussatus* (N40°37′44″E0°38′0″) and in the Fangar bay from an aquaculture bed of *Ruditapes philippinarum* (N40°47′3″E0°43′8″; **Figure 1**).

Some deviations from the sample size of the surveillance scheme (**Table 1**) were originated due to lack of adult specimens, and therefore they had to be discarded.

A total of 1459 species of bivalve mollusks were sampled: 709 and 750 bivalves from Alfacs and Fangar Bays, respectively. From the 709 individuals collected in Alfacs Bay, 283 were oysters (*Crassostrea gigas*), 306 were mussels (*Mytilus galloprovincialis*), and 120 clams (*Ruditapes* spp.). And, from the 750 individuals collected from Fangar Bay, 330 oysters, 300 mussels, and 120 clams were processed. So, similar proportional number for each species was taken from the both bays and for each year of study as shown in **Table 2**. On the whole, oyster' samples were 180, 178, 150, and 105 in 2006, 2008, 2009, and 2010, respectively. Mussels samples were 127, 179, 180, and 120 in 2006, 2008, 2009, and 2010, respectively, and clams samples were 60 for each year.

All bivalves were collected during the warmest season (July and August) each sampling year. Samples were transported in cool conditions to the laboratory. Transport lasted less than 1 h in all occasions.

Temperature and salinity data were recorded using a CTD datalogger (Sea-Bird, USA) located in the center of each bay at a depth of 2 m and recording data every 60 min. The center of each bay was selected since it coincides with the middle point of the area covered by the culture rafts.

Microbiological Analysis

Microbiological analysis was carried out as described by Roque et al. (2009). Briefly, all bivalves were individually homogenized and processed and 1 ml of the homogenate was inoculated into 9 ml of alkaline peptone water (Scharlau, Spain). Following 6 h incubation at 37°C, one loopful of the contents of each tube of alkaline peptone water was streaked onto CHROMagar vibrio plates (CHROMagar, France) and incubated for 18 h at 37°C. Mauve-purple colonies were purified, and each purified isolate was cryopreserved at −80°C for further analysis.

Molecular Tests

Total DNA was extracted from each purified isolate using the Wizard genomic DNA purification kit (Promega), following

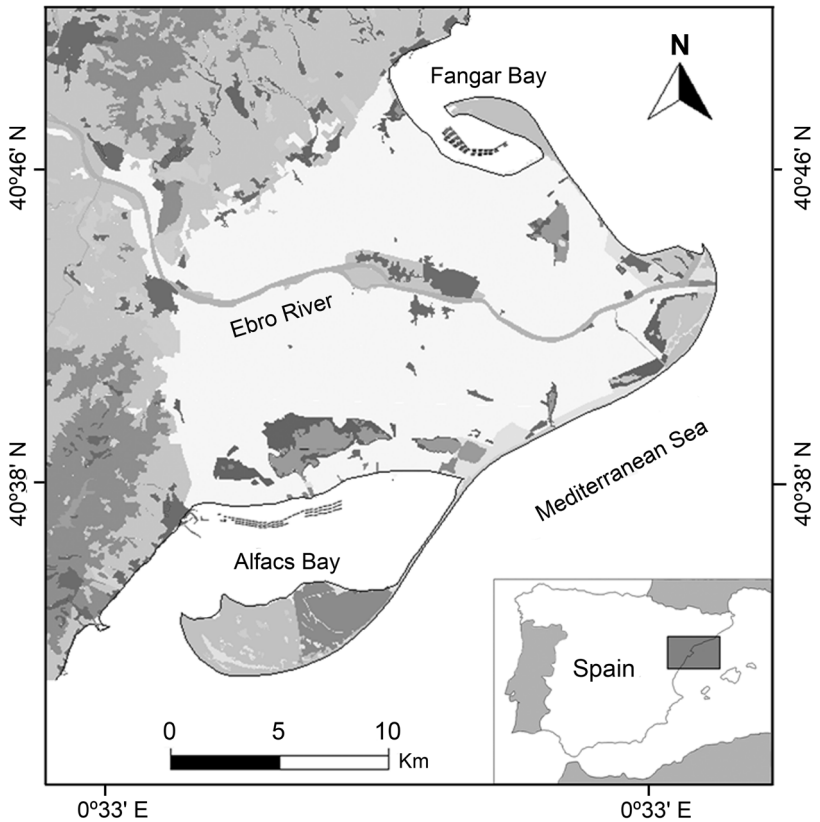

FIGURE 1 | Area of study showing: location of the sampling sites in the Ebro Delta, Spain.

TABLE 1 | Surveillance scheme in order to calculate sample size for different mollusk species in each bay.

	2006			2008			2009			2010		
Species	***n***	**CL**	**mEP**	***n***	**CL**	**mEP**	***n***	**CL**	**mEP**	***n***	**CL**	**mEP**
Crassostrea gigas	90	99%	5%	90	99%	5%	90	99%	5%	60	95%	5%
Mytilus galloprovincialis	60	95%	5%	90	99%	5%	90	99%	5%	60	95%	5%
Ruditapes spp.	30	95%	10%	30	95%	10%	30	95%	10%	30	95%	10%

n, calculated sample size; CL, confidence level; mEP, minimum expected prevalence.

TABLE 2 | Prevalence of total *V. parahaemolyticus*, stratified by years, by bays, and by species of bivalve.

	2006				2008				2009				2010			
	Alfacs		**Fangar**		**Alfacs**		**Fangar**		**Alfacs**		**Fangar**		**Alfacs**		**Fangar**	
	n	***Vp* %**	***n***	***Vp* %**	***n***	***Vp* %**	***n***	***Vp* %**	***n***	***Vp* %**	***n***	**%**	***n***	**%**	***n***	**%**
Crassostrea gigas	90	12.2%	90	25.6%	88	10.2%	90	21.1%	60	11.7%	90	11.1%	45	4.4%	60	11.7%
Mytilus galloprovincialis	67	4.5%	60	6.7%	89	11.2%	90	37.8%	90	8.9%	90	2.2%	60	5.0%	60	15.0%
Ruditapes decussatus	30	56.7%	–	–	30	30.0%	–	–	30	3.3%	–	–	30	6.7%	–	–
Ruditapes philippinarum	–	–	30	0.0%	–	–	30	40.0%	–	–	30	16.7%	–	–	30	0.0%
Total	**187**	**16.6%**	**180**	**15.0%**	**207**	**13.5%**	**210**	**31.0%**	**180**	**8.9%**	**210**	**8.1%**	**135**	**5.2%**	**150**	**10.7%**
P-value[1]	<0.001		<0.001		0.017		0.027		0.424		0.016		0.914		0.089	
P-value[2]	<0.001				0.010				0.070				0.125			

[1]*P-value: prevalences of total V. parahaemolyticus (tlh+) stratified by years and by bays;* [2]*P-value: Prevalences of total V. parahaemolyticus (tlh+) stratified by years.*

the instructions of the manufacturer. DNA concentration was verified by spectrophotometry and the concentration of each DNA was adjusted to 50 ng μl^{-1}. The PCR analysis was then performed to identify which isolates were positive for *tlh* gene (species marker for *V. parahaemolyticus*). The further detection of the *tdh* or *trh* genes was carried out on all positive *tlh* strains. All PCR analyses were carried out using the primers described by Bej et al. (1999) with the following amplification conditions on the thermocycler (Eppendorf Mastercycler Personal, Hamburg, Germany): an initial denaturation at 95°C for 8 min, followed by 40 cycles of a 1 min denaturation at 94°C, annealing at 55°C for 1 min, elongation at 72°C for 1 min, and a final extension of 10 min at 72°C. Positive and negative controls were included in all reaction mixtures: two positive controls, *tlh* and *tdh* CAIM 1400 and *trh* CAIM 1772 [Collection of Aquatic Important Microorganisms (http://www.ciad.mx/caim/CAIM.html)], and negative control DNA-free molecular grade water (Sigma-Aldrich, Spain). Expected amplicons were visualized in 2% agarose gels stained with ethidium bromide.

Statistical Analysis

The influence of different factors such as species of mollusk, location (Alfacs or Fangar) and year of study on prevalences (proportion of mollusks contaminated by *V. parahaemolyticus*) was assessed by Pearson's Chi-Square test.

A logistic regression analysis was performed using *V. parahaemolyticus* prevalence as dependent variable and year, location, mollusk species, salinity, and temperature as independent variables. Forward stepwise method was applied, and significance of the model was evaluated with omnibus test. Using coefficients of the model (Bx) Odds Ratio ($OR = e^{Bx}$) were calculated to identify significant risk factors.

Desired alpha error was established at 0.05. Statistical analysis was performed using SPSS 19.0 software (Chicago, IL, USA).

All data collected represents data points and not a continuous variable.

Results

During four summer surveillance campaigns (years 2006, 2008, 2009, and 2010), a total of 1459 bivalve mollusks (*C. gigas*, *M. galloprovincialis, R. decussatus,* and *R. philippinarum*) at commercial size, were collected and processed in the two shellfish production areas of Alfacs and Fangar Bays of the delta of Ebro river to assess the prevalences of *V. parahaemolyticus*.

Global Prevalence of *V. parahaemolyticus*

Different trends in non-pathogenic and pathogenic *V. parahaemolyticus* prevalences and their relationship with water temperature (°C) and salinity (ppt) from both sampling sites, are shown in **Figure 2**. Overall, in the two bays, *V. parahaemolyticus* was detected in 207 (14.2%) of the 1459 samples identified by targeting thermolabile hemolysin encoded by *tlh* gene. When statistical analysis was performed to compare prevalence of *V. parahaemolyticus* carrying the *tlh* gene among sampling bays, they were found to be significantly different ($P = 0.005$), being Alfacs' prevalence (11.6%, 82/709) lower than Fangar's (16.7%, 125/750). When the analysis was stratified also by year, significant differences were observed only in 2008 ($P < 0.001$), where the proportion of bivalves mollusks from Fangar with *V. parahaemolyticus* (31.0%) was higher than in Alfacs (13.5%). No differences due to the sampling site were detected in 2006 ($P = 0.679$), in 2009 ($P = 0.779$), or in 2010 ($P = 0.090$) though prevalence of total *V. parahaemolyticus* in Fangar (10.7%) were double than Alfacs (5.2%). When prevalences of total *V. parahaemolyticus* were analyzed in each bay over the sampling period, it was observed that the prevalence in Alfacs Bay decreased significantly ($P = 0.007$) over the studied period: 2006 (16.6%), 2008 (13.5%), 2009 (8.9%), 2010 (5.2%); while, the prevalence of total *V. parahaemolyticus* in Fangar bay was significantly different among years ($P < 0.001$), but fluctuating over time: 2006 (15.0%), 2008 (31.0%), 2009 (8.1%), and 2010 (10.7%; **Figure 2**).

Prevalence of pathogenic *V. parahaemolyticus* were studied considering the different trends of their virulence factors in relation with water temperature and salinity at the sampling sites as shown in **Figure 2**. The global prevalence of total mollusks contaminated by pathogenic *V. parahaemolyticus* (with virulence genes *tdh*, *trh,* or both) was estimated to be 3.8% (56/1459).

When stratified analysis of prevalence of pathogenic *V. parahaemolyticus* by bays was performed, no significant differences ($P = 0.251$) were observed between Alfacs (3.2%) and Fangar (4.4%). In both cases, proportion of bivalves contaminated by *V. parahaemolyticus* that carried pathogenic strains were similar, and the overall prevalence was 27.1%, with the following distribution: *tdh* (17.9%), *tdh/trh* (1.4%) and *trh* (7.7%), meaning that from the 212 mollusks containing *V. parahaemolyticus* (*tlh*+), a total of 57 also presented a virulence factor (*tdh, trh,* or both).

Globally, significant differences were observed ($P = 0.053$) when the presence of virulence factors was investigated over time in both bays together. Results showed higher proportion of the bivalves contained pathogenic *V. parahaemolyticus* in 2008 (6.0%, 25/417) than in other years, mainly, due to the contribution of Fangar Bay. Also, when taking into account the stratified analysis by year and by bays for the virulence factors, no significant differences were observed in Alfacs ($P = 0.747$), in contrast to Fangar. Here, significant differences were recorded ($P = 0.004$), with the highest prevalence of pathogenic *V. parahaemolyticus* being 8.6% in 2008, of which 6.7% were *tdh*-positive, 1.4% were *trh*-positive, and 0.5% were *tdh/trh*-positive (**Figure 2**).

Regarding *V. parahaemolyticus* strains harboring the *trh* gene, the highest prevalence observed was 1.4% in both bays in the whole studied period (this value was recovered in 2008 in Alfacs, and in 2008 y 2009 in Fangar).

Prevalences of total *V. parahaemolyticus*, stratified by years, by bays, and by species of bivalves are shown in **Table 2**. Prevalences among species of bivalves in Alfacs Bay, were different in 2006 ($P < 0.001$), and in 2008 ($P = 0.017$), where prevalence in *R. decussatus* (56.7 and 30.0%, respectively), was higher than in *C. gigas* or *M. galloprovincialis*, but this was not the case in 2009 ($P = 0.424$) and 2010 ($P = 0.914$).

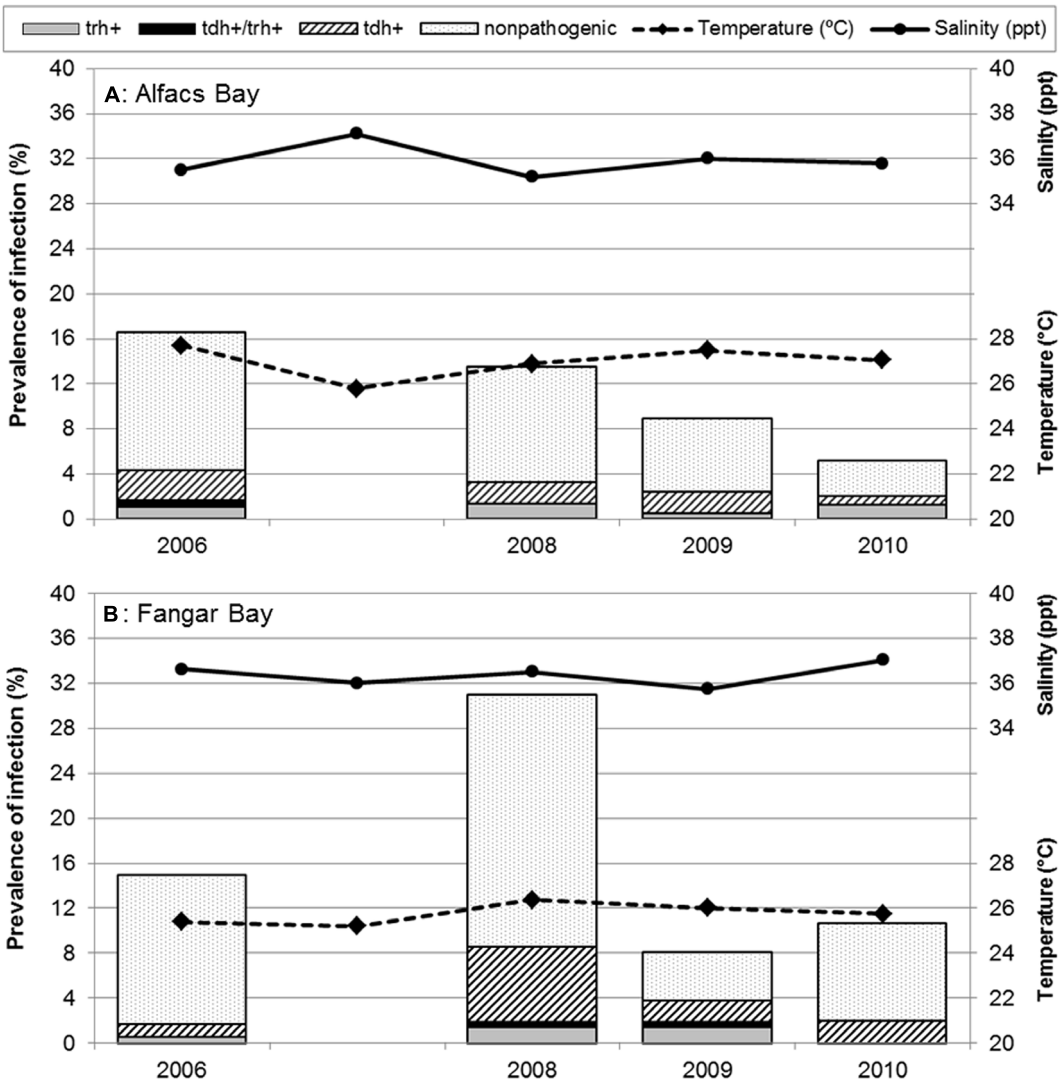

FIGURE 2 | Prevalence of pathogenic and non-pathogenic *Vibrio parahaemolyticus* in Mediterranean mollusks: stratified results in (A) Alfacs Bay and (B) Fangar Bay. Each bar represents the arithmetic mean of four species of bivalves averaged in sampling months. Solid line represents salinity (ppt); dashed line, water temperature (°C) during each campaign.

In Fangar Bay, significant difference were found in 2006 ($P < 0.001$), where higher prevalence was found in *C. gigas* (25.6%). In 2008 ($P = 0.027$), prevalences were higher in *R. philippinarum* (40.0%) and in *M. galloprovincialis* (37.8%), and in 2009 ($P = 0.016$), they were higher in *R. philippinarum* (16.7%). No significant differences ($P = 0.089$) were found in prevalences of total *V. parahaemolyticus* in mollusks in 2010.

Vibrio parahaemolyticus was not present in all sample batches in Fangar bay, e.g., *V. parahaemolyticus* was not detected in *R. philippinarum* neither in 2006 nor in 2010.

When prevalences of pathogenic *V. parahaemolyticus* by species of bivalves were analyzed for either bay, no significant differences were found (Alfacs $P = 0.065$; Fangar $P = 0.822$) during the four campaigns.

When statistical analysis was performed to study if global prevalence of total *V. parahaemolyticus* among species of bivalve were significantly different (without stratify by bay or year), results (see **Table 3A**) indicated that significant differences were found among species ($P = 0.007$). The total prevalence by mollusk species were as follows: 14.4% (88/613) in oysters (*C. gigas*), 12% (73/606) in mussels (*M. galloprovincialis*), 24.2% (29/120) in *R. decussatus*, and 14.2% (17/120) in *R. philippinarum*. In this context of stratification by species, prevalences of total *V. parahaemolyticus* were significant different within mussels ($P < 0.001$), and within both species of clams ($P < 0.001$), whereas results for oysters did not show any significant differences ($P = 0.064$).

When global prevalence and relative frequency of pathogenic *V. parahaemolyticus* (*tdh* and/or *trh*+) stratified only by

TABLE 3 | (A) Prevalence of total *V. parahaemolyticus* (*tlh*$^+$); and **(B)** Prevalence and relative frequency of pathogenic *V. parahaemolyticus* (*tdh*$^+$ and/or *trh*$^+$), both of them stratified by species of bivalves mollusks.

	Sampled mollusks	(A) Prevalence total *Vp*			(B) Prevalence pathogenic *Vp*			% Pathogenic *Vp*
	n	*n*	%	*P-value*[2]	*n*	%	*P-value*[3]	
Crassostrea gigas	613	88	14.4%	0.064	26	4.2%	0.081	29.5%
Mytilus galloprovincialis	606	73	12.0%	<0.001	19	3.1%	0.052	26.0%
Ruditapes decussatus	120	29	24.2%	<0.001	7	5.8%	0.134	24.1%
Ruditapes philippinarum	120	17	14.2%	<0.001	4	3.3%	0.009	23.5%
Total	**1459**	**207**	**14.2%**	**<0.001**	**56**	**3.8%**	**0.053**	**27.1%**
P-value[1]		0.007				0.506		0.908

[1]*P-value: prevalence of total V. parahaemolyticus, stratified by years;* [2]*P-value: prevalence of total V. parahaemolyticus (tlh+), stratified by species of mollusks;* [3]*P-value: prevalence of pathogenic V. parahaemolyticus (tdh+ and /or trh+), stratified by species of mollusks.*

species on bivalve were analyzed (**Table 3B**), the results showed also no significant differences ($P = 0.506$); being the prevalences as follows: 4.2% in oysters (26/613), 3.1% in mussels (19/606), 5.8% in *R. decussatus* (7/120), and 3.3% in *R. philippinarum* (4/120). The same happened when only contaminated bivalves with total *V. parahaemolyticus* (207/1459 total samples) were taken into account, meaning 56/207 bivalves mollusks ($P = 0.908$).

Multivariate Analysis of Influence of Different Factors on the Presence of Total *V. parahaemolyticus*

The relationship between the prevalence of total *V. parahaemolyticus* with species of bivalves, shell length (mm), year of harvest, sampling bay, water temperature, and salinity was analyzed using a logistic regression with Forward Stepwise method.

Two multivariate analysis were used, one including sampling bay and the other without it. A significant logistic regression model that included bay to explain the presence/absence (P/A) of total *V. parahaemolyticus* in bivalves was generated ($P < 0.001$; see **Table 4** for details), and it established that the presence of *V. parahaemolyticus* depends on location of the samples (Alfacs or Fangar), species of mollusks, and year of harvest. Effect of sampling bay was significant ($P < 0.001$) and the risk of finding *V. parahaemolyticus* in mollusks from Fangar Bay was 2.21 times higher than from Alfacs Bay. Moreover, results showed that year of sampling were significant using as reference year 2006. Thus, the risk (expressed as Odds Ratio) to find total *V. parahaemolyticus* varied with time. Results obtained in 2008 respect to 2006 indicated that the risk to find total *V. parahaemolyticus* in bivalves was 1.57 times higher in 2008 than 2006 ($P = 0.017$), and these results indicated that there was 57% more probable to find bivalves containing *V. parahaemolyticus* in 2008 than in 2006. However in 2009 and 2010, the risk decreased significantly and the probability to find total *V. parahaemolyticus* was 2.09 and 2.26 times less than in 2006, respectively ($P = 0.002$, in both cases). Results also showed that the risk of finding *R. decussatus* containing *V. parahaemolyticus* was higher than for other species of bivalves. The probability of finding total *V. parahaemolyticus* in *R. decussatus* was 3.39 times higher than in *C. gigas* ($P < 0.001$). On the other hand, results showed that shell length of mollusks ($P = 0.063$; data not showed), temperature ($P = 0.102$), and salinity ($P = 0.691$) had no significant effect in this logistic regression model.

A second significant model which did not include sampling bay to explain P/A of total *V. parahaemolyticus* in bivalves was generated ($P < 0.001$; see **Table 5** for details). This model established that the presence of *V. parahaemolyticus* depends on species of mollusks, year of harvest, temperature, and salinity. Results showed that the risk to find *V. parahaemolyticus in R. decussatus* was higher than for other species of bivalves, as it was

TABLE 4 | Description of the logistic regression model selected to explain the presence/absence (P/A) of total (presence of *tlh* gene) *V. parahaemolyticus* including location, species of bivalve, length, year of campaign, temperature, and salinity.

Variables included in the selected model	*B*	OR (e^B)	*P*-value
Fangar vs. Alfacs	0.793	2.211	<0.001
R. decussatus vs. *C. gigas*	1.221	3.391	<0.001
2008 vs. 2006	0.449	1.567	0.017
2009 vs. 2006	−0.738	0.478	0.002
2010 vs. 2006	−0.817	0.442	0.002
Constant	−2.159	0.115	<0.001

Method: Forward Stepwise Conditional.
Model summary: −2 Log likelihood = 1116.814; Nagalkerke R Square = 0.090; Omnibus tests of model coefficients, P < 0.001.

TABLE 5 | Description of the logistic regression model selected to explain the presence/absence (P/A) of total (presence of *tlh* gene) *V. parahaemolyticus* removing location.

Variables included in the selected model	*B*	OR (e^B)	*P*-value
R. decussatus vs. *C. gigas*	1.106	3.023	<0.001
2008 vs. 2006	0.782	2.185	<0.001
2010 vs. 2006	−0.707	0.493	0.009
Temperature (°C)	−0.241	0.786	0.001
Salininy (ppm)	0.238	1.269	0.005
Constant	−4.085	0.017	0.232

Method: Forward Stepwise Conditional.
Model summary: −2 Log likelihood = 1117.862; Nagalkerke R Square = 0.088; Omnibus tests of model coefficients, P < 0.001.

showed in the first model (above); thus the probability of finding total *V. parahaemolyticus* in *R. decussatus* was 3.02 times higher than in *C. gigas* ($P < 0.001$). Similar trends (as in the first model, **Table 4**) in total *V. parahaemolyticus* prevalence respect to the year of sampling was demonstrated using year 2006 as a reference. Results obtained in 2008 respect to 2006 indicated that the risk to find total *V. parahaemolyticus* in bivalves was 2.19 times higher in 2008 than 2006 ($P < 0.001$). In 2009, the risk was not significant respect to 2006. However in 2010, the risk decreased significantly and the probability to find total *V. parahaemolyticus* was 2.03 times less than in 2006 ($P = 0.009$).

Presence of total *V. parahaemolyticus* was significantly associated with water temperature ($P = 0.001$) and salinity ($P = 0.005$). The risk of finding bivalves containing *V. parahaemolyticus* decreased 1.27 times with each unit (°C) of increased temperature. Moreover, presence of total *V. parahaemolyticus* was directly correlated with salinity, where the risk to find bivalves containing *V. parahaemolyticus* increased 1.27 times with each unit (ppt) of salinity increased. Shell length ($P = 0.135$) had no significant effect in this logistic regression model.

Multivariate Analysis of Influence of Different Factors on the Presence of Pathogenic *V. parahaemolyticus*

In all cases, no significant model was generated when the whole population of bivalves sampled was studied. However, a significant model to explain the P/A of pathogenic *V. parahaemolyticus* in bivalves contaminated with *V. parahaemolyticus* (*tlh*+) was generated ($P < 0.001$), and it showed that isolation of pathogenic *V. parahaemolyticus* (*tdh* and/or *trh* gene) is significantly associated with water salinity ($P = 0.028$). And, the risk of finding pathogenic *V. parahaemolyticus* decreased 1.45 times with every salinity unit (ppt) increase. When a logistic regression model was performed to explain the P/A of pathogenic *V. parahaemolyticus* (*tdh* gene), no effect of variables was found. However, when pathogenic *V. parahaemolyticus* (*trh* gene) was studied ($P = 0.035$), the risk of finding pathogenic *V. parahaemolyticus* (*trh*+) in mollusks contaminated with *V. parahaemolyticus* decreased 1.64 times with every ppt salinity increase (**Table 6**). Other factors, as bivalve species, length, year, and water temperature were not significantly associated with the presence of potentially pathogenic *V. parahaemolyticus*.

TABLE 6 | Description of the logistic regression model selected to explain the presence/absence (P/A) of pathogenic (presence of *trh* gene) *V. parahaemolyticus* including location, species of bivalve, length, year of campaign, temperature, and salinity.

Variables included in the selected model	*B*	OR (e^B)	*P*-value
Salinity	−0.494	0.610	0.035
Constant	15,470	5230408	0.064

Method: Forward Stepwise Conditional.
Model summary: −2 Log likelihood = 122.733; Nagalkerke R Square = 0.044; Omnibus tests of model coefficients, P = 0.040.

Discussion

The present study examined intraseasonal relationships between selected environmental parameters (temperature and salinity) and the prevalences of total and pathogenic *V. parahaemolyticus* in four different species of bivalves (*C. gigas*, *M. galloprovincialis, R. decussatus,* and *R. philippinarum*) cultured in the Ebro delta in four different years.

In this work temperature and salinity conditions of the bays during each campaign did not suffer big fluctuations since all campaigns took place during the summer (July and August), when *V. parahaemolyticus* is more frequently present and in higher numbers. Nevertheless, differences were found between the two bays. Examination of data indicated that Alfacs Bay presented higher temperatures than Fangar Bay (1 or 2°C higher), due to the basin volume in Alfacs is about ten times larger than in Fangar, needing more time to renew its water (Camp and Delgado, 1987; Montilla et al., 1994). Alfacs bay also presented lower salinities than Fangar bay (around 1 or 2 ppt lower); the salinity of the water is influenced by differential evaporation rates and freshwater inputs from nearby agricultural (rice) fields (Camp and Delgado, 1987; Camp, 1994).

Several studies indicate that *V. parahaemolyticus* in mollusks are significantly correlated with seawater temperature; where, reported temperature ranges varied from: 10 to 33°C (DePaola et al., 2003); 9.9 to 32.7°C (Phillips et al., 2007); 14.4 to 29.2°C (Sobrinho et al., 2010); 7.7 to 29.7°C (Haley et al., 2014); and 7.9 to 25.5°C (Cruz et al., 2015).

Water temperature in Alfacs ranged between 26.93 and 27.67 and in Fangar from 25.41 to 26.37°C. In our model, the temperature was not significantly associated with total *V. parahaemolyticus* presence (**Table 4**), when the model included location. These results agree with those reported by Deepanjali et al. (2005), who observed no statistically significant correlation with tropical seawater temperature from 34 to 24°C, and, with Zimmerman et al. (2007) who did not find any correlation with temperature ranging from 22.4 to 33.8°C either. However, temperature was significantly associated (negatively) with total *V. parahaemolyticus* presence in our model (**Table 5**) when location was removed from the model, which indicated the risk of finding bivalves containing *V. parahaemolyticus* decreased 1.27 times with each unit (°C) that temperature increased. These results should be interpreted with caution because all samples were collected only during the summer season, since previous work had shown no detection of pathogenic *V. parahaemolyticus* during the other seasons of the year (data not shown), therefore it could be suggested that temperature influences *V. parahaemolyticus* levels. However, our results show that salinity in these two semi-enclosed estuarine bays is more important than summer temperature. Salinities at both sampling sites varied between 35.17 and 37.04 ppt during the sampling seasons (ranged between 35.17 and 36.01 and from 35.74 to 37.04°C in Alfacs and Fangar, respectively), which is well above the reported optimum salinity of 23 ppt for *V. parahaemolyticus* growth (Anonymous, 2005). The correlation between water salinity and total *V. parahaemolyticus* densities in bivalves suggests that salinity *per se* is an important factor

for growth of this bacterium. Therefore, this logistic regression model showed (**Table 5**, removing location) the risk of finding total *V. parahaemolyticus* increases 1.27 times with each unit (ppt) of salinity increased. And these results were corroborated in 2008 ($P = 0.001$) and 2010 ($P = 0.090$). These results on a first observation are in agreement with those obtained by Zimmerman et al. (2007), who found that when salinity increased (10 to 28 and 4 to 13 ppt), densities of total *V. parahaemolyticus* increased. On the other hand, other authors as Deepanjali et al. (2005), Martinez-Urtaza et al. (2008), Sobrinho et al. (2010), Kirs et al. (2011), Cruz et al. (2015), and Young et al. (2015), did not find correlation between these parameters; whereas, others authors found the inverse correlation (DePaola et al., 2000, 2003).

As we see above, literature reports contradictory conclusions on the association between salinity and *Vibrio* spp. This apparent contradiction could be due to a narrow range and/or optimal salinity for *V. parahaemolyticus*, so any deviation (higher or lower) from 23 ppt, has an impact in its viability. Moreover, Haley et al. (2014) suggested that salinity played a role in *V. parahaemolyticus* presence, even though it did not show up as significant in their model, and/or that an unmonitored parameter present in the estuarine environments could have influenced the *V. parahaemolyticus* presence.

In our study prevalences of total *V. parahaemolyticus* in Fangar Bay (with lower temperatures and higher salinities than Alfacs Bay) were higher (16.7%) than in Alfacs Bay (11.6%; $P = 0.005$). These results are agreement with our models (**Tables 4** and **5**) which showed the risk of finding total *V. parahaemolyticus* in mollusks from Fangar Bay was 2.21 times higher than from Alfacs Bay and the environmental conditions correspond to a narrow range of high temperatures and salinities, play an important role.

Several studies have shown that *V. parahaemolyticus* levels appear to fluctuate independently from temperature and salinity, clearly showing that these factors are not the only ones that influence the bacterium's abundance and distribution. Factors such as plankton composition, dissolved oxygen, particulate organic matter availability, and chlorophyll a, presence of fish and shellfish, as well as to levels of freshwater flows and the depth of the harvesting area though may be involved in *V. parahaemolyticus* prevalence (McCarter, 1999; Phillips et al., 2007; Zimmerman et al., 2007, Vezzulli et al., 2009; Deter et al., 2010; Julie et al., 2010; Ceccarelli et al., 2013).

Previous studies which indicated that only 1–3% of the environmental strains produce TDH or contain the *tdh* gene (Kelly and Stroh, 1988; DePaola et al., 1990; Nair et al., 2007; Cruz et al., 2015) or they are not detected (Shirai et al., 1990; Honda and Iida, 1993). In the present study pathogenic *V. parahaemolyticus* prevalences observed and analyzed by years show that higher prevalence was registered in Fangar Bay (8.6%, of which 6.7% were *tdh*-positive) in 2008, and this result may be due to an increased prevalence of total *V. parahaemolyticus* that year. No significant differences were observed in Alfacs ($P = 0.747$), in contrast to Fangar Bay ($P = 0.004$); And, although this results may be due to a type II error, failing to find differences where they exist, it is unlikely taking into account the considerable amount of data taking analyzed in this study.

Our finding are in agreement with those of DePaola et al. (2003) who indicated a higher prevalence (12.8%) of *tdh*-positive *V. parahaemolyticus* in Alabama oysters determined by direct plating, and also with those of Deepanjali et al. (2005) who found similar prevalences of *tdh*-positive *V. parahaemolyticus* (10.2%) in Oysters from India by colony hybridization. In the present study the highest prevalence observed in *V. parahaemolyticus* strains harboring the *trh* gene was 1.4% in both bays. Other studies did not detect the presence of *V. parahaemolyticus* carrying the *trh* virulence gene in their samples (Kirs et al., 2011; Cruz et al., 2015). While, Deepanjali et al. (2005) have indicated a high prevalence of *trh*-positive *V. parahaemolyticus* in oysters.

When logistic regression model to explain the P/A of each gene (*tdh*/*trh*) was performed in relation with the different factors analyzed, only *trh*-positive *V. parahaemolyticus* correlates (negatively) with water salinity (**Table 6**), suggesting that strains carrying this gene are more sensitive to salinity or that the optimal salinity for strains containing the *trh* gene is lower than for other strains of this species.

Conclusion

Our results indicate temporal and spatial variations in the prevalences of total and pathogenic *V. parahaemolyticus* in both bays and in the bivalve mollusks. Apparently, *V. parahaemolyticus* populations in bivalves are controlled quantitatively and qualitatively by different factors. It seems unlikely that selective filtration of non-pathogenic to pathogenic *V. parahaemolyticus* could affect for the magnitude of different concentrations in bivalves at the two sites (Genthner et al., 1999; Anonymous, 2005). Higher prevalences of *V. parahaemolyticus* were registered in *R. decussatus* and in *R. philippinarum*, which may indicate either a potential host effect or an effect due to culture systems, since oysters and mussels are grown in suspended ropes, clams are grown on the floor bed. It is also possible that the filtration rate of clams, under the sampling conditions, were closer to optimal when compared to mussels and oysters which would contribute to higher filtration rate in clams with consequent accumulation of bacteria.

Present study collected a considerable amount of data on the presence of total and pathogenic *V. parahaemolyticus* over a long period of time which makes this data robust. Although these data were collected in one area of the Mediterranean coast, similar environmental conditions of those of the Mediterranean coast can be found in places like California (USA), Southern Australia, Central Chile, and the western cape of South Africa. Both Australia and the USA have legislation for the safety of bivalves for consumption which includes pathogenic vibrios indicating that present data can be very useful when performing a risk analysis for assessing the consequences of *V. parahaemolyticus* presence in commercial bivalves in the Mediterranean countries.

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