Chapter 2

Review of Literature

2.1 Review of Literature

V. parahaemolyticus was the first time reported as a food born pathogen responsible for infection of 276 people after consumption of sardines in Japan in the year 1976 (Fujino, 1974). This bacterium broadly divided into two categories pathogenic or clinical strain and non-pathogenic or environmental strain. Depending upon the presence of virulent gene the pathogenic strain is again three different types *tdh* positive, *trh* positive and both *tdh*, *trh* positive strain. After this first report, this bacterium was frequently isolated from different countries of Asia associated with food borne infection.

In India the pathogenic strain of *V. parahaemlolyticus* was the first time emerged in the history as a pandemic strain in 1966 in Calcutta. This was found to be associated with a novel strain O3:K6 (Okuda et al., 1997). Since then, it moves to America, Europe, Australia, New Zeeland, Russia and Alaska (Nair et al., 2007). Trade-in live aquatic animals and their products accelerated the spread of these bacteria globally. The symptoms include dysentery, diarrhea, abdominal pain, nausea, chills, vomiting, fever, water-like stools and stools with blood (Yeung et al., 2004; Shimohata and Takahashi, 2010).

2.2 Epidemiological study on *Vibrio parahaemolyticus*

2.2.1 Asia

Vibrio parahaemolyticus was the first time recognized in the year 1951 in Osaka associated with foodborne infection. 272 infected cases and 20 deaths associated with the consumption of raw or uncooked seafood was reported from Japan (Broberg et al., 2011). 40 to 800 outbreak cases associated with *V. parahaemolyticus* contaminated seafood, affecting about 1,200 to 12,000 persons annually during 1991-2007 were reported from Japan (Infectious Agents Surveillance Report [IASR], 2008). 1500 outbreaks incidences associated with *V. parahaemolyticus* were reported in each year from 2000-2009. In 67.7 % of cases showed the presence of O3:K6 serotype of *V. parahaemolyticus* is responsible for a disease outbreak in Japan (Toyofuku, 2014). *V. parahaemolyticus* is also a leading foodborne pathogen in China since 1990. 165 clinical strain of *V. parahaemolyticus* was isolated through routine screening in Shanghai Municipal Center for Disease Control and Prevention, in China, during 2012-2014 (Guo et al., 2013). Disease outbreak caused by *V. parahaemolyticus* in Taiwan was reported by several workers at different time (Chiou et al., 2000; Guo et al., 2013). Chiou et al. (2000) found that 542 out of 850 outbreaks during 1995-1999 in Taiwan were caused by 40 different serovars of *V. parahaemolyticus.* From 1995 to 2001, 2057 cases of infection caused by *V. parahaemolyticus* in northern Taiwan were reported by Su et al. (2005). This study identified 99.4 % of *V. parahaemolyticus* strains by K serotyping, out of which 55.2 % was represented by K6 serovar. Infection by *V. parahaemolyticus* is quite common in South Thailand. In 1998, 23 pandemic strains were isolated from Songkhla Province, southern Thailand (Vuddhakul et al., 2000). Again 317 pandemic strains were isolates

from Songkhla Province, Thailand in the year 1999 (Laohaprertthisan et al., 2003). A total of 865 isolates of *V. parahaemolyticus* was obtained from patients at Hat Yai Hospital, Songkhla Province, Thailand during 2000-2005 (Wootipoom et al., 2007). Nair et al. (2007) studied the sudden outbreak of infections associated with *V.parahaemolyticus* in Calcutta in 1996. The study showed that 63 % of the strains isolated from patients between September 1996 and April 1997 belonged to serotype O3:K6. During 2001-2012, 178 strain of *V. parahaemolyticus* belongs to six different serotypes (O3:K6, O1:K25, O1:KUT, O3:KUT, O4:K8, and O2:K3) were isolated from 13,607 patients admitted in the Infectious Diseases Hospital, Kolkata (Nair et al., 2007) Association of *V. parahaemolyticus* was reported from patients suffered from diarrhea of KhanhHoa province, Vietnam during 1997 to 1999 and 523 pandemic strains of *V. parahaemolyticus* was isolated during the period (Chowdhury et al., 2004).

2.2.2 North America

V. parahaemolyticus was first identified to be a causative agent and responsible for outbreaks of 425 gastroenteritis cases after consumption of undercooked crabs in Maryland, U.S. (Molenda et al., 1972). Serotype O4:K12 was responsible for the outbreak of gastroenteritis in Washington and Oregon due to the consumption of raw oysters from Willapa Bay, Wash in 1981 (Nolan et al., 1984). Altogether 34 cases, which include one case of septicemia, 26 cases of gastroenteritis, and 6 wound infections have been reported in surveillance from four Gulf Coast states *viz.* Alabama, Florida, Louisiana, and Texas by the end 1988 (Levine and Griffin, 1993). A total of 345 cases of *V. parahaemolyticus* infection from Florida, Alabama, Louisiana, and Texas were reported to the CDC by the Gulf Coast *Vibrio* Surveillance System from 1988 to 1997

(Daniels et al., 2000). In 1997, an outbreak of 209 infection cases occurred in North America after consumption of oysters procured from coastal waters of Oregon, California, British Columbia, and Washington. The pandemic strain of *V. parahaemolyticus* (O3:K6) was identified for the first time when 416 persons from 13 states fell sick from gastroenteritis after consumption of oysters harvested from Galveston Bay in 1998 (Daniels et al., 2000). In the year 1998, another incident of the outbreak of gastroenteritis associated with O3:K6 serotype was reported from Connecticut, New Jersey, and New York (Wong et al., 2000). The presence of O3:K6 serotype was also identified in coastal waters of the United States. In 2006, a total of 177 cases of outbreak occurred in New York, Oregon and Washington of which 72 cases were confirmed due to *V. parahaemolyticus*. In Mexico and Alaska, the outbreak of gastroenteritis caused by *V. parahaemolyticus* was reported for the first time in 2004. 62 people were infected due to the consumption of raw oysters harvested from Alaskan waters. Survey over 6 years in clinical case and environmental samples detected the presence of pandemic and pathogenic strains of *V. parahaemolyticus* (Velazquez-Roman et al., 2014).

2.2.3 South America

V. parahaemolyticus (O5:K17) was identified for the first time in 1975 from a 6-year old child in Ceara, Brazil, South America which was suffering from watery diarrhea (Hofer, 1983). Later, 21 cases of gastroenteritis due to the consumption of contaminated seafood were reported in Recife, Northeast Brazil in the year 1991. In the year 1992, 18 human isolates carrying *tdh*, *trh* or both were isolated from Brazil. The presence of pandemic clone (O3:K6) was also detected in two outbreaks in northeast Brazil.

Furthermore, two cases of the outbreak in the year 2003 and one case of an outbreak in the year 2006, affecting 39 patients were reported by SobrinhoPde et al. (2010) and SobrinhoPde et al. (2011). The infection associated with *V. parahaemolyticus* was also reported from Chile and Peru. In 1998, 300 cases of infection in Antofagasta and approximately 7,000 cases of infection during 2004-2007 in Puerto Montt, Chile due to the consumption of contaminated seafood were reported by Cordova et al. (2002). Until 2006, serotype O3:K6 of *V. parahaemolyticus* was associated with the infection but later, the emergence of new serotype O3:K59 has been reported. Approximately 1500 individuals were infected with *V. parahaemolyticus*, predominantly occurred in the cold water coastal of PuertoMontt. Till 2006, most of the infection in Chile was associated with O3:K6 pandemic strain, but in 2007, 40 % of 477 cases of infection were reported to be associated with O3:K59 serotype. Furthermore, 3640 cases of gastroenteritis infection associated with different serotypes of *V. parahaemolyticus* were reported by Ministry of Health of Chile in the year 2008.

Sudden rising of infection by a pandemic strain of *V. parahaemolyticus* was observed along the entire coastline of Peru (Martinez- Urtaza et al., 2008). In 1998, 27 clinical strain of *V. parahaemolyticus* was isolated from a patient in Limaand. Pandemic strains (O3:K6 and others) were also detected during 1998-2002 in Lima and Trujillo (Gil et al., 2007). The outbreak of infection associated with O3:K59 serotype was reported in Lambayeque, Piura and Limaregions in Peru in 2009 (Zamudio et al., 2011). Later, 56 clinical strains of *V. parahaemolyticus* were isolated during the year 1994 to 2007 (Gavilan et al., 2013).

2.2.4 Europe

V. parahaemolyticus infections are common in Spain and were isolated from infected patients in Madrid (1998 and 2000), Zaragoza (1993) and Barcelona (1986, 1987, and 1999). In the year 1999, 64 cases of *V. parahaemolyticus* infection due to the consumption of contaminated oyster was reported in the year 1999 (Lozano-Leon et al., 2003). In 1997, a serious outbreak of infection associated with *V. parahaemolyticus* was reported from France. Later, eight cases of acute gastroenteritis caused by *V. parahaemolyticus* in humans were reported in Spain in 1989 (Lozano-Leon et al., 2003). In the year 1999, a total of 64 cases of acute gastroenteritis associated with the consumption of oysters were identified in Galicia (northwest Spain). Most of the clinical isolates were O4:K11 serotype. Furthermore, serotype O3:K6 and O3:K (untypeable) of *V. parahaemolyticus* associated with an outbreak of 80 cases of infection was reported from Coruña, Spain in the year 2004 (Martinez-Urtaza et al., 2005).

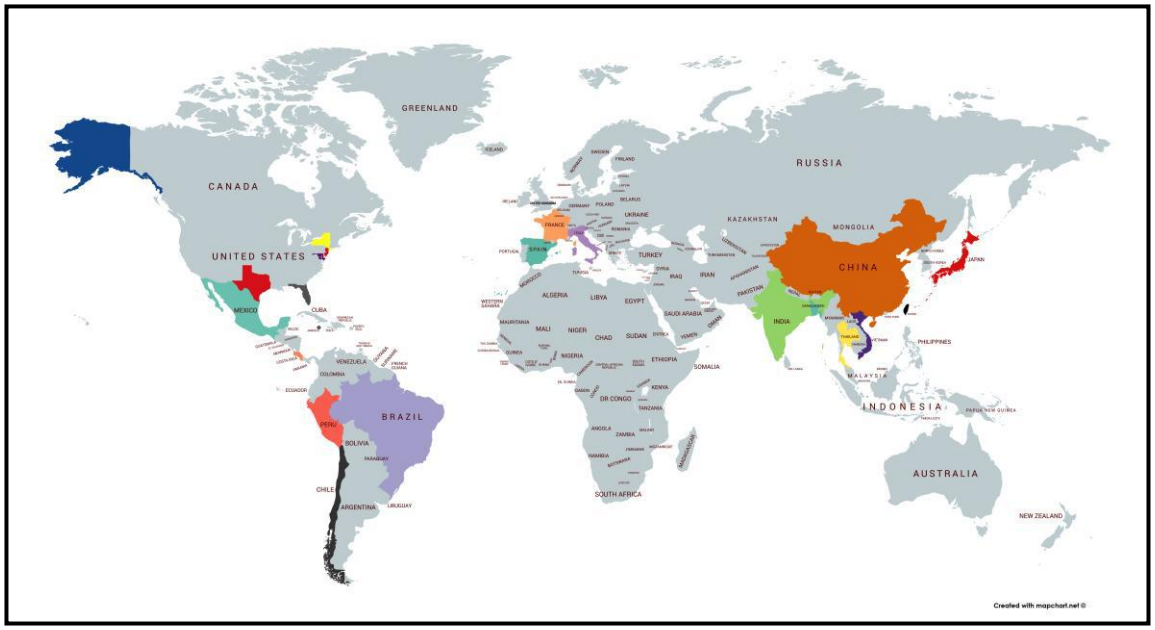

Figure 2.1 Map showing infection caused by *V. parahaemolyticus* globally. Different countries are labeled with different colors. Map prepared by using Web tool (https://mapchart.net/world.html)

2.3 Serotypes of *Vibrio parahaemolyticus*

V. parahaemolyticus can be classified based on the antigenic properties of the somatic (O) and capsular (K) antigen formed under different environmental conditions (Nair et al., 2007). 13 O serotypes and 71 K serotypes of *V. parahaemolyticus* has been identified from environmental samples (Iguchi et al., 1995). In 1996, a unique pandemic serotype O3:K6 was isolated from human in Kolkata, India. Similar serotype of *V. parahaemolyticus* was isolated from different samples in Laos (1997), Vietnam (1997), Indonesia (1997), Korea (1997-1998), United States (1997-1998), Chile (1998 and 2004), Taiwan (1996-1999), Bangladesh (1998-2000), Japan (1998), Thailand (1999), Russia (2001), Mozambique (2004), France (2004) (Nair et al., 2007). A pandemic strains with different serotypes O4:K68 and O1:K25 was reported from India (1998), Thailand (1999) Vietnam (1998-1999) Bangladesh (1999-2000) (Nair et al., 2007). In addition to O4:K68 and O1:K25, another serotype O1:KUT was reported from India (1998) and Bangladesh (2000) (Chowdhury et al., 2000; Bhuiyan et al., 2002). Five different serotypes O1:K56, O3:K75, O4:K8, O4:KUT and O5:KUT were reported from Vietnam during 1998 to 1999 (Chowdhury et al., 2004). Furthermore, nine new serotypes O5:K25, O5:K17, O2:K3, O1:K33, OUT:KUT, O3:K5, O3:KUT, O4:K10 and O4:K4 were reported from India during 2004-2006 (Nair et al., 2007). A new serotype O6:K18 was reported from Taiwan in the year 2005 (Wong et al., 2005). 21 different serotypes of *V. parahaemolyticus* were identified during 1996 to 2006 and showed unique sequences corresponding to genes like *toxRS* and open reading frame 8 (*orf8*). The serotypes O1: K25, O4: K68, O6:K18 and O1:KUT showed high molecular identity with O3:K6 serotype isolated from Taiwan (Wong et al., 2005). Before 2006, O3:K6 pandemic strain

was isolated mainly from human and shellfish in Southern Chile but Harth et al. (2009) found the emergence of a new serotype O3:K59. A survey carried out at Southern coastal region of China during 2007-2012 revealed the presence of 47 different serotypes, out of which most common serotype was O3:K6, followed by serotypes O3:K29 and O4:K8 (Li et al., 2014). 11 new serotypes O2:K28, O5:K17, O5:KUT, O4:K34, O10:KUT, O2:KUT, O1:K32. O5:K17, O2:KUT, O11:K40 and O13:KUT of *V. parahaemolyticus* was isolated from clinical and environmental samples in China from 2006 to 2014. 10 different serotypes O4:K8, O5:KUT, O3:K6, O3:K58, OUT:KUT, O6:KUT, O1:K33, O1:KUT, O3:K30 and O3:KUT was reported from Peru during the period, 1994-2007 (Gavilan et al., 2013). Pazhani et al. (2014) reported three new serotypes O3:KUT, O4:K8, and O2:K3 from India.

2.4 Genetic diversity of *Vibrio parahaemotyticus*

Genetic diversity or genetic variability within a species is a spontaneous process that led to the development of species with new traits that undergo a natural selection process. Spontaneous mutations, acquisition of mobile genetic elements, transposable elements, are the natural eventsby which bacteria acquire new traits. These new traits increased their fitness to adapt to a changing environment, increase their virulence potential and drug-resistant property. *V. parahaemolyticus* is a much-diversified species where 54 different serotypes and 21 different K serotypes have been reported throughout the world. Clinical strain, pathogenic environmental strain, and non-pathogenic environmental strain are reported from different parts of the world.

2.4.1 Diversity study by using RFLP

Restriction fragment length polymorphism (RFLP) analysis has a wide diversity of applications and in most of the cases; it is used in combination with other methods (Yeung et al., 2002; Wong et al., l999). In RFLP analysis, restriction enzymes digested genomic DNA and through agarose gel electrophoresis, the resulting fragments are separated according to size. It is similar to PFGE however; sophisticated gel-electrophoresis apparatus is not required.

Restriction fragment length polymorphism of PCR products encoding thermostable direct hemolysin (*tdh*) and thermostable direct hemolysin-related hemolysin (*trh*) of 137 strains of *V. parahaemolyticus* was used by Suthienkul et al. (1996) to understand the genetic diversity among the isolates. Marshall et al. (1999) used ERIC PCR and restriction fragment length polymorphisms (RFLP) in rRNA genes and Fla locus for differentiating and typing of *V. parahaemolyticus* and found that (RFLP) in *rRNA* genes will be useful for the evaluation of genetic and epidemiological relationships among *V. parahaemolyticus.* Bag et al. (1999) used RFLPs and pulsed-field gel electrophoresis of the rrn operons to understand the clonal diversity among the O3:K6 strains isolated in Calcutta, India. Chowdhury et al. (2000) used RFLP to understand the genetic relatedness of O3:K6 and O4:K68 strains isolated from Calcutta and Bangkok. Both the strains showed nearly similar restriction fragment patterns. RFLP of 16S rRNA was also used for the identification of *Vibrio* sp. and found RFLP of 16S rRNA is used to identify the six pathogenic *Vibrio* species (Yoon et al., 2003). Fuenzalida et al. (2006) used direct genome restriction enzyme analysis (DGREA) and RFLP-PFGE to understand the genetic diversity of *V. parahaemolyticus* isolated from shellfish and

clinical samples during epidemics and found that the *V. parahaemolyticus* isolated from clinical samples were O3:K6 pandemic clonal group which was highly diverse within the species. RFLP analysis of core OS and O-side chain gene of *V. parahaemolyticus* was used to understand the relationship between O4:K68 and O3:K6 serotype and revealed that the substitution of the putative O and K antigen genes in pandemic O3:K6 strain leads to emergence of pandemic O4:K68 strain. Later, Elola-Lopez et al. (2015) used PCR-restriction fragment length polymorphism (PCR-RFLP) of MAM-7 virulence gene to differentiate between clinical and environmental isolates of *V. parahaemolyticus* strains and found that digestion of PCR productof MAM-7 gene with *Hind*III and *Acu*I restriction enzymes generate 100 % unique band pattern of clinical isolates of *V. parahaemolyticus.*

2.4.2 Diversity study using RAPD

Random Amplification of Polymorphic DNA (RAPD) is an important molecular typing method used for understanding the genetic diversity of different bacterial species. The genetic diversity of *V. parahaemolyticus* isolated from the clinical and environmental samples in Taiwan was analyzed by Wong et al. (1999) using RAPD. Bilung et al. (2005) showed a high level of genetic diversity within the *V. parahaemolyticus* strains isolated from local cockles (*Anadara granosa*) in Malaysia using RAPD. Similarly, Sahilah et al. (2014) also used RAPD-PCR to understand the genetic diversity of *V. parahaemolyticus* isolated from cockle (*Anadara granosa*) in Malaysia. RAPD was used by Lu et al. (2006) to understand the genetic diversity of *V. parahaemolyticus* isolated from shellfish from a retail source in China. Mahmud et al. (2006) carried out a genetic diversity study of *V. parahaemolyticus* isolated from Japan by RAPD and found that all the isolates belonging

to the O3:K6 serotype generate unique band pattern. Parvathi et al. (2006) also used RAPD to understand the genetic diversity of *trh* bearing *V. parahaemolyticus* isolated from the southwest coast of India. Later, Bhowmick et al. (2008) used three different molecular typing methods *viz*. RAPD, ERIC-PCR and protein profiling for *V. parahaemolyticus*, isolated from different seafood along the southwest coast of India and found that RAPD and ERIC-PCR have a potential discriminative ability and can be used for molecular typing of *V. parahaemolyticus*. Silvester et al. (2016) used RAPD to analyze the genetic diversity of *V. parahaemolyticus* isolated from environmental samples along the southwest coast of India and tremendous genomic variationwas observed. Leal et al. (2008) used RAPD to analyzegenetic relation of two different serotypes O3:K6 and O3:KUT of *V. parahaemolyticus* involved in the outbreak of diarrhea in the northeast region of Brazil. Furthermore, genetic diversity among *V. parahaemolyticus* isolated from seafood products in two coastal areas of eastern China was analyzed by using RAPD. Based on RAPD pattern, all the *tdh* positive isolates form two groups whereas *trh* positive isolates from one group (Yang et al., 2008). Islam et al. (2004) used RAPD to understand the clonal variation among three different serotypes *viz*. O3:K29, O4:K37 and O3:K6 and exhibited three different RAPD patterns for three different serotypes, though the pandemic O3:K6 strains generate a unique RAPD pattern.

2.4.3 Diversity study using PFGE

A rapid PFGE protocol for subtyping *Vibrio parahaemolyticus* was generated using the knowledge of PulseNet protocols for *Escherichia coli* O157:H7 and *Vibrio cholera*. This protocol was evaluated in three different laboratories in the USA and found

that the protocol is reliable and reproducible for molecular subtyping of *V. parahaemolyticus* (Parsons et al., 2007)

A total of 130 selected isolates obtained from outbreaks during 1993-1994 in Taiwan were also characterized by this PFGE method and showed a clonal relationship with Japanese strain (Wong et al., 1996). Similarly, PFGE analysis with *Not*I and *Sfi*I differentiated the European isolates in two different cluster included in a homogeneous cluster and are clearly different from the Asian and North American isolates (Martinez-Urtaza et al., 2004). The majority of patients suffering from gastroenteritis in the United States were due to infection by *V. parahaemolyticus* serotype O6:K18. The PFGE analysis of the clinical isolates and the environment isolated from oysters from Alaska showed a very close relation (McLaughlin et al., 2005). The genetic diversity study of 535 strains of *V. parahaemolyticus* which consisted 336 strains isolated from Taiwan and rest of 197 strains isolated from 14 other countries by pulsed-field gel electrophoresis following *Sfi*I digestion showed that most of the strains were genetically closely related to pandemic O3:K6 strain (Wong et al., 2007). PFGE with NotI was used to characterize 95 *V. parahaemolyticus* isolates and found that all the pandemic isolates belonged to three serovars (O1:K25, O3:K6, O1:KUT) and a closely related new serovar O3:K46 (Serichantalergs et al., 2007). Subtyping of 36 well-characterized *V. parahaemolyticus* using PFGE with two restriction enzymes (*Sfi*I and *Not*I) confirmed the method has high discriminatory power (Kam et al., 2008). Wang et al. (2008) also recommended two restriction enzymes (*Not*I and *Sfi*I) for molecular typing and global surveillance of *V. parahaemolyticus*. The genetic diversity study of 246 isolates of *V. parahaemolyticus* from Norway using PFGE analysis with *Not*I discriminated into 72 clusters. All the *tdh*

and *trh* positive isolates form a separate cluster from *tdh* and *trh* negative isolates (Ellingsen et al., 2008). Antibiotic-resistant *V. parahaemolyticus* isolated from the sea the food in Hebei province of China was clustered into 5 types (Liu et al., 2009). Molecular typing by pulsed-field gel electrophoresis of 107 clinical strains of *V. parahaemolyticus* from China indicated divergence among the clinical strains (Wang et al., 2017). The intra-species variability and genetic diversity of *V. parahaemolyticus* isolated from different European countries were also analyzed by pulsed-field gel electrophoresis (PFGE) following *Not*I digestion (Suffredini et al., 2011). Genetic diversity study of *V. parahaemolyticus* was carried out using PFGE with a pandemic or non-pandemic pathogenic isolates from a specific geographical area like Thailand (Gavilan et al., 2013). The pandemic isolates collected from Thailand belonged to three different serotypes *viz*. O3:K6, O1:K25, O1:KUT and a new serotype O3:K46. All the pandemic isolates were closely related and clearly distinct from the non-pandemic isolates. Genetic diversity of *Vibrio parahaemolyticus* which were collected from the hospital in Spain and the pandemic clone isolated in Asia and North America were studied using PFGE by Martinez-Urtaza (2004). The study showed that, most of the isolates collected from the hospital in Spain belonged to serotype O4:K11 and was clearly differentiated from the Asian and North American isolates.

2.4.4 Multilocus sequence alignment

Candidate housekeeping genes were used in multi-locus sequence typing analysis for characterizing strains by their unique allelic profiles. In 1998, multi-locus sequence typing (MLST) was proposed for epidemiological surveillance and population studies of pathogenic bacteria. It was developed by Maiden et al. (1998) for the naturally

transformable Gram-negative pathogen *Neisserria meningitidis.* Later, MLST genotype were used for many pathogenic bacteria *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Campylobacter jejuni*, *Streptococcus pyogenes*, *Haemophilus influenzae*, *Helicobacter pylori*, *Salmonella typhi*, *Listeria monocytogenes*, *Escherichia coli*, *Vibrio cholerae* including *Vibrio parahaemolyticus* (Urwin and Maiden, 2003; Cooper and Feil, 2004). Genetic diversity study with MLST of *V. parahaemolyticus* strains isolated from clinical and environmental sources of Pacific and Gulf coasts of the United States shows high genetic diversity. *V. Parahaemolyticus,* isolated from the Pacific and Gulf coasts of the United States, formed separate clonal complexes and a third clonal complex which consisted of strains belonging to the pandemic clonal complex (Gonza´lez-Escalona et al., 2008). Similarly, Multilocus Sequence Typing of pandemic, pathogenic and nonpathogenic strains isolated from China was carried out by Chao et al. (2011) and found that O3:K6 pandemic strains shared the same genetic makeup with international pandemic strains and belonged to the clonal complex ST-3. Gavilan et al. (2013) used Multilocus Sequence Typing (MLST) and the presence of Variable Genomic Regions to understand the population genetics and genomic variation in pandemic and non-pandemic strains isolated from Asia Peru, and Chile. was carried out to understand the population structure of 22 Norwegian isolates of *V. parahaemolyticus* were analyzed by MLST and found 15 different sequence types (STs) consisting of 12 sequence types which were not reported earlier in the MLST database of *V. parahaemolyticus*. They also found two new clonal complexes, indicating a high genetic diversity among these isolates (Bauer Ellingsen et al., 2013). The Population structure of clinical and environmental *V. parahaemolyticus* isolated from the Pacific Northwest Coast of the United States were

characterized by using Repetitive extragenic palindromic PCR (REP-PCR) and Multilocus Sequence Typing (MLST) The Pacific Northwest population exhibited a semi-clonal structure attributed to an environmental clade clonally related to the pandemic O3:K6 complex. The clinical clade genetically closely related to endemic O4:K12 serotype. Later, the identification of five additional clinical sequence types clearly demonstrates that *V. parahaemolyticus* involve in gastroenteritis in the Pacific Northwest is polyphyletic in nature (Turner et al., 2013). Han et al. (2014) used multilocus sequence for the analysis of the genetic diversity of clinical *V. parahaemolyticus* collected from 17 coastal countries showed high-level genetic diversity. The global epidemic clone of *V. parahaemolyticus* (CC3) first emerged in India in 1996. Later, it emerged in other Asian countries, the American continent, Europe, and even in Africa. The MLST analysis of 100 clinical strains of *V. parahaemolyticus* isolating from an incidence of human illness in Canada from 2000 to 2009 resulted in 27 sequence types (STs). ST36 was most common among sequence types and is a significant sequence type which belonged to serogroup O4:27 (Banerjee et al., 2014). Furthermore, to understand the population structure, 130 environmental isolates of *V. parahaemolyticus* from various geographical locations like Sri Lanka, Ecuador, North Sea, Baltic Sea and German was subjected to MLST which resulted into 82 unique Sequence Types revealing high genetic diversity of *V. parahaemolyticus* (Urmersbach et al., 2014).

2.5 Pathogenicity of *V. parahaemolyticus*

2.5.1 Adhesion factor

Attachment to the surface of the host cell is an important criteria for translocation of effector proteins into the cytoplasm of the host cell. Again, pathogenic bacteria attach to the surface of the host cell to trigger the signaling cascade to facilitatethe pathogen invasion. Different adhesion factors were identified in *V. parahaemolyticus* that play an important role in the attachment of bacteria to host cell surface. Krachler et al. (2011) identified a multivalent adhesion molecule (MAM) which is present in many gram-negative pathogens. In the early stages of infection, MAM enables high-affinity binding to host cells. MAM7 binds with fibronectin and phosphatidic present in the host cell by protein-protein and protein-lipid interactions, respectively. Whole-genome of *V. parahaemolyticus* revealed the presence of two *icmF* family genes under type VI secretion system (vpT6SS) (icmF1) and 2 (icmF2) out of which IcmF1 play a significant role in the adhesion of bacteria to Caco-2 cells and HeLa monolayers and this clearly indicated that the virulence protein from T6SS systems plays an important role in the adhesion of *V. parahaemolyticus* to host cells (Yu et al., 2012). The mannose-sensitive haemagglutinin (MSHA) pilus was identified in *V. parahaemolyticus* by O'Boyle et al. (2013). MSHA plays a significant role in the adherence of bacteriawith Caco-2 and human intestinal epithelial cells. Jiang et al. (2014) identified enolase in the extracellular, outer membrane (OM) and cytoplasmic protein fractions of *V. parahaemolyticus*. Enolase has the ability to bind human plasminogen-binding protein and thus plays an important role in the adhesion of *V. parahaemolyticus* with epithelia. Recently, a novel adhesin protein VpadF was identified in *V. parahaemolyticus* which can be recognized and bind

both fibronectin and fibrinogen. Binding with fibrinogen helps bacteria to penetrate host barriers and spread in tissues (Liu et al., 2015).

2.5.2 Hemolysin

Hemolysin is a major virulence factor in *V. parahaemolyticus*. To date, three different types of hemolysin gene [thermolabile hemolysin (*tlh*), thermostable direct hemolysin (*tdh*) and thermostable direct-related hemolysin (*trh*)] were reported from *V. parahaemolyticus*, out of which Tdh and Trh are major virulent factors. Most of the clinical isolates carry *tdh*, *trh* or both. The Tdh has different biological activities like hemolytic activity, cytotoxicity, cardiotoxicity, and enterotoxicity (Raghunath, 2015). It is a pore-forming protein and creates ~2 nm diameter pore in the red blood cell. Tdh is a reversible amyloid toxin, inactivated when heated at 60-70 °C and reactivated by additional heating above 80 °C (Fukui et al., 2005). Trh is a heat-labile protein but shares similar antigenic properties (Honda et al., 1988). In contrast to *tdh*, *trh* positive *V. parahaemolyticus* do not produce characteristic Kanagawa phenomenon in Wagatsuma agar plate (Honda et al., 1988). The *tdh* and *trh* genes share 70 % homology (Kishishita et al., 1992). The *trh*-positive strain of *V. Parahaemolyticus* exhibited enterotoxic effects which lead to fluid accumulation in the intestines (Xu et al., 1994). The Trh protein increases the concentration of Ca^{2+} inside the cell. Ca^{2+} acts as a secondary messenger that activates chloride channels and thus increases Cl^{2-} secretion from cell. This in turn causes watery diarrhea (Takahashi et al., 2000).

2.5.3 Proteases

Extracellular bacterial proteases are also an important virulence factor. *V. parahaemolyticus* also produces different types of proteases with various pathogenic

roles like cytotoxic, collagenolytic, hemolytic and edema-forming activity (Lin et al., 2017; Miyoshi et al., 2008; Miyoshi, 2013; Osei-Adjei et al., 2018). The extracellular protease from *tdh* and *trh* negative clinical strain of *V. parahaemolyticus* was 1st time purified by Lee et al. (2002) which showed significant effects on the growth of HeLa, Chinese hamster ovary, Caco-2 and Vero and also lysed erythrocytes. Metalloproteases are able to degrade collagen, gelatin and other proteins, therefore, destroy the extracellular matrix of the host cells. *V. parahaemolyticus* expresses different types of metalloproteases like PrtV (Lee et al., 2002; Miyoshi, 2013), VppC (Kim et al., 2002; Miyoshi, 2013) and VPM (Luan et al., 2007). *V. parahaemolyticus* also produces different types of serine proteases like VPP1/Protease A (Miyoshi et al., 2012; Miyoshi, 2013), VpSP37 (Salamone et al., 2015), PrtA (Chang et al., 2017) which are able to digest collagen, an important component of the extracellular matrix. Digestion of the components of the extracellular matrix accelerates the spread of bacteria to the neighboring tissue (Miyoshi, 2013).

2.5.4 Type III secretion system

Type III secretion systems are complex molecular machine present in gram-ve bacteria. It is a syringe-like apparatus upon contact with the host cell; they inject effector proteins from the bacterial cytosol to the cytoplasm or plasma membrane of the host cell. This type of machinery was first time reported in *Yersinia pestis*. These effector proteins modify the host innate immunity, cytoskeleton and signal transduction system which helps in colonization and persistence of bacteriain within the host cell. *V. parahaemolyticus* have type III secretion system 1, Type III secretion system 2 and Type III secretion system 6.

2.5.4.1 Type III secretion system 1

Vibrio parahaemolyticus has type III secretion system 1, Type III secretion system 2 and Type III secretion system 6. Two sets of gene for type III secretion system were identified i.e., TTSS1 and TTSS2 in a clinical isolate of *V. parahaemolyticus* strain RIMD 2210633 by Park et al. (2004). A set of genes were present within TTSS1 and TTSS2 region which involve in the cell cytotoxicity. They also found that TTSS1 of *V. parahaemolyticus* has a similarity with *Yersinia* spp. There are 12 hypothetical genes that existed between *vscU1* and *vscL1* which is not present in Yersinia TTSS and 13 genes comprising *vscA1* to *vscL1*, *virG1* and *virF1* were encoding protein in a direction opposite to those of *Yersinia* spp. The study also reported that TTSS 1 genes are involved in the cytotoxicity whereas TTSS2 involve in the enterotoxicity. VopD, a protein encoded from TTSS1 region, homologous to the YopD and VopP protein encoded by pathogenic island on chromosome 2 of *Yersinia* sp. Later, Ono et al. (2006) identified four proteins namely Vp1680, VPA450, Vp1686 and Vp1656 under T3SS2. VPA450 encoded by chromosome 2 showed 93 % identity with Plu4615 protein present in *Photorhabdus luminescens*, a pathogen of insect. The protein Vp1656 which renamed as VopD showed 33 % identity with PopD protein of *P. aeruginosa* and 30 % identity with YopD protein of *Y. enterocolitica*. A study carried out by *vopD* and *vopB* deletion mutants shows VopB and VopD are involved in the contract hemolytic and cytotoxicity similar to YopD and YopB in *Y. enterocalitical* (Neyt and Cornelis, 1999; Naim et al., 2001) The N-terminal sequence of Vp 1680 and Vp 1686 showed that two proteins present in the hypothetical region of the TTSS1 gene cluster of chromosome 1. A functional study was carried out by fluorescence-activated cell shorting with fluorescein isothiocyanate-labeled annexin

and a series of deletion mutants showed that Vp1680 has involved in TTSS1 dependent cytotoxicity and the major one to cause apoptosis. Bhattacharjee et al. (2006) identified another protein Vp1686, under TTSS1 of *V. parahaemolyticus* which belongs to the Fic protein family and areable to induce Toll-Like Receptor independent apoptosis by disrupting NF-kβ activities in macrophage. Another effector protein VopQ secreted by *V. parahaemolyticus* from T3SS1, induce P13 kinase-independent autophagy (Burdette et al., 2009)**.** Matlawska-Wasowska et al. (2010) showed that, VP1680 was identified as the TTSS 1 effector protein which induces activation of JNK, P38 and ERK/MAPK pathways in human epithelial cells. Using a series of mutant strains of *V. parahaemolyticus* and inhibitors they established the pathway of cytotoxicity in epithelialcells. VP1680 (VopQ) and VepA induce activate MAPK-JNK 38, P38 and ERK by phosphorylation to promote pathogen infection. This effector protein also induces secretion of IL-8 which leads to inflammation responses that may facilitate bacterial infection and colonization. Zhou et al. (2010a) identify a protein Vp1659, encoded by a gene present inT3SS1 genomic island of *V. parahaemolyticus* showed 33 % similarity with Lcrv protein of *Yersinia* sp. and 35 % similarity with Pcrv protein of *Pseudomonas* sp. The deletion of *vp1659* significantly reduces the cytotoxicity in HeLa cell line. The Vp1659 plays multiple functions during infection. It induces membrane permeability, induces autophagy and rearranges the actin filaments. This protein is essential for efficient translocation of effector proteins into the cell.

The expression of virulent genes under Type III secretion system 1 was regulated by regulatory genes. Zhou et al. (2008) identified regulatory gene ExsA and ExsD that control the transcription of T3SS1 gene by directly interacting with the promoter

sequence of T3SS1 gene and cytotoxicity towards HeLa cells.This indicated that T3SS1 of *V. parahaemolyticus* was positively regulated by ExsA and negatively regulated by ExsD. Furthermore, Zhou et al. (2010b) demonstrated that, the interaction between ExsA, ExsC and ExsD are essential for the expression of type III secretion system 1 in *V. parahaemolyticus*. The deletion of ExsC from *V. parahaemolyticus* blocks the synthesis of T3SS1 dependent protein without affecting the expression of ExsA. The co-expression and purification of antigenically tagged ExsC, ExsD and ExsA, ExsD clearly revealed that, in absent of ExsC protein, ExsD binds with ExsA and prevent the binding of ExsA to the promoter region of T3SS1 gene leading to inhibit the expression of T3SS1 gene. But, in the presence of ExsC, it binds to Exs D and permits expression of T3SS1 genes. Another regulatory protein ExsE was identified by Erwin et al. (2012) which is associated with adhesion, swarming motility and flagella biosynthesis.Further study revealed that ExsE also involves in the negative regulation of T3SS1 system and successful translocation of effector protein.

2.5.4.2 Type III secretion system 2

V. parahaemolyticus has Type III secretion system 2 is important machinery for infection in a host cell that secret many effector proteins mainly associated with enterotoxicity. Kodama et al. (2008) identified two secreted proteins VopB2 (VPA1362) and VopD2 (VPA1361) under T3SS2 of *V. parahaemolyticus*. The functional analysis of these two protein showed that they are essential for T3SS2 dependent cytotoxicity and contact-dependent activity of pore formation in the infected cell. Further, the study showed that VopB2 and VopD2 act as a translocon protein and has a critical role in the T3SS2 dependent enterotoxicity.Two chaperon proteins VecA for VepA effector protein

and VocC for VopC were identified by Akeda et al. (2009).VocC protein is not only required for efficient secretion of VopC but also involved in the translocation of Vop C protein into the host cell. It plays an important role in the expression of VopL and VopT (Akeda et al., 2011). Zhang et al. (2012) showed VopC under T3SS2 plays a critical role for T3SS2 mediated invasion. VopC has a deamidase/transglutaminase activity and it activates small GTPase Rac and CDC42. This induces changes in actin cytoskeleton and facilitates the entry of *V. parahaemolyticus* in the nonphagocytic host cell. de Souza and Orth. (2014) described for the first time that VopC facilitates the invasion of bacteria into the epithelial cells. After internalization, the bacteria within vacuoles develop into the early endosome. The early endosome subsequently matures into the late endosome. *V. parahaemolyticus* then escape into the cytoplasm prior to vacuolar fusion with the lysosome. The vacuolar acidification is an important activator for this escape. The cytoplasm of the host cell served as the pathogen's primary intracellular replicative niche. In the year 2013, Zhou et al. (2013) discover another new effector molecule, VopZ, under type three secretion factor 2 which was found to involve in the intestinal colonization and induction of diarrhea. In vivo and in vitro analysis with wild VopZ, truncated VopZ, and mutated VopZ gene containing *V. parahaemolyticus* strain showed that, VopZ gene plays a distinct role inenabling intestinal colonization and diarrhea. Furthermore, VopZ inhibits activation of TAK1 and thereby prevents the activation of MAPK and NF-Kb signaling pathway (Zhou et al., 2013).

2.5.4.3 Type VI secretion systems

It is presumed that *Vibrio parahaemolyticus* contains two T6SS systems, VpT6SS1 and VpT6SS2 that secret many effectors and regulatory proteins involved in

the cytotoxicity, autophagy, virulence, symbiosis, interbacterial interaction, antipathogenesis and environmental fitness of bacteria. Both the T6SS1 and T6SS2 are differentially regulated by quorum sensing and surface sensing. They are also activated at different temperatures. T6SS1 present in most of the virulent *V. parahaemolyticus*is responsible for enhancing their fitness when competing for developing is own niche in the presence of other bacterial populations (Salomon et al., 2013). Ma et al. (2012) found that quorum sensing regulator OpaR, negatively regulates the transcription of T6SS1 and repress the transcription of *hcp1* that encode the structural component of T6SS1 in *V. parahaemolyticus.* The expression of T6SS2 of *V. parahaemolyticus* was positively or negatively regulated by OpaR and AphA, respectively. OpaR and AphA are two quorum sensing regulators leading to a gradient deviation of transcription levels at T6SS2 with the transition from low cell density to high cell density (Wang et al., 2013). Later, Zhang et al. (2017) revealed the binding of OpaR to the promoters of VP1388-1390, VP1400-1406, and VP1409-1407 repress the transcription of these genes. OpaR negatively regulate the transcription of VP1393-1406 genes in an indirect manner. Furthermore, AphA negatively regulate the transcription of above four T6SS1 operons in an indirect manner (Zhang et al., 2017). Salomon et al. (2014a) identified two additional transcription regulators VP1391 and VP1407 that control the expression of gene under T6SS1 of *V. parahaemolyticus*. In addition to that, they have identified H-NS, a bacterial histone-like nucleoid structured protein that serves as a repressor of T6SS1 and silences the horizontal gene transfer. They also identified two unidentified effectors under T6SS that have MIX motif (Salomon et al., 2014b). Yu et al. (2015) identified a gene *vgrG2* under T6SS2, which induce autophagy in macrophages. Nine potential T6SS1 effectors

of which five proteins belong to the polymorphic MIX-effector class have been identified by Ray et al. (2017)**.** In addition to that, they have identified another six protein which showed bactericidal activity under T6SS1. Similarly, Jiang et al. (2018) successfully retrieved several putative toxins under Type VI secretion systems, including VP1415, VPA1263, VPA0770, and VP1517 of which, VP1517 is an Rhs toxin. The N-terminal domain of this protein inhibits the DNase activity of the C-terminal domain. Ben-Yaakov et al. (2018) identified two transposon TfoY and Tmk which is essential for the activation of T6SS1 of *V. parahaemolyticus*