18 *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*

Introduction

Filippo Pacini, an Italian physician, discovered *Vibrio cholera* in 1854. In the same year, John Snow, a British physician (obstetrician), unraveled that the cholera outbreak in London that killed 616 people was due to contaminated water, linking the water for the very first time, not the air, as the primary source of contamination. Robert Koch, unaware of Pacini's work, independently isolated *Vibrio cholera* in 1884 and became the acknowledged discoverer of *Vibrio cholerae*, until the international committee on nomenclature in 1965 adopted *Vibrio cholerae* Pacini 1854 as the correct name of the cholera-causing organism. Vibrios are inhabitants of estuarine and freshwaters, and some species are pathogenic to humans and marine vertebrates and invertebrates. In humans, some species of vibrios can cause gastroenteritis following ingestion of contaminated food or water and septicemia when preexisting cuts or abrasions on the skin are exposed to contaminated water or seafood. Vibrios are of significant concern in both developed and developing countries because of their continued burden of disease resulting from contaminated water and fish products. In the USA, vibriosis causes an estimated 80,000 illnesses and 100 deaths every year. Three major species, *Vibrio cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, are responsible for the majority of human infections; however, several other species are responsible for sporadic infections.

Classification

The genus *Vibrio* is a member of the *Vibrionaceae* family and contains 63 species, and at least 11 of them are pathogenic to humans including *V. cholerae* (O1 and O139), *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus*, *V. hollisae*, *V. fluvialis*, *V. alginolyticus*, *V. damsela*, *V. furnissii*, *V. metschnikovii*, and *V. cincinnatiensis*. Among these, the first three species cause most human infections.

Biology

Vibrio species are Gram-negative curved rods with size ranging from 1.4 to 2.6 μm in length and 0.5–0.8 μm in width. They are motile and generally possess a single polar flagellum. They are facultative anaerobes, most are oxidase-positive, and utilize D-glucose as the main carbon source. *Vibrio* species produce many extracellular enzymes: amylase, gelatinase, chitinase, and DNase. Some *Vibrio* species are halophilic (tolerance up to 18% NaCl), and sodium ions stimulate their growth. Vibrios grow well in neutral to alkaline pH (~9.0) and are acid-sensitive. The optimum pH range is 8.0–8.8, and the optimum growth temperature range is 20–37 °C. Water temperatures on either side of the range severely affect bacterial growth. Nutrient deficiency, salinity, and changes in temperature promote stress

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resulting in the viable but nonculturable state (VBNC) especially for *V. cholerae* and *V. vulnificus*. Two circular chromosomes, usually one large and the other small, are present in vibrios, which provide diversity in gene structure and gene content. Chromosomes 1 and 2 in *V. cholerae* are 3.0 and 1.1 Mb, respectively, in *V. parahaemolyticus* 3.3 and 1.9 Mb, and in *V. vulnificus* 3.3 and 1.85 Mb.

Source and Transmission

Vibrio species are isolated from fresh, brackish, and marine waters. Vibrios are found as free-living in water or are associated with inanimate surfaces or aquatic organisms (zooplankton, phytoplankton), aquatic animals (seabirds), sewage water, sediments, seafood, fish, and shellfish. Vibrios are associated with chitinous zooplankton and shellfish, forming biofilms to help bacterial prolonged survival in the aquatic environment. Bivalve shellfish such as clams, oysters, and mussels accumulate bacteria because of their filter-feeding habit. Natural disasters, like floods cyclones and hurricanes, cause the failure of sewer systems and result in contamination of the aquatic environment. Foods washed in contaminated water can transmit vibrios. Water temperature, nutrient availability, salinity, and an association with marine organisms influence the *Vibrio* loads in water. *Vibrio* counts are very high during summer and autumn months, and counts appreciably diminish (or are generally absent) at temperatures below 10 °C. Vibrios are obligate halophiles (except *V. cholerae*), and depending on the species preference for water salinity varies.

Vibrio cholerae

Introduction

One of the most important members of the genus *Vibrio* is *V. cholerae*. The disease caused by toxigenic strains of the two serotypes (O1 and O139) of *V. cholerae* is known as cholera. Though Filippo Pacini and Robert Koch independently isolated *V. cholerae* in 1854 and 1884, respectively, the outbreaks of cholera dates back to 460–377 BC during the times of Hippocrates. In modern history, epidemic and pandemic cholera occur with global implications, and frequent outbreaks are reported in Asia, Africa, and South and Central America. Cholera is endemic in many parts of Asia and Africa. According to the World Health Organization (WHO), *V. cholerae* infect about 3–5 million people with 100,000–120,000 deaths each year worldwide.

Biology

Vibrio cholerae is a Gram-negative rod or curved-shaped bacterium (0.7–1.0 × 1.5–3.0 μm). It is a facultative anaerobe and produces pale-yellow, translucent colonies that are about 2–3 mm in diameter on a special medium known as thiosulfate citrate bile salts sucrose (TCBS) agar. *Vibrio cholerae* is able to grow within a temperature range of 15–45 °C, a pH range of 6–10, and a salt (NaCl) concentration of up to 6%. However, it does not require salt for growth. *V. cholerae* is often associated with zooplankton and crustaceans (Fig. 18.1). *V. cholerae* forms, biofilms on zooplankton and phytoplankton, which both contain chitin, and the bacterium can use chitin as a carbon and nitrogen source. There are 206 known serotypes based on O antigen of the lipopolysaccharide (LPS), of which two major serotypes, O1 and O139, are responsible for epidemic cholera. A major difference between O1 and the O139 is the presence of a thin capsule in O139 and its absence in O1. This difference can be observed during bacterial growth on solid agar media, where O1 produces translucent while O139 produces opaque colonies. Furthermore, the LPS of the O1 serotype is smooth while it is semi-rough in O139. The O139 LPS has a highly substituted core oligosaccharide and shorter side chains of O antigen, which are responsible for the rough phenotype. The LPS and capsule of O139 also share a unique sugar, 3,6-dideoxyhexose (colitose). The serotype O1 is subclassified into the classical and El Tor biotypes based on a set of phenotypic

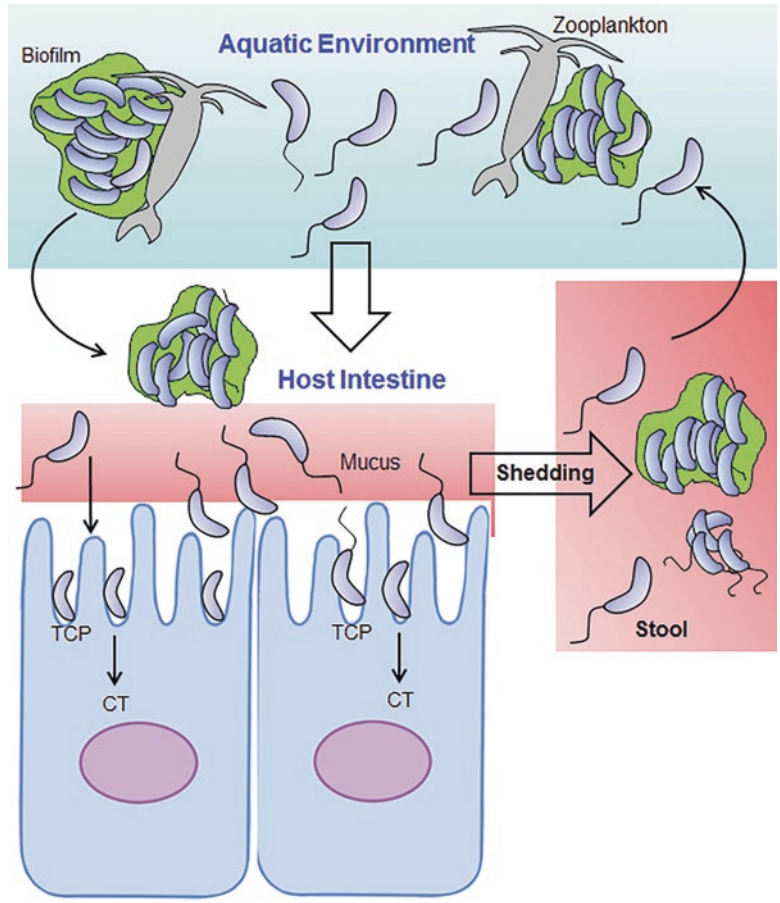

Fig. 18.1 The lifecycle of *Vibrio cholerae* in the aquatic environment and host intestine. Biofilm formation is critical for their persistence in both the aquatic environment and intestine. *TCP* toxin-coregulated pili, *CT* cholera toxin

traits. The two biotypes are further classified as Inaba, Ogawa, and Hikojima subserotypes. The El Tor biotype was originally isolated from an outbreak in El Tor, Egypt, in 1905.

All cholera-causing strains carry virulence genes for cholera toxin (CT) and toxin-coregulated pili (TCP). Virulence genes are located in *Vibrio* pathogenicity islands (VPI-1 and VPI-2). The gene (*ctxAB*) encoding CT is located in a lysogenic filamentous bacteriophage, CTXΦ (Fig. 18.2), which also contains several accessory virulence genes clustered in two regions, RS and core. The RS region constitutes *rstA*, *rstB*, and *rstR* genes that are responsible for the site-specific integration, replication, and regulation of the phage into the chromosome. The core region carries *ctxAB* which encodes CT and genes for Psh, Cep (core-encoded pilin), Ace (accessory cholera enterotoxin), and Zot (zonula occludens) which are required for phage coat synthesis and morphogenesis.

Other factors including outer membrane porins, biotin and purine biosynthetic enzymes, iron-regulated outer membrane proteins (IrgA), and O antigen of LPS are also known virulence factors. The non-O1/O139 also cause diarrhea but generally milder than O1, and these serotypes are common in the USA. *Vibrio cholerae* has a single polar flagellum, which helps the bacterium to reach the intestinal mucosa and aids in colonization. It maintains two lifestyles: one in the aquatic environment where it is free-living or attached to zooplanktons and the other is inside the host gastrointestinal tract (Fig. 18.1). The common survival strategies for *Vibrio* either in the aquatic environment or in human host include (1) the activation of stress response, (2) expression of flagella for motility and chemotaxis, (3)

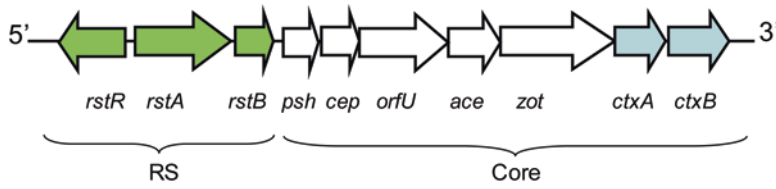

Fig. 18.2 Schematic of CTXΦ bacteriophage genome in *Vibrio cholerae* classical strain. Genes encoding cholera toxin, *ctxAB* appears at the 3′ end of the genome. The *rstR*, *rstA*, and *rstB* genes constitute the RS region (*shaded arrows*) that is responsible for the site-specific integration (*rstA*), replication (*rstB*), and regulation (*rstR*) of the phage. The core region carries *ctxAB* which encodes CT and genes for Psh, Cep (core-encoded pilin), OrfU, Ace (accessory cholera enterotoxin), and Zot (zonula occludens) which are required for phage coat synthesis and morphogenesis (Adapted and redrawn from Safa et al. 2010. Trends Microbiol. 18, 46–54)

attachment to abiotic and biotic surfaces, (4) biofilm formation, and (5) detachment from the surface. Proficient switching between the planktonic (motile) and biofilm (sessile) lifestyles in response to chemical and physical changes in the extracellular milieu is key to the survival and colonization.

Virulence Factors and Pathogenic Mechanism

Gastroenteritis

Vibrio cholerae is the most studied organism that is responsible for acute secretory diarrhea known as cholera. The infectious dose is 10^4–10^{10} cfu g^{-1} and the disease is spread through contaminated water and food, and transmission is through the fecal–oral route. Individuals with the blood group O are more susceptible to cholera than the other blood groups. Serotype O1 causes a fatal form of cholera, while the non-O1 (the O139 form) is generally less virulent. There are two biotypes, classical and El Tor, and they share a common LPS O antigen. A strain of *V. cholerae* O139 Bengal, originating around 1992, was responsible for several outbreaks in India and Bangladesh. It is capable of causing serious diarrhea. Following ingestion, *V. cholerae* overcomes low pH, bile acids, elevated osmolarity, iron limitation, antimicrobial peptides, and natural microflora and can grow to high titers in the human gut. Stress response regulators RpoS (σS) and RpoE (σE) help cope with the intrinsic stressors in the gut. Cholera patients can shed 10^7–10^9 vibrios mL^{-1} in so-called rice-water stools. *Vibrio cholerae* adherence and colonization of small intestinal mucosa are facilitated by toxin-coregulated pili (TCP), flagella, and neuraminidase. Bacteria then produce several toxins such as cholera toxin, ZOT (zona occludin toxin), ACE (accessory cholera enterotoxin), and HlyA (hemolysin), which act on mucosal cells. Toxins alter the ion balance by affecting the ion transport pumps for Na^+, Cl^-, HCO_3^-, and K^+ in the cell, resulting in extensive fluid and ion losses.

Adhesion and Colonization

Bacterial adherence and colonization are positively influenced by motility and chemotaxis. A single polar flagellum helps each bacterium to penetrate the mucus layer. Flagellin mutants are nonmotile and are less virulent. The long filamentous pili called TCP (a type IV bundle-forming pilus) help form microcolonies and are involved in colonization. The genes encoding TCP pili are regulated similarly to genes encoding for the cholera toxin. TCP mutants are avirulent in humans. TCP pilin is encoded in *tcpA* and is located in the TCP pathogenicity island. *N*-acetylglucosamine (GlcNAc)-binding protein A (GbpA: 53 kDa) encoded by *gbpA* has been shown to be involved in bacterial colonization by interacting with mucin. Other colonization factors include mannose–fucose-resistant cell-associated hemagglutinin (MFRHA: 26.9 kDa) and some outer membrane proteins (OMPs). TCP and other colonization factors are regulated by regulatory proteins (ToxR/ToxS and ToxT). Immediately adjacent to the *tcp* cluster is the *acf* gene for

accessory colonization factor (ACF), a lipoprotein. The exact role of ACF is not known, but it is believed to be involved in bacterial colonization.

Vibrio cholerae Biofilm

The ability to form biofilms on the biotic and abiotic surface is an important survival and colonization strategy for *V. cholerae* (Fig. 18.1). In the aquatic environment, biofilms enhance *V. cholerae* persistence and provide protection against stress, nutrient limitation and predation by protozoa, and attack by bacteriophages. *Vibrio cholerae* forms a biofilm on phytoplankton and zooplankton. Type IV mannose-sensitive hemagglutinin (MSHA) pili help in biofilm formation on plankton, and the extracellular matrix helps maturation of biofilms. After ingestion of biofilms or planktonic cells by humans, bacteria in the intestine express TCP and form aggregates or biofilms aiding bacterial colonization on intestinal mucosa. Biofilms also help bacteria to avoid the host's innate immune response.

Quorum-sensing molecule, cholera autoinducer 1 (CA-1), accumulates in biofilms and promotes expression of the quorum-sensing regulator, HapR, which in turn enhances expression of sigma factor, RpoS. RpoS helps bacteria to cope with the environmental stressors. During biofilm formation, cells also accumulate intracellular cyclic diguanylic acid (c-di-GMP), a second messenger that controls the transition between planktonic and biofilm lifestyles.

Cholera Toxin

Cholera toxin (CT) is the most important virulence factor in *V. cholerae*. The gene encoding CT is located in a lysogenic filamentous bacteriophage, CTXΦ (Fig. 18.2). The bacterial cell surface receptor for CTXΦ interaction is TCP. CT-mutant strains are either avirulent or may cause milder diarrhea because of the presence of other toxins. CT is the best-studied bacterial toxin. It is an A–B type "ADP-ribosylating toxin." The A subunit is a 27 kDa protein encoded by *ctxA*, and the B subunit consists of five identical proteins of 11.7 kDa and is encoded by *ctxB*. A and B subunits are secreted into the periplasm, where they are assembled.

The B subunit of toxin first binds to the host mucosal cell by binding to the ganglioside GM_1 receptor. It is a sialic acid containing oligosaccharide covalently attached to the ceramide lipid. It is found on the surface of many cells. The toxin is internalized and the A subunit is detached. The A subunit has the enzymatic activity; it ADP-ribosylates the Gs proteins (composed of three subunits: α, β, γ) also known as "GTP hydrolyzing proteins." Gs proteins regulate the activity of host cell adenylate cyclase and serve as "off" and "on" switches. Binding of A subunit to Gs subunit α locks it in the "on" position and stimulates the production of the cyclic adenylate cyclase (cAMP). cAMP activates the protein kinase A, which in turn causes phosphorylation of protein especially the CFTR (cystic fibrosis transmembrane conductance regulator) protein in the ion pump and thus alters the function of sodium and chloride ion transport, resulting in the increased Cl^- and HCO_3^- secretion by crypt cells and decreased absorption of Na^+ and Cl^- by absorptive cells (Fig. 18.3).

Regulation of Cholera Toxin Production

Cholera toxin production is regulated by transmembrane proteins ToxR, ToxS, TcpP, and TcpH. ToxR, a 32 kDa transmembrane protein, binds to a 7 bp DNA sequence located in the upstream of *ctxAB* and increases the expression of CT. ToxR, TcpP, and TcpH activate ToxT, which in turn activates CT and TCP expression. ToxR and ToxT also regulate the expression of ACF, outer membrane proteins OmpU and OmpT, and other lipoproteins. The quorum-sensing regulatory proteins LuxO and HapR also control CT and TCP expression.

Other Toxins

Vibrio cholerae also produces two other toxins, ZOT (zonula occludin toxin: 44.8 kDa) and ACE (accessory cholera enterotoxin). The ZOT disrupts the "tight junction" that binds mucosal cells together and preserves the integrity of the mucosal membrane. Normally, the tight junction maintains cellular integrity and prevents the loss of ions or water molecules. ZOT induces a reorganization of F-actin and decreases the G-actin and

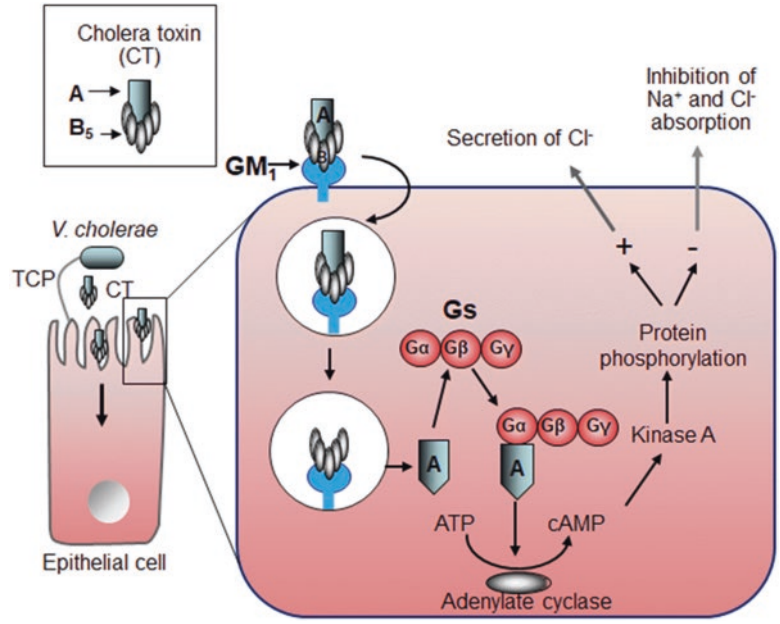

Fig. 18.3 Schematic showing the action of cholera toxin (CT) on enterocyte. *Vibrio cholerae* first bind to the cells using pili (toxin-coregulated pili: TCP) or other colonization factors and produce CT. The CT is an A–B type toxin composed of one A subunit and five B subunits. The B subunits bind to the GM1 receptor, and the CT is transported inside the cell. The A subunit ADP-ribosylate Gs protein (GTP hydrolyzing protein) increases the catalysis of ATP (adenosine triphosphate) to form cyclic adenosine monophosphate (cAMP). cAMP mediates phosphorylation of the CFTR protein, which is involved in ion transport (pump), thus affecting ion losses (Cl^-, HCO_3^-, Na^+) and fluid flow

affects the cytoskeletal rearrangements possibly mediated by protein kinase C. Consequently, the tight junction loses its barrier function and enhances pericellular permeability. ZOT also disrupts the ion balance and promotes diarrhea.

The ACE toxin is responsible for diarrhea in animals but probably has no role in human diarrhea. A hemolysin (HlyA: 65 kDa), also known as El Tor hemolysin, is responsible for enterotoxicity. It binds to cholesterol and oligomerizes in the membrane forming a pore of 1.2–1.6 nm. Other virulence factors like siderophores also help bacterial iron acquisition from host cells.

Immune Response to Cholera Toxin

Vibrio cholerae induces a strong immune response by the production of the secretory immunoglobulin A (sIgA). sIgA acts as an opsonin, and opsonization may aid in the transcytosis of *V. cholerae* through M cells by interacting with the CT receptor ganglioside GM_1 on M cells. CT aids in intestinal dendritic cell (DC) maturation through the production of prostaglandin E_2 (PGE_2) and nitric oxide (NO); thus, CT has been used as an adjuvant in various vaccine formulations. The CT also influences the immune response of lymphocytes and monocytes by altering the expression of several genes. CT induces cAMP, which regulates the expression of genes that regulate various functions including immune response.

Symptoms of *V. cholerae* Infection

The symptoms of diarrhea appear within 6 h–5 days and can last for 2–12 days. Diarrhea looks like "rice-water" with a fishy odor, and a patient may defecate up to 1 L per hour leading to hypotensive shock and death within hours. The patients show high pulse rate, dry skin, sunken eyes, lethargy, low urine volume, nausea, and

vomiting in the early phase of infection and may exhibit abdominal cramps. Fever is uncommon, but high fever is indicative of secondary infection. Watery diarrhea causes severe dehydration, loss of electrolytes, and ions causing hypertension that can be life-threatening. The death rate among untreated patients is about 70%. Infants and children are highly susceptible. Recovering patients develop immunity against cholera.

Control, Prevention, and Treatment of *Vibrio cholerae*

Contaminated water and food are the primary sources of *V. cholerae*; therefore, proper processing of water and food and sanitary hygienic practices would lessen the spread of the disease. Moreover, *V. cholerae* remain attached to zooplankton and phytoplankton as biofilms; therefore, water filtration practices that remove particles larger than 20 μm can also significantly reduce cholera cases.

Hallmark of cholera is profuse diarrhea. Loss of water and electrolytes leads to dehydration; thus, fluid therapy is the most effective treatment to prevent dehydration. Oral rehydration with boiled potable water containing table salt and glucose (glucose stimulates water uptake and is a source of energy) is effective in resource-constrained situation. Besides oral rehydration, intravenous fluid therapy is recommended in patients showing advanced signs of dehydration including sunken eyes, high pulse rate, lethargy, low urine volume, and comatose condition. Antibiotics including tetracycline, cotrimoxazole, erythromycin, doxycycline, chloramphenicol, and furazolidone can be used to treat cholera, but concern for antibiotic resistance is very high.

Cholera vaccine is used to prevent infection in the population in the endemic zone. sIgA generated from vaccination prevents bacterial colonization on the mucosal surface. Since there is no cross-protection between serotype O1 and O139, a bivalent vaccine is needed to provide protection against both serotypes. Injectable heat-killed bacterial cells were used for many years, but this vaccine exhibited toxic side effects due to the presence of endotoxin (LPS); hence, such immunization practices have been discontinued. Several strategies have been undertaken to develop a safe yet protective vaccine against cholera.

Oral vaccination with killed bacteria together with a purified B subunit of cholera toxin is widely used and is recommended by the WHO. Two killed vaccines are now used for oral administration, Dukoral (Sweden) that contains several biotypes and serotypes of *V. cholerae* O1, supplemented with 1 mg per dose of recombinant cholera toxin B subunit, and Shanchol (India), a bivalent toxin containing several O1 and O139 biotypes and serotypes without the supplemental cholera toxin B subunit. These vaccines are administered two or three times depending on the age of the patient. The overall protection is 60–85% lasting for 2–3 years. Live attenuated oral vaccine underdevelopment includes CVD 103-HgR (USA) and Peru-15 (China) use genetically modified CT-negative strains; however, their efficacy in a clinical trial has yet to be fully evaluated.

Vibrio parahaemolyticus

Introduction

Vibrio parahaemolyticus was first discovered by a Japanese scientist, Tsunesaburo Fujino, in 1950 when the organism caused an outbreak affecting 272 people, resulting in 20 deaths due to consumption of shirasu (a Japanese fish dish generally prepared with small fish, such as sardine, anchovy, etc.). *V. parahaemolyticus* is distributed in the marine environment (estuarine), and it is one of the major foodborne pathogens that are associated with seafood worldwide. Not all strains are considered pathogenic. The spread and dissemination of *V. parahaemolyticus* depend on the water temperature, zooplankton blooms, and dissolved oxygen. Countries located in a temperate climate experience higher numbers of outbreaks during summer months than the tropical countries. Countries located in the tropical zone maintain a warmer overall temperature,

which is conducive for year-round outbreaks. The outbreaks of gastroenteritis are associated with consumption of contaminated seafood including raw oysters and other shellfish, and it is responsible for 20–30% of food poisoning cases related to seafood.

Biology

Vibrio parahaemolyticus is a moderate halophilic enteropathogen and requires salt (1–9%) for survival and growth. The bacterium primarily causes gastroenteritis; however, it can cause extraintestinal infections like eye and ear infections and wound infections affecting extremities. *V. parahaemolyticus* grows at a minimum temperature of 15 °C and a maximum temperature of 44 °C. On liquid medium, the bacterium expresses single polar flagellum (*fla*) exhibiting "swimming" motility, while on solid media, it expresses peritrichous flagella or lateral flagella (encoded by the *laf* gene) exhibiting "swarming" phenotype. The bacterium also expresses capsule (K antigen). All strains of *V. parahaemolyticus* produce H_2S in triple sugar iron (TSI) medium. Urease production is an unusual phenotype for *V. parahaemolyticus*, and a majority of clinical and environmental isolates is urease-negative; however, some clinical isolates are urease-positive. There is a strong correlation between urease production and the presence of the *trh* (heat labile hemolysin) gene among clinical isolates (see below).

Vibrio parahaemolyticus strains are classified based on their somatic (O) and capsular (K) antigen patterns, and the predominant serotype is O3:K6, which is distributed globally. The O3:K6 serotype is thought to have originated from Japan, and it was responsible for a major outbreak in Calcutta (now Kolkata, India) in 1996. The serovariants of O3:K6 exist including O4:K12, O4:K68, O1:K41, O1:K25, O1:KUT, and several others which are responsible for regional outbreaks such as those that occurred in South and South East Asia. Serotype O4:K12, O6:K18, O1:K56, O4:K63, O3:K36 and O12:K12 have been associated with outbreaks in the US Pacific Northwest of which raw shellfish were associated with many of the outbreaks. Three coastal states (New York, Oregon, and Washington) were involved with 177 cases from the O4:K12 serotype without any fatalities.

Virulence Factors and Pathogenesis

Vibrio parahaemolyticus is distributed widely in the estuarine environment and is a major seafood-associated pathogen, but not all strains are pathogenic. *V. parahaemolyticus* is infectious, and a dose of about 2×10^5–3×10^7 cfu is required to cause disease. The incubation period is about 15 h (range, 4–96 h), and the disease may last for 2–3 days. Bacteria colonize the gut and produce toxins, and the pathogenesis depends on the production of a set of toxins (see below) that cause cell damage resulting in membrane pore formation and loss of fluids and electrolytes. Bacteria also induce a strong inflammatory response in the intestine, which is more severe than the infection caused by the *V. cholerae*. Though the *V. parahaemolyticus* infection induces a strong immune response, the detailed mechanism of pathogenesis is still unclear. The symptoms of *V. parahaemolyticus* infection include acute abdominal pain, nausea, vomiting, headache, low-grade fever, and diarrhea (watery or bloody). The stool is described as "meat washed" due to the presence of blood. The disease is usually self-limiting.

Adhesion and Colonization

Vibrio parahaemolyticus expresses multivalent adhesion molecule 7 (MAM7) for adhesion to host cells for colonization and delivery of effector molecules including toxins. The host cell receptor for MAM7 is fibronectin and phosphatidic acid.

Toxins

Vibrio parahaemolyticus produces four hemolysins: a thermostable direct hemolysin (TDH), a

heat-labile TDH-related hemolysin (TRH), a thermolabile hemolysin (TLH), and δ-VPH toxin and an enzyme, hemagglutinin protease (HAP). Properties of toxins and enzymes are summarized below.

1. TDH toxin production in *V. parahaemolyticus* was originally detected by the formation of β-hemolysis on Wagatsuma agar (special kind of blood agar medium), and this phenomenon was called the Kanagawa phenomenon (KP), and later, the toxin was termed the thermostable direct hemolysin (TDH). *Vibrio parahaemolyticus* acquired the *tdh* gene through horizontal gene transfer. TDH is a 21 kDa protein that causes zones of β-hemolysis on Wagatsuma blood agar. TDH is heat-stable (100°C for 10 min) and is produced by KP^+ strains. The KP^- strains generally carry the heat-labile TRH toxin. TDH acts as a porin and allows the influx of ionic species: Ca^{2+}, Na^+, and Mn^{2+} from enterocytes. The porin channels increase with increased concentrations of TDH and increase ionic influx, cell swelling, and death of cells due to ionic imbalance. TDH also disrupts the epithelial barrier function by affecting the tight junction proteins, such as claudin and occludin as well as cytosolic zonula occludin proteins (ZO-1, ZO-2, ZO-3), cingulin, and 7H6. Toxin action can also be detected by a ligated mouse ileal loop model. During gastroenteritis, a strong humoral immune response to TDH and LPS occurs, and the predominant immunoglobulin is IgM.
2. TRH is a heat-labile TDH-related hemolysin (inactivated at 60 °C for 10 min) and induces fluid accumulation when tested in a rabbit ileal loop model. TRH also induces chloride secretion, and its involvement in diarrhea has been strongly implicated.
3. TLH is a thermolabile hemolysin and possesses phospholipase A2/lysophospholipase activity. TLH consists of 2 molecular weight species of 47.5 and 45.3 kDa and is present in all *V. parahaemolyticus* strains. The role of this hemolysin in pathogenesis is unknown.
4. δ-VPH is a heat-stable hemolysin of 22.8 kDa. It is present in all strains including KP-negative *V. parahaemolyticus* strains. The role of this hemolysin in pathogenesis is also unknown.
5. Hemagglutinin protease (HAP)*: V. parahaemolyticus* has the ability to detach from damaged/sloughed mucosal cells and reattach to new mucus surfaces. The detachment factor is a zinc- and calcium-dependent protease, and it is called hemagglutinin protease because it has hemagglutination activity.

In a study, a majority of clinical isolates obtained from the Pacific Northwest (USA) belonged to serotype O4:K12, which expressed both TDH and TRH. The serotype O3:K6 that caused outbreaks in the Gulf of Mexico and the East Coast of the USA had only TDH. Environmental isolates may lack both TDH and TRH, yet those isolates may express other virulence factors, such as extracellular proteases, biofilm, and siderophore, and may exhibit cytotoxicity toward intestinal cells.

Type III Secretion System

Vibrio parahaemolyticus has two sets of genes for the synthesis of a type III secretion system (T3SS), which is necessary for injecting virulence proteins directly into the host cell to target the actin cytoskeleton, innate immune signaling, and autophagy leading to apoptosis. The first cluster (T3SS-1) is located on the large chromosome (3.3 Mb), and the second one (T3SS-2) is located on the small chromosome (1.9 Mb). Virulence factors that are secreted by T3SS-1 are responsible for cytotoxicity, mouse lethality, and induction of autophagy, while T3SS-2 is responsible for enterotoxicity and environmental persistence.

Type VI Secretion System

The genes encoding type VI secretion system (T6SS) are located in both chromosomes 1 and 2. T6SS secretes effector proteins that are involved in adhesion to host cells and may work coordinately with proteins secreted by T3SS.

Vibrio vulnificus

Introduction

Vibrio vulnificus is considered the most infectious and invasive of all the human pathogenic vibrios primarily in the immunocompromised host. It is the leading cause of seafood-related mortality causing 95% (~40 cases) of seafood-related deaths in the USA. *Vibrio vulnificus*-related infections are thought to be very high in Japan because of the warmer coastal water and increased consumption of raw seafood. In the USA, most cases are reported during the summer months (May–October). Seafood including filter-feeding mollusks (mussels, oysters, and clams), eels, and fish is the major source of this pathogen. Salinity and temperature of water have a significant impact on *V. vulnificus* persistence in water.

Biology

Vibrio vulnificus is a Gram-negative obligate halophilic bacterium (optimum salt requirement is 10–18%) and widespread in coastal warm waters (≥ 20 °C). The bacterium enters into viable but nonculturable (VBNC) state at or below 13 °C, where the bacterium remains dormant, metabolically inactive, and is unable to readily grow on media to form colonies. Climate change due to global warming has significantly influenced *Vibrio* growth and persistence and has led to an increased incidence of *Vibrio* infections in countries around the globe. A majority of *V. vulnificus* strains are considered nonpathogenic, while some are pathogenic. *V. vulnificus* has been classified into biotype 1 (BT1), biotype 2 (BT2), and biotype 3 (BT3). BT1 contains human clinical and environmental strains, BT2 is mostly eel pathogen, and BT3 is a hybrid strain that is limited to a geographic location, primarily in Israel. Classification is often misguided due to horizontal gene transfer and continuous evolution in *V. vulnificus* strains. Many strains produce several virulence factors: capsules, siderophores, and toxins such as hemolysins, collagenase, protease, elastase, DNase, mucinase, hyaluronidase, fibrinolysin, lipase, and phospholipase.

Virulence Factors and Pathogenic Mechanism

Vibrio vulnificus concentration in water could be less than 10 cells ml^{-1}, but bacteria accumulate inside the filter-feeding mollusks, such as oyster, mussels, and clams, reaching a concentration of 10^5 cfu g^{-1} of tissue. Consumption of raw and/undercooked oysters and other seafood is implicated in outbreaks. Bacteria also can penetrate through cuts or abrasions on skin during recreational or occupational activities associated with the marine environment or the seafood industry (Fig. 18.4). The infectious dose of *V. vulnificus* is unknown but is estimated to be as low as 100 cfu. The average incubation period of the disease is 26 h, but in the case of wound infection, the incubation period is much shorter, about 16 h. *V. vulnificus* is responsible for septicemia, wound infection, and gastroenteritis in humans. Immunocompromised individuals or persons with underlying conditions, such as diabetes, chronic renal disease, cirrhosis of the liver, and hemochromatosis (iron overload in the body), are predisposed to severe infection. Furthermore, males over the age of 40 are at the greatest risk (86%), possibly due to preexisting risk factors.

Adhesion Factors

Vibrio vulnificus expresses an N-acetylglucosamine (GlcNAc)-binding protein A (GbpA; 53 kDa) to attach to chitin on plankton. Filter-feeding mollusks feeding on plankton accumulate *V. vulnificus* in their tissues and serve as a vehicle for transmission to humans. GbpA may also aid in *V. vulnificus* adhesion to host mucin similar to *V. cholerae*, which also expresses GbpA.

Capsular Polysaccharide

Vibrio vulnificus produces a capsular polysaccharide (CPS) which is the primary virulence factor, helping bacteria to avoid phagocytosis by macrophages. The presence of CPS correlates with the opaque colony phenotype (Op) and

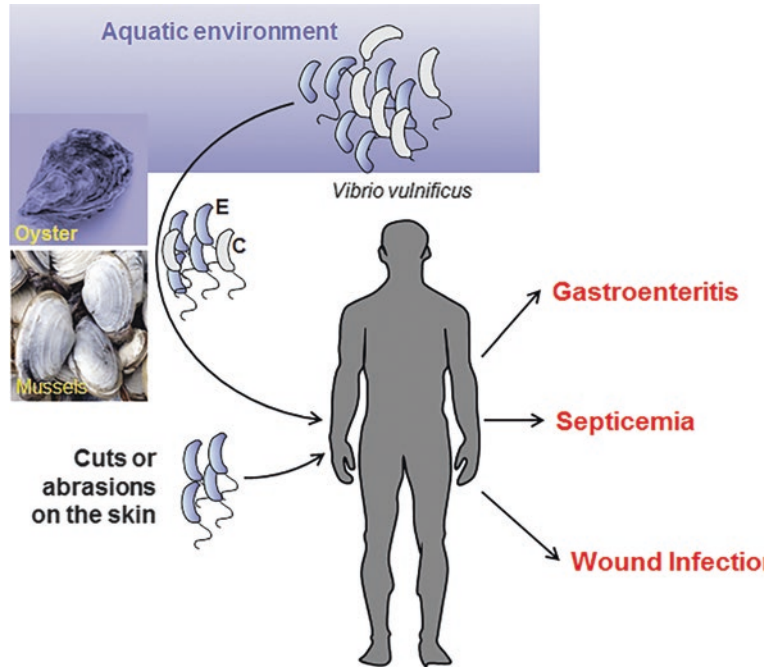

Fig. 18.4 *Vibrio vulnificus* transmission of clinical (C) and environmental (E) genotypes (strains) to humans

pathogenicity. Strains with translucent colony phenotype (Tr) are less virulent. CPS synthesis is encoded by four genes: *wcvA*, *wcvF*, *wcvI*, and *orf4*. Mutation in any of these genes results in the loss of capsule biosynthesis, translucent colony phenotype, and loss of virulence. Furthermore, spontaneous phenotypic switching from the Op phenotype to the Tr phenotype can lead to the development of a less virulent strain. All strains can potentially switch their phenotype from Op to Tr, but switching is much less frequent in clinical strains than in environmental strains.

Iron Acquisition

Vibrio vulnificus expresses siderophores, phenolate, hydroxamate, and vulnibactin to scavenge iron from the host transferrin, hemin, and lactoferrin. Host with high levels of iron such as those who suffer from chronic hemochromatosis or cirrhosis of the liver is highly susceptible to *V. vulnificus* infection. In *V. vulnificus*, *hupA* and *fur* genes regulate iron acquisition. Avirulent strains are unable to acquire iron from their host.

Flagella and Motility

The flagellum is an important adhesion and colonization factor encoded by the *flgC* gene, and it is responsible for cytotoxicity in host cells. The mutant strains exhibit defective motility and are attenuated for infection in suckling mice. Flagella-negative strains lose the ability to adhere to host cells, thus preventing bacteria from delivering toxic effectors to host cells.

Hemolysin

Vibrio vulnificus produces three types of hemolysin/cytolysins. The most widely studied hemolysin, VVH (*Vibrio vulnificus* hemolysin), is a water-soluble polypeptide (51 kDa) that binds to cholesterol on the membrane and forms small pores in the erythrocyte membrane. It can induce apoptosis by elevating cytosolic free Ca^{2+}, releasing cytochrome C from mitochondria, activating caspase-3, and degrading poly-ADP ribose-polymerase (PARP) to cause DNA fragmentation (all hallmarks of apoptosis). Toxin increases vascular permeability and causes skin damage.

Two other hemolysins, encoded by *hlyIII* and *trkA*, are not well characterized.

V. vulnificus also produces an RTX (repeat in toxin) family of the toxin. They share a repeated nine amino acid sequence motif among several Gram-negative bacterial hemolysins. RTX causes depolymerization of actin, pore formation in red blood cells, and necrotic cell death in cultured mammalian cells; however, its significance in pathogenesis remains unresolved.

Metalloprotease

Vibrio vulnificus secretes a metalloprotease, a 45 kDa zinc containing protease. The C-terminal 10 kDa segment binds to protein substrates on erythrocytes, and the N-terminal 35 kDa segment facilitates proteolysis. This protease exerts two functions: membrane permeability enhancement and tissue hemorrhage.

Septicemia

Vibrio vulnificus causes a systemic disease in hosts with underlying preexisting conditions, such as liver or kidney disease, malignancies, and diabetes. Infection is also severe in people with high levels of iron in serum caused by liver cirrhosis or genetic disorders, like hemochromatosis. From the intestinal tract, the bacterium invades epithelial cells. It first binds to epithelial cells with the aid of pili; produces hemolysin, which induces apoptosis; and facilitates bacterial invasion and translocation into the bloodstream. Bacteria acquire iron from host cells using siderophores and rapidly proliferate causing septicemia. Bacterial LPS and capsules activate the complement cascade; however, the capsule helps the bacterium escape neutrophil- or macrophage-mediated phagocytosis. From blood circulation, the bacterium can invade cutaneous tissue with the help of toxins, such as hemolysins, collagenase, protease, lipase, and phospholipase. These toxins also aid in the development of edematous hemorrhagic skin lesions, also known as buboes. Induction of proinflammatory cytokines, TNF-α, IL-1β, and IL-6, can lead to septic shock. Septicemia is manifested by fever, prostration, hypotension, chills, nausea, and occasional vomiting, diarrhea, and abdominal pain. In the USA, the mortality rate is 40–60% and is generally associated with underlying conditions.

Wound Infection

Wound infection is associated with recreational or occupational activities in seawater and the seafood industry. Preexisting cuts, skin lesions, or injuries and trauma resulting from activities from handling seafood and marine animals or from recreational activities are risk factors. *V. vulnificus* is thought to be associated with most *Vibrio*-related wound infections. Other species involved are *V. parahaemolyticus*, *V. alginolyticus*, and *V. cholerae* non-O1. The average incubation period for this type of infection is about 16 h. Vibrios produce collagenase and metalloprotease that allow the bacteria to colonize the wound. Protease evokes two types of reactions, increased vascular permeability, and hemorrhagic actions. The protease is bifunctional: the N-terminal portion mediates enzymatic activity to degrade type IV collagen located in the vascular basement membrane and causes tissue damage leading to hemorrhage. The C-terminal end binds to mast cell receptors, activating and aiding the release of histamine and bradykinin. These biologically active amines increase membrane permeability and cause wound edema. Infection results in high fever, chills, wound edema, vesicle formation, cellulitis, erythema, and tissue necrosis and may require hospitalization and amputation of extremities under severe conditions. Antibiotic therapy is needed to clear the infection. The fatality rate for wound infection is 20–30% in patients with underlying conditions.

Symptoms of *V. vulnificus* Infection

Three major conditions are associated *V. vulnificus* infection: gastroenteritis, septicemia, and wound infections. Gastroenteritis symptoms are

associated with abdominal pain, vomiting, and diarrhea. Septicemia and wound infections progress very rapidly, and a patient may die within 24 h of exposure due to toxic shock. Clinical symptoms include fever, chills, nausea, abdominal pain, hypotension, and the development of secondary lesions, which typically develop in arms and legs. The mortality rate is very low with gastrointestinal illness but about 50% for septicemia and 20–30% for wound infection.

Prevention and Control of *V. parahaemolyticus* and *V. vulnificus* Infection

Vibrios are becoming one of the most dangerous emerging foodborne pathogens, due in part of increased high-risk human population, the popularity of seafood, and global warming with the consequent bloom of bacteria and their habitats within zooplankton and phytoplankton. Aquaculture is one of the fastest growing industries worldwide. The risk of contamination of seafood is much greater in coastal waters and freshwaters than in the open ocean. Seafood safety may be enhanced by harvesting from water when the water temperature is low especially during the winter months and from unpolluted water. In addition, products need to be placed in ice or chilled, especially molluscan shellfish at harvest through shipping and processing to prevent bacterial growth. Workers' safety should be addressed especially for those who have cuts or wounds or abrasions (lacerations) of the skin. They should take precautions to avoid contact with water or seafood. Consumer education should be a part of the seafood safety program. That would include alerting consumers to the dangers of eating raw or undercooked seafood especially for persons with underlying conditions including liver disease, diabetes, kidney disease, and immunocompromised immune systems.

In diarrheal patients, loss of water and electrolytes lead to dehydration, and fluid therapy is the most effective treatment to prevent dehydration. Rehydration with water containing table salts and glucose is effective in resource-constrained situations, but intravenous fluid replacement is essential in patients showing severe signs of dehydration. Antibiotics including tetracycline and fluoroquinolones (ciprofloxacin, levofloxacin, moxifloxacin) are effective.

Detection of *Vibrio* Species

Culture and Serological Methods

Selective enrichment is performed in alkaline peptone water (APW) containing 1–3% NaCl, and colonies are isolated by streaking enriched samples onto the selective thiosulfate citrate bile salts sucrose (TCBS) agar. Bacteria have been also isolated on cellobiose polymyxin B colistin (CPC) and mannitol–maltose agar. In addition, *V. vulnificus* is also isolated using sodium dodecyl sulfate-polymyxin B-sucrose agar (SPS), *Vibrio vulnificus* agar (VVA), and modified CPC (mCPC) agar. Biochemical characterizations can be performed to determine the species of *Vibrio*. *V. parahaemolyticus* has been tested for Kanagawa phenomenon (hemolytic activity) by growing them on Wagatsuma agar containing high salt (7%) and blood for TDH activity. Identification is further accomplished by serotyping for somatic O antigen and capsular K antigens. Immunological assays including ELISA have been used that target intracellular and TDH antigens.

Molecular Techniques

Single gene or multigene-specific PCR assays have been developed targeting the 16S rRNA *tdh*, *trh*, *gyrB*, *toxR*, *ctxB*, *ctxAB*, and *tcpA* genes for detection of *Vibrio* spp. A detection limit of 10^1–10^2 cfu has been reported when used in a multiplex format targeting two to three genes. A PCR assay targeting the *vvhA* gene (specific for *V. vulnificus*) has been used to differentiate clinical (C) and environmental (E) strains. For genomic typing and identification, ribotyping, restriction fragment length polymorphism (RFLP), amplified restriction fragment length polymorphism (AFLP), randomly amplified polymorphic DNA, and enterobacterial intergenic consensus sequence-PCR (ERIC-PCR) have been used. Multilocus

enzyme electrophoresis and multilocus sequence typing of housekeeping genes have been performed for identification and typing purposes.

Summary

Although cholera is considered an old-world disease, it continues to be a serious problem in developing and economically impoverished countries. The infections caused by other vibrios are also increasing worldwide especially in developed countries and are increasingly being recognized as emerging diseases. *Vibrio cholerae* is known for its epidemic and pandemic outbreaks, especially in countries throughout Asia, Africa, and South and Central America, where the fecal–oral transmission mode spreads the disease, often through the consumption of contaminated drinking water. Upon entry into the intestine, the bacterium produces several adhesion factors including toxin-coregulated pili (TCP), flagella, neuraminidase, and accessory colonization factor (ACF) for colonization. The bacterium produces cholera toxin (CT) and zona occludin toxin (ZOT), which affect the ion transport pumps for Na^+, Cl^-, HCO_3^-, and K^+ and junctional integrity and results in extensive fluid and ion losses. Diarrhea appears within 6 h–5 days and lasts for 2–12 days. Oral vaccination with killed bacteria together with a purified B subunit of cholera toxin is widely used and is recommended by the WHO. *Vibrio parahaemolyticus* and *V. vulnificus* infections are associated with seafood harvested from estuarine or freshwaters. They produce several heat-stable (TDH) and heat-labile TDH-related hemolysins (TRH) and phospholipases, which are responsible for membrane pore formation, apoptosis, and fluid loss resulting in diarrhea. Additionally, *V. vulnificus* causes septicemia and wound infections, which could be fatal. *Vibrio vulnificus* is the most invasive of all vibrios in immunocompromised high-risk population. In addition to hemolysin, it produces collagenase, metalloprotease, lipase, and phospholipases, which promote rapid tissue destruction resulting in death within 24 h. The mortality rate of septicemic infection is about 50%, and wound infection is 22%.

Further Readings

1. Al-Assafi, M.M.K., Mutalib, S.A., Ghani, M.A. and Aldulaimi, M. (2014) A review of important virulence factors of *Vibrio vulnificus*. *Curr Res J Biol Sci* **6**, 76–88.
2. Albert, M.J. and Nair, G.B. (2005) *Vibrio cholerae* O139 Bengal - 10 years on. *Rev Med Microbiol* **16**, 135–143.
3. Ali, M., Lopez, A.L., You, Y., Kim, Y.E., Sah, B., Maskery, B. and Clemens, J. (2012) The global burden of cholera. *Bull World Health Organ* **90**, 209–218.
4. Elmahdi, S., DaSilva, L.V. and Parveen, S. (2016) Antibiotic resistance of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in various countries: A review. *Food Microbiol* **57**, 128–134.
5. Faruque, S.M., Albert, M.J. and Mekalanos, J.J. (1998) Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. *Microbiol Mol Biol Rev* **62**, 1301–1314.
6. Gulig, P.A., Bourdage, K.L. and Starks, A.M. (2005) Molecular pathogenesis of *Vibrio vulnificus*. *J Microbiol* **43**, 118–131.
7. Harwood, V.J., Gandhi, J.P. and Wright, A.C. (2004) Methods for isolation and confirmation of *Vibrio vulnificus* from oysters and environmental sources: a review. *J Microbiol Methods* **59**, 301–316.
8. Kanungo, S., Sur, D., Ali, M., You, Y.A., Pal, D., Manna, B., Niyogi, S.K., Sarkar, B., Bhattacharya, S.K. and Clemens, J.D. (2012) Clinical, epidemiological, and spatial characteristics of *Vibrio parahaemolyticus* diarrhea and cholera in the urban slums of Kolkata, India. *BMC Pub Health* **12**, 830.
9. Letchumanan, V., Chan, K.-G. and Lee, L.-H. (2014) *Vibrio parahaemolyticus*: a review on the pathogenesis, prevalence, and advance molecular identification techniques. *Front Microbiol* **5**, 705.
10. Levin, R.E. (2005) *Vibrio vulnificus*, a notably lethal human pathogen derived from seafood: A review of its pathogenicity, subspecies characterization, and molecular methods of detection. *Food Biotechnol* **19**, 69–94.
11. Levin, R.E. (2006) *Vibrio parahaemolyticus*, a notably lethal human pathogen derived from seafood: A review of its pathogenicity, characteristics, subspecies characterization, and molecular methods of detection. *Food Biotechnol* **20**, 93–128.
12. Lippi, D. and Gotuzzo, E. (2014) The greatest steps towards the discovery of *Vibrio cholerae*. *Clin Microbiol Infect* **20**, 191–195.
13. Nair, G.B., Ramamurthy, T., Bhattacharya, S.K., Dutta, B., Takeda, Y. and Sack, D.A. (2007) Global dissemination of *Vibrio parahaemolyticus* serotype O3:K6 and its serovariants. *Clin Microbiol Rev* **20**, 39–48.
14. Oliver, J.D. (2013) *Vibrio vulnificus*: death on the half shell. A personal journey with the pathogen and its ecology. *Microb Ecol* **65**, 793–799.
15. Oliver, J.D. (2015) The biology of *Vibrio vulnificus*. *Microbiol Spectrum* **3**.

16. Raghunath, P. (2015) Roles of thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) in *Vibrio parahaemolyticus*. *Front Microbiol* **5**, 805.
17. Safa, A., Nair, G.B. and Kong, R.Y.C. (2010) Evolution of new variants of *Vibrio cholerae* O1. *Trends Microbiol* **18**, 46–54.
18. Silva, A.J. and Benitez, J.A. (2016) *Vibrio cholerae* biofilms and cholera pathogenesis. *PLoS Negl Trop Dis* **10**, e0004330.
19. Teschler, J.K., Zamorano-Sánchez, D., Utada, A.S., Warner, C.J., Wong, G.C., Linington, R.G. and Yildiz, F.H. (2015) Living in the matrix: assembly and control of *Vibrio cholerae* biofilms. *Nat Rev Microbiol* **13**, 255–268.
20. Turner, J.W., Paranjpye, R.N., Landis, E.D., Biryukov, S.V., González-Escalona, N., Nilsson, W.B. and Strom, M.S. (2013) Population structure of clinical and environmental *Vibrio parahaemolyticus* from the Pacific Northwest coast of the United States. *PLoS One* **8**, e55726.
21. Whitaker, W.B., Parent, M.A., Boyd, A., Richards, G.P. and Boyd, E.F. (2012) The *Vibrio parahaemolyticus* ToxRS regulator is required for stress tolerance and colonization in a novel orogastric streptomycin-induced adult murine model. *Infect Immun* **80**, 1834–1845.
22. Zhang, X.H. and Austin, B. (2005) Haemolysins in *Vibrio* species. *J Appl Microbiol* **98**, 1011–1019.