Chapter 7

Pathogenesis of Cholera: Recent Prospectives in Rapid Detection and Prevention of Cholera

Tirumale Sharmila and Tessy Anu Thomas

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.74071

Abstract

Cholera, also known as "blue death" is a potentially epidemic and life-threatening secretory diarrhea characterized by numerous voluminous watery stools, often accompanied by vomiting and resulting in hypovolemic shock and acidosis. The causative agent of this water-borne disease belongs to certain members of the species *Vibrio cholerae* (*V. cholerae*) which can also cause mild or unapparent infections. *V. cholerae* is a facultative anaerobic, Gram-negative bacilli, which possess the characteristic feature of darting motility under wet-mound preparations. Other members of the species may occasionally cause isolated outbreaks of milder diarrhea, whereas others—the vast majority—are free-living and not associated with disease. The emergence of new, virulent, drug-resistant strains of *Vibrio* is the main cause of protracted outbreaks leading to high fatality rates. The subsequent loss of fluid volume causes a drop in blood pressure and circulatory shock. If the patient remains untreated, they become progressively weaker, sometimes to the point of death, within 12–24 h of the onset of symptoms. The severity and fatality of the disease depend on the strain of *Vibrio*. The cholera toxin-producing (CT-producing) *V. cholerae* manifests the most fatal disease known as cholera gravis. Throughout most of the twentieth century, cholera was caused by *V. cholerae* of the O1 serogroup, and the disease was largely confined to Asia and Africa. The emergence of a pandemic in 1992 was caused by an unknown serogroup of *V. cholerae* (O139) wherein the targets were India and Bangladesh. The pathogenesis and virulence of the bacteria are due to an enterotoxin it produces—cholera toxin (CT). The mechanism of action of CT is discussed in this chapter at a later stage. Attempts have been made to produce vaccines through a number of trial-and-error methods, and still the possibility of an effective vaccine which gives a good prophylactic measure is under consideration. The identification of the bacteria as well as toxin detection is one of the main elements in the clinical microbiology field. The identification and confirmation of this epidemic disease commend from the morphological identification of *Vibrio* and end with serotyping and biotyping in addition to toxin detection by various means of assays—both in vitro and in vivo. This chapter will cover all of the mentioned areas of clinical microbiology with respect to cholera infection in addition to the recent outbreaks and epidemics which occurred across the globe.

Keywords: *Vibrio cholerae*, enterotoxin, *Vibrio cholerae* O139, vibriocidal antibodies, secretory IgA, ELISA

1. Introduction

Cholera, a water-borne infectious disease (also known as "blue death"), is characterized by life-threatening secretory diarrhea often accompanied by numerous voluminous watery stools and vomiting, eventually leading to hypovolemic shock and acidosis. The term "blue death" is given to the disease as it changes the skin of the infected individuals to blue discoloration due to severe dehydration [1, 2]. It is caused by certain members of the species *V. cholerae* which can also cause mild or unapparent infections. These bacteria are found in water or food contaminated with feces. Countries with poor water and sewage treatment are likely to be the victims of cholera. This disease is very unlikely to be spread from person to person. The history of cholera began in the nineteenth century where millions of people across the continents were vanished due to six subsequent pandemics. South Asia in 1961 witnessed the seventh pandemic, Africa in 1971, and the Americas in 1991 [3]. The outcome of humanitarian crisis, like overcrowded camps, and lack of water and sanitation systems are some of the factors which increase the risk of cholera transmission. The presumptive confirmation of cholera is done on the primary clinical investigation of watery stools and severe dehydration. Nowadays the use of rapid identification kits has eased the detection of cholera within a few hours of receiving the sample in the laboratory [2, 3]. The failure to produce an effective oral vaccine against cholera is the area where the professionals and researchers are worried about. The different trials and attempts to produce a vaccine and the vaccines under trial are further discussed in the chapter. The clinical microbiology of cholera, its epidemiology and the laboratory investigations are discussed in detail in this chapter.

2. Clinical features

2.1. Symptoms

The symptoms of the cholera disease usually manifest 1–3 days after ingestion of contaminated food and/or water. In most cases, the symptoms range from mild to moderate, but in 20% of cases, severe life-threatening fatal conditions appear. The most severe manifestation of the disease, "cholera gravis," affects only minority of the patients infected with CT-producing *V. cholerae* [1]. It has been estimated that classical strains are more virulent than El Tor strains. The incubation period of cholera is dependent on inoculum size and ranges from several hours to 5 days [2]. The onset of the clinical manifestation may be sudden profuse watery diarrhea followed by anorexia and abdominal discomfort in some cases. Initially the stool will be dark brown with solid nature; gradually it turns to inoffensive whitish watery stool giving a rice water appearance [1]. This is the characteristic nature of cholera which helps to diagnose

from the other gastrointestinal infections. The massive fluid loss via vomiting and excessive diarrhea is due to the action of enterotoxins produced by the *V. cholerae* [3]. Enterotoxins are cytotoxic and alter the permeability of the epithelial cells of the intestinal wall by creating pores. As a result of osmosis, the water and other fluid substances flow out of the body leading to massive diarrhea and severe dehydration [4]. Vomiting is also present which develops after the onset of diarrhea. In cholera gravis the rate of diarrhea reaches a maximum to 600–1000 ml/h leading to hypotension, tachycardia and vascular collapse. Due to severe dehydration, the skin turgor will be poor, giving a dry appearance, with eyes sunken. The patient will be restless and thirsty, and the hands will show wrinkles as after a long immersion which is often referred to as "washer woman's hands." The drastic hypovolemic shock leads to hypokalemia resulting in painful muscle cramps and in severe cases hypokalemic nephropathy and focal myocardial necrosis. Body temperature will be quite normal except for 20% of the infected individuals which shows a slight temperature rise [4–6].

Lactic acidosis and stool bicarbonate losses associated with dehydration are manifested by decrease of blood pH and plasma bicarbonate and an increased serum anion gap (mean of 20.2 mmol/l in a study). Despite potassium loss, untreated acidosis usually leads to high potassium levels in serum. In children, hypoglycemia associated with coma and convulsions may occur [4, 6].

2.2. Mechanism of pathogenesis

A better understanding of the source of infection is very much necessary for the management and effective prophylaxis of any diseases. In the case of cholera, contaminated water and feces of persons infected with *V. cholerae* are the primary sources of infection. Planktons and zooplankton harboring the infectious agent and certain environmental factors such as surface change and terrestrial nutrient discharge are also among the factors that indirectly lead to the production of more hosts [7].

The mechanism of invasion begins as soon as the bacterium establishes itself in the bowel, following the colonization of the epithelial layer of the small intestine by penetrating the mucous. The penetration of the mucous is facilitated by mucolytic enzymes which help to destroy the mucous integrity, and also the long tail of the invading organism allows it to propel itself through the thick mucosal layer. The attachment *Vibrio* onto the microvilli of the small intestine is aided by the so-called pili on the bacterium which is one of the major factors which attributes to the darting motility [6, 8]. The production of an endotoxin by the bacteria, called CT, is the major key for the virulence mechanism. So it is proved that only toxigenic strains of *Vibrio*, i.e., *Vibrio* that produces CT, are capable of causing cholera. Studies suggest that this CT is encoded by a filamentous bacteriophage which is harbored by pathogenic vibrios [1].

The CT that is made is comprised of six protein subunits: one A subunit and five copies of B subunits, generally denoted as AB5. B subunit is also known as the binding factor which binds to the GM1 ganglioside receptor of the epithelial cells of the small intestine. Once bound with the target cells, it forms a toxin complex which is then endocytosed by the cell.

As soon as the process of endocytosis takes place, the enzymatic activation of A subunit occurs leading to increased adenylate cyclase activity, thereby increasing the concentration of cAMP to more than 120-folds. This in turns leads to increased permeability of the chloride channels subsequently mediating the efflux of more ATP-mediated chloride ions and secretion of mere H_2O, Na^+, K^+ and HCO_3^- into the lumen of the intestine [4, 5, 7]. The increased absorption of water as well as electrolytes is responsible for the massive dehydration leading to the clinical symptoms of cholera. The brief mechanism of pathogenesis is depicted in **Figure 1**.

2.3. Differential diagnosis

In places where cholera disease is found epidemic or endemic, treatment should never be delayed due to pending laboratory investigations; laboratory confirmation is very essential for the prophylaxis as well as for treatment in epidemic-endemic areas.

As outlined above, definitive diagnosis is required for which the most crucial step is the isolation of *V. cholerae* from the stool of the infected individuals. For an epidemic survey, a further characterization rather than the isolation of *Vibri*o may not be required. In other circumstances, isolates should be typed by specific serum agglutination for O139 and O1 serotypes [9].

2.4. Therapy

The key to therapy is adequate rehydration till the disease has come to its end course (usually 1–6 days in the absence of antibiotics). Rehydration can be accomplished by oral rehydration with oral rehydration solution (ORS) as in other cases of diarrhea and dysentery. In severe

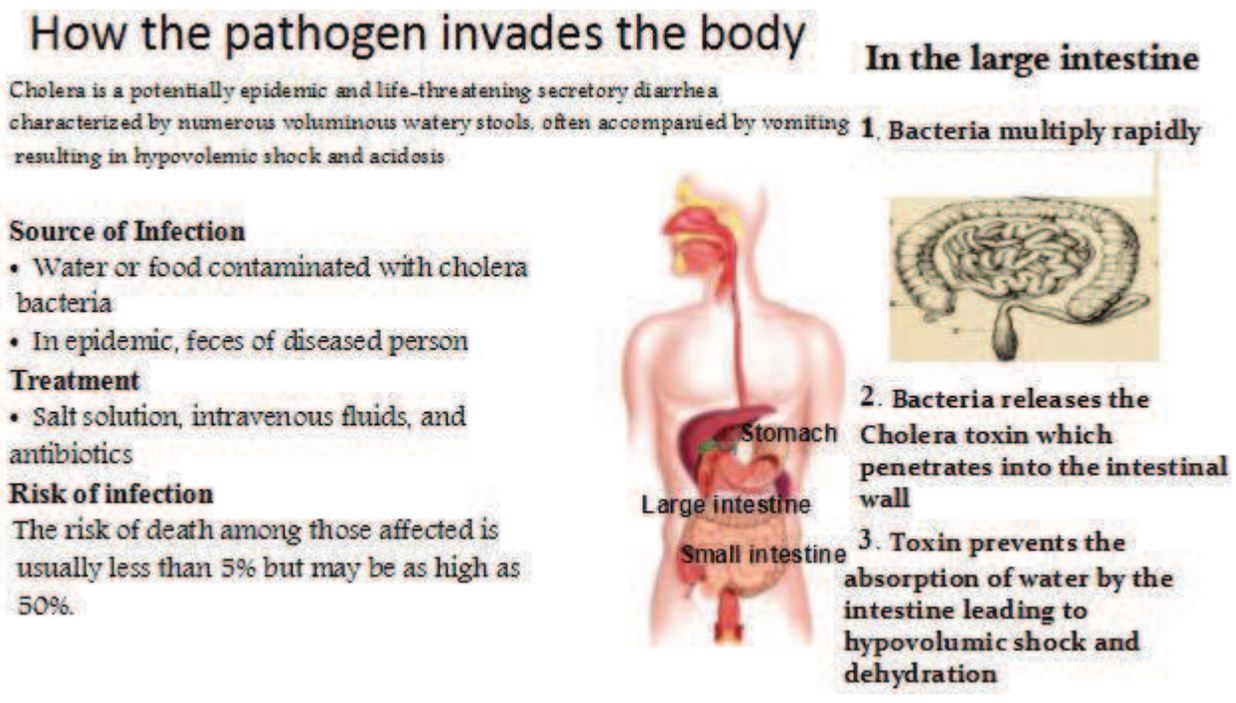

Figure 1. Mechanism of pathogenesis of *V. cholerae*.

cases, intravenous infusion of fluid will be necessary. Antibiotics help to reduce the severity of illness and the duration of excretion of the organism. In children, hypoglycemia is treated with 25 or 50% glucose administered intravenously [10].

2.5. Intravenous rehydration

For adults, intravenous rehydration is done as fast as possible; 2 liters is administered the first 30 minutes. If the condition of the patient improves at this point, the rate of infusion can be lowered to 100 ml/kg of the body weight within the first 4 hours of therapy. Children should receive 30 ml of the intravenous fluid per kg of body weight during the first hour and an additional 40 ml/kg in the following 2 hours [10, 11]. Both adults and children should receive appropriate amount of ORS during the course of illness. The rate and frequency of intravenous infusion can be varied depending on the electrolyte loss from the stool of the patient. The World Health Organization (WHO) recommends Ringer's lactate as the best rehydration solution. Isotonic saline corrects only hypovolemia, so potassium and glucose should be administered along with it for proper recovery from dehydration [12, 13].

2.6. Oral rehydration

Patients with moderate illness and dehydration can receive fluid replacement orally to compensate for the water and electrolyte depletion. In mild cases, the WHO recommends 50 ml of ORS/kg of body weight to be given in the initial 4 h. For moderate dehydration, the quantity should be increased to double, i.e., 100 ml/kg [11, 14]. Stool output should be continuously monitored depending on which the rehydration therapy is optimized. For children, who tend to have high fluid losses, it should be replaced 1:1 with ORS. The oral solution used must be the one recommended by the WHO [1]. This solution consists of appropriate amount of electrolytes to balance the fluid and electrolyte losses due to dehydration. In addition, it also contains optimum quantity of glucose to facilitate the absorption of sodium and water.

The formula for the current WHO oral rehydration solution is 2.6 grams table salt (NaCl), 2.9 grams trisodium citrate dihydrate, 1.5 grams (0.053 oz) potassium chloride (KCl) and 13.5 grams anhydrous glucose ($C_6H_{12}O_6$) per liter of fluid [1, 6, 12].

A homemade oral rehydration solution can be prepared when readymade sachet is not available. The composition of the ORS solution consists approximately of six level teaspoons of sugar and 1/2 a teaspoon of table salt in 1 liter of water. The molar ratio of sugar to salt should be 1:1, and the solution should not be hyperosmolar [9, 12].

2.7. Antimicrobial therapy

Treatment with antibiotics should start only after the initial rehydration therapy and correction of acidosis. Antibiotics play a secondary role in the treatment of cholera by shortening the length of cholera diarrhea as well as the excretion of vibrios. Though most of the *V. cholerae* strains have so far not exhibited any drug resistance, O139 strains have an intrinsic resistance to trimethoprim-sulfamethoxazole [14, 15]. Tetracycline is the usual drug of choice [7]. Rather

than the possibility of staining of teeth, tetracycline has not shown any contraindications in adult and children. Besides tetracycline, the quinolone group of antibiotics (ciprofloxacin and norfloxacin) has shown excellent in vitro activity against *Vibrio* [10, 11]. The widespread use of tetracycline as a prophylactic control is not recommended nowadays due to the emerging resistance toward it in the epidemic areas [14].

3. Epidemiology

Cholera is an indication of the prevalence of unsanitary conditions especially with the growing populations in developing countries. It is a plight that about 3–5 million cases of cholera are reported each year with the advancements of health and medical professionals across the globe [16]. When 52 countries reported 236,896 cases in 2006 with a fatality rate of 2.7%, a total of 589,854 cases were reported in 2011 from 58 countries [7]. These numbers suggest cholera being a global disease threatening worldwide.

In developing countries, where the population is high, cholera is the main cause of epidemic diarrhea. Global pandemic outbreaks are also being reported from Asia, Africa and Latin America [17]. Africa, being a country with limited medical facilities, has shown a considerable decrease in cholera outbreaks in the recent years. When 117,570 cases were reported in 2012, only 56,329 cases were reported in 2013 [16]. This shows a remarkable sanitary improvement as well as prophylactic measures adopted by the country. During 2013, Mexico and other Central African countries reported an outbreak, while 2014 witnessed a cholera outbreak in South Sudan. In an outbreak in Cuba, a total of 185 cases were reported in 2012 [18]. The WHO reports an average of 132,121 cholera cases in 2016 from 38 countries which includes 2420 fatality reports [13].

Considering the epidemiology of the different serotypes and biotypes of *V. cholerae,* serogroup O1 and O139 cause outbreaks. The biotype EL Tor and serotype Ogawa are the main causes of epidemic cholera in developing countries [18, 19]. Among *V. cholerae* serogroups, O1 is the main cause of recent outbreaks. O139 was first identified in a massive outbreak in Bangladesh in the year 1992, but as of now, it is only seen in some sporadic cases [20]. It has not been reported outside Asia. The causative strain and serotype of *Vibrio* responsible for the first four pandemic is unknown, whereas the causative agents of the fifth and the sixth pandemics were identified as classical biotypes of O1 strains and El Tor biotype, respectively [16]. In 2010, Haiti outbreak was reported which was imported by the United Nations Peacekeeping Forces from South Asia. The United Nations report says it has affected 788,000 people and claimed the lives of 9200 people [16]. These information lead us to the fact that in the progress of epidemics and pandemics, new clones of toxigenic *Vibrio* strains continue to emerge which is one of the major threat to the countries who are the victims of the life-threatening disease.

Vibrio being the native species of the aquatic environment cannot be eradicated permanently from the globe as in the case of small pox [13]. There is an increased risk of epidemic and pandemic cholera with changes in the environmental and climatic conditions. As a prophylactic measure, integrated prevention strategies and interdisciplinary actions are required.

http://dx.doi.org/10.5772/intechopen.74071

4. Immune response and vaccines

Over 75 years of research has been done on the development of an effective vaccine against cholera. Since *Vibrio* does not exhibit much of antigenic variation, the very realistic goal of a cholera vaccine is very much near and attainable. The advanced study of the intestinal immune system and the pathogenicity of cholera have brought the insight of vaccine very much near.

4.1. Infection-derived immunity

The immunity induced by cholera infection is strain and serotype specific. Studies show that volunteers experimentally infected with classical biotype *V. cholerae* O1 showed 100% protection against subsequent infection against classical biotype vibrios [12]. Similarly, infection with El Tor biotype exhibited 90% immunity against El Tor vibrios [2]. The immunity lasts for a minimum of 3 years for the similar biotype. Initial infection with either the Ogawa or Inaba serotype conferred protection against both the serotypes during reinfection.

4.2. Components of the immune system

Infection with *Vibrio* can range from a fatal diarrheal disease to a mild non-clinical/unapparent case where only serological diagnosis is only possible [1, 12]. The reason for this remains unclear. But there have been certain assumptions regarding the symptomatic difference in the infected patients. One of them is the availability of the intestinal receptors for the toxins and the cholera vibrios in patients. After natural infection, circulating antibodies are detected against most of the cholera antigens including flagellar (H) antigen, toxin and somatic (O) antigen [21, 22]. Antibodies raised against vibrios are referred to as "vibriocidal antibodies" since they lyse the bacteria in the presence of complement and other serum components. The parenteral administration of vaccines causes an apparent rise in the "vibriocidal antibodies" [2, 12]. The immune response consists of both antibacterial and antitoxic immunities, antibacterial immunity being the most predominant. An oral trial vaccine containing killed *Vibrio* conferred more resistance than parenteral vaccine consisting toxoids. A synergistic immune response consisting of both toxins and bacterial antigens showed remarkable immune response than single component [21].

It is likely that the primary immune response might be a main determinant of protection against cholera infection since it is essentially a disease of the small intestine. Moreover infants under breastfeeding are less likely to be affected due to the presence of the secretory antibodies present in the milk [19, 21]. Secretory IgA, IgM and IgG are found in the intestinal mucosa. These antibodies confer protective immunity in the presence and absence of complementary proteins. The actual protection is due to the secretory IgA and other intestinal antibodies, and it is the vibriocidal antibodies in the serum which serves as the marker for IgA antibodies to direct against the same antigens [12, 21]. *V. cholerae* is a motile bacterium which is one of the major factors in the pathogenicity. The flagellar antibodies could prevent the invasion of the bacteria by immobilizing them; as well the somatic (O) and flagellar (H)

antibodies can cause clumping and lead to arrest of motion of cells. At the same time, the antitoxins can bind with the specific toxins at the epithelial surface and prevent the binding of the toxin [2, 12].

4.3. Vaccines

The observation that natural infection confers long-lasting immunity has led to the development of cholera vaccines which can elicit protective immunity. The first attempts of vaccine development started in 1960, which focused on whole-cell vaccine preparation with parenteral administration. Ninety percent immunity was obtained, but it was not long-lasting as the antibody baseline titer waned in an year [21]. An attempt has also been made by converting cholera toxin to toxoid in the presence of glutaraldehyde and formalin, but it had elicited a very low level of protection as toxoid is a poor antigen [12].

In recent years, the importance of intestinal immune response has paved a different direction in vaccine development, i.e., a shift from parenteral to oral vaccines. Parenteral administration of antigens elicits the stimulation of secretory antibodies (IgA, IgG, and IgM), but the antibodies have a short life span and a poor memory [21]. In controversy to it, oral administration of antigens elicits immune response of a longer shelf life and good memory proving to be a good vaccine. There is not much contribution from serum antibody in cholera infection as *V. cholera* does not invade erythrocytes in the process of infection but only colonizes the intestinal mucosal surfaces. Therefore to stimulate the production of secretory antibodies, administration of oral vaccine plays a vital role when compared to parenteral vaccines [12, 21].

The FDA has approved a single-dose live oral vaccine for cholera called Vaxchora (lyophilized CVD 103-HgR) for the age group 18–64 years old [21]. Though it is not recommended in the usual immunization category, it is administered for travelers who are moving to a region actively spread with the toxigenic vibrios. As per the current scenario, there is no cholera vaccine which provides 100% protection, and vaccination is not recommended as a prophylactic measure in preventing cholera by the WHO.

The vaccines under trial consist of live attenuated whole-cell vaccines and killed bacterial suspensions, both of which are administered orally.

5. Laboratory investigations

5.1. Isolation and biochemical identification of the bacteria

Based on the somatic O antigens, currently there are about 130 serogroups of *V. cholerae*. However only O1 serogroup is involved in epidemic and pandemic cholera [12]. Isolation and identification of *V. cholerae* serogroup O1 or O139 from the stool sample of infected individuals is the gold standard in the diagnosis of the disease. The ideal transport medium is Cary Blair media; however, Alkaline Peptone Water (APW) medium is also

recommended (pH, 8.5) [1, 12]. The culture media are blood agar and MacConkey agar, the selective media being the thiosulfate-citrate-bile salts agar (TCBS). The rapid and presumptive diagnosis of cholera is the most important factor in the diagnosis, and hence the stool sample as soon as received is examined for a wet-mount preparation to look for the darting motility exhibited solely by *Vibrio* species [9, 12, 23]. Thus a presumptive identification or probability of a cholera infection is made by which further treatment is carried out.

Overnight growth (18–24 hours) of *V. cholerae* on TCBS produces large (2–4 mm in diameter) yellow-colored colonies, slightly flattened with opaque centers and translucent peripheries. The yellow color is due to the fermentation of sucrose in TCBS medium. Non-sucrose-fermenting vibrios such as *V. parahaemolyticus* produce green to blue-green colonies. On MacConkey agar, the colonies are late lactose-fermenting colonies, initially being non-lactose fermenters [23]. Yellow colonies on TCBS agar are depicted in **Figure 2**.

Table 1 is taken from Laboratory Methods for the Diagnosis of *V. cholerae*, Centers for Disease Control and Prevention [12].

5.2. Serologic identification

As discussed previously, there are more than 130 serogroups of *V. cholerae,* among which O1 serogroup is the causative agent of most of the epidemics and pandemics. Serologic identification of *V. cholera* O1 serogroup is carried out by agglutination test with the specific antisera. This is the most rapid method and specific method of identifying *V. cholerae* O1 [16].

Isolates of O1 serogroup is further divided into three serotypes, namely, Ogawa, Inaba and Hikojima (rare). Strains that are positive with polyvalent O1 antisera are further tested for agglutination with Ogawa and Inaba antisera. In some instances, there may be strains that agglutinate very strongly and equally with both Ogawa and Inaba antisera. If ever such rare reactions are suspected, those strains should be referred to a reference laboratory for further confirmation and may be referred to as "possible serotype Hikojima" [9, 12].

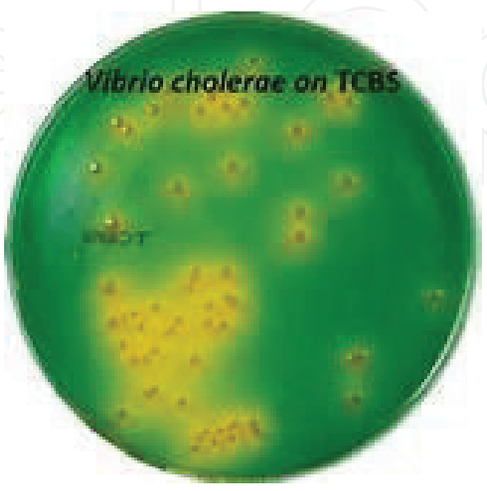

Figure 2. Colony morphology of *V. cholerae* on TCBS—yellow color colonies.

Biochemical tests	Reaction
Oxidase	Positive
String test	Positive
Kligler's iron agar	K/A, no gas, no H_2S
Triple sugar iron agar	A/A, no gas, no H_2S
Glucose (acid production)	Positive
Glucose (gas production)	Negative
Sucrose (acid production)	Positive
Lysine	Positive
Arginine	Negative
Ornithine	Positive
Voges-Proskauer	Variable*

*Most isolates of *V. cholerae* serotype O1 biotype El Tor are positive in the Voges-Proskauer test, whereas biotype classical strains are negative.

Table 1. Biochemical characteristics of isolates of ***V. cholerae*** O1.

Table 2 is taken from Laboratory Methods for the Diagnosis of *V. cholerae*, Centers for Disease Control and Prevention [12].

5.3. Tests to distinguish biotypes of *V. cholerae* O1

The typing of *V. cholerae* O1 is not necessary for the identification and diagnostic point of view but is necessary for the epidemic surveillance in helping to identify the source of infection, particularly when cholera is prevalent in a particular geographical area [12]. There are two biotypes—classical and El Tor, among which El Tor biotype is predominant throughout the world and classical is rare except in certain regions of Bangladesh.

Table 3 is taken from Laboratory Methods for the Diagnosis of *V. cholerae*, Centers for Disease Control and Prevention [12].

5.4. Toxin assays

The virulence of the O1 serogroup is marked by the production of CT toxin. The CT molecule comprises one A (active) subunit and five B (binding) subunits. The B subunit attaches to

Serotype	Major O factors present	Agglutination in absorbed sera	
		Ogawa	Inaba
Ogawa	A, B	+	–
Inaba	A, C	–	+
Hikojima	A, B, C	+	+

Table 2. Identifying characteristics of serotypes of ***V. cholerae*** serogroup.

Property	Reaction	
	Classical	El Tor
Voges-Proskauer (modified with 1% NaCl)	−	+
Zone around polymyxin B (50 U)	+	−
Agglutination of chick erythrocytes	−	+
Lysis by bacteriophage		
Classical IV	+	−
El Tor V	−	+
Hemolysis	−	+

Table 3. Differentiation of classical and El tor biotypes of ***V. cholerae*** serogroup O1.

the GM1 ganglioside receptors on epithelial cells of the intestinal mucosa during the process of infection. After this process, cleavage occurs between the subunits so as to facilitate the entry of A component into the cell. The A1 component is responsible for the stimulation of adenylate cyclase which in turn produces cyclic AMP (cAMP) [3, 12]. Increased intracellular production of cAMP leads to disruption of electrolytes across the cell membrane leading to fluid secretion into the small intestine. When the fluid entering the intestine is greater than its reabsorption capability, it leads to diarrhea [12, 23]. CT toxin is similar to *Escherichia coli* (*E. coli)* heat labile toxin (LT) both antigenically and in mechanism. So, most of the toxin-detecting assays are common for CT and LT. The mechanism of action of CT is well explained in "Mechanism of pathogenesis" and illustrated in **Figure 2**.

Before testing for the toxin, the isolate should be confirmed as *V. cholera* O1 strain. The non-O1 serogroups also produce CT or other toxins similar to heat labile enterotoxin or Shiga toxin, but these toxins are not of diagnostic value as they have not yet been associated with epidemic disease [12].

5.4.1. Bioassays

5.4.1.1. Animal methods

The first cholera enterotoxin assay was the adult rabbit ileal loop method in the 1950s in which the enterotoxin was injected into the ligated segments of the intestine (ileal loops) of experimental animals such as rabbits, pigs, dogs and calves, and this caused accumulation of fluid. This mechanism is routinely used for the study of mechanism of CT, heat labile toxin of *E. coli* and other toxins. A cell-free supernatant is injected into each of the ileal loop after the exteriorization and ligation of the rabbit's small intestine after which the abdomen is closed for 18 hours. The quantity of the accumulated fluid due to the stimulation of the toxin is measured by measuring the loops once the intestine is removed [12]. The disadvantage of this model is that it is time-consuming, cumbersome and difficult to standardize.

Another model was developed in 1955, the infant rabbit infection model which was also used for the determination of different toxins. Here, a 7-day-old infant rabbit is infected

with the test culture either by gastric intubation or by intraluminal injection. A positive assay results in watery diarrhea of the rabbit and eventual death due to dehydration. The small intestine is removed, and the loops are measured to quantify the excess fluid accumulation [8]. The drawback of this procedure is the requirement of one animal per test sample.

The vascular permeability factor assay is a dermal test wherein the activity of CT or LT is determined by the neutralization of standardized amount of specific antisera against CT. Here 20–30 supernatants can be injected per rabbit. A cell-free supernatant is injected intradermally into the rabbit, following which Evans blue dye is injected intravenously. The increased capillary permeability mediated by CT leads to perfusion of the dye in the skin causing a blue coloration with localized induration at the site of injection. The diameter of discoloration and induration is measured with a negative control [1, 12].

5.4.1.2. Tissue culture methods

Tissue culture methods are widely used in laboratories nowadays for the toxin assay. It requires skilled professionals and equipment in the laboratory with existing tissue culture facilities. It is a very sensitive assay with high reproducibility. In addition to detecting toxins, these assays are also useful in detecting toxin-neutralizing antibodies.

The Chinese hamster ovary (CHO) and the Y1 mouse adrenal (Y1) cell lines are used for the assay of CT and LT, although Vero monkey kidney cells are also used. The toxin if present in the cell-free supernatant increases the intracellular production of cAMP in the cell lines. This can be made out by the morphological changes in the cell lines (Y1 cells round and CHO cells elongate) [7, 12].

5.4.2. Immunoassays

5.4.2.1. Elisa

As CT and LT are immunogenic to both humans and animals, many immunogenic assays have been developed to detect the presence of toxins in vitro. As discussed earlier, the GM1 ganglioside is the natural receptor for CT. In ELISA method, this GM1 ganglioside receptor is purified and is used for the preparation of ganglioside-capture enzyme-linked immunosorbent assay. The cell-free supernatant in which the toxicity is to be tested is added to the microtiter wells coated with GM1 ganglioside. CT antiserum is then added on the plate to detect the binding of the toxin with the bound receptor on the plate, followed by the addition of enzyme-conjugated antiglobulin antibody [12].

5.4.2.2. Latex agglutination

In this assay, highly purified specific anti-CT is bound to latex particles. A commercial version of kit is available in the market which allows easy detection of cholera toxins in the laboratory.

5.4.3. DNA-based assays

5.4.3.1. Polymerase chain reaction (PCR)

PCR is a molecular approach based on specific DNA sequences. In PCR, DNA polymerase enzyme is used to amplify certain specific sequences of DNA (amplicons) which can be detected on an agarose gel or using specific DNA probes [23]. The toxigenicity of *V. cholerae* can be detected by using PCR and primers that specifically amplify only CT genes. The advantage of PCR is that it does not require a pure culture or even viable strains. The toxigenicity of the organism can be detected from the infected stool samples, contaminated water or food samples, thus determining the presence of *Vibrio* even without culturing it. The PCR method also has the advantage of detecting CT toxins that is produced in very low levels by certain strains, by identifying ctx genes possessed by them [12, 23]. The technical problems associated with radioisotopic labels have been solved by non-radioactive DNA labels such as biotin and digoxigenin. Apart from the fact that DNA-based assays require skilled professionals and sophisticated laboratories, these methods provide reliable, specific and rapid results unlike the conventional methods.

Table 4 is taken from Laboratory Methods for the Diagnosis of *Vibrio cholerae*, Centers for Disease Control and Prevention [12].

Assay	Sensitivity (per ml)	Type of assay	Specific target of assay	Sample tested
Rabbit ileal loop	30 ng	Bioassay	Stimulation of fluid accumulation	Culture supernatant
Infant rabbit assay	250–500 ng	Bioassay	Stimulation of fluid accumulation	Broth culture/supernatant
Rabbit skin test	0.1–3.5 ng	Bioassay	Permeability factor	Culture supernatant
Y1 mouse adrenal cells	10 pg	Bioassay	Accumulation of cAMP	Culture supernatant
Chinese hamster ovary cells	10 pg	Bioassay	Accumulation of cAMP	Culture supernatant
GM1-ELISA	10 pg	Immune	B subunit	Culture supernatant
Coagglutination	50 ng	Immune	B subunit	Culture supernatant
Reverse passive latex agglutination	1–2 ng	Immune	B subunit	Culture supernatant
DNA probe	Detects Ctx gene	Genetic	Ctx gene	DNA (colony blot)

Table 4. Commonly used assays for the detection of cholera toxin.

6. Conclusion

V. cholerae is one among the most successful emerging and reemerging pathogens that has both human and environmental components in its life cycle. The epidemic and pandemic occurrence of *V. cholerae* especially *V. cholerae* O139 has been a turning point in the history of the most dreaded diarrhoeal disease—cholera. The conventional methods of screening and identification of these Gram-negative, motile bacilli are not sufficient enough for the rapid diagnosis of cholera. Although molecular methods have evolved for the identification of most of the serotypes and biotypes of *V. cholerae*, the most important factor to control the epidemic and pandemic of the dreaded, fatal, life-threatening disease is to sanitize the food and water environment as the most important prophylactic measure. India, Bangladesh and Sri Lanka have been victims of cholera epidemics, and now it is affecting a multitude of countries. Being developing countries, the concerned governments along with medical professionals should come up with certain safety measures and evoke the necessity of sanitization and cleanliness of the food and water systems to all people. Along with the above-said preventive measures, researchers and medical professionals are on the verge of producing an effective vaccine which will enable to boost the immune system of the individuals. As mentioned in the chapter, the antigenic variation and shift of the organism are the hindrances in producing an effective vaccine. If people are immunized broadly, herd immunity results, with a decrease in the extent of contamination in the environment.

6.1. Future perspectives of the chapter

The Centers for Disease Control and Prevention (CDC) is always one step ahead in investigating any cholera outbreaks across the globe. They have trained professionals and sophisticated laboratory protocols for identification of toxigenic vibrios and emergence of any new antigenically modified strain as the Haiti outbreak. The WHO in collaboration with the CDC also provides information to the public and the health officials regarding the necessity of proper sanitization and preventive control measures to overcome the dreaded disease. The antigenic variation of the *V. cholerae* strains is one of the major limitations and hindrances in developing a 100% protective vaccination against it. The successful accomplishment of an effective vaccine will pave the way for a cholera-free globe so that the developed and the developing countries will not be able to face any more epidemics like the Haiti's and the West Bengal O139.

Author details

Tirumale Sharmila* and Tessy Anu Thomas

*Address all correspondence to: sharmila1966@yahoo.co.in

Department of Microbiology and Biotechnology, Jnana Bharathi Campus, Bangalore University, Bangalore, India

References

[1] Harris JB, Khan AI, LaRocque RC. Blood group, immunity, and risk of infection with vibrio cholerae in an area of Endemicity. Infection and Immunity. 2005;**73**(11):7422-7427. DOI: 10.1128/IAI.73.11.7422-7427.2005

[2] Leung DT, Chowdhury F, Calderwood SB, Qadri F, Ryan ET. Immune responses to cholera in children. Expert Review of Anti-Infective Therapy;**10**:435-444. DOI: 10.1586/eri.12.23

[3] Talkington D, Bopp C, Tarr C. Characterization of toxigenic vibrio cholerae from Haiti, 2010-2011. Emerging Infectious Diseases. 2011;**17**(11):2122-2129. DOI: 10.3201/eid1711.110805

[4] Nelson EJ, Harris JB, Morris JG Jr, et al. Cholera transmission: The host, pathogen and bacteriophage dynamic. Nature Reviews. Microbiology. 2009;**7**:693

[5] Silva, Anisia J, Benitez JA. Vibrio cholerae biofilms and cholera pathogenesis. PLoS Neglected Tropical Diseases. 2016. DOI: 10.2/e0004330

[6] Centers for Disease Control and Prevention (CDC). Update: outbreak of cholera—Haiti, 2010. MMWR. Morbidity and Mortality Weekly Report. 2010;**59**:1586

[7] Saulat J. Cholera: Epidemiology, prevention and control. Agricultural and Biological Sciences Significance, Prevention and Control of Food Related Diseases. 2016; 978-953-51-2277-7. DOI: 10.5772/63358

[8] Harris JB, LaRocque RC, Chowdhury F, et al. Susceptibility to vibrio cholerae infection in a cohort of household contacts of patients with cholera in Bangladesh. PLoS Neglected Tropical Diseases. 2008;**2**:e221

[9] Levine M, Nalin DR, Rennels MB, Hornick RB, Sotman S, Hughes TP, O'Donnell, Barua D. Genetic susceptibility to cholera. Annals of Human Biology. 2009;**6**(4):369-374. DOI: 10.1080/03014467900003751

[10] Sack DA, Sack RB, Chaignat CL. Getting serious about cholera. The New England Journal of Medicine. 2006;**355**(7):649-651. DOI: 10.1056/NEJMp068144. PMID: 16914700

[11] Sack RB, Nair GB, Siddique AK. Cholera. Lancet. 2004;**363**(9404):223-233. DOI: 10.1016/S0140-6736(03)15328-7

[12] Laboratory Methods for the Diagnosis of Epidemic Dysentery and Cholera (PDF). Atlanta, GA: CDC. 1999. http://www.cdc.gov/ncidod/dbmd/diseaseinfo/cholera/top.pdf. Retrieved 2010-02-01

[13] Cholera: prevention and control. Health topics. WHO. 2008. http://www.who.int/topics/cholera/control/en/index.html. Retrieved 2008-12-08.

[14] Ryan KJ, Ray CG. Sherris Medical Microbiology. 4th ed. McGraw Hill; 2004. pp. 376-377 ISBN: 0838585299

[15] Todar K. *Vibrio cholerae* and Asiatic cholera. Todar's Online Textbook of Bacteriology. http://www.textbookofbacteriology.net/cholera.html. Retrieved 2010-12-20

[16] Farmer P, Almazor CP, Bahnsen ET. Meeting cholera's challenge to Haiti and the world: A joint statement on cholera prevention and care. PLoS Neglected Tropical Diseases. 2011:5e1145

[17] Merrell DS, Butler SM, Qadri F. Host-induced epidemic spread of the cholera bacterium. Nature. 2002;**417**(6889):642-645. DOI: 10.1038/nature00778

[18] Cholera's seven pandemics. CBC News. October 22, 2010.

[19] Reidl J, Klose KE. Vibrio cholerae and cholera: Out of the water and into the host. FEMS Microbiology Reviews. 2002;**26**(2):125-139. DOI: 10.1111/j.1574-6976.2002

[20] Sehdev PS. The origin of quarantine. Clinical Infectious Diseases. 2002;**35**(9):1071-1072. DOI: 10.1086/344062

[21] Sinclair D, Abba K, Zaman K, Qadri F, Graves PM. Oral vaccines for preventing cholera. Cochrane Database of Systematic Reviews. 2007;**3**:CD008603. DOI: 10.1002/14651858.CD008603.pub2

[22] King AA, Ionides EL, Luckhurst J, Bouma MJ. Inapparent infections and cholera dynamics. Nature. 2008;**454**(7206):877-880. DOI: 10.1038/nature07084

[23] Graves PM, Deeks JJ, Demicheli V, Jefferson T. Vaccines for preventing cholera: Killed whole cell or other subunit vaccines (injected). Cochrane Database of Systematic Reviews. 2010;**8**. DOI: 10.1002/14651858.CD000974.pub2