

Lessons from Vibrio Pathogen and the Comparative Study of Vaccines Developed

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Abstract

Cholera continues to be one of the most common causes of morbidity and mortality among children and adults in developing countries. Vaccine against cholera is an approach in the control of this epidemic and pandemic disease. From the development of very early oral cholera vaccine, advances in vaccine development documented due to a good illustration of the epidemiology, outbreak strategy, and pathophysiology of the disease causing pathogen. The newer-generation oral cholera vaccines are safe and guarantee a high level of protection during outbreak settings for several years. Yet infants and young children in developing countries are hyporesponsive to vaccines and show poor protection against cholera. In this review, we survey and analyse our current knowledge on the etiology of cholera, its clinical manifestation, global epidemiology and elaborate the vaccine candidates, which are effective against the pathogen and the corresponding immune responses to the available vaccines. These reviews comprehensively cover the salient features of recent discoveries related to *Vibrio cholerae* virulence, past and present vaccine candidates and their advantages and disadvantages with their development strategies. We believe that the advances that have been included in this review will give a comprehensive insight to the prevention and control of cholera outbreaks and development of effective cholera vaccines.

Keywords

Vibrio cholerae, Serogroup O1, O139, Cholera Toxin, LPS, Vibriocidal Antibodies, IgA Antibodies, Heat-Killed Whole Cell Vaccine, Live Attenuated Oral Vaccine

1. Introduction

Cholera is a waterborne and highly infectious disease that has caused devastating outbreaks in most parts of the world [1]. It is an acute watery diarrhoea caused by the Gram-negative bacillus *Vibrio cholera*, especially of the O1 serogroup [2]. If untreated, the drastic intestinal fluid loss caused by the pathogen can often lead to death [3]. Globally, 2.8 million cases of cholera occur each year, resulting in an estimated 91,000 deaths [4]. In spite of simple and widely accessible oral rehydration treatment, small children and adults are particularly vulnerable to the extreme dehydration of severe cholera [5]. Case-fatality rates may exceed 50% for those without treatment and be less than 1% among adequately treated individuals. The establishment of adequate personal hygiene, food safety and sanitation is important for control of cholera. However, in the short term, drastic improvements in these fields are difficult to achieve in areas where cholera is endemic. In the meantime, there is an urgent need for effective vaccines as an additional public health tool for cholera prevention. Effective vaccines are critical requirements for countries of Asia and Africa where the disease is endemic and is also seen in epidemics and during natural calamities [6]. It causes substantial morbidity and mortality in children between 2 to 5 years of age [7]. In fact, the disease has spread across the boundaries of Asia and Africa and epidemics of cholera have occurred in Central and South America with imported cases in other developed countries [8]. Here, we have done a comprehensive study of the disease pathogen, body's immune responses towards it and vaccine effectiveness in conjunction with combining information on the vaccination status and disease outcomes from cholera endemic areas.

2. *Vibrio cholerae*, the Causative Bacteria

The genus *Vibrio* comprises Gram-negative straight or curved rods belonging to the family Vibrionaceae [9]. In many aspects, *vibrios* are related to enteric bacteria. *Vibrios* are different from other enteric bacteria due to their oxidase-positive attributes and their motility using polar flagella [2]. Of all the *vibrios* which are clinically noteworthy to humans, *Vibrio cholerae*, the causative pathogen of cholera, is the most important.

The name, *V. cholerae* originates from the Greek words meaning, “flow of bile” [10]. *V. cholerae* is a Gram-negative, rod-shaped, mainly water-borne bacterium carrying a single polar flagellum [11]. *V. cholerae* can be classified into serogroups based upon polysaccharides of the somatic (O) antigen [12]. It is not until 1992 while most of the epidemics of cholera were transmitted by *Vibrio cholerae* of the O1 serogroup [13]. Three serotypes and two biotypes of *V. cholerae* O1 have been described [14] [15]. Serotyping is based on somatic antigens and biotyping is according to specific phenotypic properties [16]. Ogawa (somatic antigens A and C), Inaba (A and B), and Hikojima (A, B and C) designate the serotypes [17]. E1 Tor and classic designate the biotypes. The E1 Tor biotype, originally isolated as an avirulent strain in 1905, has evolved to greater vi-

ruence and is responsible for the current pandemic [18]. In 1992, a new serogroup—a genetic derivative of the E1 Tor biotype—emerged in Bangladesh and caused an extensive epidemic [19]. It was later isolated from other parts of Asia and has been termed as *V. cholerae* O139 [20].

2.1. Global Epidemiology of Cholera

Cholera usually occurs in large epidemics or pandemics and in the 19th century pandemics frequently originated from the Ganges delta in India [21]. There have been seven pandemics of cholera in recorded history [18]. Despite the etiological agents of the first four pandemics are not identified since they occurred during the time before these agents could be known, the last three pandemics are recognized to be caused by *V. cholerae* serogroup O1 [22]. The current, seventh pandemic caused by *V. cholerae* O1 E1 Tor originated in Indonesia in 1961 and spread rapidly through most of Asia into eastern Europe [23]. In 1970, this biotype was hosted into West Africa, where it spread briskly and is now endemic in many African countries. In 1991, it was reintroduced in to Peru (South American continent), where it had been absent for over 100 years [24]. Another serogroup, *V. cholerae* O139, was discovered as being the cause of cholera epidemics in India and Bangladesh in 1992 and since then it has spread to eleven other countries in South East Asia [21]. In 1992, in Bangladesh during a 12-week period, there were approximately 220,000 cases of cholera caused by serotype O139, with over 8000 deaths, more deaths than in all of Latin America the same year [25].

2.2. Clinical Manifestation of Cholera

Cholera is one of the most rapidly fatal illnesses known till date, in its extreme manifestation [26]. A healthy person may become hypotensive within an hour of the onset of symptoms and may die within 2 - 3 hours if no treatment is provided. Universally, the disease progresses from the first liquid stool to shock in 4 to 12 hours, with death following in 18 hours to several days [3]. The clinical description of cholera begins with sudden onset of massive diarrhea [27]. The patient could lose gallons of protein-free fluids and associated electrolytes, bicarbonates and ions within a couple of hours [28]. This fluid loss ultimately leads to dehydration, acidosis and shock. The watery diarrhea is dappled with bits of mucus and epithelial cells (“rice-water stool”) and comprises huge numbers of vibrios [29]. The loss of ions particularly potassium could sometimes result in cardiac complications and circulatory failure. If untreated, cholera frequently results in mortality rates around 50% - 60% [30].

2.3. Cholera Toxin, the Main Culprit

Koch, who identified *V. cholerae* as the causative agent of cholera, had in 1887 already proposed that the disease was toxin-mediated but it was not until 1959 that the Indian scientists De and Dutta [31] convincingly demonstrated the exis-

tence of cholera toxin [32]. It is now established that *V. cholerae* adheres to and colonizes the small intestine and secrete cholera toxin-that binds to receptors on the mucosal cells [9]. Cholera toxin (CT) is a protein that is composed of five receptor binding B subunits surrounding one catalytic A subunit [33]. While the B subunits are aggregated in a ring by tight, non-covalent interactions [34], the A subunit is linked to and partially inserted in the B ring through weaker non-covalent bonds [35].

2.4. Mode of Action of Cholera Toxin

V. cholerae affects the small intestine through its secreted cholera toxin (CT) [36]. It is now known that the membrane receptor for cholera toxin is a specific ganglioside (monosialosyl ganglioside, GM1), which is ubiquitously distributed in the cell membrane of normal mammalian cells [37]. The mode of action of cholera toxin is summarized in **Figure 1**.

(1) When cholera toxin is secreted from the bacteria, it binds to the epithelial cell known as “enterocyte” in the lumen of infected intestine through the interaction of the pentameric B subunit of the toxin with the GM1 ganglioside receptor on the intestinal cell, triggering endocytosis of the toxin. (2) Next, the A subunit proteolytically cleaves into A1 and A2 peptides in order for A1 to become an active enzyme. Once inside the enterocyte, the enzymatic A1 fragment

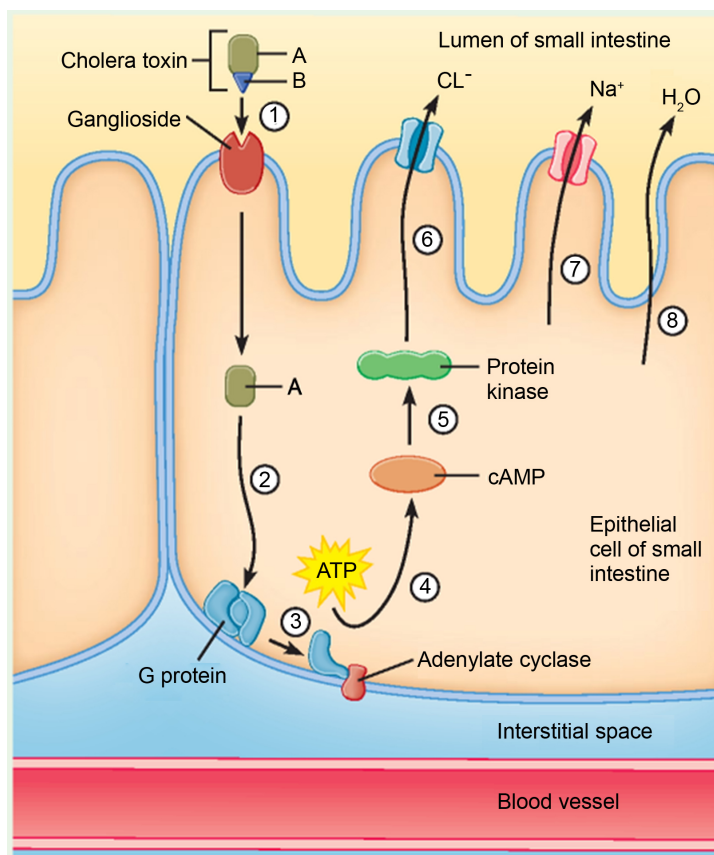


Figure 1. Mode of action of Cholera toxin (Adapted from [38]).

of the toxin A subunit enters the cytosol, where it activates the G protein G_{α} through an ADP-ribosylation reaction. (3) This ADP-ribosylation acts to lock the G protein in its GTP-bound form, thereby blocking their inherent GTPase activity. (4) This leads to constitutive activation of adenylate cyclase and the rapid elevation of cAMP levels from ATP inside the cells. (5) The high cAMP levels, in turn, phosphorylates and then activates cAMP-dependent “protein kinase A”. (6) (7) (8) Phosphorylated Protein kinase A then phosphorylates and hence activates proteins involved in the secretion of chloride ions, sodium ions and water. This dramatic efflux of ions and water from infected enterocytes leads to watery diarrhoea.

2.5. Immune Response to *Vibrio cholerae*

Epidemiological studies of cholera in endemic areas [39] and in human volunteers [40] have demonstrated that disease caused by *V. cholerae* gives rise to long-lasting protective immunity. The incidence of cholera disease is decreased with increasing age and recurrences are extremely rare in endemic areas [41] [42]. Cholera infection is associated with a rise in titer of a variety of circulating antibodies including vibriocidal antibodies [43] [44] and antibodies directed against cholera toxin (CT) [45] and cell wall lipopolysaccharide (LPS) [46].

Antibacterial antibodies, which develop in response to LPS, may protect against colonization with *V. cholerae* while antibody to CT may protect against disease in persons who are already colonized with *V. cholerae* [47] [48].

After natural infection by *V. cholerae*, circulating antibodies can be detected against several cholera antigens including CT, somatic (O) antigens, toxin co-regulated pilus (TCP) and mannose-sensitive hemagglutinin (MSHA) [49] [50]. These antibodies are also raised by parenteral injection of antigens as vaccine components. The early systemic response to somatic antigens is of the IgM class [51]. Subsequent challenges by either natural or vaccine antigens tend to induce a switch to IgG class antibodies [52]. However, in terms of protective immunity, the mucosal immune response is the most important. The intestinal IgA antibodies are the major immunoglobulin in mucosal immune response [53] [54]. These antibodies are produced locally in the intestinal mucosa and secreted onto the gut mucosal surface. The antibodies are mainly directed against bacterial components including CT, and protection is by inhibiting bacterial colonization [54] and multiplication and by blocking toxin action.

The important action of antibodies is the one directed against *Vibrio* O antigens and these are considered “vibriocidal” antibodies because they lyse *V. cholerae* cells in the presence of complement and serum components [55]. Vibriocidal antibodies which return to the baseline 2 to 7 months after the onset of clinical illness reach a peak 8 to 10 days after the infection [56]. Their presence correlates with resistance to infection; however, they may not be the mediators of this protection and their role in natural infection is unclear.

Circulating anti-CT antibodies may also confer short protection, albeit not at

the relatively low level induced by natural infection [57]. Adding the B subunit of CT to an oral vaccine stimulates mucosal formation of intestinal IgA antitoxin and contributes to protection for up to 9 months after vaccination [58].

3. Cholera Vaccine Candidates—Past and Present

3.1. Cholera Vaccines to Prevent against *Vibrio cholerae* O1 Infection

Enteric infections resulting in diarrhoeal disease from *Vibrio cholerae* remain a leading global health problem [59]. Although much sought for about a hundred years, since the identification of *V. cholerae* O1 as a causative agent of cholera, an effective and protective cholera vaccine still evades mankind [32]. The vaccine used until 20 to 30 years ago and licensed in the USA was a heat-killed whole cell parenteral vaccine, which gave short-lived protection only in adults [60]. The vaccine is no longer used because it has only limited efficacy (~50%) and duration of protection hardly exceeds 6 months [61]. Moreover, this licensed vaccine often causes pain and adverse effects at the injection site.

It has become increasingly recognized that for enteric diseases, especially those caused by bacteria, which are noninvasive, systemic immunization is not sufficient for inducing protective immunity [62]. As more knowledge of the mucosa-associated lymphoid tissue (MALT) is emerging, it has become obvious that an effective vaccine must stimulate the gut-associated lymphocytes to produce specific antibodies in the gut to inhibit colonization of the pathogen [63]. The vaccine should be capable of inducing both antibacterial and antitoxic immunity [64]. The vaccine should contain components which can stimulate memory cells comparable to natural infections. Their inadequacy to induce an appropriate memory response involves both B and T lymphocytes [65] [66].

From the beginning of the 1980s till today, much emphasis was placed by various groups of scientists in the design of an effective oral cholera vaccine that will be able to prevent disease caused by *V. cholerae* O1 [67]. Much success has been achieved in this area as a result of work carried out by two different groups of researchers [68]. Two licensed vaccines have emerged recently, which have been field-tested. Both are oral vaccines, but based on two opposite concepts: 1) One is killed, whole cell vaccine, containing a mixture of O1 bacteria of both Classical and E1 Tor biotypes and Ogawa and Inaba serotypes. 1 mg of recombinant B subunit of cholera toxin (rBS) is added [61] and is given in two oral doses. An extensive field-trial has been carried out in Bangladesh (Clemens *et al.*, 1986) and this has shown that it gives 85% protection in the first 6 months and about 60% protection over a period of 2 - 3 years. Field trials have also been carried out in Peru [69] (Concha *et al.*, 1995). Recently, the most widely used oral killed cholera vaccine is Dukoral vaccine consisting of recombinantly produced cholera toxin B subunit (CTB) and inactivated *V. cholerae* O1 whole cells [70].

2) The second vaccine which has been licensed, is new generation live vaccine

based on genetic engineering [71]. A strain of *V. cholerae* (*Vibrio cholerae* O1, classical, Inaba, strain 5698) has been attenuated so that the cholera toxin A subunit gene has been deleted [72]. As a result, the mutant strain does not produce cholera toxin. After extensive molecular biological studies, the strain has been sufficiently attenuated not to cause reactogenicity in vaccines. This vaccine candidate strain, CVD103HgR, is in addition resistant to mercury and therefore can be differentiated from naturally occurring O1 cholera strains [57] [71]. It has been tested in volunteers in the USA, Peru [63], and a large field-test has been carried out in Indonesia. The vaccine is given in a single dose. It has been developed in the USA and manufactured in Switzerland. Another vaccine named Shanchol has been prequalified by the WHO and is a formulation of killed *V. cholerae* cells (both *V. cholerae* O1 and O139). It is manufactured by Shantha Biotechnics of India, a subsidiary of the French pharmaceutical company Sanofi-Aventis [60]. It is a two-dose oral vaccine and has been established by a group of researchers from Sweden and South Korea, and its preliminary studies were carried out in Vietnam [73].

Killed cholera vaccines are safe since the fear of reacquiring genetic elements from virulent strains in the environment and in the host gut does not arise [55] [57]. On the other hand, the live cholera vaccine may be more immunogenic because it is able to colonize the gut, penetrate the M cells of the Peyer's patches and possibly stimulate better the natural course of events of the virulent *V. cholerae* organisms [74].

A second live vaccine candidate, Peru 15 is a *Vibrio cholerae* O1 E1 Tor, Inaba strain that has been engineered to be nontoxinogenic [75] (it lacks the *ctxA* and *rtxA* genes, which encode cholera toxin A subunit and the RTX toxin, respectively), nonrecombinatorial (it lacks the *recA* gene and the attachment site for the CTX phage), nonmotile, and *ctxB* positive (it makes the immunogenic, non-toxic CTB subunit) [76] [77]. It has been found to be safe and immunogenic against *Vibrio cholerae* O1 E1 Tor cholera in North American volunteers in experimental challenge studies [78]. This live attenuated oral vaccine was studied for safety and immunogenicity in Bangladeshi adults and infants [68].

3.2. Bivalent Cholera Vaccine to Protect against *Vibrio cholerae* O1 and O139

The progress made in the late 1980s in the development of an effective cholera vaccine has been jeopardized when in October 1992 a new strain of *Vibrio cholerae* serogroup O139 emerged in India and Bangladesh as an epidemic strain [21] [79]. Efforts to make a cholera vaccine that can protect against both O1 and O139 cholera has led to the development of the bivalent whole-cell O1/O139 cholera vaccine by Swedish scientists who had developed the field-tested killed O1 cholera vaccine [57] [61]. This vaccine is basically composed of the field-tested and licensed O1 vaccine plus rBS which, in addition, contains 5×10^{10} organisms of *V. cholerae* O139 [2]. Safety and immunogenicity studies on bivalent vaccine have been carried out simultaneously in Sweden, USA, Finland, and Bangladesh.

In addition, live vaccine candidates, such as Bengal 15, has been developed and is being evaluated in volunteers [80]. A live oral carrier-based O139 vaccine has been genetically engineered in the CVD103HgR strain [75]. The vaccine candidates, CH25 or CH26, express short oligopolysaccharides as well as lipopolysaccharide of *V. cholerae* O139, presumably the key protective antigens for prevention of O139 cholera [81] [82].

3.3. Immune Response to Killed Oral Cholera Vaccines

Killed oral cholera vaccines have been designed to stimulate mucosal immune responses in the intestine similar to that induced by natural exposure [57] [83]. Animal data showed that oral whole inactivated bacteria induce anti-bacterial antibodies and that of the cholera toxin B subunit induced antitoxic antibodies [84]. These antibodies gave synergistic protection against subsequent infection with cholera (Svennerholm, 1976). Effective oral cholera vaccine contains both the inactivated whole bacteria and B subunit of the toxin.

With the whole-cell/B subunit vaccine, intestinal IgA responses are seen in most vaccines [85]. However, a rise in antitoxin is generally seen after the first dose, whereas an antibacterial response frequently requires two doses to produce [45]. As the vaccine stimulates local IgA antibodies and because there is evidence of a common mucosal immune system, titers of antibody in intestinal secretions have been examined after immunization [32]. Titer rises of IgA antitoxin and anti-LPS are frequently seen in intestinal secretions [46].

3.4. Problems with Cholera Vaccines: Development Strategies

An ideal vaccine is reasonably easy to outline, however, only limited real vaccines approach the ideal. On top of that, no vaccines exist for many organisms, for which a vaccine is the only faithful protective strategy in the foreseeable future [86]. An ideal vaccine: [64] 1) should prevent disease transmission, 2) should provide life-long immunity, 3) should be broadly protective against all variants of an organism, 4) should induce effective immunity rapidly, 5) should be effective in all vaccinated subjects, including infants and the elderly, 6) would not need to be administered by injection and 7) should be cheap, stable (no requirement for cold chain) and safe [87].

The outbreaks of cholera occur in a regular seasonal pattern in developing countries. In Bangladesh, an epidemic outbreak of cholera usually occurs twice in a year and the high-risk group is children [88]. Consequently, vaccines against cholera have to be designed and formulated that the immunization scheme, route of administration and dosage should be such that the vaccine does not interfere with the response in the host to the other bacteria [87].

4. Concluding Remarks

The described vaccines are dependent on the induction of a mucosal immune response for protection. Nevertheless, for all vaccines, practically long-lasting

protection (memory) is a desirable objective despite requiring different types of immune response for protection [89]. The problems encountered in designing long-term memory response can be resolved by introducing components in the vaccine, which in natural disease, induce memory responses. Since acute watery diarrhoea caused by cholera is noninvasive, the vaccine candidates should be able to simulate the immune response by being taken up by the M cells, which are major sites for antigen uptake in the gut [62]. The introduction of CTB, which has mucosal immunopotentiating activity, has increased components which have adjuvant activity of CT, but lack the toxic properties. Since the adjuvant activity of CT is closely linked to the ADP-ribosylating action of the A subunit of CT, efforts are being made to make derivatives of *V. cholerae* strains which make cholera toxin A subunits inactive [33]. These strains, if sufficiently immunogenic and non-reactogenic, can be used as future vaccine candidates since they will have adjuvant capability as well as properties of inducing anti-toxic immunity.

5. Future Work

Establishment of an adequate sanitation and potable-water system is the most complete way to prevent and limit the spread of cholera. The promotion of WASH (water, sanitation and hygiene) practices, the creation of rehydration centres, the use of antibiotics, and the training of health personnel could drastically reduce cholera-associated mortality [90]. The dual action of oral cholera vaccine play and WASH practices could reduce the intensity of morbidities in endemic areas. Further follow-up in our study will be required to ascertain the duration of protection conferred by recently developed cholera vaccines in children and adults.

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Conflicts of Interest

The authors declare no conflict of interest that could be perceived to bias the work.

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