COMPARISON OF MEMORY B-CELL IMMUNE RESPONSES AMONG DIFFERENT AGE GROUPS AFTER ORAL CHOLERA VACCINATION

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BIOTECHNOLOGY

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Dedicated

To

My beloved parents

To whom it may concern

This is to certify that the research work embodying the results reported in this thesis entitled "Comparison of Memory B-cell Immune Responses among Different Age Groups after Oral Cholera Vaccination" submitted by **Abul Kalam Azad**, has been carried out under my supervision in the Immunology Laboratory of the Centre for Vaccine Sciences at the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr, b). It is further certified that the research work presented here is original and suitable for submission for the partial fulfillment of the degree of Master of Science in Biotechnology, BRAC University, Dhaka.

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The Author

Abstract

Infection with *Vibrio cholerae* O1causes dehydrating diarrhea, although it is now treatable disease with low case-fatality in settings with appropriate medical care. However, cholera continues to impose considerable mortality in the world's most impoverished populations. Natural infections usually give medium range immunity and it has been hypothesized that the protective immunity to *V. cholerae* infection may be mediated by anamnestic memory B-cell responses. An oral cholera vaccine, Dukoral is a killed inactivated vaccine which provides protection against the disease through the development of antigen specific memory B-cells. This study was carried out to compare memory B-cells and serological responses in Bangladeshi healthy young children (2-5 years, n=20), older children (6-17 years, n=20) and adults (18-45 years, n=32) after oral cholera vaccination. These responses to cholera antigens including lipopolysaccharide (LPS) and cholera toxin B subunit (CtxB) were assessed prior to immunization (day 0) and then on different study days using polyclonal stimulation of peripheral blood mononuclear cells (PBMC) followed by an enzyme linked immuno spot assay (ELISPOT) procedure. Adult vaccinees developed cholera toxin CT -specific IgA and IgG memory B cell responses by day 30 which remained detectable through at least for 90 days. In case of both younger and older child vaccinees, there was no significant level of CT -specific IgG memory B-cell responses, but the responses tended to be higher at day 30 (P=0.0625) post immunization. However, there was no significant elevation of the magnitude of LPS-specific IgA or IgG memory B-cell responses in any age group of vaccinees. Plasma antibody responses were measured by using the enzyme linked immunosorbant assay (ELISA) procedure. Adult vaccinees developed plasma CT-specific IgA and IgG for a longer duration of 3 months and 6 months, respectively, whereas these responses were relatively short-lived for the child vaccinees. The response to vaccination was also assessed by using the vibriocidal antibody assay. It was found that adult vaccinees developed high vibriocidal responses throughout the study period of 6 months, whereas these responses persisted for one month in both groups of child vaccinees. These results suggest that adult vacinees confer relatively long-term protection after oral cholera vaccination compared to young and older child vaccinees.

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LIST OF ABBREVIATION

ADP	**Adenosine diphosphate**
ADPR	**Adenosine diphosphate – ribose**
AEC	**3-Amino 9-Ethyl Carbazole**
APCs	**Antigen presenting cells**
ALS	**Antibody in lymphocyte supernatant**
ASC	**Antibody secreting cell**
BCIP/NBT	**5-Bromo 4-Chloro 3-Indolyl Phosphate/Nitroblue tetrazolium**
BSA	**Bovine serum albumin**
BCR	**B-cell antigen receptor**
cAMP	**cyclic adenosine monophosphate**
CI	**Confidence interval**
CT	**Cholera toxin**
CTB	**Cholera toxin B subunit**
DC	**Dendritic cell**
ELISA	**Enzyme linked Immunosorbant assay**
ELISPOT	**Enzyme linked Immunospot**
GALT	**Gut Associated Lymphoid Tissue**
GM1	**Monosialosyl ganglioside**
GTPase	**Guanosine tri-phosphatase**
GM	**Geometric mean**
HRP	**Horse-radish peroxidase**
icddr, b	**International Centre for Diarrhoeal Disease Research, Bangladesh**
Ig	**Immunoglobulin**
IL	**Interleukin**
KLH	**Keyhole Limpet Hemocyanin**
LPS	**Lipopolysaccharide**
LSD	**Laboratory Sciences Division**

LIST OF ABBREVIATION

mM	**Milli molar**
MALT	**Mucosa-associated lymphoid tissue**
MBC	**Memory B-cell**
MHC	**Major histocompatibity complex**
MW	**Molecular weight**
O antigen	**Somatic antigen**
OD	**Optical density**
OPD	**Ortho phenylene diamine**
ORS	**Oral rehydration solution**
PBMC	**Peripheral blood mononuclear cell**
PBS	**Phosphate-buffered Saline**
Peru-15	**A live vaccine candidate**
PMN	**Polymorphonuclear neutrophil**
PWM	**Pokeweed mitogen**
RBC	**Red blood cell**
rBS	**Recombinant B subunit of cholera**
rpm	**Rotation per minute**
SAC	***Staphylococcus aureas* Cowan**
SC	**Stromal cell**
SEM	**Standard error of mean**
sIg	**Secretory immunoglobulin**
Tcp-A	**Toxin coregulated pilus**
TCR	**T-cell antigen receptor**
Th	**T-helper cell**
WBC	**White blood cell**
WHO	**World Health Organization**

CHAPTER ONE

INTRODUCTION

1. Background

Diarrheal diseases caused by enteric pathogens remain a leading global health problem. Almost half of all cases of diarrhea is due to bacteria that cause disease by producing one or more enterotoxins [1]. *Vibrio cholerae* is an important cause of diarrheal morbidity and mortality. The vast majority of human disease is attributed to *V. cholerae* serogroups O1 and O139, both of which are noninvasive pathogens that colonize the small intestine and cause secretory diarrhea[2]. In countries such as Bangladesh, cholera is endemic and both the rural and urban population is afflicted with biannual outbreaks[3] with an approximate incidence of 200 cases/100,000 individuals per year, where the majority of fatal cases occur in young children [4-5]. In addition to endemic outbreaks, sporadic outbreaks can occur whenever sanitation and clean water provisions are lacking, as evidenced by the outbreak beginning in 2008 in Zimbabwe that affected over 100,000 individuals and resulted in more than 4,000 deaths [6] as well as outbreaks in 2010 in Pakistan and Haiti [7] following the collapse of infrastructure [8].

However, infection with *V. cholerae* induces protective immunity lasting from 3-7 years and the majority of patients with cholera develop robust humoral and mucosal immune responses [9]. Volunteer and epidemiologic studies demonstrate that clinically apparent infection with *V. cholerae* confers long-term protection of at least 3 years against subsequent disease [10-11]. The best-studied marker of protective immunity is the vibriocidal antibody, a complement-dependent bactericidal antibody; however, there is no vibriocidal antibody titer at which complete protection is achieved [12]. Furthermore, the vibriocidal response wanes rapidly, and it is hypothesized that the vibriocidal antibody may reflect other longer lasting, protective immune responses occurring at the mucosal surface[13].

Patients with cholera develop additional humoral immune responses to several antigens including cholera toxin subunit B (CTB), toxin-coregulated pilus major subunit A (TcpA), and LPS [14]. It has been shown that serum anti-CTB immunoglobulin A (IgA) antibody levels are also associated with protective immunity independent of the vibriocidal antibody on exposure to cholera, but serum IgA levels also wane rapidly after infection [15]. Although levels of serum anti-LPS and anti-CTB IgG antibodies increase considerably after

infection, these have not been shown to correlate with protection from *V. cholerae* infection in humans [16].

With the recognition of the fact that safe water and improved hygiene will not be immediate realities to those most affected by cholera, the World Health Organization recently issued an updated position statement on the role that cholera vaccines should play in limiting the cholera disease burden [17]. It has recently been shown that vaccinees developed immune responses that were generally comparable to those in individuals recovering from natural disease [18].

Currently, two oral cholera vaccines are licensed and available: a killed *V. cholerae* O1 vaccine supplemented with recombinant nontoxic cholera toxin B subunit (CtxB; WC-rBS; Dukoral; Crucell) and a bivalent killed *V. cholerae* O1/O139 vaccine not containing supplemental CtxB (O1/O139 WC; Shanchol-India, ORC-VAX-Viet Nam) [6, 19-20]. Both types of vaccines are safe and immunogenic and are usually administered in two doses separated by 1 to 6 week [21-22]. O1/O139 WC provided approximately 70% protection in a recent field study in Kolkata [23] and is currently being evaluated in a larger field trial in Bangladesh. The WC rBS vaccine provides 85 to 90% protective efficacy against cholera in few months following a two-dose regimen [24], but this efficacy falls toward baseline within 24 to 36 months of vaccination, especially in children who may not have had previous exposure like the adults [5]. In comparison, natural cholera induces protection that lasts for years or decades after infection [11].

Vaccine studies performed in areas in which cholera is endemic showed, older children and adults more-robust in immune responses which may have been primed by prior exposure to *V. cholerae* O1. Memory B cells (MBCs) are generated after natural infection and response upon antigenic exposure [25]. It has previously been demonstrated the presence of memory B cell responses in adults with cholera in Bangladesh [26] and that children are able to mount significant antibody responses to *V. cholerae* antigens following both natural infection and vaccination [5]. The aim of the present study was to characterize the B-cell immune response to oral cholera vaccine in children compared to those in adults.

1.1 Global epidemiology of cholera

Cholera has been endemic in southern Asia since recorded history. Cholera has spread globally in seven pandemic waves since 1817, of which the current one began in 1961. In 2008, the WHO reported 190,130 cholera cases worldwide, associated with 5143 deaths (98% in Africa), but cholera is globally under-reported and the true disease burden is estimated to be in the million [8, 27]. Cholera is a disease that occurs in low-income regions of the world where sanitation and food and water hygiene are inadequate. Imported cases occasionally occur in travelers returning from endemic areas. In areas without clean water or sewage disposal (as may occur after natural disasters or in displaced populations in areas of conflict), cholera can spread quickly and has a case fatality rate of as high as 50% in vulnerable groups with limited medical care [28]. The World Health Organization (WHO) reports the emergence of new, apparently more virulent, strains of *V. cholerae* O1 is now predominant in parts of Africa and Asia, and the emergence and spread of antibiotic resistant strains.

Cholera often occurs in large epidemics or pandemics. In the 19th century pandemics frequently originated from the Ganges delta in India, and up to the mid 20th century, were largely confined to Asia (except for a large epidemic in Egypt in 1947). The current, seventh pandemic caused by *V. cholerae* O1 El Tor originated in Indonesia in 1961 and spread rapidly through most of Asia. In 1970, this biotype was introduced into West Africa, and is now endemic in many African countries. In 1991, it was introduced into Peru where it had been absent for nearly 100 years, and from there spread throughout many countries of Latin America. Another serogroup, *V. cholerae* O139, was discovered as the cause of cholera epidemics in India and Bangladesh in 1992 and has since spread to other countries in South East Asia. Apart from a few imported cases, this serogroup is not known to have occurred outside Asia [28].

Annual global figures (2009) reported by WHO include 221,226 cases and 4,946 deaths from 45 countries. The majority of cases (98%) were reported from Africa where an outbreak, that started in 2008 and lasted for almost a year, spread to South Africa and Zambia. By the end of July 2009, over 98,000 cases and 4,000 deaths were reported in this outbreak. Asia reported an 82% decrease in cases in 2009 compared to 2008, however,

reports of acute watery diarrhea, many of which may be cholera, were not included. Recent cholera epidemics include the 2008-2009 epidemic in Zimbabwe and the 2010 epidemic following the January earthquake in Haiti [7, 29]. The *V. cholerae* strain responsible for the Haiti epidemic is nearly identical to the El Tor O1 strains predominant in southeast Asia; the ancestry is distinct from that of circulating Latin American and East African strains of *V. cholerae,* suggesting introduction of the strain from Asia [30].

1.2 Epidemiology of cholera in Bangladesh

Cholera is endemic in Bangladesh, and outbreaks occur in a regular seasonal pattern. A systematic surveillance for cholera has been carried out in Bangladesh by the International Centre for Diarrheal Disease Research (icddr, b) for more than 35 years [31-32]. A number of studies have shown that epidemic outbreaks in Bangladesh usually occur twice during a year, with the largest number of cases occurring during September to December, just after the monsoon [33-34]. A somewhat smaller peak of cholera cases is also observed in the spring between March and May.

Until 1970, more than 90% of cholera in Bangladesh was caused by the classical Inaba serotype; by 1972, 85% of all cases were due to the classical Ogawa serotype [35]. The El Tor biotype of *V. cholerae* O1 appeared in Bangladesh in 1969/1973 and since this biotype had completely replaced the classical biotype. However, in 1982, the classical biotype reemerged as the predominant epidemic biotype in Bangladesh [36] and coexisted with the El Tor vibrios until 1992. Data obtained from studies of diarrhea epidemics in nearly 400 rural subdistricts by ICDDR,B medical teams between 1985 and 1991 showed that *V. cholera* O1 was the most frequently (40%) isolated enteropathogen during the epidemics [32]. The 1991 epidemic was estimated to have caused between 210,000 and 235,000 cases and over 8,000 deaths [32]. During 1992 and 1993, an epidemic of severe and deadly watery diarrhea caused by *V. cholerae* O139 occurred in southern Bangladesh and later spread to the other parts of the country including Dhaka [37-38]. In 1992, there were approximately 220,000 cases of cholera caused by serotype O139 within a 12-week period, with over 8,000 deaths that was more deaths than in all of Latin America that same year [39].

1.3 Risk for travelers

The overall risk of cholera for travelers is extremely low and is in the order of 0.2 cases per 100,000 travelers [40-41]. For long-term travelers in areas of outbreaks the rate may be as high as 500 cases per 100,000 travelers [40], and when routine screening for *V. cholerae* is done in travelers with diarrhea who have returned from endemic areas, the rate may approach five cases per 100,000 [42]. Activities that may predispose to infection include drinking untreated water or eating poorly cooked seafood in endemic areas. Travelers living in unsanitary conditions, for example relief workers in disaster or refugee areas, are also at risk.

2. *Vibrio choleare:* The causative agent of cholera

In 1883 Robert Koch demonstrated that cholera is produced by a bacterium that he referred to as 'comma(-shaped) bacteria' [43], later designated *V. cholerae*. *V. cholerae*, a member of the family Vibrionaceae, is a facultatively anaerobic, Gram-negative, non-spore-forming curved rod, about 1.4–2.6μm long, capable of respiratory and fermentative metabolism; it is well defined on the basis of biochemical tests and DNA homology studies. The bacterium is oxidase-positive, reduces nitrate, and is motile by means of a single, sheathed, polar flagellum.

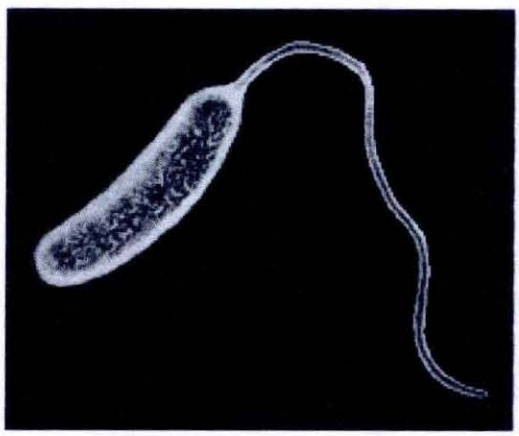


Figure 1.1: ***Vibrio cholerae***

2.1 Classification

Antigenic variation plays an important role in the epidemiology and virulence of cholera. Differences in the sugar composition of the heat-stable surface somatic "O" antigen are the basis of the serological classification of *V. cholerae* first described by Gardner & Venkatraman [44]; currently the organism is classified into 206 "O" serogroups . Until recently, epidemic cholera was exclusively associated with *V. cholerae* strains of the O1 serogroup.

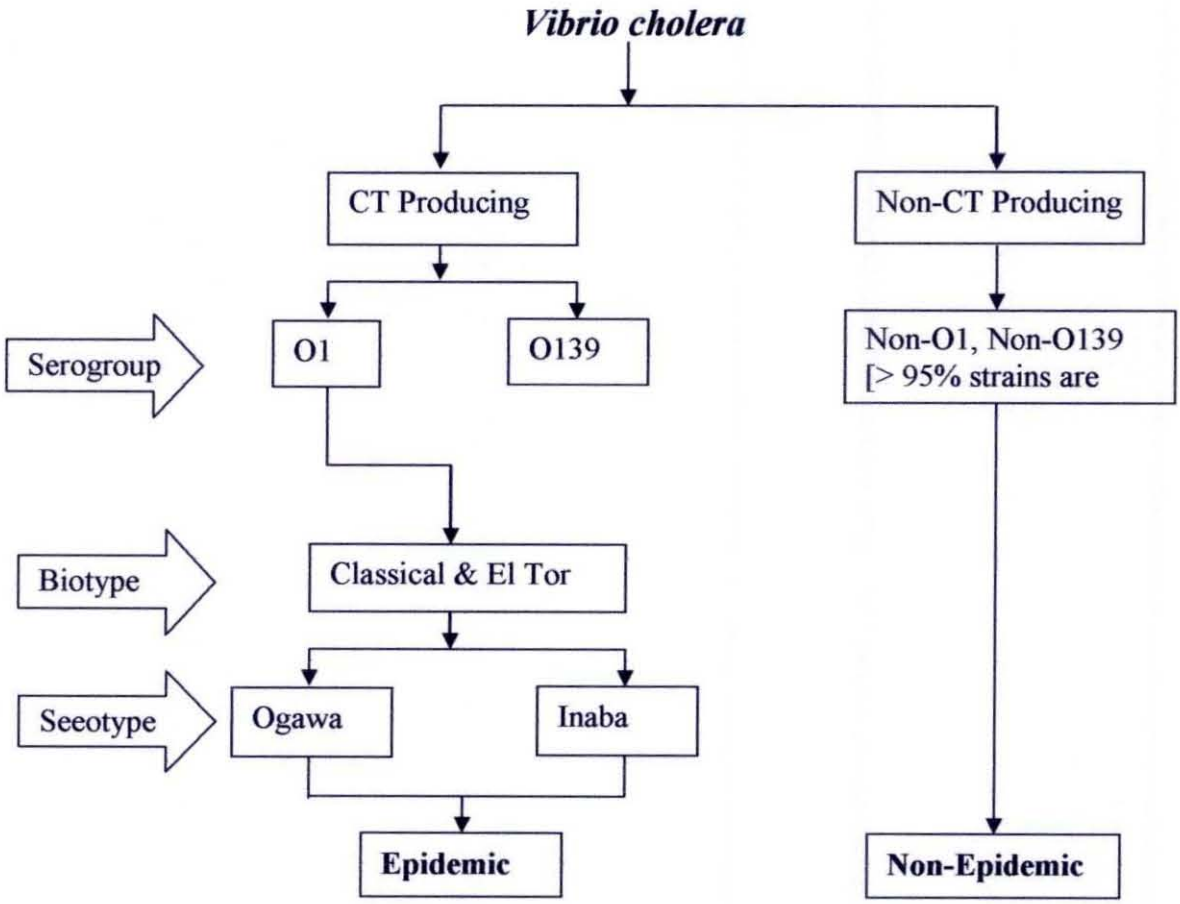


Figure 1.2: The current classification scheme of epidemic and non-epidemic strains of *V. cholerae*. Serogrouping is based on the O antigenic polysaccharide moiety of O-PS of LPS. All epidemics *V. cholerae* O1/O139 strains are CT producer.

All strains that were identified as *V. cholerae* on the basis of biochemical tests but that did not agglutinate with "O" antiserum were collectively referred to as non-O1 *V. cholerae*. The non-O1 strains are occasionally isolated from cases of diarrhea [45] and from a variety of extraintestinal infections, from wounds, and from the ear, sputum, urine, and cerebrospinal fluid [46]. They are ubiquitous in estuarine environments, and infections due to these strains are commonly of environmental origin [47]. The O1 serogroup exists as two biotypes, classical and El Tor; antigenic factors allow further differentiation into two major serotypes- Ogawa and Inaba. Strains of the Ogawa serotype are said to express the A and B antigens and a small amount of C antigen, whereas Inaba strains express only the A and C antigens. A third serotype (Hikojima) expresses all three antigens but is rare and unstable.

The simple distinction between *V. cholerae* O1 and *V. cholerae* non-O1 became obsolete in early 1993 with the first reports of a new epidemic of severe, cholera-like disease in Bangladesh [37] and India (Ramamurthy et al., 1993b). At first, the responsible organism was referred to as non-O1 *V. cholerae* because it did not agglutinate with O1 antiserum. However, further investigations revealed that the organism did not belong to any of the O serogroups previously described for *V. cholerae* but to a new serogroup, which was given the designation O139 Bengal after the area where the strains were first isolated. Since recognition of the O139 serogroup, the designation non-O1 non-O139 *V. cholerae* has been used to include all the other recognized serogroups of *V. cholerae* except O1 and O139.

2.2 Cholera Toxin: The virulence factor causing secretory diarrhea

The pathogenesis of cholera is a complex process and involves a number of factors which help the pathogen to reach and colonize the epithelium of the small intestine and produce the enterotoxin that disrupts ion transport by intestinal epithelial cells. The existence of cholera enterotoxin (CT) was first suggested by Robert Koch in 1884 and demonstrated 75 years later by [48] and Dutta, Pause & Kulkarni (1959) working independently. Subsequent purification and structural analysis of the toxin showed it to consist of A_1 and A_2 subunit and 5 smaller identical B subunits [49]. The A subunit possesses a specific enzymatic function and acts intracellularly, raising the cellular level of cAMP and thereby changing the net absorptive tendency of the small intestine to one of net secretion. The crucial role of CT in disease was clearly shown by Levine et al. [50], who fed purified CT to volunteers.

Ingestion of 25 µg of pure CT (administered with cimetidine and NaHCO3 to diminish gastric acidity) caused over 20 liters of rice water stool, and ingestion of as little as 5 µg of pure CT resulted in 1 to 6 liters of diarrhea in five of six volunteers.

2.2.1 The mechanism of action of the cholera toxin

Infection normally starts with the oral ingestion of food or water contaminated with *V. cholerae*. In human volunteer studies, the infectious dose was determined to be fairly high, and varied depending on the inocula conditions (ranging from 10^6 to 10^{11} colony-forming units). This high dose is probably needed because of the acid sensitivity of *V. cholerae* cells, which are exposed to low pH in the gastric compartment [51]. The surviving bacteria adhere to and colonize the intestinal epithelial cells, eventually producing the CT and causing cholera symptoms [52]. The B subunit is responsible for specific binding to the GM1 ganglioside receptor of epithelial cells [53-54]

Upon binding, the A subunit is translocated into the host cell cytosol, where it is activated by thiol dependent reduction, probably by thiol: protein disulfide oxidoreductases [55]. Only the resulting nicked A_1 subunit possesses an ADP-ribosylating activity that targets the host cell G-protein Gsα. ADP ribosylated Gsα in turn permanently activates adenylate cyclase activity, leading to increased levels of intracellular cAMP. cAMP inhibits active sodium chloride absorption and increases chloride and bicarbonate secretion [56]. This results in passive water loss, leading to a marked decrease in intravascular volume, hypotension and hypoperfusion of critical organs and in severe cases death ensues with a high mortality rate.

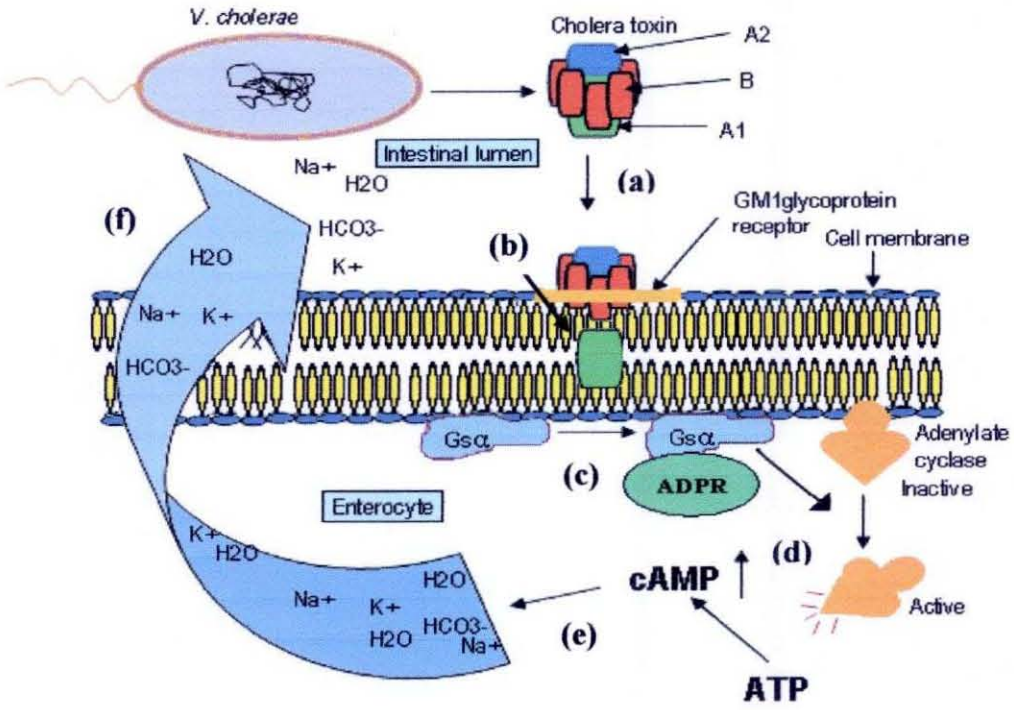


Figure 1.3: The mechanism of action of the cholera toxin. (a) The CT molecule binds to GM1 in the apical membrane of the gut epithelial cell. (b) The molecule is internalized in an endosome. (c) The A_1 (enzyme) subunit is released, and catalyses the transfer of ADPribose from NAD to α subunit of a G protein. (d) The G protein can no longer act to switch off adenylate cyclase, and the cyclic AMP level in the cell rises (e). This increase in cAMP causes ion channels in the apical membrane to open, allowing ions to escape from the cell (f).

(http://accessmedicine.net/loadBinary.aspx?name=ryan5&filename=ryan5_c032f002t.gif)

2.3 Lipopolysaccharide

The lipopolysaccharide (LPS) of *V. cholerae* represents the most abundant of exposed molecules in the outer membrane of Gram-negative bacteria, and contributes to barrier function [57-58]. It is considered one of the most important antigens from the point of view of immunogenicity in these bacteria [59] . Toxicity is associated with the lipid component (Lipid A) and immunogenicity is associated with the polysaccharide components although both act as determinants of virulence. During infection, *V. cholerae* cells are exposed to a series of changes, such as temperature, acidity, osmolarity and exposure to antibacterial agents and innate immune system components.

The cell wall antigens (O antigens) of gram-negative bacteria are components of LPS. Somatic (O) antigen or O polysaccharide is attached to the core polysaccharide. The individual chains vary in length ranging up to 40 repeat units. (Fig: 1.3) A major antigenic determinant (antibody-combining site) of the gram-negative cell wall resides in the O polysaccharide [60].

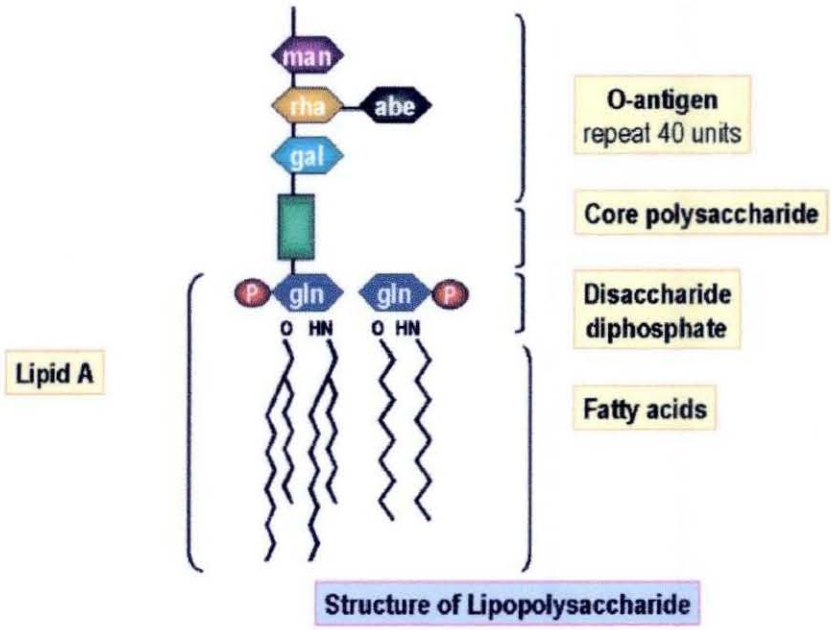


Figure 1.4: General architecture of Lipopolysaccharide in gram negative bacteria
(http://pathmicro.med.sc.edu/fox/lps.jpg)

3. Cholera toxin: A paradigm of a multifunctional protein

CT is not just another enterotoxin that causes the signs and symptoms of the dreaded disease, cholera. It is unique in many respects, starting from its structure to its functions. CT is a multifunctional protein that is capable of influencing the immune system in many ways. It not only has remarkable adjuvant properties, but also has its role in mucosal tolerization and vaccine development.

3.1 Adjuvant activity of cholera toxin

That CT possessed adjuvant activity, that was first reported in the early seventies [59]. The adjuvant activity of CT may be attributed to the enhanced antigen presentation by various types of antigen presenting cells (APCs), such as macrophages, dendritic cells (DCs) and B cells. In B cells, both recombinant CT (rCT) and recombinant CTB (rCTB) promote isotype differentiation that leads to increased IgA formation. It has been suggested that both enzymatic activity and receptor binding contribute to the stimulatory effects of the toxin [61]. CT has also been reported to upregulate various cell surface molecules such as co-stimulatory molecules and chemokine receptors in murine and human DCs as well as in other APCs [62-63]. CT also stimulates the secretion of IL-1 from macrophages, which enhances their APC function [64].

3.2 Cholera toxin B subunit for mucosal tolerization and immunotherapy

Mucosal tolerance is a mechanism whereby the immune system, upon encounter with harmless antigens through a mucosal surface, develops means to avoid reacting in a deleterious manner to the same antigen even if the antigen is encountered by a systemic route. A significant improvement has been achieved by co-administering CTB as an immuno-modulating agent to enhance the tolerogenic activity of autoantigens as well as allergens given orally or nasally. The use of antigen coupled to CTB has been found to minimize by several hundred-fold the amount of antigen/tolerogen needed and also to reduce the number of doses that would otherwise be required by reported protocols of tolerance induction by the oral route [65]. More importantly, at divergence from the use of free antigen, CTB-linked antigens have been shown to work also in an already sensitized individual. As recently reviewed [66], in experimental systems this has also resulted in effective suppression of various pathological immune responses associated with experimental autoimmune diseases, type I allergies and allograft rejection when the CTB-antigen conjugate was administered as therapy rather than for prevention.

3.3 The CTB antigen in the whole cell oral cholera vaccine

The toxicity of CT has precluded its use for human vaccination. Instead, nontoxic B subunit of CT, the CTB component has been extensively used without any side effects as a mucosal immunogen in humans. Indeed, recombinantly produced CTB is an important component of an oral cholera vaccine for human use. In addition to CTB, this vaccine also contains inactivated whole-cell cholera vibrios and is being registered (Dukoral) in more than 60 countries worldwide. The vaccine has proved to be safe and efficiently immunogenic in both adults and children. When given orally in two or three doses, the vaccine has been found to stimulate the same levels of intestinal IgA antitoxin and antibacterial (mainly anti-lipopolysaccharide) antibodies as seen in convalescence from severe form of clinical cholera. The vaccine has also been found to induce very long-lasting (more than 2 years) immunologic memory in the intestinal mucosa.

4. Treatment

4.1 Hygienic and Sanitary Control

Prevention is always better than cure. To prevent cholera some measures are recommended as follows-

- Bottled water (intact seal) or tubewell water/tap water that has been boiled or treated with sterilizing tablets should drink.
- Eating foods that are freshly prepared and cooked thoroughly. Raw or undercooked seafood should not be eaten.
- Raw vegetables such as green salads, as they may have been washed in contaminated water, should be avoided.
- Good personal hygiene should be maintained.

4.2 Rehydration therapy

The key to therapy is provision of adequate rehydration until the disease has run its course (usually 1 to 5 days in the absence of antimicrobial therapy). Rehydration can be accomplished by intravenous infusion of fluid (in severe cases) or by oral rehydration with

an oral rehydration solution (ORS) [67]. For adults, the intravenous replacement solution should be infused as rapidly as possible so that about 2 liters is given in the first 30 min. Children in shock should receive 30 ml of intravenous fluid per kg of body weight in the first hour and an additional 40 ml/kg in the next 2 h. In both adults and children, ORS (with its glucose and potassium) should be administered as soon as possible in the course of illness. Patients with mild or moderate dehydration can receive initial fluid replacement to repair water and electrolyte deficits exclusively by the oral route [68].

4.3 Antimicrobial treatment

Antimicrobial agents can shorten the duration of cholera diarrhea and the period of excretion of vibrios. Treatment should be started after vomiting subsides (i.e., after initial rehydration and correction of acidosis). Tetracycline is the drug of choice [67]. Tetracycline may provide some protection when given as a prophylactic agent within a family in which cases of cholera have occurred [69-70]. However, widespread use of tetracycline prophylaxis has been associated with rapid development of antimicrobial resistance [71-72] and should be strongly discouraged. Other antibiotics that are effective when *V. cholerae* are sensitive to them include erythromycin, cotrimoxazole, azithromycin, doxycycline, chloramphenicol and urazolidone.

4.4 Vaccine treatment

One of the main efforts at combating cholera epidemics is directed towards the development and use of modern vaccine strategies. Cholera is predicted to have a high potential for successful prevention by vaccination [73]. Efficient protection is dependent on the biotype: infection with the classical biotype shows more conserved protection against different serotypes (Inaba, Ogawa, and Hikojima) of classical strains, and El Tor-derived protection is more labile against different El Tor isolates. It was also found that naturally acquired immunity lasts for at least 3 years, whereas longer immunity depends on the individual [73]. Cholera vaccines have been used for 1100 years with varying degrees of success [74-75].

5. Natural protection against cholera

Studies to-date in patients with cholera suggest that different components of the immune system, both humoral and cell mediated, innate as well as adaptive, are activated in response to natural infection [76-77]. The best studied responses are the humoral immune responses and both mucosal and systemic antibody responses have been found to be related to protection [78]. The serological responses such as the complement mediated vibriocidal antibody response, antibody responses to lipopolysaccharide (LPS) and CT as well as to protein antigens have been found to be significantly increased in response to clinical cholera [79]. The antibacterial responses include, in addition to LPS, responses to the toxin-co-regulated pilus (TCP), which is a colonization factor and potentially protective antigen [80-81], as well as to the mannose sensitive haemagglutinin (MSHA), a type IV pilus antigen [82] which is also immunogenic and gives rise to antibody secreting cell (ASC) responses and fecal as well as plasma antibodies in patients [83].

The local secretory IgA response is believed to play a major role in protective immunity from diarrhea caused by *V. cholerae*, since cholera is a human-restricted, noninvasive mucosal infection. Cholera also induces both plasma IgG and IgA responses to *V. cholerae* antigens, but only levels of circulating *V. cholerae*-specific IgA antibodies are associated with protection [15]. However, like plasma vibriocidal-antibody titers, plasma IgA responses remain elevated for only 6 to 12 months after cholera infection [26], while protective immunity after clinical cholera infection lasts substantially longer [33].

It has been found that circulating *V. cholerae*-specific memory B-cells remain detectable for at least one year after cholera infection and persist longer than traditional measures of immunity to cholera [26] and thus may contribute to long term protection upon re-exposure.

6. The immune response to pathogens

The symptoms of an infection result from a complex interaction of microbial factors and host responses: in order to produce an infection, a pathogenic microorganism must be able to survive in the environment, be transmitted, and establish itself in its host. The immune system plays an essential role in host defense [84]. Following its introduction into a host a

pathogen faces what can be artificially divided into two types of immune responses: an innate and an adaptive response. These two responses differ in their kinetics, their effectors, and the receptors involved.

6.1 Innate and Adaptive Immunity

The innate immune system is the first line of the defense system against microbial pathogens such as Gram-positive and Gramnegative bacteria, fungi and viruses. Innate immune cells such as macrophages and DCs (dendritic cells) directly kill the pathogenic microorganism through phagocytosis or induce the production of cytokines, which aid elimination of the pathogens [85-86].

The responses of the innate immune system instruct the development of long-lasting pathogen-specific adaptive immune responses. The adaptive immune system consists of B- and T-cells, which provide pathogen specific immunity to the host through somatic rearrangement of antigen receptor genes. It functions with the following sequence of events: antigen presentation, clonal expansion, and differentiation into effector cells, either B or T lymphocytes. B-cells produce pathogen-specific antibodies to neutralize toxins produced by pathogens, whereas T-cells provide the cytokine milieu to clear pathogen-infected cells through their cytotoxic effects or via signals to B-cells [87].

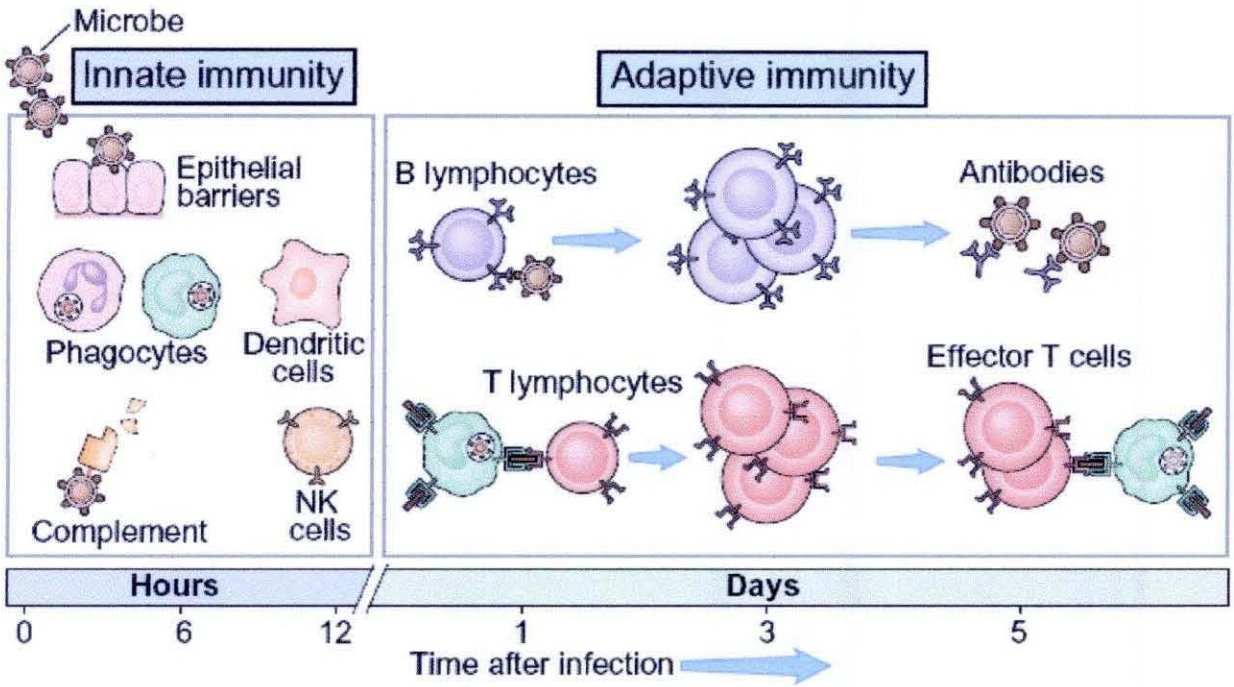


Fig 1.5: The principal mechanisms of innate and adaptive immunity: The mechanisms of innate immunity provide the initial defense against infections. Some of the mechanisms prevent infections (e.g., epithelial barriers) and others eliminate microbes (e.g., phagocytes, natural killer [NK] cells, the complement system). Adaptive immune responses develop later and are mediated by lymphocytes and their products. Antibodies block infections and eliminate microbes, and T lymphocytes eradicate intracellular microbes. The kinetics of the innate and adaptive immune responses are approximations and may vary in different infections. *(Basic Immunology: Functions and Disorders of the Immune System, 3rd Edition, Abul K. Abbas & Andrew H. Lichtman.)*

6.2 Primary and secondary immune response

The immune system mounts larger and more effective responses to repeated exposures to the same antigen. The response to the first exposure to antigen, called the primary immune response, is mediated by lymphocytes, called naive lymphocytes, which are seeing antigen for the fi rst time. The term *naïve* refers to the fact that these cells are "immunologically inexperienced," not having previously recognized and responded to antigens. Subsequent encounters with the same antigen lead to responses, called secondary immune responses, which usually are more rapid, larger, and better able to eliminate the antigen than are the

primary responses. Secondary responses are the result of the activation of memory lymphocytes, which are long-lived cells that were induced during the primary immune response. Immunologic memory optimizes the ability of the immune system to combat persistent and recurrent infections, because each encounter with a microbe generates more memory cells and activates previously generated memory cells. Memory also is one of the reasons why vaccines confer long-lasting protection against infections. Heavy chain isotype switching and affinity maturation also increase with repeated exposure to protein antigens.

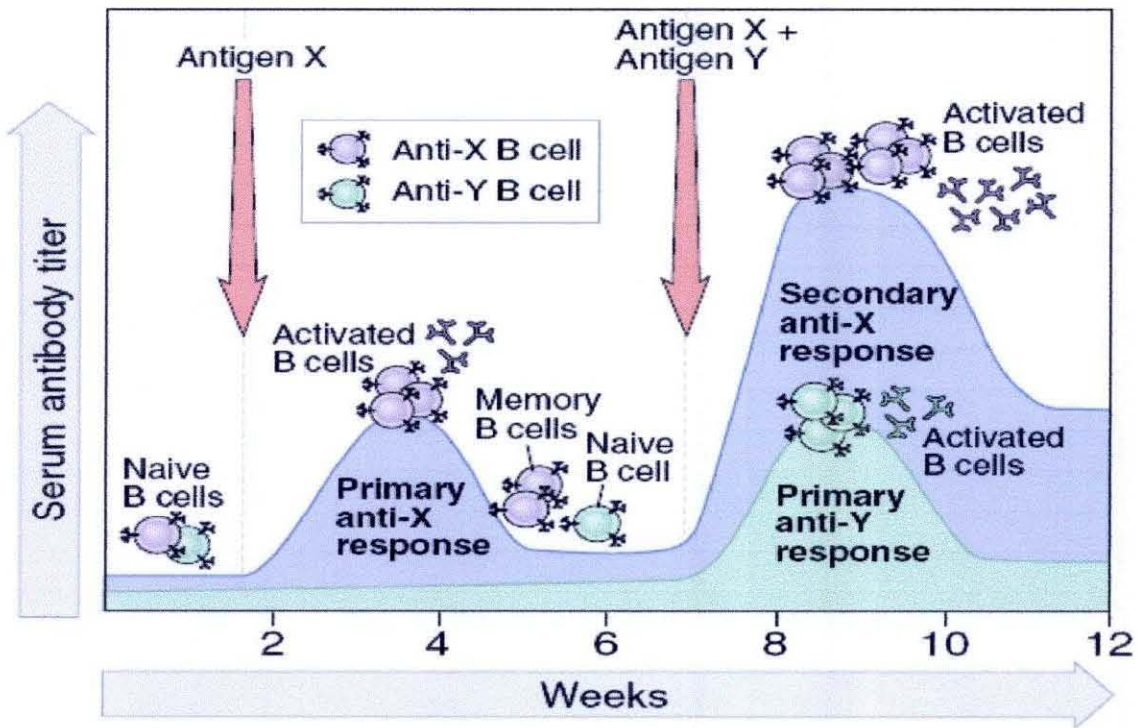


Figure 1.6: Primary and secondary immune responses. Antigens X and Y induce the production of different antibodies (a reflection of specific city). The secondary response to antigen X is more rapid and larger than the primary response (illustrating memory) and is different from the primary response to antigen Y (again reflecting specific city). Antibody levels decline with time after each immunization. *(Basic Immunology: Functions and Disorders of the Immune System, 3rd Edition, Abul K. Abbas & Andrew H. Lichtman.)*

6.3 Immunological memory

Memory lymphocytes can greatly influence immune responses against subsequent infections. The ease with which they are triggered [88-89], even at very low antigen concentrations, may explain why immune responses tend to be dominated by memory lymphocytes from previous infections, a phenomenon termed 'original antigenic sin' [90-91].

During primary pathogen encounter, the innate immune system plays a key role in determining the nature of the immune response [92]. The type of response that is induced is determined by the 'immunological context' of the pathogen, including its localization [93], the presence of conserved bacterial peptides [94-95], and the cytokines and chemokines that are locally expressed [96-97]. Based on these signals, lymphocytes differentiate to a memory phenotype and attain a certain effector mechanism. These effector mechanisms are recalled whenever effector/memory lymphocytes are re-stimulated by their specific epitope [98]. When lymphocytes differentiate, their cytokine production is somatically imprinted by chromatin remodeling and DNA demethylation. Differentiated lymphocytes thereby epigenetically transfer their mode of response to their daughter cells [99]. The immune system thus learns to associate the antigens it encounters with the appropriate types of response against them.

6.4 B-cell immunological memory

Serum and mucosal antibody levels are maintained long term by multiple mechanisms. Pathogen re-exposure or booster vaccination is clearly the most effective way to boost specific antibody and memory B-cells (MBCs). Long-lived plasma cells are responsible for the continuous maintenance of serum antibody levels [100-101]. Memory B cells are responsible for driving the rapid anamnestic antibody response that occurs after re-exposure to antigen, which is important for eliminating the pathogen and toxic antigens not cleared by pre-existing circulating antibodies. Memory B cells may also play a role in replenishing the pool of long-lived plasma cells to maintain long-term antibody levels in the absence of pathogen [102]. A latent or low-grade chronic infection in which sporadic or continuous antigenic stimulation occurs also drives B-cell receptor (BCR)-dependent differentiation of

B cells into antibody- secreting plasma cells. In the absence of antigenic re-exposure, however, long-lived plasma cells (LLPCs) and MBCs can still be maintained for decades [103-104]. Although antigen is not needed for the survival of MBCs, the presence of a BCR on MBCs as well as on naive B cells is required [105].

7. Synthesis of immunoglobulins at mucosal surfaces

In humans, more IgA is produced than all the other immunoglobulin isotypes combined [106], and high concentrations of IgA antibodies (over 1 mg per ml) are present in the secretions that are associated with mucosal surfaces in normal humans [107-108]. The protease resistance of secretory IgA (sIgA) is a result of its dimerization and high degree of glycosylation during its synthesis in mucosal plasma cells, and its association with a glycosylated fragment (the secretory component) derived from the epithelial polymeric immunoglobulin receptor (pIgR) that mediates transport of dimeric IgA across epithelial cells to the lumen [109].

sIgA has multiple roles in mucosal defense [110]. It promotes the entrapment of antigens or microorganisms in the mucus, preventing direct contact of pathogens with the mucosal surface, a mechanism that is known as 'immune exclusion'. Alternatively, sIgA of the appropriate specificity might block or sterically hinder the microbial surface molecules that mediate epithelial attachment [111], or it might intercept incoming pathogens within epithelial-cell vesicular compartments during pIgR-mediated transport [110]. Interstitial fluids of mucosal tissues that underlie the epithelial barrier contain dimeric IgA that is synthesized by local IgA-secreting plasma cells and this might prevent mucosal-cell infection, by mediating the transport of pathogens that have breached the epithelial barrier back into the lumen through pIgR [112] or by mediating antibody-dependent cell-mediated cytotoxicity (ADCC) that leads to the destruction of local infected cells [113].

Local IgG synthesis also can occur in the mucosal tissues following the administration of antigen or vaccine to mucosal surfaces [114]. Large numbers of IgG-secreting plasma cells are present in the female genital tracts of macaques and humans [114], and high concentrations of IgG as well as IgA have been measured in human cervical and vaginal secretions [108]. This IgG, as well as sIgA, could play a significant role in blocking

infection by sexually transmitted pathogens at this site, Concentrations of IgG and IgA in secretions of the female reproductive tract are affected by hormonal signals and change dramatically during the menstrual cycle, and this might be an important factor in the effectiveness of mucosal vaccines against sexually transmitted diseases. In the human intestine, 5–15% of mucosal plasma cells secrete IgG [115], but IgG is susceptible to degradation by luminal intestinal and bacterial proteases. In large intestinal secretions, for example, IgG concentrations are generally 3 to 100-fold lower than those of sIgA [116]. Nevertheless, intact IgG in mucosal tissues, whether locally produced or from serum, can potentially neutralize pathogens that enter the mucosa and prevent systemic spread. In recent study on mucosal immunologic responses, significant level of *V. cholerae* lipopolysaccharide (LPS)-specific sIgA and IgG were found for a period of one month after natural infection [117].

7.1 Mucosal immunity and vaccine development

An effectively designed mucosal vaccine must: (1) protect from physical elimination and enzymatic digestion, (2) target mucosal inductive tissues including M cells, and (3) appropriately stimulate the innate immune system to generate effective adaptive immunity. In mucosal vaccine development, it is crucial to select appropriate immunization route, and most current mucosal vaccine delivery is intended to mimic the nature encounter of mucosal inductive sites with environmental antigens and pathogens.

Mucosal immune responses are most efficiently induced by the administration of vaccines onto mucosal surfaces, whereas injected vaccines are generally poor inducers of mucosal immunity and are therefore less effective against infection at mucosal surfaces [118]. By contrast, our understanding of mucosal immunity and development of mucosal vaccines has lagged behind, in part because administration of mucosal vaccines and measurement of mucosal immune responses are more complicated. The dose of mucosal vaccine that actually enters the body cannot be accurately measured because antibodies in mucosal secretions are difficult to capture and quantitate, and recovery and functional testing of mucosal T cells is labour intensive and technically challenging. As a result, only a few mucosal vaccines have been approved for human use in the United States or elsewhere. These include oral vaccines against poliovirus, *Salmonella* Typhi, *V. cholerae* and rotavirus, and a nasal vaccine against

influenza virus [119]. However, research and testing of mucosal vaccines is currently accelerating, stimulated by new information on the mucosal immune system and by the threat of the mucosally transmitted virus, HIV [120-121].

8. Vaccines against the pathogenic diseases

Vaccines represent the epitome of a preventive strategy to control disease [122]. In the individual, they confer direct protection and, if high enough immunization coverage of a population is achieved, unimmunized people may also be protected, indirectly, through 'herd immunity' [123]. The strategic use of some vaccines, such as measles and polio vaccines, has interrupted indigenous transmission of those diseases in entire regions of the globe [124-125]. And one disease, smallpox, has been completely eradicated from the human population through the epidemiologically sound use of smallpox vaccine [126-127].

In developing countries, where two-thirds of the world's populations live, infectious diseases cause most of the mortality among children less than 5 years of age [128] and constitute major health problems in older children and adults. Vaccines are among the most promising interventions to diminish the burden of specific infections in populations in developing countries [129-130].

8.1 Cholera prevention by vaccination

Beside hygienic and sanitary control measures and cholera surveillance, one of the main efforts at combating cholera epidemics is directed towards the development and use of modern vaccine strategies. Cholera is predicted to have a high potential for successful prevention by vaccination [73]. Injectable, killed whole-cell (WC) cholera vaccines date back virtually to the discovery of the cholera vibrio in the nineteenth century. These vaccines fell from favor in the 1970s because they were found to confer low levels of efficacy of short duration and to have an unfavorable safety profile [131]. Currently, these vaccines are not recommended for use. Attention shifted from parenteral to oral vaccines against cholera with the recognition that protective immunity against cholera results primarily from local, mucosally secreted intestinal antibodies and that oral presentation of antigens is an efficient method of eliciting intestinal mucosal immune responses. In

comparison with parentally delivered vaccines, oral vaccines are easier to administer, more acceptable to recipients, and have a reduced risk of transmitting blood-borne infections [132].

Cholera vaccines fall into two broad categories: heat or formalin-inactivated whole cells (WC) and genetically attenuated live vaccines. Both vaccine types have advantages and drawbacks. For instance, field trials of the WC/CTB in Bangladesh and Peru have shown that the vaccine is safe and induced 85–90% protection after a two-dose administration regime [133]. However, protection declined rapidly after six months, particularly in children. A variant of the WC/rCTB lacking rCTB has been tested in Vietnam and shown to have 66% efficacy after eight months among all age groups [134]. Live genetically-attenuated vaccine candidates date back to the pioneering work of Finkelstein et al. who developed the *N*-methyl- *N'*-nitro-*N*-nitrosoguanidine-induced non-toxigenic and immunogenic El Tor biotype vaccine candidate Texas Star-SR [135].

8.2 WHO recommendations on vaccines

In a recent position paper of WHO [136] the following recommendation is given: "Among the new generation cholera vaccines, convincing protection in field situations has been demonstrated only with the WC/rBS vaccine. Thus, WC/rBS (Dukoral) vaccine should be considered in populations believed to be at imminent risk of a cholera epidemic". It is also recommended to travelers. Although live attenuated genetically modified strain CVD103-HgR is not recommended by WHO, for immunization of travelers to highly endemic areas its use is considered acceptable by WHO

9. Killed WC Vaccine with Cholera Toxin B Subunit: Dukoral

Dukoral is a WHO recommended cholera vaccine. This oral cholera vaccine developed by Swedish scientists was licensed in Bangladesh in 2007 by Healthcare Pharmaceuticals Limited (HPL). Dukoral has been licensed for persons two years and above. The manufacturer recommends that the vaccine be given in two doses 7-14 days apart for adults and children six years and older, and in three doses for children 2-5 years old20. Boosters

are recommended every two years for persons six and above, and every six months for 2-5 year olds.

9.1 Composition of Dukoral

Dukoral consists of a mixture of four preparations of heat- or formalin-killed whole-cell *V. cholerae* O1, representing both serotypes Inaba and Ogawa and both biotypes classical and El Tor (Table-1.1), that are then added with purified recombinant cholera toxin B subunit (rCTB) (produced in *V. cholerae* O1 Inaba, classical biotype strain 213). Because CT cross-reacts with E coli LT, the vaccine also provides short-term protection against ETEC, which is of added benefit [137-138].

Dukoral contains 1 mg recombinant non-toxic B-subunit of the cholera toxin (rCTB) and 1 x 10^{11} *vibrios* of killed whole *V. cholerae* O1 bacteria, i.e. 2.5 x 10^{10} vibrios each of:

Table-1.1: Qualitative and quantitative composition of Dukoral vaccine

Serogroup	Serotype	Biotype	Inactivation process	Number of bacteria*
Vibrio cholerae O1	Inaba	Classical	Heat inactivated	$25x10^9$ bacteria
Vibrio cholerae O1	Inaba	El Tor	Formalin inactivated	$25x10^9$ bacteria
Vibrio cholerae O1	Ogawa	Classical	Heat inactivated	$25x10^9$ bacteria
Vibrio cholerae O1	Ogawa	Classical	Formalin inactivated	$25x10^9$ bacteria

(*Bacterial count before inactivation.) Each dose of vaccine contains 3 ml suspension.

The vaccine is a whitish suspension in a single-dose glass vial. The sodium hydrogen carbonate is supplied as white effervescent granules with a raspberry flavor, which should be dissolved in a glass of water. Each dose of vaccine is supplied with one sachet of sodium hydrogen carbonate. The vaccine is taken orally with bicarbonate buffer, which protects the antigens from gastric acid.

Figure 1.7: Dukoral vaccine. (http://www.mynewsdesk.com/files)

9.2 Efficacy of Dukoral

The vaccine has been evaluated in a number of well-designed field trials; the original and largest field trial was carried out in Bangladesh [139-140]. In this trial, individuals received a total of 3 doses of the vaccine at 6-week intervals. WC-BS induced a high level of protection (85%) during the initial 6 months of the study [139]. At 12 months of follow-up, the vaccine was 62% protective. At 36 months of follow-up (the end of the study), WC-BS was 50% protective. During the first 6 months of evaluation, the vaccine provided protection in both young and older children, as well as in adults. However, the protective effect in young children rapidly decreased, and at 36 months of surveillance, the vaccine had its lowest efficacy (26%) among children aged 2–5 years, compared with 63% among individuals aged 15 years [140]. The vaccine protected equally against mild and severe cholera.

Immunologic responses after exposure to *V. cholerae* O1 vary by biotype and serotype, and although protection against disease caused by classical or El Tor biotypes was equivalent during the first 6 months of surveillance after vaccination, the longer term efficacy of WC-BS at 3 years was found to be lower against infections due to the El Tor biotype (39%) than against those due to the classical biotype (58%) [140]; the current global pandemic is caused predominantly by *V. cholerae* O1 El Tor organisms. The protective effect was also lower in individuals with blood group O (a risk factor for cholera gravis). The vaccine induced short-lived protection (67% at 3 months of surveillance and 21% at 12 months of surveillance) against heatlabile enterotoxin–producing enterotoxigenic *Escherichia coli* (ETEC) [137,

141]. Over the 3-year follow-up period, WC-BS resulted in a 25% reduction in hospital admissions of individuals with all types of diarrhea in Bangladesh, a 50% reduction in hospital admissions for life-threatening diarrhea, and a 45% reduction in mortality in women aged 115 years during a cholera epidemic [142].

Later, the production technology of WC-BS vaccine was modified: B subunit was prepared by recombinant genetic technology (WC-rBS). This vaccine was tested in multiple clinical trials in Peru in the 1990s. A trial in a cohort of adult military volunteers confirmed that the vaccine confers highgrade protection (86%) against El Tor cholera in the short term [143]. Another trial, performed in the general population, failed to find protection during the year after a 2-dose regimen but observed that a single booster dose given a year after the primary regimen elicited robust protection [144]. Because of methodological problems with the latter trial, a 2-dose regimen of WCrBS has been licensed internationally on the basis of the other cited trials [145]. A 2-dose regimen of WC-rBS was administered in a mass vaccination program in 2003 and 20004 in Beira, Mozambique, and was found to confer 84% protection to all persons aged ≥2 years and 82% protection to children vaccinated at <5 years of age [146].

9.3 Limitations of Dukoral

Unfortunately the dukoral vaccine also has some drawbacks which limit its practical application [147].

- The price is still too high to be useful in poor countries in a sustained manner.
- It must be kept in the refrigerator and the packaging is quite bulky limiting its distribution in the country.
- Finally, though rather simple for health providers to administer to patients, training and paying these health providers is a major constraint for the health system.

Due to these drawbacks the vaccine's capacity to reduce the cholera burden in Bangladesh and other cholera endemic countries has become limited. Nevertheless the launch of Dukoral in Bangladesh is a welcome step toward the eventual control of endemic cholera

10. Modified killed WC-only vaccines: ShanChol

The earlier version of the Vietnamese vaccine (ORC-Vax) was found to contain residual cholera toxin. To address these issues, a new bivalent (O1/O139) vaccine has been created in which a high toxin-producing strain (classical Inaba 569B) has been replaced by 2 alternative strains: heat-killed classical Inaba Cairo 48 and formalin-killed classical Ogawa Cairo 50. LPS content has been doubled, and modern quality control and release assays are used, including one to verify the absence of cholera toxin in the final product [148].

Several trials have evaluated a 2-dose regimen of this modified WC vaccine. The vaccine was shown to be safe and highly immunogenic against *V. cholerae* O1, with seroconversion rates of vibriocidal antibodies of 91%among adults in Vietnam [149], 53% among adults in Kolkata, and 80% among children aged ≥1 year of age in Kolkata, where high background immunity exists [19]. A phase III trial of the vaccine among ≈70,000 adults and children ≥1 year of age in slum areas of Kolkata, India, found that, during 2 years of follow-up, the vaccine conferred 67% protection against treated episodes of El Tor cholera [23]. Protection was sustained at this level during the third year, and surveillance continues. Interestingly, all cholera isolates detected in this trial exhibited the features of newly emergent modified El Tor cholera described earlier. Protection against O139 cholera was not evaluable.

Figure 1.8: ScahnChol Vaccine.

(http://cholera1.wikispaces.com/file/view/Shanchol_pic380.jpg/205284838/Shanchol_pic380.jpg)

In collaboration with the Government of Bangladesh, icddr,b is launching a five year long project to assess the feasibility of using an oral cholera vaccine, ShanChol produced in India which has whole cell components but not CTB. Evidence-based data show that increasing rates of cholera and diarrheal patients are coming to the icddr, b diarrhea hospital from the northern part of Dhaka city, and therefore this area has been chosen to receive the vaccine. ShanChol is being used in a large feasibility study in Bangladesh. About 160,000 people above the age of one year will be vaccinated, excluding pregnant women. The project has two components: vaccine provision and behavior change communication for promotion of hand washing and safe water treatment. The icddr,b is evaluating the effectiveness of vaccinating a large number of people from this area against cholera. Killed cholera vaccines have already been successfully introduced in countries like Vietnam (around 1997) and India (in 2009). The chosen vaccine Shanchol is safe, affordable and effective in preventing cholera.

11. Cholera vaccine CVD 103HgR

The Center for Vaccine Development (CVD) at the University of Maryland developed a live attenuated cholera vaccine derived from the classical Inaba 569B strain in the 1980s. It was engineered to express the cholera toxin B subunit, with most of the active A subunit deleted to remove its toxicity. The vaccine has the advantage of being administered in a single dose. Protective efficacy was studied in seven experimental challenge studies in North America involving a total of 103 vaccinees and 86 unvaccinated controls. In these studies, vaccinees were challenged with virulent El Tor Inaba (N=34), El Tor Ogawa (N=30) or classical Inaba (N=39) strains at different points in time (varying from 8 days to six months after ingestion of a single oral dose of CVD 103-HgR. Vaccine efficacy in preventing moderate or severe cholera ($\geq$3.0 liter purge) was found to be 100% against classical and 95% against El Tor cholera, and 76% in preventing diarrhea of any [150]. These studies showed the vaccine to protect North American volunteers against both classical and El Tor strains, beginning as early as eight days following vaccination and persisting for at least six months [151]. The vaccine was shown to be safe and immunogenic in a series of Phase I and II randomized, controlled clinical trials in adults and children in Asia, South America and Africa [152].

Based on the results in U.S. volunteers, the CVD 103-HgR vaccine achieved licensure in 1993 as a traveler's vaccine in several countries. It was produced by Berna Biotech (now Crucell) and sold as Orochol (or Mutachol) in single-dose, double-chambered sachets that contain the freeze-dried vaccine in one chamber and a sodium bicarbonate buffer in the other, which is required to protect the B subunit and live vibrios against gastric acid. The vaccine is reconstituted by mixing it and the buffer in 100 ml of water. Two different dosage amounts were available – one for developed countries and another, with ten times as many organisms, for developing countries [151].

12. Other cholera vaccines in pipe line

Several experimental live oral candidate vaccines are under development. A live vaccine, Peru 15 is a genetically attenuated *V. cholerae* O1 El Tor Inaba strain, originally isolated in Peru in 1991. A single-dose regimen of Peru 15 has been shown to be safe and immunogenic in the US volunteers, as well as in adults and toddlers in Bangladesh [35].

V. cholerae 638 is an attenuated O1 El Tor Ogawa strain that is being developed in Cuba. A single-dose regimen was shown to be immunogenic and protective in an experimental cholera challenge study in Cuban adults [153]. *V. cholerae* IEM 101 is an O1 El Tor Ogawa strain from China that naturally lacks the gene for cholera toxin and several other virulence factors. In human studies, it was found to be immunogenic with no adverse effects. Two additional derivatives—IEM 108 and 109—are also promising candidates, but no human data have been reported to date [154-155].

Another interesting *V. cholerae* O1 candidate is the VA1.3 from India, which is a recombinant strain able to produce CTB but which is otherwise devoid of cholera toxin. This vaccine was found to be safe and immunogenic in adults in Kolkata [156]. Two recombinant live attenuated Vibrio O139 candidate vaccines, CVD 112 and Bengal 15, have been evaluated in volunteer trials, and they provided ≈80% protection against challenge with wild-type O139 strains [157-158].

13. Objectives of the study

The main objective of the study was to evaluate memory B-cell immune responses to two major antigens, CT and LPS of *V. cholerae* O1, in children and adults after oral cholera vaccination.

Specific aims of the study were to:

- ascertain how well memory B-cells are generated after vaccination with Dukoral
- determine the longevity of memory B-cell responses after vaccination
- compare antigen specific memory B-cell responses among children and adults.

CHAPTER TWO

MATERIALS AND METHODS

1. Study site

This study was carried out in the Laboratory Sciences Divisions (LSD), International Centre for Diarrheal Disease and Research, Bangladesh (icddr, b). All the vaccinations were carried out in healthy people living in the Mirpur area in Dhaka, Bangladesh. The study was approved by the Research Review Committee (RRC) and Ethical Review Committee (ERC) of the icddr, b.

2. Study participants

The study populations were aged ranges from 2 to 17 for children and from 18 to 45 for adults. From all the study participants blood samples were obtained before vaccination (day 0) and after vaccination at day 3, 21, 30, 90 and 180 from child vaccinees and for adults after 3, 17, 30, 90 and 180 day of each dose. Participants were excluded from the study if they had a history of gastrointestinal disorder or diarrheal illness in the past 2 weeks, febrile illness in the preceding week or received antibiotic treatment at least 7 days prior to enrollment; or that were positive for common enteric pathogens were also excluded. The vibriocidal assay, enzyme linked immunosorbent (ELISA) assay for serum IgG and IgA antibodies to CTB and LPS (*V. cholerae*-specific Ogawa), and for antigen-specific IgG, IgA memory B-cells enzyme linked immunospot (ELISPOT) assays were performed for each time period.

3. Study design

3.1 Vaccination

The oral monovalent WC-O1/CTB , cholera vaccine (Dukoral) was studied (SBL Vaccine, Stockholm, Sweden). The vaccine contains 1.0×10^{11} heat- and formalin-killed O1 *Vibrios* (both Inaba and Ogawa serotypes, and classical and El Tor biotypes) together with 1.0 mg of recombinantly produced cholera toxin B (rCTB) subunit.

After venous blood was collected from participants, they were given a single dose of Dukoral. This is provided as an oral liquid suspension in a single dose glass vial with bicarbonate, citrate and ascorbic acid buffer. Vaccine was added to 150 ml of buffer, which

protects the antigens from degradation by gastric acid. The formulated vaccine was given within an hour of preparation. On the other hand the second dose was give after 14 days of first dose. Food and drink were withheld for 1h before and 1hr after vaccination.

3.2 Blood sample collection

In case of adult and child vaccinees, two doses of vaccine were given at two times interval- one at day 0 and the next one was at day 14. For adult vaccinees, 8 to 12 ml of blood was collected in a sodium heparinized vacutainer tube from each vaccine participant at 6 times point during the duration of the study. Blood samples were collected from vaccine participants at day D0 (before vaccination), and at D3, D17, D30, D90 and D180 after vaccination (Fig 2.1).

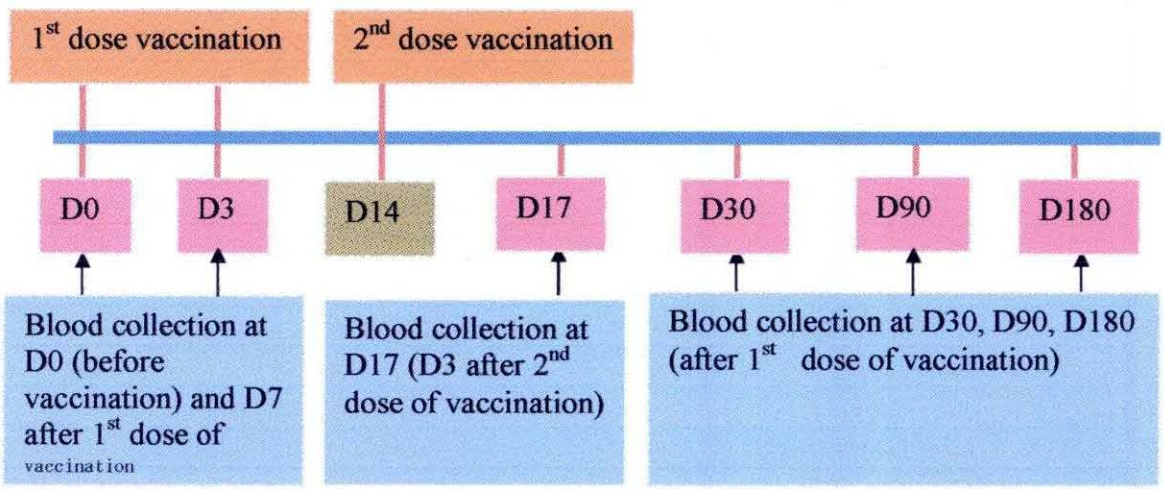


Figure-2.1: Blood collection schedule for adult vaccinees: Dukoral cholera vaccine was given at day 0 and day 14 and blood samples were collected from vaccine participants at day 0 (D0) before vaccination, and at day 3, 17, day 30, day 90, day180 after vaccination.

For child vaccinees, 8 to 10 ml of blood was collected in a sodium heparinized vacutainer tube from each vaccine participant at 6 times point during the duration of the study.

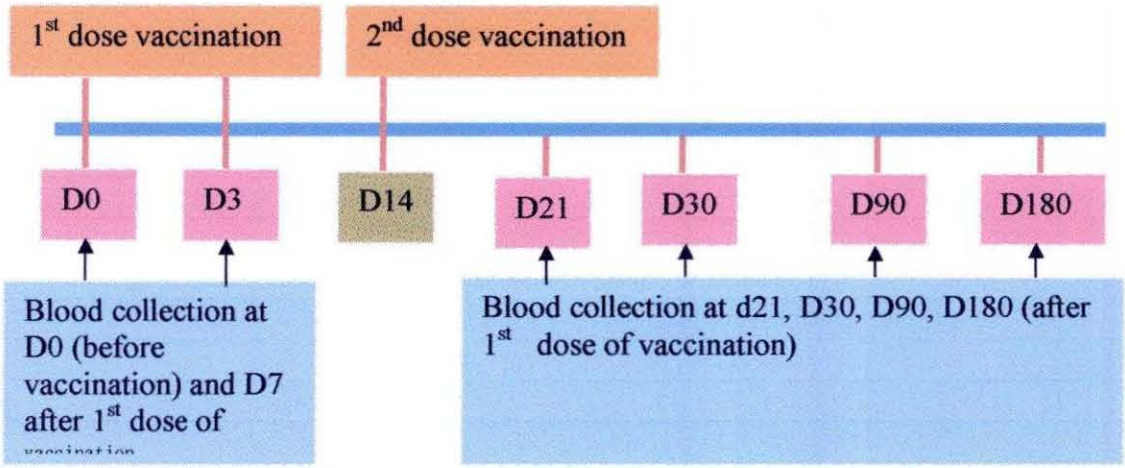


Figure-2.2: Blood collection schedule for child vaccinees: Dukoral cholera vaccine was given at day 0 and day 14 and blood samples were collected from vaccine participants at day 0 (D0) before vaccination, and at day 3, day 21, day 30, day 90, day180 after vaccination.

4. Laboratory methods

4.1 Bacteriological examination of patient stools

Stool from patients with a characteristic rice watery appearance were screened by dark-field microscopy for the observation of the darting movement by its flagella to confirm *Vibrio*s infection. Inhibition of its movement by antibodies specific for *V. cholerae* O1 or *V. cholerae* O139 confirmed the serotype. Inaba or Ogawa specific antibodies inhibition screening confirmed the biotype of the *V. cholerae* O1.

4.1.1 Dark field microscopy to diagnose cholera in diarrheal stools

Dark field microscopy (DF) is a microscope based technique to detect motile *V. cholerae* in stool sample .The genus *Vibrio* is named for the unique, rapid to- and fro- motility, which is characteristic of this group of organism. By using this property, the organisms were detected in DF. In the microscopic field, the specimen illuminates and the field around the specimen remains dark. Live and motile *V. cholerae* can be seen directly without the help of staining by this quick technique (Fig 2.3). For this technique, a small drop of the test stool sample

was placed on a clean slide by a bacteriological loop and a clean cover slip was applied on to it. Emulsion oil was applied on the lens to help the visibility of the object. The slide was examined under dark field at 40X magnifications. Organisms with typical motility were observed and sample was defined as DF^{+} for infected patients. This confirmed *V. cholerae* in the patients sample. Inhibition of motility by strain specific antibodies was used to confirm the serogroup of the pathogen.

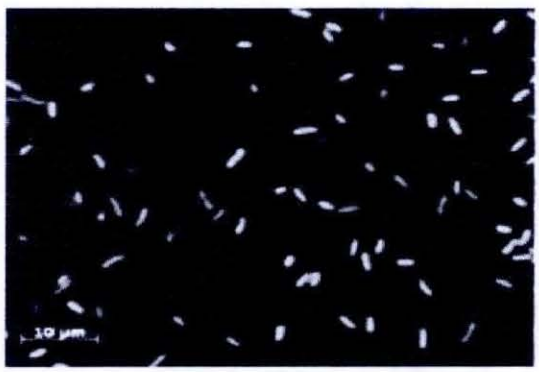


Figure-2.3: Dark field microscopy of *Vibrio cholerae.* (http://cdn.physorg.com)

4.2 Serological detection of *Vibrio cholerae* O1

Stool sample from the diarrheal patients were plated on TTGA plate. Grayish colonies with a central dark zone surrounded by a zone of opacity were observed typical for *V. cholerae* (Fig-2.4).

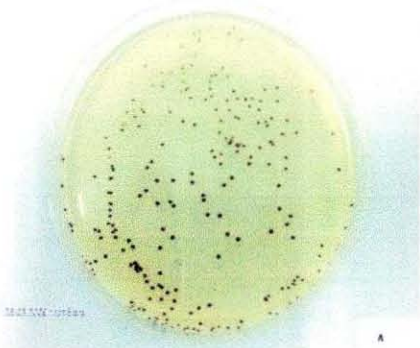


Figure-2.4: *Vibrio cholerae* colonies on TTGA plate

For serotyping, *V. cholerae* O1- or *V. cholerae* O139-specific mouse monoclonal antibody sera were used. Mouse anti-serum was raised against *V. cholerae* O1 for both Inaba and Ogawa serotypes. For serotyping, a slide was taken and divided into three parts (Fig-2.5). Then three types of antisera (*V. cholerae* 01- Ogawa, *V. cholerae* 01- Inaba and *V. cholerae* 0139-specific rabbit antisera) was dropped on each part of the slide. Bacteria from the TTGA plate were applied on the slide where antibody was present. Agglutination was observed when specific colony for the antisera was present. The occurrence of clear agglutination within 2 minutes was considered a positive reaction. Only *V. cholerae* O1 reacting samples either Ogawa or Inaba were enrolled in this study.

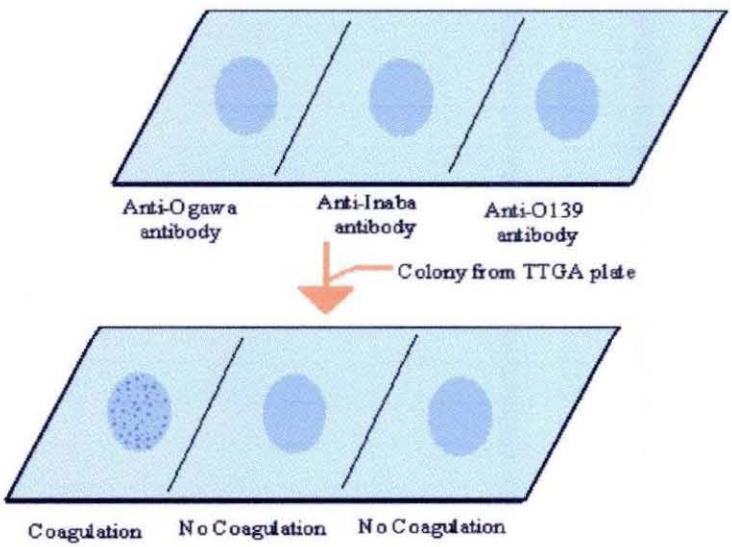


Figure-2.5: Serological detection of *V. cholerae* O1: Coagulation is seen at the first portion of the slide indicates antisera containing anti Ogawa antibody cross links *V. cholerae* and gives a spotted colour.

4.3 Isolation of peripheral blood mononuclear cells (PBMC)

a) Heparinized venous blood was diluted with equal volume of Phosphate Buffered Saline (PBS, 10 mM, pH = 7.2) in Falcon tubes.

b) Diluted blood was carefully added to the volume of Ficoll-Isopaque (in a 1:1 ratio of undiluted blood) without disturbing the Ficoll layer. Hence, two distinct layers were maintained.

c) The tube was centrifuged at 772 g for 25 minute at 20°C.

d) After centrifugation, PBMCs were remained at the interface of plasma and Ficoll. RBCs and other cell debris were precipitated at the bottom of the tube. The mononuclear cells were then removed from the top of the Ficoll layer carefully with a Pasteur pipette.

e) The PBMCs were washed once in PBS at 952 g for 10 minute at 20°C.

f) The PBMCs were resuspended in 10 ml of PBS. 25µl of cell suspension was collected into an eppendorf tube (25µl cell suspension with 25µl tryptan blue) and then MNCs were counted in the haemocytometer while the resuspended PBMCs were washed for the second time at 953 g for 10 minute at 20°C.

g) After the second wash, the cells were resuspended in RPMI Complete medium to a concentration of 10^7cells/ml. These cells were used for antigen-specific ASC-ELISPOT and for memory B cell culture.

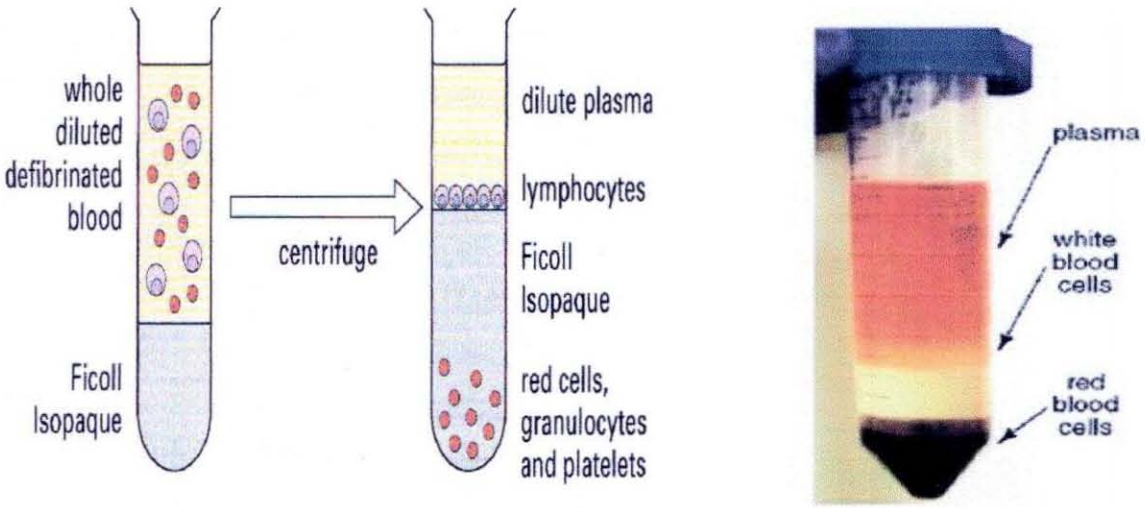
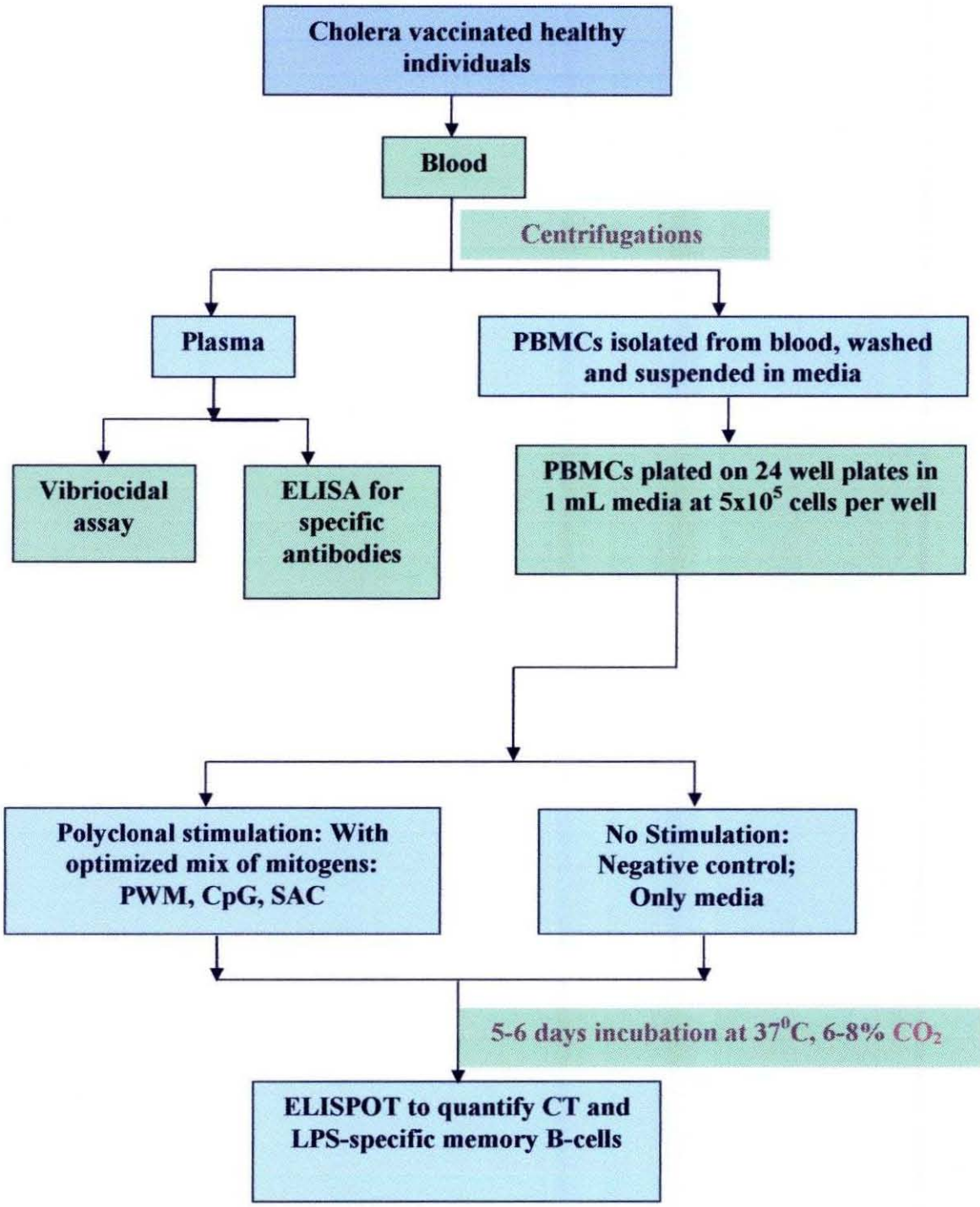


Figure-2.6: Isolation of PBMC by density gradient centrifugation on Ficoll-Isopaque. (http://www.currentprotocols.com/protocol/mca04c)

Flow Chart Describing the Study



4.4 Enzyme linked immunospot (ELISPOT) assay

The B-cell ELISPOT is a highly sensitive assay that allows the detection and enumeration of antibody secreting cells; although the number of spots are not a direct reflection of this as proliferation of cells occurs during the 6-day culture. The assay can be used for the detection of antigen-specific responses as well as for determinations of the total number of Ig-secreting cells. By use of isotype specific reagents, cells producing antibodies of different isotypes (IgG, IgA) can be analyzed separately. This assay is highly sensitive, quantitative, easy to use and amenable to high throughput.

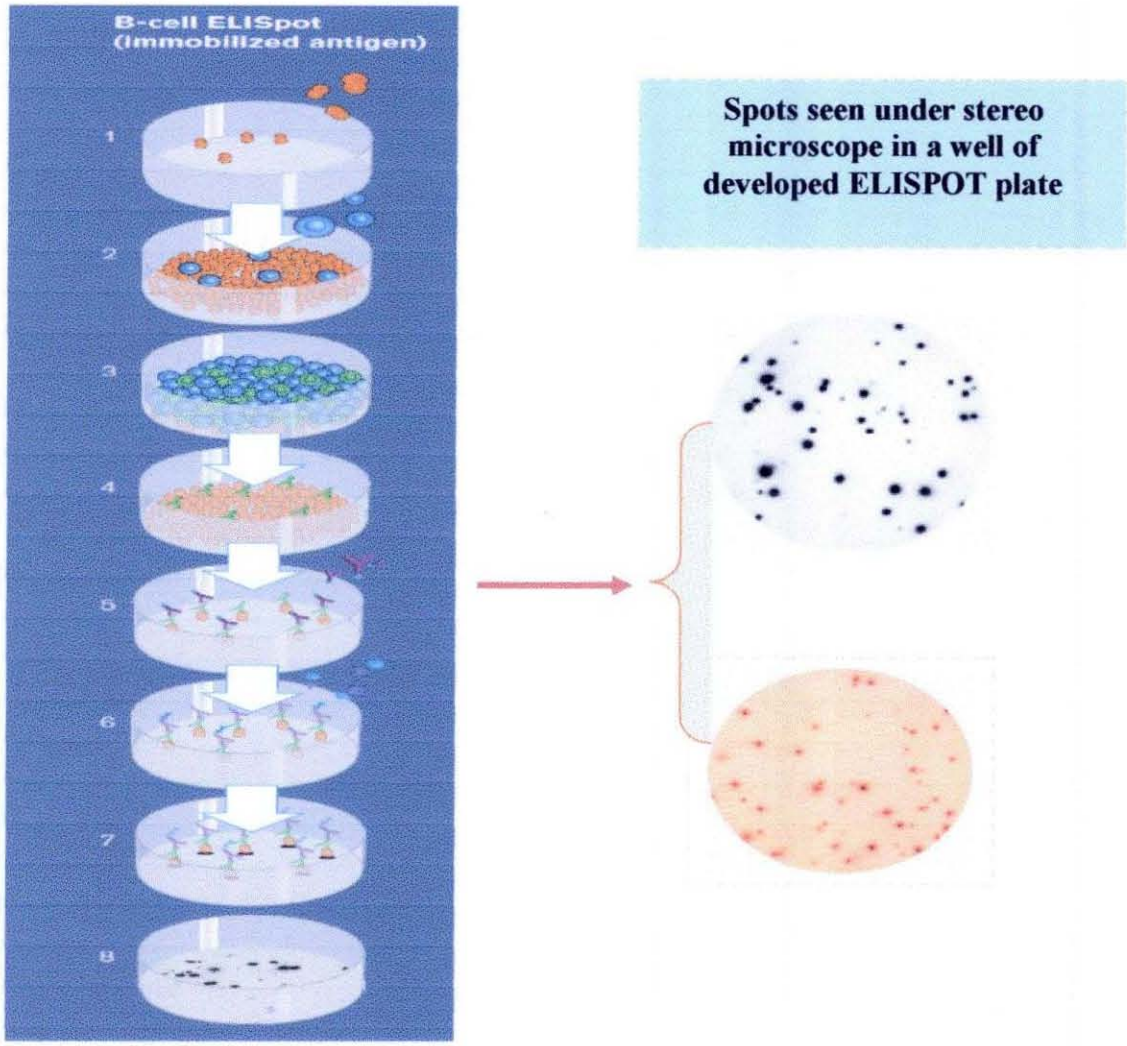


Figure-2.7: General outline of ELISPOT assay.

1) Antigen is immobilized on the membrane of a 96-well ELISPOT plate.
2-4) Cells are added and left to incubate to allow secretion and binding of antigen-specific antibodies.
5) After removal of the cells, bound antibodies are detected with HRP-cojugated anti-Ig.
6-7) Spots are visualized by the addition of AEC (amino ethyl carbazole)-H_2O_2 substrate.
8) Evaluation and counting of spots are made in a microscope

In single color ELISPOT assay separate wells are used for development of IgA and IgG secreting cells. Red spots indicate IgA specific ASC, blue spots indicate only IgG specific ASC. (http://www.biotek.com/resources/articles/automating-wash-elispot.html)

4.4.1 Coating the plates for Memory B-cell study

a) Nitrocellulose plates were coated with 100µl of:

- GM1, at 3nmol/ml in PBS for Cholera Toxin
- Goat anti-human IgG at 3.0 µg /ml in PBS for the total – Immunoglobulin plate and
- KLH, at 2.5ug/ml in PBS for negative control.

LPS (Ogawa) Plate coating:

- 50 µl of poly-L-Lysine solution (conc. 10µg/ml PBS) was added to each well. It was then kept at room temperature for at least 30 min.
- The solution was decanted and immediately 50 µl bacterial suspension (whole cell at conc. of $5.5x10^9$) or LPS (25 µg/ml in PBS) was added to each well.
- The plates were centrifuged at 2000 rpm for 5 min.
- It is recommended not to decant the content (nothing is left actually). 50 µl of 0.5% gluteraldehyde was added gently. This step should be carried out inside hood, because gluteraldehyde is toxic (put on gloves).
- Plates were kept at room temp for 15 min.
- Plates were washed twice with PBS (gently).
- 200 µl 0.1 % BSA in 0.1 M glycine was added to each well.
- Plates were kept at room temp for 30 min.
- Plates were washed twice with PBS.
- These plates are ready to use.
- To store, 200 µl 0.1% BSA-PBS was added to each well and kept at -20^0c.

b) Plates were incubated at 4^o C overnight.

c) The GM1 coating buffer was decanted and the CT plate was washed 3 times with PBS. Excessive liquid was removed. 100µl of recombinant cholera toxin B-subunit (rCTB)

(2.5μg/ ml in PBS) was added to each of the GM1 coated wells and PBS to the other wells (100μl/well). The plates were incubated for one hour at 37°C.

4.4.2 Blocking

a) Plates were washed twice gently with PBS only.

b) Next the plates were blocked for 30 minutes-1 hour with 200 μl RPMI complete media at 37°C.

4.4.3 Memory B-cell culture and ELISPOT assay for antibody secreting memory B-cells

Memory B cell (MBC) assays were performed as described below. Briefly, PBMC were placed in MBCS culture medium optimized to stimulate antigen-independent proliferation and terminal differentiation of memory B cells into ASC or medium alone as a negative control. This stimulation medium included CpG oligonucleotide, a dilution of crude pokeweed mitogen (PWM) extract, and a dilution of fixed *Staphylococcus aureus* Cowan (SAC). Cultures were incubated for 5-6 days, after which antigen-specific and total IgG, and total IgA ELISPOT assays were performed. Plates coated with keyhole limpet hemocyanin (KLH) were used as negative controls. Appropriate stimulation was defined as the stimulation medium generating greater than 4-fold increase of total IgG and IgA ASC as compared to the unstimulated cells.

4.4.3.1 Mitogens for the stimulation cocktail

I. **Pokeweed Mitogen (PWM);** diluted to a working concentration of 1:100,000 in Culture Stimulation media.

II. ***Staphylococcus aureas Cowan* (SAC)** fixed; diluted to a working concentration of 1: 10,000 in Culture Stimulation media.

III. **CpG** [T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T*T*T*G*T*C*G*T*T (* signifies phosphothionation) from operon]; diluted to a working concentration of 6μg/ml in Culture Stimulation media.

4.4.3.2 Memory B-cell culture

a) Culture wells were divided into unstimulated and stimulated wells. One ml of stimulation media was added to respective culture wells using sterile technique. Sterility was very critical in this particular step.

b) To each of these wells 5 x 10^5 PBMC were added in a biohazard hood.

c) These culture plates were incubated in 5% CO_2 at 37°C for 5-6 days.

4.4.3.3 Antigen specific memory B-Cell ELISPOT assay

Culture cell harvest:

- After 5-6 days proliferation culture cells were collected in eppendrof tubes from each culture well.
- Centrifuge at 2500 rpm for 7 minutes
- Again centrifuged at 2500 rpm for 7 min with PBS only
- Finally, cell pellet was resuspended in 200 µL RPMI complete media which contains approximately 5 x 10^5 Peripheral Blood Mononuclear Cells.

Cell loading and plate developing:

- The blocking solution was decanted. From each culture well, 20% of cells were used for total Ig's ELISPOT, while 80% were used for antigen specific ASC ELISPOT. Keeping this in mind, in the CT and LPS-plates, 40 µL of fresh RPMI complete media was added to the first row and 160 µl to subsequent rows. For the Total Ig's plate, 160 µl of fresh media was added to the first row and 180 µl to subsequent rows.
- 160 µl of the resuspended cells were added to each well for the neat well for CT, and LPS specific wells and remaining 40 µl of resuspended cells were added to the first row of total Ig's plate.

- Four 10x dilutions of the total Ig's wells (transferring 20 µl), and two 5x dilutions of the CTB, and LPS wells (transferring 40 µl) were carried out. The plates were incubated with cells for 5-6 hours at 37°C in a CO_2 (5%) incubator.
- Next the cells were decanted and the plates washed 5 times with PBS-Tween (0.05%) and 3 times with PBS. Excess liquid was shaken off in such a manner so that the nitrocellulose membrane does not become dry.
- Anti-human IgA-HRP at 1:500 and anti-human IgG-HRP at 1:500 was diluted in 1% FBS in PBS-Tween 0.05%. To each well 100µl was added. All plates were incubated overnight at 4°C. The liquid was decanted and plates washed 5 times with PBS-Tween and 3 times with PBS.
- HRP chromogen/substrate, AEC/H_2O_2, was prepared in advance up to two weeks and stored at 4°C. (AEC/ H_2O_2 preparation: 10 mg of AEC in 1 ml of DMF + 29 ml of Na-acetate then filtered through 0.2 µm). 5µl of 30% H_2O_2 was added to 10 ml of AEC solution and was vortexed well. 100µl of the chromogen/substrate was added to every well and the color reaction monitored immediately.
- When the spots were clearly visible, the reaction was stopped by washing the plates repeatedly with tap water.
- Plates were then soaked in tap water for 30 min before allowing them to dry in room temperature.
- The spots were counted under low-magnification and the plates stored away from light for automated counts later.
- Each red spot represented an IgA secreting cell in anti-human IgA-HRP conjugate added wells while every red spot indicated an IgG secreting cell in anti-human IgG-HRP conjugate added wells.
- Data was evaluated as CTB and LPS specific IgG/A ASC divided by total IgG/A ASC and per 10^6 PBMC. And the results were expressed as percentage.
- We defined appropriate stimulation of PBMC in our assay as a ≥ 4 fold increase in the number of total Ig memory cells following stimulation as compared to the

unstimulated cells. This definition included approximately 90% of all stimulated cultures for both IgG and IgA memory cells.

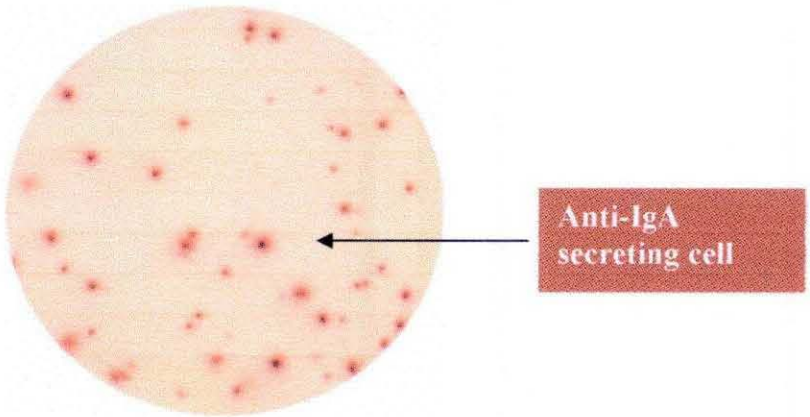


Figure-2.8: An ELISPOT plate well showing red spot as IgA. (http://www.piercenet.com)

4.4.3.4 Antibody in memory B cell culture supernatant (ALS) collection

Supernatants were collected on the day of harvest, after 5-6 days of incubation using the mitogen cocktail described above, and mixed with a protease inhibitor (10 µl/ml) cocktail consisting of aprotinin (1µg/ml), leupeptin (10µM), sodium azide (1mg/ml) and 4-(amino ethyl) benzene sulfonyl fluoride (0.2µM) and were then aliquoted and frozen at -70°C until assayed for antigen specific IgG and IgA by ELISA.

4.5 Enzyme linked immunosorbant assay (ELISA)

ELISA is a qualitative and quantitative immunoassay in which antibodies or antigens are detected by the binding of an enzyme coupled to either anti-Ig antibody or antibody specific for the antigen.

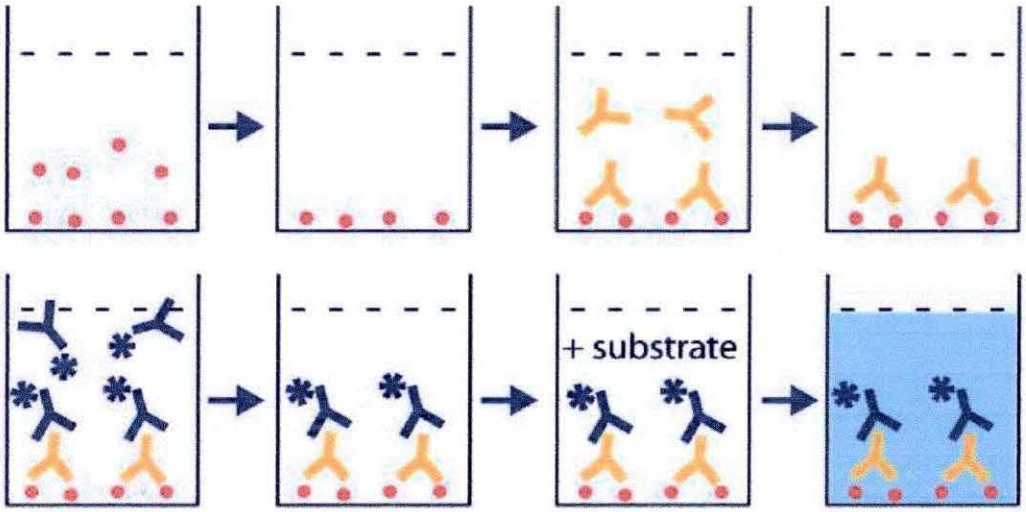


Figure-2.9: Indirect ELISA to detect presence of antibody.

(http://www.dr.marahimi.com/wpcontent/uploads/2011/09/6.jpg)

4.5.1 Detection of IgA and IgG antibodies against B subunit of cholera toxin (CT) in plasma samples using GM1-ELISA

Coating

Wells of polystyrene plates were coated with 100µL/well of GM1 at a concentration of 0.3nmol/mL or 0.5µg/ml in PBS. The plates were then incubated at room temperature for overnight. Following overnight incubation, the ELISA plates could be stored at 4°C up to 2 weeks for further use.

Blocking

The GM1-coated plates were washed thrice with PBS and blocked with 0.1% Bovine Serum Albumin in PBS (BSA-PBS), 200µL/well for 30 minutes at 37°C.

Loading of Samples and Plate developing

- The plates were washed thrice with PBS-0.05% Tween and once with PBS.
- The purified rCTB (0.5µg/ml in PBS) diluted in 0.1% BSA-PBS were added, 100µL per well and incubated for 60 min at 37°C.
- The plates were washed 3 times with PBS - Tween (0.05 %) and once with PBS.

- The plates were washed 3 times with PBS- Tween (0.05 %) and once with PBS.
- The horseradish peroxidase (HRP) conjugated rabbit anti-human IgA and anti-human IgG (Jackson ImmunResearch Laboratories Inc.) were diluted in 0.1% BSA-PBS Tween and added 100 µl per well.
    i. Anti-human IgA HRP (e.g. Jackson 309035011, 1/1000)
    ii. Anti-human IgG HRP (e.g. Jackson 309035006, 1/1000)
- The plates were incubated for 90 min at 37^0C.
- The plates were washed 3 times with PBS- Tween (0.05 %) and once with PBS.
- The plates were developed with the substrate orthophenylene diamine (OPD)-100 µl / well, prepared by dissolving 10 mg of OPD in 10 ml of 0.1 M sodium citrate buffer, (pH 4.5) to which was added 4 µl of 0.1 % H_2O_2 immediately before use.
- The plates were read kinetically at 450 nm for five minutes. The maximal rate of change in optical density in milli absorbance units per minute was normalized across plates by calculating the ratio of the test sample to a standard of pooled convalescent-phase serum from previously-infected cholera patients, which was added as a positive control on each plate. For the CTB antigen, individuals with a ≥2-fold increase in antibody titer one or three weeks post infection or vaccination were considered as responders.

4.5.2 Detection of IgA and IgG antibodies against Lipopolysaccharide in plasma samples using ELISA

Coating:

The ELISA plates were coated with 100µL/well of LPS of *V. cholerae* O1 at a concentration of 2.5µg/ml in PBS (pH 7.2-7.4). The plates were then incubated at room temperature for overnight. Following overnight incubation, the ELISA plates could be stored at 4°C for a week.

Loading of Samples and Plate developing

- The plates were washed 3 times with PBS-Tween (0.05 %) and once with PBS.
- The plasma samples (initial dilution 1:25 for IgA & IgG) diluted in 0.1% BSA-PBS containing 0.05% Tween were serially diluted threefold in microtiter plates.
- The plates were incubated at 37^0C for 90 minutes.
- The plates were washed 3 times with PBS-Tween (0.05 %) and once with PBS.
- The horseradish peroxidase (HRP) conjugated rabbit anti-human IgA and IgG were diluted in 0.1% BSA-PBS Tween and 100 µl was added to each well.
    - Anti-human IgA HRP (e.g. Jackson 309035011, 1/1000)
    - Anti-human IgG HRP (e.g. Jackson 309035006, 1/1000)
- The plates were incubated for 90 minutes at 37^0C.
- The plates were washed 3 times with PBS- Tween (0.05 %) and once with PBS.
- The plates were developed with the substrate orthophenylene diamine (OPD) as100µl / well, prepared by dissolving 10 mg of OPD in 10 ml of 0.1 M sodium citrate buffer, (pH 4.5) to which was added 4 µl of 0.1 % H_2O_2 immediately before use.
- The plates were read kinetically at 450 nm for five minutes. The maximal rate of change in optical density in milli absorbance units per minute was normalized across plates by calculating the ratio of the test sample to a standard of pooled convalescent-phase serum from previously-infected cholera patients, which was added as a positive control on each plate. For the LPS antigen, individuals with a ≥2-fold increase in antibody titer one or three weeks post infection or vaccination were considered as responders.

Significant rise: $\frac{\text{Geometric mean post immunization titer}}{\text{Geometric mean pre-immunization titer}} \geq 2$

4.6 Vibriocidal Antibody Assay

Vibriocidal antibody assays were performed using guinea pig complement and with *V. cholerae* O1 Ogawa (X-25049) as the target organism. Vibriocidal titer was defined as the reciprocal of the highest dilution resulting in >50% reduction of the optical density when compared to that of control wells without serum. Individuals showing a ≥4-fold increase in vibriocidal responses one or three weeks post infection or vaccination were considered responders.

Procedure:

- *V. cholerae* O1 (strain 19479 El Tor. Inaba) were cultured overnight on blood agar plates at 37°C.
- A loopful of bacteria from the plates was inoculated in 15 ml BHI (brain-heart infusion)-medium in a conical flask with cotton plug. This was incubated on a shaker at 37°C for 3-4 hours.
- The culture was centrifuged at 3000rpm for 10 minutes and the supernatant was thrown away. The sediment was resuspended in sterile saline.
- This was again centrifuged for another 8-10 minutes. The pellet was resuspended in sterile saline.
- Bacterial concentration was adjusted by spectrophotometer at 600 nm. For *V. cholerae* O1 OD was adjusted at 0.3
- Heat-inactivated (56° C, 30 minutes) sera was diluted 2-fold in sterile saline in flat-bottom microtiter plates (Nunc, F) as follows:
    - 25 µl of cold saline was dispensed in all wells except column #2.

equals to 1:10240). The last 25 µl was discarded from the last well on each row. The plates were kept at 4°C (on ice) until used.

- The indicator (bacteria-complement-saline mixture) was prepared. The composition for each plates is as follows:

	Sterile Saline	**Bacteria**	**Complement**
***V. cholerae* 01 (X25049 and 19479)**	2.55 ml	150 µl	300 µl

- The indicator was used immediately after preparation.
- 25 µl of the indicator was added to all wells except wells in row A, B, C and D in column #1. The plate was incubated on a shaker at 37°C for 1 hour (50 revolutions /min).
- 150 µl BHI was added to each well. This was incubated for another 3-4 hours at 37°C without shaking. The plates were read visually and spectrophotometrically. The absorbance for control wells should reach 0.20 to 0.28 at 595 nm.

Vibriocidal antibody titer is defined as the reciprocal of the highest serum dilution resulting in greater than 50% OD reduction when compared to the OD of control wells without serum.

4.7 Data analysis

Comparisons of immunologic responses were tested for significance within groups using the Wilcoxon Signed Rank test, among the groups using Mann Whitney Rank-sum test and for responder frequency comparison among different groups, we used chi-square test. All reported P values are two-tailed, with a cutoff of $P \leq 0.05$ considered a threshold for statistical significance. Analyses were performed on GraphPad Prism 4.0 and SigmaStat 3.1.

CHAPTER THREE

RESULTS

responses of the children, data were also collected from 32 adults (18-45 years) vaccinees from the earlier studies [18]. The demographic characteristics of the study participants are presented in Table 1.

Table 3.1: Demographic and serologic characteristics of the pediatric study participants

Characteristics	2-5 years (N=20)	6-17 years (N=20)	18-45 years (N=32)
Median age (years)	4.75	10.55	33
No. of subjects in Sex groups			
Male	11	10	13
Female	9	10	19
No. of subjects with blood groups			
O	9	7	12
A	5	4	7
B	4	6	11
AB	2	3	2

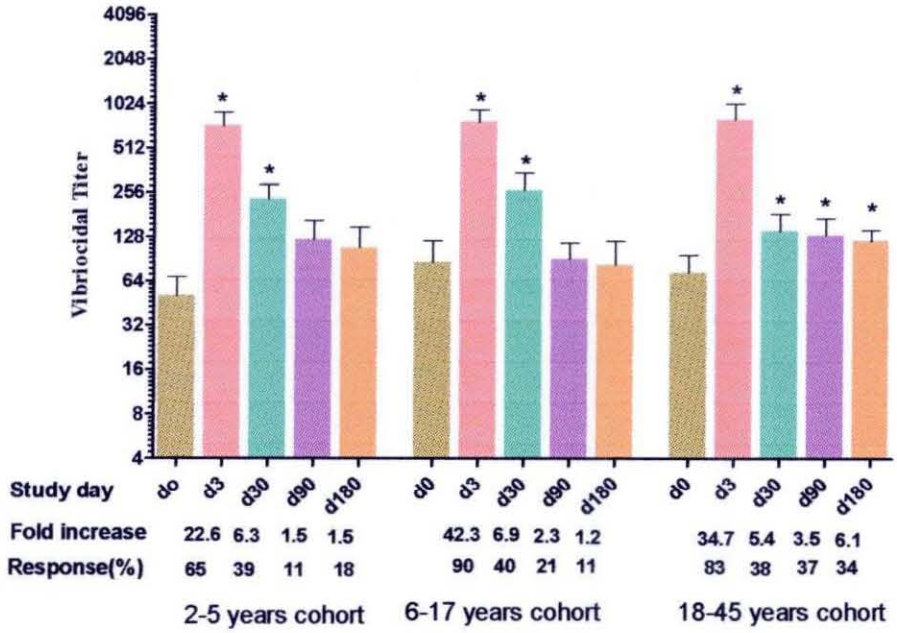


Figure-3.1: Vibriocidal responses among vaccinees of different age groups. The columns indicate mean reciprocal end titers; error bars represent the standard errors of the mean. The Wilcoxson signed-rank test was used for the analysis of data. An asteric indicates a statistically significant difference ($P<0.05$) from the baseline preimmune response. Mean fold changes and responder frequencies are also listed.

After vaccination, most of the study participants mounted strong vibriocidal responses. The vibriocidal response was represented by a ≥4-fold increase in immunoglobulin titer compared to baseline. 42.3-fold increased vibriocidal titer was observed in older children vaccinees on day 3 compared to baseline. The highest fold increase in vibriocidal titer in younger children at day 3 was 22.6 and in adults was 34.7.

0.0001), older children (GM= 452.54, 95% CI=430.5 to 1124, P= 0.0001) and in adults (GM= 305.5, 95% CI= 333.2 to 1300, P<0.0001).

For the adult vaccinees, the antibody titers remained significantly elevated up to day 180 (P= 0.007). On the other hand, the antibody titers remained significantly elevated up to day 30 for both young and older children, and subsequently declined to baseline. The vibriocidal responder frequency and magnitude of responses were comparable in both one and two dose vaccine cohorts in the respective days.

3. Plasma anti-CT and anti-LPS specific antibody responses

Plasma CT-specific IgA and IgG levels and LPS-specific IgA and IgG levels were measured at days 0, 3, 30, 90 and180.

3.1 Anti-CT IgA responses

After vaccination, plasma CT or LPS specific antibody responses were determined by a twofold or greater increase of antibody titer from the baseline. In 2-5 years cohort, 100% vaccinees were shown to mount strong CT-specific plasma IgA responses with 13.1 fold increased in immunoglobulin titer at day 3 (Figure 3.2). The most promising CT-specific plasma IgA responses were observed at day 3 in the 6-17 years old cohort, where 90% vaccines mounted 21.1- fold increased antibody titer. Strong CT-specific plasma IgA responses were also observed at day 3 in 97% adult vaccinees with a 15.4 fold increased antibody titer.

In younger and older children, these responses remained elevated up to day 30 and returned to baseline by day 90. In case of adult vaccinees, CT-specific IgA responses persisted up to day 90. No significant difference was observed in CT-specific IgA responses among the different age groups vaccine recipients in the corresponding study days.

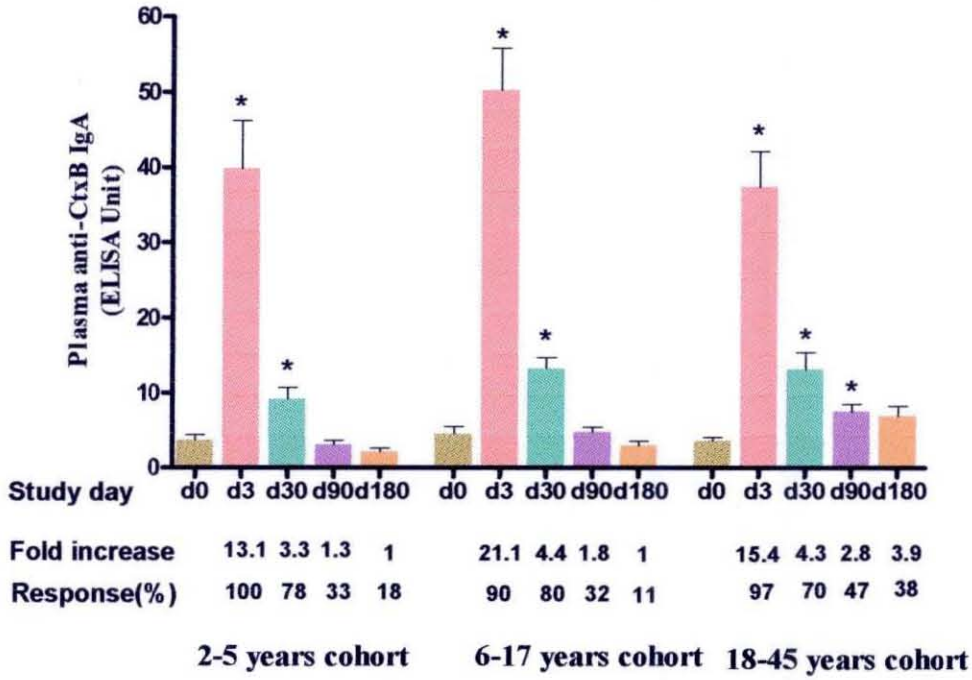


Figure-3.2: Mean normalized plasma CT-specific IgA antibody responses with (± SEM) standard error bars. The Wilcoxson signed-rank test was used for the analysis of data. An asteric indicates a statistically significant difference ($P<0.05$) from the baseline preimmune response. Mean fold changes and responder frequencies are also listed.

of vaccinees with 4.2-fold increased immunoglobulin titer. On the other hand, highest fold increase of 6.7 for CT-specific IgG was found in 91% adult vaccinees at day 3.

Anti-CT specific IgG responses were statistically significant at day 3 in younger (GM= 66.45; 95% CI= 55.74 to 77.16; *P*= 0.0005), and it remained significantly elevated up to day 90. On the other hand, in older children significant anti-CT specific IgG responses were found at day 3 (GM= 81.70; 95% CI= 70.70 to 92.70; *P*= <0.0001), which remained significantly elevated up to day 30. In case of adult vaccinees, significantly increased CT-specific IgG responses were found at day 3 (GM= 64.45; 95% CI= 54.39 to 74.52; *P*= < 0.0001), and the responses remained significantly higher up to day 180.

Statistically significant differences in anti-CT specific IgG responses were found among all 3 age groups of vaccinees. Compared to younger children and adult vaccinees, anti-CT specific IgG responses were higher in older children vaccinees at day 30 (*P*= < 0.0001).

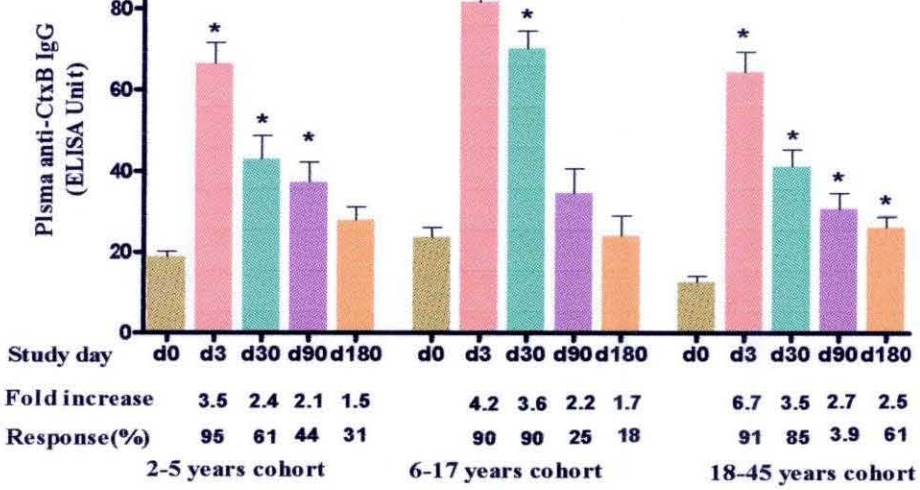


Figure-3.3: Mean normalized plasma CT-specific IgG antibody responses with (± SEM) standard error bars. The Wilcoxson signed-rank test was used for the analysis of data. An asteric indicates a statistically significant difference ($P<0.05$) from the baseline preimmune response. Mean fold changes and responder frequencies are also listed.

3.3 Anti-LPS IgA responses in vaccinees

In the adult age groups, 69% vaccinees shown to have strong LPS-specific IgA responses with 5-fold increased immunoglobulin titer at day 3. On the other hand, 60% younger and 50% older pediatric vaccinees mounted strong LPS-specific IgA responses at day 7 with 4-fold and 3.7-fold increased immunoglobulin titer, respectively (Figure 3.4).

LPS-specific IgA responses were significantly elevated at day 3 both in younger (GM= 22.00; 95% CI= 11.92 to 32.08; $P= 0.0004$) and older children (GM= 20.65; 95% CI= 12.26 to 29.04; $P= 0.0005$) vaccinees. The highest statistically significant LPS-specific IgA responses were found early in adult vaccinees at day 3 with GM= 35.93; 95% CI= 21.09 to 50.77; $P= < 0.0001$.

recipients in the corresponding study days.

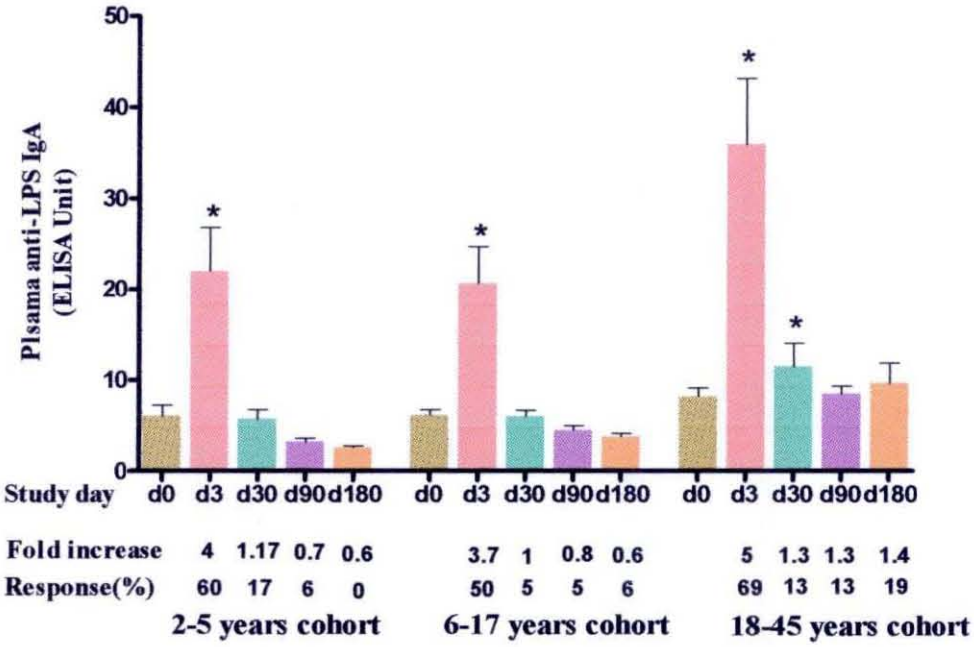


Figure-3.4: Mean normalized plasma LPS-specific IgA antibody responses with (± SEM) standard error bars. The Wilcoxson signed-rank test was used for the analysis of data. An asteric indicates a statistically significant difference ($P<0.05$) from the baseline preimmune response. Mean fold changes and responder frequencies are also listed.

3.4 Anti-LPS IgG responses

Anti-LPS IgG responses in all 3 age groups were lower than anti-CT specific IgG. The response frequencies at day 3 were 19% with 1.6-fold increased antibody titer in younger child vaccinees and in older child vaccinees it was 40% with 2-fold increased antibody titer (Figure 3.5). The highest anti-LPS IgG responses of 3.6-fold increased were found in 56% adult vaccinees.

In case of younger child vaccinees, the significant level of anti-LPS specific IgG responses was found at day 3, and which returned to baseline by day 30. In case of both older child and adult vaccinees, significant level of anti-LPS specific IgG responses were found at 3, which remained elevated up to day 30 before declining to baseline by day 90. No significant difference was observed in CT-specific IgA responses among the different age groups vaccine recipients in the corresponding study days.

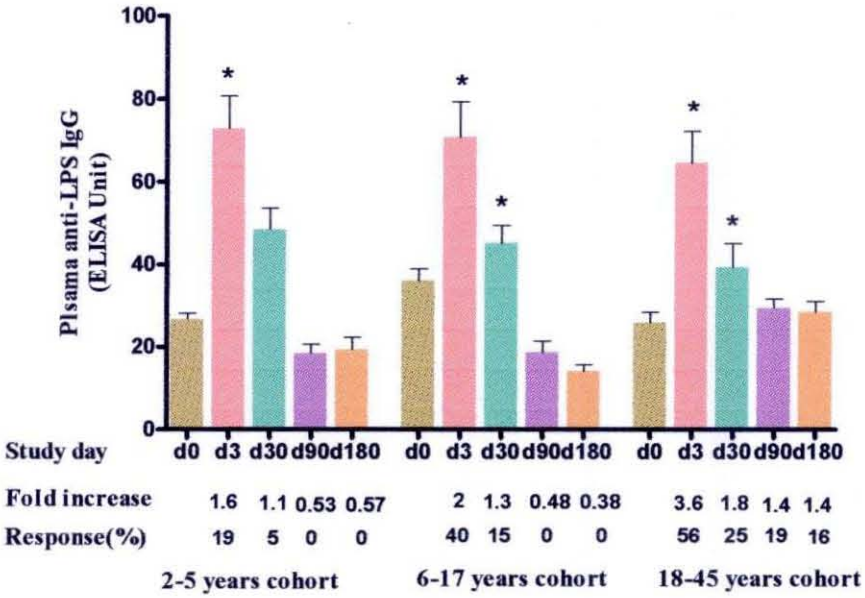


Figure-3.5: Mean normalized plasma LPS-specific IgG antibody responses with (± SEM) standard error bars. The Wilcoxson signed-rank test was used for the analysis of data. An asteric indicates a statistically significant difference ($P<0.05$) from the baseline preimmune response. Mean fold changes and responder frequencies are also listed.

0, 30, 90, and 180. After six day PBMC culture in 24 well culture plates, only those that showed stimulation of PBMC as a ≥4-fold increase in the number of total immunoglobulin memory cells following stimulation, compared to unstimulated cells, were considered for analysis.

4.1 CT-specific IgA and IgG Memory B-cells

Statistically significant increases in anti-CT-specific IgA memory B-cell responses were observed only for adult vaccinees. CT-specific IgA memory B-cell responses increased on day 30 (P =0.001) and persisted up to day 90 (P= 0.0195) and after that these responses returned to baseline by day 180 (Figure 3.6).

In young and older children vaccinees, CT specific IgA memory B-cell responses increased up to day 90. However, there was no significant memory B-cell response for the both younger and older children vaccinees. No significant difference was observed in CtxB-specific IgA memory B-cell responses among the different age groups vaccine recipients in the corresponding study days.

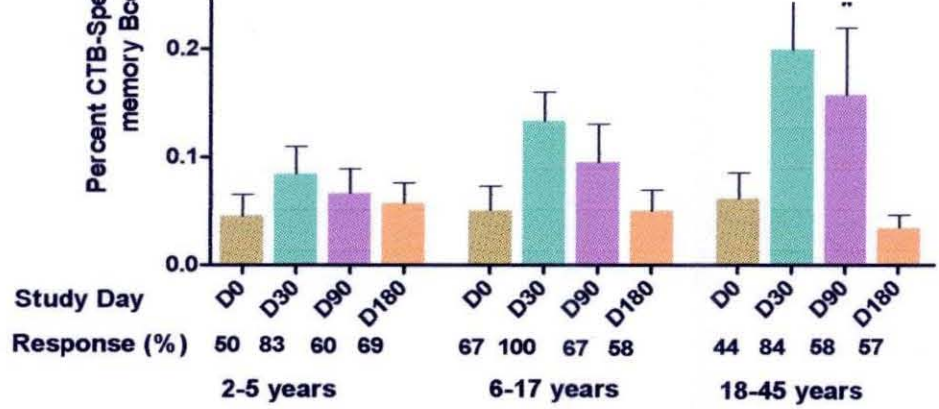


Figure-3.6: Mean CTB-specific IgA memory B-cells responses in different age groups. The columns indicate mean reciprocal end titers; error bars represent the standard errors of the mean. The Wilcoxson signed-rank test was used for the analysis of data. An asteric indicates a statistically significant difference ($P<0.05$) from the baseline preimmune response. Mean fold changes and responder frequencies are also listed.

Similarly, in adult vaccinees significant increases in anti-CT specific IgG memory B-cell responses were observed at day 30 ($P= 0.006$), and which remained elevated up to day 90 ($P= 0.008$) and subsequently these responses returned to baseline by day 180 (Figure 3.7). In case of both younger and older child vaccinees, there was no significant level of CT-specific IgG memory B-cell responses, but the response was tended to be higher at day 30 ($P= 0.0625$).

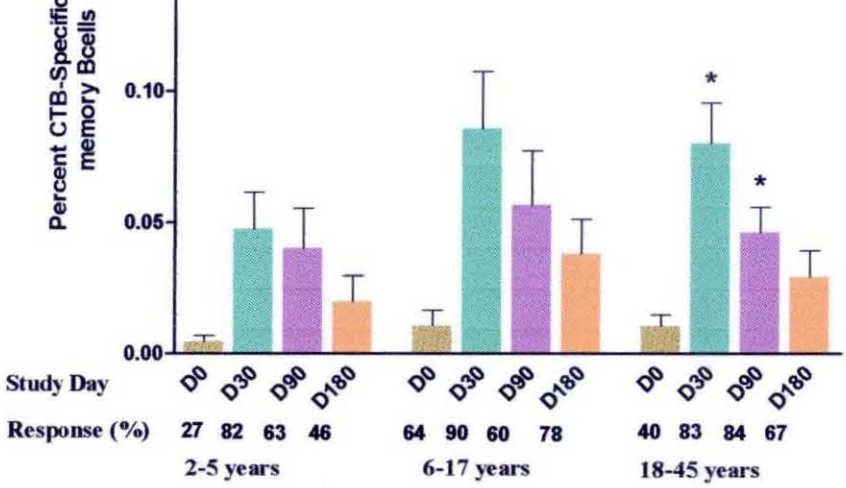


Figure-3.7: Mean CTB-specific IgG memory B-cells responses in different age groups. The columns indicate mean reciprocal end titers; error bars represent the standard errors of the mean. The Wilcoxson signed-rank test was used for the analysis of data. An asteric indicates a statistically significant difference ($P<0.05$) from the baseline preimmune response. Mean fold changes and responder frequencies are also listed.

4.2 LPS-specific IgA and IgG Memory B-cells

LPS-specific memory B-cell responses are shown in figures 3.8 and 3.9. LPS-specific IgA memory B-cell responses were comparatively higher than CT-specific IgA memory B-cell. However, no significant increase of LPS-specific IgA memory B-cell responses was observed in any age group of vaccinees. In addition, there was no difference in LPS-specific IgA memory B-cell responses among vaccinees in any corresponding study days too.

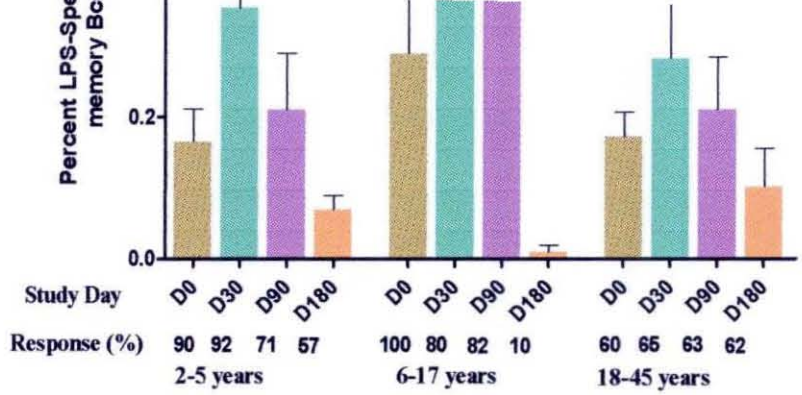


Figure 3.8: Mean LPS-specific IgA memory B-cells responses in different age groups.

LPS-specific IgG memory B-cell response is comparatively lower than CtxB-specific IgG memory B-cell. No significant increase of LPS-specific IgG memory B-cell responses was observed in any age group of vaccines and there was no difference in LPS-specific IgG memory B-cell responses among vaccinees in any corresponding study days too.

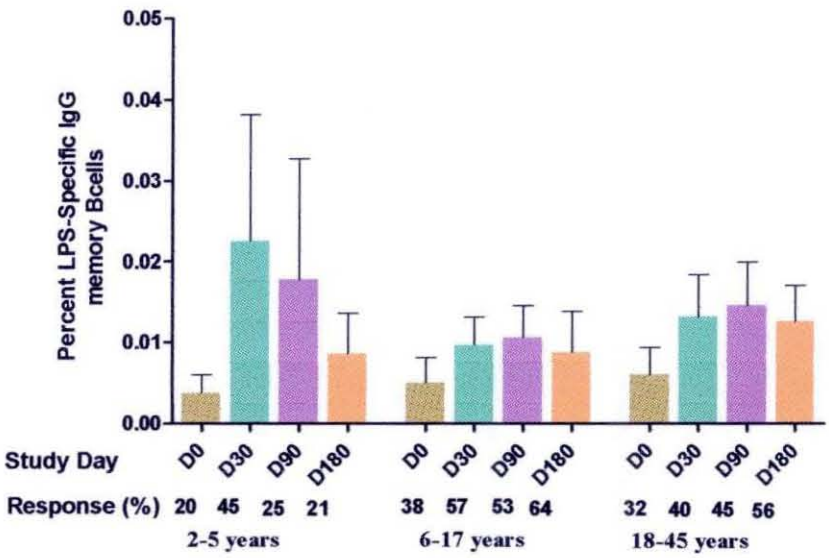


Figure 3.9: Mean LPS-specific IgG memory B-cells responses in different age groups.

CHAPTER FOUR

DISCUSSION

cell killed cholera vaccine now available in many countries including Bangladesh [159]. In addition to recombinant cholera toxin B-subunit, this vaccine also contains inactivated whole-cell cholera vibrios and has been registered in more than 60 countries worldwide [75].

One important observation in immune response against WC-rCTB (Dukoral) is that the level of vaccine-induced protection is age-dependant, being lowest in young children (a population group which is at high risk of cholera), and highest in adults, indicating that vaccines worked best in immunologically-primed populations by boosting underlying immunity. In adults and children above age 5 yr, protection remains at or above 60 per cent for another 2-3 yr. In children below age 5, the vaccine induces very high short-term efficacy (100% for the first 4-6 months) mediated by locally produced IgA antitoxic antibodies, but in this age group protection waned more rapidly than that in older children [160].

The ultimate goal of all vaccination strategy is to generate effective long-term memory. The persistence of memory B cells is of utmost importance for long-term vaccine efficacy. Memory B-cell responses, defined as rapid induction of high levels of high affinity antibody after secondary antigen challenge, are characterized by production of IgG and IgA antibodies, and by somatic mutations in the antigen-binding domains of the heavy and light chains of these antibodies [161]. Current understanding of memory B cell generation suggests that antigens such as CT lead to the activation and proliferation of naive B cells in a CD4 T cell–mediated fashion. Activated B-cells may then differentiate into short-lived, antigen-committed ASCs or undergo a germinal center reaction with CD4 T cell help, where somatic hypermutation, affinity maturation, and class switching generate high-affinity, antigen-specific memory B cells. Memory B-cells can persist for decades in an antigen-independent manner and can be further boosted by subsequent antigenic exposure [103, 162].

recipients. The vaccinees were divided into three age groups; young children (2-5 years), older children (6-17 years), and adults (18-45 years). The memory B-cell responses of these cohorts were also compared against one another.

In this study, a significant level of CT-specific IgA memory B-cell responses was found in adult vaccine participants. The increased responses were observed after one month of vaccination (P =0.001) and persisted up to three months (P= 0.0195) (Fig. 3.6). After that, CT-specific IgA memory B-cell responses returned to baseline by day 180. However, significant level of CT-specific IgA memory B-cell responses were found up to day 270 in naturally infected cholera patients in the earlier study [18]. On the other hand, CT-specific IgA memory B-cell responses in both younger and older child vaccinees were not that significant compared to adult vaccines after one month of vaccination, and these short-lived responses wane down to baseline on the subsequent days (Fig. 3.6). These findings are comparable with child patients naturally infected with *V. cholerae*, as shown recently [163]. Both of these groups of vaccinees and naturally infected patients showed relatively short-lived CT-specific IgA memory B-cell responses.

Moreover, significant level of CT-specific IgG memory B-cell responses was found at day 30 (P= 0.006) in adult vaccine participants, and these responses remained elevated up to three months (P= 0.008) (Fig. 3.7). In case of both younger and older child vaccinees, there was no significant level of CT-specific IgG memory B-cell responses, but the response was tended to be higher at day 30 (P=0.0625) (Fig. 3.7). Present study reflects that the CT-specific IgG memory B-cell responses in adult vaccinees are comparable up to day 180 to those found in the naturally infected adult patients [18]. In older child vaccinees, comparable responses were found to those of naturally infected cholera patients [163]. However, significant differences were observed in the younger children group of vaccinees and cholera patients; naturally infected patients shown to have elevated level of CT-specific IgG memory B-cell responses at day 30 [163] compared to vaccinees.

carbohydrate antigen, responses to LPS is T-cell independent [164]. On the contrary, memory B-cell response to protein antigen (e.g. CT) is dependent on T-cell responses [165]. Cytokine secretion and co-stimulation by CD4+ T-cell are the primary determinants of the quality and duration of memory B-cell responses to protein antigen like CT. The direct binding of T-cell and B- cell in secondary lymphoid tissue facilitates CD40 and CD40L interaction which is critical for B-cell proliferation and isotype switching [164]. Hence, the stability to memory B-cell responses after exposure to CTB, in contrast to T-cell independent antigens such as LPS, is likely the result of T-cell contributions to memory B-cell activation [166].

The vibriocidal antibody response is one of the most commonly used markers for assessing protection against cholera. It is thought to be a surrogate marker of protection since it detects the presence of complement binding anti-*V. cholerae* antibodies in the peripheral blood. However, there is no known vibriocidal titer above which protection is complete [12], and vibriocidal antibody levels fall to baseline within several months after infection [26]. In areas of the world in which cholera is endemic, the mean vibriocidal antibody titer increases with increasing age, presumably reflecting previous exposure to *V. cholerae* [16].

In the present study, it has been found that child vaccinees developed significantly higher magnitude of vibriocidal antibody titers at days 21 and adults at day 17 (Fig. 3.1). In case of both groups of child vaccinees, these responses remained elevated up to day 30, and which returned to baseline by day 90. On the other hand, in adult vaccinees, elevated level of vibriocidal antibody titers were found throughout the study period of 6 months (Fig. 3.1). These results are consistent to earlier studies [18, 163]. Moreover, vibriocidal antibody responses in adults are comparable to the naturally infected cholera patients [18]. In the present study, it was found that young children had the lowest baseline vibriocidal antibody levels, presumably a reflection of the fact that older children and adults might have already been exposed to *V. cholerae* infections. Despite this, the magnitudes of the vibriocidal

In case of CT specific plasma IgA, the level of antibodies significantly increased by day 7, and persisted up to 1 month in both groups of child vaccinees (Fig. 3.2). On the other hand, relatively long-lived CT specific plasma IgG responses were seen in younger child vaccinees than the older children, presumably reflecting that the younger children were recently exposed to *V. cholera* (Fig. 3.3). These responses were even longer in case of adult vaccinees; up to 3 months for IgA (Fig. 3.2) and 6 months for IgG (Fig. 3.3). Significant level of LPS-specific IgA was found only at day 7 in both groups of child vaccinees, whereas in adult it remained elevated up to 1 month (Fig. 3.4). On the other hand, significant increase in LPS-specific IgG was found at day 7 in younger child vaccinees, but it persisted up to day 30 in both older child and adult vaccine participants (Fig. 3.5). These findings support that antibody responses persist for longer period to protein antigens like CT than to non-protein antigens (LPS).

From this study it was shown that administration of oral cholera vaccine developed strong memory B-cell responses in adult vaccine participants. This might be due to the fact that adults are earlier exposed to *V. choleare* and have the efficient and robust immune systems than the children. However, the memory B-cells responses in adult vaccinees diminished after 3 months of vaccination. In older child vaccinees these responses tended to be higher than the younger children, but were not that significant to render immune protection against *V. cholerae*. This present study shows some degree of anamnestic recall, but this protective efficacy rapidly falls after oral cholera vaccination by WC-rBS. [167]. Comparing the present data with the previous data it can be said that vaccine is playing a protective role but it is not as efficient as the protection provided by the natural cholera infection.

APPENDICES

1. Eppendorf tubes and micropipette tips were taken from Eppendorf® and Sigma, and were sterilized by autoclaving at 121°C for 20 minutes.

2. Petri dishes used in the experiments were provided by either Sterilin or Gibco. Screw capped tubes and other glass wares were taken from Pyrex® Labware, USA.

3. Plastic tubes and pipettes were of Falcon®; both were the brands of Becton, Dickinson and Company. 96-well ELISA plates were obtained from Nunc™, Sweden.

4. Micropipettes were from Thermo Labsystems.

5. Mini scale centrifugations were carried out in a Sorvall® *pico* microfuge and large-scale centrifugation were carried out in a Sorvall® Legend™ X*RT* super speed centrifuge. ELISA reading was taken using ASCENT Multiskan® reader.

6. Heparin-coated sterile vacutainer tubes (Becton Dickinson, Ruther-ford, NJ)

7. Multi-channel dispenser (Lab System, USA)

Chemicals/Reagents:

1. Acetic acid, Sigma-758-12-3
2. AEC: Amino ethyl carbazole, Sigma, A-5754
3. BCIP (5-Bromo-4-Chloro-3-Indolyl Phosphate, Para Toludine Salt) – Sigma, B8503
4. DMF (N, N-Dimethyl-Formamide), Sigma, D-8654
5. FBS (Foetal Bovine Serum Albumin), Gibco BRL- 16140-071
6. Ficoll, Pharmacia LKB Biotechnology AB Uppsala, Sweden.
7. Goat anti human IgG $F(ab)_2$, Jackson Immuno Research 109-005-097
8. Goat anti-human IgA-HRP, Southern Biotechnology Associates, Inc. 2050-05
9. Goat anti-human IgG-AP, Southern Biotechnology Associates, Inc. 2040-04
10. H_2O_2 (Hydrogen Peroxide), Fisher Scientific, H-325
11. $MgCl_2$ (Magnesium chloride), Sigma- 7786-30-3

15. NaCl (Sodium Chloride) *Fischer Scientific, Pittsburgh, PA, USA)*

16. KH_2P0_4 (Potassium Phosphate)

APPENDIX B

Buffers and Substrate Solutions

1. Preparation of phosphate buffered saline (PBS) (Vacutainer System; Becton Dickinson, Rutherford, NJ) (pH 7.2)

NaCl (0.136mM Fischer Scientific, Pittsburgh, PA, USA)----------80.00g

Na_2HPO_4---------- 27.50g

KH_2PO_4 ----------2.75 g

KCL (Fischer Scientific, Pittsburgh, PA, USA)----------2.00 g

Deionized water ----------1000.0 ml

The concentrated solution (10xPBS) was diluted ten times and was used as working solution.

2. Preparation of 0.05% PBS-Tween (1000ml)

Tween ----------0.5ml

PBS ----------1000ml

3. Preparation of 1% BSA in PBS (500 ml)

Phosphate Buffer Saline (PBS) ----------500 ml

Bovine Serum Albumin (BSA) ----------5 g

4. Preparation of 1% BSA in PBS-Tween (500 ml)

Phosphate Buffer Saline (PBS) ----------500 ml

Bovine Serum Albumin (BSA) ----------0.5 g

Tween ----------250 µl

Phosphate Buffer Saline (PBS) ----------500 ml

Bovine Serum Albumin (BSA) ----------0.5 g

Tween----------250 µl

7. Preparation of Sodium citrate buffer (pH 4.5) (1000ml)

Tri-natrium citrate ($Na_3C_6H_5O_4.2H_2O$) ----------29.4 g

H_2O (deionized) ----------1000.0 ml

8. Preparation of Orthophenylene diamine - H_2O_2 substrate (10 ml)

OPD----------10.0 mg

0.1 M sodium citrate (pH 4.5) ----------10.0 ml

30% H_2O_2 ----------4.0 µl

9. Preparation of 1% FBS in PBS-Tween (500 ml)

Phosphate Buffer Saline (PBS) ----------500ml

Fetal Bovine Serum (FBS) ----------5.0 ml

Tween ----------250 µl

10. Preparation of BCIP (4ml)

DMF----------4ml

BCIP----------60mg

11. Preparation of NBT (4ml)

70% DMF----------4ml

NBT----------120mg

Deionized water ----------1000ml

13. Preparation of ALP chromogen/substrate: BCIP/NBT 50ml

BCIP (60 mg/4 ml 100% DMF) ----------0.5 ml

NBT (120 mg/4 ml 70% DMF

[2.8ml DMF+1.2ml dH_2O])----------0.5 ml

Carbonate Buffer ----------50 ml

14. Preparation of HRP chromogen /substrate: AEC/H_2O_2 10ml

AEC ----------10 mg

Dimethyl formamide (DMF) ----------1 ml

The mixture is then dissolved in 30mL of 0.1M sodium acetate buffer (16.4 g of 0.2M Na-acetate dissolved in 1000 ml of distilled water, and then the pH was adjusted to 5.0 with 0.2 M acetic acid). The AEC solution was filtered through 0.2µm Millipore filter and stored in a 50 ml plastic tube wrapped with aluminium foil. Stable at 4° C for a week.

15. Preparation of tryphan blue (100ml)

NaCl ----------0.81g

KH_2PO_4 ----------0.06 g

Tryphan blue----------0.4g

Deionized water----------100.0ml

APPENDIX C

Media

1. Preparation of RPMI complete medium (220 ml)

RPMI 1640 (1X) ----------200 ml

Fetal Bovine Serum (FBS- 10%) ----------20 ml

2. Preparation of RPMI–MBCS (Basic Culture Media) (220 ml)

RPMI 1640 (1X) ----------200 ml

Fetal Bovine Serum (FBS- 10%) ----------20 ml

Pcn/Strp (Penicillin-Streptomycin- 2%) ----------4ml

L- Glutamine (1%) ----------2ml

β- mercaptoethanol (1 : 1000 dilution) ---------- 20µl

3. Preparation of Stimulation media (20 ml)

Basic Culture Media ----------20 ml

Pokeweed mitogen (PWM – 1%) ----------20 µl

Staphylococcus aureus Cowan (SAC – 0.1%) ----------2.0µl

CpG (6%) ----------120.0µl

4. Preparation of BHI media (1000ml)

BHI----------37.0g

Deionized water----------1000ml

5.Preparation of TTGA culture media

Composition of TTGA:

Reagent	**Amount**
Trypticase (Casein) (BD) ……..….	10 g
Nacl (BDH/MERCK)……..	10 g
Sodium taurocholate (Sigma)……..	5g
Sodium carbonate (Sigma)……	1.2-1.5g
Gelatin (BDH) …….	30g
Agar (BD) ……	16g

Before pouring the plates coal about 50 C, add potassium tellurite (K_2TeO3)(5ug/ml).

APPENDIX D

Softwares:

1. **MS word**
2. **Microsoft Excell**
3. **GraphPad Prisom** (Graph PAD Software, 2236 Avenida de la Playa, La Jolla, CA 92037, USA)
4. **Endnote X**

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