'O antigenic' Polysaccharide Specific Memory B Cell Responses in Bangladeshi Cholera Patients Infected with *Vibrio cholerae* O1

A DISSERTATION SUBMITTED TO THE BRAC UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIRMENTS FOR THE DEGREE of MASTERS OF SCIENCE IN BIOTECHNOLOGY

Submitted By

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Dedicated To

My Beloved

Parents

TO WHOM IT MAY CONCERN

This is to declare that the research work embodying the results reported in this thesis entitled "'O antigenic' polysaccharide specific Memory B cell responses in Bangladeshi cholera patients infected with *Vibrio cholerae* O1" submitted by Md. Israk Nur Sami, has been carried out by the under signed joint supervision Professor Dr. Naiyyum Choudhury, Co-ordinator, Biotechnology and Microbiology program, Department of Mathematics and Natural Sciences, BRAC University and Professor Dr. Firdausi Qadri, Senior Scientist and Head in the Immunology Laboratory of the Centre for Vaccine Sciences at the International Centre for Diarrheal Disease Research, Bangladesh (icddr,b). It is further declared that the research work presented here is original and submitted in the partial fulfillment of the degree of Masters of Science in Biotechnology, BRAC University, Dhaka and has not be submitted anywhere else for a degree or diploma.

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ABSTRACT

Cholera, caused by *Vibrio cholerae* O1/O139, is an acute dehydrating enteric disease with high mortality rate if untreated. Several efforts have been made to develop effective vaccines, and effort is still continuing. The available oral cholera vaccines provide protection for 3-5 years in adult and for shorter duration in younger children. However, it was observed that natural infections in cholera endemic areas provide longer term protective immunity lasting for 7-10 years, but the mechanism is not completely understood. It was hypothesized that this protective immunity may be mediated by memory B cell responses. In this study 60 hospitalized cholera culture confirmed patients were evaluated for their immune response up to 180 days after onset of illness. A method of polyclonal stimulation of peripheral blood mononuclear cells followed by an enzyme-linked immunospot assay was used to study memory B cell responses specific to *V. cholerae* O1 antigens, lipopolysaccharide (LPS) and O-specific polyscaccharide (OSP) of LPS. To observe circulating plasma antibody responses enzyme-linked immuno assay accompanied with memory B cell studies were carried out at different study points. All patients exhibited LPS and OSP-specific memory B cell proliferation by day 30. LPS-specific IgG and IgM memory B cell responses were elevated throughout the follow up days, while LPS IgA response magnitude waned down by day 90, but persisted up to day 180. However, OSP-specific IgA, IgG and IgM memory B cell response were found at day 30. The OSP-specific IgA and IgG memory B cell responses were elevated throughout the whole study period, while IgM responses started to wane down by day 180. Significant level of LPS and OSP-specific circulating plasma antibody for all the antibody isotypes were observed. Results suggested that such memory B cells might persist for longer period providing protective immunity against subsequent exposure to cholera. A vaccine, targeted to generate memory B cell, preferably OSP-specific will be important for evaluating effectiveness of vaccines and for design and formulation of future effective cholera vaccines.

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Abbreviations

ADP	Adenosine Diphosphate
AEC	3-Amino 9-Ethyl Carbazole
ALS	Antibody in Lymphocyte Supernatant
APCs	Antigen Presenting Cells
ASC	Antibody Secreting Cell
BCIP/NBT	5-Bromo 4-Chloro 3-Indolyl Phosphate/Nitroblue Tetrazolium
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CI	Confidence Interval
CT	Cholera Toxin
CTB	Cholera Toxin B Subunit
ELISA	Enzyme Linked Immunosorbant Assay
ELISPOT	Enzyme Linked Immunospot
ERC	Ethical Review Committee
g	Gram
GALT	Gut Associated Lymphoid Tissue
GM	Geometric Mean
GM_1	Monosialosyl Ganglioside
GTP	Guanosine Tri-Phosphate
HRP	Horse-Radish Peroxidase
ICDDR, B	International Centre For Diarrheal Disease Research, Bangladesh
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
Kda	Kilo Dalton
KLH	Keyhole Limpet Hemocyanin

LPS	Lipopolysaccharide
LSD	Laboratory Sciences Division
LT	Leukotrienes
MHC	Major Histocompatibity Complex
mM	Milli Molar
MMC	Mucosal Mast Cells
MW	Molecular Weight
NAD	Nicotinamide Adenine Dinucleotide
OD	Optical Density
OPD	Ortho Phenylene Diamine
ORS	Oral Rehydration Solution
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-Buffered Saline
PMN	Polymorphonuclear Neutrophil
PWM	Pokeweed Mitogen
RBC	Red Blood Cell
rBS	Recombinant B Subunit Of Cholera
Rpm	Rotation Per Minute
RRC	Research Review Committee
SEM	Standard Error Of Mean
SHM	Somatic Hypermutation
Tc	Cytotoxic T Cell
Tcp-A	Toxin Coregulated Pilus A
TCR	T-Cell Antigen Receptor
Th	T-Helper Cell
TNF	Tumor Necrosis Factor
WBC	White Blood Cell
WHO	World Health Organization

Chapter 1

Introduction

1.1. Background

Cholera, an ancient and devastating diarrheal illness continues to be a primary cause of morbidity and mortality in developing countries like Bangladesh (1, 2). It is the second leading cause of death among children's under five years of age globally. Approximate incidence of cholera is 3-5 million per year in Bangladesh. Cholera is an infection of intestine caused by ingestion of food or water containing *Vibrio cholerae*, gram negative bacteria resulting in acute secretory diarrhea (3, 4). Symptoms include loose or watery stools more frequently than normal for a healthy individual and vomiting which can result in severe dehydration or water loss. When left untreated, death can occur rapidly sometimes within hours. Cholera is transmitted through contaminated food or water and person to person contact through fecal-oral route (5). Although person to person direct transmission lacks proof, *Vibrio cholerae* from patient stool is hyper infectious (9).

The serogroup of the causative agent is differentiated by the O-specific polysaccharide (OSP) portion of *V. cholerae* lipopolysaccharide (LPS) in the outer membrane of the bacterium. Among characterized more than 200 serogroups, only serogroups O1 and O139 are known to cause epidemic cholera (6). Natural infection by *V. cholerae* provides serogroup specific long term protection against subsequent disease, however the gold standard marker for this protection remain unknown (7). Long term protection refers to antibody production due to memory B cell response to subsequent exposure to the antigen OSP or LPS (8). Memory B cells response towards OSP still needs investigation vastly which may play crucial role in vaccine development.

1.2. Cholera and clinical features

Being diarrhea a bacterial disease, *Vibrio cholerae* is associated with most severe case of cholera with up to 30 liter of fluid loss per day with death rate of 30% (10). The sudden onset of profuse, watery stools with occasional vomiting is characterized as cholera (11). Incubation period is usually 2–5 days but may be only a few hours. In severe cases about 5–10% of infected, dehydration, metabolic acidosis, and circulatory collapse may rapidly develop. If left untreated, over 50% of the most

severe cases patients may die within several hours. Proper treatment may reduce mortality to less than 1% (12). In its 18 h to 5 d incubation period, it may also be associated with muscle cramps, and complications related to dehydration and metabolic acidosis. In its extreme manifestation, within 3-4 h of the onset of symptoms, a healthy person may become hypotensive and may die within 6-8 h. More commonly fatal cases progress to shock within 6-12 h with death (1).

1.3. Salient features of *Vibrio cholerae*

The *Vibrio cholerae* is a gram-negative rod from the 'Vibrionaceae' family, characterized by single polar flagellum, because of which they are highly motile. The organism grows best in saline condition, although it can grow in low salinity when it is warm and contains sufficient nutrients, as found in coastal water and estuaries, associated with zooplankton and shellfish in water. It uses chitin as a carbon and nitrogen sources, which induces natural competence in *V. cholerae*, suggesting that lateral gene transfer occurs in water, especially during zooplankton blooms. In water, *V. cholerae* enters a viable yet a nonculturable form, also called conditionally viable environmental cells (1, 4).

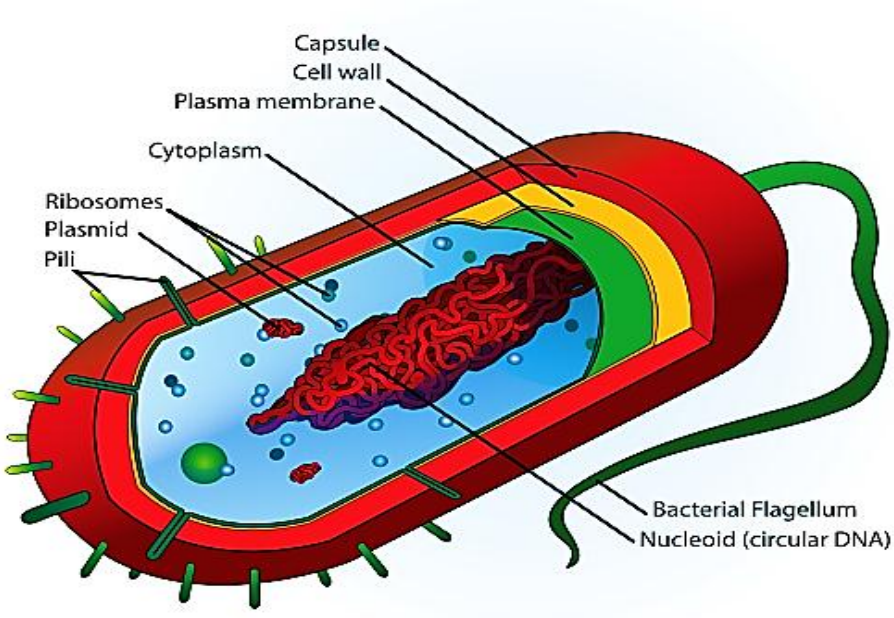


Figure 1.1: structure of *Vibrio cholerae*
http://www.biologyguide.net/biol1/3b_exchange.htm

V. cholerae is a facultative anaerobe, non-spore-forming curved rod, about 1.4–2.6 mm long, capable of respiratory and fermentative metabolism; it is well defined biochemically and in DNA homology. The bacterium is oxidase positive and nitrate reducer with sheathed flagellum. 1% sodium chloride (NaCl) can stimulate growth of it.

However, the ability of *V. cholerae* to grow in nutrient broth without added NaCl made it distinct from other *Vibrio* spp (14). It grows in alkaline conditions up to a maximum of pH 10 but is inhibited when the pH drops to 6 or below. *V. cholerae* requires 5-15mM Na+ for optimum growth (15).

1.3.1. Classification of *Vibrio cholerae*

Vibrio cholerae has been serogrouped on the basis of 'O' antigen of lipopolysaccharide and classified into more than 200 serogroups. Among these serogroups O1 and O139 were known to be the cause of cholera in epidemic form (1, 4). Serotype O1 again is subdivided into two major serotypes Inaba and Ogawa, and two biotypes El tor and Classical. The prevalence of biotype varies with time (4, 16). Presence of a 2-O-methyl group in the non-reducing terminal sugar of the Ogawa OSP, that is absent in Inaba OSP differentiate between the two major serotypes Ogawa and Inaba. Ogawa serotype expresses antigen A, B, with small amount of C while Inaba expresses A and C (17, 18). Till date among the 7 pandemics, 6 occurred by Classical biotype but the 7th and the last which caused by El Tor. There is also another unstable and rare serotype Hikojima the third (4).

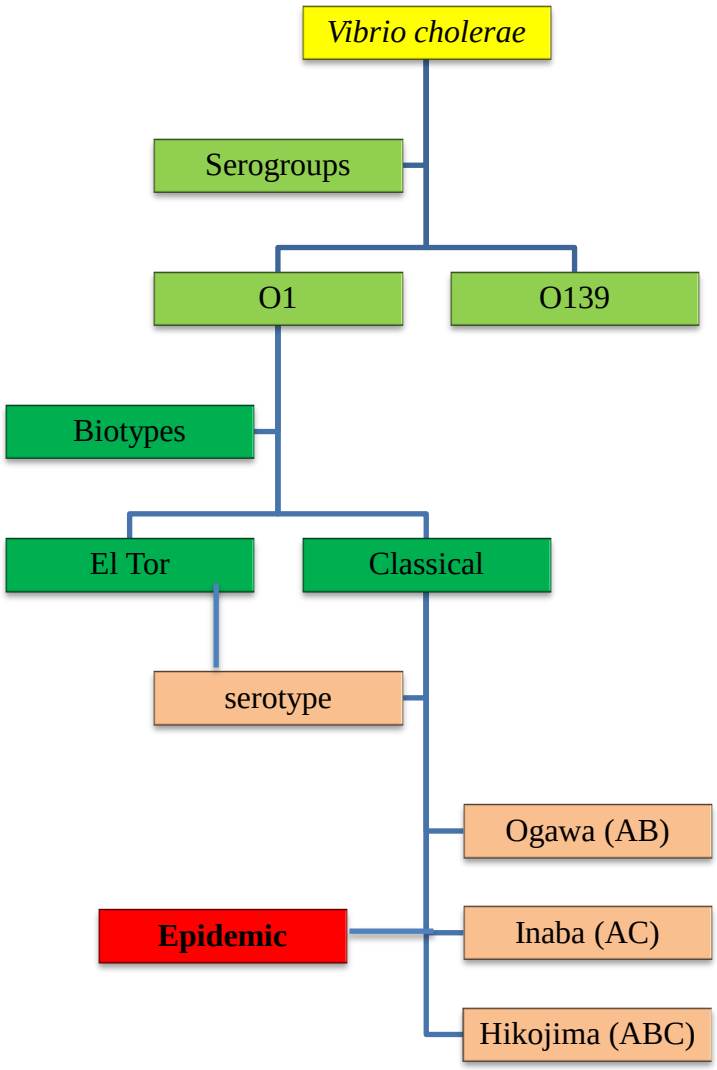


Figure 1.2: Schematic presentation of *Vibrio cholerae* classification.

1.3.2. Cholera Toxin

Cholera toxin (CT) is the virulence factor responsible for the massive secretory diarrhea seen in dehydrating cholera (19). Robert Koch was first to suggest CT as cholera causative agent in 1884 (20). The discovery of the three-dimensional structure of CT enhanced our understanding of this remarkable toxin. The structure of CT is shown in Figure 3. CT consists of two types of subunits heterodimeric A & homopentameric B. The larger A subunit (240 amino acids; MW 28 kD) forms the center, while the five B subunits (103 amino acids; MW 11 kD each; aggregate MW ~56 kD) are located peripherally. Identical B (green, blue, purple, white, red, pink) monomers are arranged in a ring-like configuration with each a single binding site for

the plasma membrane receptor of the jejunal intestinal epithelial cells, the monosialoganglioside GM_1. The A subunit (yellow) consists of two distinct polypeptide chains tide chains CT-A_1 (MW~22,000 and CT-A_2 (MW~5,400) linked by a single disulfide bridge.

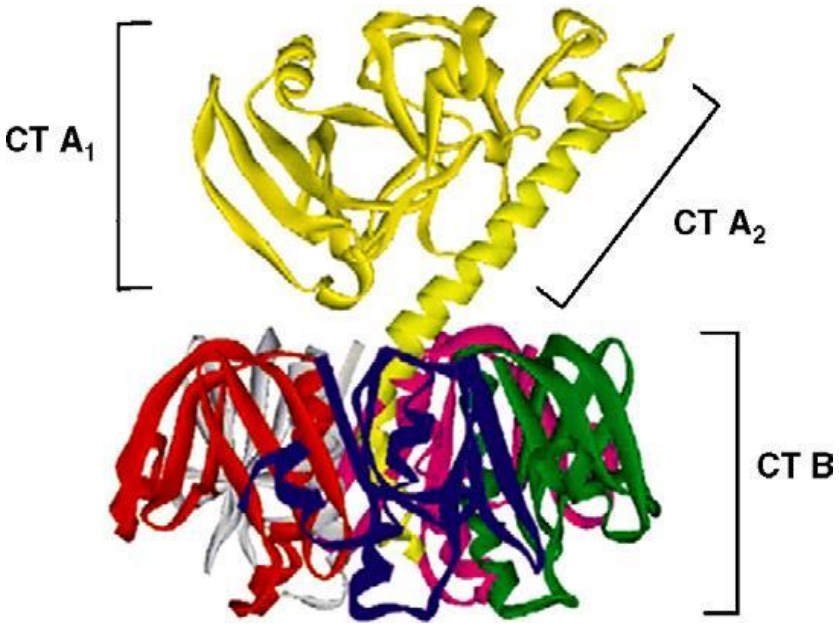


Figure 1.3: Three dimensional structure of Cholera Toxin (CT) (22)

The CT-A_2 M polypeptide links and occupies the central channel and goes through the doughnut-like structure of the CT-B pentamer, tethering CT-A and CT-B subunits (19, 22). The A subunit of CT has over 82 per cent sequence identity with the *Escherichia coli* heat-labile enterotoxin (LT), while the B subunits of the former shares over 83 per cent sequence identity with the latter (21).

1.3.3. Lipopolysaccharide (LPS) and O-Specific Polysaccharide (OSP)

Lipopolysaccharides (LPS) are endotoxins invariably associated with gram-negative bacteria irrespective of pathogenicity. In bacteriology the term endotoxin refers lipopolysaccharide complex on the outer membrane of gram-negative pathogens, for example: *Escherichia coli*, salmonella, shigella etc. LPS participate in the physiological functions (24). Like all Gram-negative bacteria, LPS of *Vibrio cholerae* polysaccharide attached to a lipid component, named lipid A. The polysaccharide component is made of a core and a polymer of oligosaccharide molecules (O-antigen), which is immensely variable and specific to each bacterial strain. A sugar 2-keto-3-deoxyoctulosonic acid (KDO) bounds the polysaccharide to lipid A. Lipid A is a peculiar bacterial lipid consisting of a phosphorylated β-1, 6-linked glucosamine disaccharide, to which long fatty acids are attached. Expression of cytokines by LPS is assumed to be induced by Lipid A (25).

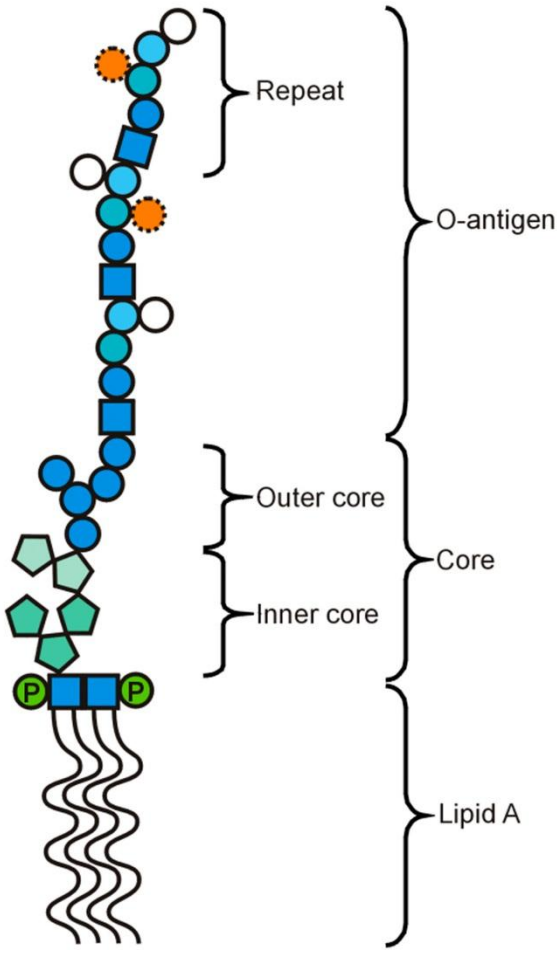


Figure 1.4: Schematic Lipopolysaccharides http://www.beilstein-institut.de/glycobioinf2O11/Proceedings/Stenutz/Stenutz.html

LPS toxicity is associated with the lipid component (Lipid A) while immunogenicity is associated with the OSP components. LPS elicits a variety of inflammatory responses in an animal and it activates complement by the alternative (properdin) pathway, so it may be a part of the pathology of gram-negative bacterial infections (23).

Toxicity is associated with lipid A component and that is why holo LPS antigen are not normally used as components of parenteral and injectable vaccines while the antigenic OSP on LPS is a virulence factor at the same time an important protective

agent of *Vibrio cholerae* and many other bacteria. LPS can be detoxified in many ways such as: mild hydrolysis with dilute acetic acid, separating the OSP core antigen from the Lipid A component (26). Small changes in LPS side chain sugar molecule result in major change in virulence while the O antigenic polysaccharide probably allows the organism to adhere to specific tissues in the mucoal surface. Smooth variants of bacteria probably allow resistance to phagocytosis, whereas rough mutants are more readily engulfed and destroyed by phagocytes. The 'O' antigens could provide protection from damaging reactions with antibody and complement that makes it an important target for vaccine development.

O antigen is the basis of antigenic variation among many important Gram-negative pathogens-including *Vibrio cholerae,* which guarantees the existence of multiple serotypes of the bacterium, so it offers multiple opportunities to infect its host if it can bypass the immune response against a different serotype (23).

1.3.4. Pathogenesis of *Vibrio cholerae*

Pathogenesis of *Vibrio cholerae* is a complex process (27). Most ingested bacteria are killed by gastric acid. The infectious dose of *Vibrio cholerae* with water is 10^3 -10^6 and with food is 10^2-10^4. Organisms those can survive colonize the small intestine and secret Cholera Toxin (CT) the major virulence factor for cholera. There are a number of virulence genes encoding factor which aids the pathogens to reach the epithelium of small intestine, colonizes and produces enterotoxin. In addition to CT, pathogenicity of *Vibrio cholerae* depends primarily on the expression of a pilus colonization factor known as toxin co-regulated pilus (TCP), under same genetic control as CT (28). The action mechanism of cholera toxin (Figure: 5) that leads to acute diarrhea or cholera schematically described below:

1. CT binds with high affinity GM_1 receptor and actively gets internalized by host cell machinery into the early and recycling endosomes.

2. It is transported as holotoxin in a retrograde manner to the Golgi and further to ER, where CT dissociates into CT-A_1 and CT-A_1/CT-B driven by PDI, that is also responsible for unfolding of CT-A_1.

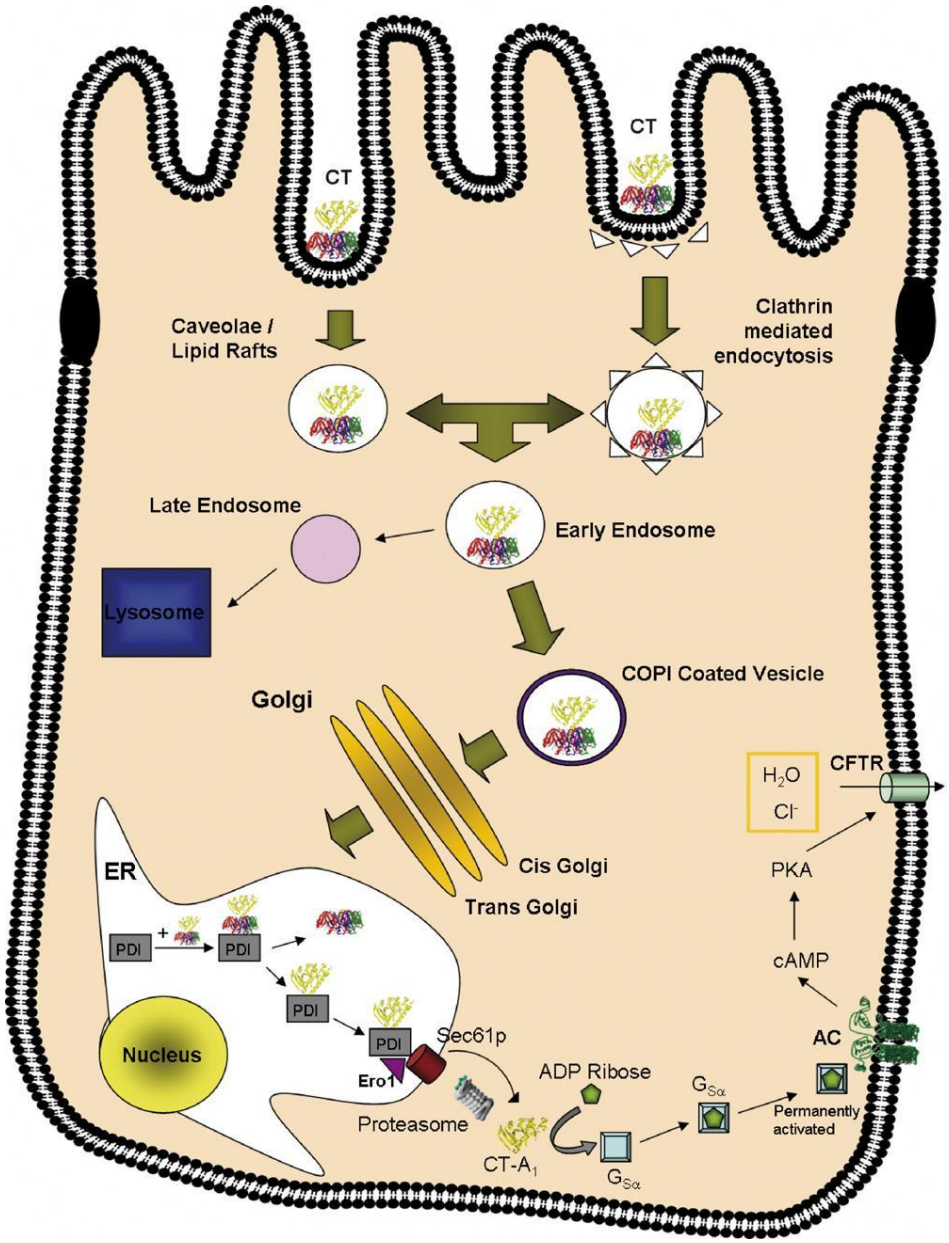


Figure 1.5: Cholera toxin mode of action (22)

3. The CT-A_1 is further translocated via sec61 complex to the cytosol, where it associates with basolateral (Adenylate Cyclase) AC avoiding proteasomal degradation.
4. Mono-ADP-ribose get transported from NAD^+ to GS_α constitutively triggers the AC, resulting in substantial increase in intracellular concentration of cAMP, followed by a protein kinase (PKA)-mediated phosphorylation of the major

chloride channel of intestinal epithelial cells. This leads to increased Cl^- secretion, which is accompanied by osmotic movement of a large quantity of water into the intestinal lumen, resulting in severe diarrhea (22).

1.4. Transmission

Generally cholera infection is caused and transmitted by contaminated food and water. In developing countries the lack of proper sanitary facility is counted as a major reason of infection. In human stool *Vibrio cholerae* might be present as individual planktonic form or as biofilm aggregate (9, 29). This converts *Vibrio cholerae* to conditionally viable state in environment (30).

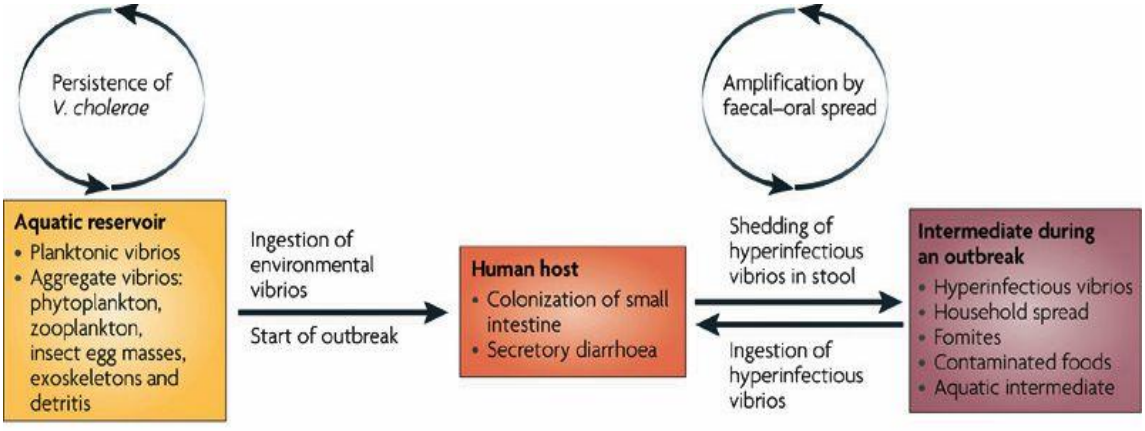


Figure 1.6: Transmission of Cholera (31)

1.5. Susceptibility and Risk Factors

Previous studies suggested individual's susceptibility to cholera including other diarrheal diseases is influenced by their blood types. People with AB blood type are most resistant while with O blood is most susceptible. Among these two extremes blood types, type A are more resistant than type B (32, 33). Typically 10^3 -10^6 is infectious dosage for cholera onset for healthy adult but people with lack of immunity and nutrition, gastric acidity is more susceptible (4, 34). Absence of several host factors is also responsible for increased risk of cholera (33, 35). Cholera outbreak also depends on three major factors, those are: contamination of source of water, rainfall and natural disaster (36, 37).

1.6. Epidemiology of cholera

1.6.1. Global Epidemiology

Cholera is known as both endemic and pandemic disease (4). There are records suggesting that it affected people's health for decades. Human kind already faced 7 major pandemics in last two centuries (1, 4). Cholera is endemic in many areas of Asia and Africa. Devastating cholera occured in Angola, Ethiopia, Zimbabwe, Pakistan, Somalia, Sudan, Vietnam, and Haiti. Cholera can be highly fatal in epidemic form irrespective of age groups, i.e. neonates to old are in equal risk (40). Among the seven devastating epidemics first five were reported to be originated from Bangladesh by classical O1 *Vibrio cholerae* biotype, but the last and seventh one reported was caused by O1 El Tor (1). Cholera outbreak causes estimated at 120,000 deaths annually worldwide and many more each year, among these the vast majority of affected are children. The cholera case reports increased by 16 percent over the previous year in 2009, as reported by WHO (41). WHO reported an estimation of cholera cases of about 500000 to 700000 in year 2O11. But this estimation was considered as significantly underestimated (43). Cholera epidemics occur in long cycle being superimposed on existing endemic disease that leads to declining level of immunity from a previous outbreak (4).

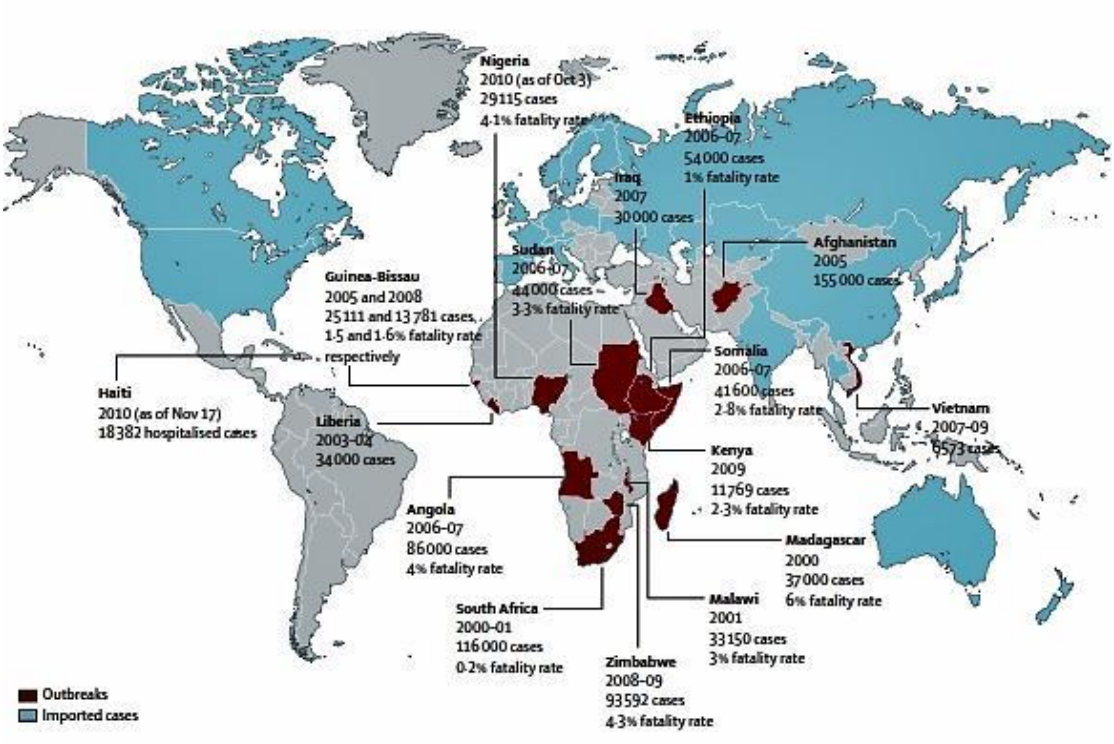


Figure 1.7: Global outbreak of cholera since 2000 (37)

1.6.2. Epidemiology of cholera in Bangladesh

As Bangladesh is a developing country, cholera is an important public health concern due to lack of basic infrastructure with regard of safe water access and proper sanitation. Cholera continues to be a threat and a challenge to the growing population living in unsanitary condition such as shanty towns and refugee or displaced population camp (3). In Bangladesh cholera attacks show seasonality. There are two peaks every year, before the warm season and after the monsoon rains (42). Cholera infection strain in Bangladesh varied time to time. Until 1970, more than 90% cholera was caused by classical ogawa type, but in 1992 *Vibrio cholerae* O139 emerged and caused epidemic in the following year 1993 in southern area of Bangladesh including India and neighboring countries and spread to the other parts of the country (44). In 1992, Approximately 220,000 cases were reported in a 12-week period, with over 8,000 deaths (45).

1.7. Prevention and Control

Prevention of cholera is simply a practice of healthy living with hygiene. And control of cholera refers to rapid action against any cholera outbreak at any place with proper medical facilities and supplies for reducing death rates. Vaccination might be most effective prevention and control over this cholera nightmare, although more investigation in this field yet to be done.

Household practices that help to prevent Cholera to a great extents, are as follows:

- Firstly, drinking pure water or boiled tube well water or after treating water with sterilizing tablets or chlorine or iodine.
- Secondly, one should eat freshly prepared or cooked foods, taking green vegetables is good to health. Any kind of food must be cleaned thoroughly before eating.
- Finally and most important, one should develop a regular practice of good hygiene (46).

1.7.1. Oral Rehydration Solution Therapy

Cholera is a disease in which rapid fluid and salt loss occur weakening the patient and thus can lead to death. Rapid restoration of fluid with salts is a common treatment for any patient and also effective in cases of cholera outbreak. Medically patients are treated with oral rehydration solution (ORS) a package mixture of salts and sugars, which is drunk in a large amount mixing with water. This treatment is accepted

Figure 1.8: Oral rehydration Salts

worldwide in cases of diarrhea and cholera but in cases where patients are severely dehydrated they must be treated with intra venous fluids (IVF) (47).

1.7.2. Antibiotic therapy

Although antibiotic therapy is not a part of emergency treatment but it is a good addition to reduce duration of diarrhea and period of *Vibrio cholerae* excretion with proper rehydration treatments. But *Vibrio cholerae* is getting resistant to available antibiotics like: tetracycline, erythromycin, cotrimoxazole. More effective antibiotics include: azathromycin, doxycycline, chloramphenicol and furazolidone (48).

1.7.3. Vaccination

An effective vaccine with proper vaccination program would be very effective in prevention of infectious Cholera and reduction of morbidity and mortality rates. The available vaccines in present days are not effective for long term protection against cholera (47).

There are two major types of vaccines available now. They are:

1. Killed WC-based vaccines and
2. Genetically attenuated live vaccines

There are two licensed commercial oral cholera vaccines:

a. **WC-rBS (Dukoral):** Killed whole cells of *V. cholerae* O1 (Inaba and Ogawa, classical and El Tor) plus recombinant cholera toxin B subunit.
b. **Modified WC-only vaccines (mORCVAX and Shanchol):** Killed whole cells of *V. cholerae* O1 (Inaba and Ogawa, classical and El Tor) and O139 (bivalent).

1.8. Immunity

Having the state of sufficient biological defenses to avoid infection, disease, or other unwanted biological invasion is immunity. Both specific and non-specific components include immunological components. The non-specific components act either as barriers or as eliminators of pathogens irrespective of antigenic specificity while, other components of the immune system adapt themselves to fight each new disease encountered by generating pathogen-specific immunity known as adaptive immunity. Immunity to any kind can be divided into two categories, which are: Innate immunity and Adaptive immunity.

1.8.1. Innate immunity

Innate immunity is natural and first line of defense mechanism in human body that is achieved at birth. When encountered with new pathogen response occurs in three phases (Figure: 9). First phase is where some preformed soluble molecules (antimicrobial enzymes, peptides and a complement system of plasma proteins) present in blood, extracellular fluids and epithelial secretions kill or weaken effect of pathogen by cell lysis or lyse the bacterial cell membrane. And in the second phase innate immunity components sense the presence of pathogen recognizing molecules those are not shared by host cell but of typical of a microbe and eliminate the infection by effector cells (76).

Cellular components include:

- **Neutrophils and macrophase:** Identify microbes and ingest them for intracellular digestion.
- **Dendritic Cells:** Produce cytokines important in recruitment of leukocytes, one of many bridges between innate and adaptive immune systems.
- **Natural Killer Cells:** Effector cells of innate immunity. They recognize infected cells and then kill them.
- **Other Classes of Lymphocytes:** Like other lymphocytes, their antigen receptors are somatically rearranged (like B and T cells). However, in contrast to classical

lymphocytes, they have limited diversity. Examples include γδ T cells and B-1 cells.

Humoral Components

- **Complement System:** A group of proteins that defend the body against microbe.
- **Cytokines of Innate Immunity:** Products of dendritic cells, macrophase and other cells that act as mediator for cellular component of innate immunity. Examples include TNF, Interleukin-1 and IFN-γ.
- **Plasma Proteins of Innate Immunity**: Acute phase proteins, for example C-reactive protein and surfactant.

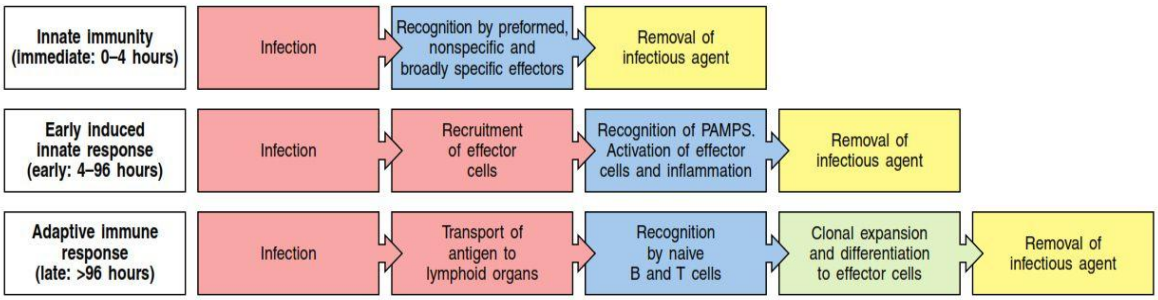


Figure 1.9: innate immunity and progression to adaptive immunity (76)

1.8.2. Adaptive immunity

If pathogen passes previously described two lines of defenses, the third phase takes the action by generating antigen specific lymphocytes that target the pathogen specifically to form long lasting memory cells (Figure: 9) (76).

Adaptive immune system components are normally silent; however, when activated, they "adapt" to the presence of infectious agents by activating, proliferating, and creating potent mechanisms for neutralizing or eliminating the microbes. Adaptive immune responses are classified in two groups: **Humoral immunity**, mediated by antibodies produced by B lymphocytes, and **Cell-mediated immunity**, mediated by T lymphocytes.

Two major groups of cells are important for effective immune response, T-lymphocytes and Antigen presenting cell. Immune response is generated when

Antigen Presenting Cell (APC) represent the antigen to helper T-lymphocytes (Th) and signals B-cells to release antigen specific antibody as response. This process can generate both humoral and cell mediated immune response.

1.8.2.1. Antigen presenting cells (APC)

APCs trap the processed antigens (may be bacteria or bacterial part) from the invading organism on surface groove of Major Histocompatibility complex (MHC) class-II. This makes the antigen identifiable to the Th-cells (Figure: 10) (49).

1.8.2.2. T-lymphocytes

T-lymphocytes arise from bone marrow and migrate to thymus. There are two major subpopulations of T-lymphocytes: T-helper (Th) and T-cytotoxic (Tc) cells. Th-cells are termed as "middleman" of adaptive immunity. Th-cells signals B-cells when identifies the antigen in MHC Class-II groove by T Cell Receptor (TCR). T-cells also release cytokines (IL-2 and IL-4, 5, 6 or IFNγ) which stimulates B-cells to proliferate and differentiate into Antibody Secreting Cells (ASC) (49, 50).

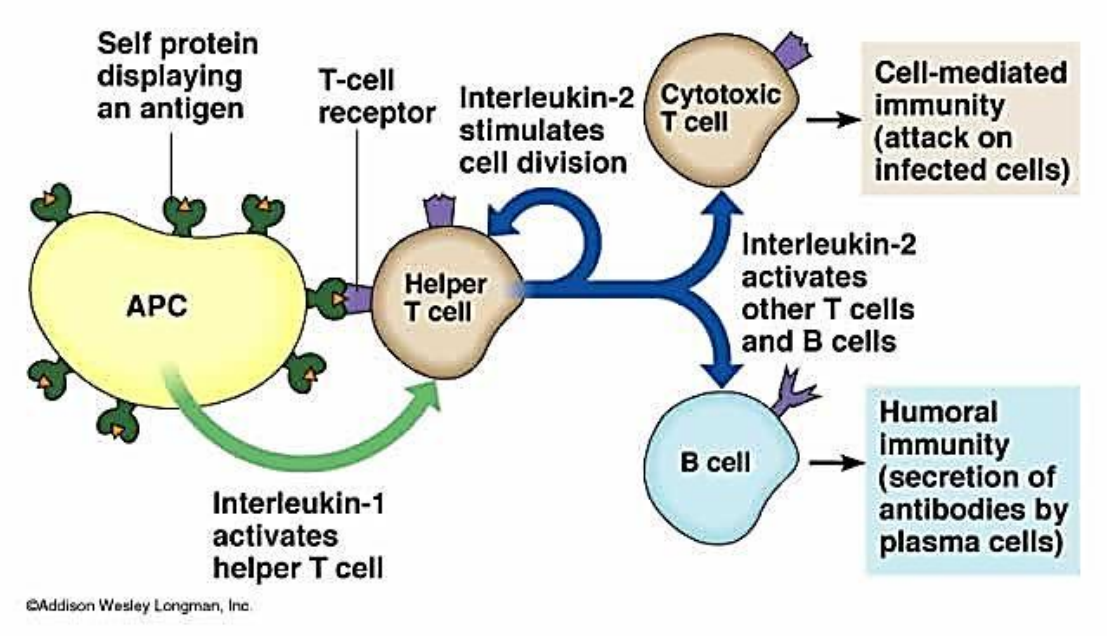


Figure 1.10: Activation of T cells by APC
http://www.anselm.edu/homepage/jpitocch/genbio/immunenot.html

1.8.2.3. B-lymphocytes

Producing antibodies against specific antigen of invading organism is primary duty of B-lymphocytes. Protective immunity require co-operation between T and B lymphocytes. Helper T-cell interaction required to produce high-affinity antibodies. Approximately 10^9 B-cells produced daily. Mature B-cells carry antibodies (IgD and IgM) on their surface. There are about 10^7 different specificities. Another important function of B-cells is to differentiate in Plasma cells (51).

1.8.2.4. Plasma B cells

Plasma cells are also known as "Antibody Factories". These are the most abundant lymphoid cells with more than 80% presence in Intestinal Mucosa. Plasma cells from lamina propria are effector cells programmed to produce IgA and IgM. They are rich in endoplasmic reticulum responsible to produce large amount of antibodies (52, 53).

1.8.2.5. Memory B cells

Long lived plasma cells are known as Memory B-cells generate humoral immunity by continuous maintenance of serum antibody level. Rapid anamnestic antibody response that occurs after re-exposure to antigen is action of Memory B cells, which is important for eliminating the pathogen and toxic antigens. These cells also clear pre-existing toxicity and maintain the pool of long-lived plasma cells in absence of pathogen. Memory B-cells originate from activated B-cells which are antigen specific produced during primary immune response. These cells are quick respondent in re-exposure to that specific antigen (54, 55).

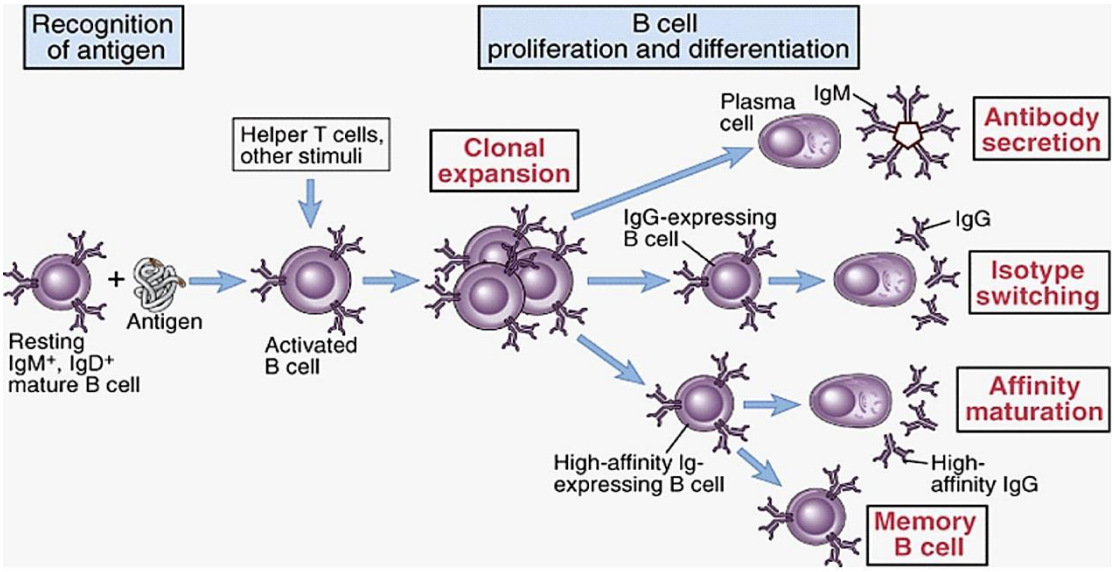


Figure 1.11: Clonal selection, expansion, proliferation and differentiation of B-cells (52)

1.8.2.6. Antibody production

Antibodies, therefore Immunoglobulins (Igs) constitute the gamma globulin part of the blood proteins. Gamma globulin constituents are: IgG-76%, IgA-15%, IgM-8%, IgD-1%, and IgE-0.002%. Among these, IgG is the predominant antibody for secondary response and poses an important defense against bacteria and viruses. IgA is the major immunoglobulin in colostrum, saliva, tears, and respiratory, intestinal and genital secretions. IgM is produced early in the primary response. IgE defends host against certain parasites and mediates anaphylaxis (50).

1.8.2.7. Immunological memory

Activation of lymphocytes during primary response generates long lived memory cells, which may survive for years after the infection. Memory cells represent an expanded pool of antigen-specific lymphocytes (52).

In the course of infection, pathogen must reach to its target sites through blood and tissue fluids but in its way they are susceptible to different immune components. Immune system provides immediate or innate and acquired immunity that is more

specific, rapid, efficient and regulated. The principal of immune system is to provide protection against foreign agents by distinguishing self from non-self (54).

1.8.2.8. Primary response

After encounter of an antigen, antibodies are detectable in the serum after a lag phase, of 5-7 days. A small clone of B-cells and plasma cells specific for that invading antigen is formed depending on the nature and dose of the antigen and the route of administration. The serum antibody concentration continues to rise for several weeks, as primary response of infection as innate immunity and then starts to decline. The first antibodies to appear are IgM, followed by IgA or IgG. IgM levels decline earlier than IgG levels. (55).

1.8.2.9. Secondary Response

There are cells of at least three stages of maturation: naïve cells, effectors cells and memory cells in peripheral lymphoid organs. During primary response some cells are stimulated to proliferate and differentiate into effectors cells, which are actively engaged in making a response (effectors B cells secrete antibody, while effectors T cells kill infected cells or help other cells fight the infection), some naïve cells are stimulated to multiply and differentiate into memory cells named as clonal expansion, that are not themselves engaged in a response but are more easily and more quickly induced to become effectors cells by a later encounter with the same antigen known as secondary response as adaptive immunity (57, 58). Secondary immune response (second or subsequent exposure to an antigen) is not only faster but produces antibody with up to a 10,000 fold increased binding affinity. Re-exposure to antigen is most likely to cause clonal expansion of memory cells to produce the highest affinity antibody. Although the early systemic response is of IgM, subsequent challenges, tends to induce a switch to IgG antibodies. Also, since many if not all of the memory B cells will have switched to IgG (IgA or IgE) production, IgG is produced earlier in a secondary response (56).

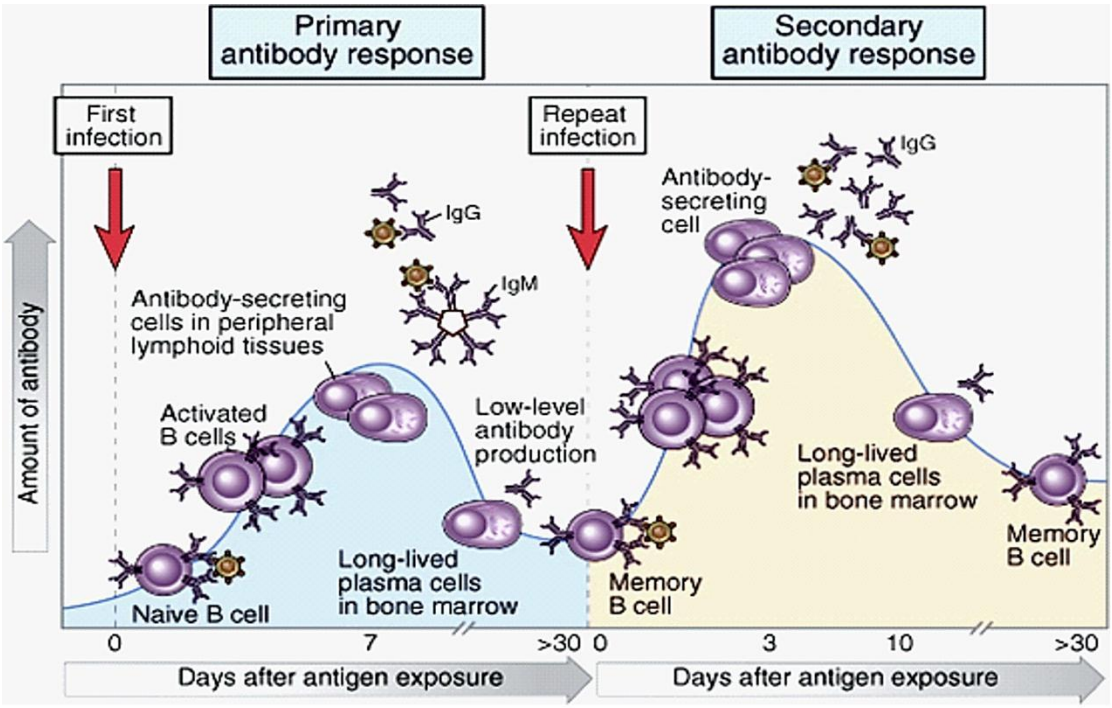


Figure 1.12: Primary and secondary immune response (52)

1.9. Immunologic response against *Vibrio cholerae*

The mechanism of immunity against *Vibrio cholerae* infection is not clearly understood yet. Immunologic response following infection can be classified into several types and classes.

1.9.1. First line of defense

Innate immunity refers to body's pre-existing protection mechanism against invading agents, which include physical barriers (the skin and mucosal surfaces) and chemical substances (mostly proteins) that neutralize microorganisms and other foreign particle. Following infection by both *Vibrio cholerae* O1 and O139, innate cells are activated and mediators remain elevated at the mucosal surface. Mucosal mast cells (MMC) play a crucial defense against immediate infection. These cells are activated by binding with of IL-3 and Stem Cell Factors (SCF), which produced from T-cells and epithelial cells (59). Mast cells produce IL-4 that is an early response cytokine to activate Th-cells. Presence of IL-3 and SCF in duodenal secretion explains MMC

activation and production of Th-2 type cytokines IL-4 and IL-5 (59) in adult cholera patients. As primary host defense, mobilization of neutrophil polymorph is stimulated by mast cell derived TNF-α at the site of infection. In acute stage of cholera increased level of TNF-α, suggest that it may act as an early response cytokine to up regulate Polymorpho Nuclear (PMN) and initiate bactericidal proteins. Mast cell produced leukotriene (LT) LTB-4 and LTC-4 are rich in intestinal secretion following infection indicates migration and recruitment of neutrophils (60, 61). Mast cells are found with increased level of lipid bodies suggesting increased production of arachidonic acid meatbolites (66). Arachidonic acid Prostaglandin E_2 found to be increased in cholera infection (61, 62). Increased level of leucocytes, including PMN, found in gut (62, 63). Among others bactericidal proteins- lactoferrin, MPO, as well- defensin were also elevated during the acute stage, further highlighting the functional role of PMN in cholera. Not only neutrophils but eosinophils also remain elevated in acute cholera cases which necessary to activate mast cells to produce IL-5 (64, 65). Innate immunity also rises in rectal areas in patients with natural cholera infection (66).

1.9.2. Serum antibody responses

Vibrio cholerae being a non-invasive, serological immunity against it is very important. Serological immune response varies with day points in both naturally infected patient and vaccinated individuals. Significant increases of LPS-specific IgA and IgG antibody levels were found in duodenal extracts on day 30 and decreased to baseline by day 180. LPS-specific IgA in plasma remained elevated longer. Levels of mucosal CTB antibodies also peaked on day 30, but were significant only for IgG (67). In duodenal fluids, mucosal secretory IgA (sIgA) antibodies were detected but these also returned to baseline within 6 months (67).

1.9.3. Vibriocidal responses

Vibriocidal is an assay that measures the ability of serum antibodies to lyse *V. cholerae* in the presence of complement. This is the best-characterized marker of protective immunity to cholera is the plasma, a complement-dependent bactericidal

antibody that increases with age in areas in which cholera is endemic. But vibriocidal antibodies directly mediate protection is very unlikely because there is no threshold vibriocidal-antibody titer at which protection against cholera is achieved (67, 68). Vibriocidal antibodies (mainly, but not entirely directed against LPS) have been inversely correlated with susceptibility to infection (69, 70). The vibriocidal-antibody titer peaked by day 7 and remained significantly elevated through day 90 before declining to baseline levels at day 180 (67).

1.9.4. Antibody secreting Cell (ASC) responses

Individuals infected with *V. cholerae* developed IgA ASCs specific for LPS and CTB, which peaked 7 days after the onset of disease (40, 73) and expressed the gut-homing receptors α4β7 (74). Circulating IgA ASCs are typical markers of mucosal priming after enteric infection. Mucosal LPS-specific IgA ASCs were demonstrated in duodenal biopsy specimens from cholera-infected patients. The cells remained detectable for 6 months, even in the absence of detectable anti-LPS IgA in secretions. A significant CTB-specific IgA levels were found in duodenal extracts on day 30. These cells may resume antibody production when appropriately stimulated (67).

1.9.5. Memory B cell responses

Protective immunity mediators against cholera are yet to be known, but memory B-cells response may play a central role in facilitating long-term and anamnestic responses against *Vibrio cholerae*. It was reported that individuals with natural cholera developed plasma anti-cholera toxin B subunit (CtxB) and lipopolysaccharide (LPS) IgA responses, which returned to baseline by 1 year of follow-up (40). Again CtxB-specific IgG memory B cell responses are detectable in the circulation at least 180 days following *V. cholerae* O1 infection and remain measurable even after serum antibody titers have declined to considerably lower levels (71). Dukoral vaccinated individuals showed IgA and IgG memory B cells responses to CTB for 1 to 6 months while there was no response to LPS (40). But IgG memory B cells responses against

V. cholerae O1 LPS and protection against infection in household contacts of cholera patients was found (72).

1.10. General objectives of the study

To assess whether natural infections in cholera endemic areas can provide longer term protective immunity for long and the role of memory B cells in this regard.

1.11. Specific objectives of the study

A. To investigate antigen specific memory B cell proliferation and its persistency and it's potential to generate protective immunity in naturally infected cholera patients.
B. To compare circulating memory B cell specific to OSP and LPS up to day 180.

Chapter 2

Methodology

2.1. Place of study

The study was carried out in the Laboratory Sciences Division (LSD), International Centre for Diarrhoeal Diseases and Research, Bangladesh. All the patients were enrolled from icddr,b Hospital. The study was approved by the Research Review Committee (RRC) and Ethical Review Committee (ERC) of icddr,b.

2.2. Study participants

Participant were about 60 enrolled *V. cholerae* positive patients at icddr,b hospital. Written informed consent for the study was obtained from them following infection. Blood samples were collected during acute infection (the second day of hospitalization) and again on days 7, 30, 90 and 180 following illness onset. For each day point, ELISA for plasma IgG, IgA and IgM antibodies to OSP, LPS and CTB and also vibriocidal assay were performed. OSP, LPS and CTB specific IgA, IgG and IgM antibodies were assayed on day 2, 30, 90, 180 in Memory B cell culture supernatant.

2.3. Study design and sample collection

Admitted patients with characteristic rice watery appearance stools were screened by dark-field microscopy to observe darting movement of *V. cholerae* by its flagella. To confirm cholera infection followed by serotype and biotype test by specific antibody after culturing in TTGA plates (75). Blood had drawn from confirmed cholera positive patients at day points 2, 7, 30, 60 and 180 and separated into Plasma and PBMC. Vibriocidal assay and ELISA for specific antibodies were performed for plasma of each day points.

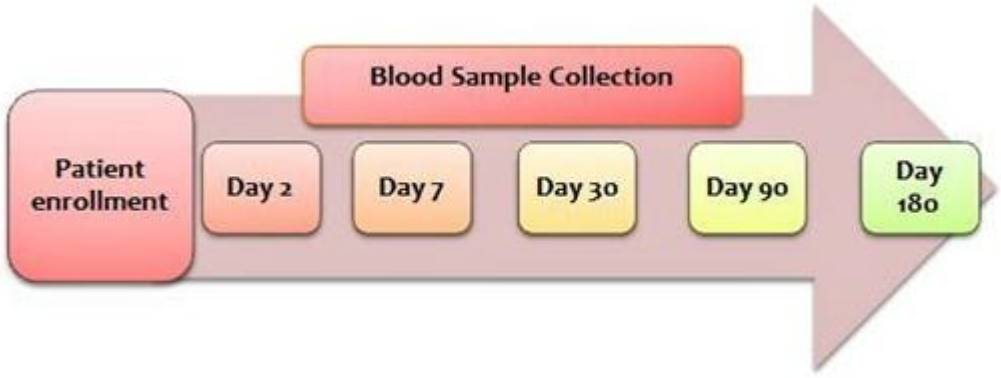


Figure 2.1: Blood Sample collection at different day points

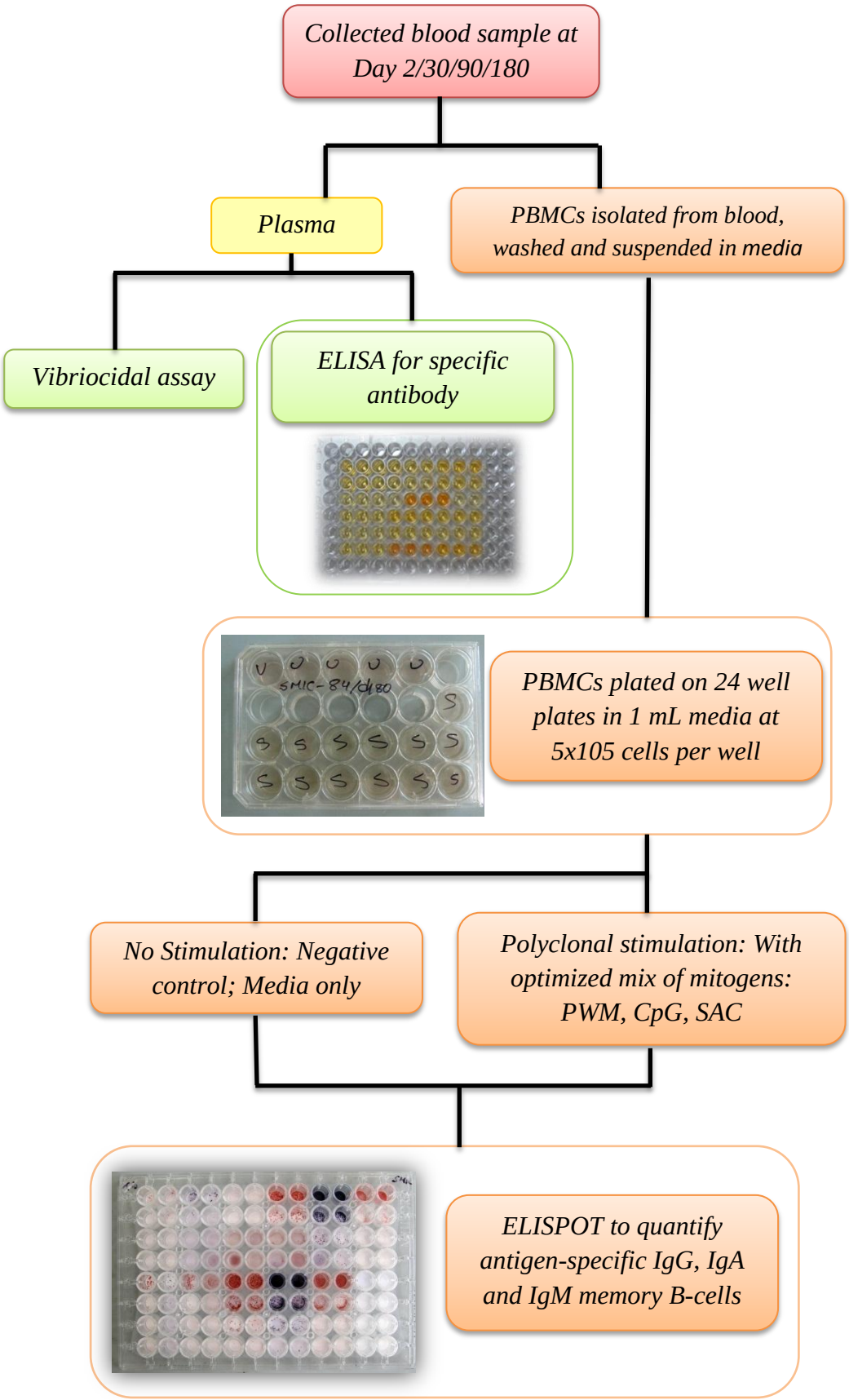


Figure 2.2: Assays with blood sample

2.4. Laboratory Methods

2.4.1. *V. cholerae* positive patients are confirmed by three different tests

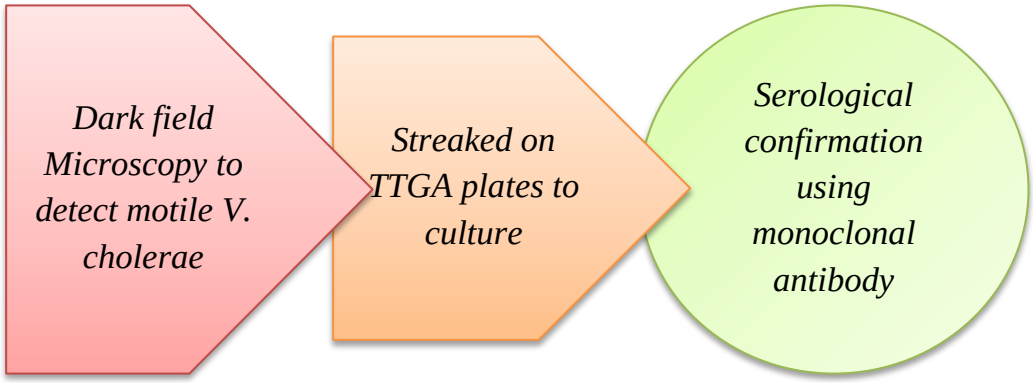


Figure 2.3: Serological confirmation of *V. cholerae* infected patients

2.4.1.1.Dark field microscopy to detect *V. cholerae*

Stool from patients with characteristic rice watery appearance were observed using dark field microscopy for motility of *V. choleare* that confirms vibrios infection. The movement is rapid to- and fro-, which is characteristic and unique to the organisms of this genus "Vibrio" (77). By using this property *V. cholerae* is easily detected without help of any stain. For this quick technique, one drop of stool sample is dropped on a clean slide and cover slip applied on it. Emulsion oil was applied on the lens to aid the visibility. Then the slide was observed at 40X magnification (Figure 2.4). Stool samples with motility are defined as DF^{+} and enrolled as cholera positive patient (77).

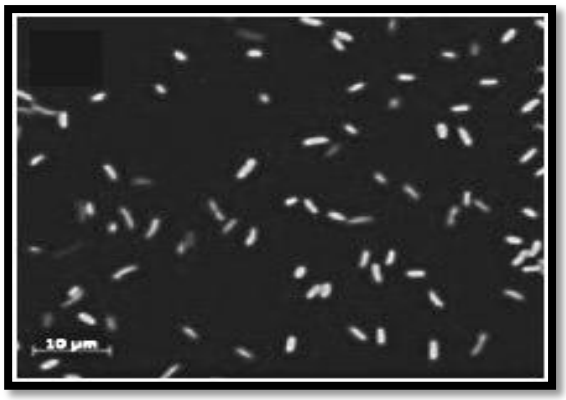


Figure 2.4: *Vibrio cholerae* in dark field microscopy

2.4.1.2.Serological detection of *Vibrio cholerae*

Antibodies were used to inhibit motility in stool sample, which confirms the serogroup of the pathogen. Stool specimens from the DF^+ patients were streaked on TTGA (taurocholate-tellurite-gelatin agar) plate. DF^+ stool makes a transparent colony with a dark zone of potassium tellurite on TTGA plate**.**

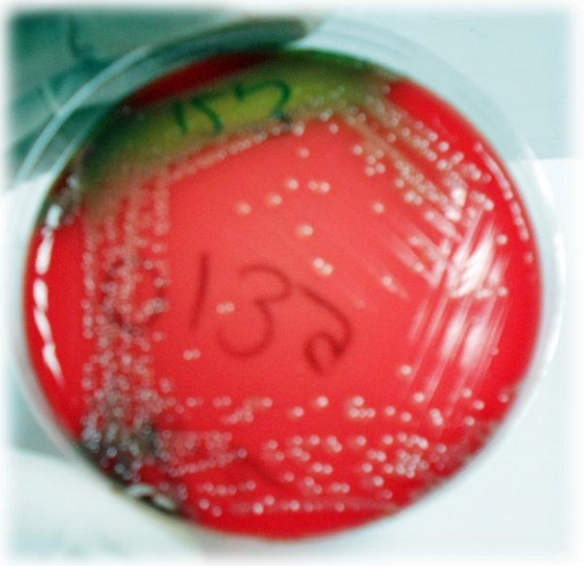


Figure 2.5: *Vibrio cholerae* colonies in TTGA plate

V. cholerae O1 or *V. cholerae* O139-specific mouse antisera were used for serotyping. Mouse anti-serum was raised against *V. cholerae* O1 for both Inaba and Ogawa serotypes. A slide was taken and divided into three blocks and marked. Then three types of antisera (*V. cholerae* O1 Ogawa, *V. cholerae* O1 Inaba and *V. cholerae* O139-specific mouse-rabbit antisera) were dropped on each block of the slide (Figure 18). Bacterial colony from the TTGA plate was applied on each block of the slide. Slide was observed with bright light for agglutination in blocks. The occurrence of clear agglutination within 2 minutes was considered a positive reaction. Only *V. cholerae* O1 reacting samples Ogawa were enrolled in this study.

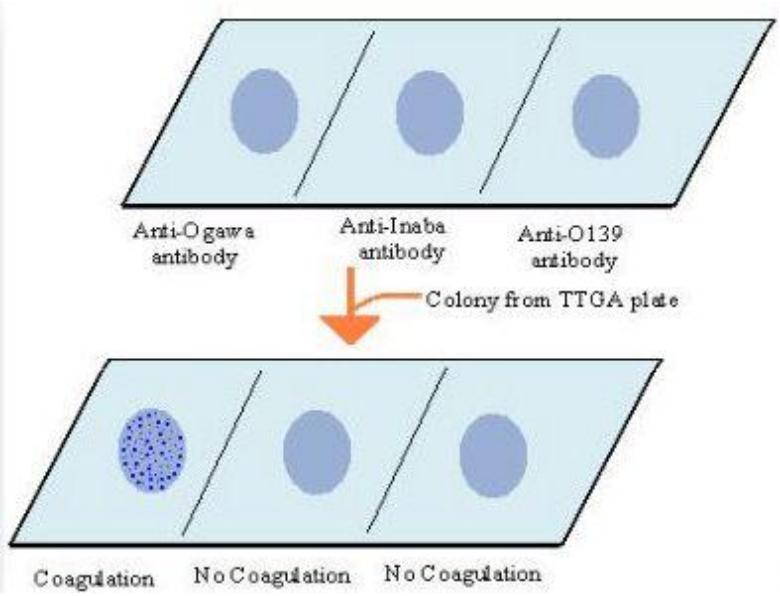


Figure 2.6: Serological detection of *V. cholerae* O1, Coagulation was seen at the first portion of the slide indicates antisera containing anti Ogawa antibody cross links *V. cholerae* and gives a spotted colour.

2.4.2. 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 50 mL Falcon tubes.
b. Diluted blood was carefully added to the volume of Ficoll-Isopaque (Pharmacia, Uppsala, Sweden) (in a 1:1 ratio of undiluted blood) without disturbing the Ficoll layer. Hence, two distinct layers were maintained.
c. The tubes were centrifuged at 700 g for 30 minute at 25°C (in a Sorvall® Legend™ XRT super speed centrifuge)
d. After centrifugation, PBMCs 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 500 g for 15 minute at 25°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 trypan blue) and

the MNCs were counted in a haemocytometer while the resuspended PBMCs were washed for the second time at 500 x g for 5 minute at 25°C.

g. After the second wash, cells were resuspended in RPMI Complete medium such that 100 µL contained 1×10^5 cells (e.g. 1 mL for 10 million cells).

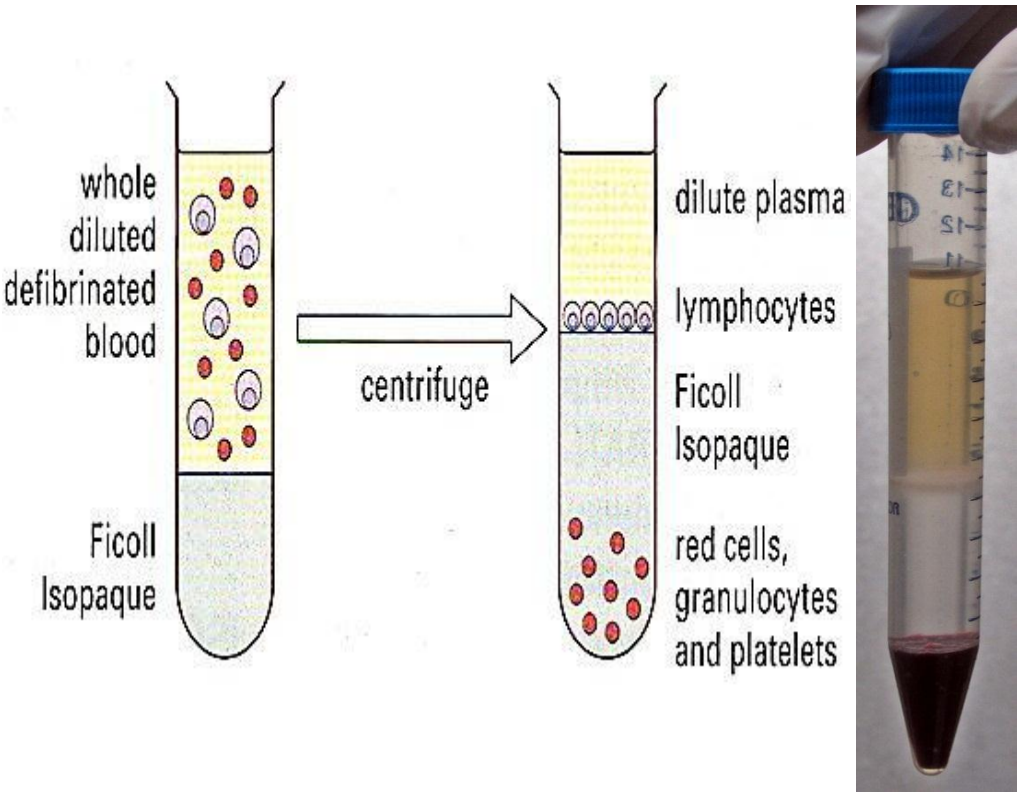


Figure 2.7: Isolation of PBMC by density gradient centrifugation on Ficoll-Isopaque

2.4.3. Antigen preparation

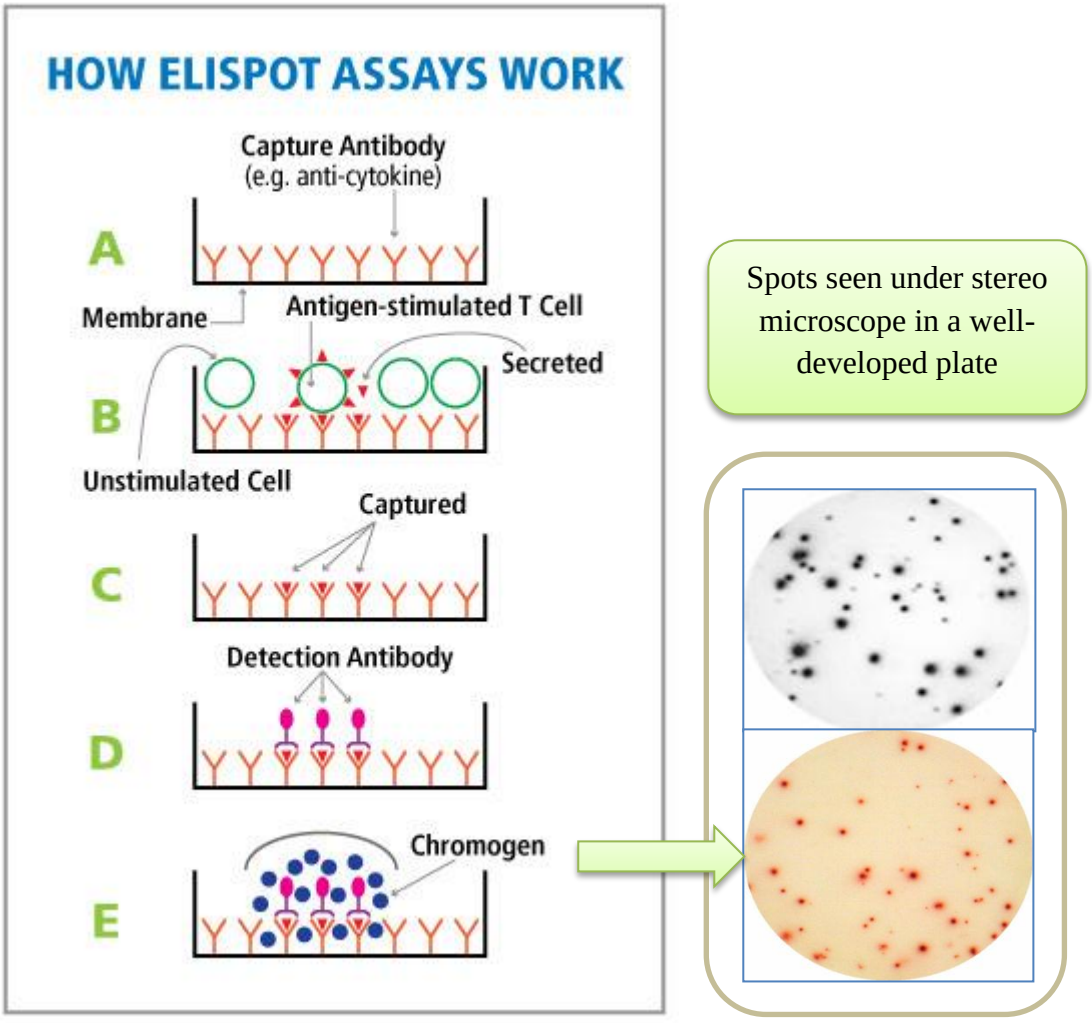
To measure immunological responses *V. cholerae* specific purified LPS, OSP and rCTB were used as antigens. LPS was obtained from *V. cholerae* O1, Ogawa (strain X-25049) by protein denaturation by hot phenol-water extraction (78, 79) followed by enzymatic treatment (DNase, RNase and protease), and ultracentrifugation (100,000 × g for 3 h) (80). OSP was recovered by acid hydrolysis of LPS, generating OSP attached to core oligosaccharide (OSPc) (78, 79, 80) and same followed to generate OSPc:BSA conjugates. To facilitate binding of OSP to immunological plates, and to permit display of OSP in a sun-burst pattern consistent with single point attachment similar to that occurring on wild type *V. cholerae*, we assessed immune responses targeting OSP using OSPc:BSA (henceforth referred to as OSP in immunonologic assays). To assess responses targeting CTB, we used recombinant antigen supplied by Professor A. M. Svennerholm, Gothenburg University, Sweden.

2.4.4. Determination of OSP, LPS and CT specific memory B cell responses by ELISPOT assay

2.4.4.1. Overview of ELISPOT assay

The Enzyme-Linked ImmunoSpot (ELISPOT) assay is a very sensitive immunoassay which measures the frequency of antibody-secreting cells at the single-cell level. Cells are cultured on a nitro cellulose membrane surface coated with a specific capture antibody in the presence or absence of stimuli. Antibodies which are secreted by the cells will be captured by the specific antibodies on the surface.

After incubation time, cells are removed by washing and the secreted molecule is detected by using a substrate (BCIP/NBT/AEC) with a precipitating rather than a soluble product, the end result is seen as visible spots on the surface where each spot corresponds to an antibody-secreting cell.



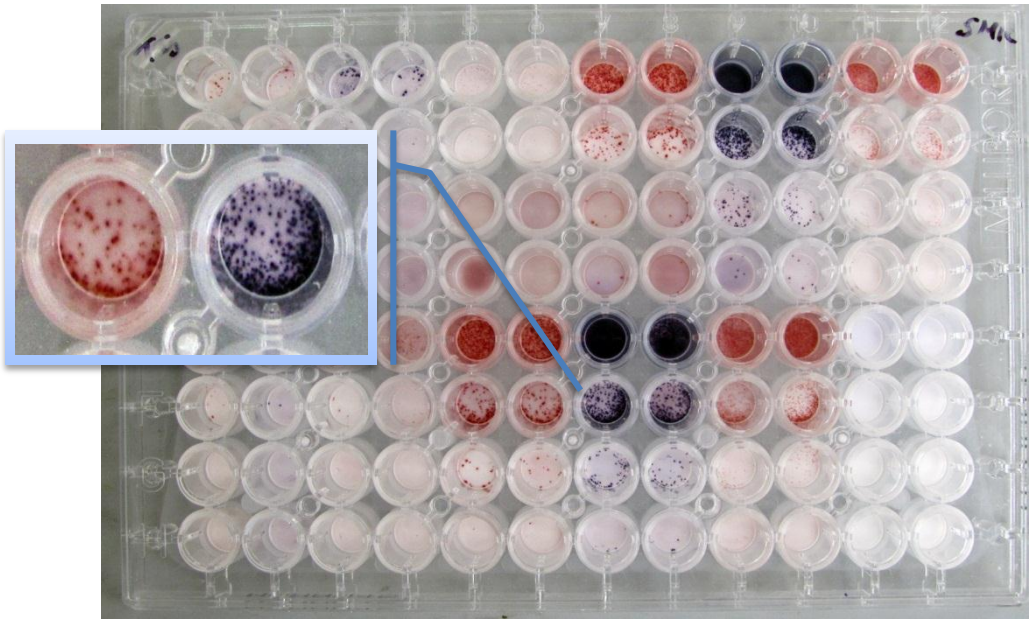


Figure 2.8: ELISPOT Assay. (a) Red spots correspond to IgA or IgM and (b) Blue spots corresponds to IgG. http://www.immunospot.com/index.php?id=514

2.4.4.2. ELISPOT assay protocol for Memory B cell culture

Coating of plates

I. Nitrocellulose wells were coated with

- Affinipure Goat anti-human IgG ((Jackson Immunology Research Cat 109-005-097)) at 5.0 µg/ml in PBS for the total – Immunoglobulin plate (positive control)
- KLH Keyhole Limpet Hemocyanin (KLH) (Pierce Biotechnology, Rockford, IL), at 2.5 µg/ml in PBS for negative control and
- OSPc:BSA OSP, at 10.0 µg/ml in PBS.
- 100 µl of GM1 (Ganglioside Monophosphate) (Sigma, cat#G-7641), at 3 nmol/ml in PBS followed by r recombinant Cholera Toxin B subunit (rCTB) at 2.5 µg/ml

LPS (Ogawa) Plate coating

a. 50 µl of poly-L-Lysine solution (conc.10µg/ml PBS) was added to each well and kept at room temperature for at least 30 min.
b. The wells were decanted and immediately 50 µl bacterial suspension (whole cell at conc. of $5.5x10^9$) or LPS (25 µg/ml in PBS) 50 µl was added to each well.
c. The plates were centrifuged at 2000 rpm for 5 min.
d. The content (nothing is left actually) was not decanted and gently 50 µl of 0.5% gluteraldehyde was added. (carried out inside hood) [Gluteraldehyde is toxic, so gloves is recommended]
e. Kept at room temperature for 15 min.
f. The plate was washed twice with PBS (gently).
g. 200 µl 0.1 % BSA in 0.1 M glycine was added to each well.
h. Kept at room temperature for 30 min.
i. Washed twice with PBS.
j. These plates were then ready to use.
k. To store the plates, 200 µl 0.1% BSA-PBS was added to each well and kept at –20 ºC

II. Plates were incubated at 4°C overnight.

III. The GM1 coating buffer was decanted and the CT plate was washed 23 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 then incubated for one hour at 37°C.

Blocking

a. All the plates were washed twice gently with PBS only.
b. Next they were blocked with 200 µl RPMI complete media per well at 37°C for 2 hours.

Memory B cell culture of collected blood sample

Reagents for stimulation media

- Crude Pokeweed Mitogen extract (1:100,000 dilutions)
- Staphylococcus Aureas Cowan (SAC), fixed. (Sigma Cat #P7155). (1:10000 dilutions)
- CpG oligonucleotide. [TCGTCGTTTTGTGTTTTGTCGTT from operon] (6 µg/ml)

Memory B cell culture

a. Culture wells were divided into unstimulated and stimulated wells.
b. To each of these wells, 5 x 10^5 peripheral blood mononuclear cells were added. Sterility was critical.
c. 1 mL of unstimulation culture media/well and 1 mL stimulation culture media/well were added to respective culture wells using sterile technique. Sterility was very critical in this particular step.
d. These culture plates were incubated in 5% CO_2 at 37°C for 5-6 days.

Culture cell harvest

a. After 5-6 days of proliferation, culture cells were collected in eppendorf tubes from each culture well.
b. All the eppendorf tubes were centrifuged at 2500 rpm for 7 minutes.
c. Washed cells with PBS again centrifuged at 2500 rpm for 7 min.
d. Finally, cell pellet was resuspended in 200 µL RPMI complete media which contains approximately 5×10^5 peripheral blood mononuclear cells.

Cell loading and developing

a. 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 memory B cell ELISPOT. Keeping this in mind, in the LPS, CT and OSP-plates, 40 µL of fresh RPMI complete media was added to the first row and 160 µL to subsequent rows for dilution. For the Total Ig's plate, 160 µL of fresh media was added to the first row and 180 µL to subsequent diluting rows.
b. 160 µL of the re-suspended cells were added to each well for the neat well for LPS, CT and OSP- specific wells and remaining 40 µL of resuspended cells were added to the first row of total Ig's plate.
c. Three 10 x dilutions of the total Ig's wells (transferring 20 µL), and a 5 x dilutions of the LPS, OSP and CT wells (transferring 40 µL) were carried out. The plates were incubated with cells for 5 hours at 37°C in a CO_2 (5%) incubator.
d. Next the cells were decanted and the plates were 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.
e. Anti-human IgA-HRP, anti-human IgG-AP and anti-human IgM-HRP at 1:500 were diluted in 1% FBS in PBS-Tween 0.05%. To each well 100 µL was added. All plates were incubated overnight at 4°C. The liquid was decanted and plates were washed 5 times with PBS-Tween and 3 times with PBS.
f. 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 vortexed well. 100µL of the chromogen/substrate was added to every well and the color reaction monitored immediately.

g. When the spots were clearly visible, the reaction was stopped by washing the plates repeatedly with tap water.
h. Plates were then soaked in tap water for 30 min before allowing them to dry in room temperature.
i. The spots were counted under low-magnification and the plates were stored away from light to count later.
j. Each red spot represented an IgA secreting cell and every blue spot indicated an IgG secreting cell in anti-human IgA-HRP conjugate and anti-human IgG-HRP conjugate added wells respectively while each red spot represented an IgM secreting cell in anti-human IgA-HRP conjugate added wells.
k. Data was evaluated as antigen specific IgG/A/M memory B cell divided by total IgG/A/M memory B cell and per 10^6 PBMC. And the results were expressed as percentage.
l. Appropriate stimulation of PBMC in this assay was defined as a $\geq$ 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 IgG, IgA and IgM memory cells.

2.4.5. ELISA for detection of IgA, IgG and IgM antibodies against Lipopolysaccharide (LPS), O-specific polysaccharide (OSP) and Cholera Toxin (CT) in plasma samples

2.4.5.1. Enzyme-Linked Immunosorbent Assay (ELISA) Principle

The ELISA is a specific and highly sensitive method for quantification of antibodies and other analytes in solution. The set-up consists of a specific antigen, coated to a microtiter plate, which is able to capture the protein of interest. After addition of the sample, the specific antigen on the plate will capture. A secondary monoclonal antibody, used for detection, binds a different epitope on the primary antibody. The detection antibody is conjugated with Horseradish peroxidase. Any unbound reagents

are washed away. When substrate is added, a color reaction will develop which is proportional to the amount of protein bound. The concentration of protein in the sample is determined by comparison with a standard curve with known concentrations of protein.

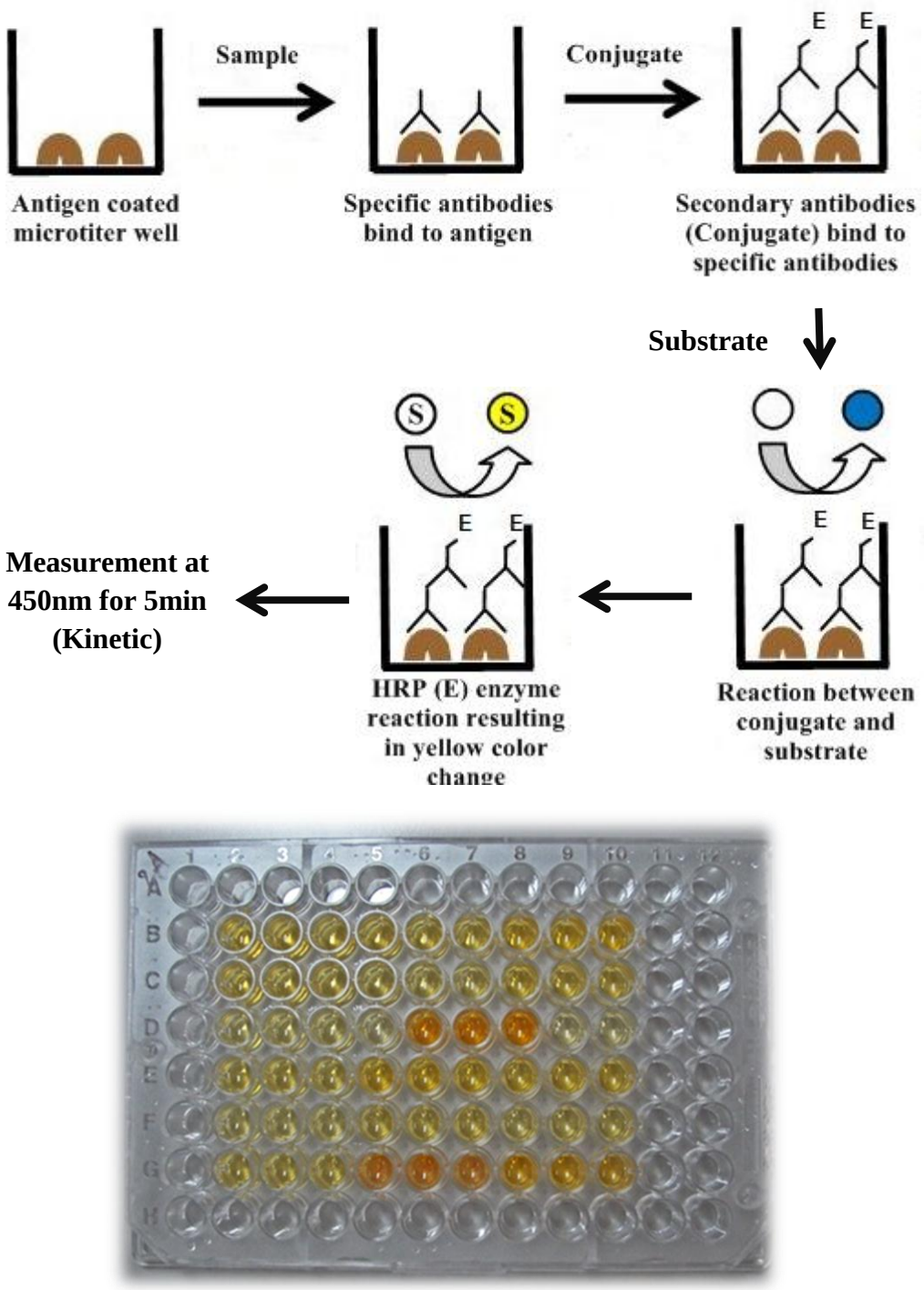


Figure 2.9: Principle of Enzyme-Linked Immunosorbent Assay (ELISA)

2.4.5.2. ELISA to detect IgA, IgG and IgM antibodies against Lipopolysaccharide (LPS) and O-Specific Polysaccharide (OSP) in plasma

Coating

The ELISA plates (Nunc F, Denmark) were coated with *V. cholerae* LPS about 100µL/well at a concentration of 2.5 µg/ml in PBS (pH 7.2-7.4) and OSPc:BSA at a concentration of 1µg/ml in carbonate buffer (pH 9.8). 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.

Blocking

The LPS and OSP-coated plates were washed thrice with PBS and blocked with 1.0% Bovine Serum Albumin in PBS (BSA-PBS), 200 µL/well for 45 minutes at 37°C.

Loading of Samples and Plate developing

a. The plates were washed 3 times with PBS-Tween (0.05 %) and once with PBS.
b. The plasma samples (initial dilution 1:50 for IgA, IgG & IgM) diluted in 0.1% BSA-PBS containing 0.05% Tween were loaded 100 µl per well of the plates.
c. The plates were incubated at 37°C for 90 minutes.
d. The plates were washed 3 times with PBS-Tween (0.05 %) and once with PBS.
e. The horseradish peroxidase (HRP) conjugated rabbit anti-human IgA, IgG and IgM (Jackson Immune Research Laboratories Inc.) were diluted in 0.1% BSA-PBS Tween and added 100 µl per well.
f. The plates were incubated for 90 minutes at 37°C.
g. The plates were washed 3 times with PBS- Tween (0.05 %) and once with PBS.
h. The plates were developed with the substrate orthophenylene diamine (OPD) as 100µl/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.

i. 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 and OSP antigen, individuals with a ≥2-fold increase in antibody titer one or three weeks post infection or vaccination were considered as responders.

2.4.5.3.ELISA to detect IgA, IgG and IgM antibodies against Cholera Toxin (CT) B subunit

Coating

The ELISA plates (Nunc F, Denmark) were coated with 100 µL/well of GM1 (Sigma, cat#G-7641) at a concentration of 0.3 nmol/mL or 0.5 µg/mL in PBS. The plates were then incubated at room temperature for overnight. Following overnight incubation, the ELISA plates were stored at 4°C up to 2 weeks for further use.

Blocking

The GM1-coated plates were washed twice with PBS and blocked with 1% Bovine Serum Albumin in PBS (BSA-PBS), 200µL/well and incubated for 30 minutes at 37°C.

Loading of Samples and Plate developing

a. The plates were washed thrice with PBS-0.05% Tween and once with PBS.
b. 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.
c. The plates were washed 3 times with PBS - Tween (0.05 %) and once with PBS.
d. The plasma samples (initial dilution 1:100 for IgA, IgG & IgM) diluted in 0.1% BSA-PBS containing 0.05% Tween were loaded 100 µl per well of the plates.

e. The plates were incubated at 37°C for 90 minutes.
f. The plates were washed 3 times with PBS- Tween (0.05 %) and once with PBS.
g. The horseradish peroxidase (HRP) conjugated rabbit anti-human IgA, anti-human IgG and goat anti-human IgM (Jackson ImmunResearch Laboratories Inc.) were diluted in 0.1% BSA-PBS Tween and added 100 µl per well.
h. The plates were incubated for 90 min at 37°C.
i. The plates were washed 3 times with PBS- Tween (0.05 %) and once with PBS.
j. 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.
k. 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 infections were considered as responders.

2.4.6. Vibriocidal 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 were considered responders.

Procedure

a. *V. cholerae* O1 (strain X-25049 El Tor Ogawa) were cultured overnight on blood agar plates at 37°C.
b. A loop full 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.
c. The culture was centrifuged at 3000 rpm for 10 minutes and the supernatant was discarded. The sediment was resuspended in sterile saline.
d. This was again centrifuged for another 8-10 minutes. The pellet was resuspended in sterile saline.
e. Bacterial concentration was adjusted by spectrophotometer at 600 nm. For *V. cholerae* O1, OD was adjusted at 0.3.
f. Heat-inactivated (56°C, 30 minutes) sera were 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 no. 2.
    - 45 µl of cold saline and 5 µl of test sera were dispensed in column no. 2.
g. The sera was serially diluted (initial dilution 1:10) 2-fold by using a multi-channel dispenser. The dilution was accomplished by mixing the solution in column #2, aspirating 25 µl and dispensing and mixing the sample in column #3 and so on, till column #12 (this 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.
h. The indicator (bacteria-complement-saline mixture) was prepared. The composition for each plate is as follows:

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

i. The indicator was used immediately after preparation.
j. 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).

k. 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.

2.5. Statistical analysis

Comparisons of immunologic responses were tested for significance among the groups using Mann Whitney Rank-sum test. All reported P values are two-tailed, with a cutoff of $P \leq 0.05$ considered a threshold for statistical significance. All the analyses were performed on GraphPad Prism 5.0 (GraphPad Software, Inc.,La Jolla, CA).

Chapter 3

Results

3.1. Study subjects

A total of 60 cholera patients were enrolled for the study and 87% of the patients completed the follow up to day 180. Among them, 66.7% were male. Median of patients' ages was 13 years. All the patients were *Vibrio cholerae* O1 ogawa serotype infected patients. The demographic characteristics of the patients were presented in table 3.1.

Characteristics		Frequency	
Median of ages		13 years	
Sex of patient	Male	20	**N=60**
	Female	40	
Blood group of patients	O+ ve	19 (31.6%)	
	O- ve	1 (1.7%)	
	A+ ve	15 (25%)	
	B+ ve	20 (33.3%)	
	B- ve	1 (1.7%)	
	AB+ ve	4 (6.7%)	

Table 3.1: Demographic and serological characteristics of the study subjects

3.2. Vibriocidal response

Vibriocidal antibody response specific to *V. cholerae* 01 ogawa were measured at days 2, 7, 30, 90, 180.

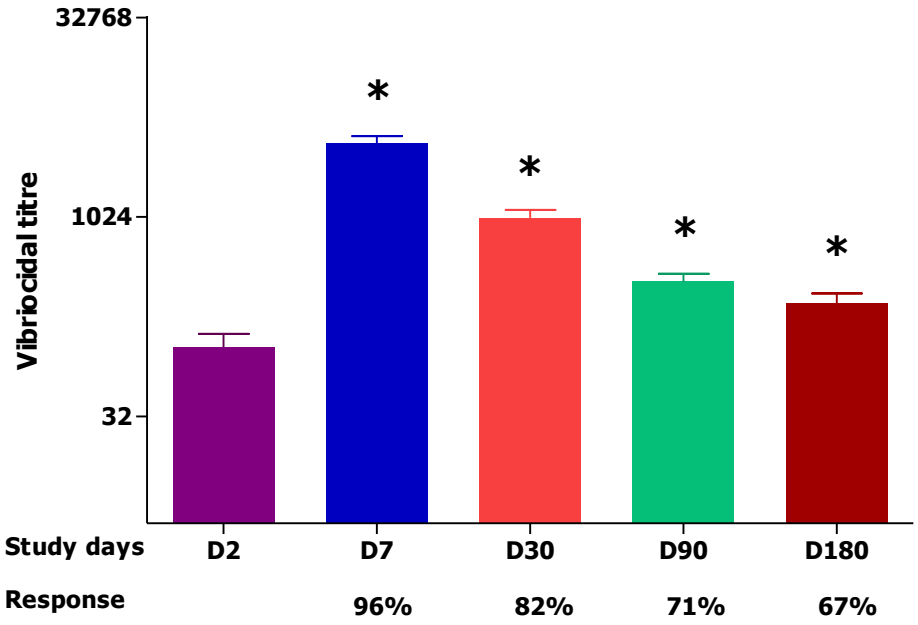


Figure 3.1: Mean serum Vibriocidal antibody response with (±SEM) standard error bars. An asterisk indicates significant difference compared to baseline values (P<0.05).

All patients presented strong vibriocidal response on day 7 and represented at least four fold increases in vibriocidal titer compared to baseline; and remained elevated up to day 180. The geometric mean (GM) of vibriocidal titer on day 2 was 28.46 (95% confidence interval [CI], 18.30 to 44.25). The day 7 vibriocidal titer (GM, 2100; 95% CI, 1459 to 3023; P<0.0001) in the patients were significantly higher than the titer on day 2. On day 30 and day 90 the geometric mean were 593.4 (95% CI, 437.2 to 805.4; P<0.0001) and 204.5 (95% CI, 150.9 to 277.2; P<0.0001) respectively and showed a lowest GM of 118.2 (95% CI, 81.13 to 172.2; P=0.0007) on day 180 during the follow up period. The titer waned down continuously after day 7 however it was significantly higher when compared to day 2 vibriocidal responses. Responder frequency was found highest on day 7 (96%) as well, while the lowest was on day 180 (67%) (Figure 3.1).

3.3. Anti-OSP and Anti-LPS specific antibody response in plasma and serum

ELISA was performed to study Anti-OSP and Anti-LPS specific antibody response and two fold increase in responses were considered as respondent.

3.3.1. Anti-OSP and Anti-LPS specific IgA antibody response

Compared to baseline responses, anti-OSP specific IgA plasma antibody response was significantly increased on day 7 with GM 65.53 (95% CI, 42.53 to 101.0; P<0.0001) with response rate of 74%. Response also increased on day 30 (GM, 26.88; 95% CI 18.84 to 38.33; P<0.0001) and 90 (GM, 16.12; 95% of CI 12.79 to 20.33, P=0.0015), but showed a decreased response rate of 52% to 46% in the patients. The lowest response rate was 39%, and seen on d180 (GM, 10.63; 95% CI, 8.84 to 13.32; P=0.1654) (Figure 3.2).

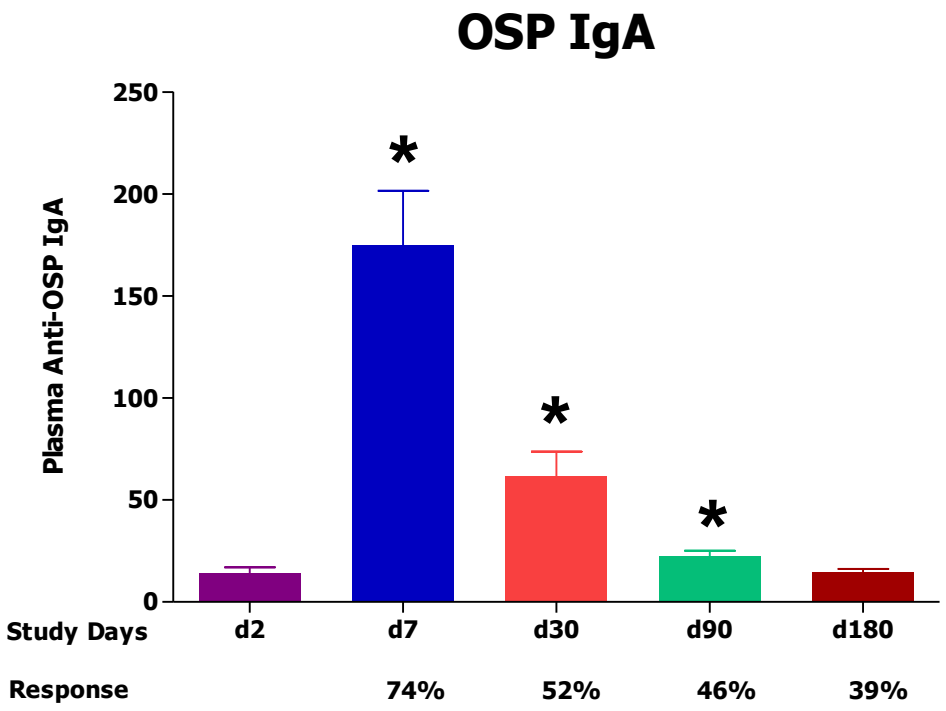


Figure 3.2: Mean plasma Anti-OSP specific IgA antibody response (with standard error). An asterisk indicates significant difference compared to baseline values (P<0.05).

Anti-LPS IgA antibody response was significantly developed on day 7, 30, 90 and 180, when compared to baseline responses. Highest result was achieved on day 7 (GM, 57.71; 95% CI, 37.43 to 88.96; P<0.0001), day 30 (GM, 22.43; 95% CI 16.49 to 30.52; P<0.0001) and responses rate were 75% and 60% respectively. Response on day 90 (GM, 19.12; 95% CI, 15.59 to 23.46; P<0.0001) and day 180 (GM, 15.15; 95% CI, 12.42 to 18.42; P=0.0032) were also significant with decreasing response rate seen in 51% to 38% of patients (Figure 3.3).

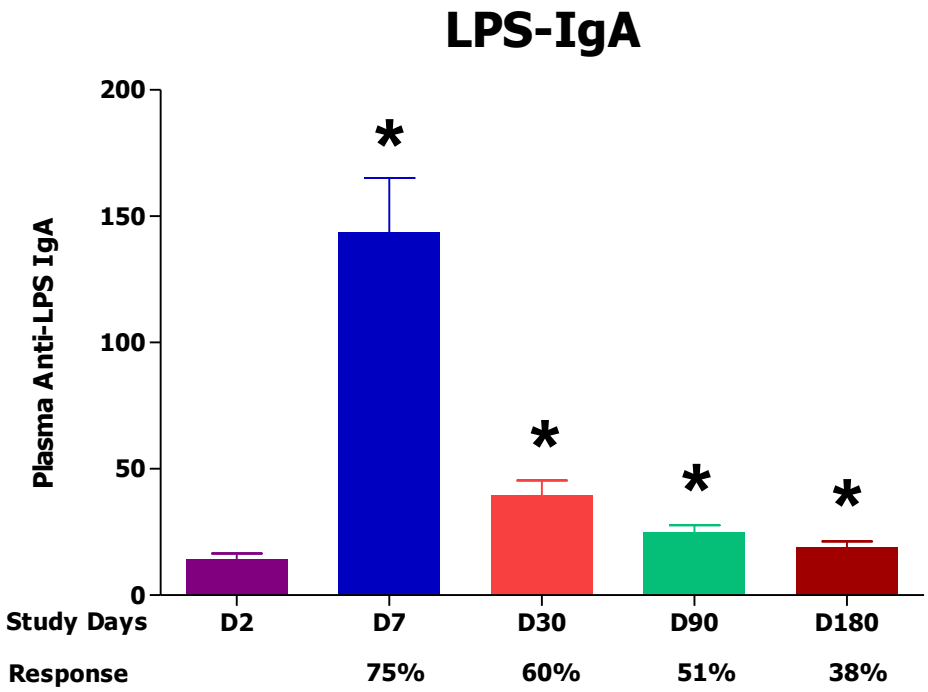


Figure 3.3: Mean plasma Anti-LPS specific IgA antibody response (with standard error). An asterisk indicates significant difference compared to baseline values (P<0.05).

3.3.2. Anti-OSP and Anti-LPS specific IgG antibody response

Anti-OSP specific IgG antibody response significantly increased on day 7 and persisted up to day 180 (P<0.0001). The geometric mean titre (GM) was maximum at day 7 (100.4, 95% CI, 80.75 to 124.9) with maximum response in 76% of patients. Gradually decreased responses were observed on following days 30 (GM, 83.28; 95% CI, 66.71 to 104; 54%), 90 (GM, 64.0; 95% CI, 52.67 to 77.93; 65%) and 180 (GM, 49.29, 95% CI, 42.28 to 57.46; 46%) (Figure 3.4).

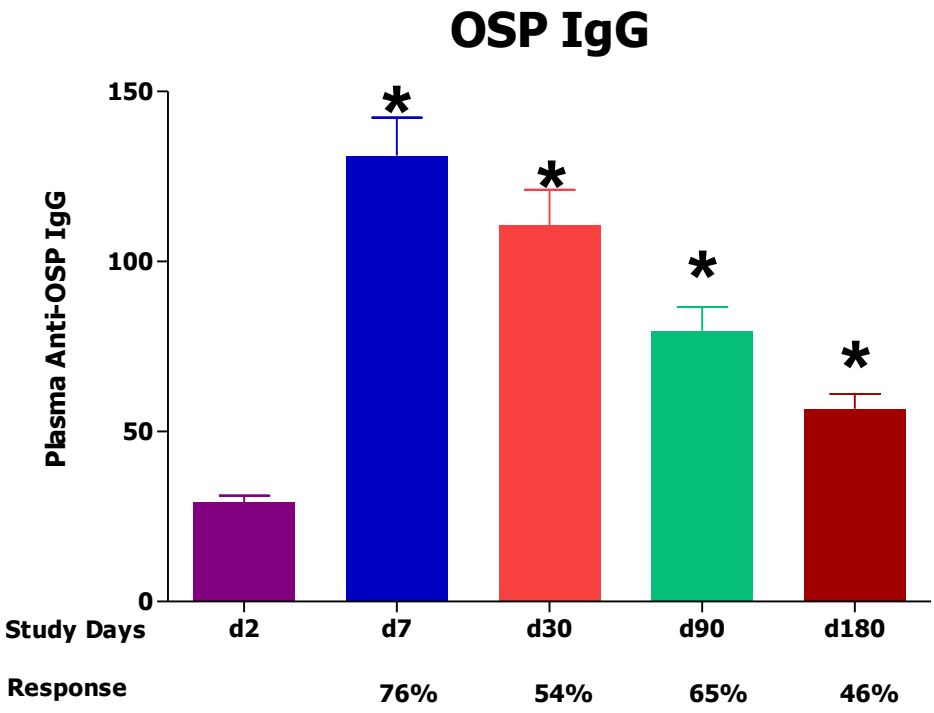


Figure 3.4: Mean plasma Anti-OSP specific IgG antibody response (with standard error). An asterisk indicates significant difference compared to baseline values (P<0.05).

IgG antibody response specific to LPS was also found to be elevated. The showed results like anti-OSP IgG. Compared to baseline response significant OSP-IgG responses (P<0.0001) on 7, 30, 90 and 180 were observed with response in 64%,

56%, 43% and 45% on day 7 in patients. A maximum GM of 88.35 was obtained on day 7 (95% CI, 73.14 to 106.7) which decrease gradually at different study days, i.e. on day 30 (GM, 72.49; 95% CI, 61.11 to 85.98), day 90 (GM, 61.25; 95% CI, 53.34 to 70.33) and day 180 (GM, 57.59, 95% of CI, 51.58 to 64.29) (Figure 3.5).

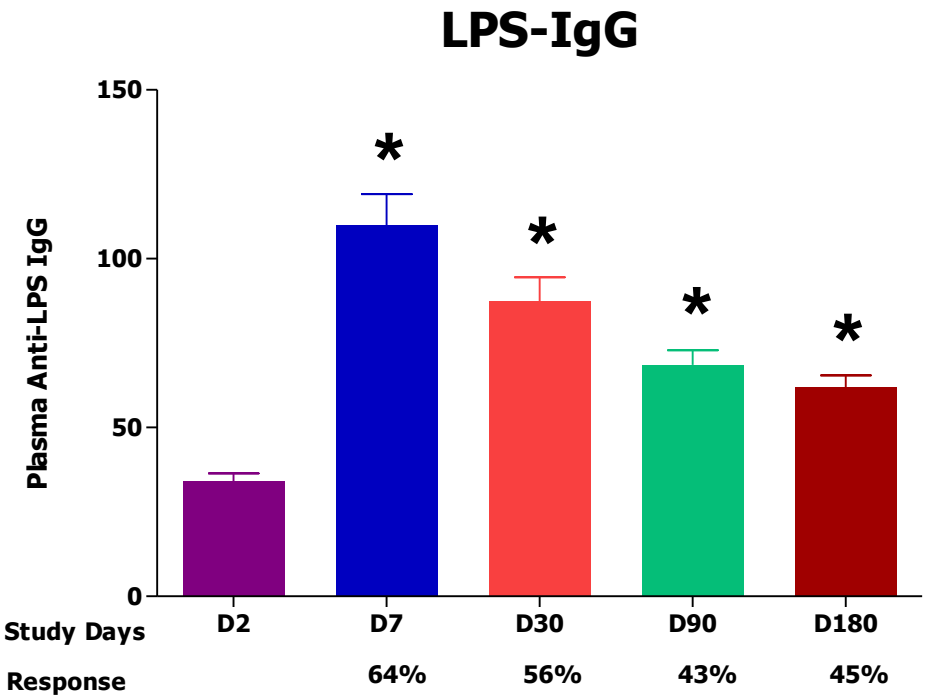


Figure 3.5: Mean plasma Anti-LPS specific IgG antibody response (with standard error). An asterisk indicates significant difference compared to baseline values (P<0.05).

3.3.3. Anti-OSP and Anti-LPS specific IgM antibody response

Anti-OSP specific IgM response was most significant (P<0.0001) on day 7 and day 30. Maximum GM 157.5 was obtained on day 7 (95% CI 123.1 to 201.4) with maximum response rate in 71% of patients. Although response was significant on all day points it waned during convalescence. The same was observed for response at the different time points i.e. day 30 (GM, 96.14; 95% CI, 77.70 to 119.0; 50%), day 90 (GM, 65.10; 95% CI, 53.78 to 78.81; 40%) and day 180 (GM, 56.96; 95% CI, 47.20 to 68.75; 35%) (Figure 3.6).

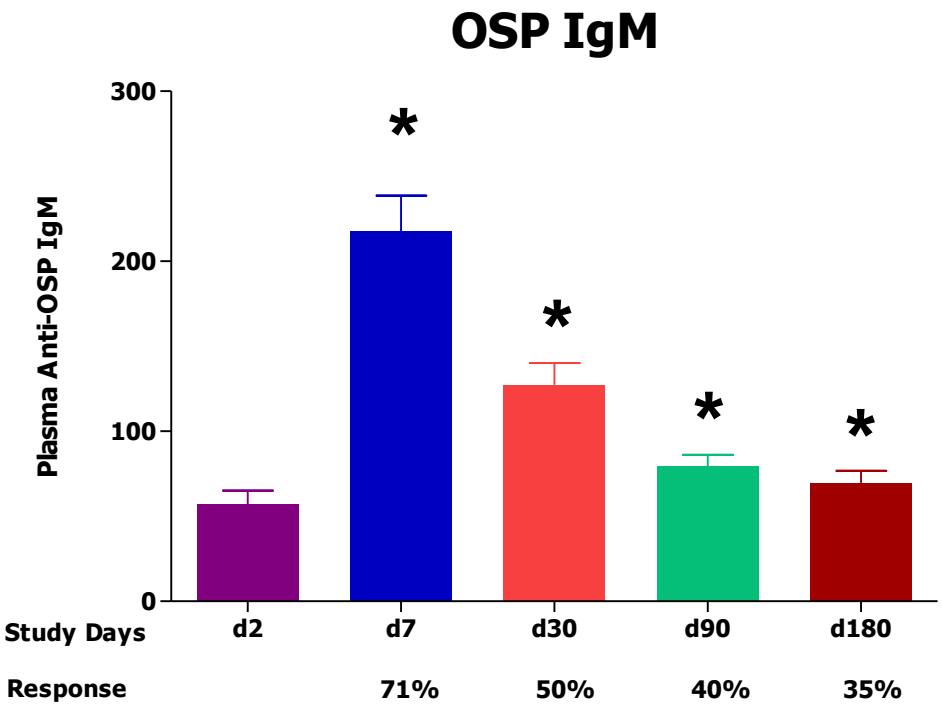


Figure 3.6: Mean plasma Anti-OSP specific IgM antibody response (with standard error). An asterisk indicates significant difference compared to baseline values (P<0.05).

Anti-LPS specific IgM responses were significant (P<0.0001) for the all study days. The maximum response was 73% with highest GM of 139.3 on day 7 (95% CI, 117.9 to 164.4). The GM for following day points were almost consistent having 94.98, 75.49 and 76.60 respectively with responses of 53% on day 30 (95% CI, 81.16 to 111.2), 42% on day 90 (95% CI, 61.65 to 92.42) and 38% on day 180 (95% CI, 62.80 to 93.45) (Figure 3.7).

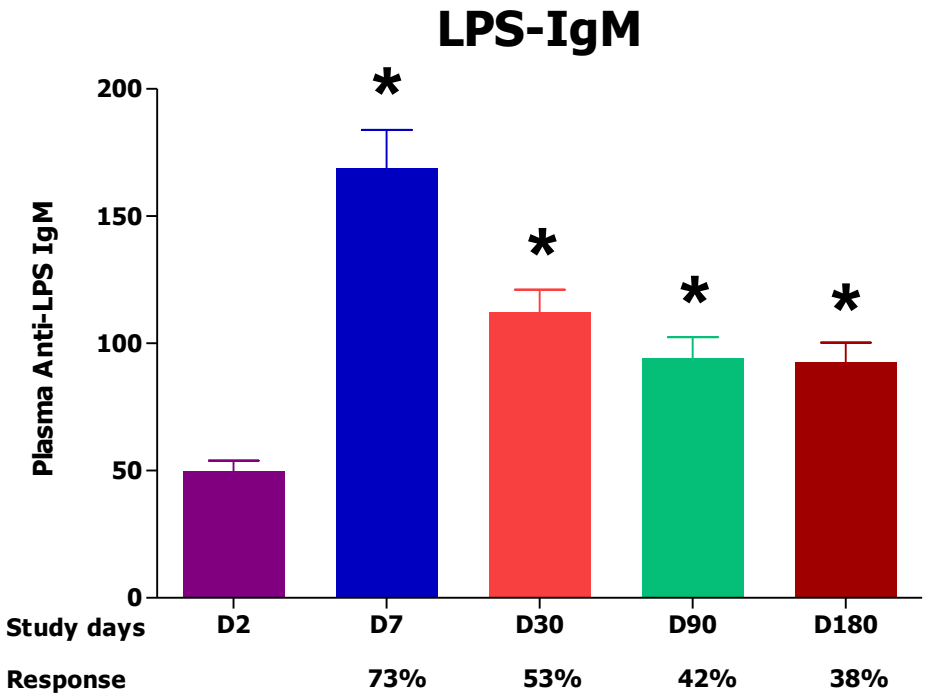


Figure 3.7: Mean plasma Anti-LPS specific IgM antibody response (with standard error). An asterisk indicates significant difference compared to baseline values (P<0.05).

3.4. Anti-CtxB specific IgA and IgG antibody response in blood plasma and serum

IgA antibody response specific to rCtxB was significant increased throughout the whole study day points, with a peak response on day 7 (GM 32.76; 95% CI 25.29 to 42.44; P<0.0001) with 93% response rate. Although responses waned in different time points, it was significantly high on day 30 (67%; GM 11.24; 95% CI 8.653 to 14.63). The responses were also significantly on day 90 (GM 6.600; 95% CI 5.287 to 8.239; P=0.0009) and day 180 (GM 5.317; 95% CI 4.287 to 6.693; P=0.0230) (Figure 3.8).

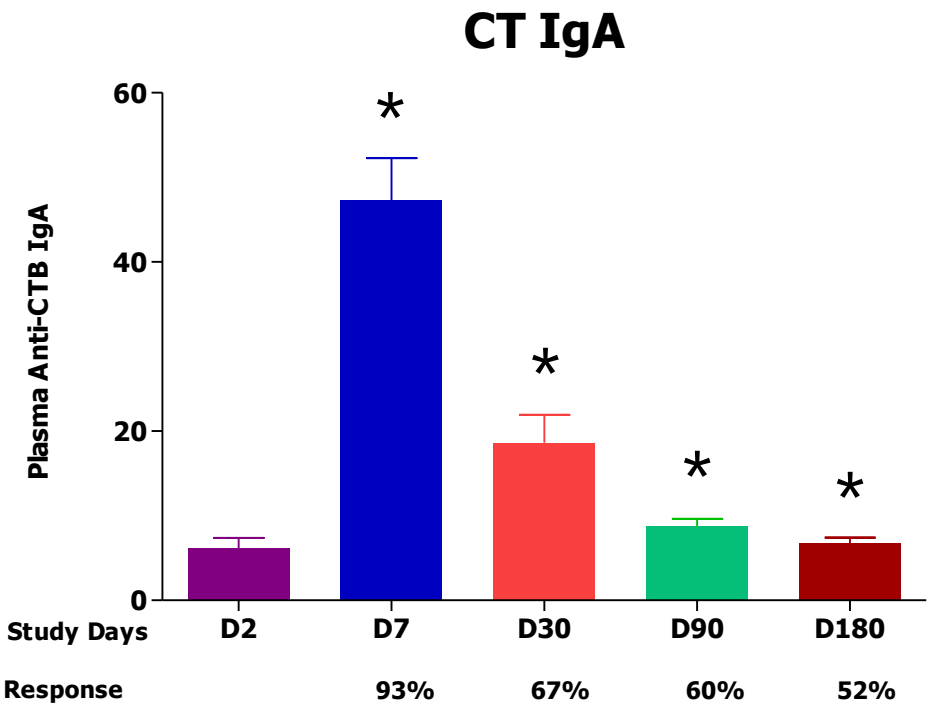


Figure 3.8: Mean plasma Anti-CtxB specific IgA antibody response (with standard error). An asterisk indicates significant difference compared to baseline values (P<0.05).

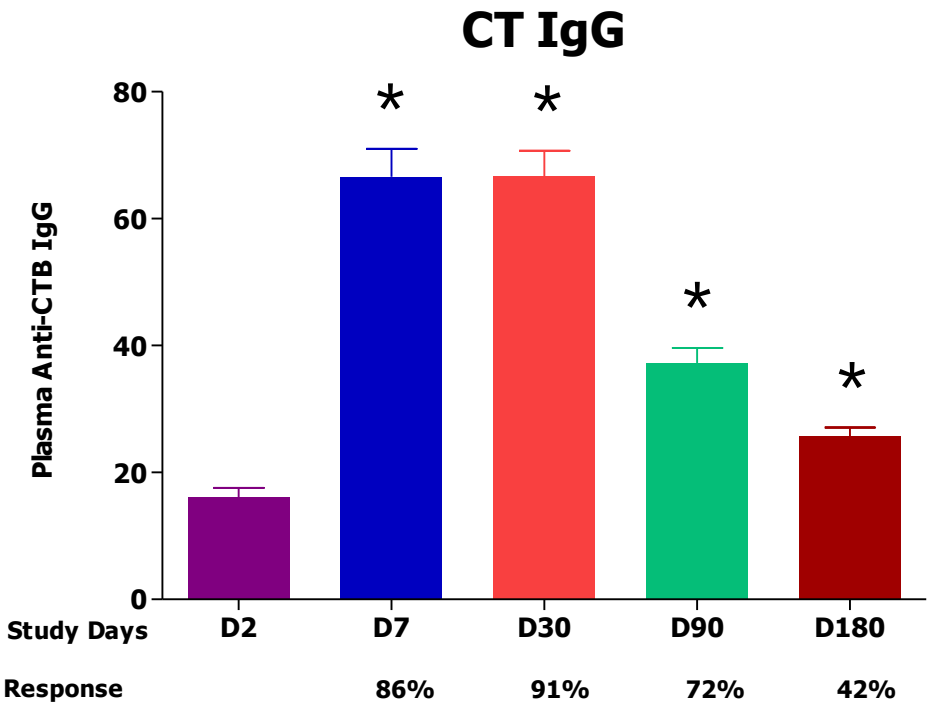


Figure 3.9: Mean plasma Anti-CtxB specific IgG antibody response (with standard error). An asterisk indicates significant difference compared to baseline values (P<0.05).

Unlike the CT-IgA response, the CT-IgG response peaked on day 30 (GM 58.39; 95% CI 49.56 to 68.79%; P<0.0001) with 91% responders. Responses on all study days showed significant responses. The response rate on day 7 was 86% (GM 58.10; 95% CI 50.04 to 67.45) and was highest when compared to the other time points. Responses started to wane by day 30 and decreased further on day 90 (GM 32.39; 95% CI 28.32 to 38.30; P=0.5084) and day 180 (GM 23.69; 95% CI 20.99 to 26.74; P<0.0001) with a responder frequency of 72% and 42% respectively (Figure 3.9).

3.5. OSP and LPS-specific IgA, IgG and IgM memory B cell response

OSP and LPS specific IgA, IgG and IgM memory B cell using the ELISPOT technique was carried out assay at day 2, 30, 90 and 180 post onset of illness. After six day PBMC culture, only those that showed ≥3 fold increase in the number of total Immunoglobulin as compared to unstimulated cells were considered for analysis.

Memory B-cells data were excluded from the analysis due to following reasons:

4. The averaged total Ig samples for each patient-sample did not have appropriate stimulation.
5. Patient samples had four or more antigen-specific ASC spots prior to stimulation.
6. Patient samples had four or more ASC spots to the negative control antigen KLH.

3.5.1. OSP and LPS- specific IgA memory B cell response

It was observed that 58% patients responded with a significant OSP specific IgA memory B cell on day 30 when compared to baseline responses. Statistically significant response was also seen at day 180 (50%, P=0.0203) and day 90 (22%, P= 0.0332) (Figure 3.10).

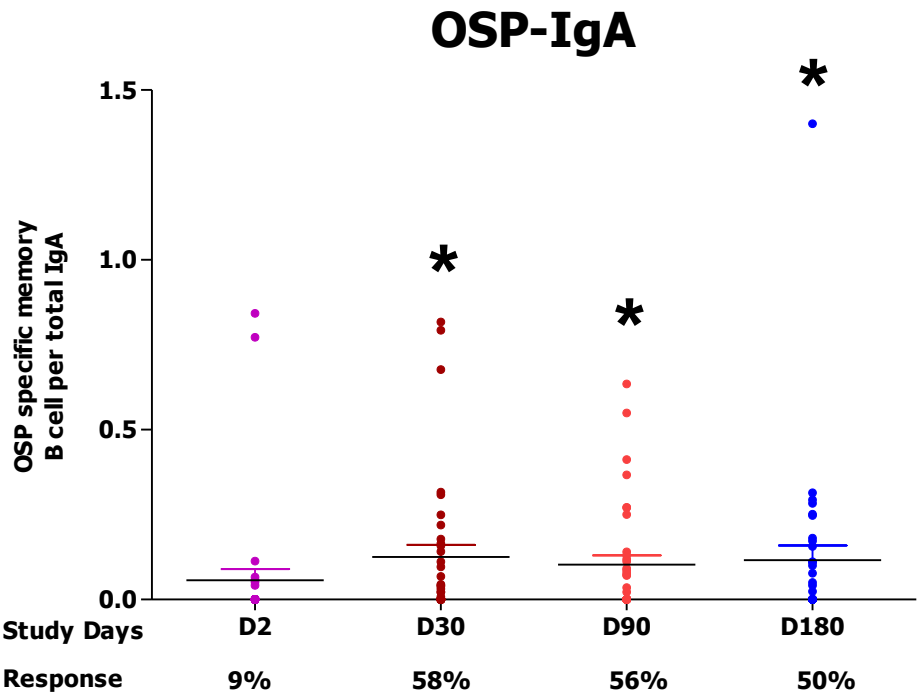


Figure 3.10: Mean OSP-specific IgA memory B-cell response with (standard error). An asterisk indicates a statistically significant difference compared to baseline values (P<0.05).

LPS specific IgA memory B cell response was significantly increased only at day 30 (P= 0.0003) and respondent rate was about 97%. Response on day 90 (83%, P= 0.0914) and 180 (87%, P= 0.1031) was higher comparing to day 2 responses (Figure 3.11). But IgA memory B cells for LPS showed lowest respondent on day 2 (55%).

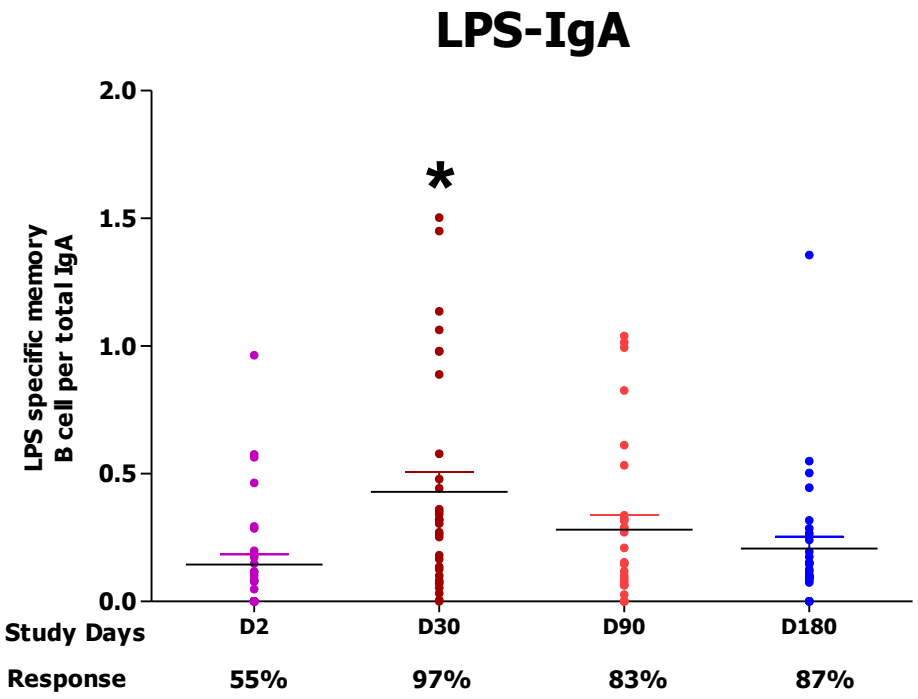


Figure 3.11: Mean LPS-specific IgA memory B-cell response (with standard error). An asterisk indicates a statistically significant difference compared to baseline values (P<0.05).

3.5.2. OSP and LPS-specific IgG memory B cell response

IgG memory B cells specific to OSP showed significant increase with gradually rising response. Although lower detectable response was seen day 2, an increased magnitude of response rose gradually on day 30 (53%, P=0.0079) and day 90 (46%, P=0.0054). Interestingly, the response remained significantly elevated up to day 180 (36%, P=0.0353) (Figure 3.12).

On the other hand, LPS specific IgG memory B cell response were statistically significantly increased only on day 180 (P=0.0318) compared to the baseline response. Although detectable memory was 78% at day 2, it increased in the following day points on 30, 90 and 180 and it was 94%, 94% and 90% respectively (Figure 3.13).

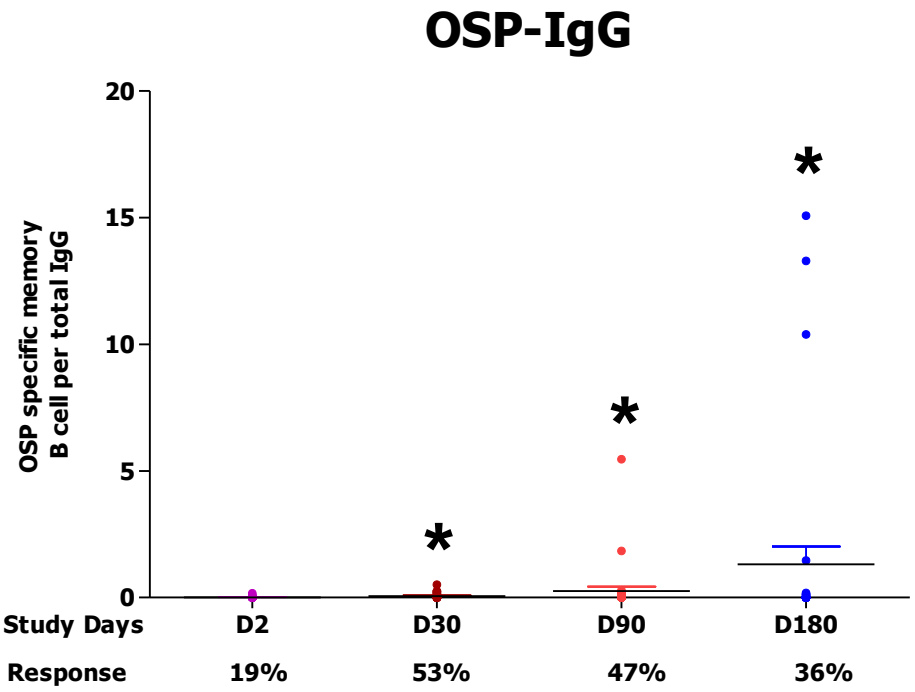


Figure 3.12: Mean OSP-specific IgG memory B-cell response (with standard error). An asterisk indicates a statistically significant difference compared to baseline values (P<0.05).

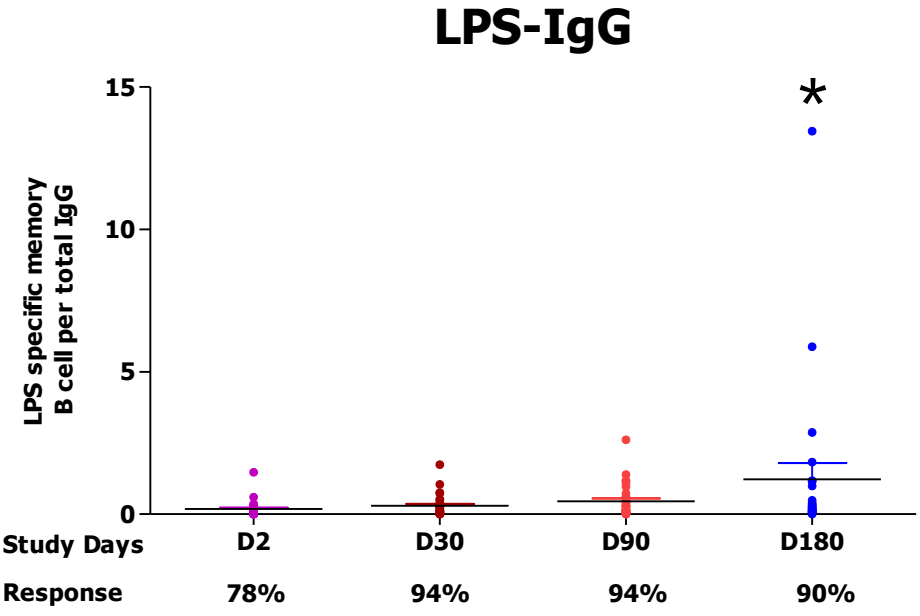


Figure 3.13: Mean LPS-specific IgG memory B-cell response (with standard error). An asterisk indicates a statistically significant difference compared to baseline values (P<0.05).

3.5.3. OSP and LPS specific IgM memory B cell response

The OSP specific memory B cell response was significantly increased at day 30 (P=0.0186) and day 90 (P=0.0137) when compared to the day 2 responses; it decreased to the baseline level by day 180. At day 2, detectable OSP specific IgM memory response was observed in 40% of patients and these rates increased on the following day points on 30, 90 and 180, it was seen in 72%, 94% and 53% of patients respectively (Figure 3.14).

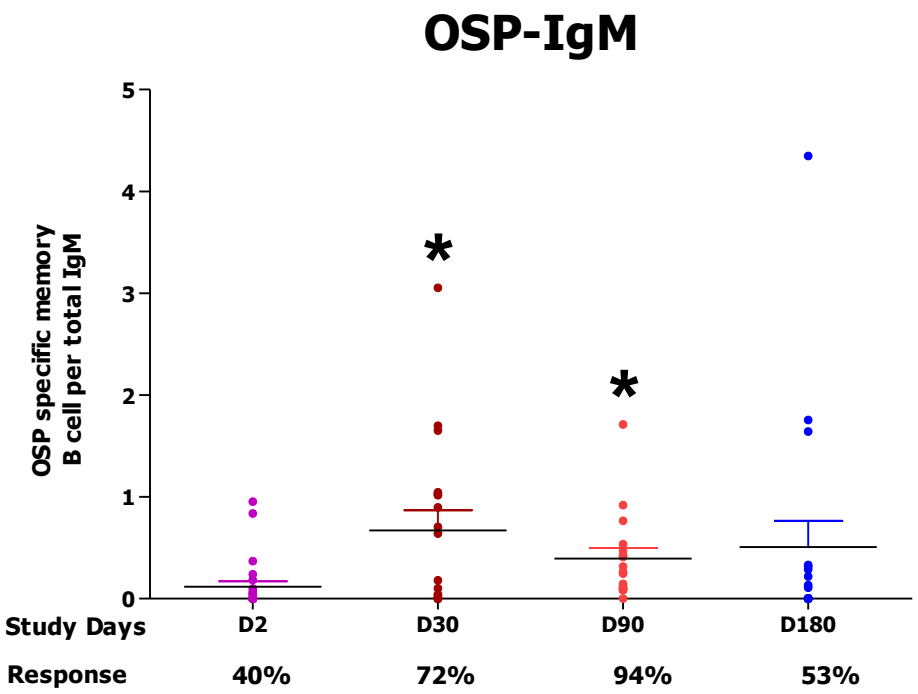


Figure 3.14: Mean OSP-specific IgM memory B-cell response (with standard error). An asterisk indicates a statistically significant difference compared to baseline values (P<0.05).

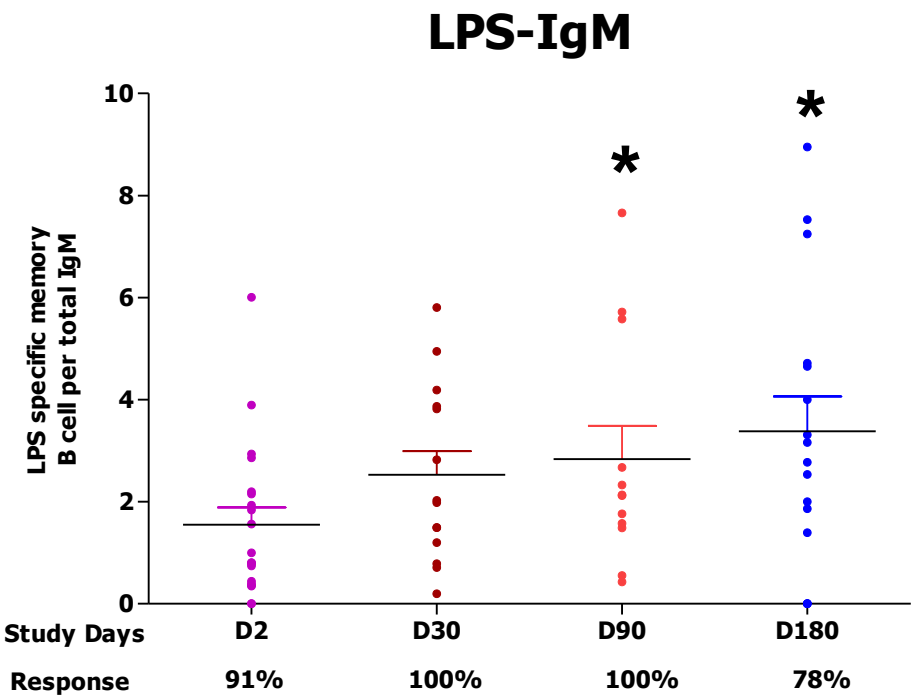


Figure 3.15: Mean LPS specific IgM memory B-cell response (with standard error). An asterisk indicates a statistically significant difference compared to baseline values (P<0.05).

The highest IgM memory B cell response for LPS was seen on day 90 (100%, P=0.0078). Response at day 180 was also significant (P=0.0391) with 74% respondent compared to day 2. High LPS specific IgM with 100% response observed at day 30 (P=0.0537) comparing to day 2, although was not statistically significant (Figure 3.15).

Chapter 4

Discussion

Studies in endemic areas of cholera have shown that the natural infection generates protective immunity to symptomatic disease on re-exposure for at least 3 years or perhaps as long as 10 years while available vaccine provides protection for 3-5 years in adults or shorter for younger children (6, 7). As the underlying mechanism of afforded longer protection by natural protection is not completely understood, an effective vaccine production is yet to be carried out. Although we know that LPS plays an important role in memory responses but available oral killed vaccines do not generate LPS targeted memory response (40, 81).

Currently the most used marker for immunity to cholera is vibriocidal antibody titer which is yet to be defined (82), how this complement dependent antibody derived from plasma constituting mainly IgM antibody provides protection for the patients who have previously had cholera (33, 83). Although there is no such cut off vibriocidal titer at which complete protection against cholera is achieved (82), all the patients showed four fold increase in vibriocidal titer on all the study day points in this study, supporting previous findings (38,82, 83). Vibriocidal titer also correlates with LPS and OSP specific antibody responses where higher vibriocidal titer is associated with higher LPS and OSP specific antibody responses. This indicates that vibriocidal titer is a surrogate marker for antibody response (79). Cholera infection generates significant memory B cell responses to *V. cholerae* antigens LPS, CTB and TcpA (40). And in the majority of patients' memory B cells remained detectable for at least 1 year with increased levels of IgA and IgG. Thus, antigen specific memory B cells may be useful markers for long term protection against cholera. Memory B cells are responsible for rapid immune response on re-exposure to the antigens. Furthermore, it is important to eliminate the antigens and pathogens that are not cleared by circulating antibodies even after the patient recovers from illness. Memory B cells may play an important role in replenishing the pool of long lived plasma cells to maintain long term antibody level in circulation after removal of pathogens resulting in long lasting acquired immunity. But it is not clear how long they are maintained in the absence of re-exposure to antigen. Measurement of antibody in memory B cells and availability of circulating plasma antibodies may be a better alternative than designing multiple antigen derived vaccine design. This study attempted to study memory response and circulating plasma antibody response to V. cholerae O1 and antigenic part of LPS (OSP) up to day 180.

However, LPS specific IgA antibody in duodenal biopsy may provide immunity to naturally infected cholera patient (83). Moreover, LPS can also develop memory B-cell responses that may persist up to 180 days post infection, but wanes down by 1 year (6). Kendall et al previously investigated LPS specific-IgM memory B cells response in memory B cell culture supernatants of cholera patients at day 2, day 30 and 90 following onset of disease; and found that LPS specific antibody increased significantly at day 30 and 90 compared to the baseline. Furthermore, LPS specific IgA, IgG and IgM memory B cell responses were found up to day 180 in this current study. IgA memory B cell response was significantly elevated on day 30 among the study day points. Responses waned down in the following days but were higher compared to the baseline. In addition, LPS specific IgG memory B cell responses remained higher up to day 180 compared to the baseline. The presence of IgG specific memory B cell in the circulation was significantly higher on day 180. Anti-LPS IgM memory B cells were significantly detectable in circulation on days 90 and 180; responses were almost consistently high during the whole study period. LPS specific IgA, IgG and IgM plasma antibody response were also significantly detectable throughout the study point due to persistence of circulating memory B cells.

LPS was also shown to generate immunity among household contacts of infected patients, and is thus an important candidate for vaccine design (72) or an antigenic component of it would be a more attractive one. Analysis for immune responses targeted to LPS was complicated because of its heterogeneous nature. O-specific polysaccharide (OSP) of *V. cholerae* defines serogroup specificity, most likely to contribute to the immune response to *V. cholerae* LPS. It has already been shown that the OSP component of LPS was sufficient to detect immune responses comparable to those against LPS in patients infected with cholera (6) and was found to be associated with increased levels of vibriocidal antibody titer. The plasma antibody responses were also found correlated with vibriocidal antibody response in the same patients, indicating that responses against LPS, more specifically the antigenic O specific polysaccharide, OSP, mediate the observed vibriocidal antibody responses. In earlier studies, it was shown that there is a strong relationship between LPS and OSP response in the same patients (26). This indicates that the immune response to cholera infection specially targets the OSP component of *V. cholerae* LPS and also contribute to the vibriocidal antibody response.

As an antigenic part of LPS, the protective immunity generated by OSP was studied in this study. Interestingly OSP was found to generate memory B cells and was comparable to LPS generated memory B cell response. Unlike LPS, OSP specific IgA and IgG memory B cell generation was significant and responses remained elevated up to day 180 after onset of disease compared to baseline. Circulating plasma antibody levels for IgA and IgG were found to support findings for the circulating memory B cell responses. OSP specific IgM memory B cell response remained elevated up to day 90 and waned by day 180 but, was higher compared to the baseline response. Plasma IgA, IgG and IgM antibody response specific to OSP remained elevated and significant compared to the baseline response in all the study days after onset of cholera.

Previous studies have shown that Anti-CTB (Cholera toxin B subunit) specific antibodies remain elevated in naturally cholera infected individuals and develop humoral immunity (85). There was no strong correlation between LPS and CTB specific plasma antibody elevation. But anti-LPS and CTB specific IgA antibodies may be associated with natural immunity against cholera infection (33). In earlier studies IgA, and IgG antibodies were found to be elevated up to day 30 while there was no significant increase in IgM levels (81) and suggested not to be associated with long term protection (40). This study revealed gradual decrease in plasma circulating IgA and IgG antibody after day 7 as was anticipated.

In summary, currently available oral killed vaccines are not capable of providing long lasting immunity against cholera for subsequent re exposure over 5 years. A vaccine design capable of generating long lived memory B cells may be able to provide long lasting protective natural immunity in cholera endemic areas. As circulating OSP specific memory B cell response was found to be significantly persistent up to day 180 and comparable to LPS specific memory B cell response when compared to all isotypes, a vaccine targeted to OSP would be an attractive option. Moreover, results were interesting enough to investigate OSP specific memory B cell persistence for long term protective immunity. Since OSP is the antigenic part of LPS and free of endotoxic portion the lipid A component, inclusion of OSP would be a safer and more reliable approach to design an effective vaccine capable of providing robust protection against cholera in the future.

Limitations of present study:

1. As enrolled patients for this study were V. cholerae O1 ogawa confirmed, response against Inaba is yet to study.
2. Although 60 subjects were enough to study, study with greater subject numbers would produce result with more significance.

Recommendation for future work

1. Memory B cell responses against OSP can be demonstrated in vaccinees after vaccination with oral cholera vaccine.
2. Determination of antibody avidity to OSP will be a worth target.
3. Immune responses to OSP can be observed using biopsy specimen of cholera patients.

Chapter 5

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Appendices

a. Used common laboratory apparatus

Name	Description	Source
ELISA plates	96-well	Nunc™, Sweden
EON Microplate Reader	ELISA reader	Bio-tek
Eppendorf tubes	sterilized by autoclaving at 121°C for 20 minutes	Eppendorf®
Micropipette tips	sterilized by autoclaving at121°C for 20 minutes	Sigma
Micropipettes		Thermo Lab systems
Multi-channel Dispenser		Lab System, USA
Petri dishes		Sterilin or Gibco
Plastic tubes and pipettes		Falcon®, Becton, Dickinson and Company
Screw capped tubes and other glass wares		Pyrex® Labware, USA
Sorvall® Legend™ XRT super speed centrifuge	large-scale centrifugation	Thermo Scientific
Sorvall® *Pico* microfuge	Mini scale centrifugations	Thermo Scientific
Vacutainer tubes	Heparin-coated, sterile	Becton Dickinson, Ruther-ford, NJ

b. Reagents Used

1	**Acetic acid** Sigma-758-12-3
2	**BCIP (5-Bromo-4-Chloro-3-Indolyl Phosphate, Para Toludine Salt)** Sigma, B8503
3	**DMF (N, N-Dimethyl-Formamide)** Sigma, D-8654
4	**Ethydium Bromide** Invitrogen, Scotland, UK
5	**FBS (Foetal Bovine Serum Albumin)**

	Gibco BRL- 16140-071
6	**Ficoll** Pharmacia LKB Biotechnology AB Uppsala, Sweden
7	**Goat anti-human IgA-HRP** Southern Biotechnology Associates, Inc. 2050-05
8	**Goat anti-human IgG-AP** Southern Biotechnology Associates, Inc. 2040-04
9	**Goat anti-human IgM-HRP** Hybridoma (Cat#HP6083)
10	**H_2O_2 (Hydrogen Peroxide)** Fisher Scientific, H-325
11	**$MgCl_2$ (Magnesium chloride** Sigma-127-09-3
12	**Na-acetate** Sigma-127-09-3
13	**NaCl (Sodiun Chloride)** Fischer Scientific, Pittsburgh, PA, USA)
14	**NaHCO3 (Sodium bi-carbonate)** Fisher Scientific- S233-500
15	**NBT (Nitro blue tetrazolium)** BioRad, 170-6532
16	**Rabbit anti-human IgA, anti-human IgG and anti-human IgM** Jackson Immune Research Laboratories Inc.
17	**Tween 20 (polyoxietylensorbitanmonolaurat)** Sigma chemical Co., St. Louis, MO, USA

c. Buffer Solutions and Substrates

Phosphate Buffer Saline (PBS) (10X)	
KCl (2.68 mM)	2.0 g
$Na_2HPO_4.12\ H_2O$ (7.7 mM)	27.5 g
NaCl (0.136 M)	80.0 g
KH_2PO_4 (2 mM)	2.75 g
pH	7.2 – 7.4
For PBS- Tween 0.05% Tween 20 was added in 1X PBS	

1M Sodium Citrate (1000 mL)	
Trinatrium Citrate ($Na_3C_6H_5O_4.{}_2H_2O$)	29.4 g
H_2O (Deionized)	1000 mL
pH	4.5

Orthophenylene Diamine (OPD)-Hydrogen peroxide (Substrate) (10mL)	
OPD	10.0 mg
0.1 M Sodium Cictrate	10.0 mL
30% H_2O_2	4.0 mL

0.1% BSA PBS (500 ml)	
Phosphate Buffer Saline (PBS)	450 ml
1% BSA in PBS	50 ml

1% FBS PBS Tween (500 ml)	
Phosphate Buffer Saline (PBS)	450 ml
1% BSA in PBS	50 ml

BCIP (4 ml)	
DMF	4ml
BCIP	60 g

NBT (4 ml)	
70% DMF	4 ml
NBT	120 mg

Carbonate buffer (1000 ml)	
0.1M $NaHC0_3$	8.4 g
5mM $MgCl_2.6H_20$	1.0 g
Deionized water	1000 ml

ALP chromogen/substrate: BCIP/NBT (50 ml)	
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	49 ml

HRP chromogen /substrate: AEC/H2O2 (10 ml)	
AEC	10.0 mg
DMF	1.0 ml
The mixture is then dissolved in 30 mL of 0.1M sodium acetate buffer (16.4 g of 0.2M Na-acetate dissolved in 1000 ml of distilled water, and then 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. It remains stable at 4^0C for a week.	

Tryphan blue	
NaCl	0.81 g
Tryphan blue	0.4 g
KH2PO4	0.06 g
Deionized water	100.0 ml

d. Media Composition

RPMI Complete (220 ml)	
RPMI 1640 (1X)	194 ml
Fetal Bovine Serum (FBS- 10%)	20 ml
Pcn/Strp (Penicillin-Streptomycin- 1%) Pen-10,000 IU/mL, Strep-10,000 ug/mL	2 ml
Na-Pyruvate 100 mM (1%)	2 ml
L- Glutamine 200 mM (1%)	2 ml

MBCS media (220 ml)	
RPMI 1640 (1X)	194 ml
Fetal Bovine Serum (FBS- 10%)	20 ml
Pcn/Strp (Penicillin-Streptomycin- 2%) Pen-10,000 IU/mL, Strep-10,000 ug/mL	4ml
L- Glutamine 200 mM (1%)	2 ml
BME	22 µl

TTGA Culture Media	
Trypticase (Casein) (BD)	10 g
Nacl (BDH/MERCK)	10 g
Sodium taurocholate (Sigma)	5 g
Sodium carbonate (Sigma)	1.2-1.5 g
Gelatin (BDH)	30 g
Agar (BD)	16 g
D/W	1 L

pH	8.5-9.0

Boil, then sterilize at 15 Ibs pressure for 15-20 minutes. Before pouring the plates cool about 50^0C, add potassium tellurite (K_2TeO_3)(5µg/ml).

e. Software

1. MS word
2. Microsoft Excell
3. Instat2 (Graph PAD Software 10855, USA)
4. Endnote X7
5. Ascent software