# Immune Response of Oral Cholera Vaccine, Shanchol in Bangladeshi Recipients

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**Dedication**

*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 "Immune Response of Oral Cholera Vaccine, Shanchol in Bangladeshi Recipients" submitted by Sultana Rownok Jahan, has been carried out by the under signed under 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, an acute and severe dehydrating diarrheal disease caused by *Vibrio cholerae* O1 remains a major public health concern which has significant morbidity and mortality worldwide. Several efforts have been made to develop cholera vaccines that could confer long term protection. T cells play an important role in immunity to cholera and may contribute to the activation of B cells. So, understanding the memory T cell responses is important for optimizing design of oral cholera vaccine or immunization strategies. It had been investigated that immunization with the oral cholera vaccine, Shanchol, induces *V. cholerae* antigens specific CD4+ and CD8+ memory T cell responses in Bangladeshi adult participants. The study enrolled 45 adult healthy participants and divided them into three groups (In each group n=15) where two groups received double doses of the vaccine (14 days and 30 days apart) and another group received single dose of the vaccine alone. The main objective of the study was to examine *V. cholerae* antigens, including membrane preparation (MP) and mutant CT (mCT) specific T cell responses by the flow cytometric assay of the specific cell-mediated immune responses in activated whole blood (FASCIA). After vaccination stimulation with MP showed better proliferative CD4+/CD45RO+ and CD8+/CD45RO+ memory T-cell responses than mCT at day 7 compared to baseline in all vaccine cohorts. However, one month after the last dose of the vaccine, the responses were all most diminished over the period of day 90 in all vaccination regimens. Overall a high baseline proliferative CD4+ and CD8+ T cell responses to mCT was found in all vaccinee groups but it did not show increased memory T cell responsiveness significantly to mCT after vaccination. This is because the absence of cholera toxin (CT) and presence of Lipopolysaccharide (LPS) of different *V. cholerae* strains in the vaccine. Again, *V. cholerae* O1 Ogawa and Inaba specific vibriocidal antibody responses were also observed maximum at day 7 after administration of one dose of vaccine in all vaccine cohorts. After one month of last dose of the vaccination, the responses started to wane gradually and became vastly reduced at subsequent study days in all vaccination regimens. In contrast the two doses given 30 days apart showed significantly increased responses in comparison to baseline over the period of day 90. These findings suggested that single dose vaccine regimen was as effective as double dose vaccine regimens. So it can be concluded that this early proliferative T cell response

is a potent tool that provides help for the generation of subsequent memory B cell responses. However, additional studies are needed to determine whether these early T-cell-mediated events would help to design a more effective vaccine as well as provide long-lasting immunity.

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# ABBREVIATIONS

| | |
|---|---|
| AMI | Antibody-mediated immunity |
| ADP | Adenosine diphosphate |
| ADPR | Adenosine diphosphate – ribose |
| APC | Allophycocyanin |
| APCs | Antigen presenting cells |
| BD | Becton Dickinson |
| BSA | Bovine serum albumin |
| BHI | Brain Heart Infusion |
| CFTR | Cystic fibrosis transmembrane conductance regulator |
| CTLs | Cytotoxic T lymphocytes |
| cAMP | Cyclic adenosine monophosphate |
| CMI | Cell-mediated immunity |
| CCR7 | Chemokine receptor 7 |
| CCR9 | C-C chemokine receptor 9 |
| CCL-25 | CC-chemokine ligand 25 |
| CD | Cluster of differentiation |
| CF | Clonization factors |
| CSR | Class switch recombination |
| CT | Cholera toxin |
| CTA | A-subunit of cholera toxin |
| CTB | B- subunit of cholera toxin |
| CXCR5 | Chemokine Receptor 5 |
| DC | Dendritic cell |
| DMEM | Dulbecco Modified Eagle Medium |
| EDTA | Ethylene diamine tetraacetic acid |
| ERC | Ethical review committee |
| FACS | Flurochrome activated cell sorter |

| | |
|---|---|
| FCS | Flow cytometry Standard |
| FITC | Fluorescein isothiocyanate |
| g | Gram |
| GALT | Gut Associated Lymphoid Tissue |
| GC | Germinal center |
| GM1 | Monosialosyl ganglioside |
| GTP | Guanosine tri  phosphate |
| GTPase | Guanosine tri  phosphatase |
| HRP | Horse-radish peroxidase |
| icddr,b | International Centre for Diarrhoeal Disease Research, Bangladesh |
| IEL | Intra-epithelial lymphocytes |
| IFN | Interferon |
| Ig | Immunoglobulin |
| Ig G | Immunoglobin G |
| IL | Interleukin |
| LPS | Lipopolysaccharide |
| LSD | Laboratory Sciences Division |
| LNs | lymph nodes |
| M cell | Membrane epithelial cell |
| MAdCAM-1 | Mucosal vascular–addressin cell-adhesion molecule 1 |
| mCT | Mutant cholera toxin |
| MALT | Mucosa associated lymphoid tissue |
| MHC | Major histocompatibity complex |
| Mf T cells | Multifunctional T cells |
| min | Minute |
| mL (ml) | Mililiter |
| mM | Milimolar |

| | |
|---|---|
| MP | Cholera membrane preparation |
| MW | Molecular weight |
| NKT cells | Natural Killer T Cells |
| NAD | Nicotinamide adenine dinucleotide |
| NALT | Nasopharinx-associated lymphoid tissue |
| ORS | Oral rehydration solution |
| OD | Optical density |
| PBS | Phosphate Buffer Saline |
| PE | Phyco erythrocyanin |
| PerCP | Peridinin-chlorophyll-protein Complex |
| pT | Precursor T cells |
| RBC | Red blood cell |
| rCTB | Recombinant cholera toxin B subunit |
| rpm | Rotation per minute |
| RRC | Research review committee |
| SEM | Standard error of mean |
| sIg | Secretory immunoglobulin |
| $T_c$ | Cytotoxic T cell |
| $T_{reg}$ cells | Regulatory T cells |
| TCP | Toxin-coregulated pilus |
| TCR | T-cell antigen receptor |
| $T_{EM}$ cells | Effector memory T cells |
| $T_{EMRA}$ cells | Effector memory RA |
| $T_{fh}$ | Follicular helper T cell |
| $T_H$ | T helper cell |
| TNF | Tumor necrosis factor |
| *V. cholerae* | *Vibrio cholerae* |

# Chapter 1

# Introduction

## 1.1. Cholera- the oldest epidemic prone disease

Cholera, a severe diarrheal disease, has been well known from the nineteenth century and still remains the most feared and stigmatized disease, particularly in the developing countries of Asia, Africa, and recently, the Caribbean islands. It is an acute intestinal infection characterized by profuse watery diarrhea; vomiting and rapid dehydration. Cholera is one of the oldest and best understood of the epidemic prone diseases (John D. Clemens 2014). It is endemic in more than 50 countries causing 3 to 5 million cases of secretory diarrhea and over 100,000 deaths annually (Zuckerman, Rombo et al. 2007) (WHO 2010).

*Vibrio cholerae* is the causative microorganism of this disease and human body is the only known natural host of this organism. The disease is spread mainly by fecal-oral contamination of water and food. But direct transmission from person to person is not uncommon (Nelson EJ 2011) (Weil, Khan et al. 2009).

Though naturally acquired infection has been shown to protect against subsequent symptomatic disease for 3 to 8 years (Koelle, Rodo et al. 2005) (Levine, Black et al. 1981), currently available oral killed cholera vaccines (OCVs) provide protection for 6 to 36 months, depending on which OCV is studied and the age of the recipient (Sinclair, Abba et al. 2011) (Sur D 2011 ) (WHO 2010).

It has been shown that the systemic and mucosal memory play an important role in protective immunity induced by vaccines (Sallusto, Lanzavecchia et al. 2010) and T cells play an important role in immunity to cholera (Kuchta, Rahman et al. 2011) (Harris, Bhuiyan et al. 2009) (Weil, Khan et al. 2009) (Bhuiyan, Lundin et al. 2009). Therefore, understanding the relationship between the developments of memory T cells response could be critical to optimizing oral cholera vaccine design or immunization strategies.

## 1.2. *Vibrio cholerae*- The etiologic agent of cholera

**1.2.1. General Characteristics:** *Vibrio cholerae* is a curved, Gram-negative rod, facultative anaerobic, comma-shaped bacterium belong to the family vibrionaceae and genus *Vibrio.* It comprises of a microscopic size around 1 µm in width and 2-3 µm in length and motile by at least one polar flagellum (Baumann 1984). *Vibrio*s display a wide variation in colony morphology and color (Stoll 1982).

**Figure 1.1:** ***Vibrio cholerae*** **(http://www.futura.sciences.com)**

Although *V. cholerae* can grow in water of lower salinity, these organisms grow best in the presence of salt and warmer weather (Colwell 1996).

### 1.2.2. Genomic Structure:

*V. cholerae have* two circular chromosomes, together with 4 million base pairs of DNA sequence and 3,885 predicted genes (Heidelberg, Eisen et al. 2000). The genes for cholera toxin are carried by CTXphi (CTXφ), a temperate bacteriophage inserted into the *V. cholerae* genome. CTXφ can transmit cholera toxin genes from one *V. cholerae* strain to another through horizontal gene transfer. The genes for toxin coregulated pilus are coded by the VPI pathogenicity island (VPIφ) (Control 2013). *V. cholerae* contains a genomic island of pathogenicity and is lysogenized with phage DNA. Therefore the genes of a virus were integrated into the bacterial genome which made the bacteria pathogenic (Control 2013).

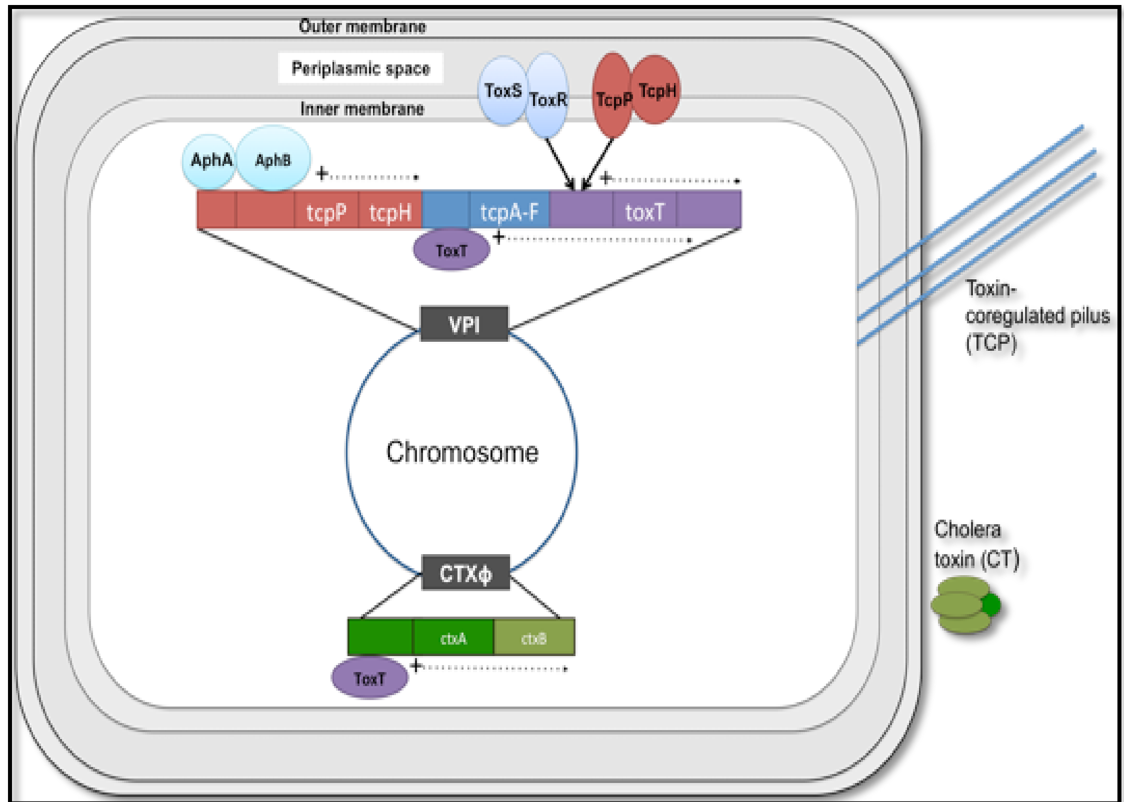


**Figure 1.2: Genomic Structure of *V. cholerae*.** The expression of the primary *V. cholerae* virulence factors, toxin-coregulated pilus and cholera toxin, occurs via a transcriptional cascade involving several activator proteins and serves as a paradigm for the regulation of bacterial virulence (Control, C. f. D. 2013).

### 1.2.3. Classification

*V. cholerae* is classified into more than 200 serogroups based on the O antigen of the lipopolysaccharide (LPS) antigen (Morris 2003). Among these, only O1 and O139 serogroups cause epidemic cholera. *Vibrio cholerae* O1 is further classified into two biotypes, classical and El Tor (Sack, Sack et al. 2004). Two major serotypes exist, Ogawa and Inaba, which vary in prevalence with time (Longini, Nizam et al. 2007).

Strains of the Ogawa serotype express the A and B antigens and a small amount of C antigen, whereas Inaba strains express only the A and C antigens. A third serotype (Hikojima) expresses all three antigens but it is rare and unstable (Sack, Sack et al. 2004). The O1 El Tor biotype predominates in most current infections. These two

serogroups- O1 and O139 are pathogenic due to their production of an enterotoxin, cholera toxin (CT) that promotes the secretion of fluids and electrolytes into the intestinal lumen, resulting in diarrhea. The sixth pandemic and presumably the fifth were caused by *V. cholerae* O1 of the classical biotype (Pazzani, Scrascia et al. 2006), but the seventh pandemic was due to the El Tor, which started in 1961 and even continues today (WHO 2010).

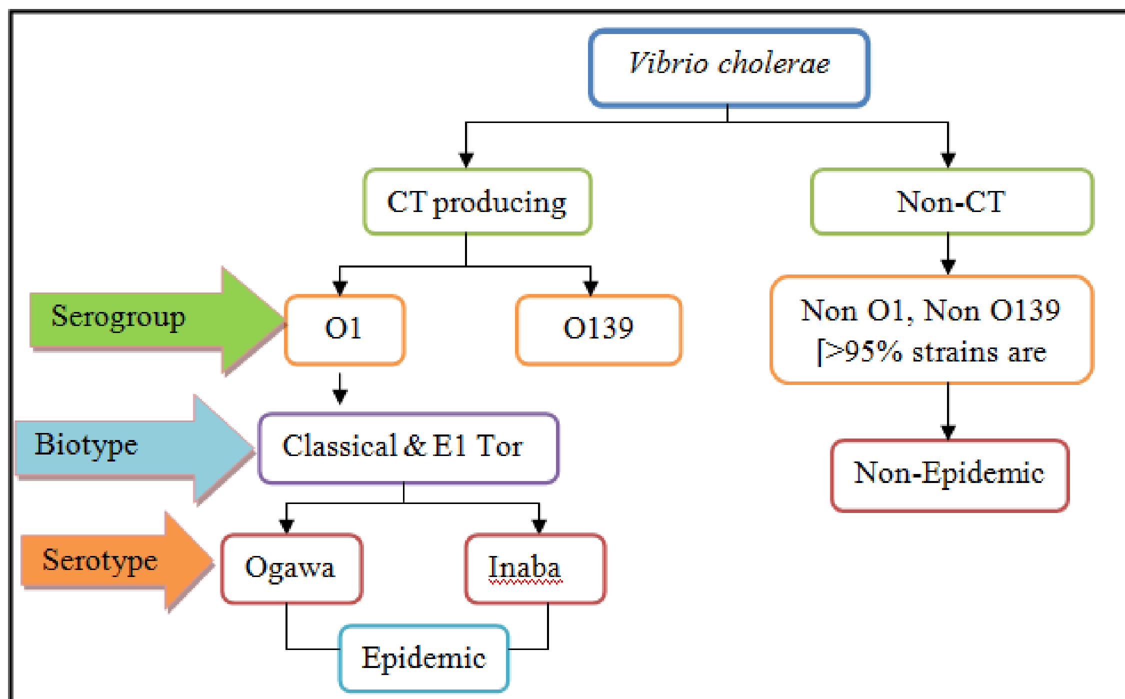


**Figure 1.3: The current classification scheme of epidemic and non-epidemic strains of *V. cholerae***

*V. cholerae* O1 and O139 cause clinical disease by producing Cholera toxin (CT) that promotes the secretion of fluid and electrolytes into the lumen of the small intestine.

## 1.3. Virulence factors

### 1.3.1. Cholera toxin – an enterotoxin

The cholera toxin is an oligomeric complex made up of six protein subunits: a single copy of the A subunit (part A, enzymatic), and five copies of the B subunit (part B,

receptor binding), denoted as $AB_5$. A1 and A2 are connected by a single disulfide bond (Zhang, Scott et al. 1995). The B subunits are responsible for binding to a ganglioside (monosialosylganglioside, GM1) receptor located on the surface of the cells that line the intestinal mucosa (Shamini, Ravichandran et al.).

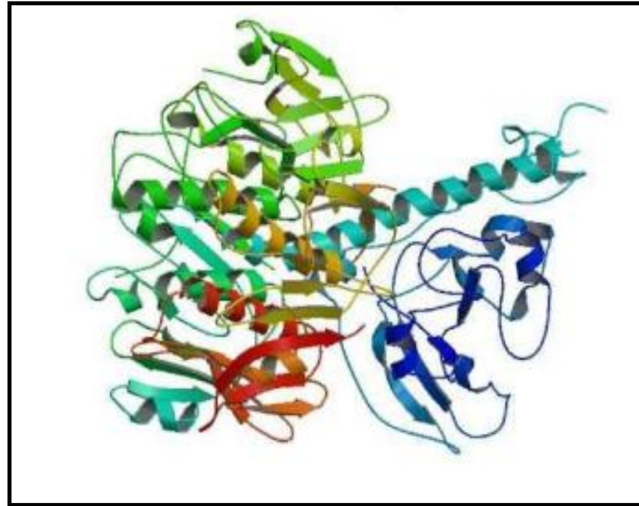

**Figure 1.4: 3D structure of Cholera toxin**
**(http://www.rcsb.org/pdb/explore/explore.do?structureId=1xtc)**

Although being synthesized as a single polypeptide chain, CTA is post-translationally modified through the action of a *V. cholerae* protease that generates two fragments, CTA1 and CTA2, which however still remain linked by a disulphide bond. The toxic (enzymatic ADP-ribosylating) activity of CTA resides in CTA1, whereas CTA2 serves to insert CTA into the CTB pentamer. The CTB pentamer is held together by approximately 130 hydrogen bonds and 20 salt bridges which render stability of pentameric CTB to proteases, bile components and other factors in the intestinal milieu. It has been suggested that pentamer-pentamer interactions may possibly further add to the stability (Sanchez and Holmgren 2011).

### 1.3.2. Lipopolysaccharide (LPS) - an endotoxin

All Gram-negative bacteria express a glycolipid component called endotoxin, lipopolysaccharide (LPS), which participate in the physiological functions of the

bacterial outer membrane. The LPS is composed of lipid region, core region and variable polysaccharide region (Fig-1.5). Toxicity is associated with the lipid component (Lipid A) and immunogenicity is associated with the polysaccharide components and both act as determinants of virulence. Polysaccharide attached to core region (O side chain) consists of repeating oligosaccharide subunits made up of 3 - 5 sugars unit and vary up to 40 repeat units. *V. cholerae* O1 differs from other *V. cholerae* O serogroups by O side chain orientation. O antigen and capsule play important role for intestinal colonization (Chiang and Mekalanos 1999) (Nesper, Lauriano et al. 2001) (Waldor, Colwell et al. 1994) (Iredell, Stroeher et al. 1998) (Angelichio, Spector et al. 1999) (Attridge, Fazeli et al. 2001). LPS core oligosaccharide (core-OS) also has such role (Herrington 1988) (Lee 1999).

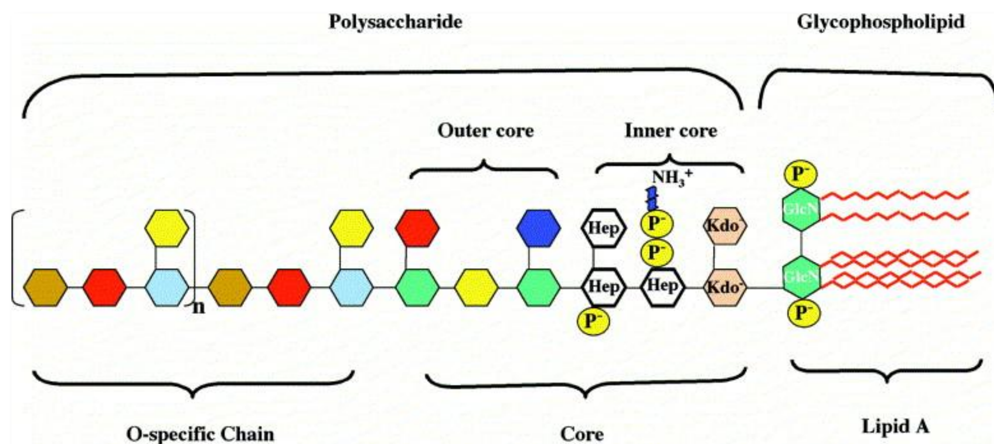


**Figure 1.5:** Lipopolysaccharide of gram negative bacteria: LPS consists of O side chain, core polysaccharide and lipid Aregion (http://origin-ars.els-cdn.com/content/image/1-s2.0-S128645790201612X-fx1.jpg)

In addition to causing endotoxic shock, LPS is pyrogenic, can activate macrophages and complement, and is mitogenic for B lymphocytes that induce interferon production. It also causes tissue necrosis and tumor regression and has adjuvant properties. The endotoxic properties of LPS reside largely in the lipid A components (Finkelstein 1962).

## 1.4. Pathogenesis of Cholera

Although the cholera toxin (CT) is directly responsible for the manifestation of diarrhea, cholera pathogenesis associated with a number of factors acting synergistically to promote virulence. The pilus colonization factor, toxin-coregulated pilus (TCP) mediate colonization of brush borders of epithelial cell of the small intestine (Faruque, Nair et al. 2004). Colonization is a prerequisite to establish a productive infection by *V. cholerae.*

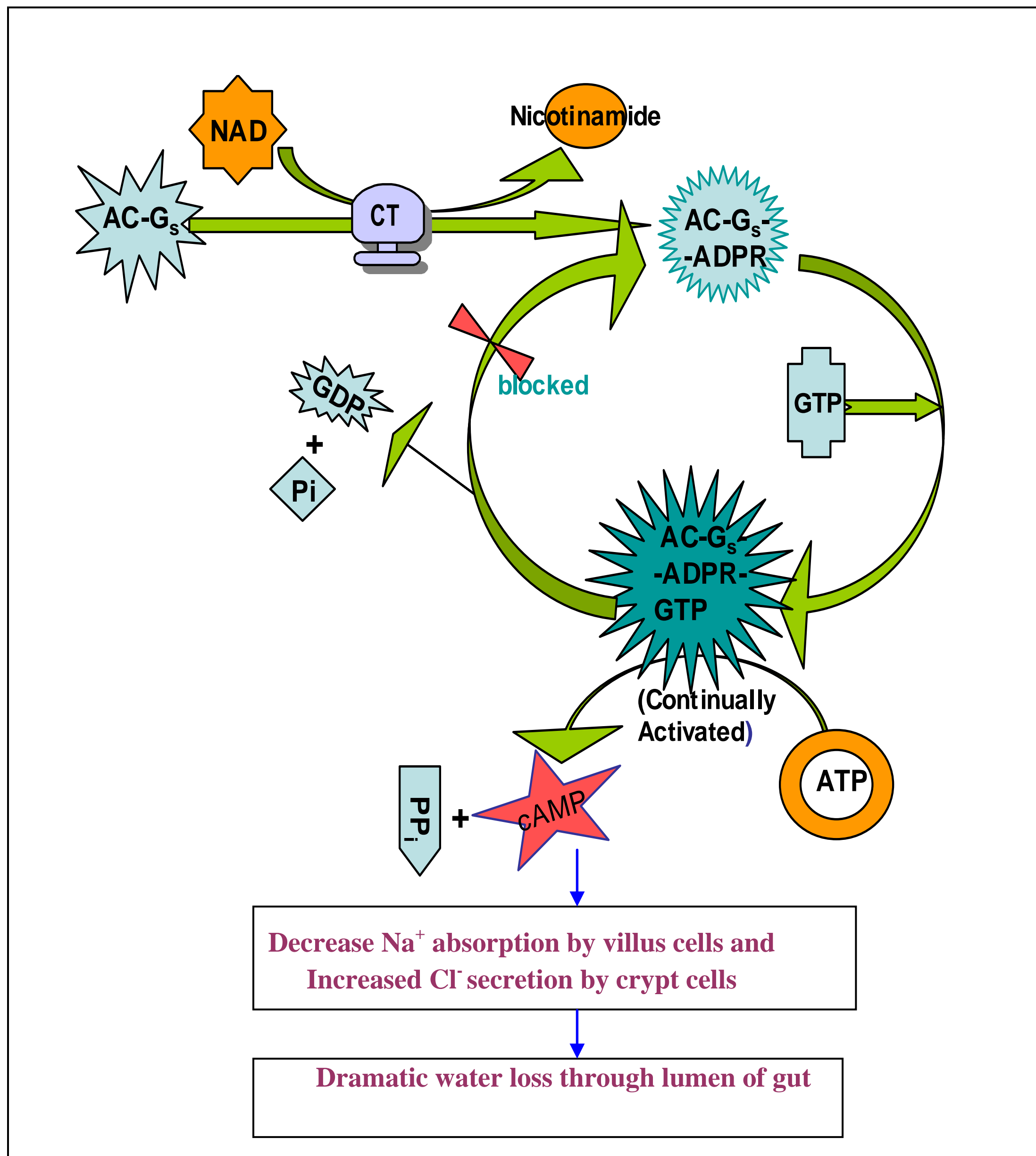


**Figure 1.6: Mode of action of cholera toxin**

After successful colonization, *V. cholerae* secrete a potent enterotoxin (CT). This enterotoxin is an 87-KD protein, consisting of five B subunits (103 amino acid residues in each) and one A subunit (240 amino acid residues in which the B subunits form a pentagonal ring surrounding the A subunit (Kenneth Todar 2012).

The toxin starts to exert its effect by irreversibly binding to its cell surface receptor monosialosylganglioside (GM1 ganglioside) present on the surface of intestinal mucosal cells (Kovach 1996 ). CT is taken into the cell, possibly via receptor mediated endocytosis. This process is accompanied by cholera toxin activation through the proteolytic cleavage and disulfide bond reduction of the A subunit to two fragments, A1 (~195 residues) and A2 (~ 45 residues) where upon A1 is released into the cytosol (Kaper 1994). Once inside the cell, A1 catalyzes the transfer of the ADP-ribose unit from $NAD^+$ to an Arginine side chain of $G_s\alpha$, ADP ribosylated $G_s\alpha$GTP can activate adenylate cycalse but is incapable of hydrolyzing its bound GTP. As a consequence, Cholera toxin activated adenylate cyclase remains "locked" in its active state (Kaper 1994). This leads to uncontrolled production of intracellular cAMP (Fig 1.6).

Elevated cAMP level in turn activates protein kinase A, which opens the luminal cystic fibrosis transmembrane conductance regulator (CFTR) $Cl^-$ channel and inhibits the $Na^+/H^+$ exchanger by protein phosphorylation. Thus increase secretion of $H_20$, $Na^+$, $K^+$, $Cl^-$, and $HCO_3^-$ into the lumen of the small intestine. The net result is voluminous, life threatening intestinal electrolyte and fluid secretion in cholera patient.

## 1.5. Epidemiology

Cholera occurs in both endemic and epidemic patterns. In the past decade, devastating epidemics of cholera occurred in Angola, Ethiopia, Zimbabwe, Pakistan, Somalia, Sudan, Vietnam, and Haiti. Among immunologically naive populations, cholera affects all age groups, and epidemics can be associated with high case-fatality rates (Harris 2008). In Asia, cholera occurs seasonally before and after the monsoon rains (Sack, Sack et al. 2004). The incidence is highest in children, and the disease can occur in neonates (Deen, von Seidlein et al. 2008) (Khan, Hossain et al. 2009). This pattern was recorded in Haiti, where cholera had been notably absent before 2010 (Harris 2012). The first

cholera pandemic occurred in the Bengal region of India starting in 1817 through 1824. The disease dispersed from India to Southeast Asia, China, Japan, the Middle East, and southern Russia. The second pandemic lasted from 1827 to 1835 and affected the United States and Europe particularly due to the result of advancements in transportation and global trade, and increased human migration, including soldiers (Harris 2012).

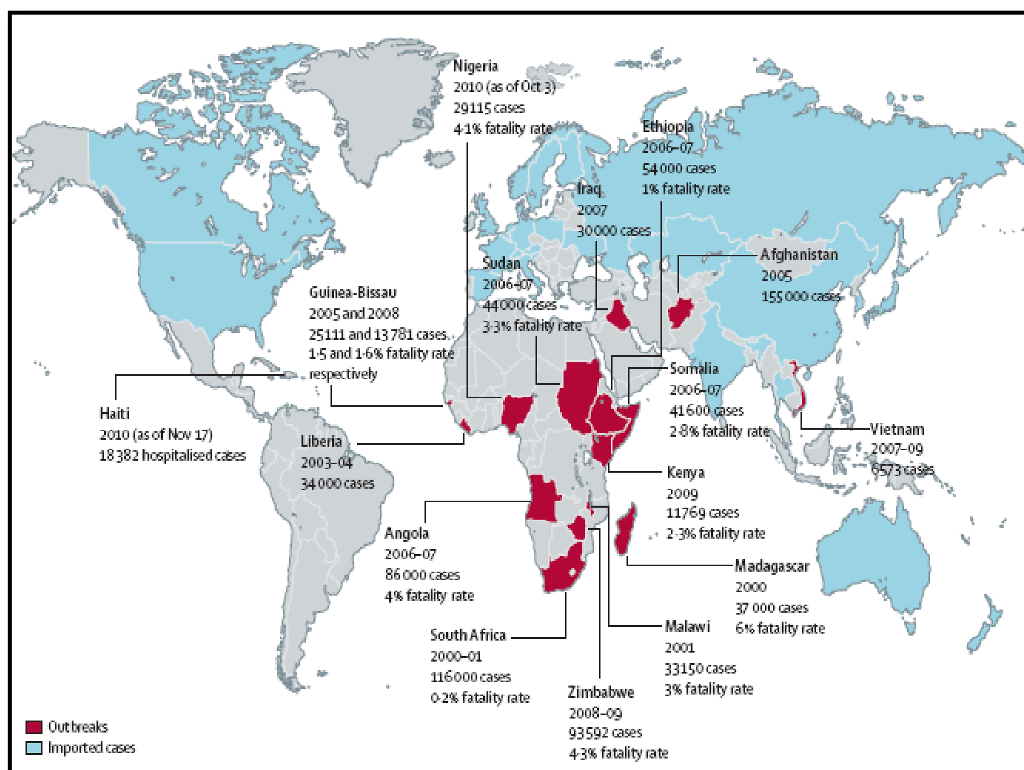


**Figure 1.7: Major cholera outbreaks after 2000 (Harris 2012)**

The third pandemic erupted in 1839, persisted until 1856, extended to North Africa, and reached South America, for the first time specifically infringing upon Brazil. Cholera hit the sub-Saharan African region during the fourth pandemic from 1863 to 1875. The fifth and sixth pandemics ranged from 1881–1896 and 1899-1923.

The final seventh pandemic originated in 1961 in Indonesia and is marked by the emergence of a new strain, nicknamed *El Tor*, which still persists today in developing countries (Bishop 2011) (Harris 2012).

Late in October 2010, a cholera outbreak was reported in Haiti (Times 2010). A recent study concluded that the Haitian cholera epidemic was most likely a result of an

introduction of a strain from a distant geographic location and derived from human activity. The examined genomic diversity of Haitian isolates was presented an effort to contribute to the understanding of the origin.

## 1.6. Clinical manifestations of cholera

### 1.2.1. Infectious Dose

The infectious dose of *V. cholerae* O1 has been estimated to be $10^5 - 10^8$ CFU in human volunteers, but may be as low as $10^3$ CFU in the presence of achlorhydria (Nelson, Harris et al. 2009).

### 1.2.2. Incubation Period

The incubation period ranges between 12 hours to 5 days (Nelson, Harris et al. 2009)

### 1.2.3. Signs and Symptoms

Onset of cholera is sudden with profuse, watery diarrhea with or without symptoms such as anorexia and abdominal discomfort (Sack, Sack et al. 2004). The stool color becomes pale gray with an inoffensive, slightly fishy odor. Mucus in the stool imparts the characteristic rice water appearance (Sack, Sack et al. 2004). Vomiting is often present, occurring a few hours after the onset of diarrhea (Clemens, Sack et al. 1986). Muscle cramps may occur as water and electrolytes are lost from body tissues. Loss of skin turgor, scaphoid abdomen, and weak pulse are characteristic of cholera. The initial stool may exceed 1 L, and several liters of fluid may be secreted within hours, leading to hypovolemic shock (Finkelstein 1996).

## 1.7. Prevention and diseases management

The treatment for cholera recommended by WHO is oral rehydration solution (ORS), which reduces mortality from 50% down to 1%. In severe cases intravenous fluid replacement is necessary. Antibiotics can be used to treat severe cholera and may shorten the duration of disease and thereby decrease the risk for further spread of the disease. Mass administration of antibiotics is not recommended, as it has no effect on the spread of cholera and contributes to increasing antimicrobial resistance (WHO 2012)

(WHO 2009). The best way to avoid cholera is to have access to safe water supplies, and avoid contaminated foods. However, safe water supplies are not available to a great proportion of the world population. Therefore, an effective cholera vaccine is an urgent need to prevent illness and deaths of thousands of people around the world and this is why the effectiveness of Shanchol vaccine is investigated in this study.

## 1.8. The Immune System

The immune system is an interactive network of lymphoid organs, cells, humoral factors, and cytokines. The essential function of the immune system in host defense is best illustrated when it goes wrong; under activity resulting in the severe infections and tumors of immunodeficiency, over activity in allergic and autoimmune disease. The immune system responds in a specific way to pathogens and displays a long-term memory of earlier contacts with the disease agents (Parkin and Cohen 2001).

### 1.8.1. Innate & Adaptive Immune System

Immunity is divided into two “lines of defense” determined by the speed and specificity of the reaction. The first, representing a non-specific (no memory) response to antigen (substance to which the body regards as foreign or potentially harmful) known as the innate immune system; and the second, the adaptive immune system, which displays a high degree of memory and specificity (Parkin and Cohen 2001).

**The innate immune system** represents the first line of defense to an intruding pathogen. The innate immunity is sometimes used to include physical, chemical, and microbiological barriers, but more usually encompasses the elements of the immune system (neutrophils, monocytes, macrophages, complement, cytokines, and acute phase proteins) which provide immediate host defense. The highly conserved nature of the response, which is seen in even the simplest animals, confirms its importance in survival (Parkin et al. 2001) .

**Adaptive immunity** is the hallmark of the immune system of higher animals. This response consists of antigen-specific reactions through T lymphoctyes and B

lymphocytes. Whereas the innate response is rapid but sometimes damages normal tissues through lack of specificity, the adaptive response is precise, but takes several days or weeks to develop. The adaptive immune response has memory, so that subsequent exposure leads to a more vigorous and rapid response, but this is not immediate {Parkin, 2001 #31}

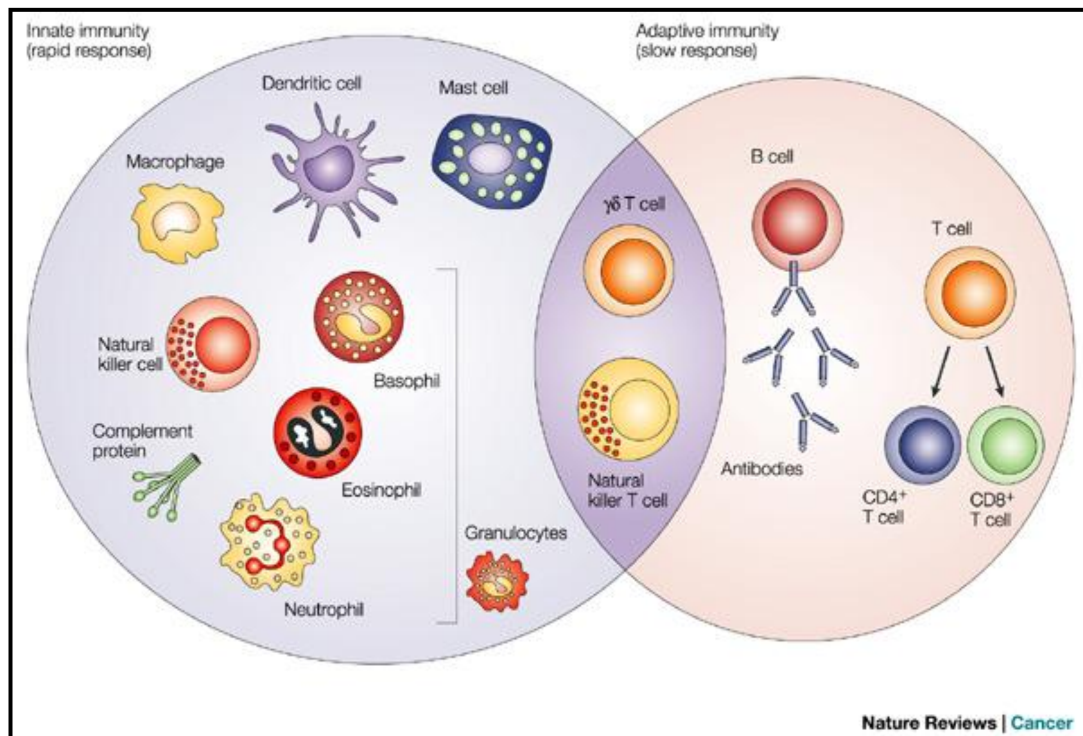


**Figure 1.8: Innate and adaptive immunity (Dranoff 2004)**

### 1.8.2. Types of adaptive immune responses

There are two types of adaptive immune responses (Abul K. Abbas 2007):

a) Humoral immunity or Antibody-mediated immunity (AMI)
b) Cell-mediated immunity

**Antibody-mediated immunity** (AMI) is the type of adaptive immunity that is mediated by soluble host proteins called antibodies or immunoglobulins. It is largely due to the presence of circulating antibody molecules in the serum and is also called circulating immunity or humoral immunity. If a naive (unstimulated) B cell encounters an antigen,

it is stimulated to develop into a plasma cell which produces the antibodies that will react with the stimulating antigen. They also develop into clones of identical reactive B-cells called memory B-cells (Kenneth Todar 2012).

**Cell-mediated immunity** (CMI) is the type of adaptive immunity that is mediated by specific subpopulations of T-lymphocytes called effector T-cells. In non immune animals precursor T-cells (pT cells) exist as "resting T cells". They bear receptors for specific antigens. Stimulation with antigens results in their activation.

After that, the cells enlarge, enter into a mitotic cycle, reproduce and develop into effectors T-cells whose activities are responsible for this type of immunity. They also develop into clones of identical reactive T-cells called memory T-cells (Kenneth Todar 2012).

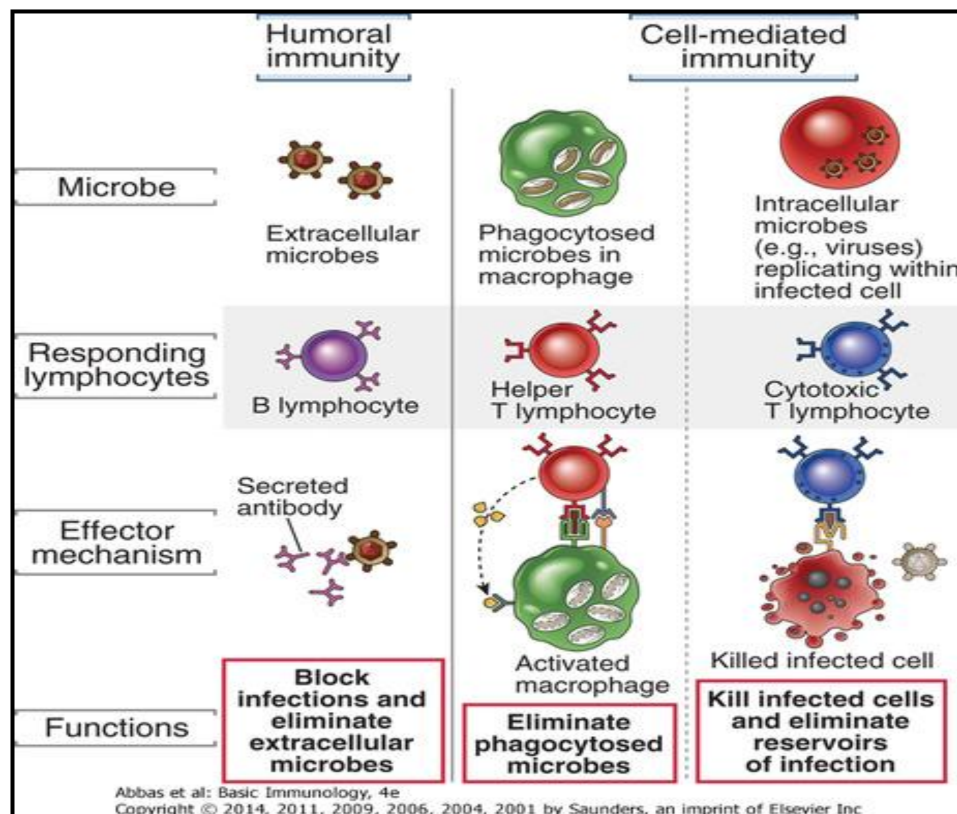


**Figure 1.9: Types of adaptive immunity (Abbas 2010)**

Both activated T helper ($T_H$) cells and cytotoxic T lymphocytes (CTLs) serve as effectors cells in cell-mediated immune reactions. Cytokines secreted by $T_H$ cells can activate various phagocytic cells, enabling them to phagocytose and kill microorganisms more effectively. CTLs participate in cell-mediated immune reactions by killing altered self-cells; they play an important role in the killing of virus infected cells and tumor cells (Richard A. Goldsby 2006).

### 1.8.3. Cellular components of the Immune System

The immune system has developed to protect the host from pathogens and other foreign substances. The immune system consists of many cells that take part in the response against pathogen  (Male 2006).

#### 1.8.3.1. B-cell

About 5-15% of the circulating lymphoid pool is B cells, which are defined by the presence of the surface immunoglobulin. These immunoglobulins function as membrane bound antigen receptors on B cell and soluble circulating antibodies (Roitt Ivan 2001). Depending on the structure of the heavy chain, immunoglobulins have multiple classes and sub-classes: IgG (G1-G4) - 76%, IgM-8%, IgA (A1, A2)-15%, IgD-1%, IgE-0.002%. B-cells perform two important functions; they differentiate into plasma cells and produce antibodies, and they serve as APCs. When $T_H$ cells sensitizes or primes the naive B cell, it undergoes clonal selection, which means the antigen specific surface Ig (sIg) containing B-cell is selected and activated to expand into a clone of cells with the same antigen specificity. Most of the family of clones becomes plasma cells. These cells, after an initial lag, produce highly specific antibodies at a rate of as many as 2000 molecules per second for four to five days. The other B cells become long-lived memory cells (Finkelstein 1962).

#### 1.8.3.2. T Cells

T-lymphocytes belong to a group of white blood cells (WBC) known as lymphocytes are defined by expressing the T cell receptor (TCR), a complex of trans-membrane proteins

able to recognize a peptide excised from a protein-antigen, if this peptide is presented on MHC. They are called T cells because they mature in the thymus (although some also mature in the tonsils) (Alberts B 2002) (Ward 2014).

There are two major types of T cells, Helper T cells ($CD4^+$) and cytotoxic T cells (CD8+). CD4+ T cells account for 45% (900/µL) of lymphocytes while CD8+ T cells account for 30% (600/µL). There are several subsets of T cells, each with a distinct function.

#### 1.8.3.2.1. Subsets of T-Cells

- **Helper T Cells ($T_H$)**

As this population of T cell expresses CD4 glycoprotein on their surface, these cells named as CD4+ T cells. They recognize antigens when presented along with Class II MHC molecules. T helper cells ($T_H$ cells) assist other white blood cells in immunologic processes, including maturation of B cells into plasma cells and memory B cells, and activation of cytotoxic T cells, inflammatory leukocytes and macrophages and secretes cytokines (Gutcher and Becher 2007). These cells can differentiate into one of several subtypes, including $T_H1$, $T_H2$, $T_H3$, $T_H17$, Th9, or $T_{FH}$, which secrete different cytokines to facilitate a different types of immune responses (Gutcher and Becher 2007).

**Types of Helper T Cells**

**$T_H1$:** These cells participate in cell-mediated immunity. They are essential for controlling such intracellular pathogens such as viruses and certain bacteria. $T_H1$ cells help B cells to produce antibody and they activate macrophages by secreting interferon γ (IFNγ). They provide cytokine-mediated "help" to cytotoxic T cells possibly the body's most potent weapon against intracellular pathogens (Abbas 2010).

**$T_H2$:** $T_H2$ cells produce IL-4, IL-5, IL-6, IL-10 and IL-13. These provide help for B cells and are essential for the production of IgE antibodies and perhaps assist in the production of other classes as well (Abbas 2010).

**$T_H$17:** $T_H$17 cells are named for the cytokine IL-17 they produce. These protect surfaces (skin, lining of the intestine) against extracellular bacteria. They seem to enhance neutrophil action early in an adaptive immune response. Effector $T_H$17 cells leave the lymph node and migrate to the site of infection. There, they re-encounter their antigenic peptides presented on macrophage MHC II and respond with secretion of IL-17 and IL-22. Most cells express IL-17 receptors (Abbas 2010).

- **Follicular Helper T cell**

These cells provide help to B cells enabling them to develop into antibody-secreting plasma cells. This occurs in nests of lymphoid cells called follicles in the lymph nodes.

- **Cytotoxic T Cells ($T_C$)**

Cytotoxic T cells ($T_C$ cells or CTLs) destroy virally infected cells and tumor cells, and are also implicated in transplant rejection. These cells are also known as $CD8^+$ T cells since they express the CD8 glycoprotein at their surface. These cells recognize their targets by binding to antigen associated with MHC class I molecules, which are present on the surface of all nucleated cells. Through IL-10, adenosine and other molecules secreted by regulatory T cells, the $CD8^+$ cells can be inactivated to an anergic state, which prevents autoimmune diseases (Willinger, Freeman et al. 2005).

- **Memory T cells**

Memory T cells are a subset of antigen-specific T cells that persist long-term after an infection has resolved. They quickly expand to large numbers of effector T cells upon re-exposure to their cognate antigen, thus providing the immune system with "memory" against past infections. Memory T cells comprise three subtypes: central memory T cells ($T_{CM}$ cells) and two types of effector memory T cells ($T_{EM}$ cells) and effector memory RA ($T_{EMRA}$ cells) (Willinger, Freeman et al. 2005).

Memory cells may be either $CD4^+$ or $CD8^+$. They typically express the cell surface protein CD45RO (Akbar, Terry et al. 1988). Long-term immunological protection depends on both the quantity and quality of the memory T cells that are formed. Vaccine boosters most probably enhance immunological protection by affecting the quality and quantity of memory T cells. Therefore, memory T cell is used as a parameter to evaluate Shanchol vaccine in this study.

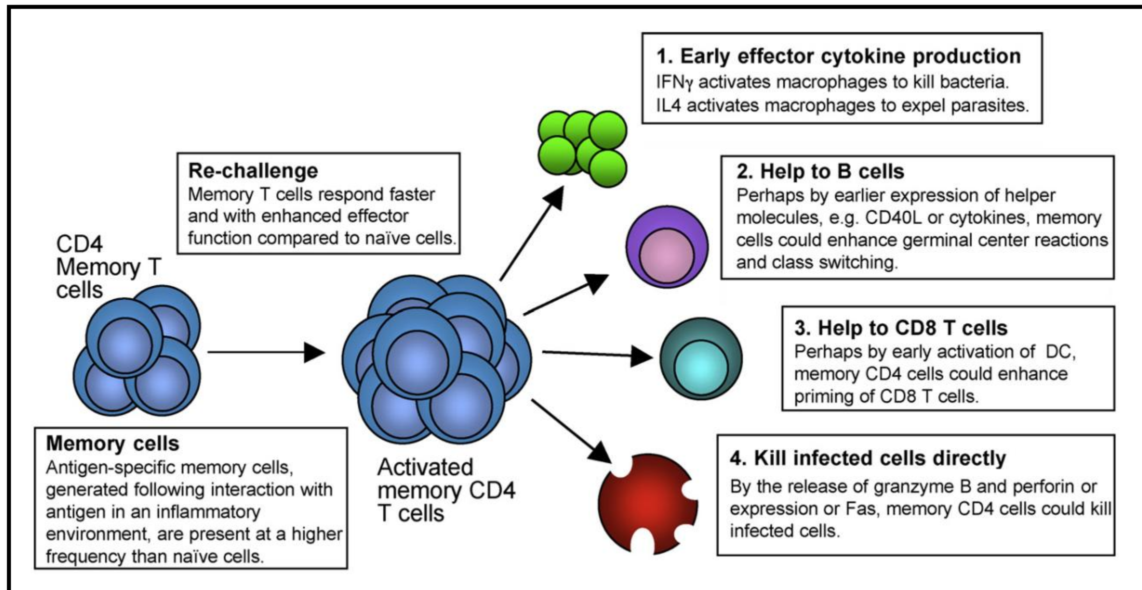


**Figure 1.10:** The different ways of memory CD4+ T-cells by which it can protect the host (MacLeod 2009)

- **Regulatory T cells**

Regulatory T cells ($T_{reg}$ cells), formerly known as suppressor T cells, are crucial for the maintenance of immunological tolerance. Their major role is to shut down T cell-mediated immunity toward the end of an immune reaction and to suppress auto-reactive T cells that escaped the process of negative selection in the thymus. Two major classes of $CD4^+$ $T_{reg}$ cells are naturally occurring $T_{reg}$ cells and Adaptive $T_{reg}$ cells.

- **Natural killer T cells**

Natural killer T cells bridge the adaptive immune system with the innate immune system. Unlike conventional T cells that recognize peptide antigens presented by major

histocompatibility complex (MHC) molecules, NKT cells recognize glycolipid antigen presented by a molecule called CD1d. Once activated, these cells can perform functions ascribed to both $T_H$ and $T_C$ cells (i.e., cytokine production and release of cytolytic/cell killing molecules). They are also able to recognize and eliminate some tumor cells (Terabe and Berzofsky 2008).

### 1.8.4. The Mucosal Immune System

The mucosal immune system is the part of the immune system juxtaposed to the mucosal surfaces and in direct contact with the external antigenic environment. It is composed of the lymphoid tissues that are associated with mucosal surfaces (MALT or mucosa-associated lymphoid tissue) which respond to antigens at the mucosal surfaces and can be separated into several components: gut associated lymphoid tissue (GALT), bronchus-associated lymphoid tissue (BALT), nasopharinx-associated lymphoid tissue (NALT), the MALT is mainly composed of gut-associated lymphoid tissues (GALT) lining the GI tract including the lamina propria (contain B cells, plasma cells, activated $T_H$ cells, and macrophages) and Peyer's patches (contain both B cells and CD4+ T-cells) (Chandran, Satthaporn et al. 2003). Peyer's patches is composed of specialized epithelial cells called M cells. The epithelial cell plays an important role in promoting the immune response by delivering foreign antigen from the lumina of the respiratory, digestive, and urogenital tracts to the underlying mucosal-associated lymphoid tissue. This antigen transport is carried out by specialized M cells (Thomas J. Kindt 2000). After encountering by antigen, lymphocytes migrate to the systemic circulation, where their further maturation and differentiation completes (Cheroutre and Madakamutil 2004). These differentiate deffectors memory mammary and salivary glands and the genitourinary organs (Kelsall BL 1996) cells migrate back to the mucosa and wait for antigen. This is regulated by the coordinated interaction various cell surface molecules on the T cell and respective ligands on the contraluminal surface of GI tract (Cheroutre and Madakamutil 2004). T cells expressing α4β7- integrin, αEβ7-integrin and C-C chemokine receptor 9 (CCR9) are perhaps the best characterized in terms of homing potential to the GI tract.

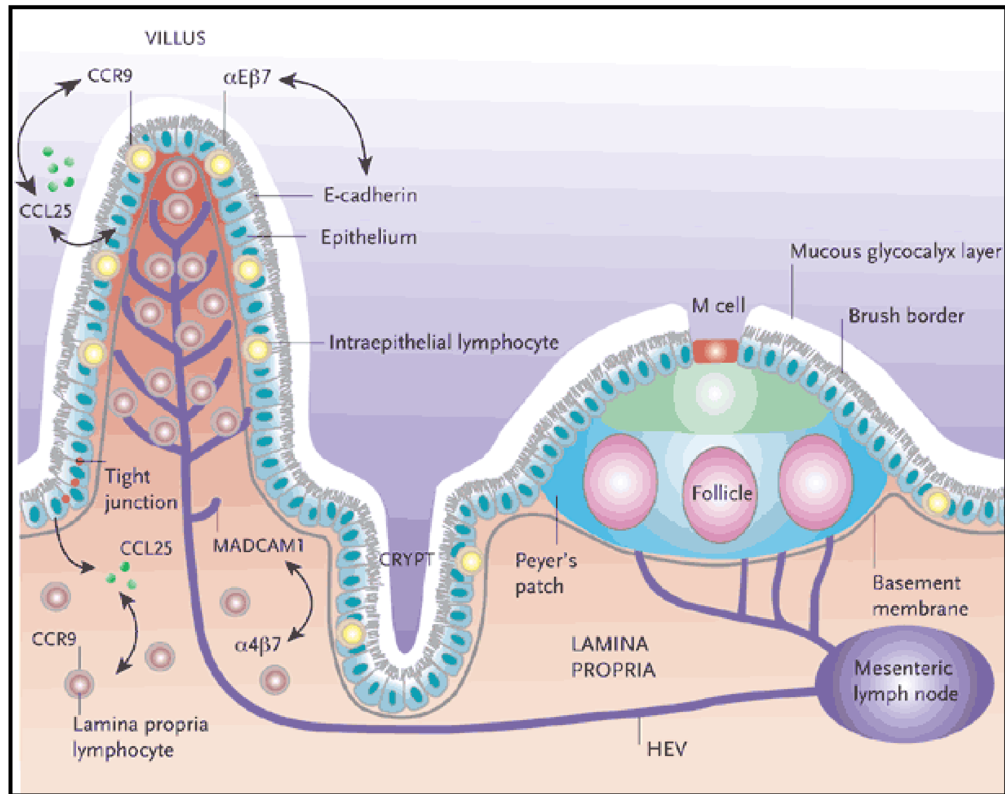


**Figure 1.11: Organization of the Mucosa Associated Lymphoid Tissues (MALT)** (*Nature reviews Immunology* April 2004; 4(4):290-300.3). In this figure, HEV (High Endothelial Venule), the ligand for α4β7-integrin is mucosal vascular–addressin cell-adhesion molecule 1 (MAdCAM-1), ligand for αEβ7-integrinis E-cadherin and C-C chemokine receptor 9 (CCR9) binds with CC-chemokine ligand 25 (CCL-25).

The mucosal immune system has three main functions (Holmgren and Czerkinsky 2005): to protect the mucous membranes against colonization and invasion by potentially dangerous microbes that may be encountered, to prevent uptake of undegraded antigens including foreign proteins derived from ingested food, airborne matter and commensal microorganisms, and to prevent the development of potentially harmful immune responses to these antigens if they do reach the body interior. As *V. cholerae* is an intestinal pathogen, mucosal immunity plays an important role against this pathogen.

### 1.8.5. Immunological Memory

Immunological memory can be defined as the altered response of a host that follows re-exposure to the same antigen. It is an operational property of the host and the immune system. Generation of immunological memory following contact with pathogens is antigen-specific and reflects a combination of humoral (‘antibody’) and cellular immunity, which is often lifelong (van Leeuwen, Sprent et al. 2009). Memory B cell and Memory T cells are two major component of immunological memory.

#### 1.8.5.1. Memory B cell

Memory B cell pathway is a complex developmental process that occurs in germinal centers and emerges after isotype switching and hypermutation of the immunoglobulin genes (R. W. Dutton 1998 ). Thus, after initial contact with antigen, some of the B cells participating in the primary response mature into memory cells; generation of these cells is the end result of clonal expansion, differentiation and affinity maturation. These ‘primed’ B cells are more efficient than naïve B cells and give heightened humoral responses on secondary contact with the antigen concerned. Memory B cells may also play a role in replenishing the pool of long-lived plasma cells to maintain long-term antibody levels in the absence of pathogen (Kenner JR 1995) (Cabrera, Martinez et al. 2006). Long-lived plasma cells are responsible for the continuous maintenance of serum antibody levels (Sanchez and Holmgren 1989) (Kenner JR 1995) (Longini, Nizam et al. 2007). Therefore, memory B cells and long-lived plasma cells are responsible for the long-term humoral immunity elicited by most vaccines (Levine and Kaper 1993).

#### 1.8.5.2. Memory T cell

T cells have an essential role in protection against a variety of infections. Since the case for a memory T cell is less clear, no special anatomical site has been identified where memory T cells develop; no isotype switching of the T cell receptor genes occurs; and no advantageous somatic mutations selected for higher affinity have been observed (van Leeuwen, Sprent et al. 2009). It has been studied that the CD4+ T cell population may be divided into four major subsets based on surface phenotype and function: resting, primed, effector and multifunctional (Mf) (Bell and Westermann 2008). After migration

from the thymus, CD4+ T cells join the recirculating pool and represent a population of resting CD4+ T cells (inexperienced, naive) (Bell and Westermann 2008). During the first encounter with antigen, specific T cells turned to short-lived, metabolically active primed T cells. It is also clear that the frequency of memory T cells specific for particular antigenic epitopes correlates closely with the extent of proliferation (clonal burst size) of the precursor cells during the primary response (Hou, Hyland et al. 1994). A second encounter with antigen transforms primed T cells into terminally differentiated; cytokine-secreting effector T cells (Seddon, Tomlinson et al. 2003). On the contrary, if primed T cells fail to find specific antigen, they modify their cytokine capabilities to become Mf T cells with a slightly extended lifespan. Mf T cells may respond rapidly to new or residual antigen, proliferate rapidly and add to the number of primed T cells. These intermediate T cells produce several different cytokines in different quantities as cytokine mRNA decays and new transcripts emerge (Anderson 2008). In the absence of antigen, Mf T cells default to antigen experienced (Ag-Exp) resting T cells that retain no memory of a previous antigen encounter. The Mf T cells reacquire the potential to recirculate through lymph nodes (LNs) (Springer 1994) (Zaph, Rook et al. 2006) and respond rapidly should they re-encounter antigen (Sallusto, Lenig et al. 1999). Such T cells are currently named as central memory.

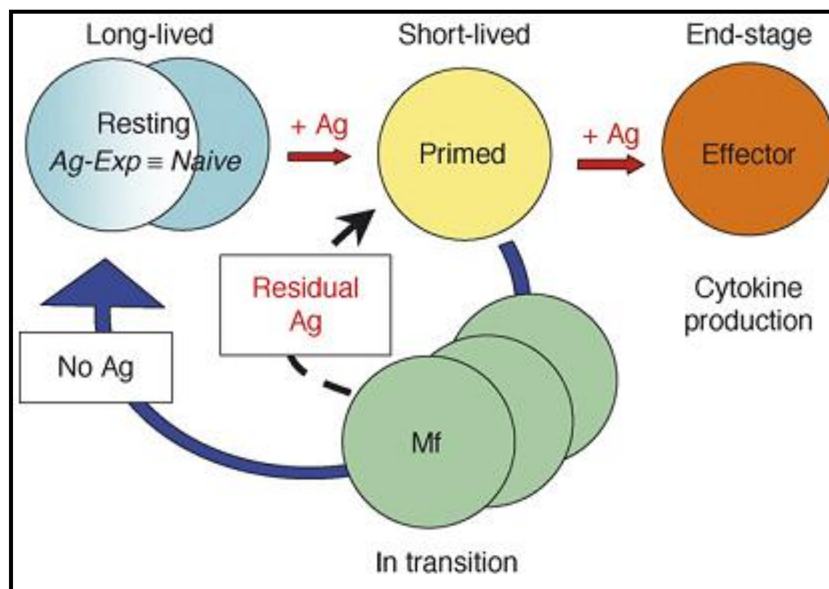


**Figure 1.12: Generation of Memory T-cell (Bell and Westermann 2008)**

### 1.8.6. Effects of Natural Infection to *V. cholerae*

Natural infection to *V. cholerae* provides long-term protection against subsequent disease (Cash, Music et al. 1974) (Koelle, Rodo et al. 2005) (Levine, Black et al. 1981). But the mechanism of protective immunity is not well understood yet. The protection may be mediated by anamnestic immune responses to *V. cholerae* antigens (Harris, Bhuiyan et al. 2009) (Nelson, Harris et al. 2009) (Svennerholm, Jertborn et al. 1984). Different components of the immune system, both humoral and cell mediated become activated in response to natural *V. cholerae* infection, suggested by studies to-date in patients with cholera (Qadri, Mohi et al. 1995) (Asaduzzaman, Ryan et al. 2004) (Qadri, Bhuiyan et al. 2004).

#### 1.8.6.1. Innate Immune Response to *V. cholerae*

Cholera is thought to be a prototypical non-inflammatory infection. During acute cholera infection a broader range of innate immune mechanisms are activated in the intestinal mucosa, both in the epithelium and in lamina propria cells (Flach, Qadri et al. 2007). Already it has been noted that increased infiltration of neutrophils, degranulation of mast cells and eosinophils, and production of some innate defense molecules occur during acute cholera infection although there is no pronounced inflammation during cholera (Mathan, Chandy et al. 1995) (Qadri, Raqib et al. 2002) (Asaduzzaman, Ryan et al. 2004). It has been showed that innate factors, including myeloperoxidase, lactoferrin, nitric oxide metabolites, and eicosanoids are also induced to increase in *V. cholerae* infection (Cash, Music et al. 1974) (Qadri, Raqib et al. 2002).

#### 1.8.6.2. Adaptive Immune Responses to *V. cholerae*

After the initiation of immune response to cholera by presenting the antigen in the Payer's patches of the gastrointestinal mucosa, stimulated antigen specific B cells migrate to the regional lymph node and differentiate into specific antibody secreting cells (Qadri 1997). Furthermore, robust systemic and mucosal antibodies are produced to the *V. cholerae* lipopolysaccharide, to cholera toxin, and to colonization factors, including the major subunit of the toxin-coregulated pilus, TcpA (Qadri 1997) (Levine 1979) (Asaduzzaman, Ryan et al. 2004) where the intestinal IgA antibodies are the

major immunoglobulin in mucosal immune response (Jertborn 1984 ) (Brandtzaeg 1986). This locally produced antibody in the intestinal mucosa further secret onto the gut mucosal surface. However, after natural infection, the serum levels of these antibodies wane more rapidly than protective immunity (Harris 2008).

#### 1.8.6.2.1. Humoral Immune Response to *V. cholerae*

The vibriocidal antibody is considered as a surrogate marker of a protective mucosal immune response. Antibodies directed against *Vibrio* O polysaccharide antigens are considered "vibriocidal" antibodies because they will lyse *V. cholerae* cells in the presence of complement and serum components (Levine MM 1993) (Tacket CO and G 1999) (Harris 2008) (Saha D 2004) . It can be used to determine if a person has developed antibodies against *V. cholerae*, either by exposure to infectious *V. cholera*e or as a result of oral cholera vaccine administration. 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 (Kenneth Todar 2012).

Patients with cholera respond with LPS-specific antibodies in the IgG, IgM, and IgA isotypes (Qadri, Ahmed et al. 1999) (Qadri F 1997) (Provenzano D 2006). It has been studied in Bangladesh that every 2-fold increase in vibriocidal titer is associated with a 44% decrease in the subsequent risk of cholera (Mosley WH et al. 1969).

#### 1.8.6.2.2. Cell-mediated Immune Response to *V. Cholerae*

Cholera patients mount a primed T-cell response in the mucosa after *V. cholerae* O1 infection (Bhuiyan, Lundin et al. 2009). The protection from cholera may be mediated by memory B cells which is capable of an anamnestic response in the gut mucosa and may depend on stimulation provided by memory T cells for their development and maintenance. T cells may contribute to the activation of B cells during *V. cholera*e infection by secreting stimulatory cytokines and direct contact with B cells in lymph nodes. Thus, T cells may have an important role in protective immunity to *V. cholera*e infection (Weil, Arifuzzaman et al. 2009).

It has been shown that the mucosal immune response to cholera toxin is T-cell dependent where, CD4+ T helper cells have an important role and it is established by studies with experimental animals (Elson 1987) (Hirabayashi 1991). Though the increased proliferative responses of CD4+ and CD8+ T cell has been shown at the acute stage of infection , their differences in the kinetics is not clear . However, it is possible that these differences may be due to the requirement for CD4+ T cells to help for activativation of CD8+ T cells (Bhuiyan, Lundin et al. 2009). They mediate their performance by enhanced production of IFN-γ (Th1 response) and IL-13 (Th2 response) (Bhuiyan, Lundin et al. 2009). Memory B-cell responses to T-cell-dependent antigens (such as CTB) persist for at least 1 year, whereas response to lipopolysaccharide, a T-cell-independent antigen, wane more rapidly after infection (Weil, Arifuzzaman et al. 2009). Therefore, the induction of CD4+ T-cell responses might be critical to the subsequent development of memory-B-cell responses and for long-term protective immunity following cholera.

## 1.9. Vaccination

### 1.9.1. Development of vaccines to prevent Cholera

An ideal vaccine for cholera would be very beneficial in the control of morbidity and mortality secondary to infection. Present vaccines in the market cannot give protection efficiently as natural infection. Natural infection with *V. cholerae* provides greater than 90% protection against subsequent disease for at least 3 years in U.S. volunteer studies (the maximum period studied) and an average of 3 to 8 years on the basis of epidemiological studies in endemic areas (Cash 1974) (Koelle, Rodo et al. 2005) (Levine and Kaper 1993) (Black 1982). Therefore, a vaccine which will render long lasting immunity is necessary for immune protection of cholera. The aim of cholera vaccination is to reduce the case fatality rate to less than 1%. An added prequisite is that the vaccine should contain components which can stimulate an appropriate memory response which involves both B and T lymphocytes.

### 1.9.2. T Cell based Immunization

As newly emerging and well-established infectious diseases have rekindled a drive to explore CD4+ T-cell memory, there is now an urgent need to understand the fundamental mechanisms that underpin CD4+ T-cell memory and to develop more effective vaccines (Bell and Westermann 2008).

Moreover, studies with experimental animals have shown that the mucosal immune response to cholera toxin is T cell dependent and that $CD4^+$ helper T cells have an important role (Elson and Ealding et al. 1984) (Hirabayashi 1991) (Hirabayashi, Tamura et al.; Holmgren 1987) (Holmgren, Svennerholm et al.). $CD4^+$ and $CD8^+$ T cells also increase in the circulation of cholera patients (Bhuiyan, Lundin et al) (Flach, Qadri et al. 2007).

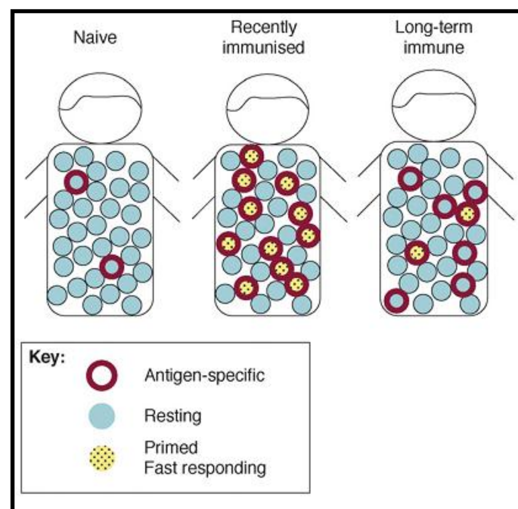


**Figure 1.13: Individuals at different stages of immunity. (Bell and Westermann 2008)**

Development of humoral responses to protein antigens is dependent on T-cell responses (Qadri, Mohi et al. 1995). Memory T cell response also produced in systemic circulation of *V. cholera* O1 infected patients against VCC and MP (Weil, Khan et al. 2009).

Before immunization the naive CD4+ T cells are found in low frequency. After vaccination (recently immunized), the naive T cells proliferate and becomes primed providing individuals a long term immunity (Fig.1.13).

Thus, long-term immunological memory is primarily supported by increased numbers of long-lived, resting, antigen-specific CD4+ T cells (Homann, Teyton et al. 2001) (Varga and Welsh 1998) of specific CD4 T cells (Bell and Westermann 2008). Epitope plays an important role in the development of a candidate vaccine. So the identification of the epitope recognized by CD4+ T cell or CD8+ T cells can be used as a tool to identify new antigen for the development of vaccine (Buus 1999) (De Groot, Ardito et al. 2009) (Doolan, Southwood et al. 2003) (Lauemoller, Kesmir et al. 2000) (Khan, Zaman et al. 2014).

### 1.9.3. Oral Cholera Vaccine

To date, two types of oral cholera vaccines are available, which have been shown to be safe, immunogenic and effective- inactivated vaccines (containing killed whole cells of *V. cholerae)* and live attenuated vaccines (containing genetically modified non-pathogenic strains of *V. cholerae*) (Sallusto, Lanzavecchia et al. 2010). These vaccines have been licensed in some countries and are mainly used by travelers, but are now under consideration for use in public health (Glass, Becker et al. 1982).

- **WC-rBS(Dukoral®):** A monovalent inactivated vaccine containing killed whole cells of *V. cholerae* O1 plus additional recombinant cholera toxin B subunit (Finkelstein 1996) (bacteriology) (Bradford A. Kay) (Snyder JD 1981).
- **BivWC (Shanchol®):** A bivalent inactivated vaccine containing killed whole cells of *V. cholerae* O1 and *V. cholerae* O139 (Anh, Canh do et al. 2007) (Mahalanabis, Lopez et al. 2008).
- **BivWC (mORCVAX®):** A bivalent inactivated vaccine containing killed whole cells of *V. cholerae* O1 and *V. cholerae* O139. Produced by VABIOTECH, Vietnam and only available in Vietnam (WHO 2009).

- **CVD103-HgR:** A live attenuated genetically modified *V. cholerae* O1 Inaba strain and engineered to produce CTB but not the A subunit of CT (Richie, Punjabi et al. 2000).

- **Peru-15:** Peru-15 is a *Vibrio cholerae* O1 E1 Tor, Inaba strain that has been engineered to be non-toxinogenic (it lacks the *ctxA* and *rtxA* genes, which encode cholera toxin A subunit and the RTX toxin, respectively), non-recombinatorial (it lacks the *recA* gene and the attachment site for the CTX phage), non-motile, and *ctxB* positive (it makes the immunogenic, nontoxic CTB subunit (Richie, Punjabi et al. 2000).

### 1.9.4. Shanchol™

Shanchol™ is a new oral, killed, bivalent, whole-cell cholera vaccine developed through International Vaccine Institute (IVI) with funding from the Bill & Melinda Gates Foundation, the Government of Korea, and the Swedish International Development Cooperation Agency (SIDA) by simply modifying a vaccine used and produced in Vietnam, in response to the need for a low-cost cholera vaccine for the developing world International Vaccine Institute (IVI) transferred the technology to Shantha Biotechnics, India and it was licensed in 2009 as Shanchol and prequalified by the WHO in 2011 so that it meets international Good Manufacturing Practice (GMP) standards and WHO production guidelines (Sanofi 2009).

The vaccine has some major advantages over the only other cholera vaccine currently on the market (Dukoral®). Firstly, it contains no cholera toxin B subunit. There is no chance of degradation of antigen by gastric acid.

So it does not require administration with a buffer, thereby it greatly simplifying its use under field conditions and other post-crisis situations. Secondly, it will be available to governments and international agencies at low cost $1.85 per dose. Thirdly, a phase III

trial taking place in India is showing that the vaccine is more effective and lasts 67% efficacy in young children (1-5 years old) comapared to Dukoral® (Sanofi 2009).

In comparison, within 6 months of vaccination the protective efficacy of two- and three-dose regimens of the Dukoral vaccine was 85% in Bangladesh (Clemens, Sack et al. 1986) and Peru (Sanchez, Vasquez et al. 1994), however this protection falls to 50% within 3 years (Clemens, Sack et al. 1990).

**Figure 1.14: Shanchol Vaccine** (https://www.ivi.org)

On the contrary, Kolkata field trial of Shanchol vaccine revealed comparable levels of protective efficacy in children <5 years and adults during 2-year period of observation (Sur, Lopez et al. 2009).

In Bangladesh, previous study had been done to show the temperature stability of Shanchol vaccine and it was found that the vaccine was effective without maintaining the controlled temperature. Currently this study conducted to show the immune response of oral cholera vaccine Shanchol in Bangladeshi adult recipients.

## 1.10. Objectives of the Study

### 1.10.1. General Objective

To assess the efficacy of Shanchol vaccine to generate immunological responses in the vaccine administered volunteers

### 1.10.2. Specific Objectives

The specific objectives of this study are as follows:

- To evaluate the involvement of different subsets of T cells like CD4+ T cells, CD8+ T cells and their memory responses in vaccinees after administration of one and two doses of vaccine.
- To compare the memory T cell responses in vaccinees using different vaccine administration strategies like administration at 14 days or 30 days apart in case of two doses of vaccination.
- To evaluate B-cell mediated long lasting protection efficacy of vaccine.
- To evaluate the durability of vaccine specific memory T-cell responses.

# Chapter 2

## Methods & Materials

## 2.1. Study Design

The study was conducted from November 2012 to January 2014.

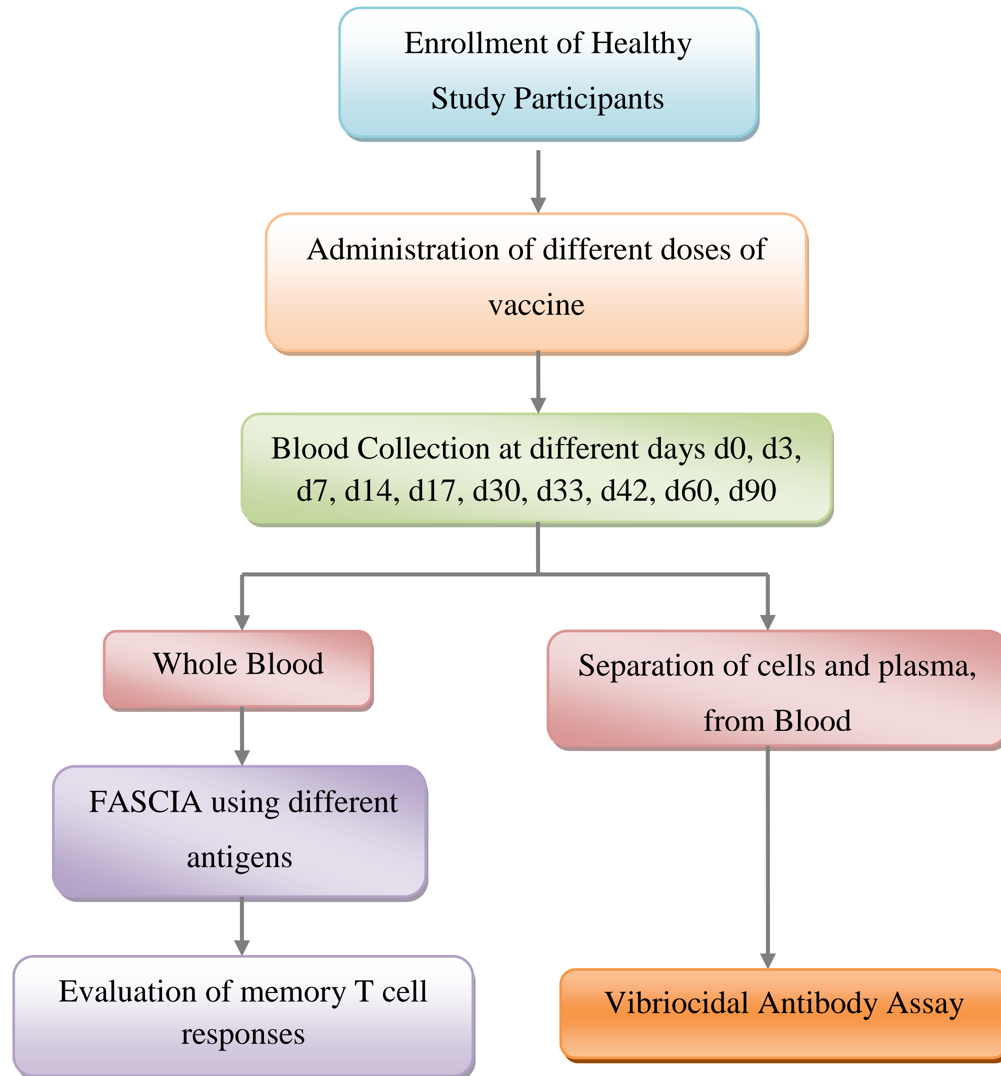


**Figure 2.1:** Flow chart of the Study

### 2.2.1. Location of the Study

Blood sample was collected from urban slum of Mirpur, Dhaka, Bangladesh. The immunological work resembling flow cytometric assays with heparinized whole blood for measuring T-cell responses and plasma for vibriocidal assay were carried out in the immunology laboratory unit of the Centre for Vaccine Sciences (CVS), icddr,b, Dhaka.

The Research Review Committee (RRC) and Ethical Review Committee (ERC) of icddr,b approved the study protocol. The human experimentation guidelines of the U.S. Department of Health and Human Services were followed during the study. Oral and written informed consent were obtained from adult participants in all instances.

### 2.2.2. Study Participants

A total number of 45 healthy adults were enrolled in this study. The healthy participants were divided into 3 groups depending on vaccine doses. 15 participants were given a single dose of the Shanchol vaccine, and 15 participants were given two doses at 14 days interval, while the rest 15 participants were given two doses at 30 days interval.

## 2.3. Vaccination

Before vaccination, participants were excluded from the study if they had a history of gastrointestinal disorder or diarrhoeal illness in the past 2 weeks, febrile illness in the preceding week or having history of receiving antibiotic treatment at least 7 days prior to enrolment, immunocompromising condition or therapy, intake of anti-diarrheal medication or acute disease in the past week or that were positive for common enteric pathogens. Participants who had ever taken killed OCV or any live or killed enteric vaccine in the last month prior to vaccination were also excluded.

### 2.3.1. Vaccine components

Shanchol™ is an oral, killed, bivalent whole-cell cholera vaccine developed by Shantha Biotechnics, India and it was licensed in 2009 in India and prequalified by the WHO in 2011. Again, it meets international Good Manufacturing Practice (GMP) standards and WHO production guidelines (Shantha 2011).

**Figure 2.2: Shanchol Vaccine**. (https://www.ivi.org)

Each dose of the Shanchol (Shantha Biotechnics) vaccine in 1.5 mL contained heat killed and formalin killed whole cell bacteria consisting of 600 ELISA Units (EU) of lipopolysaccharide (LPS) of formalin-killed *V. cholerae* O1 Inaba, El Tor biotype (strain Phil 6973). It also contains 300 EU LPS of heat-killed *V. cholerae* O1 Ogawa classical biotype (Cairo 50); 300 EU LPS of formalin killed *V. cholerae* O1 Ogawa classical biotype (Cairo 50); 300 EU LPS of heat killed *V. cholerae* O1 Inaba, classical biotype (Cairo 48) and 600 EU LPS of formalin killed *V. cholerae* O139 (4260B) (Sur, Lopez et al. 2009) (Mahalanabis, Lopez et al. 2008).

### 2.3.2. Administration of vaccine

The participants included in the study were instructed not to eat for 1 h before and after intake of vaccine and were requested to wait at the field clinic for half an hour to monitor the side-effects. Before opening the vaccine vial, field assistant rotated the vial gently to disperse the cellular contents. Following administration, the participants were offered half a cup of water {(Saha et al. 2009).

## 2.4. Blood Collection Scheme

Administration of vaccine was done using different dose patterns to evaluate the immunogenicity in study participants which are as follows:

### 2.4.1. Single Dose Vaccination

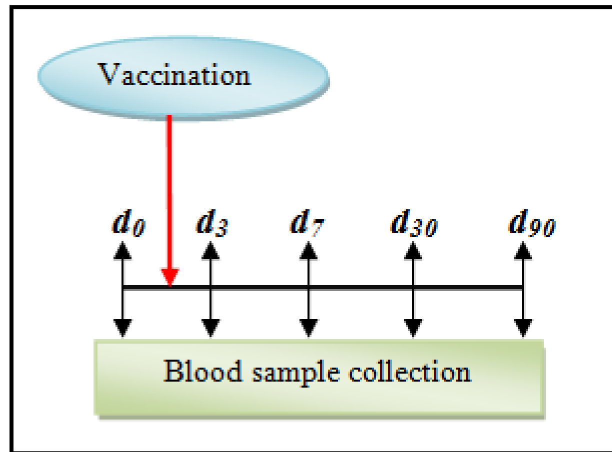


**Figure 2.3:** Before vaccination blood was collected from study participants and marked as day point 0 (d0). After administration of single dose of vaccine blood samples were collected at d3, d7, d30, d90.

### 2.4.2. Double Dose Vaccination 14 Days Interval

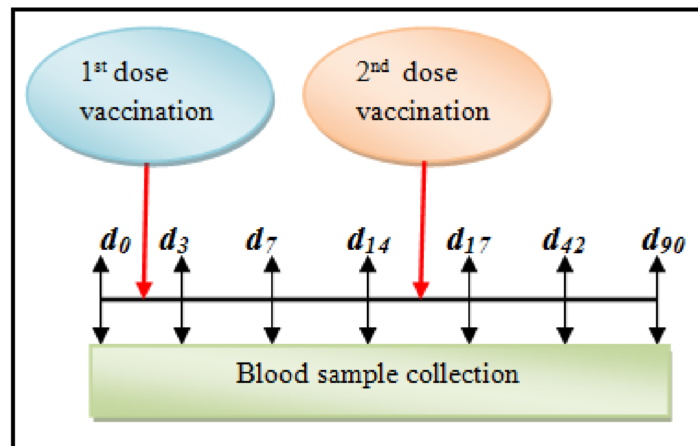


**Figure 2.4:** After administration of $1^{st}$ dose of vaccine at day 0, second dose of vaccine was given at 14 days apart and blood was collected at different day points d0, d3, d7, d14, d17, d42, d90.

### 2.4.3. Double Dose Vaccination 30 Days Interval

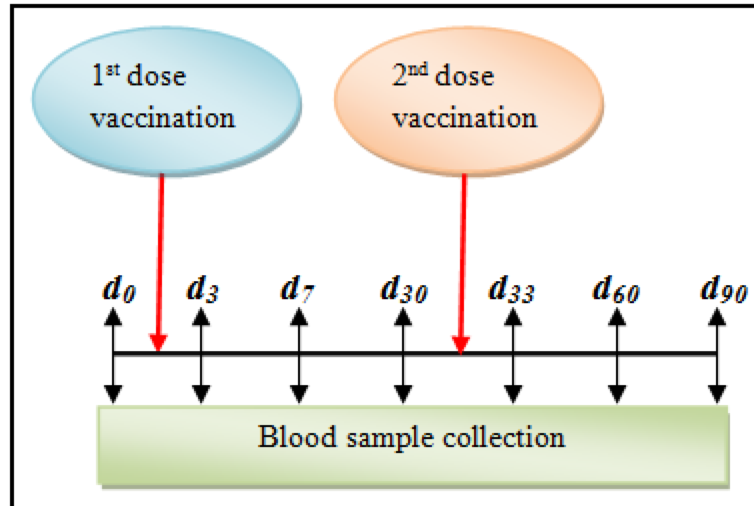


**Figure 2.5:** After administration of 1st dose of vaccine at day 0, second dose of vaccine was given at 30 days apart and blood was collected at different day point's d0, d3, d7, d30, d33, d60, d90.

Approximately, 0.5ml venous blood sample was collected in lithium heparin tube to assess memory T cell responses. About 3 ml of blood sample was taken in vials, not containing additives, for serum collection at day 3 of each vaccination and plasma was collected on other days. Using density gradient centrifugation at 750g, 20°C for 10 mins and was stored at -20°C for ELISA and vibriocidal assay.

## 2.5. Laboratory Methods

### 2.5.1. Flow Cytometric Assay of Specific Cell-mediated Immune Response in Activated Whole Blood (FASCIA)

FASCIA (flow cytometric assay of specific cell-mediated immune response in activated whole blood), a simple and reproducible method was used to determine lymphoblast formation in response to antigenic stimulation (Parment, Svahn et al. 2003) (Weil, Arifuzzaman et al. 2009).

T- Cell stimulating antigens *V. cholerae* O1 specific membrane preparation (MP) and mutant cholera toxin (mCT) was used. A plant derived protein phytohaemagglutinin (PHA) (1 µg/ml; Murex, Remel, Sweden) was used as a positive control and nonstimulated culture was used as negative controls (Kuchta, Rahman et al.).

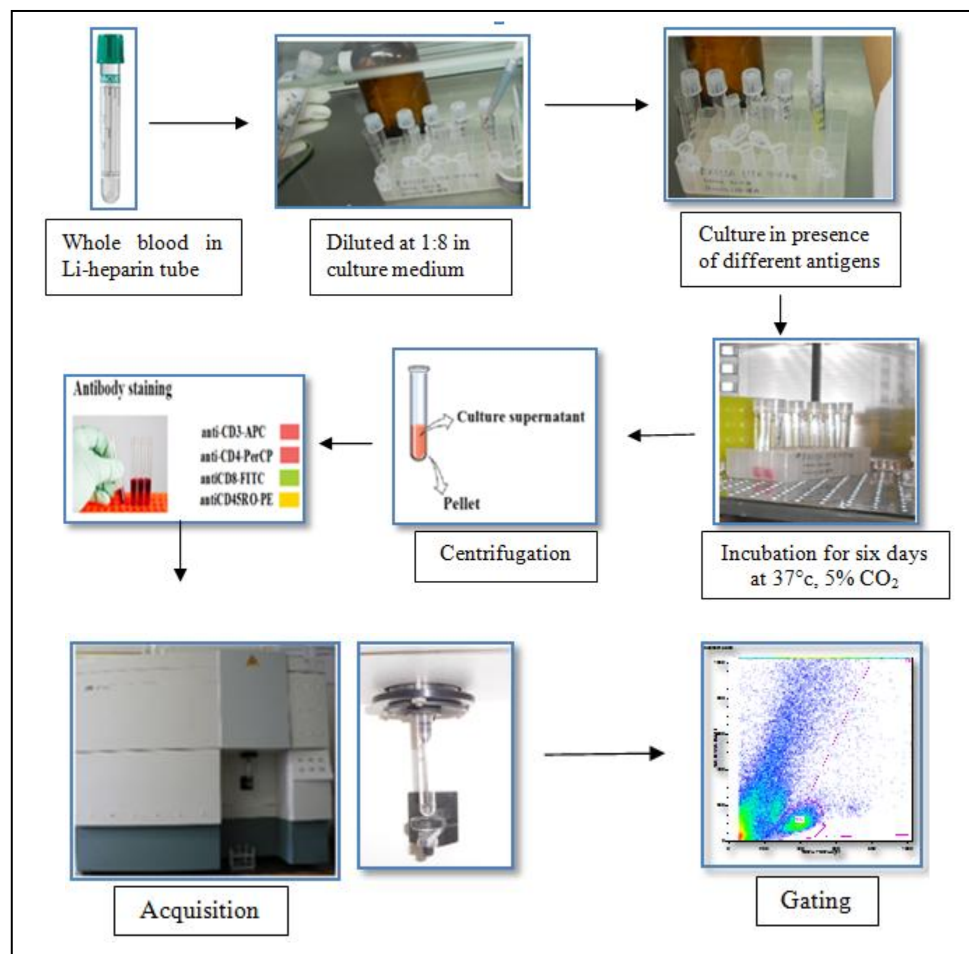


**Figure.2.6: Graphical representation of FASCIA method**

#### 2.5.1.1. Antigen Preparation

**Membrane Preparation (MP)**

MP contains a mixture of bacterial proteins and it is composed of mostly outer membrane protein U precursor porin OMPU, elongation factor, chaperonin, ATP synthase subunit α, β, some other different enzymes etc. (Weil, Arifuzzaman et al. 2009).

*V. cholerae* MP was made from the sequenced O1 El Tor strain N16961 grown in vitro in AKI medium (Iwanaga and Yamamoto 1985). Specifically, a culture of N16961 was inoculated into 1 liter of AKI medium (Iwanaga and Yamamoto 1985). The culture was grown in nonaerating conditions to an optical density of 0.3 at 600 nm and then transferred to shaking conditions for growth to stationary phase. Bacterial pellet was collected after centrifugation and then sonicated. The sonicated mixture was centrifuged at 1,400 × *g* for 10 min, and the remaining supernatant was then centrifuged at 14,900 × *g* for 30 min. The pellet containing the membrane fraction was then suspended in $MgCl_2$-Tris buffer (5 mM $MgCl_2$, 10 mM Tris [pH 8.0] ) for subsequent experiments.

**Mutant Cholera Toxin (mCT)**

Mutant cholera toxin contains the G33D variant homopentameric B subunit (mCT) (Wolf, Jobling et al. 2008). The mCT protein contains a G33D substitution in the B subunit binding domain that prevents the molecule from binding GM1 ganglioside (Wolf, Jobling et al. 2008). The mutant Cholera Toxin antigen (G33D holotoxin) has a single amino acid substitution (Gly-33→Asp) in the binding site for GM1 ganglioside (Jobling and Holmes 1991) due to a single point mutation. The FASCIA response is stronger to mutant CT (mCT) B subunit than unaltered CT (Ahmed, Arifuzzaman et al. 2009).

**PHA**

Phytohaemagglutinin (PHA) is a lectin found in plants, especially in legumes. PHA actually consists of two closely related proteins, called leucoagglutinin PHA-L and PHA-E. The letters 'E' and 'L' indicate these proteins agglutinate Erythrocytes and Leukocytes. Phytohaemagglutinin has carbohydrate-binding specificity for a complex oligosaccharide containing galactose, N-acetylglucosamine, and mannose (Summers et

al. 2002). It is used as a mitogen to trigger cell division in T-lymphocytes. Hence lymphocytes cultured with Phytohemagglutinin can be used for *ex vivo* T-cell proliferation.

**NS**

Dulbecco modified Eagle (DMEM) medium with supplementation with 10% FBS, 1% Gentamycin and 1% Mercaptoethanol is used as non stimulating medium.

#### 2.5.1.2. Protocol

1. Whole blood was collected in a lithium heparin-coated tube and diluted 1:8 in Dulbecco modified Eagle medium (DMEM) (Gibco, NY) supplemented with 1% gentamycin (Sigma), 1% mercaptoethanol, 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL- 16140-071) and DMEM-rest.
2. 0.4 ml of diluted blood was added to each 5 ml polystyrene FACS tubes (Becton Dickinson, San Jose, USA).
3. 100µL of stimulating antigen, control antigen, or additional medium was added to 400 µL of diluted blood. The final concentrations of each stimulatory antigen and the control antigen were 10 and 1 µg/ml, respectively.
4. The tubes were incubated at 37$^{o}$C in 5% $CO_2$ for 6 days.
5. After 6 days of *in vitro* culture in an atmosphere containing 5% CO2 at 37°C, the tubes were centrifuged for 5 min at 400×g.
6. We recovered the culture supernatant by centrifugation, mixed it with a protease inhibitor and then stored this at -70°C for subsequent cytokine analysis.
7. The pellet of whole blood cells were stained directly with fluorochrome conjugated monoclonal antibody cocktail having anti-CD3-FITC, anti-CD4-PerCP, anti-CD8-APC, and anti-CD45R0-PE (Becton Dickinson Immunocytometry Systems [BD], Stockholm, Sweden) and incubated for 15-20 min in darkness at room temperature.
8. 2 ml of an erythrocyte lysing solution of ammonium chloride (sigma) containing potassium chloride and EDTA was added to each tube. Tubes were vortexed and

another 1 ml of ammonium chloride was added. Then tubes were incubated for 5 min in dark at room temperature.

9. At 400×g, tubes were centrifuged for 5 min and the supernatants were removed immediately.
10. 3 ml of FACS buffer was added to each tube and mixed by vortexing. Afterward, tubes were centrifuged for 5 min at 400×g and the supernatants were discarded.
11. 400 µl cell fixative (Becton Dickinson, San Jose, USA) was added to each tube, mixed and stored in the dark until acquisition.
12. Acquisition was conducted by fluorescence-activated cell sorting (FACS Calibur, Becton Dickinson, San Jose, USA) for standardized acquisition time of 2-min using CellQuest Pro software (Becton Dickinson, San Jose, CA) within 12h. All events acquired during 120 s acquisition time were saved.
13. Analysis was performed with FlowJo software (TreeStar, Inc. Oregon). The results are presented as the ratio of lymphoblast count with antigenic stimulation to the count without stimulation (stimulation index). A value of "1" indicates that stimulation is equal in samples with or without *V. cholerae* antigenic stimulation.

**Table 2.1: FASCIA Antigen preparation**

| ***Antigen*** | **Stock concentration** | **Working concentration** | **5X Amount (in DMEM complete) (For FASCIA)** |
|---|---|---|---|
| ***MP*** | 8.3 mg/ml | 10 µg/ml | 3.0 µl for 500 µl |
| ***G33D Holotoxin*** | 8. 04 mg/ml | 10. 0 µg/ml | 3.2 µl for 500 µl |
| ***PHA*** | 2° stock-200 µg/ml (1° stock-2 mg/ml) | 1 µg/ml | 5.0 µl for 500 µl |
| ***NS*** | | | |

**Table 2.2: Fluorochrome tagged antibody specifications**

| Fluorochrome tagged antibody | Fluorescence emission color | Ex-max[a] (nm) | Em-max[b] (nm) | Company |
|---|---|---|---|---|
| anti-CD3-allophycocyanin ***(anti-CD3-APC)*** | Red | 650 | 660 | BD[c] |
| anti-CD4-peridinin chlorophyll protein ***(anti-CD4-PerCP)*** | Red | 482 | 678 | BD |
| anti-CD8-fluorescein isothiocyanate ***(anti-CD8-FITC)*** | Green | 494 | 519 | BD |
| anti-CD45RO-phycoerythrin ***(anti-CD45RO-PE)*** | Yellow | 496, 546 | 578 | eBioscience |

Ex-max[a]- Escitation maxima, Em-max[b]- Emission maxima,
BD[c-] Becton Dickinson, San Jose, USA

### 2.5.1.3. An Overview of the Flow Cytometry

Flow cytometry is a technique for counting, examining, and sorting microscopic particles suspended in a stream of fluid. In the flow cytometer, particles are carried to the laser intercept in a fluid stream. Any suspended particle or cell from 0.2–150 micrometers in size is suitable for analysis. The properties measured include a particle’s relative size, relative granularity or internal complexity, and relative fluorescence intensity. Flow cytometers use the principle of hydrodynamic focusing for presenting cells to a laser (or any other light excitation source). A flow cytometer is made up of three main systems: fluidics, optics, and electronics. The fluidics system transports particles in a stream to the laser beam for interrogation. The optics system consists of lasers to illuminate the particles in the sample stream and optical filters to direct the resulting light signals to the appropriate detectors. The electronics system converts the detected light signals into electronic signals that can be processed by the computer. For some instruments equipped with a sorting feature, the electronics system is also capable of initiating sorting decisions to charge and deflect particles.

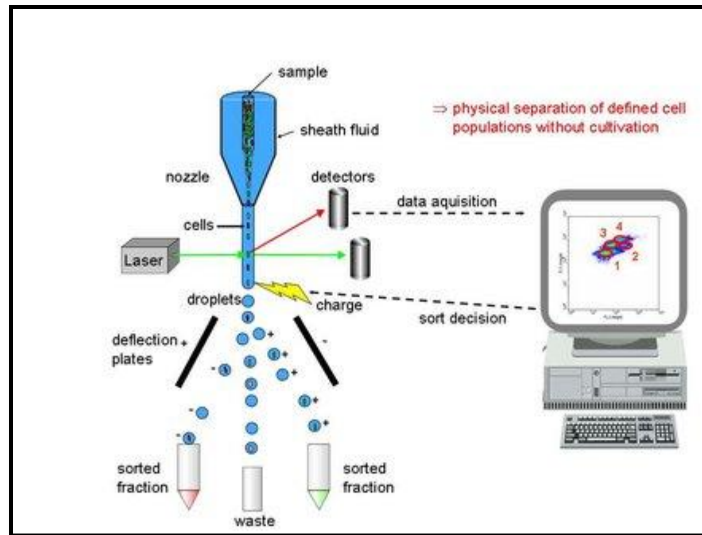


**Figure 2.7: Acquisition of FASCIA cultured lymphocyte populations** (© 2014, Max Planck Institute for Marine Microbiology)

A fluorescent compound absorbs light energy over a range of wavelengths that is characteristic for that compound. This absorption of light causes an electron in the fluorescent compound to be raised to a higher energy level. This energy is released as a photon of light with specific spectral properties unique to different fluorochromes. This transition of energy is called fluorescence. The argon ion laser is commonly used in flow cytometry because the 488-nm light that it emits excites more than one fluorochrome.

**Table 2.3: Quanta of Fluorochrome Fluorescence** (http://www.seci.info/.pdf)

| Dye | Excitation | Emission | Molecular Weight |
|---|---|---|---|
| FITC | 488 nm | 520 nm | 389 Da |
| PE | 488 nm | 578 nm | 240 000 Da |
| PerCP | 488 nm | 688 nm | 35 000 Da |
| APC | 613 nm | 665 nm | 105 000 Da |

#### 2.5.1.4. Acquisition of Cells

Following staining, cells were washed and fixed in cell fix (Becton Dickinson, San Jose, USA) to stabilize the cells before flow cytometry was performed. Cells were stained with fluorescent markers, allophycocyanin-(APC), peridinin chlorophyll protein- (PerCP), fluorescein isothiocyanate – (FITC), phycoerythrin-(PE) conjugated with anti-CD3, anti-CD4, anti-CD8 and anti-CD45RO respectively. Acquisition was performed on a FACS caliber flow cytometer (Becton Dickinson, San Jose, CA, USA) and viable cells were analyzed with CellQuest Pro multipurpose software (version 3.3; Becton Dickinson, San Jose, U.S.A).

Sorting allows us to capture and collect cells of interest for further analysis. To sort particles or cells, the cytometer first needs to identify the cells of interest, then separate out the individual cells. Once the population of interest has been identified on a data acquisition plot, a region is drawn around that population. A logical gate is created from the regions. This gate is then loaded into the cytometer's software as the sort gate. The sort gate identifies cells of interest to be sorted out of the stream.

Flow cytometric data is stored according to a standard format, the flow cytometry standard (FCS) format, developed by the Society for Analytical Cytology. According to the FCS standard, a data storage file includes a description of the sample acquired, the instrument on which the data was collected, the data set, and the results of data analysis. When multiplied by the approximately 10,000 events collected for a single sample, an FCS data file typically contains 80 kB of data.

### 2.5.1.5. Analysis of Gating Strategies

**Gating Principles**

A gate in cytometry is a set of value limits (boundaries) that serve to isolate a specific group of cytometric events from a large set of data. It is a numerical or graphical boundary that can be used to define the characteristics of particles to include for further analysis.

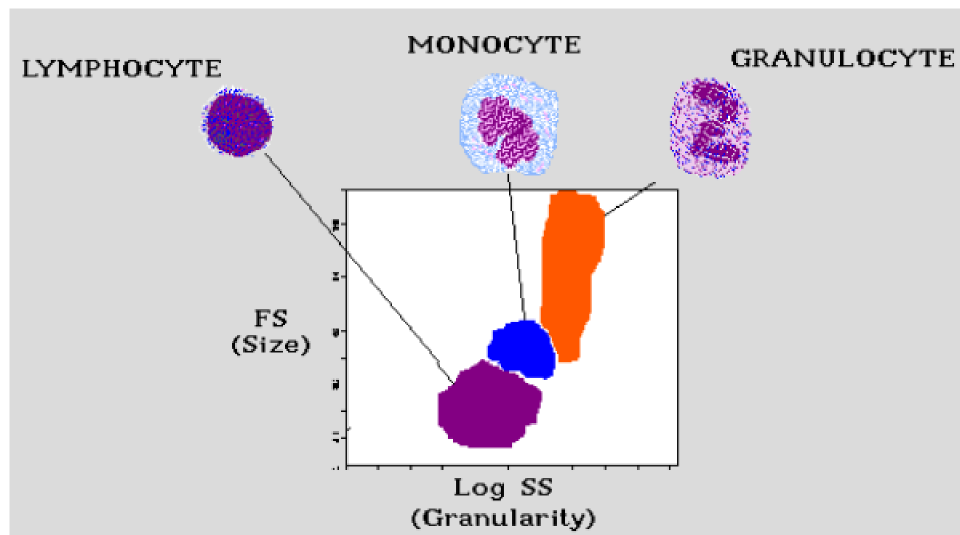


**Figure 2.8: Scatter Diagram Morphology** (http://www.seci.info/.pdf)

An important principle of flow cytometry data analysis is to selectively visualize the cells of interest while eliminating results from unwanted particles e.g. dead cells and debris. Cells have traditionally been gated according to physical characteristics. For instance, sub cellular debris and clumps can be distinguished from single cells by size, estimated by forward scattering and side scattering light. Also, dead cells have lower forward scatter and higher side scatter than living cells. These same principles can be used to distinguish different cell populations.

The Flow cytometry (FCM) data were analyzed with FlowJo software (Tree Star Inc., version 8.5.3). The lymphocyte population was identified in a forward- versus side-scatter plot, the different subpopulations were gated according to different staining

patterns and results expressed as frequency of expression. T cells were identified based on their expression of the CD3+ cell surface marker. Among the CD3 cells, CD4+ and CD8+ subpopulation was gated followed by identification of the CD45RO memory cells. The ratio is referred to as the stimulation index (SI). An SI value equal to “1” indicates that stimulation is equal in samples with or without a *V. Cholerae* antigen, and “1” indicates *V. Cholerae* antigen-specific stimulation.

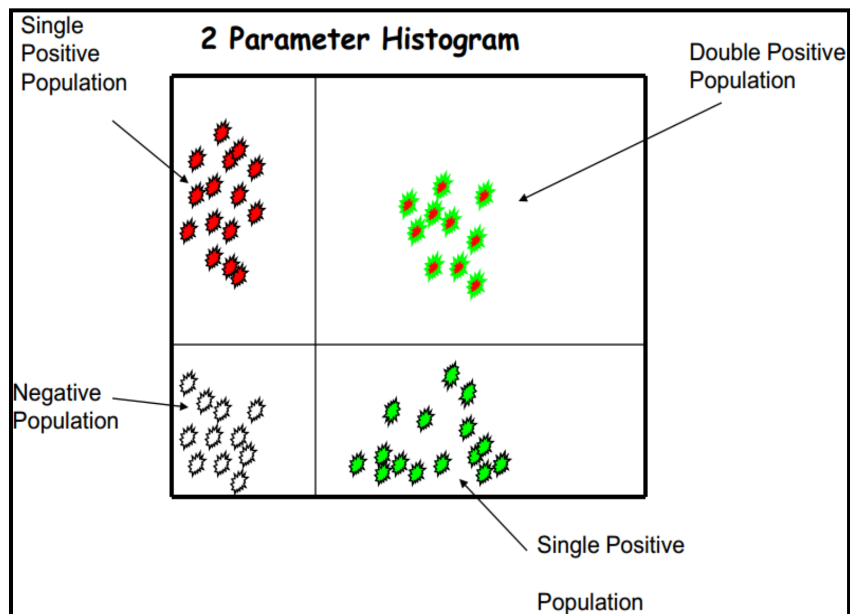


**Figure 2.9: Two Parameter Histogram Plot** (http://www.seci.info/.pdf)

A quadrant marker divides two-parameter plots into four sections to distinguish populations that are considered negative, single positive, or double positive. The lower-left quadrant displays events that are negative for both parameters. The upper-left quadrant contains events that are positive for the y-axis parameter but negative for the x-axis parameter. The lower-right quadrant contains events that are positive for the x-axis parameter but negative for the y-axis parameter. The upper-right quadrant contains events that are positive for either parameters, or double positive.

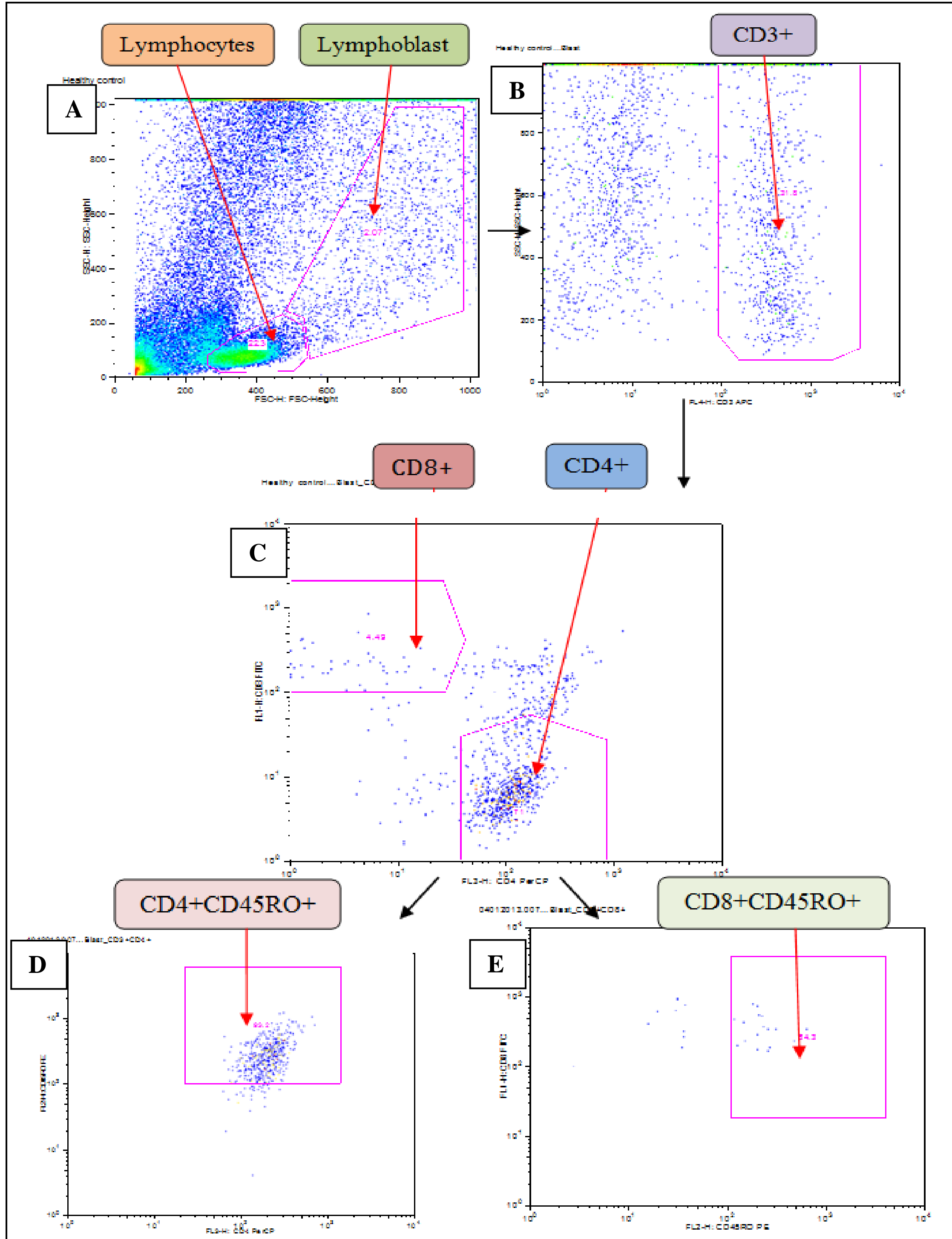


**Figure 2.9:** FACS Plot gating strategy after stimulation with antigens: (A) Cells are separated according to forward scattering cells versus side scattering cells. B) T-cells are separated by surface marker CD3 (C) Subpopulation of T-cell (Helper-T-cell and Cytotoxic T-cells) are separately gated by using surface marker CD4 and CD8 respectively (D) Memory T-cells (defined as $CD4^{+}CD45RO^{+}$) are separated , finally (E) Memory T-cells (defined as $CD8^{+}CD45RO^{+}$) are separated.

## 2.5.2. Vibriocidal Antibody Assay

### 2.5.2.1. Vibriocidal Assay

Vibriocidal antibody assay is considered as the surrogate marker. Vibriocidal antibody assay was performed using guinea pig complement with *V. cholerae* O1 Ogawa (X-25049 El Tor), *V. cholerae* O1 Inaba (19479 El Tor. Inaba) and *V. cholera* O139 as target organisms. 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 antibody titer at day 7 or day 21 after vaccination were considered as responders.

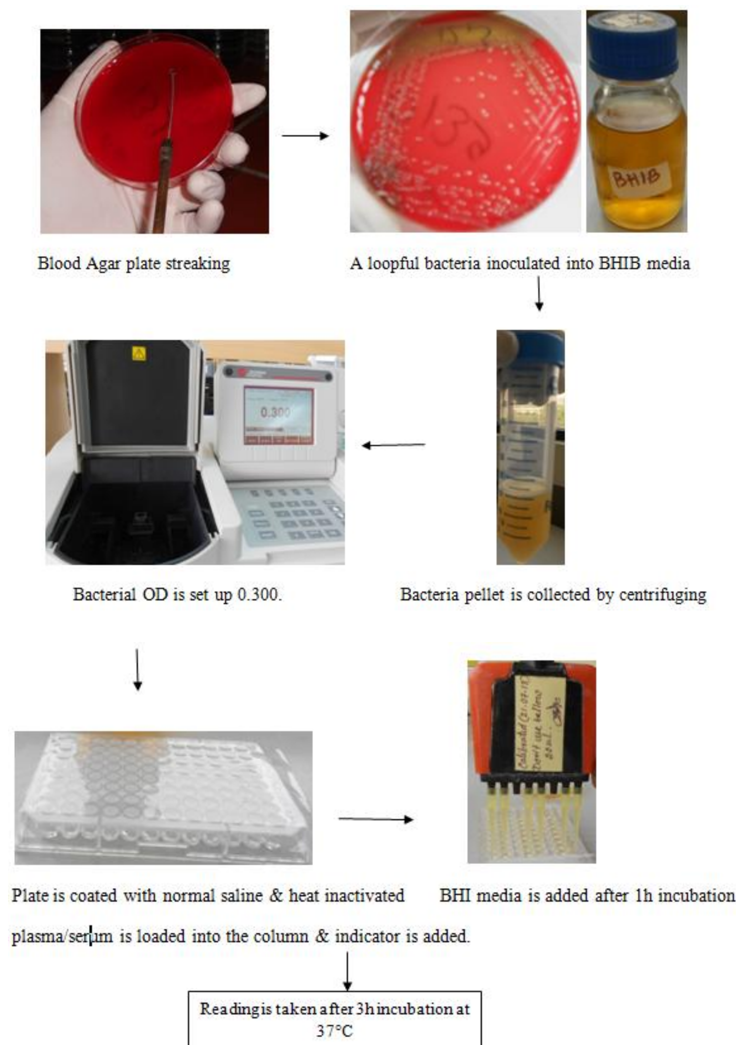


**Figure 2.10:** **A schematic representation of vibriocidal assay.**

#### 2.5.2.2. Procedure

- *Vibrio cholerae* O1 (Ogawa: strain 25049 El Tor, Inaba: strain 19479 El Tor) were cultured overnight on blood agar plates at 37$^{o}$C.
- A loopful of bacteria from the plates was inoculated in 15 ml Brain Heart Infusion (BHI) medium in a conical flask covered with cotton plug. This was incubated on a shaker at 37$^{o}$C for next 3-4 h.
- The culture was centrifuged at 3000 rpm for 10 min and the supernatant was discarded. The sediment was further suspended in sterile saline.
- The suspension was centrifuged for another 8-10 min and pellet was resuspended in sterile saline.
- Bacterial concentration was adjusted by spectrophotometer at 600 nm wavelength. For *V. cholerae* O1 (Ogawa: strain 25049 El Tor, Inaba: strain 19479 El Tor) OD was adjusted at 0.3.
- Heat-inactivated (56$^{o}$C plus 30 min) plasma was diluted 2-fold in sterile saline in flat bottom micro titer plates (Nunc, F) as stated below:
  - 25 µl of cold saline was dispensed in all wells except column #2.
  - 45 µl of cold saline and 5 µl of test sera were dispensed in column #2. 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$^{o}$C (on ice) until used.
- The indicator (bacteria-complement-saline mixture) was prepared and used immediately after preparation. The composition for each plate was as following:

**Table 2.4: Composition of indicator system**

| | Sterile Saline | Bacteria | Complement |
|---|---|---|---|
| *V. cholerae* O1 (X25049 or19479) | 2.55 ml | 150 µl | 300 µl |

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 h (50 rpm).

- 150 µl BHI was added to each well. This was incubated for another 3-4 h at 37°C without shaking. The plates were read visually and spectrophotometrically. Optical density (OD) was measured at 595 nm in the Multiscan Ascent Reader (Thermo Labsystems, Denmark). The absorbance for control wells should reach 0.20 to 0.28 at 595 nm.
- Vibriocidal antibody titer was 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.

## 2.6. Statistical Analysis

Comparisons of immunological response for significance were tested for significance within groups using Wilcoxon Matched Paired t-test, among groups using Mann Whitney Rank-sum test. All reported $P$ values were two-tailed and $P \leq 0.05$ was considered a threshold for statistical significance. Analyses were performed with Graph Pad Prism 5.0 and Microsoft Excel.

# Chapter 3

# Results

# Chapter 3

# Results

## 3.1. Demographics of Study Participants

The study was conducted to characterize the memory T-cell responses after administration of the oral cholera vaccine, Shanchol using different dose pattern such as single dose, double dose given at 14 days and 30 days intervals. In total 45 adult vaccinees aged between 18 to 45 years (median age, 30 years) were enrolled and were divided into three groups, each group embedded by 15 participants. Same group of vaccinees were assessed to study both of their T-cell responses and serological immune responses. The number of male and female participants were 2 (5%) and 43 (95%) respectively. Among them only two participants were Rh negative. Participants of this study did not show any adverse effect after the administration of vaccine. The demographic characteristics of the study participants are presented below (Table 3.1).

**Table 3.1: Demographics of Study Participants**

| **Sex** | | | **No. of Vaccinees (%)** |
|---|---|---|---|
| Male<br>Female | | | 2(5%)<br>43(95%) |
| **Median Age (years)** | | | **Range (years)** |
| 30 | | | 18-45 |
| **Blood Group** | **Rh Factor** | | **No. of Vaccinees (%)** |
| | **+ve** | **-ve** | |
| O<br>A<br>B<br>AB | 9<br>8<br>18<br>8 | 1<br>1<br>None<br>None | 10 (22)<br>9 (20)<br>18 (40)<br>8 (18) |

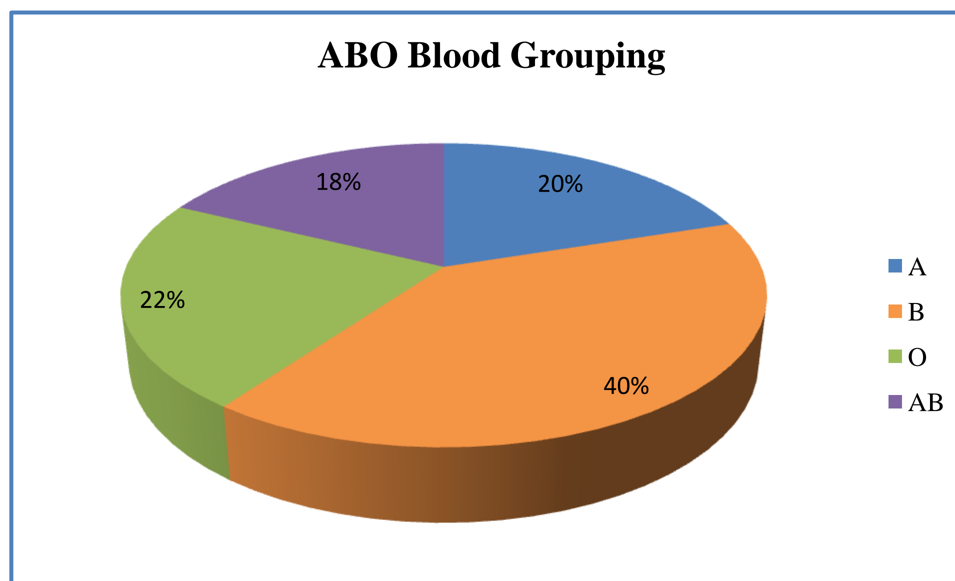


**Figure 3.1: ABO Blood Grouping of Study Participants.**

## 3.2. Memory T-cell Responses by FASCIA

Memory T-cell responses to multiple *V. cholerae* antigens of Bangladeshi participants, given the oral cholera vaccine Shanchol and its subsets were analyzed by using multi parameter flow cytometric assay of specific cell mediated immune response in activated whole blood (FASCIA). The FASCIA assay was used to investigate the lymphoblast proliferation and responses were observed at different day points using different dose patterns of vaccine administration. The results are presented as the ratio of lymphoblast count with antigenic stimulation to the count without stimulation (stimulation index). A value of "1" indicates that stimulation is equal in samples with or without *V. cholerae* antigenic stimulation (Weil, Arifuzzaman et al. 2009) .

### Selection of antigens for T-cell stimulation

The study included stimulation with *V.cholerae* O1 specific membrane preparation (MP) and modified cholera toxin (mCT) as antigen since previous study showed that MP and mCT are highly immunogenic (Weil, Arifuzzaman et al. 2009).

### 3.2.1 Antigen specific CD3+ T-cell responses in vaccinees using different dose patterns

#### 3.2.1.1. CD3+ T-cell responses to MP and mCT in single dose vaccinees

Proliferative responses of CD3+ T-cell at day 7 to MP were (1.38±1.43) significantly ($P$=0.002) higher ($P$=0.002) than the day 0 response (0.54±0.33) after the single dose of vaccine. On the contrary, CD3+ T-cell stimulation decreased at day 30 and day 90 after the single dose. The mean value against MP antigen at day 90 was 0.56±0.24 which was significantly lower than day 0 (0.81±0.33). Similarly, the mean value of same pattern of stimulation was observed in case of similar T-cell responses against mCT at Day 7 (1.58± 1.23) which was significantly ($P$=0.33) higher than day 0 response (0.82±0.36). After day 7 of vaccination, response to mCT wane down (Fig.3.2).

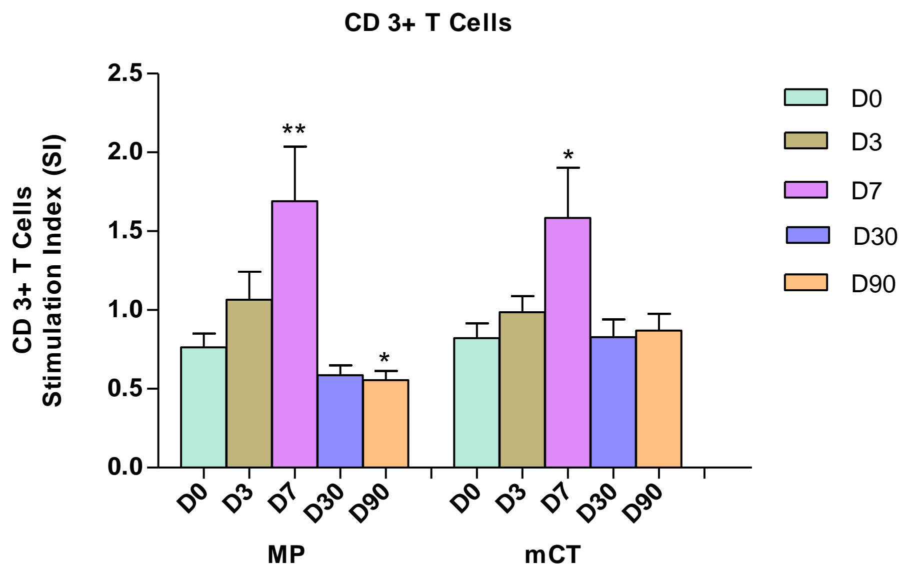


**Figure 3.2:** Comparison of lymphoblast T-cell response (blast CD3+) to *ex vivo* stimulation of whole blood by *V. cholerae* MP and mCT antigens at different study days in study participants. The response was expressed as Stimulation Index (SI). The study days are plotted on X- axis and stimulation index on Y-axis. Each bar represents the mean value and the error bar represents the standard error of means (SEM). Paired *t*-test was used for statistical evaluation *(*P < 0.05).*

### 3.2.1.2. CD3+ T-cell responses to MP and mCT in double dose 14 days apart vaccinees

In case of double dose 14 days apart vaccines, proliferative responses to MP antigen was significantly higher at day 7 (1.24±1.14) (*P*=0.006) than the baseline at day 0 (0.75±0.27) (*P*=0.76). But response decreased after day 7. Similar, responses were observed for CD3+ T-cell lymphoblast to mCT antigenic stimulation at day 7 and decreased gradually (Fig.3.3)

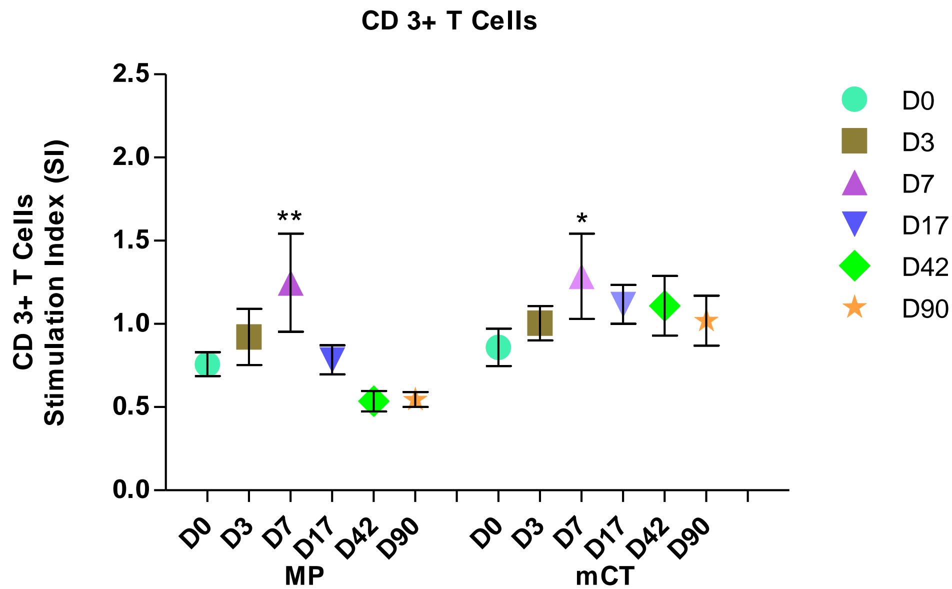


**Figure 3.3:** Comparison of lymphoblast T-cell response (blast CD3+) to *ex vivo* stimulation of whole blood by *V. cholerae* MP and mCT at different study days in study participants. The study days are plotted on X- axis and stimulation index on Y-axis. The response was expressed as Stimulation Index (SI). The dot represents the mean SI value and the error bar represent the standard error of mean (SEM). Paired *t*-test was used for statistical evaluation *(*P < 0.05)*

### 3.2.1.3 CD3+ T-cell responses to MP and mCT in double dose 30 days apart vaccinees

In this group, double dose of the vaccine was administered 30 days apart and CD3+ T-cell lymphoblast response was increased gradually up to day 7. The response at day 7 (1.54±0.85) was significantly (*P*=0.0003) higher than the baseline value (0.68±0.31) to MP antigen. The response was also found significantly (*P*= *0.025*) higher than 3 days after second dose of vaccine at day 33 (1.04±0.36). Stimulation index was decreased gradually over the period of day 60 and day 90. Similar responses to mCT was not observed (Fig.3.4)

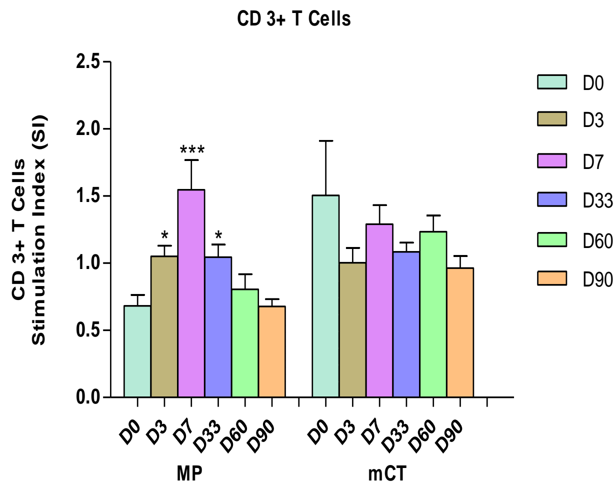


**Figure 3.4:** Comparison of lymphoblast T-cell response (blast CD3+) to ex vivo stimulation of whole blood by *V. cholerae* MP and mCT antigens at day 0 (before vaccination), day 3 (3 days after first dose of vaccination), day 7, day 33 (3 days after second dose of vaccination), day 42, day 90 in adult participants. The study days are plotted on X- axis and stimulation index on Y-axis. The response was expressed as Stimulation Index (SI). Each bar represents the mean value and the error bar represents the standard error of mean (SEM). Paired t-test was used for statistical evaluation (*$P < 0.05$).

## 3.2.2 Antigen specific CD4+ T-cell responses in vaccinees using different dose patterns

### 3.2.2.1. CD4+ and CD4+/ CD45 RO+ T-cell response to MP and mCT in single dose vaccinees

In response to MP antigen, the lymphoblast helper T cell (CD4+) and memory helper T cell (CD4+ CD45RO+) responses were increased after intake of the first dose of Shanchol vaccine. The baseline mean value for lymphoblast CD4+ T cell (1.25± 0.91) (1.67± 0.92) was significantly increased at day 3 (*P=0.047*) and day 7 (*P=0.001*) respectively. Similar pattern of response was found in lymphoblast CD4+/CD45RO+ T-cell elevated day 3 (*P= 0.030*) and day 7 (*P=0.0003*) responses were observed in response to MP. After day 7 responses was wane down in both lymphoblast CD4+ T cell and CD4+/CD45RO+ T -cell over the period day 90. Similar responses were not observed to mCT at any day points. (Fig.3.5)

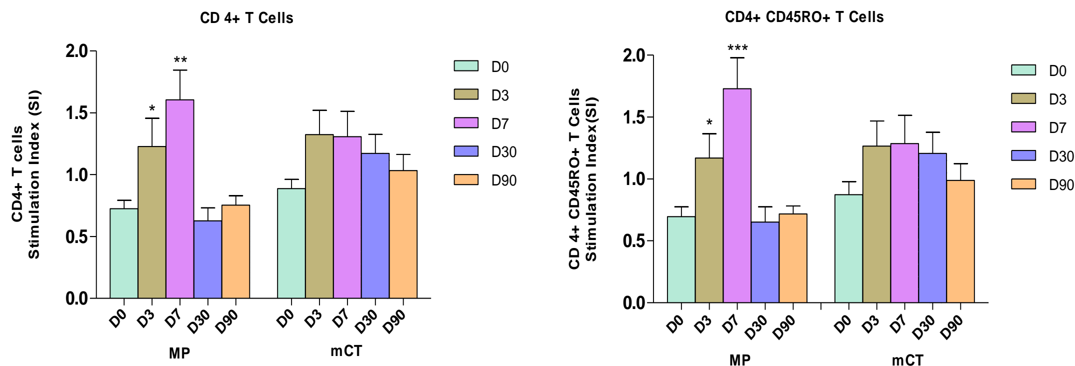


**Figure 3.5:** Comparison of lymphoblast T-cell response (blast CD4+ and CD4+ CD45RO+) to *ex vivo* stimulation of whole blood by *V. cholerae* MP and mCT antigens at day 0 (before vaccination), day 3 (3 days after first dose of vaccination), day 7, day 30, day 90 in study participants. The study days are plotted on X- axis and stimulation index on Y-axis. The response was expressed as Stimulation Index (SI). Each bar represents the mean value and the error bar represents the standard error of mean (SEM). Paired *t*-test was used for statistical evaluation *(*P* $< 0.05$).

### 3.2.2.2. CD4+ and CD4+/ CD45 RO+ T-cell responses to MP and mCT in double dose 14 days apart vaccinees

The baseline SI to MP antigen for lymphoblast CD4+ T-cell and the CD4+ CD45RO+ T-cell were 0.77±0.44 and 0.74±0.46 respectively. Three days after the first dose of the vaccine, the SI for lymphoblast CD4+ T-cell showed increasing trend (1.62±1.23) which eventually became significant at day 7 (1.42±0.83) compared to baseline. Similarly, the memory helper T cell (CD4+/CD45RO+ T cell) response was significantly (*P=0.03*) higher (1.42±0.83) at day 7 to MP antigen. Three days after the second dose of vaccine, SI for both helper (*P=0.02*) and memory helper (*P=0.04*) types of lymphoblast were also found significantly higher. But, in both cell types, responses decreased. However, no response was observed to mCT at any study day points compared to baseline (Fig.3.6).

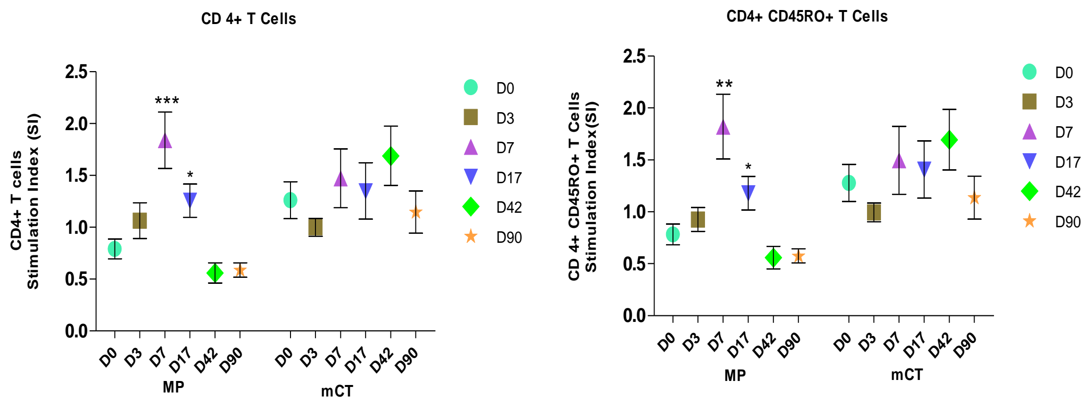


**Figure 3.6:** Comparison of lymphoblast T-cell response (blast CD4+ and CD4+ CD45RO+) to *ex vivo* stimulation of whole blood by *V. cholerae* MP and mCT antigens at day 0 (before vaccination), day 3 (3 days after first dose of vaccination), day 7, day 17 (3 days after second dose of vaccination), day 42, day 90 in adult participants. The study days are plotted on X- axis and stimulation index on Y-axis. The response was expressed as Stimulation Index (SI). The dot represents the mean SI value and the error bar represent the standard error of mean (SEM). Paired *t*-test was used for statistical evaluation *(*P < 0.05).*

### 3.2.2.3. CD4+ and CD4+/ CD45 RO+ T-cell response to MP and mCT in double dose 30 days apart vaccinees

A gradually increasing trend of responses for CD4+ lymphoblast and the CD4+/CD45RO+ T cell lymphoblast was found at day 3, day 7 in vaccinees after administration of first dose of the vaccine. The baseline SI for CD4+ lymphoblast and the CD45RO+ lymphoblast were (0.67± 0.32) and (0.64±0.33 ), three days after administration of first dose vaccine, the SI for CD4+ T cell and the CD4+/CD45RO+ T cell lymphoblast were (1.22±0.57) and (1.16± 0.58) respectively which were significant in comparison to the baseline. At day 7, more significant SI for both populations were observed with the mean value (1.71±1.28) for CD4+ lymphoblast and (1.70± 1.33) for CD4+/CD45RO+ lymphoblast. After the second dose of the vaccine, vaccinees also showed significant elevated responses for lymphoblast CD4+ T cell and lymphoblast CD4+/CD45RO+ T cell to MP antigen. However, in both helper and memory helper cells cellular responses to MP and mCT decreased after three days of second dose of vaccine. mCT responses at different days were elevated than MP specific responses. (Fig.3.7).

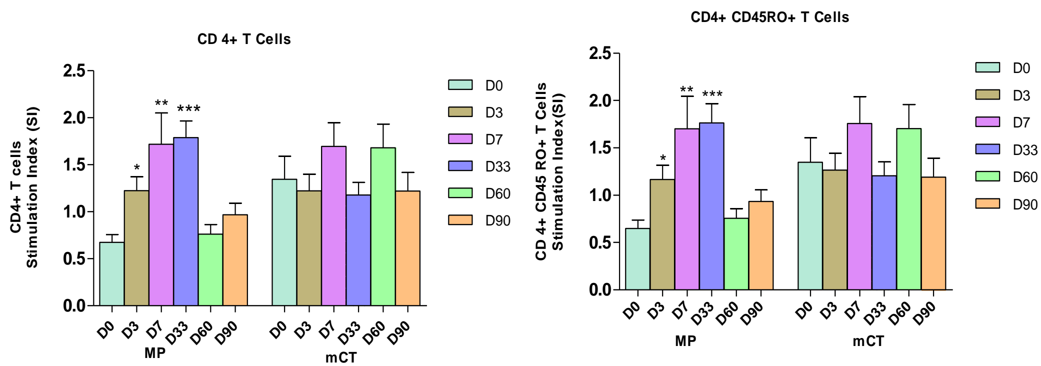


**Figure 3.7:** Comparison of lymphoblast T-cell response (blast CD4+ and CD4+ CD45RO+)) to *ex vivo* stimulation of whole blood by *V. cholerae* MP and mCT antigens at day 0 (before vaccination), day 3 (3 days after first dose of vaccination), day 7, day 33 (3 days after second dose of vaccination) day 60 and day 90 in adult participants. The study days are plotted on X-axis and stimulation index on Y-axis. The response was expressed as Stimulation Index (SI). Each bar represents the mean value and the error bar represents the standard error of means (SEM). Paired t-test was used for statistical evaluation (*P < 0.05).

## 3.2.3 Antigen specific CD8+ and CD8+/CD45RO+ T-cell response at different dose pattern

### 3.2.3.1. CD8+ and CD8+/CD45RO+ T-cell response to MP and mCT in vaccinees receiving single dose of the vaccine

Proliferative response to MP antigen CD8+T cell lymphoblast response was increased at day 7 (1.66±1.14) compared to baseline (0.84±0.37) at day 0 after administration of single dose vaccine. Over the study period, the response was maximum at day 7 which was found gradually decreased at day 30 (0.97±0.41) and day 90 (0.73±0.37). In case of memory cytotoxic T-cell (CD8+/CD45RO+) lymphoblast, similar pattern of response were observed and the SI for CD8+/CD45RO+ T cell lymphoblast has the highest peak at day 7 (1.38± 1.43). After that the responses gradually wane over the period of day 30 and day 90. Although no significant change in SI for CD8+ T cell lymphoblast to mCT was observed in this group, there is a significant elevated CD8+/CD45RO+ T cell lymphoblast specific SI was found at day 3 and day 7. But the response did not persist at later stage over the period of day 30 and day 90 (Fig.3.8).

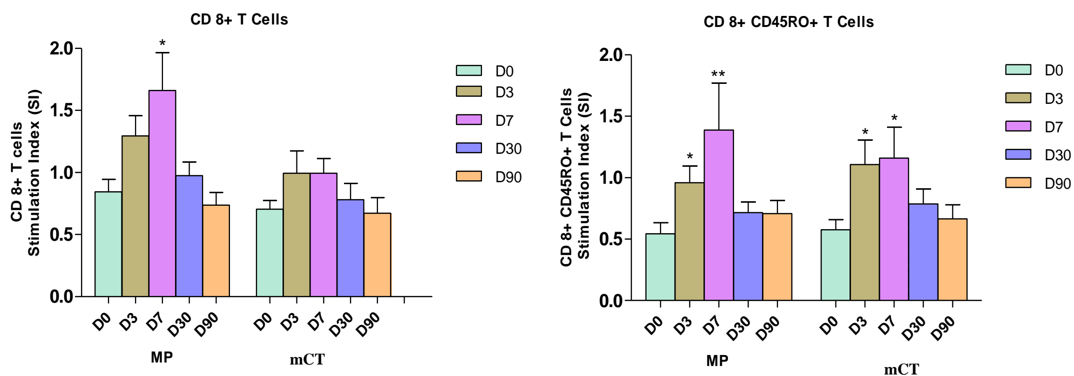


**Figure 3.8:** Comparison of lymphoblast T-cell response (blast CD8+ and CD8+ CD45RO+) to *ex vivo* stimulation of whole blood by *V. cholerae* MP and mCT antigens at day 0 (before vaccination), day 3 (3 days after first dose of vaccination), day 7, day 30, day 90 in adult participants. The study days are plotted on X- axis and stimulation index on Y-axis. The response was expressed as Stimulation Index (SI). The response was expressed as Stimulation Index (SI). Each bar represents the mean value and the error bar represents the standard error of mean (SEM). Paired *t*-test was used for statistical evaluation *(*P < 0.05).*

### 3.2.3.2. CD8+ and CD8+/CD45 RO+ T-cell response to MP and mCT in double dose 14 days apart vaccinees

Cytotoxic and memory cytotoxic lymphoblast cell significantly increased at day 7 (*P*=0.01) and day 17 (*P* < 0.0001) when stimulated with MP after administration of double dose 14 days apart vaccine. The responses of CD8+ cytotoxic T cell at day 7 (1.16±0.36) was increased significantly and remain elevated up to day 17(1.50±0.68). Similar responses were observed for memory cytotoxic T cells. Response was elevated at day 3 and remained up to day 17 (1.12±0.49), (0.93±0.44), (1.08±0.66) respectively. No change in SI for CD8+ T cell lymphoblast to mCT was observed in this group; there is a significantly elevated CD8+/CD45RO+ T cell lymphoblast specific SI was found at day 3, day 7 and day 17. But the response did not persist longer. (Fig.3.9).

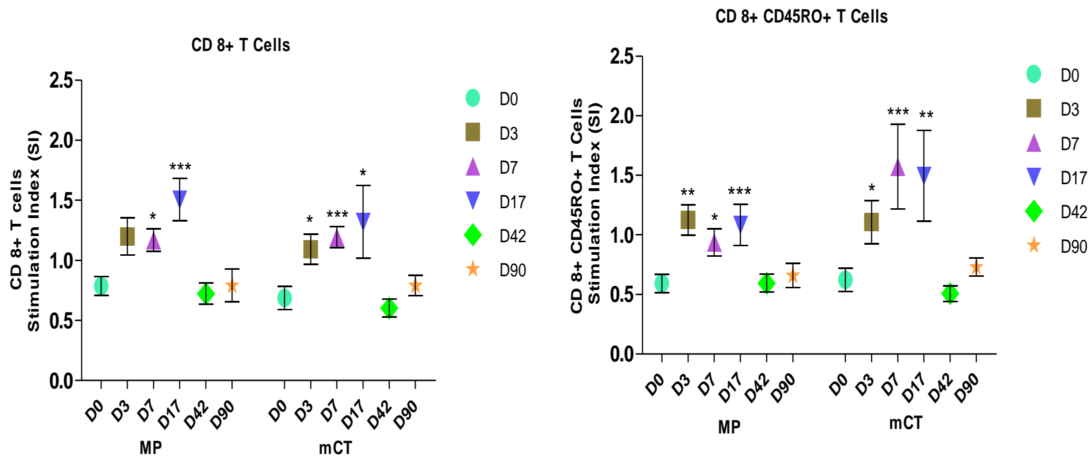


**Figure 3.9:** Comparison of lymphoblast T-cell response (blast CD8+ and CD8+ CD45RO+) to *ex vivo* stimulation of whole blood by *V. cholerae* MP and mCT antigens at day 0 (before vaccination), day 3 (3 days after first dose of vaccination), day 7, day 17 (3 days after second dose of vaccination), day 42, day 90 in adult participants. The study days are plotted on X- axis and stimulation index on Y-axis. The response was expressed as Stimulation Index (SI). The dot represents the mean SI value and the error bar represent the standard error of mean (SEM). Paired *t*-test was used for statistical evaluation *(*P < 0.05).*

### 3.2.3.3. CD8+ and CD8+/CD45 RO+ T-cell response to MP and mCT in double dose 30 days' apart vaccinees

Proliferative response to MP antigen, the CD8+ T cell lymphoblast specific was significantly increased (*P*=0.0006) at day 7 (1.41± 1.13) compared to baseline (0.80± 0.52). After administration of second dose of the vaccine, further elevation of the SI was found at day 33. But after that, the SI started to decline gradually although it was significantly elevated one month after the second dose of the vaccine (at day 60) which become almost comparable to baseline (0.54± 0.33) at day 90 (0.70± 0.40).

Similar pattern of SI for MP stimulation was also observed in case of CD8+/CD45RO+ lymphoblast population and significantly increased response was at day 3 and remained elevated up to day 60 in comparison to baseline. On the contrary, no significant change in CD8+ T cell and CD8+/CD45RO+ T cell lymphoblast specific SI to mCT were observed because of the high baseline SI to mCT in comparison to those for MP (Fig.3.10).

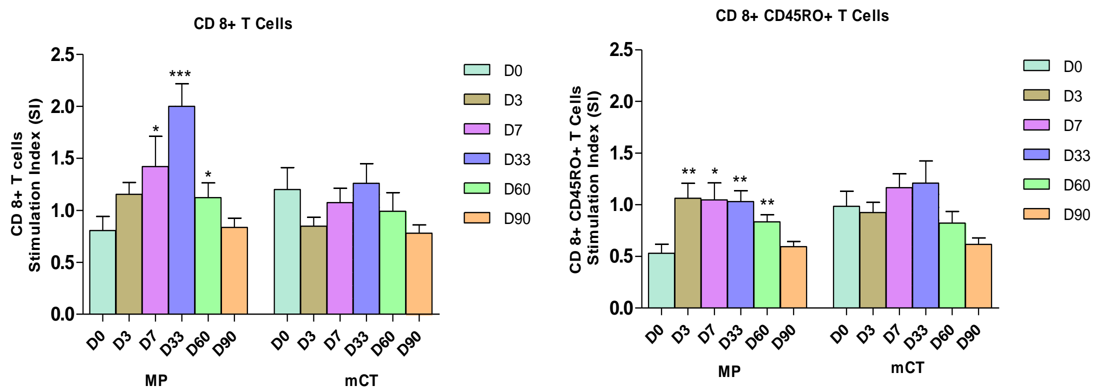


**Figure 3.10:** Comparison of lymphoblast T-cell response (blast CD8+ and CD8+ CD45RO+) to *ex vivo* stimulation of whole blood by *V. cholerae* MP and mCT antigens at day 0 (before vaccination), day 3 (3 days after first dose of vaccination), day 7, day 33 (3 days after second dose of vaccination), day 60, day 90 in adult participants. The study days are plotted on X- axis and stimulation index on Y-axis. The response was expressed as Stimulation Index (SI). Each bar represents the mean value and the error bar represents the standard error of mean (SEM). Paired *t*-test was used for statistical evaluation *(*P < 0.05).*

## 3.3. Vibriocidal Antibody Response

### 3.3.1. *Vibrio cholerae* O1 Ogawa specific vibriocidal antibody response in vaccinees

Overall 60% of single dose vaccinees, 67% of two dose 14 days apart vaccinees and 73% of two dose 30 days apart vaccinees responded with vibriocidal antibodies against *Vibrio cholerae* O1 Ogawa.

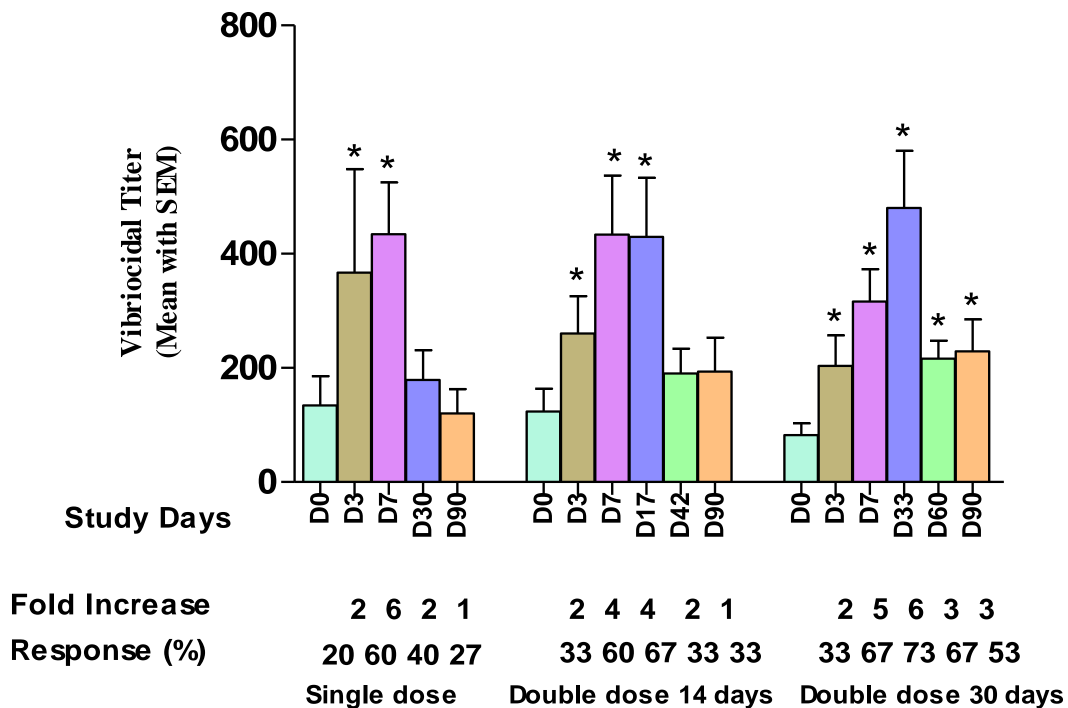


**Figure 3.11: Vibriocidal antibody titer using *V. cholerae* O1 Ogawa as target bacteria in different groups receiving vaccine.** The bars indicate mean reciprocal end titers, and error bars represent the standard errors of the mean (SEM). Asterisks indicate statistically significant differences between different day points *(*P<0.05)*. The Wilcoxon signed-rank test and Mann Whitney test was used for analyses of the data. Mean fold changes and responder frequencies are also listed.

The baseline mean vibriocidal antibody titer of the single dose vaccinees was 134.6; and two dose (14 days and 30 days apart) vaccine cohorts were 123.7 and 82.00 , respectively

(Figure 3.3.1). After first dose of vaccine, all vaccine regimens showed significantly increased Ogawa specific vibriocidal antibody response at day 7 (single dose, *P =0.002;* two doses 14 days apart, *P=0.002;* two dose 30 days apart, *P= 0.0007*). In single dose regimen, this response became highly diminished within in one month of vaccination. However, in two doses regimen, the responses also stayed significantly higher 3 days after 2nd dose of vaccine at day 17 (two dose 14 days apart, *P=0.006*) and at day 33(two dose 30 days apart, *P=0.001*). After that the response gradually went down over the period of day 90 in case of two doses 14 days apart vaccine regimen. On the contrary, in two dose 30 days apart vaccine regimen, the response was found significantly increased over the period of day 90 (*P=0.006*). However, the vibriocidal antibody response was also compared between day 17 and day 33 of both two dose vaccine regimens, but no significant difference in their response (*P=0.57)* was observed (Fig 3.11)

### 3.3.2. *Vibrio cholerae* O1 Inaba specific vibriocidal antibody response in vaccinees

Similar trend of vibriocidal antibody response was observed to *V. cholerae* O1 Inaba in all vaccine regimens. Overall 87% of single dose vaccinees, 80% of two dose 14 days apart vaccinees and 87% of two dose 30 days apart vaccinees responded with *V. cholerae* O1 Inaba specific vibriocidal antibodies.

The baseline vibriocidal titer in single dose vaccinees was 127.7; and two dose 14 days apart and 30 days apart vaccines were 53.33 and 133.3 respectively (Figure 3.3.2). After 1st dose of vaccine, similar increment of *V. cholerae* O1 Inaba specific vibriocidal antibody responses were observed at day 7 in single dose (*P =0.0007)* and two doses 14 days (*P=0.0007)* and 30 days apart (*P=0.001*) vaccinees. In case of two dose 14 days apart recipients, the response was declined 3 days after 2nd dose of vaccination and it gradually became diminished over the study period. On the contrary, the *V. cholerae* O1 Inaba specific vibriocidal antibody response was found significantly increased over the period of day 90 in two dose 30 days apart vaccination regimen.

However, comparison of the responses also between day 17 and day 33 of both double doses vaccination regimens was also done and significant difference in their response (*P= 0.0077*) was found (Fig.3.12).

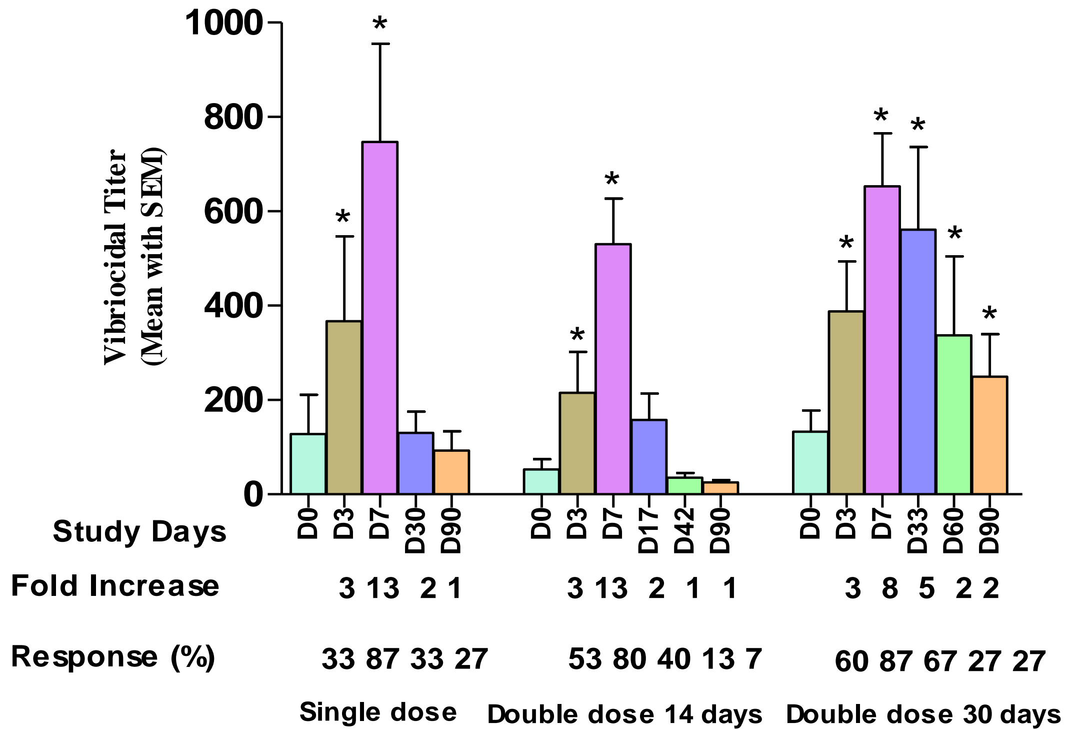


**Figure 3.12: Vibriocidal antibody titer using *V. cholerae* O1 Inaba as target bacteria in different groups receiving vaccine.** The bars indicate mean reciprocal end titers, and error bars represent the standard errors of the mean (SEM). Asterisks indicate statistically significant differences between different day points *(*P<0.05)*.The Wilcoxon signed-rank test and Mann–Whitney rank sum tests were used for statistical evaluation. Mean fold changes and responder frequencies are also listed.

# Chapter 4

# Discussions

Immunological memory which is an important feature of adaptive immune responses generates specific memory cells after infection or vaccination (Megan KL MacLeod 2010). It has been assumed that protection from cholera may be mediated by memory B cells capable of an anamnestic response in the gut mucosa and these memory B cells may depend on stimulation provided by memory T cells for their development and maintenance (Weil, Arifuzzaman et al. 2009). Experimental animals study has also confirmed that the mucosal immune response to cholera toxin is T cell dependent and CD4+ helper T cells have an important role in this regard (Elson 1987) (Hirabayashi 1991) (Holmgren 1987). Therefore, understanding the memory T cell responses is important for optimizing design of an oral cholera vaccine or immunization strategies.

Previous study in Bangladeshi participants showed a satisfactory results of Shanchol as a promising vaccine to be safe with adequate immune responses in all ages (Saha, Chowdhury et al. 2011)and thus encouraged continued study of this vaccine. The present study was conducted to evaluate the immunogenicity of this vaccine in terms of adaptive immune responses. We have investigated if immunization with the oral cholera vaccine, Shanchol, induces cholera antigen specific CD4+ and CD8+ memory T cell responses in adult participants using different dose strategies.

In a previous study, it was shown that the effector T-cells significantly increases after 6 days of in vitro stimulation of lymphocytes (Weil, Arifuzzaman et al. 2009). Therefore cells are being cultured for six days to amplify the lymphoblastic T cells. To stimulate the T cells *in vitro*, *V. cholerae* specific immunogenic antigens, *V. cholerae* membrane preparation (MP) and G33D mutant CT (mCT) were selected. *V. cholerae* MP contains numerous of cell membrane proteins, outer membrane protein U precursor porin (OMPU) most abundantly. Although Shanchol does not contain any detectable cholera toxin (CT), proliferative responses to the mCT has been evaluated in this study as it has been done earlier with Dukoral, another oral cholera vaccine. Although CT is a potent mucosal-vaccine adjuvant in experimental animals (Northrup and Fauci 1972) (Elson and Ealding 1984) (Freytag and Clements 2005), it is already hypothesized that anti-CT immunity is not sufficient or even necessary for protection from cholera (Apter, Lencer et al. 1993) (Bishop, Tarique et al.). Only DMEM complete medium has been used as control and

Phytohaemagglutinin (PHA), a known polyclonal activator was used as positive stimulatory antigens that helped to ensure the proliferation of lymphocytes in the *in vitro* culture.

To assess T-cell memory, flow cytometric analysis was used to detect CD45RO+ T cells which is a well established marker for T-cell memory (Ahmed and Gray 1996) (Sprent and Surh 2002). FASCIA technique was employed rather than other currently available methods as it only requires small amounts of blood.

In this study, Shanchol vaccine was administered using different dose patterns as single dose, double dose 14 days apart and double dose 30 days apart. It was observed that there is a gradual increase in CD4+ T cell and CD8+ T cell response to MP which become significantly maximum at day 7 after administration of one dose of the vaccine in all groups. Similar proliferative response was found for CD4+/CD45RO+ and CD8+/CD45RO+ memory T-cell response to *V. cholerae* MP. In a previous study, increased proliferative memory T cell response to *V. cholerae* antigens MP, TcpA, VCC was observed at day 7 in adult patients due to exposure to *V. cholerae* (Weil, Arifuzzaman et al. 2009). It is hypothesized that early T cell responses is required for generation and maintenance of subsequent durable memory B cell responses (Weil, Arifuzzaman et al. 2009). So, we assume that the significantly elevated memory T cell responses observed by day 7 after vaccination, a time prior to and concurrent with the development of B cell responses, may play a major role in development of memory B cell response to this vaccine.

In the present two dose vaccination strategies, significantly proliferative CD4+/CD45RO+ and CD8+/CD45RO+ memory T-cell responses to *V. cholerae* MP have also been observed 3 days after second dose of vaccination (at day 17 and day 33) which was found to be absent in one dose of vaccination regimen. This can happen for the re-exposure of the vaccinees in two dose vaccination scheme that causes immediate proliferative response.

Although in a previous study, significant T-cell responses were observed to mCT in young children after administration of Dukoral vaccine (Ahmed, Arifuzzaman et al.

2009), no such significantly elevation of memory T cell responsiveness could be detected to mCT in adult participants after Shanchol vaccination. It is assumed that the response to mCT is absent as the vaccine contains LPS of different *V. cholerae* strains and an undetectable level of CTB.

However, a high baseline response to mCT has been found in all vaccinee groups of this study. Similarly, high baseline responses to mCT were found even in children after administration of Dukoral vaccine in a previous study (Ahmed, Arifuzzaman et al. 2009). Previous priming of study participants living in areas of endimicity with *V. cholerae*, *E. coli* can be responsible for this as the ETEC strains express the heat labile toxin (LT) which is highly homologous with cholera toxin (CT) and is responsible for the non-specific responses (Ahmed, Arifuzzaman et al. 2009).

In the present study, within one month of vaccine administration, the proliferative CD4+/CD45RO+ memory T-cell response gradually wanes down causing no significant response after one month of intake of last vaccine dose in all vaccination schemes and at day 90 the proliferative response was found highly diminished. This correlates with earlier findings where it was explained that protective immunity generated by vaccine candidates is partial and short-lived which declines more quickly in people living in Cholera endemic zone in contrast to natural infection (Weil, Arifuzzaman et al. 2009).

Vibriocidal antibody assay is a surrogate marker that is widely used to evaluate the efficacy of cholera vaccine (Saha D 2004). This *in vitro* assay has been shown to correlate with protective immunity to oral cholera vaccine (Clemens 1987) and there is no threshold at which protection is complete (Saha D 2004).

In our study, the *V. cholerae* O1 Ogawa and Inaba specific vibriocidal antibody responses were found maximum at day 7 after administration of one dose of vaccine in all vaccine cohorts. After one month of last dose of the vaccination, the responses declined gradually and it was found highly diminished over the period of day 90 in all vaccination regimens except for the double dose 30 days apart vaccination regimen where the responses were found lower but significantly increased in comparison to baseline. This correlates with previous findings where it was shown that the titer falls to baseline within several months

after natural infection (Harris, Bhuiyan et al. 2009). So, it can be suggested that similar to natural infection with *V. cholerae*, the long-term immunity following vaccination may be mediated through memory and anamnestic responses (Alam, Riyadh et al.).

This study shows that the memory T cell response is important in cholera and also provides a tool to understand the effectiveness of vaccine. This study may help to understand how this new vaccines may need to be modified to elicit an immune response that will provide long-lasting protection. The findings of this study may also explain that the relatively short-term protection is afforded by administration of Shanchol oral cholera vaccines.

As it is found in our study that the vaccine is safe, immunogenic and effective following a single-dose; it will have intense implications in case of emergencies where a double dose regimen is difficult to set up. The delivery system and uptake of the vaccine is also feasible and other associated public health costs of immunization is minimized when the single dose vaccination is available. Thus, the findings of this study imply that the single dose may be as effective as double dose (given 14 days apart and 30 days apart).

From the present study it may be concluded that

i. Early proliferative T cell response is a potent tool that provides help for the generation of subsequent memory B cell responses.

ii. The long-term immunity following vaccination may be mediated through memory and anamnestic responses.

iii. Early T-cell-mediated events would help to design a more effective vaccine and single dose vaccine is as effective as double doses.

**Recommendation for future work**

As this study has been carried out in small number of participants, further study with a large sample size should be conducted to confirm robust efficacy and durability of Shanchol single dose vaccine and to conclude that single dose is as effective as the two dose regimen of vaccination in cholera endemic areas like Bangladesh.

# Chapter 5

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# Appendices

# APPENDIX A

## Laboratory Apparatus

1. Fluorescent-activated cell sorter *(FACSCalibur, Becton Dickinson, San Jose, CA)*
2. Mini scale centrifugations were carried out in a *Sorvall®* *pico* microfuge and large-scale centrifugation were carried out in a *Sorvall®* Legend™ X*RT* super speed centrifuge.
3. ELISA reader (*ASCENT Multiskan® reader.*)
4. Spectrophotometer- BECKMAN COULTER$_{TM}$
5. Water Bath
6. Incubator Thermo Scientific
7. Eppendorf tubes and micropipette tips were taken from Eppendorf® and Sigma, and were sterilized by autoclaving at 121°C for 20 minutes.
8. Petri dishes used in the experiments were provided by either Sterilin or Gibco. Screw capped tubes and other glass wares were taken from *Pyrex® Labware*, *USA*.
9. Plastic tubes and pipettes were of Falcon®; both were the brands of Becton, Dickinson and Company, *BD*.
10. 96-well ELISA plates were obtained from *Nunc*™, *Sweden*
11. Polystyrene FACS tubes *(Becton Dickinson, San Jose, USA)*
12. Micropipettes were from Thermo Labsystems, *Denmark*.
13. Multi-channel dispenser *(Lab System, USA)*
14. Microtiter plates *(Nunc, Roskilde, Denmark)*
15. BD Falcon™ round-bottom tubes
16. Filter- Minisart® *Sartorius stedim biotech, Germany.*
17. Aluminum foil
18. Paraffin paper (BEMIS® *Neenah WI, U.S.A)*
19. Heparin-coated sterile vacutainer tubes *(Becton Dickinson, Ruther-ford, NJ)*

## Chemicals/Reagents

1. FBS (Fetal Bovine Serum Albumin), Gibco BRL- 16140-071
2. $H_2O_2$ (Hydrogen Peroxide), *Fisher Scientific, H-325*
3. $MgCl_2$ (Magnesium chloride), *Sigma- 7786-30-3*
4. Na-acetate, *Sigma-127-09-3*
5. $NaHCO_3$ (Sodium bi-carbonate), *Fisher Scientific- S233-500*
6. EDTA (*Titriplex$^R$ II Merck 1.0841*)
7. Sodium azide, $NaN_3$
8. BD Stabilizing Fixative
9. Guinea pig complement
10. Dulbecco Modified Eagle Medium, DMEM (*Invitrogen, USA*)
11. FACS flow *(Becton Dickinson)*
12. FACS antibody :
    1. Anti-CD3 APC (*Becton Dickinson*)
    2. Anti-CD4 PerCP (*Becton Dickinson*)
    3. Anti-CD8 FITC (*Becton Dickinson*)
    4. Anti-CD45RO PE (*eBioscience*)
13. Antigen used in FASCIA method:
    1. G33D mutant holotoxin (mutant form of cholera toxin)
    2. MP (Cholerae membrane protein)
    3. PHA (Phytohemagglutinin)

# APPENDIX B

**1. Preparation of phosphate buffered saline (PBS) (Vacutainer System; Becton Dickinson, Rutherford, NJ) ($P^H$ between 7.2 to 7.4)**

| Reagents | Amount |
|---|---|
| NaCl (0.136mM Fischer Scientific, Pittsburgh, PA, U.S.A ) | 80.00 gm |
| $Na_2HPO_4$ | 27.50 gm |
| $KH_2PO_4$ | 2.75 gm |
| KCL (Fischer Scientific, Pittsburgh, PA, USA) | 2.00 gm |
| Deionized water | 1000 mL |

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

**3. Preparation of FACS Buffer (1000 mL) ($P^H$ between 7.2-7.4)**

| Reagents | Amounts |
|---|---|
| EDTA (Titriplex$^R$ II Merck 1.0841) | 0.375 gm |
| Albumin (Bovine serum albumin) | 2.0 gm |
| Sodium azide, $NaN_3$ | 1.0 gm |
| PBS | 1000 mL |

No heating ($P^H$ between 7.2-7.4)

Sterilization should be done by filtration, not autoclaved.

Kept for 3 months

Store at 2-8$^{\circ}$ C

**4. Preparation of Normal Saline**

| Reagent | Amount |
|---|---|
| Distilled Water | 1L |
| Sodium Chloride ( NaCl ) | 9gm |

**5. Preparation of $NH_4Cl$ Lysing Solution (5X) (1000 mL)**

| Reagent | Amount |
|---|---|
| $NH_4Cl$ | 41.45 g |
| $KHCO_3$ | 5.00 g |
| EDTA-disodium salt | 0.185 g |

All the above reagents are transferred to 1L D/W, mixed, filtered by 0.2µl filter and then it is ready for use. Working conc. is 1X.

**6. Preparation of Gentamycin (10mg/mL)**

| Reagent | Amount |
|---|---|
| ISCOVE media | 15 mL |
| Gentamycin | 0.15 g |

- 0.15g Gentamycin was measured and taken in ISCOVE media and final volume was made to 15mL in a beaker was filtered with 0.2um filter.
- 1mL gentamycin was aliquoted in each eppendorf and stored at -20°C.

**7. Preparation of Mercaptoethanol**

| Reagent | Amount |
|---|---|
| DMEM complete medium | 50 mL |
| Mercaptoethanol | 17.5 µL |

- 17.5uL ME was taken in 50mL DMEM complete medium.
- Finally 1mL ME was aliquoted in each eppendorf and stored at -20°C.

**8. Preparation of Indicator Solution**

| | Sterile Saline | Bacteria | Complement |
|---|---|---|---|
| V. cholerae O1 (X25049 & 19479) | 2.55 ml | 150 µl | 300 µl |

# APPENDIX C

## Preparation of Media

### 1. Preparation of DMEM complete medium (50mL)

| Reagent | Amount |
|---|---|
| DMEM media | 44 mL |
| Fetal Bovine Serum (10% FBS) | 5 mL |
| Gentamycin (1%) | 0.5 mL |
| Merchaptoethanol (1%) | 0.5 mL |

### 2. Preparation of Iscove (1X) Medium (1000mL)

| Reagent | Amount |
|---|---|
| Iscove | 17.77 gm |
| Deionized Water | 1000 ml |

### 3. Preparation of BHI medium (Brain Heart Infusion medium) (1000 ml)

| Reagent | Amount |
|---|---|
| Bacto™ Brain Heart Infusion | 37 gm |
| Aotoclaved deionized Water | 1000 ml |

# APPENDIX D

## Software's

1. CELL QUEST PRO *(version 3.3; Becton Dickinson)*
2. Flow Jo *(Tree Star, Inc., version 8.5.3)*
3. Prism 5.0 *(Graph PAD Software 10855 Sorrento Valley Road #203, San Diego, CA 92121 USA)*
4. Microsoft Word and Excell