Characterization of the Immune Response to *Vibrio cholerae* O1 Antigens and Its Impact on Vaccine Development Strategies

Ph.D Thesis

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Declaration

This is to certify that the thesis entitled "Characterization of the Immune Response to *Vibrio cholerae* O1 Antigens and Its Impact on Vaccine Development Strategies" contains the findings of Firoz Ahmed's Ph.D. research works. He has fulfilled the ordinance and regulations required for the degree Doctor of Philosophy (Ph.D.) from the Department of Microbiology, University of Dhaka, Bangladesh.

It may be mentioned here that based on his thesis work, one research paper has already been published in a peer reviewed international journal and two more papers are in the pipeline to be published soon.

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Declaration

This is to certify that the thesis entitled "Characterization of the Immune Response to *Vibrio cholerae* O1 Antigens and Its Impact on Vaccine Development Strategies" contains the findings of Firoz Ahmed's Ph.D. research works.

During my tenure at Institut Pasteur, Paris, France, Firoz has carried out studies and training at my laboratory "Unite du Cholera et des Vibrions" under my supervision as part of the collaborative research leading to Ph.D. He finished all the designed research works of first and third year to determine the antigenic epitope of *Vibrio cholerae* O1 Inaba serotype by a crystallographic method as well as cloning and sequencing of the antibody molecules generated against different cholera immunogens after immunization to determine the genetic variability. The findings of his work will have an excellent impact in the cholera vaccine research and are producing a number of manuscripts which have been accepted or to be submitted to several peer reviewed journals. The research methodology adapted for his research work implicated intrinsic application of immunological, molecular and biotechnological approaches.

He has fulfilled the ordinance and regulations required for the degree Doctor of Philosophy (Ph.D.) from the Department of Microbiology, University of Dhaka, Bangladesh.

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ABSTRACT

Cholera persists as one of the major causes of mortality and morbidity globally. Prevention using different approaches are needed, one of which is vaccination. Improving immunogenicity of oral cholera vaccines and production of effective single dose schedules suitable for all age groups and populations is the prerequisite. Historically injectable cholera vaccines have been replaced in the last three decades by oral mucosal vaccines but neither fulfills all the requisites. The aim was to evaluate potential vaccine immunogens which have not been tested earlier for generating protective immunity. The concept of a one dose vaccine using bacterial antigen(s) through a "needle free approach" of immunization via the transcutaneous route (TCI) for enteric vaccines was explored in this work.

The lipopolysaccharide (LPS) of *Vibrio cholerae* O1 is the most consistently described protection associated immunogen and has been identified as the most important of the antigens described so far. Epidemiological studies of natural infection in cholera suggest that Inaba serotype is more protective against subsequent attacks of cholera than the Ogawa serotype. Crystallographic studies have revealed antigenic determinants of LPS Ogawa and binding pattern of monoclonal antibodies (mAbs) using synthetic fragment of Ogawa in the O-specific polysaccharide (O-PS) region. Previous studies with other mAbs have indicated involvement of both the core and the O-specific polysaccharide as common antigenic determinants. This dissertation describes the first crystal structure of a mAb F-22-30 that recognizes a common epitope present in both the Ogawa and Inaba serotypes of *V. cholerae* O1. Modeling calculations and preliminary NMR results suggest that binding pocket at the center of antibody recombination site could bind side-chain of a perosaminyl residue from the O-PS with higher accessibility compared with core-PS implying a lateral recognition in contrast to antibodies specific for the Ogawa serotype.

Mechanism of the immune response elicited by transcutaneous route of immunization (TCI) with potential *V. cholerae* antigens was evaluated as alternative modes of vaccination compared with oral (ORI) and subcutaneous route (SCI) with the same antigens i.e. recombinant cholera toxin B subunit (CTB), cholera toxin (CT), recombinant toxin co-regulated pilus A (TcpA), LPS Ogawa and Inaba and in-house prepared membrane extract in Balb/c mice to determine the immunoglobulin isotype patterns and its properties linked with protection. Both humoral and cellular immune responses induced by the immunization were studied using ELISA and flow cytometric procedures to assess and phenotypic changes in the mucosal and systemic compartments were analyzed. Among the three routes of immunization, SCI showed robust elevation pattern with superiority of IgG compared toIgM and IgA respectively. TcpA in the TCI route did not up-regulate IgA, while CT and CTB were seen to respond with all three antibody isotypes. CTB immunized mice showed reduced serum antibody titer in the zinc supplemented but CT and TcpA individually as well as co-administered together had no effect. When CT in the TCI and SCI route generated all four IgG subclasses, TcpA and LPS-specific antibody response was only IgG1 subclass where membrane extract has overcome the inherent limitation of LPS. However, sera obtained from both CT and TcpA immunized were negative for vibriocidal antibody responses. Membrane extract displayed more than 10 times elevated IgM and IgG titers with vibriocidal response than LPS with comparable positive effect of zinc supplementation. Moreover, ALS antibody titers were comparable with ELISA and vibriocidal assays and expressed the recently formed status of the humoral response. Expression of increased CD19+ve B cells in blood and lamina propria as well as CD4+ cells in the lamina propria were detected following TCI. Significant elevations of the co-stimulatory marker CD40 in the CD19+ve B cell population as well as CD28 and CD154 in the CD4 population were observed in spleen and

lamina propria. The expression of homing markers, cutaneous lymphocyte antigen (CLA) was increased in blood and lamina propria (CD19, CD4), whereas an increase of the gut homing marker (β_{7}) was not seen in blood or the mucosal surface in the different cell types. The results of TCI immunization were compared with those obtained after subcutaneous (SCI) where similar or discordant results were seen but interestingly the magnitude of responses by TCI was equal to that seen by SCI.

The isotype switch and antibody maturation patterns elicited by the TCI route of immunization incorporating zinc as another confounder with the potential immunogens, applying a novel in-house immunogenetic method, were studied. Finally, bioinformatics approaches were applied to compare position specific similarities and dissimilarities of LPS specific 'membrane extract' immunized antibody sequences with experimentally characterized mAb F-22-30 for better understanding the binding affinity of the carbohydrate antigen. Considering the percentages of antigen-specific cellular representation at different compartments, CTB specific cells was the highest compared to TcpA and LPS specific pool. With the novel *in situ* positive selection immunogenetic technique, first murine B-cell VH and VL chain repertoire specific for protein and polysaccharide antigens were reported. VH and VL amino acid sequence comparison demonstrated variable expression in co-administration than individual immunogens. Amino acids expressed through immunization of polysaccharide immunogens were more stable than protein immunogens specific sequences. Moreover, CDRH3 length of antibody sequences to protein immunogens varied between 8 and 19 amino acids with CTB specific shorter sequences containing more charged and hydrophobic residues. Further bioinformatics approaches with Inaba specific sequences obtained through TCI confirmed ligand binding with similar nature amino acids to be identical groove type binding pattern. Findings presented here suggest that the newer, needle-free TCI route is suitable for inducing humoral and cellular immune responses in the systemic and mucosal compartments and should be considered as an option in vaccine development strategies.

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µl/ml	Microliter per milliliter
Å	Angstrom
ALS	Antibodies from lymphocyte secretion
APC's	Antibody presenting cells
ASC	Antibody secreting cells
cAMP	Cyclic adenosine monophosphate
CBP	Choline binding protein
CD	Cluster of differentiation
cDNA	cyclic Deoxy-ribonucleic acid
CDR	Complementarity determining region
CF	Control feed
CLA	Cutaneous lymphocyte antigen
CMF-HBSS	Calcium magnesium free Hank's balanced salt solution
CSR	Class switch recombination
CT	Cholera toxin
CTB	Cholera toxin subunit B
CTX Φ	Cholera toxin phage
DC	Dendritic Cells
DEAE	Diethylaminoethyl
ELISA	Enzyme Link Immune Sorbent Assay
Fab	Fragment antibody binding
FACS	Fluorescence Activated Cell Sorters
Fc	Fragment crystallization
FITC	Fluorescein isothiocyanate
FR	Framework Region
Fv	Fragment variable
GALT	Gut Associated Lymphoid Tissue
gm	Gram
GM1	Monosialo-tetrahexosyl-ganglioside
ICAM	Intracellular adhesion molecule
IEL	Intraepithelial lymphocytes
IFN	Interferon
IgA	Immuneglobulin A
IgG	Immuneglobulin G
IgG1	Immuneglobulin G1
IL	Interleukin
JH	Heavy chain junction
LBW	Low birth weight
LPL	Lamina propria lymphocytes
LPS	Lipopolysaccharide
LT	Heat liable toxin
Mabs	Monoclonal antibodies
MAdCAM-1	Mucosal vascular addressin cell adhesion molecule 1
MHC	Major histocompatibility complex
ml/h	Milliliter per hour
ml/l	Milliliter per liter
MSHO	Mannose sensitive hemagglutinin operon
MSHP	Mannose sensitive hemagglutinin protein
NF-κB	Nuclear Factor-kappa B
NMR	Nuclear Magnetic Resonance
OD	Optical density

ORFs	Open reading frames
ORI	Oral immunization
ORIG	Oral immunization group
ORS	Oral rehydration saline
P	Prabability
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll proteins
PNAd	Peripheral node addressin
rCTB	Recombinant Cholera toxin B subunit
R-PE	R-Phycoerythrin
rpm	Rotation per minute
RPMI	Roswell Park Memorial Institute
SCI	Sub cutaneous immunization
SCIG	Sub-cutaneously immunized group
SDS-PAGE	Sodium dodecyl sulfate-poly acrylamide ael electrophoresis
SHM	Somatic hyper mutation
sIgA	secretory Immuneglobulin A
STD-NMR	Saturation transfer difference-nucleotide magnetic resonance
TCBS	Thiosulfate citrate bile-salt sucrose
TCI	Trans-cutaneous immunization
TCIG	Trans-cutaneously immunized group
TCP	Toxin coregulatedpilus
Th	T helper cell
VH	Variable heavy chain
VL	Variable light chain
WC/rBS	Whole cell/recombinant B subunit
WC-OM	Whole cell-outer membrane
ZnM	Zinc deficient
ZnP	Zinc supplemented

1 Introduction and Review of Literature

Cholera is a clinical syndrome that can quickly annihilate thousands of life with explosive epidemics and persists with significant mortality at endemic region (Kaper *et al.*, 1995; Provenzano *et al.*, 2006). Although cholera is predominant in developing countries, a marked increase of nearly 100% of cholera cases was seen on all continents at the end of the 20th century(WHO, 1999).World Health Organization (WHO) reported worldwide 85% increase of total cholera cases with a total of 589854 cases and 7816 deaths (WHO, 2012). Global trends in the incidence of cholera have increased steadily since the beginning of the millennium as noted a 24% increase of cases in 2004 to 2008 with 2000 to 2004(WHO, 2008) while gross underreporting is present elsewhere(WHO, 2009).In recent years, devastating epidemics of cholera have occurred in Angola, Ethiopia, Zimbabwe, Pakistan, Somalia, Sudan, Vietnam, and Haiti. Importantly the epidemics in Zimbabwe and Haiti have been extensive resulting in high mortality and morbidity and resulting in the disease becoming endemic in these countries (Harris *et al.*, 2010; WHO, 2011).

Cholera remains one of the most feared infectious diseases in public health which poses great threat to individuals with lower immunity, such as malnourished children (Palmer *et al.*, 1976) or people living with AIDS, are at greater risk of death if infected by cholera (WHO, 2012). About 80% of the infected people present mild to moderate acute watery diarrhea, while the other 20% develop rapidly severe dehydration leading to deaths(Krishna *et al.*, 2003).In spite of simple and widely accessible oral rehydration treatment, young children and the elderly are particularly vulnerable to severe cholera (WHO, 2001). Man-made and natural disasters as well as poor sanitary conditions have always favoured the spread of cholera epidemics(WHO, 2001).The situation has become absolutely complex increasing cholera outbreaks due to the emergence of new, apparently more virulent, antibiotic resistant strains of *V. cholerae* O1(Ansaruzzaman *et al.*, 2007; Nair *et al.*, 2006; Siddique *et al.*, 2010) combining rising sea levels and increases in water temperature (Patz *et al.*, 1996). At this situation targeted vaccination with attending modest hygienic improvement reduces cholera and prevents deaths (Chao *et al.*, 2011)**.** The WHO and other international agencies have prioritized vaccination as appropriate short term strategy for the prevention of cholera in Asia, Africa and Latin American countries where cholera isbecoming endemic.

Vaccine development approaches started in the late 18th century (Plotkin, 2009) through the development of prophylactic human anti-cholera vaccine by Waldemar Mordecai Haffkine (Haffkine, 1895) followed by a toxoid vaccine against both bacillary dysentery and cholera (Felsenfeld & Young, 1945). A widespread use of parenteral cholera vaccines trial was seen during 1960s in Bangladesh, India and the Philippines(Levine, 2010) but disfavored on grounds of efficacy (Bhadra *et al.*, 1994) and sufficient intestinal immunity to prevent infection (Levine, 2010) during the 1970s. This failure has prompted in the search for an appropriate vaccine that stimulate the gut-associated lymphocytes so as to produce specific antibodies to inhibit colonization of the pathogen with extended memory response by appropriate vaccine components as induced in natural disease. This led to a shift in interest from injectable vaccine to oral vaccine, focusing mainly on formulations that stimulate the mucosal secretory immune system. Three oral cholera vaccines have undergone large-scale tests in humans (Harris *et al.*, 2012; WHO, 2011)

A large number of field trials have been conducted by the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) over the last 50 years of which five were on the injectable and the last one with oral vaccine containing inactivated bacterial cells and the B-subunit of cholera toxin (CTB), was tested from 1985 to 1988(Clemens *et al.*, 1990; Holmgren J *et al.*, 1994). This field trial although showed 85% protection for 6 months in all age groups with an additional short term (3 months) cross-protection (60-65%)against the heat-labile toxin of enterotoxigenic *Escherichia coli* (LT-ETEC) (Clemens *et al.*, 1988b; Peltola *et al.*, 1991; Scerpella *et al.*, 1995), protection level for cholera declined rapidly after 6 months in young children with about 60% in older children and adults after 2 years. WHO recommended thisWC/rBS vaccine to use preemptively during crisis (WHO, 1999) and (Anonymous ,1999).Over the time, numerous hypotheses of potential benefits conferring herd protection has been developed after re-analysis of the trial data (Ali *et al.*, 2005; Longini *et al.*, 2007)but merely attained global acceptability (Chaignat & Monti, 2007; Chaignat, 2008).

The other locally manufactured, ORC-Vax based on *V.cholerae*O1 and O139 but without CTB, has been evaluated among individuals >1 year through wide-spread vaccination in Vietnam during an epidemic and achieved 66% efficacy after 2 doses (Trach *et al.*, 1997). This vaccine, in cooperation with the International Vaccine Institute in Korea, ORCVAX was significantly

reformulated in 2004 and prequalified fulfillingWHO standards(Horowitz *et al.*, 2004) and has been licensed as mORCVAX in Viet Nam while Shanchol in India and international market(WHO, 2010) which are comparably cheaper to use in developing world.

Due to short-lived effectiveness of internationally licensed killed cholera vaccine,(Clemens *et al.*, 1990), live oral cholera vaccines has been considered advantageous as tested so far (Sack et al. 1997) on several new live oral bacterial vaccines (Levine and Kaper, 1993; Kenner et al. 1995). The live attenuated, CVD 103-HgR initially showed excellent immunogenicity and protection after a single dose in U.S. volunteers (Levine et al. 1988) but failed to show protective efficacy and cross protection in a large field trial in Indonesia (Leyten *et al.*, 2005; Richie *et al.*, 2000). Moreover, there is a major safety concern associated with reversion to virulence due to possible horizontal transfer of genes and recombination (Kimsey & Waldor, 1998; Waldor & Mekalanos, 1996).

Another live-attenuated oral *Vibrio cholerae* O1 vaccine candidate of the El Tor biotype and Inaba serotype; CholeraGarde, also well known as Peru-15with the genetically modified characteristic of almost remote reversion to a virulent phenotype (Kenner *et al.*, 1995) has been tested and displayed a good safety and immunogenicity profilein the USA (Cohen *et al.*, 2002) as well as in Bangladesh (Qadri *et al.*, 2005; 2007). While phase II studies incorporating CholeraGarde with parenteral measles vaccineandstudies on HIV-positive individuals are ongoing to determine safety, immunogenicity and contraindications, if any (Chowdhury *et al.*, 2009), phase III studies are being targeted to determine the protective efficacy of CholeraGarde to customize into a single-dose vaccine.

The epidemiological studies of Wiley H. Mosley in Bangladesh in the 1970s (Mosley, 1969; Mosley *et al.*, 1969) provided evidence for the involvement of systemic antibodies in protection against cholera while others opposedit as only markers of protection (Levine & Pierce, 1992).To address the nature and origin of protective antibodies against cholera, monoclonal antibodies (mAbs) were found to recognize the polysaccharide moiety of LPS with vibriocidal and agglutination propertiesand also bound the LPS exposed on the surface as well ason flagella of *V. cholerae* O1, which viewed through electron microscopy confirming immobilization properties. These antibodies also displayed serotype-specific protection in neonatal mouse and were seen to be transferred from blood vessels to the intestinal lumen(Bougoudogo *et al.*, 1995), which

confirms numerous reports demonstrating that IgG is transferred to human intestinal fluid and interpreted the correlation between serum vibriocidal antibody concentration and protection (Bougoudogo *et al.*, 1995; Glass *et al.*, 1985b). The vibriocidal antibodies increase after natural infection with *Vibrio cholerae* O1 and O139 (Qadri *et al.*, 1995b; 1997) and after oral (Jertborn *et al.*, 1986; Svennerholm *et al.*, 1984) or parenteral vaccination and to date has been found to be the most appropriate indirect correlate of protection.

These results stimulated efforts to develop glycoconjugate vaccines composed of the polysaccharide moiety of the LPS of *V. cholerae* O1 or *V. cholerae* O139 covalently linked to a carrier protein, with the aim of inducing a long-lasting, thymus-dependent immune response (Boutonnier *et al.*, 2001; Chernyak *et al.*, 2002)and has been studied in mice to determine immunogenicity. Synthetic antigens that mimic the terminal hexa-saccharide epitope of Ogawa, conjugated to bovine serum albumin, have been prepared and tested, which showed high titers of IgG and protection in mice (Chernyak *et al.*, 2002). Glycoconjugates with polysaccharide of Inaba, linked to either the B subunit of cholera toxin or to tetanus toxoid, have been prepared by Robbins's group and Fournier's group (Gupta *et al.*, 1992; 1998; Ins.Pasteur, 2004). However, titers of anti-O1 Inaba IgG induced by these conjugates were lower than those induced by O139 or O1-Ogawa glycoconjugates and urges further studies.

Cholera which is considered as non-invasive (Fresh *et al.*, 1964) and thought to be relatively non-inflammatory infection(Satchell, 2003; Silva *et al.*, 1996) but reports of inflammation suggestthe involvement of other components associated with the disease (Qadri *et al.*, 2004b). In humans,although*V. cholerae*-specific antibody surely plays a role in protection, no direct evidence of contributing isotype or antibody subclass is available. Early reports indicated cell-mediated immunity (Chaicumpa & Rowley, 1973) while the involvement of innate immunity in cholera is now being reported (Mathan *et al.*, 1995; Qadri *et al.*, 2004b; Saha *et al.*, 1997).In addition, involvement of neutrophils with sIgAfor phagocytosis at the luminal surface was predicted(Shen *et al.*, 1994), which ultimately demands reassessment of immune profiling against cholera. Humans, whether infected or immunized with whole *V. cholerae*, mostly generate antibodies to LPS epitopes. Antibodies that bind cholera toxin(CT), outer membrane proteins(Sengupta *et al.*, 1992) and fimbriae including TcpA (Sun *et al.*, 1990)are induced following cholera and are protective in animal models. The utility of CT specific antibodies

induced by infection or by some oral cholera vaccines has been debated for years. Killed oral cholera vaccines with CT do not perform better (long-term protective efficacy) than cholera vaccines without CTB (CTB) subunit, (Attridge *et al.*, 2002; Mahalanabis *et al.*, 2008; Trach *et al.*, 2002) but greater short-term (8 months) protection was seen in the group that received CTB as part of the cholera vaccine (Clemens et al., 1988b). The antibody response of humans to TcpA, is described (Asaduzzaman *et al.*, 2004; Attridge *et al.*, 2004; Provenzano *et al.*, 2006), and is immunogenic for humans during natural infection (Asaduzzaman *et al.*, 2004; Attridge *et al.*, 2004; Hall *et al.*, 1991). The protective potential of rabbit anti-TcpA (classical or El Tor) was reported (Sun *et al.*, 1990) but no report is apparent on humans challenge which also lacks for TcpF and CBP-A in the biomedical literature.

Effectively immunizing all vulnerablegroups with an ideal cholera vaccine may not be realistic, because the conditions necessary to prime naïve B cells are different from those needed to activate a memory response to *V. cholerae* antigens. The gut flora and the gut physiology of the Western population are different from those living in developing countries (Lagos *et al.*, 1999). Nutritional status and the number of current infections are factors that affect response to cholera vaccines and thus make those in the third-world more difficult to effectively vaccinate (Albert *et al.*, 2003; Cooper *et al.*, 2001). Moreover, individuals living in endemic areas acquire immunity to cholera with age (Chongsa-nguan, 1986).

1.1 General Background:

Cholera is considered as the pioneer of epidemiological study which causes explosive epidemics rendering significant mortality at endemic regions with profuse watery diarrhea, vomiting, and leg cramps assymptoms while severe dehydration can also lead to death(CDC, 2011). It is mainly acquired through ingestion of contaminated food and water with a very short incubation period of two hours to five days(Finkelstein, 1996). Moreover, cholera is extremely dreadfulfor both children and adults(VRD:WHO, 1995).

1.2 Epidemiology

1.2.1 Transmissions

Cholera is often described as the classic waterborne disease because the major source for the transmission of cholera is contaminated water when a common water body is used for washing, bathing, and drinking, involvingthe fecal-oral route. Foods such as shellfish, coconut milk, raw vegetables, cooked rice, lentils and other grains in contact with contaminated water play secondary role, especially when these are reheated. Nosocomial transmission is possible but is not a major source of infection(Lashley & Durham, 2007). Cholera is not usually spread through person-to-person contact, but in some instances this does occur(Albert & Morris, 2000). In developing world where fresh water supply and sanitation facilities are distant, the source of the contamination is typically other cholera patients when their untreated diarrhealdischarges are allowed to get into waterways or into groundwater or drinking water supplies.Both toxic and non-toxic strains of *V. cholerae* harbors naturally in the zooplankton of fresh, brackish, and salt water, attached primarily to their chitinous exoskeleton(Kirn *et al.*, 2005). Non-toxic strains can acquire toxicity through a lysogenic bacteriophage(Boyd & Waldor, 1999). Coastal cholera outbreaks typically follow zooplankton blooms, thus making cholera a zoonotic disease. For more developed countries, contaminated food (especially undercooked seafood) is the usual vehicle for transmission and contaminated water is more common in less developed countries(Glass *et al.*, 1991; Hughes *et al.*, 1982; Shapiro *et al.*, 1999).

1.2.2 Potential human contribution to transmissibility

Cholera bacteria grown in vitro, encounter difficulty subsequently growing in humans without additional stomach acid buffering. In a collaboration study in 2002 between icddr,b and Tufts University School of Medicine, it was found that stomach acidity serves as the principal factor for creating hyper-infective state of the disease through human colonization that contributes to epidemic spread(Merrell *et al.*, 2002). These bacteria possess a unique physiological and behavioral state, characterized by high expression levels of genes required for bacterial chemotaxis.

Seasonality is observed in endemic areas that are related in India and other parts of Asia to the monsoon onset and to warm weather(Albert & Morris, 2000). A role has been postulated for a relationship between cholera and the El Ni`no–Southern Oscillation that is a source of interannual variation in climate in Bangladesh(Anyamba *et al.*, 2006). In Bangladesh, where the disease is endemic, two peaks occur each year corresponding to the warm seasons before and after the monsoon rains (Glass *et al.*, 1982; Sack *et al.*, 2003; Siddique *et al.*, 1992). Elevated ocean surface temperatures of the coast of Peru, due in part to El Ni`no, correlated with cholera incidence in Peru(Gil *et al.*, 2004) and epidemics are strictly confined to the warm season(Tauxe *et al.*, 1995). The seasonality seems to be related to the ability of vibrios to grow rapidly in warm environmental temperatures. Other than shellfish and plankton, there are no animal reservoirs. In endemic areas, annual rates of disease vary widely, probably as a result of environmental and climate changes.

1.2.3 Susceptibility

Although the typical clinical picture is severe diarrhea, most individuals infected with *V. cholerae* have no symptoms or only mild diarrhea, indistinguishable from other mild diarrhoeal diseases with a case ratio of one in three to one in hundred (Glass *et al.*, 1982; Khan & Shahidullah, 1980). Severity depends on many factors, especially local intestinal immunity, size of the inoculum ingested, adequacy of the gastric-acid barrier, and patient's blood group. Epidemiologic research suggests that people of blood group O are more susceptible to severe cholera from El Tor vibrios than are those of other blood groups(Barua & Paguio, 1977; Clemens *et al.*, 1989; Glass *et al.*, 1985a; Harris *et al.*, 2005).

A high infectious dose (10^8 bacteria) is needed to cause severe cholera in healthy volunteers, but lower dose (10^5 bacteria) is sufficient if given with antacids to neutralize stomach acid (Hornick *et al.*, 1971; Sack *et al.*, 1998). Under natural field circumstances, the inoculum size to cause cholera may be even lower, because attack rates are lower than in volunteer studies, and many patients do have low gastric-acid production(Sack *et al.*, 1972).

In cholera-endemic areas, the highest attack rates are in children aged 2-4 years(Glass *et al.*, 1982) while it is rare in children below 1 year(Albert & Morris, 2000). The elderly may be susceptible because of decreased immunity or less gastric acid(Tauxe, 1998). Women of

childbearing age, individuals with lower immunity and malnourished children are particularly susceptible (Palmer *et al.*, 1976).

1.2.4 Clinical Features

The incubation period for cholera may range between 12 and 72 hours, with 18 to 40 hours being the average, depending on the inoculum ingested and the susceptibility of the host(Levine *et al.*, 2006). After ingestion, the bacteria pass through the stomach to the small intestine, where they produce the cholera toxin that binds to receptors in the mucosa of the small intestine; affects a variety of functions; and ultimately causes the intestines to fill with an alkaline, salty fluid, greatly facilitating the growth of *V. cholerae*(Sack *et al.*, 2004; Tauxe & Barrett, 1998). Although the majority of cholera infections are either asymptomatic or mildly symptomatic, in the full clinically manifested cases, the first symptom is usually diarrhea that begins when the diarrheal fluid accumulates in the small intestine and causes distention, increased intestinal motility, and decreased transit time(Albert & Morris, 2000). After an incubation period of between about 18h and 5 days, symptoms are generally abrupt and include watery diarrhea and vomiting(Sack *et al.*, 2004). The most distinctive feature of cholera is the painless purging of voluminous stools resembling rice-water. The stools are sometimes described as having a fishy odor. The vomitus is generally a clear, watery, alkaline fluid. In adults with severe cholera, the rate of diarrhea may quickly reach 500-1000 ml/h, leading to severe dehydration with the signs of absent or low-volume peripheral pulse, undetectable blood pressure, poor skin turgor, sunken eyes, and wrinkled hands and feet. At first, patients are restless and extremely thirsty, but as shock progresses, they become apathetic and may lose consciousness. Many patients also show respiratory signs of metabolic acidosis with Kussmaul, gasping breathing. Most patients have no urine output until the dehydration is corrected. The fluid loss may be so rapid that the patient is at risk of death within a few hours of onset, and most deaths occur during the first day. However, if rehydration fluids are provided in insufficient quantities, the patient may survive temporarily, but there is a chance of mortality within a few days.

Several complications can occur with cholera, but these are generally from improper treatment. They include acute renal failure from protracted hypotension if insufficient fluids are given. Most cholera patients have low blood glucose concentrations, and a few have severe

hypoglycemia(Butler *et al.*, 1989). Electrolyte imbalance, especially hypokalaemia, can occur if the intravenous fluids are not appropriate(Chhabra *et al.*, 1995). Miscarriage or premature delivery although infrequent, can occur in pregnant women as a complication of shock and poor perfusion of the placenta(Khan, 1969) which to be managed by proper hydration. Severe muscle cramps of arms and legs are common probably due to the electrolyte imbalance, although the exact explanation is not known. They subside within a few hours of treatment. Fever is not a prominent feature of cholera.

1.2.5 Prevention

Hence contaminated food and water are the main vehicles of transmission of *V. cholerae,* measures include ensuring a safe water supply, (especially for municipal water systems), improving sanitation, making food safe for consumption by thorough cooking of high-risk foods (especially seafood), and health education through mass media can keep transmission rates to a minimum. The WHO and CDC have developed simple strategies for use in developing countries with increased potential for cholera outbreaks. Prevention of cholera transmission appears to be relatively clear-cut for an individual with strictly following basic hygienic proceduresand appropriate disposal of human waste. As *V. cholerae* are exquisitely sensitive to acid, simple modifications in food preparation including the use of lemons, limes, tomatoes, tamarinds, and yogurt can acidify foods so that *V. cholerae* cannot survive(Levine *et al.*, 2006; Rodrigues *et al.*, 2000). Training of street vendors in appropriate sanitation and educating consumers regarding food vendors are also important(Tauxe, 1998).

1.3 Vibrio cholerae

1.3.1 Clinical Microbiology

Vibrio cholerae is a gram negative, polar monotrichous, oxidase-positive, asporogenous curved rod that ferments glucose, sucrose, and mannitol and is positive in the lysine and ornithine decarboxylase tests. The organism is classified by biochemical tests and is further subdivided into 200 diverse serogroups based on the somatic O antigen (Yamai *et al.*, 1997). Only the O1 and O139 serogroups cause epidemic and pandemic disease. The non-epidemic serogroups though are not involved in cholera epidemics, but can be pathogenic(Morris *et al.*, 1990), and are

infrequently associated with small outbreaks of diarrheal disease(Aldova *et al.*, 1968; Dakin *et al.*, 1974). They occasionally cause a variety of severe extra intestinal infections, including wound infections and acute sepsis, especially in people with liver disease or immunosuppression (Ko *et al.*, 1998).

Vibrio cholerae survives well in faecal specimens if kept moist. If there is a delay of more than a few hours then Cary-Blair transport medium is to be used for transportation. The feces, either fresh or in the transport medium, should be plated onto TCBS (Thiosulphate citrate bile salts sucrose) agar, a medium that inhibits most other normal flora but supports the growth of the vibrios. In addition, the specimen should also be inoculated into alkaline peptone water, a high-pH enrichment broth, which preferentially supports the growth of vibrios. After 6-12 hours of incubation, a second TCBS plate is inoculated. These plates are incubated for 18-24 hours, and *V. cholerae* colonies appear as smooth yellow colonies with slightly raised centers. Presumptive identification of *V. cholerae* O1 or O139 can be made on the basis of typical colonies, which are oxidase positive and agglutinate with O1 or O139 antiserum. Agglutination should be carried out with subcultures onto non-selective medium, because colonies can auto agglutinate from TCBS medium, giving false-positive results.

Rapid tests include dark-field microscopy in which a wet mount of liquid stool is examined for the appearance of "darting" organisms that are halted by the addition of O1 or O139 antiserum(Benenson *et al.*, 1964). Rapid immunoassays are also available (Hasan *et al.*, 1994; Qadri *et al.*, 1995a).

The rapid immunological assays can be especially useful for monitoring of epidemiological patterns in remote areas where cultures are not readily available, but new outbreaks must be confirmed by cultures(Bhuiyan *et al.*, 2003; Kalluri *et al.*, 2006; Ley *et al.*, 2012; Mukherjee *et al.*, 2010; Nato *et al.*, 2003; Page *et al.*, 2012).Molecular methods, including PCR and DNA probes, are also available but are not widely used and not practicable in many areas where cholera is common.

1.3.2 Subtypes of *V. cholerae*

The*Vibrio cholerae* O1 serogroup is divided into two biotypes, classical and El Tor, based on their distinct biochemical properties. The latest approach, however, is to use biotype-specific

genes (e.g. tcpA, rtxC) to differentiate between the two biotypes. Each of the O1 biotype can be further subdivided into two major serotypes, Ogawa and Inaba. Ogawa strains produce the A and B antigens and a small amount of C, whereas Inaba strains produce only the A and C antigens. A third serotype, Hikojima, produces all the three antigens but is rare and unstable.

V. cholerae strains of the same biotype and serotype can be differentiated by a phage-typing scheme. There are 145 phage types for O1(Chattopadhyay *et al.*, 1993) and five for O139(Chakrabarti *et al.*, 2000). Multilocus enzyme electrophoresis can distinguish between classical and El Tor strains and has grouped the toxigenic El Tor biotype strains into four major clonal groups or electrophoretic types (ET) representing broad geographical areas (Cameron *et al.*, 1994; Momen & Salles, 1985). These include the Australian clone (ET1), the Gulf Coast clone (ET2), the Seventh Pandemic clone (ET3), and the Latin American clone (ET4) (Chen *et al.*, 1991; Salles & Momen, 1991; Wachsmuth *et al.*, 1993). In addition, a standard ribotyping scheme for *V. cholerae* O1 and O139 can distinguish seven different ribotypes among classical strains, 20 ribotypes and subtypes among El Tor strains, and six distinct ribotypes among O139 strains(Faruque *et al.*, 2000; Popovic *et al.*, 1993). These ribotypes have been especially useful for molecular epidemiological studies. For example, molecular analysis of epidemic isolates of *V. cholerae* between 1961 and 1996 in Bangladesh revealed clonal diversity among different epidemic strains(Faruque *et al.*, 1995; 1997). These studies demonstrated the transient appearance and disappearance of more than six ribotypes among classical vibrios, at least five ribotypes of El Tor vibrios, and three different ribotypes of *V. cholerae* O139. Different ribotypes showed different CTX genotypes resulting from differences in copy number of the CTX element and variations in the integration site of CTX element in the chromosome (Faruque *et al.*, 1997). Molecular epidemiological studies have shown that many strains are in circulation but most outbreaks are caused by a restricted number of clones (Koblavi *et al.*, 1990; Lizarraga-Partida & Quilici, 2009; Tamayo *et al.*, 1997).

1.3.3 Clinical pathophysiology

Ingested vibrios from contaminated water or food must pass through the acidic environment ofstomach before they are able to colonize the upper small intestine. Colonization is aided by way of fimbria, filamentous protein structures called toxin co-regulated pilus (TCP) extending

from the cell wall, that attach to receptors on the mucosa(Taylor *et al.*, 1987), and by the bacterium's motility, which helps to penetrate the mucus overlying the mucosa. Concentrations of vibrios on the mucosal surface rapidly increase to 10^7 or 10^8 cells/gm. With this high concentration of vibrios closely attached to the mucosa, enterotoxin can be efficiently delivered directly to the mucosal cells.

Formerly cholera was thought to cause sloughing of the intestinal mucosa by an inflammatory process. However, the intestinal mucosa is now known to remain intact and without inflammatory changes (Sprinz *et al.*, 1962). The previous findings were shown to be spurious, based on autolytic postmortem changes. Koch first postulated in 1884 that the bacteria produce a toxin and that this stimulates the massive outpouring of fluid from the intestine. De and Dutta were the first to demonstrate this toxin (now called cholera toxin) by use of culture filtrates in rabbits (De, 1959) and (Dutta *et al.*, 1959). The toxin was later purified and sequenced (Finkelstein & LoSpalluto, 1969; Holmgren *et al.*, 1971). It has a molecular mass of 84,000 kDa and consists of five binding subunits B and one active subunit A (Gill, 1976; Lonnroth & Holmgren, 1973). As we now understand the mechanism of action, the B subunits are physiologically inactive but bind the holotoxin to the GM1 ganglioside receptors in the small-intestinal mucosa, and the A subunit is transported into the cell where it activates adenylate cyclase (Holmgren *et al.*, 1973; Van Heyningen *et al.*, 1971). This activation leads to an increase in cyclic AMP, followed by an increase in chloride secretion in the crypt cells, and inhibition of neutral sodium chloride absorption in the villus cells, which in turn leads to a massive outpouring of fluid into the small intestine(Field *et al.*, 1972). The volume secreted exceeds the normal absorptive capacity of the bowel and results in watery diarrhoea. Most of the secretions come from the small intestine, although the toxin also inhibits water absorption by the colon(Speelman *et al.*, 1986). The diarrhoeal fluid contains large amounts of sodium, chloride, bicarbonate, and potassium, but little protein or blood cells(Molla *et al.*, 1981). The loss of electrolyte-rich isotonic fluid leads to blood volume depletion with attendant low blood pressure and shock. Loss of bicarbonate and potassium leads to metabolic acidosis and potassium deficiency. The stools of cholera patients contain high concentrations of cholera vibrios (up to 10^8 bacteria/g), and they are highly infectious. When passed into the environment, they can contaminate water sources and food and may continuously seed an environmental reservoir.

1.3.4 Virulence factors

The pathogenesis of cholera is a multi-factorial process involving multiple genes encoding virulence factors that aid the pathogen in its colonization, coordinated expression of virulence factors and toxin action. In *V. cholerae*, the major virulence genes required for pathogenesis are in clusters and can apparently propagate laterally and disperse among different strains. Genetic analyses have revealed the presence of two important genetic elements that distinguish a pathogenic *V. cholerae* from an innocuous one. These are the previously called CTX genetic element, which is the genome of a lysogenic bacteriophage designated CTXϕ that carries the genes encoding cholera toxin, and the vibrio pathogenicity island (VPI), which carries genes for the pilus colonization factor TCP(Taylor *et al.*, 1987) and (Waldor & Mekalanos, 1996).

The typical CTXϕ genome has a modular structure composed of two functionally distinct domains, the Core and RS2 regions (Waldor & Mekalanos, 1996). The core region encodes cholera toxin, which does not contribute to virion formation, and the other genes encode proteins (Psh, Cep, OrfU and Ace) that are involved in phage packing and secretion and one (Zot) required for CTXϕ assembly. The products of zot and ace genes also show enterotoxic activity and increase short-circuit current across rabbit intestinal tissue in Ussing chambers(Fasano *et al.*, 1991; Trucksis *et al.*, 2000). The RS2 region encodes genes required for replication (rstA), integration (rstB), and regulation (rstR) of CTXϕ (Waldor *et al.*, 1997).

Within *V. cholerae* cells, the CTXϕ genome can exist either as a replicating plasmid or as a prophage integrated into the chromosome (Waldor & Mekalanos, 1996). Under appropriate conditions, toxigenic *V. cholerae* strains can be induced to produce extracellular CTXϕ particles (Faruque *et al.*, 1998a; Waldor & Mekalanos, 1996). Cultures of *V. cholerae*harboring the replicating form of CTXϕ produce high titers of the phage in their supernatants. Non-toxigenic environmental strains can be converted by phage transduction with CTXϕ(Faruque *et al.*, 1998a), and this event could conceivably take place in the gastrointestinal environment, yielding new toxigenic strains(Reidl & Klose, 2002).

TCP mediates bacterial colonization of the intestine by facilitating microcolony formation via pilus-mediated bacterial interactions and perhaps direct attachment to the intestinal brush border (Kirn *et al.*, 2000). The genes for TCP form part of the 40 kb VPI segment that is generally

absent from nonepidemic strains (Karaolis *et al.*, 1998). Biogenesis of TCP requires the activities of at least 11 accessory proteins, most of which are encoded by genes located in the TCP operon(Kovach *et al.*, 1996). The structural features of the VPI include the presence of groups of virulence genes, a regulator of virulence genes, a transposase gene, and specific (att-like) attachment sites flanking each end of the island. The presence of an integrase with homology to a phage integrase gene suggests that the VPI was also derived from a bacteriophage (Karaolis *et al.*, 1999; Manning, 1997). As remarkable examples of evolutionary co-adaptation, the CTXϕ virion uses TCP as a receptor during infection (Kovach *et al.*, 1996). Colonization is a prerequisite to establishing a productive infection. Other colonization factors such as the mannose-fucoseresistant cell-associated haemagglutinin, the mannose-sensitive haemagglutinin, and some outer-membrane proteins are suspected from findings in animals to have roles in increasing adhesion and colonization (Franzon *et al.*, 1993; Jonson *et al.*, 1994; Sengupta *et al.*, 1992). The exact roles of these factors in the virulence of *V. cholerae* in human beings are still uncertain, but the mannose-sensitive haemagglutinin type IV pilus has been identified as one factor involved in the adherence to the chitin of zooplankton(Chiavelli *et al.*, 2001).

The entire genome sequence of *V. cholerae* O1 (biotype El Tor) was described at the inception of the millennium (Heidelberg *et al.*, 2000). The genome consists of two circular chromosomes (Heidelberg *et al.*, 2000; Trucksis *et al.*, 1998)where the large chromosome contains most of the metabolic and growth-associated genes and the small chromosome contains genes for several essential and metabolic pathway.

Vibrio cholerae can activate or inactivate a set of genes including those encoding colonization factors or toxins as an appropriate response to changing environmental conditions. ToxR, a 32 kDa transmembrane protein, binds to a tandemly repeated 7 bp DNA sequence found upstream of the ctxAB structural gene and increases transcription of this gene resulting in higher expression of cholera toxin. The coordinated regulation of several genes through the toxR regulon shows that the organism has developed a mechanism of sampling and responding to its environment. ToxR regulates the expression not only of ctxAB but also of at least 17 distinct genes that constitute the ToxR regulon(Hughes *et al.*, 1994; Parsot *et al.*, 1991; Peterson & Mekalanos, 1988). Except for the ctxAB genes, other genes in the ToxR regulon are controlled through another regulatory factor called ToxT, a 32 kDa protein. ToxR controls the transcription

of the toxT gene, which encodes one of the AraC bacterial transcription activators. The resulting increased expression of the ToxT protein then leads to activation of other genes in the ToxR regulon. Thus, ToxR is at the top of the regulatory cascade that controls the expression of several other genes, and the expression of ToxR itself remains under the control of environmental factors (Parsot & Mekalanos, 1990; Skorupski & Taylor, 1997).

The emergence of the O139 epidemic strain of *V. cholerae* resulted from horizontal gene transfer of a fragment of DNA from another serogroup into a strain of the seventh pandemic *V. cholerae* O1, El Tor strain. This transfer occurred in the region that brings about O-antigen biosynthesis(Bik *et al.*, 1995; Comstock *et al.*, 1996; Stroeher *et al.*, 1995) DNA hybridization analysis of the O-antigen biosynthesis gene in O139 showed that it has homology with the gene of several non-O1 serogroups, but especially with serogroup O22. Thus, O22 is the likely origin of the genes for O139 biosynthesis (Dumontier & Berche, 1998; Yamasaki *et al.*, 1999). Molecular epidemiological studies support these findings and show that O139 strains have genetic backbones very similar to those of the O1, El Tor Asian seventh pandemic strains(Berche *et al.*, 1994; Johnson *et al.*, 1994; Waldor & Mekalanos, 1994). However, unlike *V. cholerae* O1, serogroup O139 has a capsule distinct from the lipopolysaccharide antigens and has 3, 6-dideoxyhexose (abequose or colitose), quinovosamine, and glucosamine, and traces of tetradecanoic and hexadecanoic fatty acids (Comstock *et al.*, 1995).

1.4 Ecology of *V. cholerae*

Studies of the aquatic environment, however, have shown that *V. cholerae*, including strains of O1 and O139, are normal inhabitants of surface water, particularly brackish waters, and survive and multiply in association with zooplankton and phytoplankton quite independently of infected human beings(Colwell *et al.*, 1977; Huq *et al.*, 1983; Islam *et al.*, 1990; Nair *et al.*, 1988). Because global climate changes affect the growth of plankton, growth of the vibrios associated with plankton could also be modified. The continuing presence of cholera in the Indian subcontinent and the re-emergence of cholera in other continents may be highly dependent on environmental factors (Colwell, 1996; Pascual *et al.*, 2000). The movement of the bacteria in association with plankton has led to the suggestion that ship ballast may be a cause of its global spread(McCarthy & Khambaty, 1994).

The life cycle of *V. cholerae* consists of two distinct phases. Outside of the host and in the aquatic phase, *V. cholerae* can be found as free swimming cells, attached to surfaces provided by plants, filamentous green algae, copepods, crustaceans, insects(Colwell, 1996; Islam *et al.*, 1994), and egg masses of chironomids(Broza & Halpern, 2001). Biofilm formation(Watnick *et al.*, 2001) and entry into a viable but non-culturable state in response to nutrient deprivation(Colwell, 2000) are thought to be important in facilitating environmental persistence within natural aquatic habitats during periods between epidemics(Reidl & Klose, 2002). Neither the genetic events that help the organism to lead a life in association with plankton nor the biofilm ecology of vibrios on abiotic surfaces are completely understood.

Although *V. cholerae* is part of the normal estuarine flora, toxigenic strains are mostly isolated from the environment in areas probably contaminated by infected individuals. Environmental isolates from areas that are distant from regions of infection do not generally have the cholera toxin genes (Faruque *et al.*, 1998b).

There are two crucial sequential steps in the evolution of a pathogenic *V. cholerae*. First, strains have to acquire the VPI (which most environmental strains do not have); second, having acquired the CTXϕ receptor, the TCP positive strains are infected with and lysogenised by CTXϕ (Faruque *et al.*, 1998a; Mekalanos *et al.*, 1997; Waldor & Mekalanos, 1996). Experiments in animals have shown that the intestinal milieu is the site where strains can acquire these mobile elements efficiently (Lazar & Waldor, 1998; Waldor & Mekalanos, 1996). Thus, *V. cholerae* can be visualized as an autochthonous marine bacterium that colonizes and thrives in the human gut during phases of infection and spends the time between epidemics in its "original" habitat, the estuary.

1.5 Immunity to cholera

Initially it was believed that only humoral immunity and not cellular immunity was important for protection against cholera. Since *V. cholerae* does not cross the epithelial barrier and is a non-invasive pathogen, the antibodies must provide the protection required for the disease. *V. cholerae*-specific antibody surely plays a role in protection but no direct evidence has demonstratedthat a particular isotype or antibody subclass is required for immunity against the disease(Provenzano *et al.*, 2006). Even though, it is known that *V. cholerae* does not survive

intracellularly as other pathogens do, nevertheless, cell-mediated immunity has been suggested to be protective in the infant mouse model(Chaicumpa & Rowley, 1973). The claim was further supported by the demonstration that the transfer adult peritoneal exudate cells in association with anti-O17 antibody provided protection in 3-day old mice. Another experiment in which it has been demonstrated that neutrophils can migrate to an area of inflammation has also established a role of innate immunity in cholera immunity (Mathan *et al.*, 1995; Qadri *et al.*, 2004b; Saha *et al.*, 1997). Neutrophils and sIgA via the IgA Fc-receptor might act additively to control *V. cholerae* at the luminal surface by increased phagocytosis(Shen *et al.*, 1994).

1.6 Cholera Vaccine

Waldemar Mordecai Haffkine is credited for the development of the first effective prophylactic human cholera vaccine (Haffkine, 1895; Hawgood, 2007), which although could not prevent few deaths, proved to be efficient in culminating the infectivity of the disease during an epidemic(Haffkine, 1895). Later on, a toxoid vaccine was developed to impart immunity against both bacillary dysentery and cholera(Felsenfeld & Young, 1945). During the 1960s, there was a widespread use of cholera vaccines after a series of large trial in Bangladesh, India and the Philippines(Kaper *et al.*, 1995), which were composed of *V. cholerae* serogroup O1 cell, usually a mixture of biotypes and serotypes, killed by either formalin, phenol, or heat(Kaper *et al.*, 1995). However, these whole cell vaccines were provided parenterally and felt out of favor on grounds of efficacy during the 1970s(Bhadra *et al.*, 1994). Though they presented high titers of serum vibriocidal antibodies, they did not provide sufficient intestinal immunity to prevent infection(Kaper *et al.*, 1995). This led to a shift in interest from injectable vaccines to oral cholera vaccines (OCV).

Presently, there are two main types of oral vaccines that have been investigated in clinical trials(Harris *et al.*, 2010): inactivated vaccines (containing killed whole cells of *V. cholerae*) which are given in two or three doses at one week intervals to produce adequate immunological response and live attenuated vaccines (containing genetically modified, non-pathogenic strains of *V. cholerae*) which are usually given as a single dose. In addition, sub-unit vaccines have been tested which consist only of cell components (antigens). The following vaccine formulations are currently available in the market:

1) WC-rBS (Dukoral®): A monovalent inactivated vaccine containing killed whole cells of *V. cholerae* O1 plus additional recombinant cholera toxin B sub-unit. SBL Vaccine/Crucell, Sweden, presently produces it.
2) BivWC (Shanchol®): A bivalent inactivated vaccine containing killed whole cells of *V. cholerae* O1 and *V. cholerae* O139. It is produced by Shantha Biotechnics, India.
3) BivWC (mORCvAX®): A bivalent inactivated vaccine containing killed whole cells of *V. cholerae* O1 and *V. cholerae* O139. Presently, this vaccine is being produced by Vabiotech, Vietnam and only available in Vietnam.

1.7 Protective Antibody Isotypes

1.7.1 Serum Immunoglobulin A (sIgA)

Two contending views exist on which isotype of antibody is protective in human cholera. Because cholera is an enteric disease, it has long been surmised that *V. cholerae*-specific sIgA, which is secreted into the gut lumen, provides the protective response and thus should be the goal of immunization(Keren *et al.*, 1988). Indeed, increased levels of sIgA specific against *V. cholerae* LPS have been found in convalescent sera and intestinal secretions of patients recovering from cholera (Levine *et al.*, 1981; Migasena *et al.*, 1989; Qadri *et al.*, 2003b). Those individuals living in endemic cholera areas have anamnestic sIgA response to *V. cholerae*(Svennerholm *et al.*, 1977; Svennerholm *et al.*, 1980). The presence of sIgA specific for *V. cholerae* antigens does not demonstrate an absolute role of that antibody isotype in protection from cholera.The percent of the world's human population deficient in IgA ranges from 0.001 to 0.1%(Weber-Mzell *et al.*, 2004), the decrease of their sIgA is compensated by increased IgM and IgG levels(Friman *et al.*, 1994)and even can respond to oral cholera vaccines with an increase in gut lumen IgG(Nilssen *et al.*, 1993).The vaccines were not challenged, so it is not known if the antibody response was protective. Experiment results in mice studies with the defects of transcytosis or IgA gene or J chain when infected with mucosal pathogens including *Helicobacter pylori*, and influenza virus(Blanchard *et al.*, 1999; Johansen *et al.*, 1999; Lycke *et al.*, 1999; Mbawuike *et al.*, 1999) suggest that sIgA is not absolutely required for gut immunity.

The proposed mechanism for sIgA before establishment of infection and perhaps in limiting disease could be i) immune exclusion, a non-inflammatory process to prevent attachment of infectious organisms to host receptors, is thought to be the main role for sIgA in the gut(Bollinger *et al.*, 2003; Brandtzaeg, 2003), ii) bactericidal killing by binding receptors on neutrophils or macrophages(Rodriguez *et al.*, 2001), iii) obstruct colonization by the interference of anti-LPS sIgA with flagellar sheath function(Fuerst & Perry, 1988) and thus reduce movement and access to preferred areas of the gut, and iv) interfere with LPS function of *V. cholerae* byanti-LPS sIgA, rendering the cells more sensitive to the bactericidal activity of bile when protection from bile is thought to be one of LPS's roles in *V. cholerae* pathogenesis(Nesper *et al.*, 2001). Another role for sIgA after infection could be to bind micro colonies and prevent shedding by reducing the number of free-swimming, exiting vibrios in the gut (Provenzano *et al.*, 2006).

1.7.2 Immunoglobulin G (IgG)

Another isotype of antibody that could protect against cholera is *V. cholerae* LPS-specific serum IgG, which is produced in response to *V. cholerae*infection (Losonsky *et al.*, 1996; Qadri *et al.*, 1997; 1999). The Robbins group (Gupta *et al.*, 1992; 1998)have put forward the concept of IgG being protective against cholera forth. In support of this perspective, parenteral vaccination with killed WC *V. cholerae* induced protective anti-LPS antibodies in older individuals(Mosley *et al.*, 1968b). Recently it was discovered that the human neonatal Fc-receptor could transport IgG from interstitial spaces into the gut lumen as well as retrieve IgG and possibly retrieve antigen back into the host(Yoshida *et al.*, 2004). People have argued that IgG is more susceptible to proteases as evidenced by IgG fragments in fecal material and thus IgG is less effective than sIgA. This idea obviates the activity of Fab or F(ab'2) which has been established, and also the activity of IgG in the proximal area of the gut epithelium which could provide a protected environment for the time it takes the antibodies to be effective(Bouvet & Fischetti, 1999). Also the argument that IgG is degraded by intestinal enzymeswas disproved by the isolation of functional IgG (binds rotavirus) in intestinal fluid samples(Watanabe *et al.*, 1978). The utility of anti-LPS IgG in protection from cholera needs to be established as does that of sIgA.

1.7.3 Secretory Immunoglobulin M (sIgM) and IgM

Regardless of the *in vivo* protective isotype of antibody, serum and intestinal vibriocidal antibodies can be measured following infection or vaccination. The *in vitro* "vibriocidal" capability of cholera antiserum is the standard marker of immunity to cholera (Clemens *et al.*, 1991a; Mosley *et al.*, 1968a). Vibriocidal antibodies are quantified by in vitro tests that measure complement-mediated lysis of virulent *V. cholerae*(Boutonnier *et al.*, 2003). Mercaptoethanol reduction of IgM quantitatively reduces the vibriocidal titers of patient sera indicating a major role for IgM and not IgG in vibriocidal activity of the sera(Ahmed *et al.*, 1970). Thus, while the IgM fraction of sera provides the vibriocidal activity in vitro, the role of IgM or sIgM as a component of cholera immunity remains unknown. To infer that IgM, which is the vibriocidal antibody in vitro, is not functional in vivo would be an over simplification. Clearly, IgM and complement can be found in the gut (Bernatowska *et al.*, 1989; Halstensen *et al.*, 1992). A recent report indicated that children with acute diarrhea have more IgM than IgA in whole gut lavage(Croft & Hodges, 2005). The vibriocidal activity of serum is a reflection of in vitro complement-mediated lysis of *V. cholerae*, but does not certify that this protective mechanism operates in vivo. The anti-LPS specificity of the vibriocidal antibodies may well be functional on IgG or sIgA and thus vibriocidal antibodies are protective but not necessarily via the complement system.

Clearly, sIgM can have many of the same effects of sIgA. There are however differences between the two antibody isotypes because white blood cells lack IgM Fc receptors and IgM is the most efficient isotype at fixing complement but protease sensitivity might limit the location of the protective effect of sIgM to the epithelial surface when serum transudates are present(Wernet *et al.*, 1971). In addition, early parenteral cholera vaccines that were protective in certain individuals did not prevent bacterial shedding; suggesting that serum IgG or IgM may not have a role once *V. cholerae* cells are no longer closely associated with the gut epithelium(Azurin *et al.*, 1967). However, efficacy of anti-LPS IgA suggests that complement is not required for protection in the infant mouse model (Apter *et al.*, 1993; Steele *et al.*, 1975). Hence, the role for serum IgG and IgM in cholera might not be to prevent colonization but to reduce the severity or shorten the clinical disease. As a whole, the effector mechanism of IgG and IgM, once in the tissues or the gut lumen, could include complement-mediated lysis, antibody-dependent cellular cytotoxicity,

agglutination, opsonization, and phagocytosis (Kirimanjeswara *et al.*, 2005). Although these results do not prove the notion that preventing colonization is the only protective mechanism available to humans to prevent cholera and the role of complement-mediated killing in human cholera remains unknown, antibodies from several species (mouse, rabbit, and human) of multiple isotypes (IgM, IgA, IgG1 and IgG3) specific for multiple *V. cholerae* antigens (LPS, TcpA, TcpF, fimbriae) are protective in animal models of cholera(Chernyak *et al.*, 2002; Ehara *et al.*, 1993; Kirn & Taylor, 2005; Taylor *et al.*, 2004). As often is the case, multiple antibody isotypes are likely to play some role in disease protection, may be overlapping or perhaps unique. The major antibody isotype in mucosal secretions, sIgA, does not fix complement in the classical manner. If protective antigens can be identified, it is clearly possible to manipulate the form of the antigen, the adjuvant, or the route of inoculation to induce the antibody isotype required for protection of humans.

1.7.4 Cellular markers related to humoral immunity

Activation of B lymphocytes involves binding of antigen to the specific receptor and signaling through several co-receptors, of which CD19 has been found to play a pivotal role as a response regulator for the interaction of T and B cells. Study of CD19-deficient mice suggests that CD40L-CD40 interactions are more important for Th2 than for Th1 co-ordinated B-cell differentiation (Gardby *et al.*, 2001). Engagement of CD40 on these B cells by CD40 ligand (CD154) expressed by activated T cells is a critical interaction that occurs during T-B cells collaboration and initiates many steps of B cell activation, including induction of proliferation, differentiation, and immunoglobulin (Ig) production(Cerutti *et al.*, 1998; Van Kooten & Banchereau, 1996). Besides these, CD19 stimulation can upregulate the co-stimulatory molecules B7.1 (CD80) and B7.2 (CD86) which indirectly influence T- and B-cell interactions (Kozono *et al.*, 1998). These CD80 and CD86 interact with CD28 and deliver co-stimulatory signals required for T cell activation (Jaffar *et al.*, 1999). Co-stimulatory signal mediated by CD28 is expressed on $CD4^+$ and most $CD8^+$ T cells is required for the activation and production of various cytokines, including IL-2(Thompson *et al.*, 1989).

Tissue-specific homing is believed to control the extent and scope of immune responses and to account for the regional compartmentalization of immune system functions(Butcher & Picker, 1996). Effector lymphocytes often function in nonlymphoid sites of inflammation and infection.

Moreover, effector and memory lymphocytes generally display selective tropism for specific peripheral tissues such as the skin or intestinal lamina propria(Butcher *et al.*, 1999). Little is known about the development of these tissue-specific lymphocyte subsets. Expression of specific homing phenotypes may be directed by specific microenvironments within cutaneous versus intestinal secondary lymphoid organs during initial T cell activation (Picker *et al.*, 1993). Alternatively, homing and chemo attractant receptors may be upregulated stochastically in secondary lymphoid organs and subject to selection during effector and memory cell recirculation by survival of cells trafficking through antigen-rich tissues(Bode *et al.*, 1997). Indeed, even the kinetics with which lymphocytes acquire tissue-specific tropism during the primary immune response remains unexplored(Campbell & Butcher, 2002).

T lymphocyte molecule thought to participate in 'skin selective' lymphocyte homing is called the cutaneous lymphocyte associated antigen (CLA), a sialyl Lewis^x-related carbohydrate dependent epitope, which interacts with E-selectin in endothelial cells. On the other hand, lymphocytes expressing the integrin α4β7 bind the mucosal vascular addressing MAdCAM-1(Andrew *et al.*, 1994; Berlin *et al.*, 1993)selectively expressed on HEV in mesenteric lymphnodes, intestinal lamina propria and Peyer's patches(Bargatze *et al.*, 1995) allowing the selective homing to both intestinal secondary lymphoid tissue and intestinal effector sites(Scala *et al.*, 2005). In addition, L-selectin deficiency in mice or treatment of wild type animals with mAbs to L-selectin or peripheral node addressing (PNAd), the high endothelial venule (HEV)-specific ligand for L-selectin, resulted in impaired lymphocyte homing to peripheral lymph nodes(Scala *et al.*, 2005).

1.8 Protective antibodies in response to *V. cholerae* antigens

Historically, cholera researchers have claimed that identification of *V. cholerae* virulence factors and their regulation should lead to a universal and hopefully one dose cholera vaccine. Much knowledge has been gained on virulence regulation in *V. cholerae*(Matson *et al.*, 2007) and its environment as well as host links that facilitate transmission to humans along with suggestive cholera vaccine candidates or interdiction points(Matson *et al.*, 2007; Pruzzo *et al.*, 2008).

1.8.1 *Vibrio cholerae* LPS specific antibodies in response to infection

Humans, whether infected or immunized with whole *V. cholerae*, in large part generate antibodies to LPS epitopes. Significant levels of anti-*V.cholerae* LPS antibodies are found in intestinal secretions, saliva, tears, fecal material, and in serum. Immunization of rabbits with killed *V. cholerae* cells-induced antibodies that react with at least 17 species, including LPS in a western blot analysis of *V. cholerae* antigens(Provenzano *et al.*, 2006). Antibodies that bind CT, outer membrane proteins(Sengupta *et al.*, 1992), and fimbriae including TcpA(Sun *et al.*, 1990) are induced following cholera and they are protective in animal models.

Although antibodies to LPS are generally considered as marker for immunity to cholera by some immunologists with the notion that increases in anti-LPS antibody titers only correlate with response to (an)other antigen(s) that is (are) associated with protection against *V. cholerae*along with the gradual increase of age for elevation anti-*V. cholerae* LPS titers and immunity to cholera(Chelvarajan *et al.*, 2005; Glass *et al.*, 1982; Mosley *et al.*, 1968b), attenuated *Salmonella typhi*, Ty21a, transfected with the genes to synthesize *V. cholerae* LPS reported 25% protection against O1 *V. cholerae* challenge, suggesting that anti-LPS antibodies alone can provide protection(Tacket *et al.*, 1990). Moreover, field trials that used vaccine of purified Ogawa or Inaba LPS clearly showed protection, although they were not as effective as LPS provided by killed WC vaccines(Benenson *et al.*, 1968; Mosley *et al.*, 1970; 1973). The importance of serotype-specific anti-LPS antibody is highlighted by the fact that older individuals, who were immune to O1 *V. cholerae* were as susceptible to the emergent O139 serogroup of *V. cholerae* as younger, O1 immunologically naive individuals(Qadri *et al.*, 1995b; 1997).

Individuals recovering from cholera (classical O1) can have protective immunity for 33-36 months(Levine *et al.*, 1981). Anti-LPS antibody-secreting cells isolated from blood of individuals recovering from O1 or O139 secreted IgA1 independent of the infecting serogroup. Other isotypes e.g. IgG1, IgG2 and IgG3 were also secreted(Qadri *et al.*, 1999). Anti-LPS IgA1 and IgA2 have been found in fecal material, which is indicative of a mucosal response, but the percent of those that responded with mucosal antibody was lower than those who responded with serum IgA.

1.8.2 Cholera toxin-specific immunological response to cholera

Cholera toxin (CT) is a potent immunogen and induces antigen-specific sIgA and serum IgG antibody responses(Elson & Ealding, 1984) while also acts as adjuvant to a mucosal co-administered antigen(Vajdy & Lycke, 1995). Following intestinal administration, CT binds to intestinal epithelial cells and secretes cytokines (Bromander *et al.*, 1993; McGee *et al.*, 1993) and is reported to influence in increasing intestinal permeability of macromolecules(Lycke *et al.*, 1991) which was indicated controversial in some reports(Jackson *et al.*, 1993; Nedrud & Sigmund, 1990).

The interaction of CT through GM1 ganglioside(Kensil *et al.*, 1991)with leukocytes is thought to be of major importance for mediating their adjuvant effects. Especially dendritic cells (DCs) seem to be the principal cell type by which CT mediates their adjuvant effect in vivo as reports are available on its luminal antigens uptake(Rescigno *et al.*, 2001), phenotypic and functional maturation as well as coligands upregulation regarding humoral immunity(Bagley *et al.*, 2002; Eriksson *et al.*, 2003). In addition, a cAMP-dependent upregulation of the chemokine receptors CXCR4 and CCR7 occurs (Gagliardi & De Magistris, 2003), enabling the migration of DCs to lymph nodes and interaction with naive T cells(Iwasaki & Kelsall, 2000; Shreedhar *et al.*, 2003). In vitro assays also demonstrated the CT-matured DC's are able to direct immune response towards the Th2 phenotype and inhibition of Th1-response (Bagley *et al.*, 2002; Braun *et al.*, 1999; de Jong *et al.*, 2002).

In addition, CT exerts several effects on MØ such as enhanced expression of Th2 related coligands(Cong *et al.*, 1997; Yamamoto *et al.*, 1999; Yamamoto *et al.*, 2000)interleukins(Bromander *et al.*, 1991; Feng *et al.*, 2000; Foss *et al.*, 1999). CT also suppresses TNF-α production in response to LPS (Burkart *et al.*, 2002; Chen *et al.*, 2002; Cong *et al.*, 2001) and as a consequence the NO production is reduced(Cong *et al.*, 2001).

In mouse model, the effects of CT on T cells displayed to promote a Th2 response staring from initial event in upregulation of IL-4(Okahashi *et al.*, 1996; Vajdy & Lycke, 1995; Yamamoto *et al.*, 2000)which leads to the secretion of IL-5, IL-6 and IL-10, a typical helper signals in CD4+ T cells for the induction of antigen specific sIgA as well as serum IgG1, IgA and IgE responses(Hornquist & Lycke, 1993; Xu-Amano *et al.*, 1993). CT was also shown to selectively

inhibit proliferation and IFN-γ synthesis of Th1 clones (Munoz *et al.*, 1990; Yamamoto *et al.*, 1999) and abrogates IL-12R expression by T cells (Braun *et al.*, 1999). Moreover, CT has been reported to induce selective apoptosis of CD8+ T cells and naïve cells are more sensitive than activated cells(Elson *et al.*, 1995).

When studied B cells responses, binding of CT leads to the upregulated expression of MHCII, B7.1 and B7.2, CD40, ICAM-1 and IL-2Rα(Anastassiou *et al.*, 1990; Francis *et al.*, 1992) which enhances their role as MHC II-restricted antigen presenting cells and favors the induction of Th2-dominated responses. Another in vitro studies indicate that CT facilitates B cell switching to IgA through the action of TGF-β1 and increases the effects of IL-4 and IL-5 on IgG1 and IgA synthesis in lipopolysaccharide (LPS)-triggered spleen B cells(Kim *et al.*, 1998; Lycke *et al.*, 1990) and is independent of the A subunit(Kim *et al.*, 1998).

However, the utility of cholera toxin (CT) antibodies induced by infection or by some oral cholera vaccines have been debated for years(Muse *et al.*, 2012). Killed oral cholera vaccines with CT do not perform better (long-term protective efficacy) than cholera vaccines without CT B (CTB) subunit, but greater short-term (8 months) protection was seen in the group that received CTB as part of the cholera vaccine(Clemens *et al.*, 1988b). The licensed Vietnamese killed cholera vaccine, mORCVAC, and another vaccine, Shanchol, the new entry for killed cholera vaccines do not contain CTB, yet both are proven to provide immunity against cholera(Mahalanabis *et al.*, 2008; Trach *et al.*, 2002).

1.8.3 TcpA specific immunological response to cholera

A number of observations suggest that immune response to TcpA may contribute to protection against *V. cholerae* infection. TcpA has been shown to be essential for *V. cholerae* colonization in both mice and humans(Herrington *et al.*, 1988; Taylor *et al.*, 1987), its mRNA is up-regulated during early stages of human infection(Larocque *et al.*, 2005), systemic and mucosal anti-TcpA responses occur in over 90% of individuals infected with *V. cholerae* O1,El Tor in Bangladesh (Asaduzzaman *et al.*, 2004; Hang *et al.*, 2003), passive administration of both polyclonal and monoclonal antibody to TcpA in mice is fully protective against *V. cholerae* challenge(Sun *et al.*, 1990; 1991) and active parenteral immunization of adult female mice with a TcpA peptide,

along with an immune adjuvant, induces protection against *V. cholerae* challenge of mice born to immunized mother(Wu *et al.*, 2001).

1.8.4 Outer membrane (OM) immunogens specific immunological response to cholera

Knowing the complete Whole Cell Outer Membrane (WC-OM) profile and its structural variability with*V. cholerae* life cycle stages is a first step to engineering a better cholera vaccine in any form of formulation. Antibodies that bound PilA, TcpA, and OM-associated structures, PilQ, MSHO, MSHP, and CapK were found in cholera convalescent serum (Hang *et al.*, 2003). TcpA is required for human and mouse colonization while MSHA and PilA to be specific for humans(Attridge *et al.*, 1996; Fullner & Mekalanos, 1999; Taylor *et al.*, 1987) and protective capacity of anti-OM structure Abs ultimately needs to be assessed in humans which is clearly a rate limiting step in cholera vaccine development (Wade, 2011).

Several identified *V. cholerae* virulence factors are protective antigens in animal models, but only a detoxified-Inaba LPS-CT conjugate has been tested in humans(Gupta *et al.*, 1998). Chitin binding protein A (CBP-A) contributes in chitin-based *V. cholerae* colonization and induces rabbit protective Abs, but its immunogenicity in humans has not been reported(Kirn *et al.*, 2005). LPS and TCP, both colonization factors, induce synergistic vibriocidal and protective Abs in mice(Osek *et al.*, 1994)yet there are no immunogenicity and protective potential reports in humans combining*V. cholerae* immunogens (*e.g.,* TcpA, CBP-A, LPS, flagella, OmpU) has been generated even though multivalent vaccines are prevalent(Knuf *et al.*, 2006). Efficacy of other *V. cholerae* antigens, (*e.g.*cell wall antigens or cell wall associated antigens) combined with LPS as cholera vaccine immunogens have been studied(Eubanks *et al.*, 1977; Guentzel & Berry, 1974; Guentzel & Berry, 1975; Guentzel *et al.*, 1977) which established non-W-C, flagella containing vaccine preparation as superior immunogen in mice compared to WC *V. cholerae* vaccine preparations(Eubanks *et al.*, 1977; Guentzel *et al.*, 1977). In addition, anti-flagellar Antibodies (to crude flagellar fractions) inhibited access of *V. cholerae* to the deep ileal crypts, thus effectively reducing the infecting inoculums (Guentzel *et al.*, 1977). Later reports by Bishop and Camilli supported that motility-related antigens are important targets for Abs that would reduce colonization (Bishop *et al.*, 2010). Aquatic-based *V. cholerae* antigen may represent a subset of uncharacterized potentially protective antigens as*V. cholerae* binds chitin expressing biota in part

through CBP-A, TcpA, and MSHA, which induce Abs that prevent mouse colonization (Kirn *et al.*, 2005; Osek *et al.*, 1992; Pruzzo *et al.*, 2008; Taylor *et al.*, 2004). The complete subset has not been identified and characterized for protective efficacy in animals let alone in humans.

Many reviews have been published on cholera vaccines and *V. cholerae* pathogenesis which emphasize different aspects of the biology; the immunology, the virulence, and the ways forward to a better cholera vaccine(Bishop & Camilli, 2011; Chowdhury *et al.*, 2009; Levine, 2010; Lopez *et al.*, 2008; Matson *et al.*, 2007; Pruzzo *et al.*, 2008; Ritchie & Waldor, 2009; Ryan & Calderwood, 2000). However, intensive integration of the immunology focusing the realities of protection at the gut mucosal surface given the B cells that are there, and considering the antigens expressed by *V. cholerae* at different times during its life cycle is the right approaches for a one dose cholera vaccine that will work for all, at any time in their lives(Wade, 2011).

1.9 Zn Supplementation and its impact on cholera and vaccine in the developing world

Zinc supplementation impacted with beneficial effects on the clinical course of dehydrating acute and persistent diarrhea(Roy *et al.*, 1992) as well as in malnourished children with acute watery diarrhea and recommended as an adjunct therapy to ORS(Dutta *et al.*, 2000).Further studies(Altaf *et al.*, 2002) validated its contribution to improving the physiologic status of the small intestine through decreased NF-κB DNA binding activity and reduced nitric oxide metabolites that potentially reduce the risks of recurrent diarrhea episodes.

Randomized, double-blind, community-based intervention studies conducted in rural children indicated that zinc supplementation was effective in reducing diarrhoeal morbidity when administered either daily or in a weekly schedule(Gupta *et al.*, 2003) and had a beneficial impact on the incidence of diarrhea and also weight gain among low birth weight infants while another double-blind, randomized, placebo controlled trial among children with diarrhea and moderate dehydration confirmed the clinical benefit of zinc in children with diarrhea as it was found that supplementation of a combination of micronutrients and vitamins was not superior to zinc alone (Dutta *et al.*, 2011). Similarly, a different double blind, randomized, placebo controlled trial study carried out by in Bangladesh (Roy *et al.*, 2008) on zinc supplementation significantly

reduced the duration of diarrhoea and stool output in children with cholera and slacken the severity.

1.9.1 The mouse model

The utility of the mouse infection model system is good as their gut environments induce 65% of the *V. cholerae* transcripts isolated from *V. cholerae* in cholera victim's stool. Infant mice also reproduce an important aspect of human cholera pathogenesis-the increased infectivity phenotype of gut-passaged *V. cholerae*(Alam *et al.*, 2005). *V. cholerae* transcription, measured 21 hours after mouse infection, revealed 60% of the transcripts were important for *V. cholerae* persistence in stool or the aquatic environment(Schild *et al.*, 2007). Ninety-three percent of *V.cholerae*'s ORFs (core genome) are expressed *in vitro* (nutrient-rich culture) and in infected humans (nutritional or immune stress), with 42 *V. cholerae* genes (1.0%) being expressed preferentially late in infection (Larocque *et al.*, 2005). Environmental *V. cholerae* isolates share 71-88% of the core gene set with virulent O1 *V. cholerae*(Keymer *et al.*, 2007). Environmental *V. cholerae* does not express approximately 7% of the genes that virulent, stool-resident *V. cholerae* does. While many of the unique and common genes expressed are internal antigens, those that are not need to be evaluated relative to vaccine targets that could function at different points in *V. cholerae*'s life cycle.

The mouse model is reasonable because it reproduces the physiology of cholera. Infant mice (5-6 days of age) are susceptible to *V. cholerae* as the developmental state of their intestinal flora and innate immunity permits colonization(Klose, 2000). The animal model is routinely used to test the efficacy of various cholera anti-sera by passive immunization (Falklind-Jerkerus *et al.*, 2005; Meeks *et al.*, 2004; Mukhopadhyay *et al.*, 2000; Taylor *et al.*, 2004). Anti-sera from vaccinated individuals or experimental animals are combined with virulent *V. cholerae* and gavaged into the stomach of neonatal mice. The mice become moribund or die based on the antisera's ability to protect.

The infant model, however, does not provide information about the potential role of specific Ig isotypes in the natural infection where antibody is not admixed with the infecting inoculum, but must localize actively to the site of infection. The mouse model does, however, provide information about the potential protective efficacy of antibodies to a particular *V.*

*cholerae*antigen. If protective antigens can be identified, it is clearly possible to manipulate the form of the antigen, the adjuvant, or the route of inoculation to induce the antibody isotype required for protection of humans.

1.10 Cholera vaccine delivery route

Several cholera vaccines have been developed over the last four decades, but none have achieved the level of immunity seen in natural infection. It may be that these vaccines do not induce robust immune responses against important virulence factors as TcpA and cholera holotoxin (CT) or LPS for safety reasons. Immunization strategies that augment immune responses to critical virulence factors may, thus, contribute to the development of an optimal cholera vaccine. Based on this, efforts are being made to explore different vaccination strategies as well as test the transcutaneous route of immunization (TCI) to determine its appropriateness as an alternative path of vaccination. TCI involves the topical application immunogens directly on the skin surface (Sheikh *et al.*, 2010; Sur *et al.*, 2011) delivering target antigens to the Langerhan's cells in the epidermis which then carry antigens to the regional draining lymph nodes to initiate immune response(Enserink, 2011). TCI has been shown to elicit strong systemic and mucosal IgG and IgA antibody responses in the presence of immune adjuvants such as LT or CT (Ahmed *et al.*, 2009; Enserink, 2011; Scharton-Kersten *et al.*, 2000; Sur *et al.*, 2011).One study has demonstrated that higher systemic and mucosal immune responses are obtained with oral priming and TCI boosting over either TCI or oral immunization alone, suggesting that supplementing oral vaccines with a transcutaneous boost with a substance such as TcpA, may induce mucosal immunity(Waldor & Mekalanos, 1996). Another TCI evaluation was carried out with a colonization factor (CS6) of ETEC and LT to induce immune responses and protection in experiment animals and human volunteers (Guerena-Burgueno *et al.*, 2002). The TCI route has also been used for testing a *V. cholerae* antigen, the toxin co-regulated pilus A (TcpA), co-administered with cholera toxin (CT) where it has found to be immunogenic and protective (Rollenhagen *et al.*, 2006). Moreover, a recent work has demonstrated that TCI with a *V. cholerae* O1 Ogawa synthetic hexasaccharide conjugate following oral whole-cell cholera vaccination boosts vibriocidal responses and induces protective immunity in mice (Tarique *et al.*, 2012) and supports to delineate the immune mechanism involved for robust immune response.

1.11 Immunoglobulin Structure

Antibodies were characterized electrophoretically as γ-globulin(Tiselius & Kabat, 1939). Antibody consist of two identical light chain and two identical heavy chains(Porter, 1959). This has been deduced by the X-ray crystallography of crystalline antibody fragments.

An antibody molecule can be divided into two fragments – the variable fragment (Fab) and the constant fragment (Fc). The Fc fragment is mostly responsible for the antibody to binding onto the cell surface for phagocytosis. It also presents some enzymatic functions which takes part and activates the classical complement pathway. Irrespective to that of the Fc fragment, in an antibody, there are two Fab fragments. Hence, it is represented as $F(ab')_2$ in case of bivalency. An example for it is the IgG molecule. The Fab fragment actually binds to the antigen through the antigen binding pocket. The presence of two Fab fragments in an antibody helps it in cross-linking the antigen. This is particularly helpful as it restricts the organism to a locality, hindering its spread throughout the body.

There are two regions in the Fab fragment – light chain and heavy chain. The light chain in humans can be of two types – λ chain and γ chain. A single antibody or antibody-producing cell can present anyone of the two light chains but never both together. However, irrespective of the types of light chain, it is divided into two regions, which are namely the variable region (V) and the junction region (J). These two parts play an important role regarding the specificity that antibody confers, moreover, these two parts containing the complementary-determining region (CDR). There are three CDR regions in a light chain of the antibody. These regions confer a high mutability. These regions are termed as CDR-L1, CDR-L2, and CDR-L3.

Unlike the light chain, the heavy chain is divided into three parts, namely variable (V) region, diversity (D) region, and the junction (J) region. However, there are also complementary-determining regions (CDR) within this heavy chain region. These three are represented as CDR-H1, CDR-H2, and CDR-H3.

Both the light chain and the heavy chain together of the Fab region of the antibody is the main part that binds to the antigen. It has been observed that the CDR regions present high mutability in the antibody structure. Interestingly, amongst all the six CDR regions, the CDR-H3 shows the

highest rate of mutability. The advantages of mutation within these regions are that it helps in strong binding with the antigen. Many studies have been carried out in the characteristics of the CDR-H3 region where its structure, its optimum length, and its spatial arrangement have been observed.

In addition to the CDR-H3 region, it has also been observed that the other CDR regions take on specific structure based on the amino acids sequence and their spatial arrangement. This has led to the study of certain common conformation of the loop structure and its classification.

The CDR regions (loops) are of great interest as they participate in the binding of the antibody with the antigen. Interestingly, it has been observed that the accessible surface area (ASA) differs with the type of antigen that the antibody detects. In case of proteins, since they are larger in size, the ASA of the CDR regions are flat while that of small molecules, such as lipids and carbohydrates, the ASA region is cleft shaped. Moreover, the type of antigen that the antibody binds to also regulates the canonical structure of the CDR regions.

1.12 Constraints and future challenges with LPS and other confounders tocholera vaccine

The evaluation of the current cholera vaccine preparations, vaccination strategy and final protective efficacy outcome and to explore alternative approaches needed to find a solution to the complex problems in the field of cholera vaccine which although initiated over 100 years ago has not resulted in an effective vaccine suitable for all age groups and different populations. These approaches include reassessment of the antigenic components of *V. cholerae* O1 which are being used or have the potential to be candidates in different vaccine formulations in terms of the different routes of immunization. In spite of all the efforts, further improvement is needed to augment the protective efficacy of cholera vaccines especially in nutritionally immuno-compromised populations of children in developing countries using mucosal adjuvants and micronutrients to improve efficacy and immunogenicity of vaccines are being tested.

According to previous studies, LPS although generally considered marker for immunity to cholera,the Ogawa and Inaba serotypes of *V. cholerae* O1 contain three antigenic determinants, A, B and C(Nato *et al.*, 2003). The B determinant is present only on Ogawa cells. The A and C

antigenic determinants are present on both Ogawa and Inaba cells. However, only about half as much C antigenic determinant is present on Ogawa cells as on Inaba cells, whereas the A antigen is more abundant on Ogawa cells (Bhuiyan *et al.*, 2003). The antigenic determinant of *V. cholerae* O1 Ogawa serotype has been characterized by immunochemical and physicochemical methods. The binding of monoclonal immunoglobulins specific for the Ogawa serotype (clone S-20-4) was studied by ligand-induced fluorescence titration using synthetic saccharides showed that the terminal monosaccharide bearing the 2-*O*-methyl group to be the serotype-specific determinant for the Ogawa strain. The crystal structure of the Fab from this monoclonal antibody was then further determinedthe complementary water-excluding hydrophobic interface and five antibody-antigen hydrogen bonds crucial for carbohydrate recognition(Villeneuve *et al.*, 2000). As stated above, the upstream terminal O-SP monosaccharide from the Ogawa LPS, bearing the 2-*O*-methyl group structure determined accounts for the serotype specificity of anti-Ogawa antibodies and provides a rational basis for the development of a synthetic carbohydrate-based anti-O1 Ogawa vaccine(Chernyak *et al.*, 2002), has been shown to be the B antigenic determinant.

Studies of binding the core and the O-specific polysaccharide of Ogawa and Inaba LPS to a monoclonal antibody that recognizes both the Ogawa and Inaba serotypes (clone I-24-2) in ELISA has shown that both the core and the O-specific polysaccharide are involved in this common antigenic determinant(Villeneuve *et al.*, 1999) and suggested to be the C antigenic determinant. However, the precise chemical structure of this antigenic determinant has yet to be determined. Moreover, the location and structure of the A antigenic determinant are unknown.Epidemiological studies carried out at the icddr,b field site at Matlab have shown that natural infection with *V.cholerae* O1 of the Inaba serotype is more protective than *V. cholerae* O1 of the Ogawa serotype against subsequent attacks of cholera(Sack *et al.*, 2003).

It is now clear that the B antigenic determinant is specifically expressed by cholera vibrios of the Ogawa serotype. However, it is less clear whether the Inaba serotype expresses a specific, as yet unidentified antigenic determinant. In the 1980s, Gustafsson described a monoclonal antibody (clone C6) specific to the Inaba serotype(Gustafsson *et al.*, 1982;Gustafsson, 1984;Gustafsson and Holme, 1983, 1985). However, as these authors concluded that this monoclonal antibody was directed against the C antigenic determinant, it remains a question of debate whether this

antibody is specific to the Inaba serotype because the C antigenic determinant has been reported to be common to both the Ogawa and Inaba serotypes.

Preliminary studies of these antibodies showed that mouse polyclonal sera react with Inaba LPS in ELISA, but have no vibriocidal or agglutinating activities(Wang *et al.*, 1998). In contrast, the monoclonal antibody 1G12 agglutinates Inaba cells, is weakly vibriocidal, but does not react with Inaba LPS in ELISA. Moreover, immobilizing activity *in vitro* and the protective activity of these antibodies in the neonatal mouse assay have not yet evaluated. Characterization of the antigenic determinants expressed by the Inaba serotype of *V. cholerae* O1 is of particular importance for the preparation of a glycoconjugate inducing high titers of anti-Inaba IgG antibodies.

There are different approaches for design of vaccines for protection from enteric infections. These include the oral whole cell bacteria killed, live vaccines, parenteral, and transcutaneous vaccines. All these approaches are being tested in the field of cholera vaccinology. The whole cell bacterial cholera vaccines have been tested in Phase I to III studies and have also progressed to effectiveness trials. Another concept of using polysaccharides conjugated proteins is being evaluated. Various immunization strategies that augment immune responses to critical virulence factors may, thus, contribute to the development of an optimal cholera vaccine. One way to augment the protection afforded by these vaccines would be through the application of antigens via transcutaneous immunization (TCI). In spite of all these efforts, there still seems to be improvements needed to augment the protective efficacy of cholera vaccines especially in nutritionally immuno-compromised populations in developing countries of mucosal adjuvants and micronutrients like vitamin A and zinc to improve efficacy and immunogenicity of vaccines are being tested. As it is established that zinc plays a critical role in the normal functioning of the immune system and recent findings suggests that zinc has a role to play in infectious diseases such as shigellosis and cholera, as well as on enteric vaccines, there is a need to understand the mechanism by which zinc acts in the immune system.

1.13 Objectives

1.13.1 General objective

To determine the alternative route of immunization for cholera vaccination using membrane extract and other available immunogens of *V. cholerae* O1.

1.13.2 Specific Objectives

Part I

1. To ascertain, the structure of the antigenic determinants recognized by the mAbs including 1G12 and immune polyclonal sera obtained from mice and rabbits.
2. To evaluate the protective activity of these antibodies (polyclonal and monoclonal) in the neonatal mouse assay.
3. To identify the parameters measured *in vitro* (agglutinating activity, vibriocidal activity, immobilizing activity, ELISA titers, and if necessary, other parameters that remain to be defined) that are correlated with the protective activity of these antibodies *in vivo* in the neonatal mouse assay.
4. To determine the antigenic epitope of *V. cholerae* O1 Inaba serotype by a crystallographic method.

Part II

1. To determine humoral and cellular responses for assessing seroconversion pattern and the phenotypic changes respectively in the systemic and mucosal compartments

2. To develop method for measuring and isolation of antigen specific B cells in the spleen, blood and lamina propria using positive selection magnetic bead system after subcutaneous (SCI), oral immunization (ORI) and transcutaneous immunization (TCI).

3. To define the effect of zinc supplementation on the immunogenicity of *V.cholerae*antigens in the murine model.
4. To assess of the level of genetic variability and somatic hypermutation (SHM) by cloning and sequencing possible antibody isotypes of antigen specific B cells after TCI immunization.

2 Methods and Materials

As programmed in the research proposal, the overall experimental design was linked with the evaluation of the current cholera vaccine preparations, vaccination strategy and final protective efficacy outcome and to explore alternative approaches needed to find a solution to the complex problems in the field of cholera vaccine suitable for all age groups and different populations. The approaches planned to be included were: i) experimental protocols targeting for elucidation of the antigenic determinant common to both the serotype Ogawa and Inaba of *V. cholerae* O1, ii) reassessment of the immunogenic components of *V. cholerae* O1 which are being used or have the potential to be candidates in different vaccine formulations in terms of the different routes of immunization, and iii) improvements needed to augment the protective efficacy of cholera vaccines especially in nutritionally immuno-compromised populations in developing countries, like Bangladesh.

2.1 Experimental protocols targeting for elucidation of the antigenic determinant common to both Ogawa and Inaba of *V. cholerae*O1

Monoclonal antibodies (mAbs) and rabbit polyclonal sera produced at Institut Pasteur, Paris, France, International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) and National Institute of Cholera and Enteric Diseases (NICED), Calcutta, India were collected for selection of the best clone. Both on *in vitro* parameters such as immunoagglutination, vibriocidal antibodies and ELISA as well as *in vivo* protective activity were assessed for the immunochemical and physicochemical characteristics. This would facilitate to explore further in the description of a new antigenic determinant specific to the Inaba serotype of *V.cholerae* O1 LPS and provide a rational basis for the development of a glycoconjugate vaccine directed specifically against the Inaba serotype.

2.1.1 Immunoagglutination

This test was carried out with the freeze-dried and freshly prepared ascites of the monoclonals of icddr,b, Bangladesh, NICED, Kolkata and Institut Pasteur, France. Freeze-dried ascites were dissolved in deionized water at actual volume before freeze drying. The ascites were then diluted in sterile saline and were tested in slide agglutination against overnight culture of *V. cholerae* O1 Inaba (strain CNRVC 950707) and *V. cholerae* O1 Ogawa (strain CNRVC 960250) grown at

37°C in alkaline nutrient agar medium. List of the monoclonal antibodies with specificity and immunoglobulin(Ig) types has been given in Table 1.

Table 1: Different monoclonal and polyclonal antibodies produced in mice and rabbit at three collaborating laboratories

mAb ID and Ig Type	Ig Isotype	Specificity
ICL-18[a]	IgG3	Inaba+Ogawa
ICL-28[a]	IgG3	Inaba+Ogawa
ICL-17[a]	IgG1	Ogawa
ICL-26[a]	IgG1	Ogawa
ICL-29 (Lot –95)[a]	IgG3	Ogawa
ICL-29 (Lot –990919)[a]	IgG3	Ogawa
1G12[b]	IgG3	Inaba
S-20-4[c]	IgG1	Ogawa
I-24-2[c]	IgG3	Inaba+Ogawa
F-22-30[c]	IgG1	Inaba+Ogawa
Rabbit Sera[c]	IgG	Inaba+Ogawa

[a]Monoclonal antibodies produced at icddr,b, Bangladesh
[b]Monoclonal antibody produced at NICED, India
[c]Monoclonal and Polyclonal antibodies produced at Institut Pasteur, France

2.1.2 Vibriocidal Assay

Strains of *V. cholerae* O1 Inaba (CNRVC 950707) and *V. cholerae* O1 Ogawa (CNRVC 960250) were inoculated in hypersaline alkaline peptone broth from the stock culture preserved at -70°C and incubated at 37°C for overnight. These cultures were then inoculated in Columbia Agar Plate (Difco) by pouring with 1.0 ml overnight culture in alkaline peptone broth and mixed homogeneously in the plate. Discarding extra liquid, plates were incubated for 90 minutes at 37°C. Cultures of Columbia Agar after incubation were transferred to sterile normal saline by adding 2.5 ml of sterile normal saline in each plate and the growing cells were collected in tube to measure the optical density (OD) and was adjusted to 0.8 at 600 nm (OD_{600}) which corresponded to $3X10^8$ cfu/ml. Monoclonal ascites and serum samples were simultaneously incubated at 56°C for 30 minutes in water bath for serum complement inactivation before

vibriocidal assay dilutions. The de-complemented sera were then diluted at 1:20 and added in 50 µl/well of the first column with sterile normal saline in all wells except the second row with normal saline for positive control (Microtest^{TM} Tissue Culture Plate, 96 well, U-Bottom with Low Evaporation Lid, Becton Dickinson). Two-fold serial dilutions from the second column to the end were performed to dilute the samples up-to 1:40960 and the plates were kept at 4°C to be ready with the preparation of the strain-complement suspension which contained one volume of bacterial suspension mixed with two volumes of guinea pig serum traditionally used as complement (Sigma) for vibriocidal assay and seven volumes of sterile normal saline after adjustment of OD_{600} at 0.8. The suspension was kept on ice for 20 minutes and added in 25 µl/well to all wells of the microtiter plates containing the diluted samples. The plates were incubated at 37°C for one hour and 150 µl/well of Luria-Bertani broth was dispensed to all wells. The plates were incubated uncovered at 37°C for 2 hours in a humidified chamber. After completing incubation, a sterilized solution containing one volume of 0.1% neotetrazolium mixed with nine volume of 2.7% sodium succinate in distilled water was added in 25 µl/well of the plates with the absence of color indicating lysis of bacteria and violet coloration of the live vibrios and were kept inside a humidified chamber at 4°C to take a second reading after overnight incubation for confirmation of complete quantitative determination.

2.1.3 Specific ELISA againstLPS of*V. cholerae* O1 Inaba and Ogawa

To assess the specificity and antibody titer against LPS of *Vibrio cholerae* O1 Inaba and Ogawa, 60 µg of both LPS were dissolved in 1.2 ml of 1.0 M $NaHCO_3$ buffer containing 10.8 ml deionized water by vortexing to make the working concentration 5 µg/ml. Both diluted Inaba and Ogawa LPS were then dispensed in 100 µl/well for coating on the Maxisorp 96 well microtiter plate (Immuno Microwell, Cat. No. 439454, Batch No. 062938, Nunc, Nalgene Corporation, Finland) and incubated at 37°C for 2 hours at 400 rpm shaking. The plates were washed twice with 0.01% Tween-20 (Sigma) in phosphate buffer saline (PBS) and were quenched with 0.5% gelatin (Prolabo) in PBS, 200 µl/well and were incubated at 37°C for 30 minutes with 400 rpm shaking. After washing twice with 0.01% PBS-Tween again, the diluent prepared with 0.05% gelatin in 0.01% PBS-Tween (Phosphate-Gelatin-Tween, PGT) was added, 100 µl/well, in all the wells except for column 2. The wells of column 2 were added with prediluted mAbs in PGT based on immunoagglutination titer, 200 µl/well and serial two-fold

dilutions were made and the plates were incubated at 37°C for 1 hour at 400 rpm shaking. The plates were then washed twice with 0.01% PBS-Tween and peroxidase conjugated mouse anti-IgG (H+L), 1 :1000 dilution in PGT was added in 100 µl/well to all the wells of the plates and were incubated at 37°C for 1 hour with 400 rpm shaking. After washing twice with PBS-Tween 0.01%, substrate OPD (O-Phenylene-Diamine, 10 mg/ml) was added in 100 µl/well to the all wells of the plates and the plates were then incubated in a dark place at room temperature for 10 minutes. The reaction was stopped with 3N HCL (50 µl/well) just before reading. The plates were read spectrophotometrically (EL 800, Bio-Tek) at 490 nm with 630 nm as reference filter. The cutoff was set at 0.4 the optical density (OD) over background.

2.1.4 Inhibition ELISA against *Vibrio cholerae* LPS O1 Inaba and Ogawa

The amount of antigen giving 50% inhibition of antibody binding was determined by a two-step ELISA. In step 1, mAb was incubated with solutions of LPS and in step 2, the resulting mixtures of free and bound antibody were added to microtiter plates coated with serotype Ogawa or Inaba LPSs. At the first step of the experiment, end point dilutions of mAbs at cutoff (OD_{492} 0.5) were incubated with two fold serially diluted LPS started from 0.1 mg/ml to the end 0.04785 ng/ml and in the second step, the resulting mixtures of free and bound antibody were added to microtiter plates coated with serotype Ogawa and Inaba LPSs of *Vibrio cholerae* to determine the percentage of inhibition.

Flat-bottom microplates (Immuno Microwell; Nunc) were blocked by incubation with 0.5% gelatin (Prolabo) in PBS for 1 h at 37 °C and washed with PBS containing 0.1% Tween-20. Polysaccharide dilutions (100 µl) and 100 µl mAb in PBS-Tween-gelatin were added to the wells, at a dilution, determined by direct ELISA titration, giving an A_{492} of 0.5. The mixture was incubated for 1 h at 37 °C and 100 µl samples from each well were transferred to a second plate, that had been previously coated by with purified serotype Ogawa or Inaba LPSs (5 µg ml^{-1}) in carbonate/bicarbonate buffer (0.1 M, pH 9.5), blocked with gelatin and washed with PBS-Tween. This plate was incubated at 37 °C for 1 h and washed with PBS-Tween. An anti-mouse peroxidase-conjugated IgG (heavy- and light-chain specific) (Sanofi Diagnostic, Pasteur) diluted 1 in 1000 in PBS-Tween containing 0.5% gelatin was added to the wells. The plate was incubated at 37 °C for 45 min and washed with PBS-Tween. The enzyme substrate (*o*-

phenylenediamine dihydrochloride, Sigma: 100 µl at 0.4 mg ml^{-1}) in 0.1 M sodium citrate (pH 5.2), containing 0.02% hydrogen peroxide, was added to each well and the plate incubated for 10 min at room temperature. The reaction was stopped by adding 3 M HCl (50 µl per well) and the A_{492} was read in an EL 800 spectrophotometer (Bio-Tek Instruments). The degree of inhibition was calculated as percentage inhibition$[(A_{-\text{inhibitor}} - A_{+\text{inhibitor}})\}/ A_{-\text{inhibitor}})] \times 100$.

2.1.5 Protective activity of purified IgG1 mAb

Suckling Swiss mice of 5 days old and weighing 3.3 to 4.4 g were used for oral challenge experiments with strains of *V. cholerae* O1 Inaba (CNRVC 950707) and *V. cholerae* O1 Ogawa (CNRVC 960250) and selected for its capacity to produce high levels of cholera toxin (5 mg/ml), was used for oral challenge in mice. The experiment was carried out to define the concentration of purified IgG1 from F-22-30 ascites to be efficient to demonstrate significant protection.After removing secreted cholera toxin, a dose of 3.5×10^8 *V. cholerae* cells (10 times the 50% lethal dose), preincubated for 30 min at 37°C with purified IgG1 from F-22-30 ascites at various dilutions in 0.1 ml was delivered into the stomach with a blunt-tip feeding needle. Groups of mice that received vibrio suspension alone, phosphate-buffered saline alone, or vibrio suspension with non-immunized mouse serum served as controls. Mice were maintained at 30°C for 48 h or until death, and all surviving mice were scored as well or ill at 48 h. Mice were considered ill if they met all of the following criteria: diarrhea, markedly reduced skin turgor, and poor response to stimuli. The level of protection decreased as the dilution of the purified IgG1 antibody increased: protection was therefore dependent on dose. No protection was observed in mice that received pooled non-immune control sera.

2.2 Chromatographic techniques performed for purification of antibody from ascites and Fab purification

Monoclonal Mouse Ab F-22-30 (IgG1, κ) was purified from the ascitic fluid by protein-G-agarose affinity chromatography. The molecule was eluted with glycine-HCl (100 mM, pH 2.5) and equilibrated against 2.0 M Tris-base at pH 9.0. The Fab fragment was produced by papain digestion with an enzyme to substrate molar ratio of 1:30 in 0.1 M phosphate buffer, pH 7.2, 2.5 mM EDTA, 10 mM L-cysteine at 37°C. The Fab fragment was separated by ion-exchange chromatography and further purified by gel filtration on a Superdex TM G75 column

(Amersham Biosciences). As isoelectric focusing revealed the presence of heterogeneity, homogeneous Fab peaks were separated by an additional step of ion-exchange chromatography in a Mono-Q DEAE column. The purified Fab fragment was concentrated to 10 mg/ml in 40 mM Tris-HCl, pH 7.6, for crystallization trials.

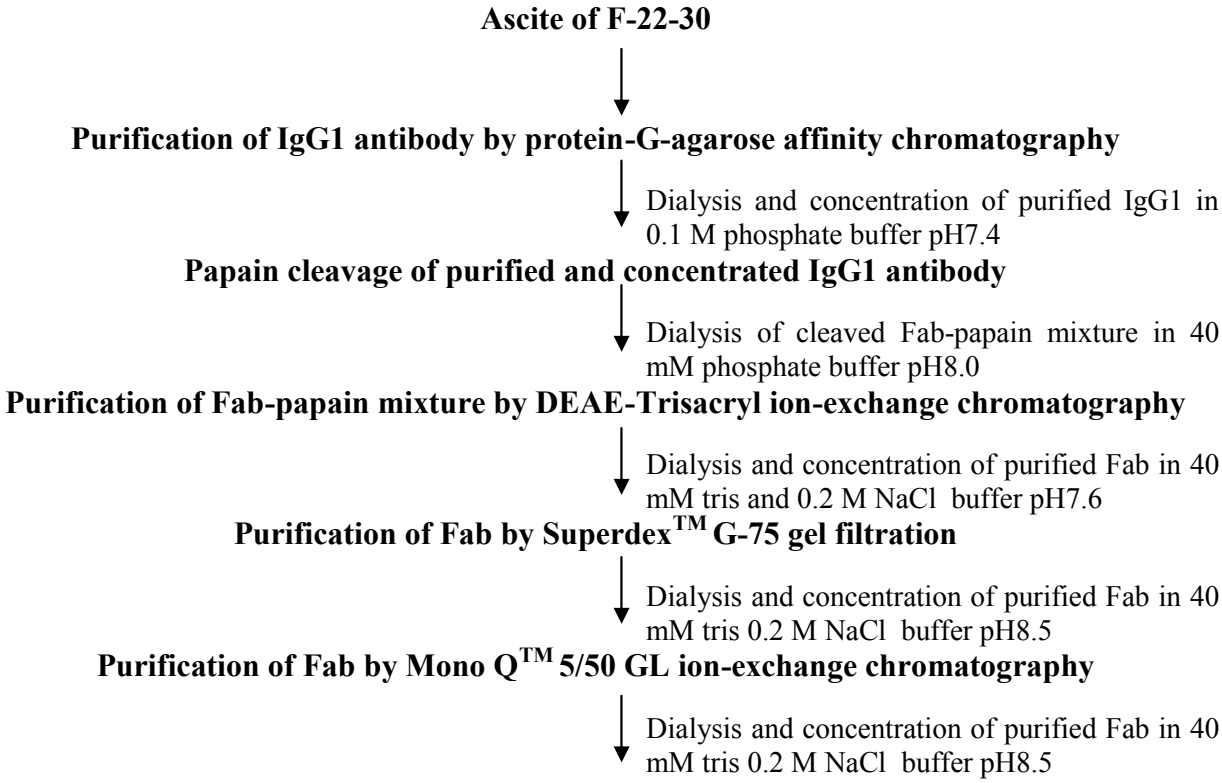


Figure 1: Flow Chart of chromatographic methods for the purification of ascite and Fab Preparation.

2.3 Studies on mice model for elucidating the role of zinc supplementation and different cholera immunogens in the different routes of immunization

The aim was to study in a mouse model for better understanding the role of zinc in modulating the immune response with major components of *V. cholerae* immunizing through different routes of immunization. The study was designed to evaluate the effect of supplementation with zinc in the immunogenicity and focusing on the serum isotype patterns and its serological properties

linked with protection, antibody secreting cells (ASC) and antibody in lymphocyte supernatants (ALS) responses of mature B cells, flow cytometry for phenotypic changes of the homing receptors and co-stimulatory markers of the antigen presenting cells (APCs), mature B and T cells and determination of class switch recombination (CSR) and somatic hypermutation (SHM) in mature antigen specific B cells in mouse immunized with individual antigens or with different combinations was aimed for better selection of antigenic components with their route of immunization (Figure 2).

2.3.1 Mice and feed

Female BALB/c mice bred in the animal resources branch of icddr, bwas used for immunization in the zinc-free stainless steel cage by washing with 1N HCl before the experimental feeding started. At 6 weeks of age, mice were matched for weight and divided into different groups for antigenic challenge with controls. Based on zinc status in the experimental feeding, the groups of mice selected as; i) Regimen A: Mice fed with zinc free diet (Altromin c 1040, Lage, Germany) supplemented with 2.0 mg/kg elemental zinc designated as deficient zinc (ZnM) group, ii) Regimen B: Mice given the diet (Altromin c 1040, Lage, Germany) supplemented with 25.0 mg/kg elemental zinc as zinc adequate group (ZnP) and iii) Regimen C: Mice fed with standard diet with free access to deionized water for all three regimen/ groups (Table 2). Experimental feed supplementation started 14 days prior to immunization and continued up to the day 77 for completion of five immunizations in 14 days interval (Figure 3). Sets of 6 mice per group were tested in the immunized or non-immunized controls. The control group was referred to as non-immunized.

2.3.2 Antigens

Cholera antigens (Table 2) were recombinant major subunit toxin TcpA (Asaduzzaman *et al.*, 2004), CT (List Biological Laboratories, Campbell, CA), recombinant cholera toxin B-subunit (rCTB) (Qadri *et al.*, 2003a; Taylor *et al.*, 1999) and purified LPS obtained from *V. cholerae* O1 El Tor Ogawa (strain 25049) or *V. cholerae* O139 (strain 4260B) (Jertborn *et al.*, 1996; Qadri *et al.*, 1997). Crude membrane extract of *V. cholerae* was prepared with in-house protocol.

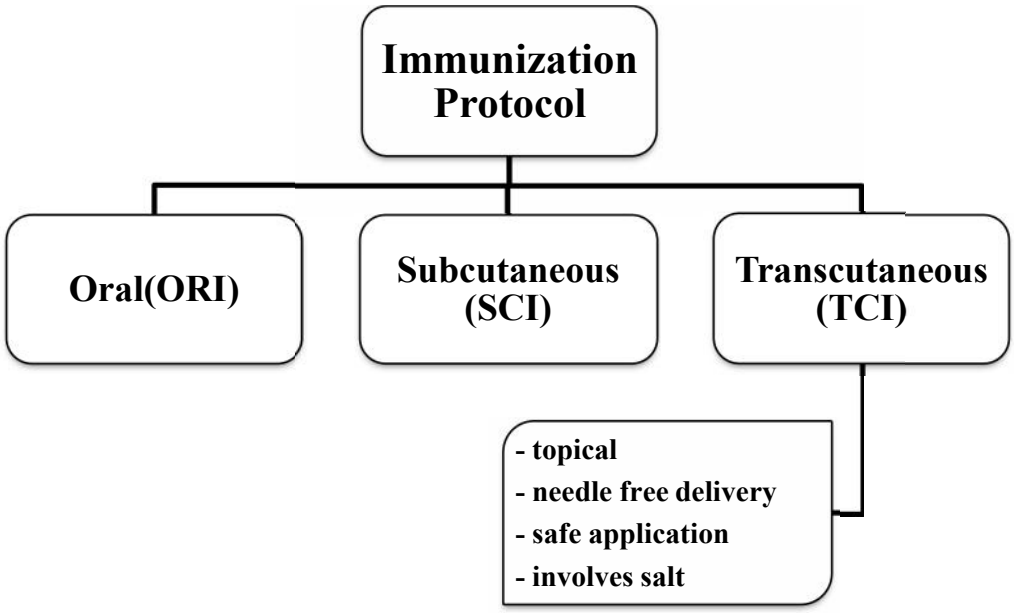


Figure 2: Evaluation of potential cholera immunogens through different route of immunization.

Table 2: Cholera immunogens doses in different route of immunization and the quantity of $ZnSO_4$ added in the Altromin Zn free diet

Animal: BALB/c mice, 4-6 weeks of age, 5 mice/group **Immunization Dose :**			
Antigens	**ORI(µg)**	**TCI(µg)**	**SCI(µg)**
CT	0.5	100	1
TcpA	100	100	10
LPS Inaba	100	100	10
Membrane Extract	Not Done	100	10

Control	**Zn free diet (Altromin, Germany)**	
Normal Feed	Zn Supplemented (ZnP)	Zn Deficient (ZnM)
	$ZnSO_4$(25 mg/kg)	$ZnSO_4$(2 mg/kg)

2.3.3 Immunization protocols and collection of serum samples

Groups of female Balb/c mice (6 mice/ group) were shaved on a $3\times5cm^2$ area on the dorsum using a clipper with no. 40 blade (Wahl Clipper Corp., Sterling, IL) and kept in rest around 24 h prior to immunization. During immunization, mice were anesthetized with 2,2,2-tribromoethanol (Avertin; Aldrich, St. Louis, MO) administered intraperitoneally at 0.4 mg/g of body weight to prevent grooming. Shaved area was swabbed with acetone followed by sterile water and allowed

to dry. Antigens were prepared at specific concentrations. The site was hydrated and abraded mildly by brushing with emery papers. Immunizing solutions (100 µl) containing 100 µg LPS or 100 µg membrane extract or 100 µg TcpA plus 100 µg CT in saline (0.9% NaCl) were applied on the shaved skin between 30 min to 2 h until consciousness was regained. Mice were washed extensively, tail down, under running lukewarm tap water for approximately 30 seconds and patted dry (Figure 4). Oral and subcutaneous route of immunization followed the scheme as ORI was carried out using a feeding needle (Fuchigami, Japan) with 100 µg LPS or 100 µg TcpA plus 1 µg CT in 0.25 ml PBS (10 mM, pH 7.2) mixed with 0.25 ml $NaHCO_3$ (100 mg/ ml, pH 8.5) prior to the instillation of each dose (Lycke & Holmgren, 1986) and SCI with 1 µg CT plus 10 µg TCpA or 10 µg LPS or 10 µg membrane extract in saline (Table 2) for each dose. Mice received different feed supplementation for 14 days and received immunization on day 0 followed by booster immunizations on days 14, 28, 42 and 56 by the same route with the same dose and formulation of antigens. The study was approved by the animal ethics committee of icddr,b.

2.3.4 Collection of specimen

Mice immunized at intervals of two weeks with 5 doses with the same dose and formulations of antigens in different routes and blood was sampled individually from each mouse at intervals of one week after each immunization i.e. on days 0, 7, 21, 35, 49 through tail bleed (1:10 dilution in PBS, 10 mM, pH 7.2) and through heart puncture on day 63. In brief, mice were placed in separate small cage with a lid and the tail was warmed with the heat lamp. Individual mouse was taken on the bench, covered with the box and the tail was extended through the opening. A sampling site was visualized on the lateral tail vein in the distal one-third of the tail and swiftly incised across the lateral vein with a new scalpel blade. Blood drops were collected with the transfer pipette and transferred it into the micro-fuge tube. A bit of towel was applied with gentle pressure to the wound. On day 63, after a short anesthetic exposure in chloroform (BDH Laboratory, Poole, BH151TD, England), blood was drawn in EDTA-vial (BD Vacutainer, BD Franklin Lakes, NJ) through heart puncture and spleen and gut were put in sterile cold PBS and processed immediately (Figure 3). Serum was separated by centrifugation in 3000 rpm for 10 minutes and preserved at -20°C.

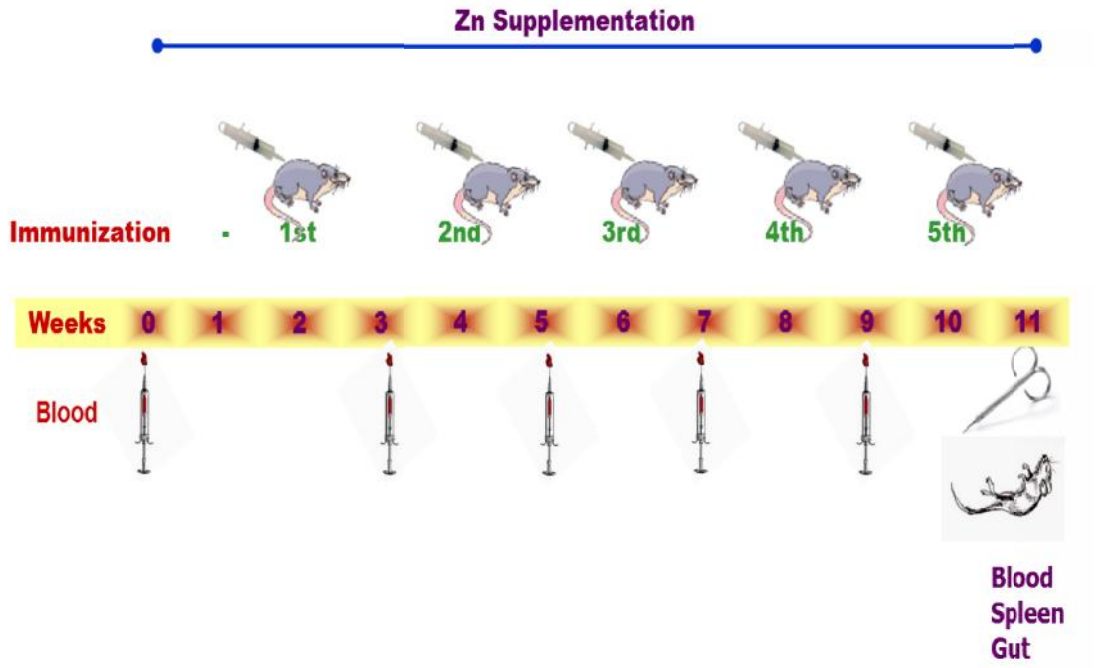


Figure 3: Optimized immunization protocol for different route of immunization.

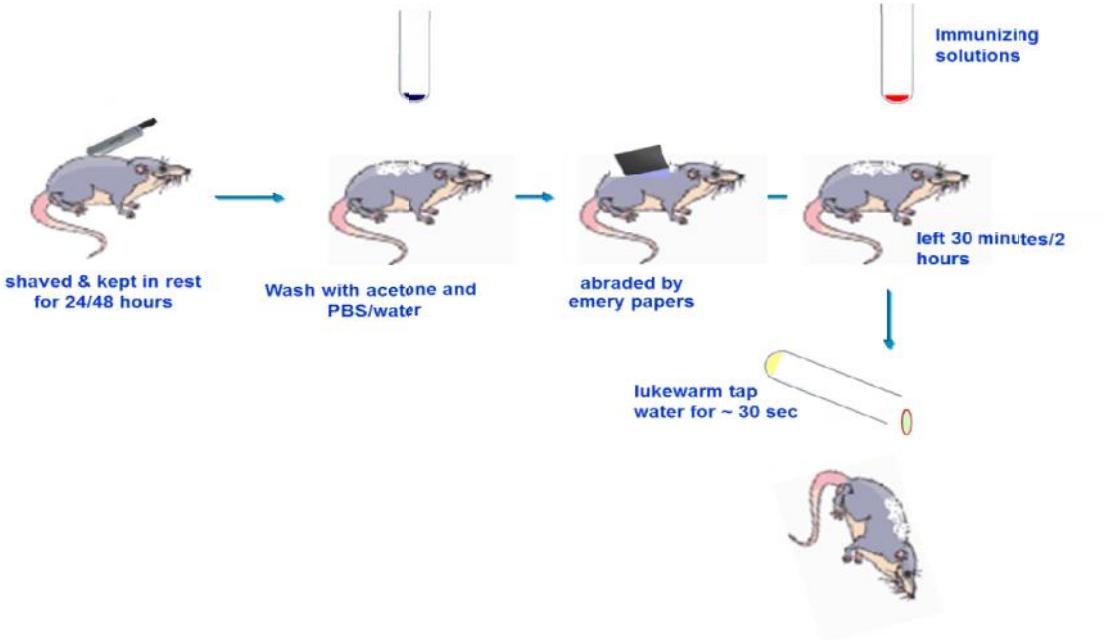


Figure 4: Transcutaneous immunization protocol.

2.4 Preparation of cell suspension and antibodies in lymphocyte supernatants (ALS) from spleen

Spleen cells were prepared according to the standard techniques. Spleens were placed on a Filtering Mesh (Sigma) in a petri dish, containing 2.0 ml PBS. Using a circular motion, the spleen was pressed through the screen with a plunger of syringe. The suspension was centrifuged for 5 min and washed twice with PBS. Cells were centrifuged (1200 g; 5 min) and the pellet was resuspended in ammonia lysing buffer (NH_4Cl, $KHCO_3$, EDTA di-sodium, pH 7.2) and incubated for 10 min at 37°C. Cells were washed with PBS and centrifuged again after which the pellet was resuspended in 10.0 ml volume of PBS and the cell number determined. After final washing, the cells were suspended in RPMI medium to desired concentration for different studies described below. For antibodies in lymphocyte supernatants (ALS), 5×10^6 cells/ml were cultured in vitro without stimulation in complete RPMI for 48 h and antibodies produced by these lymphocytes and secreted in the culture supernatant was collected for detection of immune responses to the four antigens (Rollenhagen *et al.*, 2006).

2.4.1 Antibody response in sera and ALS

The ALS and **s**erum samples from different groups of mice were tested for CT and TcpA and *V. cholerae* O1 LPS Ogawa and Inaba specific antibodies for IgM and IgG and IgA isotypes. For detection of CT-specific responses, the GM1-CT procedure (Qadri *et al.*, 1999) was used, with rCTB as the coating antigen in microtiter plates (Nunc, Roskilde, Denmark). For TcpA-specific responses, microtiter plates were coated with recombinant TcpA (1.0 µg/ml) (Asaduzzaman *et al.*, 2004). LPS Ogawa and Inaba specific responses were carried out with microtiter plates coated with purified LPS Ogawa and Inaba (2.5 µg/ml). Tenfold-diluted serum samples or two-fold diluted ALS samples were added in duplicate wells. Goat Anti-mouse IgM (1020-05), IgG (H+L) (1031-05), IgA (1040-05), and IgG1 (1070-05), IgG2a (1080-05), IgG2b (1090-05), IgG3 (1100-05) conjugated to horseradish peroxidase (HRP) (Southern Biotech, Birmingham, AL) were added in appropriate wells at a 1:2,000 dilution in PBS-Tween and incubated at 37°C for 90 minutes. Plates were developed using 10 mg of ortho-phenylene diamine (Sigma) in 10 ml of 0.1 M sodium citrate buffer (pH 4.5) to which 4 µl of 30% hydrogen peroxide was added just before use. Optical densities were measured kinetically at 450 nm. Plates were read for 5 min at 19-s

intervals, and maximum slope for an optical density change of 0.2 U was expressed as milli-optical density absorbance units per min(mOD/min) (Rollenhagen *et al.*, 2006).

A positive response in plasma at convalescence was defined as twofold or greater increase in the specific antibody response between acute (day 0) and convalescence phase (day 7, 21, 35, 49 and 63) samples were considered positive responses.

2.4.2 Isolation of intestinal lamina propria lymphocytes (LPL)

Intestinal LPL was prepared with some modifications of the method first described earlier (Davies & Parrott, 1981; Lycke & Holmgren, 1986). The small intestine (from the end of the stomach to the cecum) taken in cold saline and Peyer's Patches were excised with a sharp pair of pincers from the serosal side. The fecal contents were removed mechanically from the lumen and the intestine was opened longitudinally and cut into 5-mm 15-20 pieces and washed thoroughly in CMF-HBSS (Sigma, St. Louis, MO) kept at 37°C. Tissues were transferred into 25 ml glass bottle with magnet containing 6.0 ml EDTA (5mM) HBSS medium (initially pre-warmed to 37^0C). The suspensions were incubated on a magnetic stirrer with slow stirring at room temperature for 15 min to remove the epithelial cells and intraepithelial lymphocytes (IEL). Supernatant containing IEL were collected in 6.0 ml of cold PBS. This process was repeated for 4 times and supernatant pooled (≅ 48 ml) and kept in cold at 4°C. To isolate LPL, the remaining pellet was suspended in collagenase (0.6 mg/ ml, Sigma, St. Louis, MO)-HBSS and further incubation was carried out at 37^0C with vigorous stirring with a magnet to homogenize for 2 h. The suspension was filtered through the sieve nets to remove small particles of the tissues and was taken in a tube for centrifugation at 1600 g rpm for 10 min at 10°C. The pellet was suspended in RPMI containing 10% FBS and layered over Lympholyte-M (Cedarlane Laboratories Ltd, Burlington, Canada) for density gradient centrifugation and centrifuged (1800 g for 20 min at room temperature). The lymphocyte layer at the interface was collected and washed twice. The lymphocytes were suspended in RPMI medium to desired concentration for further experiments.

2.4.3 Vibriocidal assay

Serum samples from different groups of mice were tested for vibriocidal antibody response against the homologous serotype of bacteria using strain *V. cholerae* O1, El Tor Ogawa (strain

25049) or Inaba (strain 19479) as the target organism in procedures previously optimized (Qadri *et al.*, 1995b). A reference pooled serum specimen from *V. cholerae* O1-infected patients was included in each test to control for variation between analyses (Qadri *et al.*, 2003b).

2.4.4 Flow cytometry with immunocytes

Spleen cells and LPL fraction were suspended in RPMI containing 1×10^5 cells/ staining group in V-bottom polystyrene plate (Nunc) (100 µl/ well) and 100 µl FACS buffer was added. The plate was centrifuged at 1200 g for 5 min at 4°C and the antibodies added in different combinations for staining. Rat-anti-mouse CD19 PerCP-Cy5.5 (BD Biosciences, San Jose, CA) was added separately or with rat-anti-mouse CD40 FITC (BD Pharmingen) as well as CD19 PerCP (BD Pharmingen) with hamster-anti-mouse CD80 FITC (BD Pharmingen) and rat-anti-mouse CD86 R-PE (BD Pharmingen) as combinations. FITC conjugated rat anti-mouse CD3 (BD Pharmingen) was added as individually or with hamster anti-mouse CD28 PE (eBioscience, San Diego, CA) as well as hamster-anti-mouse CD154 PE (BD Pharmingen) as different combinations. The same scheme of FACS staining was applied for APC conjugated rat-anti-mouse CD4 (BD Pharmingen) and CD28 as well as CD154 (BD Pharmingen) coligands as described above. To determine the homing phenotypes, FITC labeled rat-anti-mouse β_7 (BD Pharmingen), FITC labeled rat-anti-human CLA (BD Pharmingen) and R-PE labeled rat-anti-mouse CD62L (BD Pharmingen) were used in appropriate combinations with CD19, CD3 and CD4 antibodies. To determine cutaneous marker, CLA, we used Heca-452 antibody assuming both mouse and human share this antigen (Borges *et al.*, 1997). R-PE conjugated rat-anti-mouse CD8a (BD Pharmingen) and APC conjugated hamster-anti-mouse CD11c (eBioscience) was used for determination of cytotoxic T cells and dendritic cells respectively. The plate was incubated at 4°C for 30 min. Cells were washed and resuspended in 100 µl FACS buffer for acquisition using FACS Caliber (BD Biosciences). For analyses of whole blood, 100 µl was diluted (1: 10 in PBS) and centrifuged at 1800 g for 5 min. The pellet was resuspended with specific fluorescent labeled antibodies as described earlier and incubated at 4°C for 30 min. The tubes were washed with PBS and pellet resuspended in 1.0 ml ammonia lysing buffer (NH_4Cl, $KHCO_3$, EDTA di-sodium salt, pH 7.2). The cells were washed and suspended and phenotyping carried out as described earlier (Table 3).

Table 3: Assays carried out to determine humoral and cellular responses

Humoral Immune Response	Cellular Immune Response with Flow Cytometry
Vibriocidal assay and specific antibody responses • ELISA for serum antibody level • ALS assay for antibody responses	Determine antigen presenting cells, T and B cells, homing receptors (β7, CLA and CD62L) and co-ligands of both T and B cells.
	Antibodies used for FACS: CD19 PerCP, β7 FITC, Streptavidin APC, Heca452 FITC, CD4 APC, CD62L PE, β7 FITC, CD8 PE, CD11c APC

2.4.5 Separation of antigen (Ag) specific B-cells

Lymphocytes from spleen were fractionated to detect the antigen specific cells. Splenocytes, 5×10^6 cells/ ml were used to isolate the antigen specific B cells using the biotin-streptavidin bead based positive selection method (Cato *et al.*, 2011; Safarik & Safarikova, 1999; Shinohara *et al.*, 1999). After washing with cold PBS, the cells were resuspended in complete RPMI medium containing 0.5 M NaCl and incubated for 15 min at 4°C without shaking to block the endogenous biotin molecules, which was followed by another wash step. Biotinylated CtxB, TcpA, Ogawa and Inaba LPS (biotinylation produced by Advanced Targeting Systems, San Diego, CA); 50 µg each, were added to tubes containing enriched splenic lymphocytes. The mixtures were incubated at 4^oC for 1 h with gentle shaking. After the final washing, the cells were resuspended in PBS containing 2% FBS for magnetic separation. Magnetic beads (~1.7×10^7; Dynabeads M-280 Streptavidin, Dynal Biotech ASA, Oslo, Norway) were aliquoted in a glass tube and placed in the Magnetic Particle Concentrator (Dynal MPC-6, Dynal Biotech ASA, Oslo, Norway) to discard the supernatant and washed with PBS containing 2% FBS. The antigen-biotin bound cells were mixed with beads and incubated for 30 min at 4° C with gentle shaking for binding of the streptavidin beads with the positive cells. The tubes were placed in the Dynal MPC-6 and the supernatants were collected. Four consecutive washing with PBS containing 2% FBS was carried out to remove the negative cells. Positive cells with beads were dissolved in Trizol reagent

(Invitrogen, Auckland, New Zealand) for RNA preparation from the antigen specific B cells and kept at −70°C until used for RNA extraction (Figure 5).

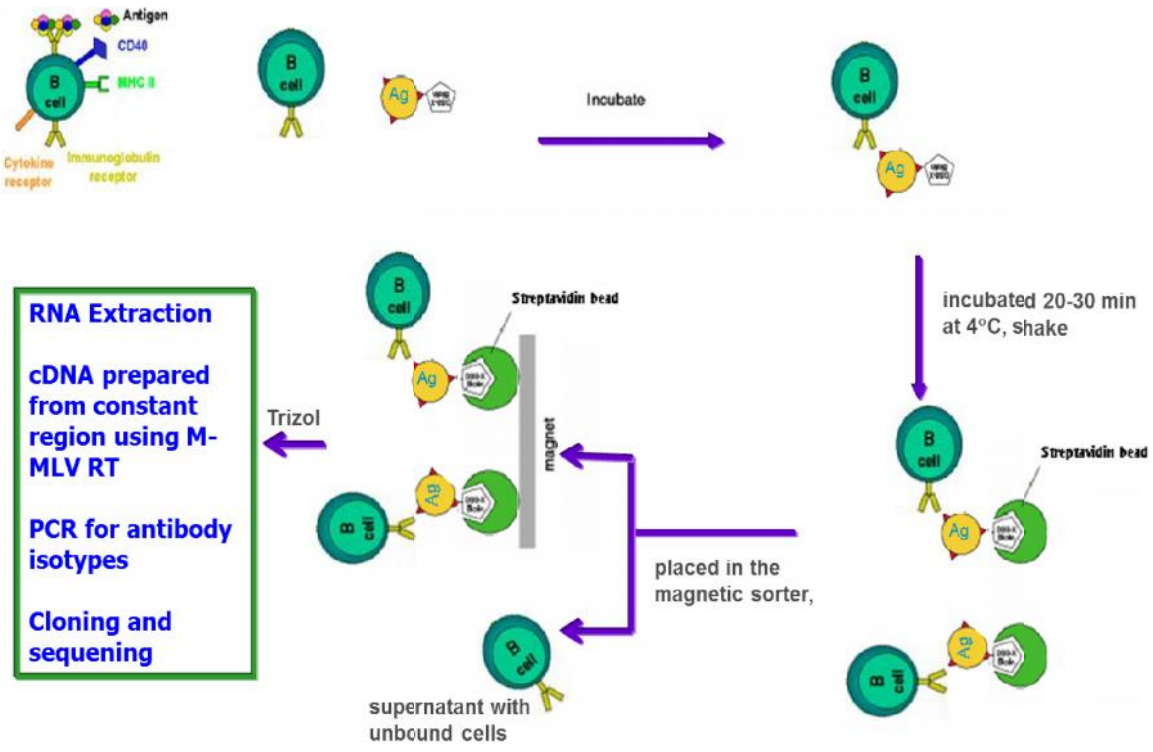
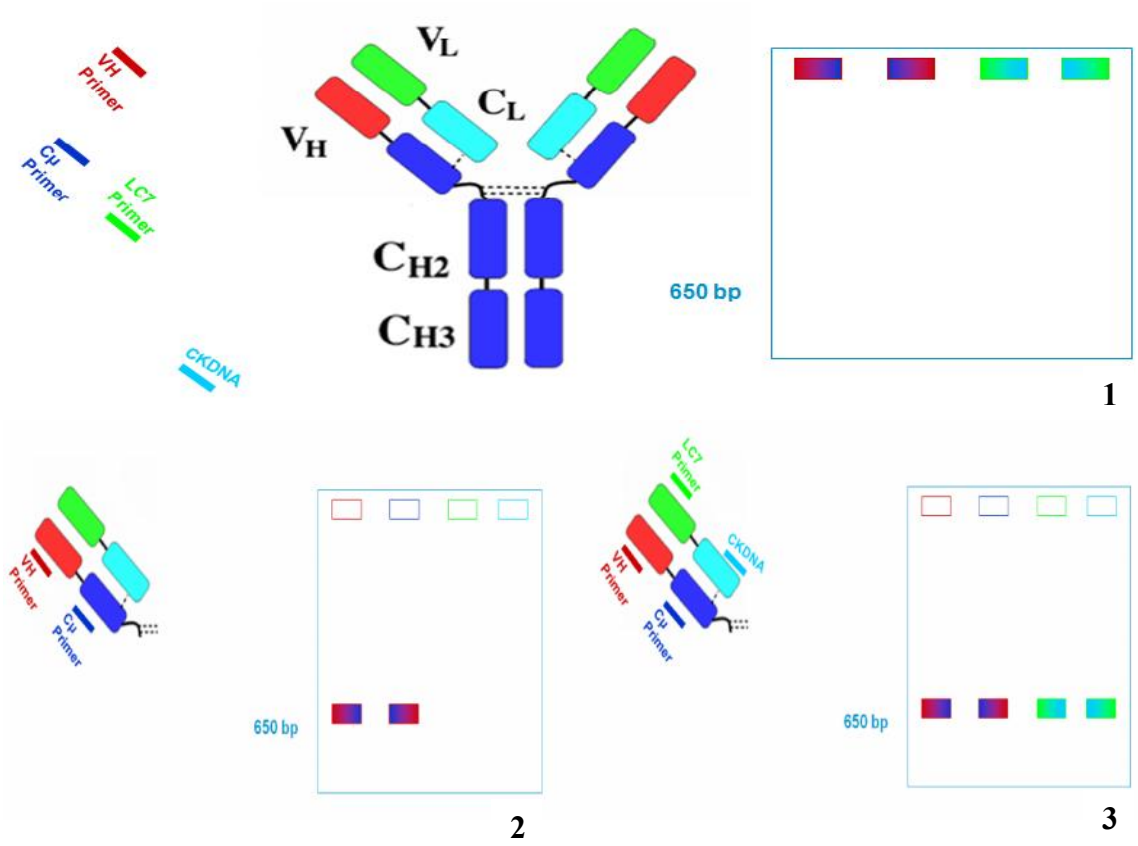


Figure 5: Flow diagram of extraction of Ag-specific B cells for molecular assays.

2.5 Cloning of the heavy (VH) and light (VL) chains of the antigen-specific B cells

Trizol samples containing the lysed antigen specific B cells were placed on ice, chloroform was added, centrifuged and the supernatants were collected to mix with an equal amount of isopropanol and incubated at 4°C for overnight. Following the sequential washing steps with ethanol and 0.2 M NaCl, the RNA pellets were dissolved in DEPC water. cDNA was produced via reverse transcription using 10 pg to 10 µg of RNA template, specific primers for constant regions (5 µm, 10 µl) and M-MLV reverse transcriptase (Invitrogen). Reactions were incubated at 37°C for 50 min, followed by inactivation of the reverse transcriptase at 70 °C for 15 min. RNA was removed from the reactions by incubating them with RNasin Plus RNase Inhibitor (Promega, WI, USA) at 37°C for 2 min. The resulting cDNA was used as a template for PCR amplification using Taq polymerase (Invitrogen) and various 5′ and 3′ primers specific for VH and VL genes. The primers used are as follows (in 5′–3′ orientation), and are described in detail

elsewhere (Barbas III *et al.*, 2001; Berry *et al.*, 1999; Dattamajumdar *et al.*, 1996). VH-specfic, 5': VHI: GAG GTG CAG CTC GAG GAG TCA GGA CC; VHIIA: GAG GTC CAG CTC GAG CAG TCT GGA CC; VHIIB: CAG GTC CAA CTC GAG CAG CCT GGG GC; VHIIC: GAG GTT CAG CTC GAG CAG TCT GGG GC; VHIIIA: GAG GTG AAG CTC GAG GAA TCT GGA GG; VHIIIB: GAG GTA AAG CTC GAG GAG TCT GGA GG; VHIIID: GAA GTG CAG CTC GAG GAG TCT GGG GG; VHV: GAG GTT CAG CTC GAG CAG TCT GGA GC. C_H-specific, 3': IgGl (muGIL): GCA AGG CTT ACT AGT ACA ATC CCT GGG CAC AAT TTTCTT GTC CAC; cM-Mu: ATG CAG ACT AGT GTT TTT GCC TCC GTA GTG G. V_L-specific, 5': LC7: CCA GTT CCG AGC TCG TGA TGA CAC AGT CTC CA. C_L-specific, 3': CKDNA: GCG CCG TCT AGA ATT AAC ACT CAT TCC TGT TGA A. PCR reactions were incubated at 94°C for 15 min, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72 °C for 1 min. A final extension at 72 °C for 10 min completed the reactions. Approximately 1–5 μg of the PCR products were resolved on 1% agarose gels and stained with ethidium bromide (Figure 1). PCR products of the appropriate size (650 base-pairs) were inserted into the Invitrogen pCR2.1-TOPO-TA™ vector and transformed into TOP-10 cells, according to the manufacturer's instructions. Colonies containing the plasmid with insert were selected by overnight growth, at 37 °C, on Luria–Bertani agar plates containing ampicillin (50 μg/ml) and X-gal (40 mg/ml). White (positive) colonies were incubated in 5 ml of Luria–Bertani broth containing ampicillin (50 μg/ml) for 12–16 h with vigorous shaking at 37 °C, and plasmids were isolated using QIAprep® Spin Miniprep DNA isolation kits, according to the manufacturer's instructions (QIAGEN®). *Eco*RI digestion of 1–5 μg of the resulting plasmids and resolution of the products via agarose gel electrophoresis, as described above, confirmed the presence of the appropriately sized inserts. One to 10 putative positive clones were sequenced with T7 (5′-TAA TAC GAC TCA CTA TAG GG-3′) and M13Rev (5′-CAG GAA ACA GCT ATG AC-3′) primers, using Chignin-1403-019 DNA sequencer of Genome Express Company, Grenoble, France (Figure 6). Consensus sequences were generated using the APE software and submitted to GeneBank using BankIt (www.ncbi.nlm.nih.gov/WebSub/?tool=genbank) online submission tool. GenBank (www.ncbi.nlm.nih.gov/genbank) accession numbersare (i) KC906585 to KC906636 for the VL sequences, (ii) KC906637 to KC906662 for the VH-Mu (μ) sequences, and (iii) KC 906663 to KC 906700 for the VH-Gamma (γ) sequences.



Immunogen	Ab Isotype	Variable Primer	Ab Isotype	Variable Primer	Ab Isotype	Variable Primer	Constant Primer
CTB	IgG1	VHIIA	IgM	VHIIA	L-Chain	LC7	CKDNA
LPS Inaba		VHV		VHIIc, VHV			
LPS Ogawa		VHV		VHV			
TcpA+CT		VHIIA, VHIIIA VHV		VHIIa, VHIIIa, VHV			
TcpA		VHIIA, VHIIIA VHV		VHIIA, VHIIIA VHV			

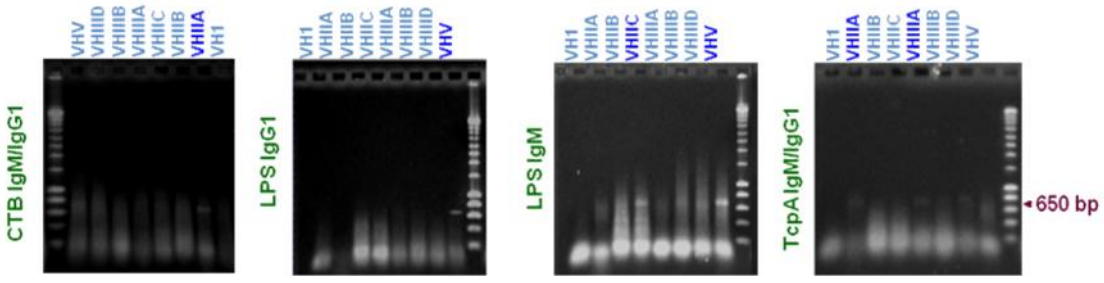


Figure 6: Immunogen and corresponding variable region family for heavy and light chain of the immunoglobulin isotypes and the PCR products.

2.6 Identification of Ig germline sequences and assignment of relevant regions

Consensus nucleotide sequences were compared against the *Mus musculus* immunoglobulin (Ig) set database using VBASE2 (http://www.vbase2.org/cgi-bin/vsearch_vbase2.pl). The sequences were concurrently compared against the *M. musculus* Ig germline V-gene database using VBASE2 (IMGT). This allowed the identification of the complementarity determining region (CDR) and framework (FR) regions of the VH and VL sequences, and provided numbering to the inferred amino acid sequences according to (Kabat & Wu, 1991). Similarly, the VBASE2 results allowed for the identification of the most closely related murine Ig germline V-genes currently available in these databases. In all cases, the entire sequence, including those at the 5′ end of each sequence imposed by the specific primers used in the original PCR amplification, were examined.

2.6.1 Sequence alignments

The inferred amino acid sequences were trimmed to remove all residues encoded by the 5′ primer regions, to eliminate potential artefactual matches caused by the primers employed in PCR amplification of the heavy and light chains. The sequences were then aligned with ClustalW (Thompson *et al.*, 1994) using standard parameters (gap open penalty: 10; gap extension penalty: 0.05; no weight transition; hydrophilic gaps allowed; weight matrix: Blosum). The alignments were then analyzed using GeneDoc version 2.6.002 (Nicholas *et al.*, 1997) to produce textual alignments and quantify the relatedness of the sequences.

2.6.2 Analyses

Acquisition data were analyzed with software FlowJo (Tree Star, Inc., Ashland, OR) in FACS Caliber (BD Biosciences). The Wilcoxon signed rank test and the Mann-Whitney U test were used for statistical analysis. The Fisher exact or chi-square test was used where applicable to evaluate the statistical significance of differences between groups. A P value of ≤ 0.05 was considered the criterion for a significant difference. Analyses were carried out with the statistical software SigmaStat (Jandel Scientific, San Rafael, Calif.). The geometric means (GM), medians and SEMs of the values were calculated.

2.6.3 Model building

Three-dimensional structure modeling of the Fv region of the monoclonal antibody M2-Inaba-Fv and M4-Inaba-Fv was performed by using the PIGS (Prediction of Immunoglobulin Structure) Server at *http://arianna.bio.uniroma1.it/pigs*(Marcatili *et al.*, 2008). PIGS is a web based tool to build the structure of immunoglobulin variable region relies upon a database of known immunoglobulin structures and of their structural alignment that is regularly updated. Among the four available criteria for template selection (Same Antibody, same canonical structure, same antibody+canonical structure, best H and L chains), "same antibody" is chosen for template selection that is, selection of structure that can provide a template for both the heavy and light chain as this can provide the best model suggested by the program itself. For side chain modeling, PIGS uses three different strategies (Backbone only, Transfer conserved residues and Transfer conserved plus SCWRL 3.0). We have used Transfer conserved plus SCWRL method in which conformation of the side chains of residues conserved between the target and the template is maintained and the non-conserved residues side chains are modeled using SCWRL 3.0 (Canutescu *et al.*, 2003) as this program uses conserved residues side chains as constraints when modeling remaining side chains. Further energy minimization of the model is performed by using the GROMOS96 (van Gunsteren *et al.*, 1996) force field parameter implemented in Swiss-PDB Viewer (Guex & Peitsch, 1996).

For modeling of M2-Inaba-Fv, the template was selected PDB Code 1PKQ with 3.0 A° resolution which shows 81.98% homology for Light chain and 64.96% for Heavy chain. For modeling of M4-Inaba-Fv, template was selected PDB code 1D5I with 2.0A° resolution which shows 78.1% homology for light chain and 88.98% homology for heavy chain.

2.6.4 Evaluation of the Model

To assess the quality of the structural model of both M2-Inaba Fv and M4-Inaba Fv, coordinates were submitted to online structural bioinformatics toolkit iMOLTALK (Diemand & Scheib, 2004). From this web server, Ramachandran plot of each heavy and light chain of the model is calculated. Further validation of the model is performed using the program ProSa-Web (Wiederstein & Sippl, 2007) at *https://prosa.services.came.sbg.ac.at*. ProSa-Web is a web-based implementation of the widely used PROSA program for the recognition of errors in three-

dimensional structure of proteins. This interactive program displays Z-score and energy plots that highlight potential problems in protein structures. The *Z*-score indicates overall model quality. Its value is displayed in a plot that contains the *Z*-scores of all experimentally determined protein chains in current PDB. In this plot, groups of structures from different sources (X-ray, NMR) are distinguished by different colors that can be used to check whether the *Z*-score of the input structure is within the range of scores typically found for native proteins of similar size.

2.6.5 Prediction of interaction interface on antibody

The program ProMateat *http://bioportal.weizmann.ac.il/promate* is used for prediction of potential interacting amino acids on protein surface(Neuvirth *et al.*, 2004). ProMate calculates binding site based on several biophysical properties such as atom distribution, chemical character, amino acid pair distribution, evolutionary conserved positions, non-regular secondary structure length, sequence distance within a circle, secondary structure, hydrophobic patch rank etc.

2.6.6 Perosamine Ligand preparation and Docking

Structure of perosamine ligand is obtained from the crystal structure PDB ID 1F4X (Villeneuve *et al.*, 2000). PDB coordinate file is then modified by removing terminal 2-O-methyl group that is the characteristic of Inaba-specific Perosamine.

This structure was then computationally docked into the binding site of both M2-Inaba Fv and M4 Inaba Fv using the program ArgusLab 4.0, Planaria Software LLC, Seattle, WA (Thompson, 2004). The ArgusDock docking engine, implemented in ArgusLab4.0, approximates an exhaustive search method, with similarities to DOCK and Glide. The binding site on the protein is defined by using results obtained from Promate and in such a way that most of the residues in CDR including some adjacent framework residues come in contact. Argusdock exhaustive search docking engine was used, with grid resolution of 0.40 Å. Docking precision was set to 'high precision' and 'rigid ligand docking' mode was employed for each docking run.

Visualization of the ligand binding site and the binding site surface was performed by using Accelrys Discovery Studio program version 2.0 *(*http://www.accelrys.com/discovery studio 2.0*)*.

2.6.7 Multiple Sequence alignment

Multiple sequence alignment of the M2-Inaba-Fv, M4-Inaba-Fv and crystal structure 2uyl(Ahmed *et al.*, 2008) was performed using the program ClustalW 2.0(Larkin *et al.*, 2007).

2.7 Expected Benefits

Studies of the immunochemical and physicochemical characteristics of monoclonal antibodies were carried out with the expectation to:

- determine a new antigenic determinant specific to the Inaba serotype of *V.cholerae* O1 LPS
- elucidate the structure of this putative Inaba-specific antigenic determinant
- provide a rational basis for the development of a glycoconjugate vaccine directed specifically against the Inaba serotype.
- re-evaluate cholera immunogens using different routes of immunization toselect as any of them individually or in combination (as prime boost concept) to be screened as potential vaccine candidate
- assess the impact of zinc on cholera immunity and vaccine research

3 Results

Studies on the immunochemical and physicochemical characteristics of monoclonal antibodies have opened the way to the description of new antigenic determinants. Characterization of the antigenic determinants expressed by the Inaba serotype of *V. cholerae* O1 is of particular importance to address these issues concerning the nature and origin of protective antibodies against cholera. Monoclonal and polyclonal antibodies of the IgM, IgG1 and IgG3 isotypes specific against lipopolysaccharide (LPS) of *V. cholerae* O1 Ogawa and Inaba serotypes were produced in mice and rabbit, at the Institut Pasteur, Paris, France and Immunology Laboratory, icddr,b, Dhaka, Bangladesh as well as NICED, Kolkata, India. These antibodies were screened to identify the parameters measured *in vitro* (agglutinating activity, vibriocidal activity, ELISA titers) with the ascites in the preliminary study. Assays were carried out to determine*V. cholerae* O1Ogawa and Inaba LPS-specific antibody titers, agglutination and vibriocidal titers against *V. cholerae* O1 Inaba (strain CNRVC 950707) and *V. cholerae* O1 Ogawa (CNRVC 960250) (Table 4). As it was of special interest to select the best clone from the existing monoclonals (mAbs) positive for both Inaba and Ogawa, they were further tested for specificity in ELISA with heat-killed whole cell (WC) and acetone-treated extract (ATE) of *V. cholerae* O1 strain X-25049 for Ogawa and strain 19479 for Inaba (Table 5). All the clones showed specificity to either Ogawa or both Inaba and Ogawa, of which clone F-22-30 was chosen for its optimal response to all the parameters assayed *in vitro*. This F-22-30 clone was produced in mice using a series of conjugates of the polysaccharide moiety of the lipopolysaccharide of *V. cholerae* O1, serotype Inaba which recognizes the common antigenic determinants of Ogawa and Inaba LPS and is able to facilitate purification and maximum concentration becoming IgG1 isotype which is a prerequisite to the Fab preparation and X-ray crystallography. This F-22-30 (IgG1,κ) clone was further assessed for other immunological parameters to determine the inhibition properties as well as the *in vivo* protection assay in suckling mice model. All these both *in vitro* and *in vivo* immunological parameters were also assayed after purification of the monoclonal antibody from ascites and after final preparation of Fab from purified antibody through a series of column chromatography of the clone F-22-30 (Table 6).

Table 4: Characterization of different monoclonal and polyclonal antibodies produced in mice and Rabbit

mAb ID and Ig Type	Ig Isotype	Specificity	Agglutination Titer Against		Vibriocidal Titer Against		Initial Dilution (ELISA)	ELISA Titer at 0.5 Optical Density Against	
			In	Og	In	Og		LPS In	LPS Og
ICL-18[a]	IgG3	In+Og	½	1/160	20	20	1:20	UD	$1.1X10^3$
ICL-28[a]	IgG3	In+Og	-	-	20	20	1:20	UD	UD
ICL-17[a]	IgG1	Og	-	1/320	-	320	1:20	UD	$1.1X10^4$
ICL-26[a]	IgG1	Og	-	1/2	-	20	1:20	UD	UD
ICL-29 (Lot –95)[a]	IgG3	Og	-	1/40	-	40	1:100	UD	UD
ICL-29 (Lot – 990919)[a]	IgG3	Og	-	Neat	-	20	1:20	UD	UD
1G12[b]	IgG3	In	1/20	-	20	-	1:10	UD	UD
S-20-4[c]	IgG1	Og	1/160	-	-	1280	ND	ND	ND
I-24-2[c]	IgG3	In+Og	1/80	1/80	20	20	1:100	$1X10^4$	$2X10^4$
F-22-30[c]	IgG1	In+Og	1/3200	1/3200	20480	20480	1:100	$1X10^5$	$9X10^4$
Rabbit Sera[c]	IgG	In+Og	1/5	1/2	80	160	ND	ND	ND

In-Inaba, Og-Ogawa, UD-Undetected, ND-Not Done

[a]Monoclonal antibodies produced at ICDDR, B, Bangladesh
[b]Monoclonal antibody produced at NICED, India
[c]Monoclonal and Polyclonal antibodies produced at Institut Pasteur, France

Table 5: Monoclonal antibody (mAb) titers obtained with killed whole cell (WC) and acetone-treated extract (ATE) prepared with Ogawa strain X-25049 and Inaba strain 19479

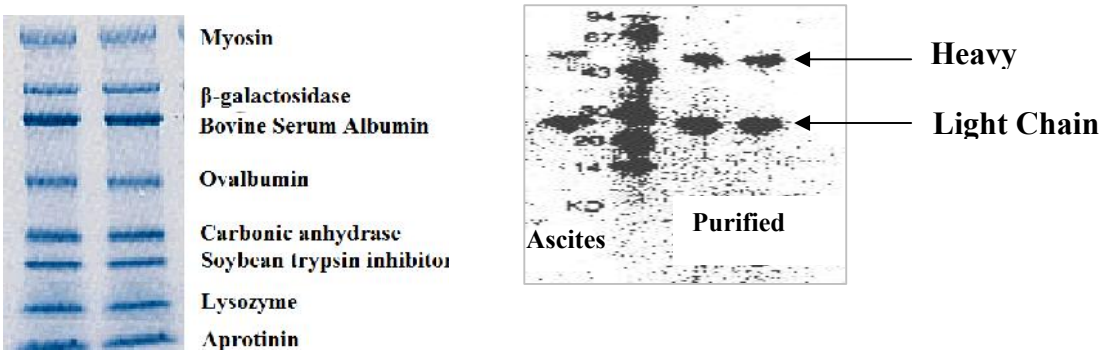
mAb ID	Results Against Serotypes	ELISA Titer Against WC 1/100 Dilution (at O.D 0.5)	ELISA Titer Against WC 1/1000 Dilution (at O.D 0.5)	ELISA Titer Against ATE (at O.D 0.5)
F-22-30	Inaba	$1.4X10^4$	$4.0X10^4$	$2.3X10^4$
	Ogawa	$2.0X10^4$	$5.0X10^4$	$8.0X10^3$
1G12	Inaba	Undetected	Undetected	$1.5X10^1$
	Ogawa	Undetected	Undetected	Undetected
ICL-17	Inaba	Undetected	Undetected	Undetected
	Ogawa	$8.0X10^2$	$1.5X10^3$	$5.0X10^2$

Table 6: *In vitro* and *In vivo* immunological parameters observed in ascites, purified antibody and Fab of the F-22-30 clone

Immunological Assay	Results Against Serotypes	Ascites	Purified Antibody	Fab
Agglutination	Inaba	1/3200	6.25 µg/ml	6.25 µg/ml
	Ogawa	1/3200	6.25 µg/ml	6.25 µg/ml
Vibriocidal Assay	Inaba	25600	1.56 µg/ml	Not Done
	Ogawa	12800	1.56 µg/ml	Not Done
Specific ELISA	Inaba	$1X10^5$	50-65 ng/ml	6.0 µg/ml
	Ogawa	$9X10^4$	60 ng/ml	3.0 µg/ml
Inhibition ELISA	Inaba	25.0 µg/ml	40 µg/ml	Not Done
	Ogawa	100.0 µg/ml	<100.0 µg/ml	Not Done
Protection Assay	Inaba	Protective	12.5 µg/ml	Not Done
	Ogawa	Protective	12.5 µg/ml	Not Done

The mAb F-22-30 (IgG1, κ) was purified from the ascitic fluid by protein-G-agarose affinity chromatography, concentrated up to 10.0 mg/ml. The level of purity and uniformity of the IgG1, heavy chain (50,000 daltons) and κ, light chain (25,000 daltons) were assessed by SDS-PAGE using electrophast gel electrophoresis (Fig. 7). The Fab fragment was prepared from the purified antibody by cleavage using the enzyme, papain, in its most favorable reaction condition. It was optimized through gel filtration column by computer based GILSON method overseeing the preparative digestions at different time points, t-0 hr, t-1 hr, t-2 hr and t-4 hr respectively (Fig. 8). The Fab-papain mixture after completion of incubation and after bringing to a stable condition by stopping the reaction was introduced in the chromatographic Fab purification protocol. The mixture was dialyzed against 40 mM potassium phosphate buffer, pH 8.0 and passed through DEAE-trisacryl ion-exchange column with the same buffer followed by re-saturation with 0.5 M potassium phosphate buffer as an optimized protocol and the eluent peaks were collected in tubes using a fraction collector (Fig. 9). The eluent corresponding to Fab was confirmed by ELISA and concentrated and dialyzed simultaneously against 40 mM Tris 0.2 M NaCl buffer, pH 7.6 and passed through a Superdex^{TM} column for gel filtration using the same buffer to get purified Fab free of impurities (Fig. 10). The eluents corresponding Fab peaks were collected, dialyzed and concentrated in 0.04 M Tris buffer, pH7.6 to purified Fab. Although papain digestion conditions

were optimized to produce as few Fab specifies as possible, for the clone F-22-30, at least four major species were always produced differing in their isoelectric points showing heterogeneity of Fab populations (Fig. 11). The level of heterogeneity of the purified Fab was also confirmed by mono QTM 5/50 GL ion-exchange chromatography at pH 7.6 (Fig. 12) and in the purified Fab Mass (m/z) calculation during Mass Spectroscopy (Fig. 13). The purified Fab preparation containing heterogeneous species of population were eluted through mono QTM 5/50 GL column to carry out ion-exchange chromatography with a gradient of NaCl (Fig. 14 and 15). Different fractions of Fab species were collected, dialyzed and concentrated in 0.04 M Tris buffer, pH 7.6 and assessed for functional activity by ELISA and immunoagglutination (Table 6) which indicated that peaks 3-8 could be chosen for further assays. These peaks were assayed again for their isoelectric points which showed homogeneity of peak 3 and peak 6 (Fig. 16) and was used to go forward with crystallography experiments.



Prestained SDS-PAGE Standards were used to assess the efficiency of electrophoretic transfer on Western Blots. Broad range prestained SDS-PAGE standards; 5 µl, were run on 4-20% Ready Gel precast gel and transferred to nitrocellulose in the Mini Trans-Biot cell.

Calibrated molecular weight of prestained SDS-PAGE Standards

Protein	High Range	Low Range	Broad Range
Myosin	204,000	-	209,000
β-galactosidase	123,000	-	124,000
Phosphorylase b	-	103,000	-
BSA	80,000	77,000	80,000
Ovalbumin	48,000	50,000	49,100
Carbonic anhydrase	-	34,300	34,800
Soybean trypsin inhibitor	-	28,800	28,900
Lysozyme	-	20,700	20,600
Aprotinin	-	-	7,100

Figure 7: Purity and uniformity of the IgG1, heavy chain and κ, light chain assessed by SDS-PAGE using Electrophast gel electrophoresis after purification from ascitic fluid of F-22-30 clone by protein-G-agarose affinity chromatography.

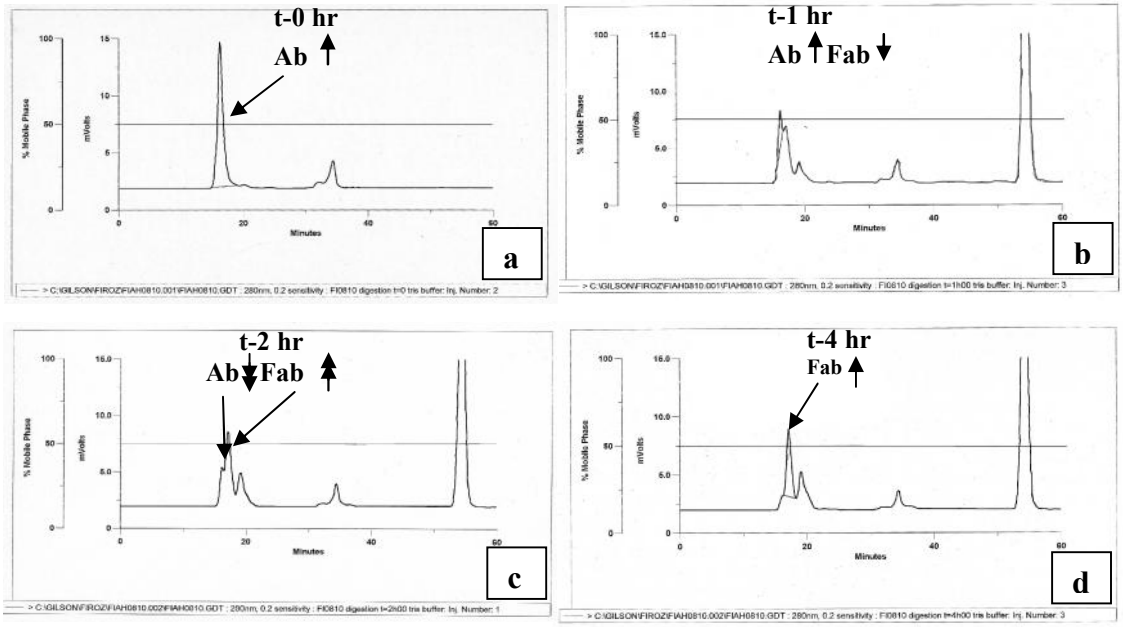


Figure 8: Papain cleavage monitoring and optimization by gel filtration during Fab preparation from purified antibody of the clone F-22-30. a. indicates no cleavage of Ab,b. initiation of cleavage, c. continuation of cleavage and d. complete cleavage of Ab to Fab.

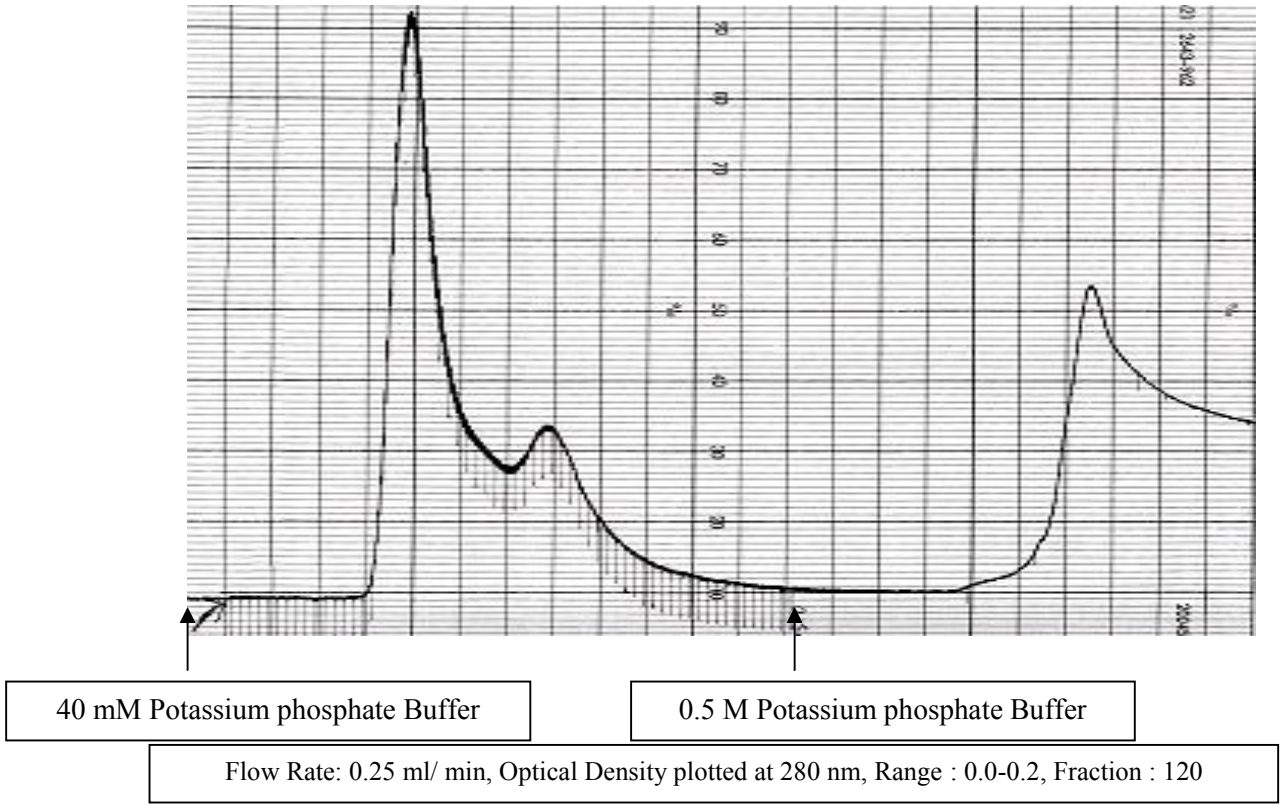


Figure 9: Purification of Fab-papain mixture by DEAE-trisacryl ion-exchange chromatography.

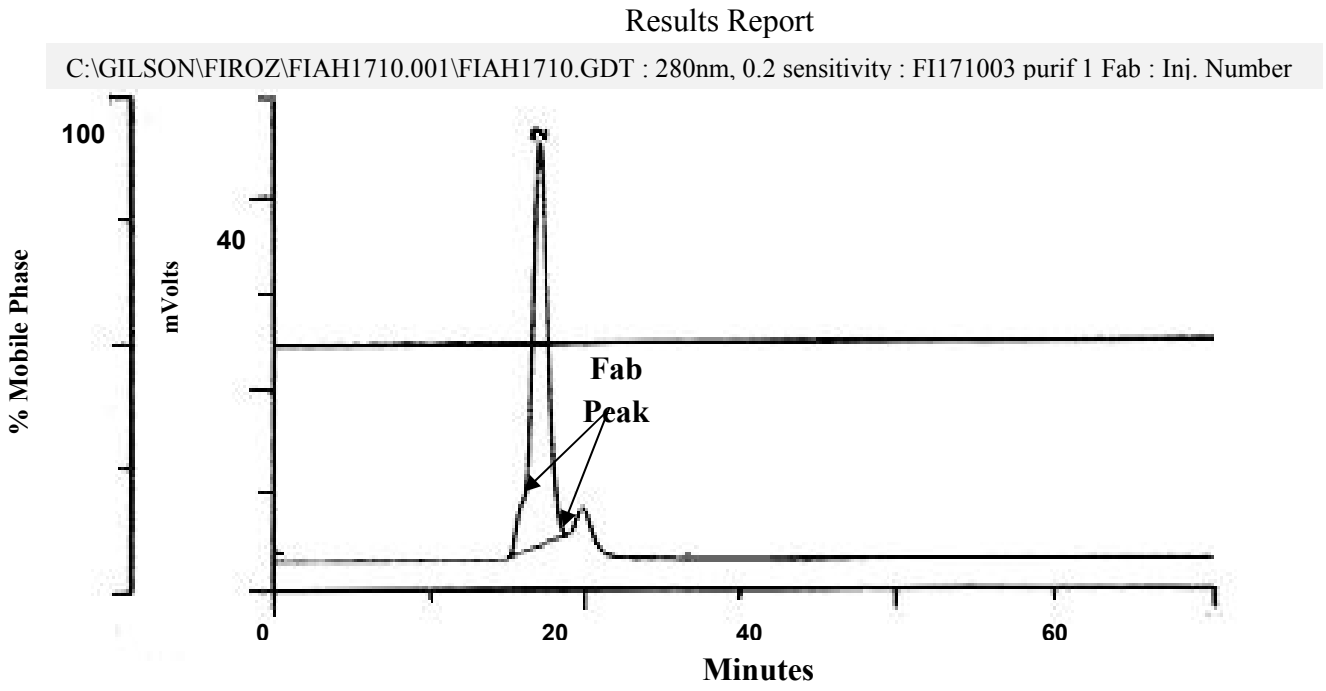


Figure 10: Purification of Fab by SuperdexTM G-75 gel filtration.

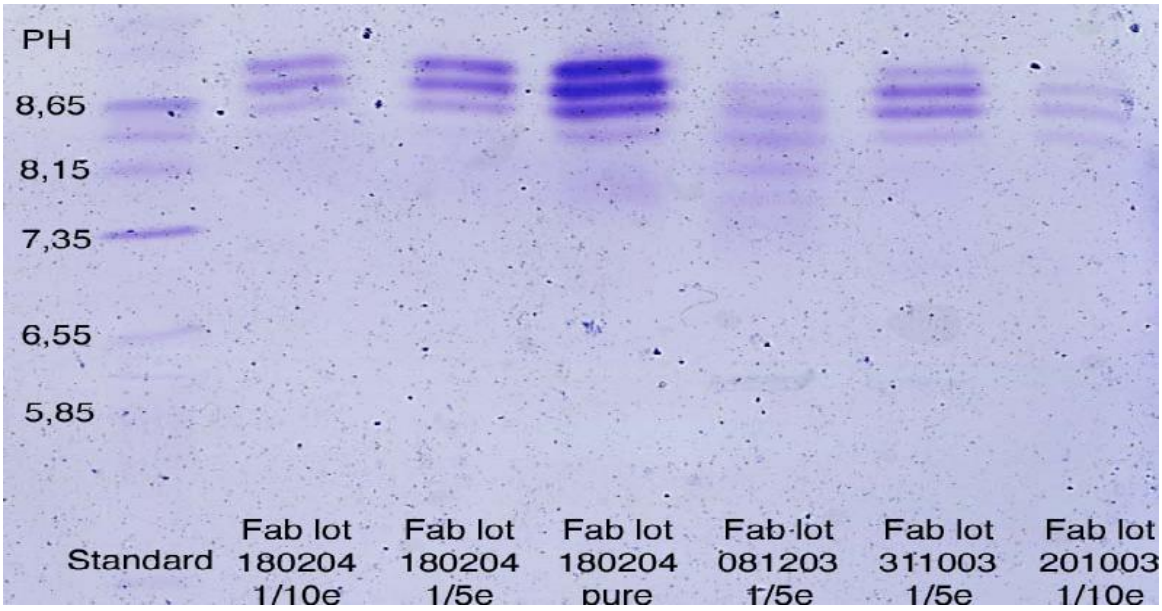


Figure 11: Determination of pH gradient profile and heterogeneity of Fab populations.

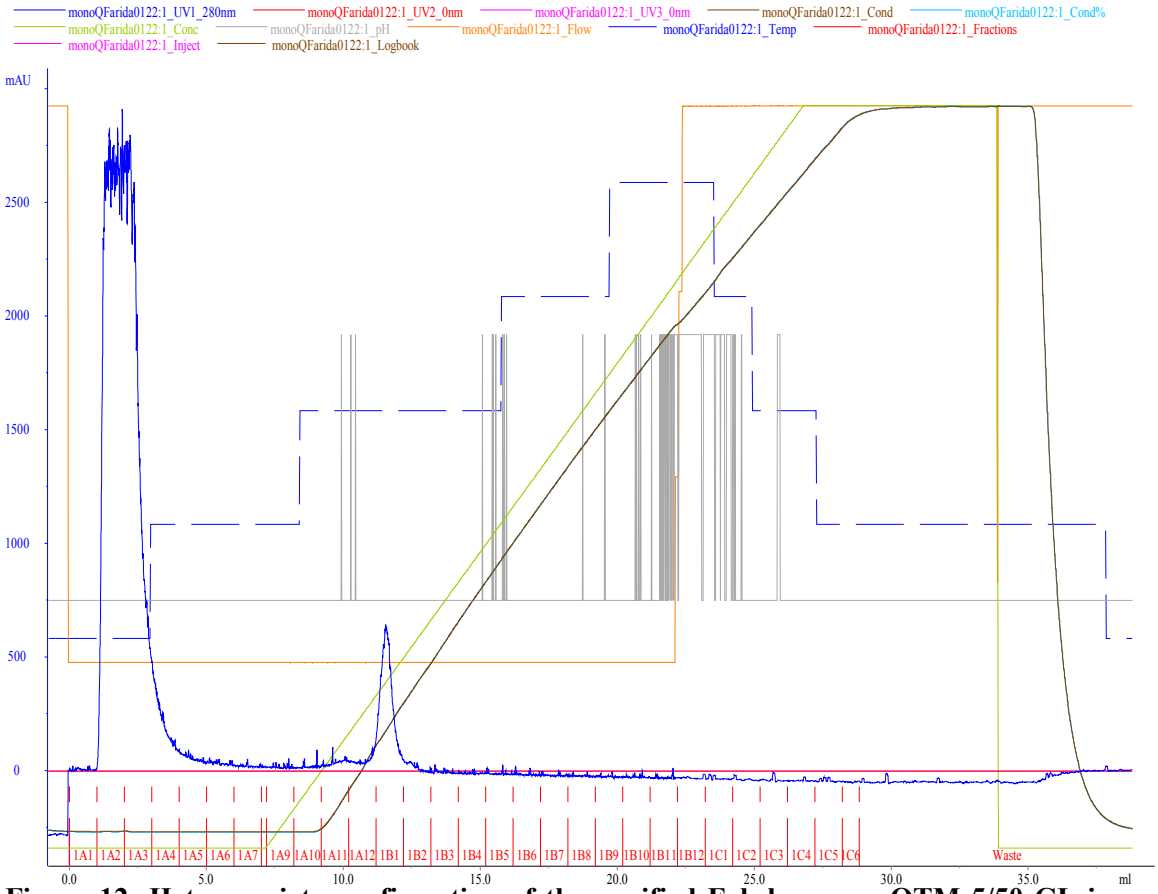


Figure 12: Heterogeniety confirmation of the purified Fab by mono QTM 5/50 GL ion-exchange chromatography at pH 7.6.

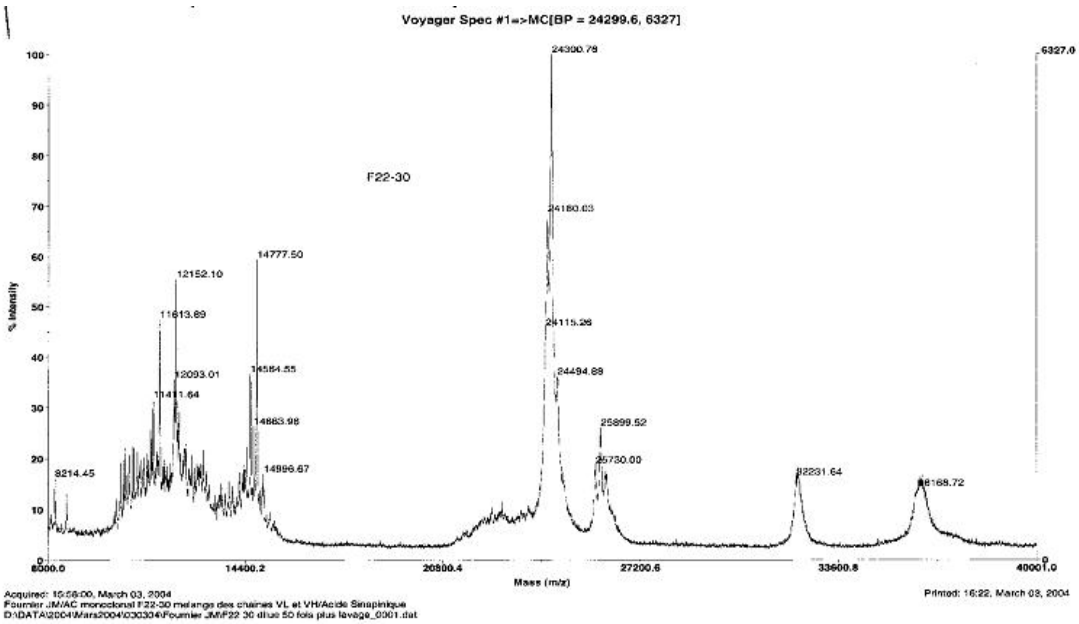


Figure 13: Heterogeneity observed in the purified Fab mass (m/z) calculation during Mass Spectroscopy.

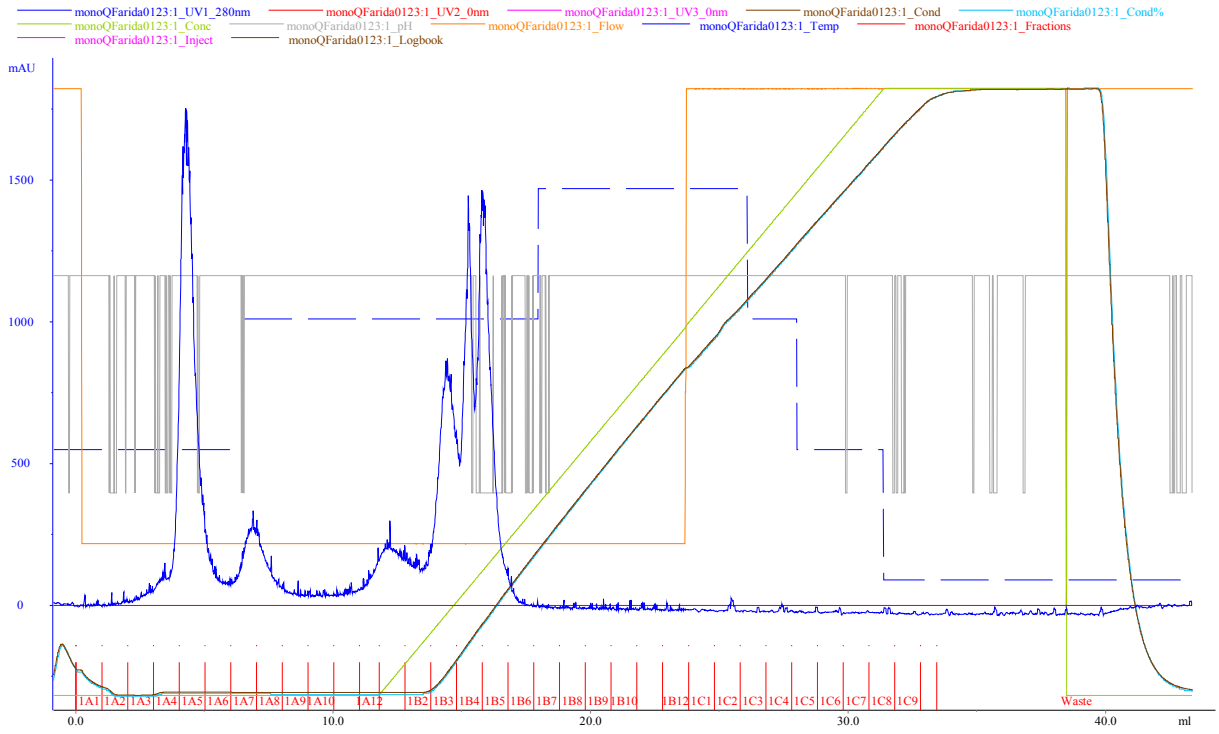


Figure 14: Purification of Fab by mono QTM 5/50 GL ion-exchange chromatography at pH 8.5.

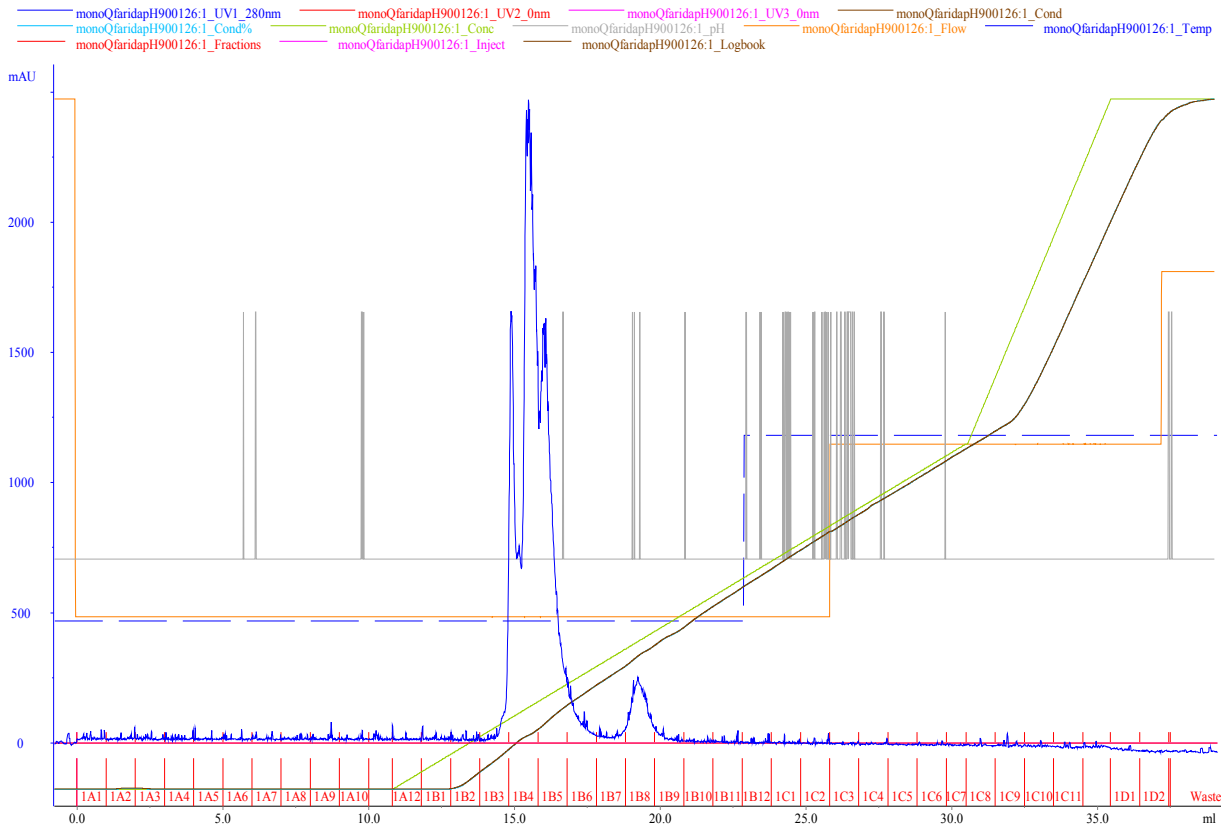


Figure 15: Purification of Fab by mono QTM 5/50 GL ion-exchange chromatography atpH 9.0.

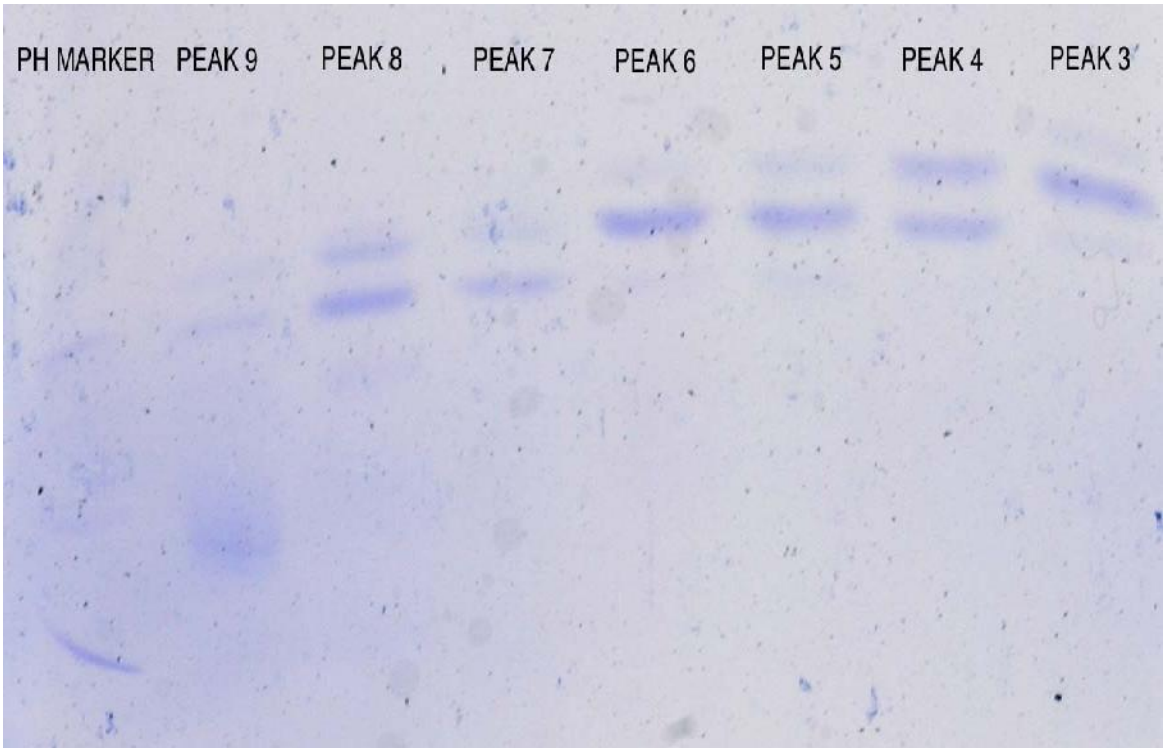


Figure 16: Determination of pH gradient profile and homogeneity of Fab populations.

The crystal structure of the Fab fragment from monoclonal antibody F-22-30 directed against *V. cholerae* O1-LPS was determined at 2.5 Å resolution (Fig. 17a) and refined to a final crystallographic R-factor (R-free) of 0.211 (0.257) (Table 7). The four independent molecules in the unit cell werevery similar to each other, with rmsd values of 0.1-0.2 Å. The model displays a good stereochemistry, with a single protein residue (ser85H) falling out of the allowed regions in the Ramachandran Plot(Emsley *et al.*, 2010). However, this residue had the same unfavorable conformation and a well-defined electron density in all four independent Fab molecules, suggesting that this is a genuine feature of the structure.

The antigen-binding site displays a rather shallow surface, with two bulges corresponding to the tips of the L1 and H2 hyper variable loops and a small deep cavity primarily defined by residues from the L1, L3 and H3 hyper variable loops (Fig. 17b). This putative binding pocket, delimited by the aromatic face of Tyr37L, the main-chain residues of both L3 and H3, and the side chains of salt-bridged residues Arg101L and Glu99H, appears to be occupied by an unidentified molecule in the crystal (Fig. 17c). The size and shape of the pocket suggests that it could accommodate a single (terminal) monosaccharide from the O1-LPS. The core-PS contains four

such putative epitopes, namely α-glucose, α-glucosamine, α-heptose and β-fructose(Chatterjee & Chaudhuri, 2003; Vinogradov *et al.*, 1995). Another possible ligand for this pocket could be the side-chain (3-deoxy-L-*glycero*-tetronic acid) that acylates the amino group of perosaminyl residues from the O-PS (Table 8).

Preliminary molecular modeling calculations were carried out for these five putative ligands, as described in materials and methods section. The results suggest that all five molecules could occupy the observed pocket, in some cases with the sugar ring making stacking interactions with Tyr37L. In all predicted models, a few protein residues (His31L, Arg101L, His50H, Glu99H and Thr101H) could be involved in protein-ligand hydrogen bonding interactions. As shown in Table 5, the best binding affinity was predicted for the D-perosamine side-chain. This ligand was completely anchored in the binding pocket, where it could interact with the aromatic face of Tyr37L and form several hydrogen bonding interactions with residues from the light and heavy chain of the antibody (Fig. 18). Interestingly, the perosamine side-chain was also the only compound predicted to form a hydrogen bond with Glu39L, which is located at the bottom of the cavity (Fig. 17c) and cannot interact with the other ligands.

NMR experiments provided some additional support for the model predictions. Indeed as shown in Fig. 19, the perosamine resonances are clearly observed in the STD-NMR spectra obtained with on-resonance irradiations of antibody resonances either in the methyle proton region (0.5 ppm;Fig. 19b) or in the aromatic proton region (7 ppm;Fig. 19c). It was interesting to note that protons from both the ring and the side-chain (2.05 ppm and 1.87 ppm), although much weaker, were observed, suggesting in agreement with the molecular modelisation that the whole perosamine is in contact with the antibody. The unfavorable ratio of perosamine units to core units precludes deeper analysis of the observed peaks in the STD-NMR spectra and cannot exclude interaction with the core residues.

Table 7:Data Collection and Refinement Statistics

Data collection	
Data resolution (Å) *	45 – 2.5
	(2.64 – 2.5)
Measured reflections *	127748 (18592)
Unique reflections *	64354 (9355)
Completeness (%) *	97.5 (97.1)
R_{sym} (%) *†	0.046 (0.226)
<I/σ> *	17.6 (5.7)
Refinement	
R_{cryst} ‡‡ [N° refs]	0.211 [57803]
R_{free} ‡‡ [N° refs]	0.256 [3275]
Rms bonds (Å)	0.021
Rms angles (degrees)	1.978
Protein atoms	13125
Overall B factor (Å^2)	23.4

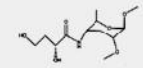
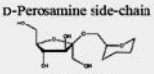
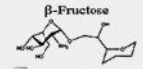
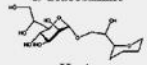
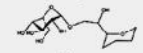
* Values in parentheses apply to the high resolution shell.

† $R_{sym} = \sum_{hkl}\sum_{i}|I(hkl) - \langle I(hkl)\rangle| \Big/ \sum_{hkl}\sum_{i} I(hkl)$

‡ Anomalous phasing power = $\langle|Fh(calc)|\rangle$phase integrated lack of closure.

‡‡ $R = \sum_{hkl}|F(h)obs - F(h)calc| \Big/ \sum_{hkl}|F(h)obs|$. Rcryst and Rfree were calculated from the working and test reflection sets, respectively.

Table 8: Total energies of Fab-ligand complexes (referred to that of the Fab-perosamine complex)

Ligand	Total energy of the complex (kcal/mol)	Contribution of light / heavy chains (%)
 D-Perosamine side-chain	0	51 / 49
 β-Fructose	11.2	37 / 63
 α-Glucosamine	23.0	27 / 73
 α-Heptose	25.0	53 / 47
 α-Glucose	63.4	36 / 64

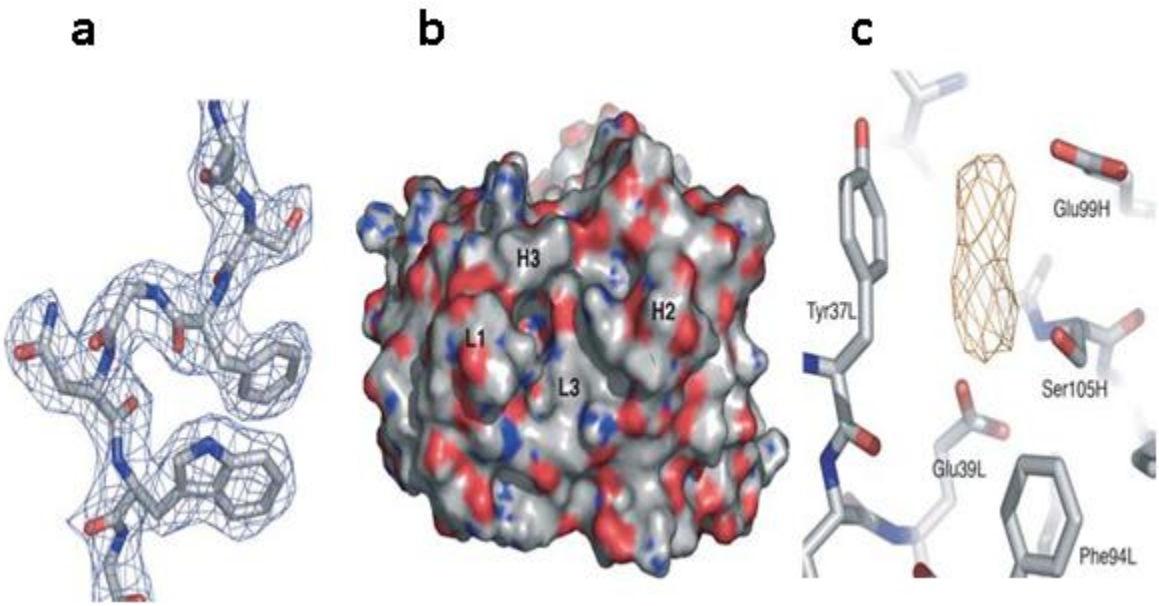


Figure 17: Crystal structure of Fab F-22-30. a. Final electron density map (contoured at 1.2 σ) of a segment from the H3 hypervariable loop (Ser105H-Trp109H). b. Molecular surface of the antibody recombination site. The positions of some hypervariable loops are indicated. Note a deep pocket delimited by the L1, L3 and H3 hypervariable loops. c. A difference fourier (Fo-Fc) map contoured at 3σ suggests that an unidentified molecule binds to this pocket in the crystal.

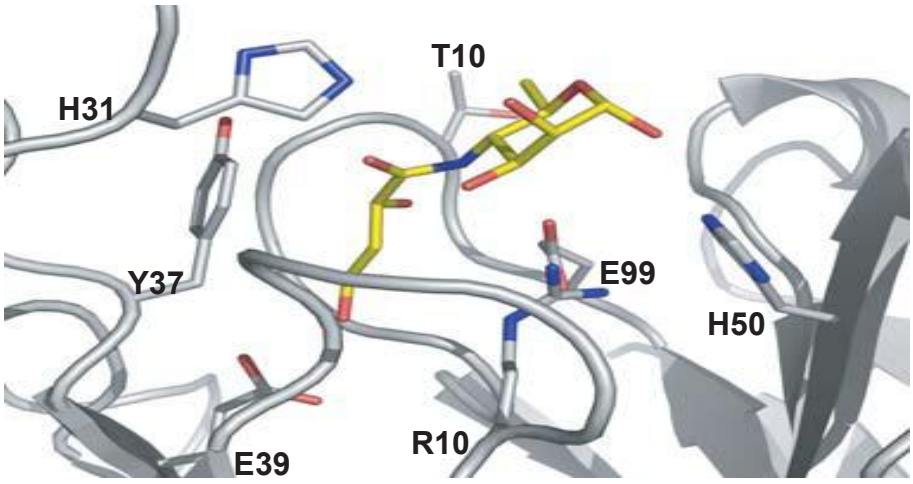


Figure 18: Putative model of 3-deoxy-l-glycero-tetronic acid docked into the antigen-binding site. Amino acid residues that could participate in protein-ligand H-bonds are indicated. The direction of the O-PS homopolymer would be roughly perpendicular to the plane of the Figure.

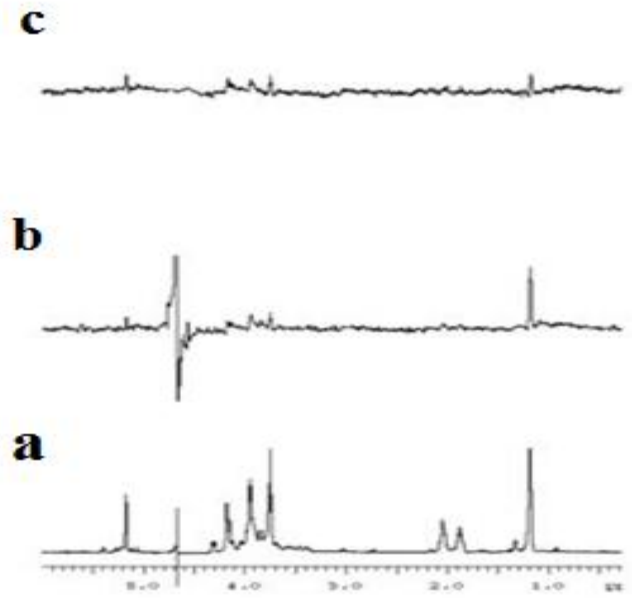


Figure 19: 1D STD-NMR of*V.cholerae* Inaba LPS to IgG at a 50 to 1 LPS to IgG ratio binding site. (a) 1D reference spectrum acquired with a saturation pulse applied at 20 ppm (32 scans; phosphate buffer 50 mM; pH 6.5; 35 °c; 500 MHz), (b) 1D STD spectrum with on-resonance irradiation of IgG protons at 0.5 ppm and off-resonance irradiation at 20 ppm (8192 scans; phosphate buffer 50 mM; pH 6.5; 35 °c; 500 MHz) (c) 1D STD spectrum with on-resonance irradiation of IgG protons at 7 ppm and off-resonance irradiation at 20 ppm (12 000 scans; phosphate buffer 50 mM; pH 6.5; 35 °c; 500 MHz).

3.1 Antibody response to different immunogens of *V. cholerae* after Transcutaneous Immunization (TCI),Subcutaneous Immunization (SCI), and Oral Immunization (ORI).

As described in the Method and Materials section, transcutaneous immunization (TCI) was compared with subcutaneous immunization (SCI) and oral immunization (ORI) routes to assess the antibody responses with functional properties specific to the probable vaccine candidate immunogens, commercially available cholera toxin B subunit (CTB), cholera toxin (CT), major subunit toxin co-regulated pilus antigen(TcpA) while LPS O1 Inaba and Ogawa as well as membrane extract which was extracted using an in-house protocol described in the Methods section. All immunizations and experimental protocols were prepared, optimized and followed through standard guidelines of published references. All three immunizations and further immunological experiments incorporating serological, cellular and molecular methods required

to assess immunogen potentials were initially carried out with protein immunogens s CTB, CT and TcpA either through individual application or co-administration of two immunogens due to its capacity to generate early antibody response when mice were given normal control feed (CF).

3.1.1 Anti-CT and anti-TcpA antibody responses.

The CT specific responses increased in all three isotypes; IgM, IgG and IgA in mice that received immunogen via the transcutaneous immunization route with TcpA plus CT (Fig. 20A). The mice developed a prominent anti-CT response in the IgM, IgG and IgA isotypes following the first dose of immunization (P= 0.002) which remained elevated until 7 days after the last immunization (P= 0.010). The same pattern was observed for mice in the SCIG, which developed prominent anti-CT specific IgM, IgG and IgA responses (P= 0.002) (Fig. 20B). In the mice given oral immunization (Fig. 20C), the magnitude of response were comparable to those in the TCIG and SCIG routes. After the final immunization, responses were seen in all three antibody isotypes. Higher magnitude of CT responses were seen in SCIG than in the TCIG or the ORIG in the IgM (P= 0.024-0.036), IgG (P= 0.017-0.048) and IgA (P= 0.024-0.036) isotypes.

As noted the TCI route was comparable to the SCI route and could perform better than ORI through initial experimentations.Stepwise analysis of antibody isotypes and their magnitude with multiple doses were explored which was further assessed with the variations of feed supplementation. When analyzed CT specific IgM, IgG and IgA responses in sera of the CTB only and TcpA-CT immunized mice after the final immunization on day 56 and sacrificed on day 63, no significant difference in the magnitude of responses were observed between the CT and CTB specific IgM and IgG (P= ns) but IgA showed significant difference (P= 0.040) with a mean magnitude of 30.4 and 74.0 for CTB and TcpA-CT immunized CF mice (Table 9). In contrast, co-administration and both Zn supplementation and depletion impacted differently than Control Feed (CF) mice. TcpA-CT co-administered ZnP and ZnM TCIG IgM and IgA isotypes were seen with significant mean increase (P= 0.001; ZnM IgM, 0.045; ZnP IgM and P= >0.001; ZnM IgA, 0.003; ZnP IgA) than CTB only immunized mice of the same feed supplemented group. Although CTB CF IgM and IgA antibodies displayed higher magnitude than ZnP and ZnP group, TcpA-CT ZnP IgM titer was more elevated than ZnM and CF groups while IgA and IgG titers were comparable (Table 9).

Transcutaneous immunization of mice resulted in significant anti-TcpA responses in blood in the IgM and IgG but not in the IgA isotype (Fig. 20D). The group showed significant increases in IgM (P= 0.002) and IgG responses (P= 0.026) following the second dose, which peaked after the fifth immunization. The TcpA specific IgM responses in the SCIG significantly increased (Fig. 20E) following the first dose of immunization (P= 0.038) with further increases with corresponding doses (P= 0.002). A similar trend was observed in the IgG isotype with a significant elevation following the second dose of immunization (P= 0.026) which showed the highest peak after the fifth dose (P= 0.002). In case of ORIG (Fig. 20F), responses in both the IgM (P= 0.029) and IgG (P= 0.05) isotypes increased following the first dose of immunization no further increases were seen after intake of the fifth dose (Fig. 20B-20C). Responses although lower in magnitude were seen in the IgA isotype to TcpA in the different groups studied (Fig. 20D-20F), however, higher than seen in the control, CTRG mice (no response seen to TcpA; data not shown).

Unlike CT specific IgM, IgG and IgA responses, no significant difference was found between CTB only and TcpA-CT immunized mice after final immunization with the fifth dose. In addition, changes in the feed supplementation did not impact in the TcpA and TcpA-CT immunized groups (Table 9).

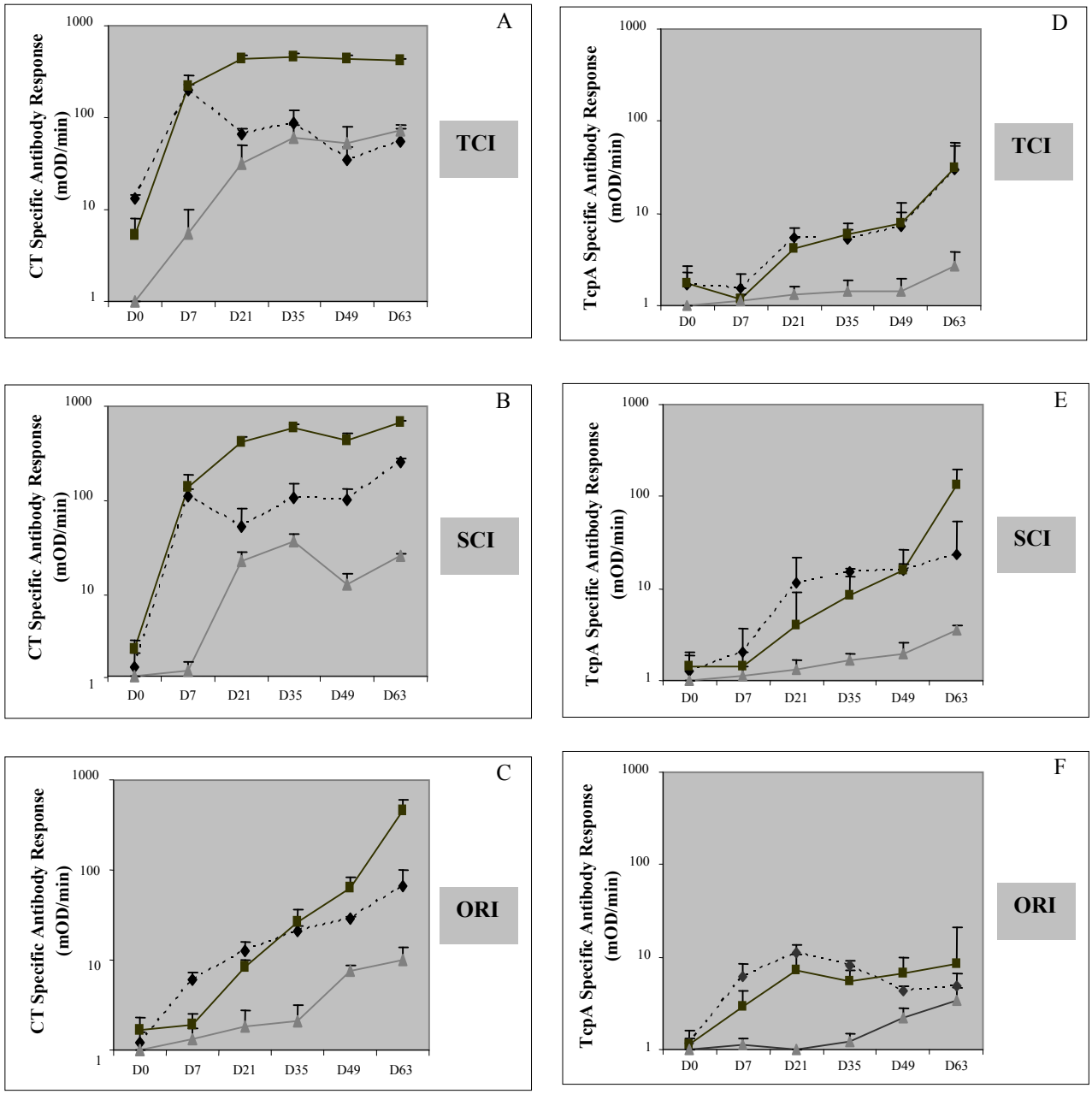


Figure 20: TcpA and CT specific of antibody response with gradual increase shown in IgM (-◆-), IgG (—■—) and IgA (—▲—) isotypes in mice after immunization on days: 0, 14, 28, 42 and 56, ELISA was carried out on days: 0, 7, 21, 35, 49 and 63 respectively.

Table9: CT and TcpA specific antibody titers in sera of the CTB and TcpA only and TcpA-CT immunized mice with different feed supplementations

Mice Immunized with	Feed Status	Serum Antibody Titer (mAb/Min) against CTB			Mice Immunized with	Feed Status	Serum Antibody Titer (mAb/Min) against TcpA		
		IgM	IgA	IgG			IgM	IgA	IgG
CTB	ZnP	44.2	15.2	425.5	TcpA	ZnP	17.8	0.0	87.1
	ZnM	18.8	11.7	336.4		ZnM	30.7	1.0	112.5
	CF	58.9	30.4	438.4		CF	23.4	0.5	99.0
TcpA-CT	ZnP	108.5	66.2	486.3	TcpA-CT	ZnP	19.0	2.1	122.8
	ZnM	66.3	69.7	503.4		ZnM	24.6	2.5	182.0
	CF	55.9	74.0	416.0		CF	14.5	0.5	107.3

3.1.2 LPS-specific Inaba and Ogawa antibody responses

Mice when transcutaneously immunized with LPS Inaba and assessed for Inaba specific antibody responses (Figure 21), IgM titer, in contrast, significantly elevated following the first and second dose of immunization (P= 0.026) but remained as plateau with no rise in magnitude after the next three doses of immunization. IgG isotype demonstrated significant rise in titer after the first dose (P= 0.003), remained almost unchanged after the second dose and further increased with the corresponding doses (P= 0.001). IgA isotype, on the other hand, showed peak slightly after the fifth dose of immunization (Figure 21A).Whenmice were immunized withLPS Inaba through transcutaneous route and assessed for antibody responses, Ogawa specific IgM and IgG antibodies showed an increase (P= 0.045, 0.05) after the second dose of immunization which slightly decreased after the third dose but remained elevated significantly after the fourth (P=0.003, 0.02) and fifth doses (P= 0.001, 0.001) although IgM showed a higher magnitude than IgG isotype. IgA isotype, on the other hand, showed peak slightly above the baseline after the third dose which remained stable after the fourth dose but decreased to the baseline after the fifth dose of immunization (Figure 21B).

TCI mice immunized with membrane extract displayed robust Inaba specific immune response with elevation of IgG isotype magnitude over IgM and IgA isotypes with the increase of the

number of immunization doses (Figure 21). Inaba specific IgM were significantly increased following the first dose of immunization (P= 0.010) and further increased up-to the fourth dose but slightly decreased in magnitude after the fifth dose. IgG isotype significant increased after the second (P= 0.010) and third (P= 0.003) dose of immunization, remained unchanged after the fourth dose and again displayed elevation in titer after the fifth dose which was higher in magnitude than IgM titer after the final dose (P= 0.041). Inaba specific IgA isotype elevated after the fourth and continued up-to the final fifth dose of immunization (Figure 21C). LPS specific Ogawa IgM antibody after the TCI immunization withthe membrane extract showed significant peak (P= 0.023) on day 7 after the first dose and continued elevation up-to third dose (P= 0.001) while did not increase further after the last two doses on day 42 and 56 (Fig). LPS Ogawa specific IgG responses in the TCIG immunized with membrane extract significantly increased (Fig. 2B) following the first dose of immunization (P= 0.038) with further increases with corresponding doses (P= 0.002). IgA specific responses however did not increase in the first four doses but slightly elevated after the fifth dose (Figure 21D).

When compared with the feed supplemented CF, ZnM and ZnP TCIG group IgM, IgG and IgA responses, Inaba specific CF, ZnM and ZnP membrane extract immunized mice, the IgM and IgG titers were significantly higher compared to the LPS Inaba responses (Table 10). Although for both LPS Inaba and membrane extract immunized Inaba, the ZnM IgM showed the lowest titers; ZnM and CF IgM titers were comparable (P= ns) but ZnP IgM increased significantly than ZnM IgM (P= 0.034, >0.001) for LPS Inaba and membrane extract immunized mice respectively. In addition, LPS Inaba immunized IgG titer was very low compared to the membrane extract immunized Inaba specific IgG (P= >0.001). Both LPS Inaba and membrane extract immunized three different feed supplemented IgG responses within each group were comparable (P= ns). When compared LPS Ogawa specific IgM, IgG and IgA responses between LPS Inaba and membrane extract immunized CF, ZnM and ZnP TCIG mice, no significant difference was observed in the IgM isotype magnitudes although membrane extract immunized ZnP IgM showed the highest peak. LPS Ogawa specific membrane extract immunized IgG titers of the CF, ZnM and ZnP TCIG displayed significant rise (0.002- >0.001) than LPS Inaba immunized CF, ZnM and ZnP IgG titers and were comparable (P= ns) within each group. IgA titers in both groups were insignificant.

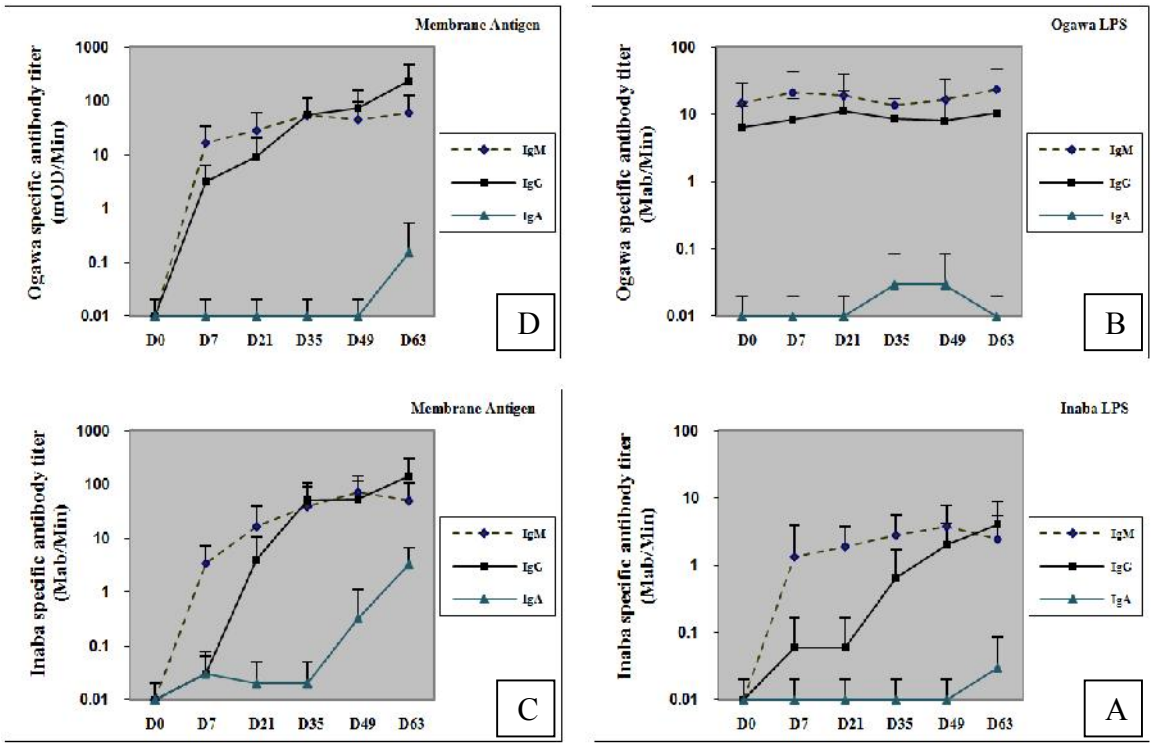


Figure 21: 1. Inaba and Ogawa LPS specific antibody responses with gradual increase shown in IgM (-◆-), IgG (—■—) and IgA (—▲—) isotypes in mice after immunization on days; 0, 14, 28, 42 and 56, ELISA was carried out with on days; 0, 7, 21, 35, 49 and 63 respectively.

Table 10: LPS Inaba and Ogawa specific antibody titers in sera of the membrane extract immunized mice with different feed supplementation

Mice Immunized with	Feed Status	Serum Antibody Titer (mAb/Min) against						Vibriocidal Antibody Titer against	
		LPS O1 Inaba			LPS O1 Ogawa				
		IgM	IgA	IgG	IgM	IgA	IgG	Inaba	Ogawa
LPS Inaba	ZnP	45.0	3.5	9.0	95.0	1.5	59.0	320	1280
	ZnM	17.0	1.5	4.0	55.0	1.0	63.0	40	320
	CF	30.0	2.0	5.5	71.0	0.6	51.0	80	320
Membrane Extract	ZnP	166.5	5.0	254.5	145.0	2.0	177.5	10240	10240
	ZnM	45.5	0.5	235.5	104.0	1.0	234.5	5120	5120
	CF	90.5	4.5	214.0	61.0	0.5	234.0	5120	5120

3.2 Comparison of TcpA, CT and LPS Inaba as well as Ogawa specific immune responses in the IgG subclass.

In the TCIG CF, the TcpA specific IgG1 responses were elevated. Poor responses were seen in other 3 subclasses (Figure22A). An IgG1 based response was seen in the SCIG and ORIG cohorts of mice. The magnitude of response was however lower when mice were given the immunogens orally than by the other routes (P=0.036-0.024). No response was seen in the CTRG. Responses to CT in the TCIG mice were elevated in all four subclasses (IgG1, IgG2a, IgG2b, IgG3). Similar responses were seen in mice immunized via the SCI route (Figure22B). Both the subclass distribution and the magnitude of responses were similar (Figure 22B). However those given CT via the oral route showed a similar IgG1 response but significantly lower IgG2a (P= 0.036-004), IgG2b (P= 0.05-0.017) and IgG3 (P=0.05-0.018) responses compared to that seen in the other two groups. Both CTB and TcpA specific subclass responses in the TCIG were although lower in magnitude than TcpA-CT immunized CT and TcpA specific subclass responses but comparable (P= ns). LPS Inaba specific IgG subclass responses were not determined due to the low titer of IgG isotype after multiple doses. However, for membrane extract immunized mice, both LPS Inaba and Ogawa specific IgG subclass response was seen in theIgG1 subclass while were not detectable in IgG subclasses.

3.3 TcpA and CTB specific antibody responses in culture supernatant of lymphocytes (ALS) from mice in the TCIG experiments

Specific anti-TcpA responses in the IgG (GM= 5.6 mOD/min, P= 0.004) isotype was observed in ALS specimens obtained from spleen cells which was higher in contrast to that seen in the controls (GM= 1.1 mOD/min). Significant increases (P= 0.036) were observed for antibody responses to CT in the IgM (GM= 13 mOD/min) and IgG (GM= 204 mOD/min) isotype but not in the IgA (GM= 1.8 mOD/min) isotype when compared to the responses of controls (IgM: IgG: IgA= 1.0: 1.25: 1.0 mOD/min).

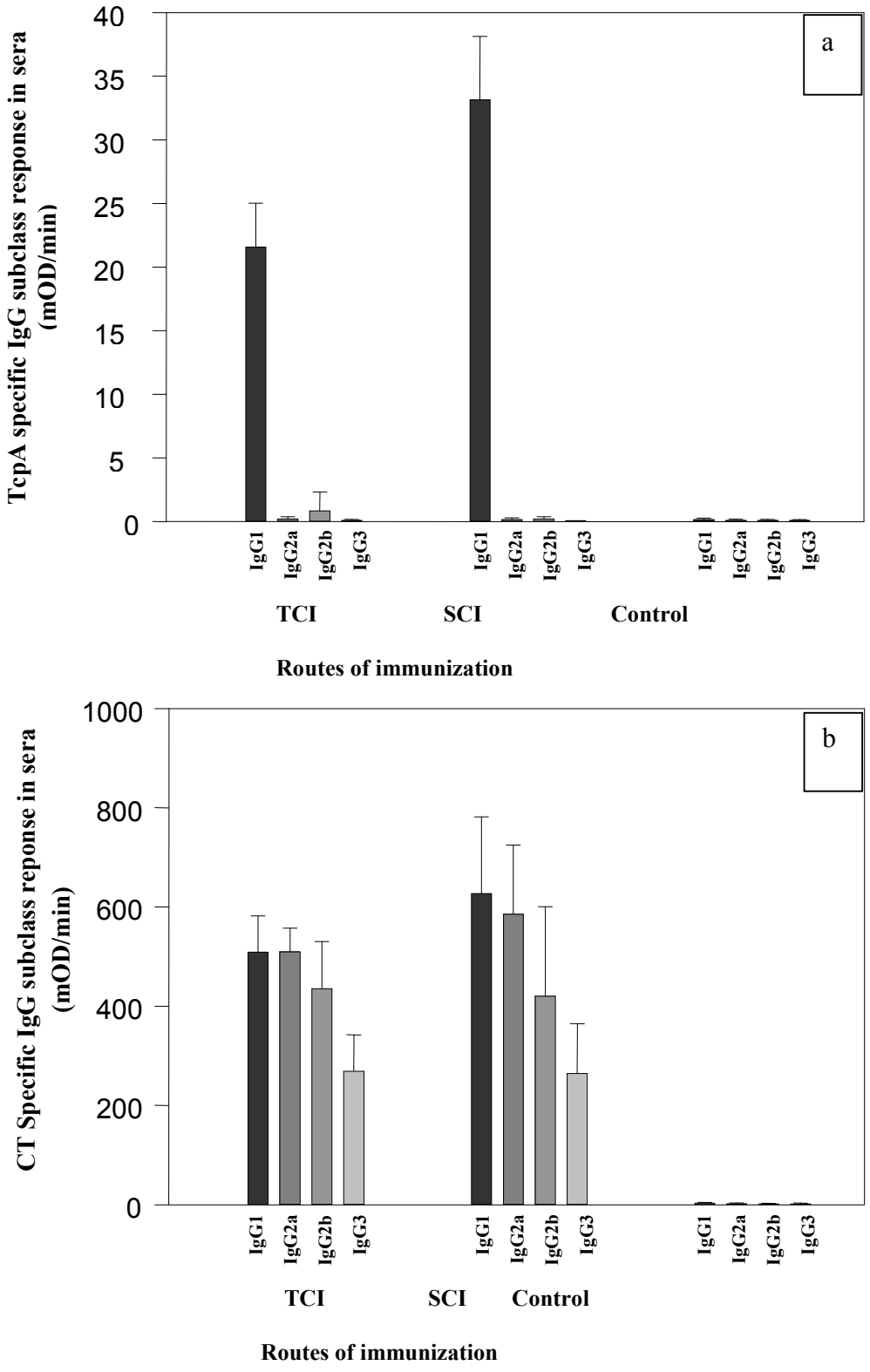


Figure 22: TcpA (a) and CT (b) specific response in different IgG subclasses in mice immunized with TcpA plus CT. Responses shown at day 63 of the immunization scheme.
Responses shown in the IgG1(■), IgG2a (■) and IgG2b (■) IgG3 (■) isotypes.

3.4 Vibriocidal antibody response to the different *Vibrio cholerae* antigens

Vibriocidal capabilities of the immunized mice were determined in sera after the immunization (Table 10).No vibriocidal antibody titer was found in the serum from the individually immunized CTB, TcpA and TcpA-CT co-administered mice. Vibriocidal responses were observed in the LPS Inaba and membrane extract immunized mice sera when tested for bactericidal capability against *V. cholerae* Inaba strain 19479 and *V. cholerae* Ogawa strain 25049. Vibriocidal antibody titer in the sera of LPS Inaba Immunized CF, ZnM and ZnP were 80, 40 and 320 against Inaba strain 19479 while 320, 320 and 1280 against LPS Ogawa strain 25049 respectively. When tested with the membrane extract immunized CF, ZnM and ZnP sera, the highest bactericidal titer of 10,240 was observed for ZnP serum against both Inaba 19479 and Ogawa 25049 strains while CF and ZnM sera showed the bactericidal titer of 5120 against both Inaba and Ogawa strains. As a result, membranes extract immunized sera displayed significant bactericidal capabilities (P= >0.001) in all three feed supplemented TCIG mice sera when compared with that of LPS Inaba immunized sera.

3.5 Immune cells and co-stimulatory markers status

Immune cells and their co-stimulatory markers as well as homing receptors of the immune cells were assessed using the flow cytometer in the TcpA-CT co-administered TCIG and SCIG mice. ORIG mice were not included to determine the immune cells and their coligands and homing receptors status due to its entirely different mechanism of immunization involving the Peyer's Patches and the Gut Associated Lymphoid Tissue (GALT) system for immunological responses as well as poor serological responses in comparison to SCIG and TCIG mice.

Bothtranscutaneous and subcutaneous immunization of mice showed varied expression of CD markers on different immune cells, their co-ligands and homing receptors. A variation of expression was seen based on the site of isolation of the cells. The percentage of the expression of CD19 positive immunocytes significantly increased in TCIG blood (P= 0.001) and in the lamina propria (P= 0.003-0.007) in both TCIG and SCIG when compared with CTRG. In addition, significant elevation of the co-stimulatory marker CD40 was seen in the CD19 cell population in the lamina propria in both TCIG and SCIG (P= 0.010- 0.035) (Table. 11) whereas cutaneous lymphocyte antigen (CLA) showed variation as it was increased in the spleen (P=

0.05) and lamina propria (P= 0.004) in TCIG but in blood (P= 0.048) and in lamina propria (P= 0.032) in the SCIG (Fig. 3). No increase was observed for the systemic marker CD62L and gut homing receptor, β_7 in any of the three compartments in both groups of mice (P= NS) (Figure23).

Expression of CD3 positive population in the TCIG, SCIG as well as CTRG were comparable (P=NS). However, the percentage of co-stimulatory markers CD28 in CD3 cells showed significant elevation in the spleen (P= 0.014-0.021) and lamina propria (P= 0.020-0.030) in both TCIG and SCIG while in TCIG blood (P= >0.001) in comparison to CTRG. Another important co-stimulatory marker CD154 level in CD3 cells increased significantly in the spleen (P= 0.022-0.026) in both TCIG and SCIG and in the lamina propria (P= 0.020) in TCIG mice compared to its level in controls (Table 11). The expression of CD62L in the CD3 population was seen to be increased significantly in lamina propria (P= 0.030) in TCIG and in the spleen (P= 0.043) in SCIG mice. β_7 expression in the CD3 population did not show increase in any of the compartments (P= NS) (Figure 23).

Following the TCI route of immunization, the CD4 subpopulation increased in the lamina propria (P= 0.015-0.050) in both TCIG and SCIG although levels in spleen and blood were not significantly elevated (P= NS). The expression of the co-stimulatory marker CD28 on CD4+ve cells were elevated in the spleen (P= 0.036) in TCIG and in blood (P= 0.026) in the SCIG; CD154 increased in spleen (P= 0.008-0.026) and in blood (P= 0.020-0.030) in both TCIG and SCIG and in the lamina propria (P= 0.039) in TCIG when compared to CTRG (Table 11). The CLA marker on CD4 positive cell population was elevated in blood (P= 0.009-0.048) in the TCIG and SCIG and in spleen (P= 0.009) and in lamina propria (P=0.003) in the TCIG mice. No Increase of the gut homing receptor β_7 expression (P= NS) was seen although the systemic homing receptor CD62L expression was seen in SCIG spleen (P= 0.043) and in the lamina propria (P= 0.030-0.05) in both TCIG and SCIG when compared to CTRG (Figure23).

In the CD8 expression pattern, a similar trend was observed in the TCIG and SCIG as well as CTRG mice (P= NS) in all the three compartments (Table 11). The dendritic cell population in spleen was increased in the SCIG group compared to that seen in the TCIG (P=0.019) and CTRG (P=0.004) mice (Table 11). The levels were similar in the TCIG and the CTRG mice.

3.6 Comparison of the TCI cellular response with that seen after subcutaneous immunization

Comparisons were carried out in mice immunized by the TCI and SCI routes in the spleen, lamina propria and blood. The CD19 distribution in all three compartments were similar in the TCIG mice when compared to those in the SCIG (P= NS). The CD3 expression in blood was significantly more elevated in the SCIG than TCIG mice (P= 0.003). The CD4, CD8 and CD11c expression patterns of the two immunized groups were also comparable (P= NS).

3.6.1 Comparison of co-stimulatory marker for B cells

The constitutive marker, CD40 was elevated in the spleen, blood and lamina propria for TCIG as was in the SCIG mice (Table 11). No changes were seen in the expression of the inducible co-stimulatory marker B7.1 (CD80) level in mice immunized by any of the two routes in spleen and lamina propria while in blood an increase was seen in the SCIG (GM= 11.0, P= 0.036) mice when compared to CTRG (GM= 5.8). Another co-stimulatory marker B7.2 (CD86) expression on CD19 cells were comparable in the spleen, blood and lamina propria when comparisons were made with controls (P= NS). Overall levels were comparable in the experimental groups.

3.6.2 Co-stimulatory marker for pan T cells and subpopulation

The constitutive co-stimulatory marker CD28 in CD3 and CD4 positive cells increased in immunized mice with a significant elevation in the spleen in SCIG (P= 0.026) compared to TCIG. The ligand for CD40 on B cells CD40L (CD154) expression was increased in CD3 and CD4 subpopulation in the spleen, lamina propria and blood in immunized mice when compared with control (P=0.002-0.026) and the intergroup differences of expression between TCIG and SCIG were comparable (P= NS).

As the immune cell status showed significant increase in all three compartments; spleen, blood and lamina propria in the TCIG and SCIG mice and almost all immune cells were comparable in both TCIG and SCIG mice. Results obtained stepwise in the antibody responses with the three routes of immunization with all probable vaccine candidates and elucidation of the cellular changes of the immune cells validated transcutaneous immunization as another alternative immunization protocol to approach in resolving the complex problem in cholera vaccination to achieve long term efficacy.

Table 11: Cellular response of immune cells in mice after immunization via the TCI and SCI route

Cll Type	Markers	Parameters	Presence of Immune Cells (%) in								
			Spleen			Lamina Propria			Blood		
			TCI	SCI	Control	TCI	SCI	Control	TCI	SCI	Control
CD19	CD19	GM (%)	42.22	41.9	42.46	28.64	30.63	18.07	6.89	3.46	3.33
		P*	NS	NS		**0.007**	**0.003**		**0.001**	NS	
	CD19+CD40	GM (%)	88.72	78.89	80.17	85.67	90.93	66.68	26.16	18.29	9.23
		P*	NS	NS		**0.039**	**0.01**		NS	NS	
CD3	CD3	GM (%)	46.24	47.8	47.2	45.32	49.46	52.84	74.8	82.89	76.56
		P*	NS	NS		NS	NS		NS	NS	
	CD3+CD28	GM (%)	92.09	95.08	87.49	92.9	83.71	71.28	90.36	95.57	71.94
		P*	**0.021**	**0.014**		**<0.001**	NS		**0.03**	**0.02**	
	CD3+CD154	GM (%)	3.56	3.43	0.64	5.38	3.64	2.4	1.6	1.52	0.57
		P*	**0.026**	**0.022**		**0.02**	NS		NS	NS	
CD4	CD4	GM (%)	66.27	58.98	57.41	51.28	44.83	27.8	69.61	76.62	66.07
		P*	NS	NS		**0.015**	**0.05**		NS	NS	
	CD4+CD28	GM (%)	84.23	80.44	75.33	75.92	76.09	71.45	86.52	98.21	78.62
		P*	**0.036**	NS		NS	NS		NS	NS	
	CD4+CD154	GM (%)	3.27	3.43	1.42	7.29	4.92	2.64	2.96	2.8	0.65
		P*	**0.008**	**0.026**		**0.039**	NS		**0.02**	**0.03**	
CD11c	CD11c	GM (%)	2.99	4.62	2.57	5.87	5.38	5.97	1.65	1.84	1.39
		P*	NS	**0.004**		NS	NS		NS	NS	

3.6.3 Immunogen specific B cells after TCI

As mentioned in the Methods and Materials section, a novel method was adapted and standardized to determine the percentage of immunogen specific B cells through flow cytometry. CTB specific B cells were prominent in both the systemic and mucosal compartments after immunization via TCI route with TcpA-CT (Figure 24). Mean percentage of CTB specific cells in spleen, blood and lamina propria were 42.9, 30.7 and 9.54 respectively which were significantly higher than negative controls (P= >0.001). TcpA specific cells were with very scanty percentages as correspondingly observed 1.37, 0.67 and 0.16 in the spleen, blood and lamina propria (Figure 25). However, these minor percentages of cells might contribute in effector functions and protection. With these much appreciated findings, novel molecular experimentations were designed to assess the genetic variability of the different immunogen specific cells.

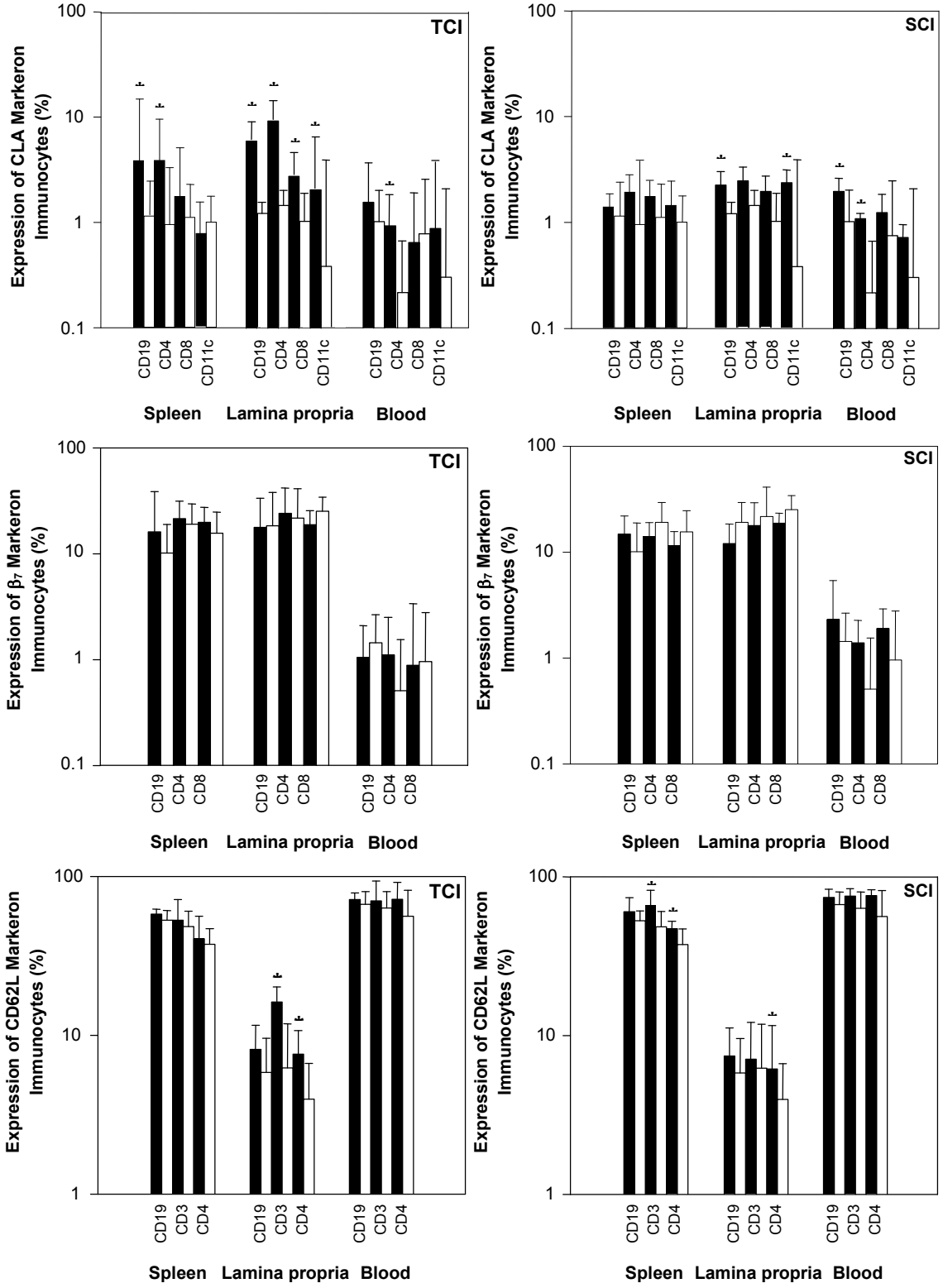
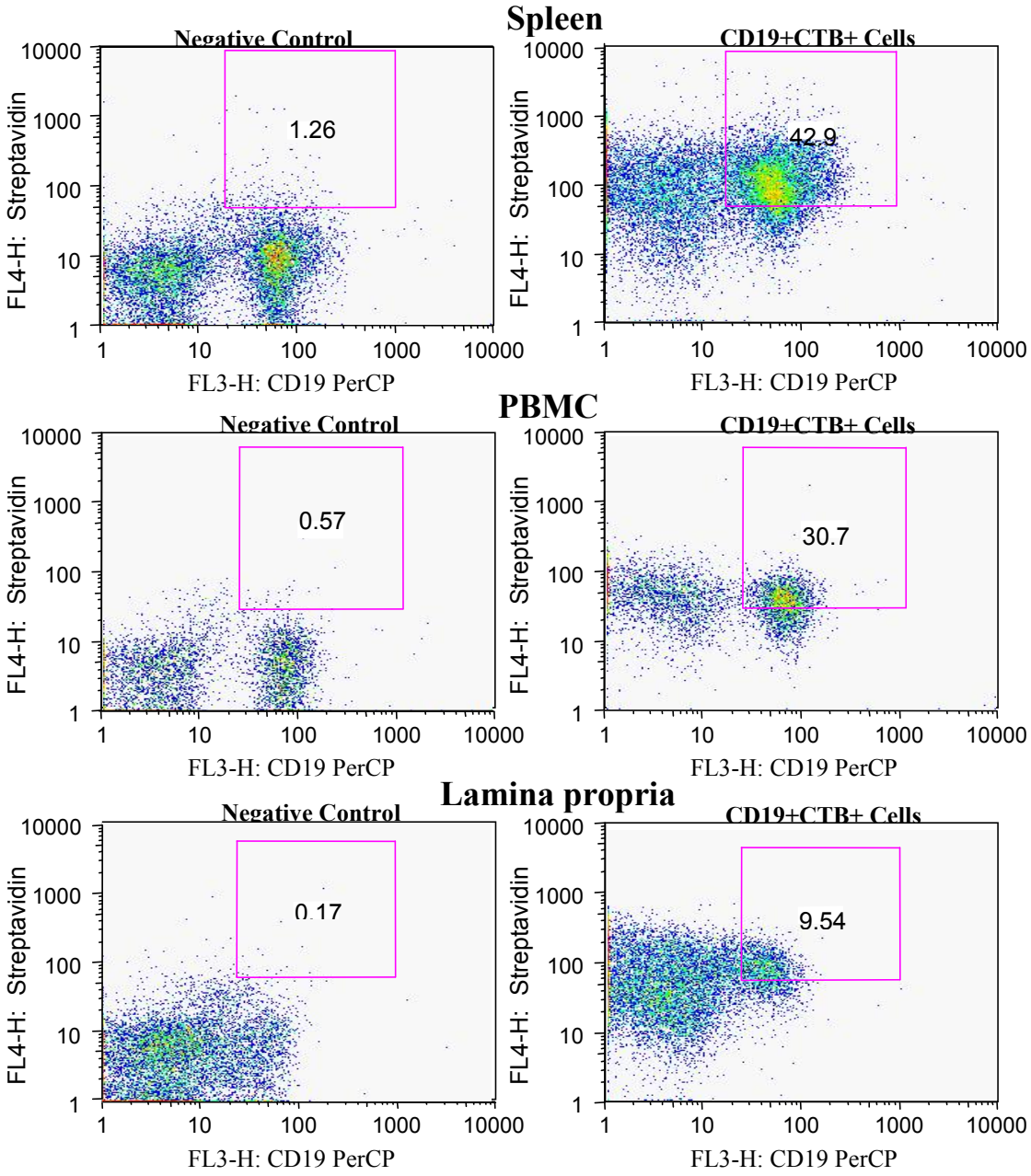
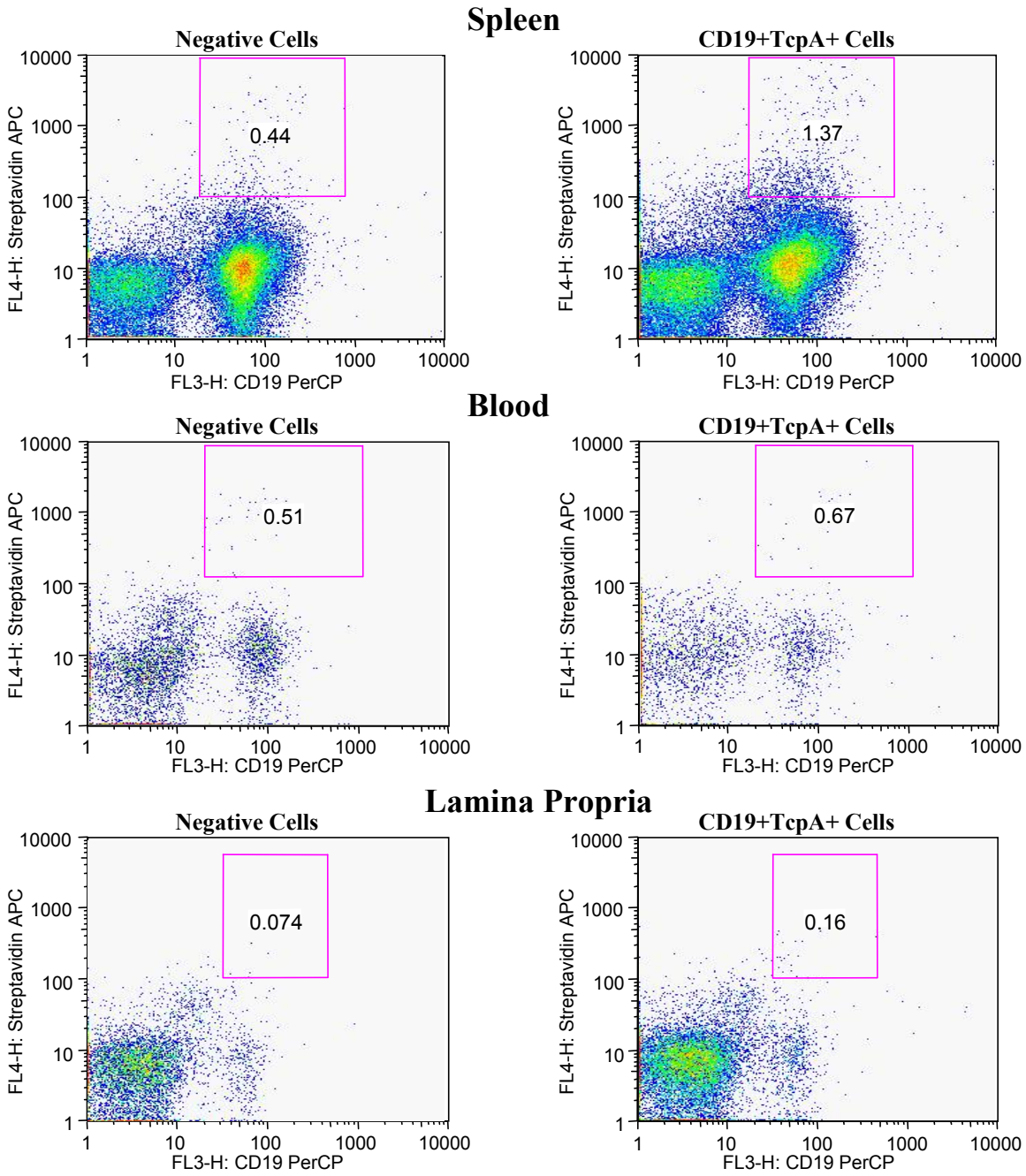


Figure 23: Distribution of cutaneous homing marker, CLA, gut homing receptor, β_7 and systemic marker, CD62L in mice. Results were compared between mice immunized with TcpA plus CT and controls. Responses shown were TcpA plus CT (■) and control (□). * indicate P< 0.05-0.001.



Compartments	Mean (%) of specific cells		P Value
	Negative control	Antigen specific	
Spleen	1.26	42.9	<0.001
Blood	0.57	30.7	<0.001
Lamina Propria	0.17	9.54	<0.001

Figure 24: CTB specific CD19 positive cells in systemic and mucosal compartment after immunization via the TCI route with TcpA plus CT. Antigen specific cells were labeled by CTB-biotin to analyze by FACS Caliber using streptavidin-APC-Cy5.5 and CD19-PerCP-Cy5.5 to determine percentage of antigen bound biotinylated cells.



Compartments	Mean (%) of specific cells		P Value
	Negative control	Antigen specific	
Spleen	0.29	0.86	0.001
Blood	0.24	0.42	0.050
Lamina Propria	0.11	0.25	0.045

Figure 25: TcpA specific CD19 positive cells in systemic and mucosal compartment after immunization via the TCI route with TcpA plus CT. Antigen specific cells were labeled by TcpA-biotin to analyze by FACS Caliber using streptavidin-APC-Cy5.5 and CD19-PerCP-Cy5.5 to determine percentage of antigen bound biotinylated cells.

3.7 Analysis of Variable (V) gene in mice immunized with different *V. cholerae* antigens

3.7.1 Distribution of Heavy (H) chain V-gene families in mice immunized with different *V. cholerae* antigens

Two types of heavy chain were analyzed: μ-chain and the γ1-chain. Each of these chains was again sub-divided into different families. Sequences of the VH5-V7183 and VH4-VX24 gene families were restricted within the μ-chain while sequences clustered in the VH9-VGAM3.8 and VH4-VSM7 gene families were found mostly restricted within the γ1-chain. In addition, sequences under VH1-VJ588 family were distributed in both groups. The diversity (D) genes originated were DFL16.1 and DSPs in IgM, DSPs in IgG1 for both CTB and LPS and the junction (J) genes responded were JH1, JH2 and JH4 in CTB-IgM, JH4 in CTB-IgG1, JH2 and JH3 in LPS-IgM, and JH3, JH4 in LPS-IgG1.

Out of 25 CTB genes sequenced (Table 12), 18 resulted through TCI immunization with CTBand the rest 7 resulted through TcpA-CT application through TCI. This CTB application as immunogen resulted in two μ-chain family classes based on the type of immunogen provided such as VH5-V7183 in case of pure CTB as immunogen and VH4-VX24 in case of CT conjugation with TcpA. Upon class switching, transition from μ-chain to γ1-chain resulted in VH1-VJ558 predominant in case of pure CTB application and VH9-VGAM3.8 in case of CT conjugated with TcpA.

However, out of 12 genes sequenced for TcpA (Table 12), 8 resulted from TcpA-CT immunization while the rest 4 resulted through immunization of TcpA only through TCI. VH4-VX24 was the predominant μ-chain family while VH1-VJ558 as the predominant γ1-chain family emphasizing a class switches recombination (CSR) from VH4-VX24 to VH1-VJ558.

No change of significant nature was observed in case of Ogawa and Inaba immunization through TCI (Table 12). All Inaba and Ogawa specific sequences attained VH1-VJ558 family very early in the development. The gene family class was reflected within the IgM sequences after which there was no change at all when the transition from IgM to IgG1 through CSR happened there.

Table 12: Heavy chain Variable (V) sequences and their corresponding gene families with Diversity (D) and Joining (J) chains identified from immunogen specific splenocytes of mice

TCI Protocol	Ag Tested	No. of Seq.	Heavy Chain V-Gene Usage					
			μ Chain			γ1 Chain		
			VH5-V7183	VH4-VX24	VH1-VJ558	VH1-VJ558	VH9-VGAM3.8	VH4-VSM7
CTB	CTB	18	10	--	--	5	3	--
TcpA-CT	CTB	7	--	2	--	1	4	--
	TcpA	8	--	2	1	5	--	--
TcpA	TcpA	4	--	--	--	3	1	--
Inaba	Ogawa	12	--	--	5	6	--	1
	Inaba	11	--	--	6	5	--	--
	Total	60	10	4	12	25	8	1

Diversity Gene: DFL16.1 and DSPs in IgM DSPs in IgG1 for both CTB and LPS J-Gene: JH1, JH2 & JH4 (IgM CTB), JH4 (IgG CTB), JH2, JH3 (IgM, LPS), JH3, JH4 (IgG1 LPS)

3.7.2 Analysis of all V_H chain sequences

All CTB, TcpA, TcpA-CT and LPS Inaba as well as LPS Ogawa specific sequences were considered as individual group per immunogen excluding the disparities with the feed supplementation and analyzed to delineate the similarities and dissimilarities between the protein and polysaccharide immunogens, the effect of co-administration through alignment of the sequences as well as relatedness among the groups at different positions as summarized in Table 13-15.

FR-H1. LPS Inaba and Ogawa specific sequences might have capabilities to respond earlier to ensure protection due to its constitutive genetic make-up which CTB and TcpA specific sequences gained after transition to IgG1 were at positions 6, 20 and 23 but turned into different amino acids than the unique amino acids in LPS IgM and IgG1 sequences at positions 09, 10, 17 and 18. In transition from IgM to IgG1, replacement occurred in all three CTB, TcpA and LPS

immunogen specific sequences which displayed identical amino acids at positions 13 and 16 while disparate in position 12. Hydrophilic and/or charged amino acids were placed at positions 6, 10, 13, 17 and 23 in transition from IgM to IgG1. However, hydrophobicity increased at positions 9, 16 and 20 and partly in 13 among LPS specific sequences. Co-administration of immunogen might have contribution in shifting of amino acids as noted transition from serine (S) to threonine (T) at position 17. Co-administration of immunogen with minimum or no effect at positions 06, 09, 10 but disparate expressions at positions 12, 13 and 16 in CTB and 23 in TcpA specific sequences.

CDRH-1.LPS Inaba and Ogawa specific sequences might have capabilities to respond earlier to ensure protection than CTB and TcpA which was noted at positions 27 and 35. Hydrophilic amino acids were placed at positions 27, 30, and 35 in transition from IgM to IgG1. In addition, polar hydrophilic threonine (T) displayed majority but discrete variability's observed for all immunogens at position 28. However, transition at position 30 could be a typical instance of CSR for the protein immunogens specific sequences which increased hydrophilicity while LPS Inaba and Ogawa specific sequences showed unresponsiveness. On the other hand, hydrophobicity was seen to be increased in the LPS and TcpA-CT co-administered TcpA specific sequences while most of the CTB specific sequences remained indifferent during transition from IgM to IgG1 at position 34. Replacement in transition from IgM to IgG1 fully or partly occurred in all position (27, 28, 30, 31, 33, 34 and 35) among sequences specific to protein immunogens but partly occurred (28, 31, 33, 34 and 35) in LPS. Co-administration of TcpA-CT responded differently than CTB only immunized group among CTB IgM and IgG1 sequences at position 28, showed influence progressively at positions 31, 33 and 35 in both CTB and TcpA IgG1 sequences while demonstrated unresponsiveness in CTB IgG1 but increase hydrophobicity in TcpA IgG1 at position 34.

FRH-2. Shifting of amino acids occurred among almost all CTB and majority of TcpA specific sequences at positions 38 and 40 while partly occurred at positions 41, 42, 43, 44, 46, 48, 49, 50 and 41,43, 44, 46, 50 with 43, 50 among CTB, TcpA and LPS sequences respectively during transition from IgM to IgG1. When considered individual immunogen, shifting was noticed with CTB specific CTB only immunized sequences at positions 42, 43, 44, 48 and 49. IgG1 sequences of TcpA along with Inaba and Ogawa IgG1 sequences displayed positively

charged histidine (H) in some of the sequences at position 43. LPS specific sequences achieved stability at positions 38, 40, 41, 42, 44, 46, 48, 49. Co-immunization with TcpA-CT might have favorable contribution at position 38, 42 and 44, demonstrated disparate expression in CTB and TcpA sequences than Individual immunogens at positions 40, 41, 43, 46 and 50 while suppressive effect in reducing hydrophobicity at position 48 with no-effect at position 49. When considered expression within individual immunogens, distinct expression in all immunogen specific IgG1 was observed at position 42, 43 and 49 while slightly variable in CTB and TcpA but distinct in LPS at positions 38, 41, 44 with slightly variable in CTB but distinct in TcpA and LPS at position 48, variable in TcpA and CTB but distinct in LPS at position 40 whereas most variable within all immunogen at position 50.

CDR-H2. Shifting occurred in the CTB specific CTB only immunized sequences at position 52 and 53. Moreover, shifting from hydrophilic amino acid to hydrophobic amino acid was observed in the LPS-specific sequences at position 52 while hydrophilic to positively charged amino acid at position 60. Among the CTB only and TcpA-CT immunized CTB and TcpA specific sequences, shifting from hydrophobic and negatively charged amino acids to hydrophilic amino acid were remarkably observed at position 54. In both CTB and TcpA specific sequences, changing from hydrophilic to hydrophobic occurred at position 56. Shifting also noted with extreme variability with the protein-specific sequences at position 57 and 59. In contrast, LPS-specific sequences existed shiftless from early development during transition from IgM to IgG1 at 53, 56 and 58. TcpA only and LPS specific sequences remained unaffected in displaying hydrophobic amino acid glycine (G) in both IgM and IgG1 sequences at position 54 while Inaba specific IgM and IgG1 sequences mostly displayed negatively charged aspartate (D) at position 55. Although no specific amino acid was found to dominate at position 57 and 59, Inaba and Ogawa specific IgM and IgG1 sequences expressed polar hydrophilic amino acid. In general, expression of amino acids in the protein specific sequences were different than polysaccharide immunogen derived sequences at positions 52, 54, 55, 56, 57, 58 and 59.

FR-H3. LPS Inaba and Ogawa specific sequences might have capabilities to respond earlier to ensure protection due to its constitutive genetic make-up than CTB and TcpA specific sequences which displayed almost same amino acids at positions 61, 63, 64, 67, 68, 69, 71, 75, 76, 79, 81, 82, 83, 85, 86, 87, 88, 91, 93 but turned into different amino acids than the unique

amino acids in LPS IgM and IgG1 sequences at positions 72 and 84. Positions at which replacement occurred in all three CTB, TcpA and LPS immunogen-specific sequences which displayed amino acids as identical at positions 62, 70, 95 while disparate in 74 and 77. Mostly hydrophilic and/or charged amino acids were placed at positions 61, 62, 63, 67, 69, 71, 74, 75, 76, 77, 82, 84, 85, 87, 88, 91, 95 in transition from IgM to IgG1. However, positions at which hydrophobic amino acids were mostly positioned and /or hydrophobicity was increased at positions 64, 68, 70, 72, 79, 81, 83, 86, 93 and 95. Diversity in all immunogens specific sequences noted at positions 62, 70, 77, 83, 95. Co-administration of immunogen with minimum or no effect at positions 64, 70 (CTB), 75, 86, 88 (TcpA), 93 (TcpA) but might have contribution in shifting of amino acids as facilitated expressions than manifestation with individual immunogens at positions 61, 68 (CTB), 69 (TcpA), 70 (CTB), 71 (TcpA), 76 (TcpA), 77 (TcpA), 79 (TcpA), 81 (TcpA), 85 (TcpA), 87 (TcpA), 88 (CTB), 91 (CTB), 95 (TcpA) while disparate expressions at positions 62, 63, 67, 68 (TcpA), 69 (CTB), 70 (TcpA), 72, 74, 76 (CTB), 77 (CTB), 81 (CTB), 82, 83, 84, 85 (CTB), 87 (CTB), 91 (TcpA), 93 (CTB). Relatedness firmly found between Inaba and Ogawa but irregularly between CTB and TcpA at 61, 63, 67, 68, 69, 71, 72, 77, 81, 82, 83, 84, 85, 87, 88, 91, 93 but partial relatedness among the immunogen specific sequences at positions 70, 74, 76, 95 while relatedness among the sequences specific to three immunogens: CTB, TcpA and LPS at positions 64, 75, 79 and 86.

Table 13: Comparison of expressed amino acids at different positions in the CTB, LPS and TcpA specific antibody sequences

Sequence Region	Distance in Sequences	Positions at which Replacement in CTB and TcpA Specific Sequences Turned into **Identical Amino Acids** in LPS IgM and IgG1 Sequences	Positions at which Replacement in CTB and TcpA Specific Sequences Turned into **Different Amino Acids** than Predominant one in LPS IgM and IgG1 Specific Sequences	Positions at which Replacement Occurred in CTB, TcpA and LPS and Displayed Amino Acids as		Diversity in Expression
				Identical	**Disparate**	
FR-H1	1-25	6, 20, 23	9, 10, 17, 18	13, 16	12	Nil
CDR-H1	26-37	27, 28	30, 31, 33	Nil	34, 35	Nil
FR-H2	38-50	38, 40, 41, 42, 44, 46, 48, 49	Nil	Nil	43, 50	Nil
CDR-H2	51-60	53, 56, 58	54	Nil	52, 60	55, 57, 59
FR-H3	61-96	61, 63, 64, 67, 68, 69, 71, 75, 76, 79, 81, 82, 83, 85, 86, 87, 88, 91, 93	72, 84	62, 70, 95	74, 77	Nil

3.8 Light chain sequence comparison for all antigens

3.8.1 Distribution of Light (L) chain V-gene families in mice immunized with different *V. cholerae* antigens.

In case of light chain, it was observed that the selection of family is more diverse than that for the heavy chain (Table 14). The most diverse among these antigens is TcpA applied in conjugation with CT. It is spread across all the 7 families, the most predominant being V-38c. In case of only TcpA application as vaccine, there were only two families observed – V8, V-38c. The second most diverse antigen is CTB. It is spread along five families with the V9/10 being the most predominant followed by V8 family. The LPS antigens, Ogawa and Inaba showed the least amount diversity. The most predominant family observed in this case is the V8 family, which is also the most common family, observed for the entire antigen vaccination.

Table 14: Light chain Variable (V) sequences and their corresponding gene families with Joining (J) chains identified from immunogen specific splenocytes of mice

TCI Protocol	Ag Used	No. of Seq.	Light Chain V-Gene Usage						
			V4/5	V8	V-9/10	V-12/13	V21	V23	V-38c
CTB	CTB	10	--	3	4	--	--	2	1
TcpA-CT	CTB	10	--	3	4	1	--	1	1
	TcpA	13	1	1	1	1	1	3	5
TcpA	TcpA	4	--	2	--	--	--	--	2
Inaba	Ogawa	5	__	3	--	--	1	1	--
	Inaba	6	--	3	2	--	1	--	--
	Total	48	1	15	11	2	3	7	9

V-Gene		Predominant J-Chain
V8	:	jk1, jk2 and jk5
V9/10	:	jk1 and jk2
V23	:	jk1
V38c	:	jk1

3.8.2 Representation of amino acids at specific position and relatedness with VL family

All analyzed 48 sequences were differentiated into seven VL gene families based on similarities and relatedness among themselves. As distributed, V8 gene family incorporated 15 sequences followed by V9B, V38C, V23, V21, V12/13, V4/5 and V1 included sequences 11, 09, 07, 02, 02, 01 and F-22-30 respectively (Table 15). During analysis of the sequences through alignment, each gene family was characterized by unique representation of distinct amino acids at different positions of the VL sequences. Relatedness between two gene families was only considered with the expression of identical amino acids at the same positions.

Table 15: Representation of relatedness of the analyzed sequences with VL family

Immunogen Applied for Immunization	Seq. Specific to	Sequences Segregated in the Gene Families							
		V1	V4/5	V8	V9B	V12/13	V21	V23	V38c
CTB	CTB (10)	Nil	Nil	M1-CF, M4-CF, M2-ZnM	M2-CF, M4-A-CF, M3-ZnP, M4-ZnP	Nil	Nil	M3-CF, M3-ZnM	M5-ZnP
LPS Ogawa	LPS Ogawa (05)	Nil	Nil	M4-CF, M4-A-CF, M5-CF		Nil	M1-CF	M2-CF	Nil
LPS Inaba	LPS Inaba (05)	F-22-30	Nil	M1-CF, M1-A-CF, M2-CF	M5-CF, M4-ZnM	Nil	Nil	Nil	Nil
TcpA	TcpA (07)	Nil	Nil	M4-ZnM, M4-A-ZnM	Nil	Nil	Nil	Nil	M4-ZnP, M4-A-ZnP, M5-ZnP, M5-ZnM, M5-A-ZnM
TcpA-CT	CTB (10)	Nil	Nil	M1-CF, M1-ZnP, M2-A-ZnP	M1-A-CF, M2-CF, M1-A-ZnP, M3-ZnM	M3-A-ZnM	Nil	M2-A-CF	M2-ZnP
TcpA-CT	TcpA (10)	Nil	M3-AA-ZnM	M2-CF	M3-A-ZnM	M1-CF	M2-A-CF	M1-A-CF, M1-A-ZnP, M2-A-ZnP	M3-ZnM, M2-ZnP

3.8.3 Summary of relatedness of the gene families

When analyzing the relatedness between the two VL gene families, V1 gene family mostly inclined to V8 family at five positions (23, 30, 58, 64 and 87) followed by V23 in three positions (15, 18 and 95) while V21 at position 17. V4/5 gene family showed closeness with all gene families although mostly with V21 at positions 37, 64 and 82 along with V8 at positions 19 and 31, V38c at positions 55 and 60 while with V9B and V23 at positions 09 and 44 respectively. Other than the relatedness with V1 and V4/5 correspondingly at five and two positions, V8 gene family sequences presented resemblance with V21 at three positions (10, 29 and 59) and V12/13 at two positions (13, 83). Among these three families, V1 and V8 gene families did not display affiliation at any position with V9B and V38c families. V9B gene family displayed similarities at four positions (26, 28, 93 and 96) with V38c gene family along with V21 in two positions (40 and 97) while with V4/5 and V12/13 through only positions 09 and 60 respectively. As mentioned earlier about the relatedness of V12/13 with V4/5 (position 59), V8 (positions 13 and 83), V12/13 gene family was also seen to demonstrate similarities with V21 and V23 gene families correspondingly at positions 36 and 21. V21 family did not show any kinship with V23 and V38c but as indicated, it maintained most closeness with V4/5 and V8, moderate similarities with V9B and nominal resemblance with V1 and V12/13 gene families. Except all stated relatedness of V23 with mostly V1 (03 positions) followed by V4/5 (01 position) and V12/13 (01 position), V38c gene family showed association at positions 49 and 78. Relationship of V38c was already conferred as noted at positions with V9B followed by two positions withV4/5 and V23 gene families.

The sequences incorporated in various gene families with their distinct amino acids at different positions and the amino acids contributed in clustering of gene families might have significant role in effector activities and functions such as binding to the immunogen and bacterial clearance through immunological mechanisms (Table 16).

Table16: Positions with distinct amino acids representing V gene families of the L chain sequences

Seq. Region	Distance in Seq.	Positions representing distinct amino acids specific to the VL Gene Families							
		V1 (n= 01)	V4/5 (n= 01)	V8 (n= 15)	V9B (n=11)	V12/13 (n= 02)	V21 (n= 02)	V23 (n= 07)	V38c (n= 09)
FR-L1	1-24	07 (P), 10 (P), 16 (Q)	8 (I)	Nil	10 (Y)	16 (T)	Nil	13 (P)	15 (G)
CDR-L1	25-36	28 (V), 29 (H), 33 (G), 34 (D)	33 (V), 34 (N)	27 (L), 28 (L), 33 (Q), 34 (K)	Nil	25 (G), 33 (I), 34 (Y)	25 (E), 27 (V), 28 (D), 34 (I/N)	28 (S), 35 (D)	35 (K)
FR-L2	37-53	38 (E), 41 (L)	38 (Y), 45 (D), 46 (A), 51 (W)	Nil	38 (S), 50 (T)	44 (E/Q), 49 (Q), 52 (V)	Nil	45 (H), 46 (E), 53 (K)	42 (H), 47 (G), 53 (H)
CDR-L2	54-56	54 (K), 55 (V)	Nil	Nil	54 (R), 56 (N)	56 (K)	Nil	Nil	Nil
FR-L3	57-92	59 (F), 78 (E), 80 (T), 81 (R)	73 (K), 74 (S), 84 (G), 87 (A), 89 (A)	67 (T/I)	57 (R), 59 (V), 73 (Q), 84 (Y), 87 (M), 89 (I)	74 (Q), 78 (K), 87 (F)	81 (P)	57 (Q), 58 (S), 59 (I), 73 (S)	59 (Q), 73 (R), 77 (F), 81 (N), 87 (I)
CDR-L3	93-102	93 (F), 97 (H), 101 (R)	96 (T)	96 (Y?)	Nil	94 (H)	95 (S)	94 (N)	Nil

3.9 Analysis of Heavy and Light chain sequences with that of the immunological results

Attempts to compare the magnitude of antibody response obtained with that of the above-analyzed sequences. There are six canonical structures, namely CDR-L1, CDR-L2, CDR-L3, CDR-H1, CDR-H2 and CDR-H3 that were involved in the binding of the antigen with the antibody. As the whole structure of the molecule was not involved in binding but a few residues from the structures actually bind with the antigen, the most prominent positions predicted were 50, 95, 99, and 101 of the heavy chain while positions 32, 34, 37, 39, 50, and 91of the light chain.

On analysis of the above mentioned positions, it was observed with the Inaba-specific sequences that position 50 was occupied by either glutamate (E) or glutamine (Q). Both are amino acids of the same group with a single minor difference in the side chain in which glutamine contains amino functional group instead of carboxyl group in case of glutamate. When compared with the antibody responses, it was noted that both the amino acids were equally important for the efficiency of effector function. However, glutamine and glutamate represented two Inaba specific sequences; M2 and M5 respectively which showed higher titer of IgG. Strangely, a similar trend was observed at position 95, where phenylalanine (F) and tyrosine (Y) were present with phenylalanine being prominent. The rest of the position corresponds to the CDRH3 region. Within that region based on the arrangement type described by Ahmed et al. (Ahmed *et al.*, 2008), anti-Inaba antibodies presented mostly glutamate (E) at position 99 (107). In addition to glutamate (E), there was proline (P) in M3 and glycine (G) in M5 presented by the antibody sequence. However, in relation to the ELISA results observed M3 presented the lowest antibody titer while M5 presented the highest antibody titer.

3.10 Analysis of the binding site of the antibodies of CTB, TcpA, Inaba and Ogawa

The binding site of the antibody molecules to the immunogen relates to the type of immunogen that is present. The canonical regions of the antibodies were detected using the software Vbase2 (www.vbase2.org). The canonical structures of the CDR-L1, -L2, -H1, -H2 and -H3 was determined. The length of CDRH2 and CDRL1 actually determines the size of the antigen-binding groove on the antibody which represents the size of the immunogen to be bound inside the groove. The CDRH2 region represented a length of eight amino acids irrespective of the antibody molecules generated through immunization of either protein or polysaccharide immunogens. The sequences that represented in the CDRH2 region of CTB only immunized CTB specific sequences were 'IDPYYVST', 'INTYTGEP', and 'IYPGDGST'. While in case of TcpA-CT immunized CTB specific sequences, the major sequences for the said region contained 'INTYTGEP' and 'INTYSGET' which were also observed among the Zn supplemented CTB specific sequences in addition to 'INTYTGQP'.

However, although TcpA specific sequences displayed a similar length of CDRH2 region, there were disparities in the selection of the sequences. The primary sequences observed were 'INTHSGVP', 'IDPYNGGT', 'IFPGSGSI', and 'IYPGSGST' among the TcpA only immunized

sequences. In addition to 'IDPYNGGT', TcpA-CT immunized TcpA-specific sequences also presented 'INPYNDGT' and 'INPYNGGN' which were prominent among the Zn-supplemented sequences as well.

When considered Ogawa and Inaba specific sequences, the predominant sequences observed in the CDRH2 region were 'IYAGTGAT', 'ILPGDGNT', 'ILPGSGST', 'ILLGSGST', and 'IYPGDGNT' among the Ogawa and 'ILLGTGDT', 'IYPGDGNT' and 'ILPGSGST' among the Inaba specific sequences.

Although all the three immunogen-specific sequences presented the same length of CDRH2 sequence, there were significant variation between the sequence length of CDRL1 region among CTB, TcpA and LPS specific sequences. CTB specific sequences presented mainly 06 amino acids length sequences distributed mostly as 'QDINSY' among M2-CF, M4-A-CF and M4-ZnP of the CTB only and M1-A-CF, M2-CF, M3-ZnM and M1-A-ZnP of the TcpA-CT immunized sequences, followed by 'QSISDY' among M3-CF and M3-ZnM of the CTB only and M2-A-CF of the TcpA-CT immunized sequences while correspondingly QDINKF, QDIKSY and QDINKY in the M2-ZnP, M3-ZnP and M5-ZnP CTB only sequences but exceptionally 'GNIHNY' in the M3-A-ZnM TcpA-CT immunized CTB specific sequence. However, CTB specific CDRL1 sequences containing 12 amino acids which, exceeded the 6 amino acid length observed, in general, were 'QSLLYSSNQKNY' in M1-CF CTB only and M1-ZnP TcpA-CT immunized sequences while correspondingly QSLLNSSNQKNY and QSLLNSGNQKNY in the M4-CF and M2-ZnM CTB only but QSLLNSSTQKNY' and 'QSLLNSGDQKNY' in the M1-CF and M2-A-ZnP of the TcpA-CT immunized CTB specific sequences respectively.

Similarly, TcpA-specific L chain sequences predominantly presented sequences within the 06 amino acids range in 13 sequences while displayed 12 amino acids 02 sequences followed by 10 amino acids in 01 sequence with 05 amino acids in another 01 sequence. Among the 06 amino acids sequences; 'QDINKY' represented 04 sequences (M5-ZnM, M5-A-ZnM, M4-A-ZnP, M5-ZnP) of the TcpA only and 02 sequences (M3-ZnM and M2-ZnP) of the TcpA-CT immunized TcpA specific sequences while 'QSISDY' demonstrated 02 sequences (M1-A-ZnP, M2-A-ZnP) of the TcpA-CT immunized while 'QDINNY' characterized M4-ZnP of the TcpA only but 'GNIYSF', 'QNISDY' and 'QDINSY' were correspondingly present in M1-CF, M1-A-CF and M3-A-ZnM sequences with only 05 amino acids containing 'SSVNY' in M3-AA-ZnM

immunized with TcpA-CT. Likewise CTB specific sequences, 12 amino acids containing CDR-L1 sequences were observed as 'QSLLYSSNQKNY' in M4-A-ZnM of TcpA only and M2-CF of TcpA-CT immunized while 'QSLLNSRTRKNY' in M4-ZnM of TcpA only sequences. In addition, another 10 amino acids containing 'ESVDGYGNSF' sequence was also found in M2-A-CF of the TcpA-CT immunized sequences.

In contrast to the observation among the CTB and TcpA specific sequences, most of the LPS Ogawa and Inaba sequences presented 12 amino acids sequence while 06 amino acids sequences were also present in both Inaba and Ogawa specific sequences. Among the 12 amino acids sequences, 'QSLLNSSNQKNY' represented M1-CF Inaba and M4-CF Ogawa sequences while 'QSLLNSGNQKDY' characterized M1-A-CF Inaba and M4-A-CF Ogawa sequences with 'QSLLYSSDQKNY' was present in the M5-CF Ogawa. LPS Inaba and Ogawa CDR-L1 sequences containing 06 amino acids sequences observed were 'QDINSH' in M5-CF Inaba, 'QDIKSY' in M4-ZnM Inaba while 'QSISDY' in M2-CF Ogawa sequences. An exception of 10 amino acids sequence 'ESVDNYGISF' was observed in the M1-CF Ogawa sequence.

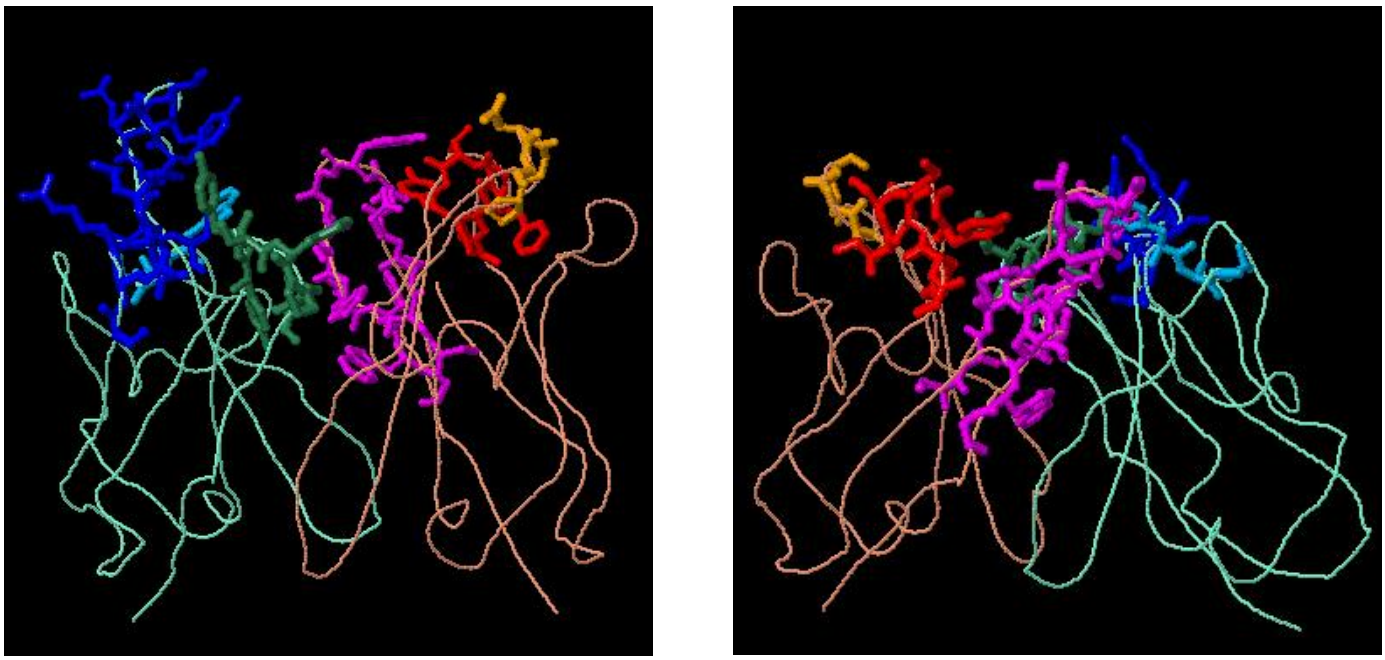
The above data were consistent with the fact that CTB and TcpA specific sequences predominantly recognized protein immunogens as their antigen-binding surface which was flat in conformation while LPS Inaba and Ogawa sequences participated in binding with small molecules signified with the fact that the antigen-binding surface for LPS was cleft in conformation.

3.11 Homology model of the Fv region of M2-Inaba and M4-ZnM-Inaba

In case of M2-Inaba-Fv, PIGS identified light chain residues 26-33 (L1), 50-53 (L2), 91-96 (L3) and heavy chain residues 26-32 (H1), 52-54 (H2), 96-101 (H3) as the CDR forming amino acids (Figure 26a). All the canonical structures of L1, L2 and L3 loops are belong to the known canonical structure class. In case of heavy chain, H1 and H2 loops fall into the known canonical structure class and for H3 loop; non-bulged canonical structure class is found. H3 loop is constructed based on the H3 loop of PDB structure 1C5D as it shows significant homology.

In case of M4-ZnM-Inaba-Fv, light chain residues 26-33 (L1), 50-53 (L2), 91-96 (L3) and heavy chain residues 26-32 (H1), 52-54 (H2), 96-101 (H3) as the CDR forming amino acids (Figure 26b). All the L1, L2 and L3 loops fall into the known canonical structure classes. On the other

hand, H1 and H2 loop fall into known canonical structure class and for H3 loop, bulged canonical structure class is found. H3 loop is constructed based on the H3 loop of PDB structure 1UCB as it shows significant homology with target.



(a) **(b)**

Figure 26: (a) Fv model of M2-Inaba is predicted by the PIGS server. Locations of different loops are shown in different color. L1 (26-33) blue, L2 (50-53) light blue, L3 (91-96) light green, H1 (26-32) red, H2 (52-54) orange, H3 (96-101) purple. (b) Fv model of M4-Inaba is predicted by the PIGS server. Different loops on heavy and light chain is displayed in different color. L1 (26-33) blue, L2 (50-53) light blue, L3 (91-96) light green, H1 (26-32) red, H2 (52-54) orange, H3 (96-101) purple.

3.12 Evaluation of the structural model

Ramachandran plot (Table 17) using the iMOLTALK program showed good compliance with known geometrical constraints in protein backbone structures. In case of M2-Inaba-Fv light chain, approximately 99% residues fall in the favored (core+allowed) region and only 1 % residues in the generous region. No residues are found in the disallowed region. On the other hand, 97% residues of heavy chain fall in favored region, 2% residues fall in generous region and only one residue (Arg 65) of FR-H3 falls in disallowed region but this residue is not the part of CDR region and does not cause any problem in further modeling calculation.

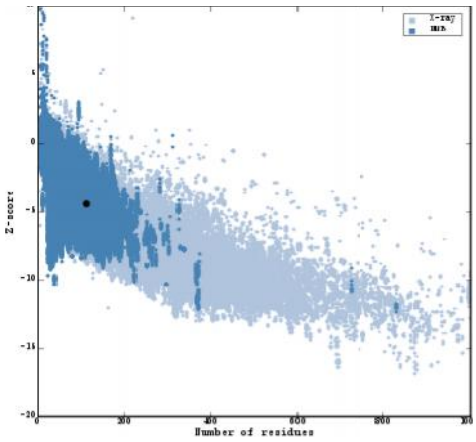
In case of M4-ZnM-Inaba-Fv light chain (Table 17), 99% residues fall in the favored region and only one residue (Ala51) of FR-L2 fall in disallowed region but this residue does not take part in the interaction. On the other hand, all (100%) the residues fall in the favored region.

For M2-Inaba-Fv light chain, Z-score from the program ProSA was found -4.41 which indicates that the overall model quality is good. This Z-score falls in the range typical for native fold. The combined (paired plus surface) energy graph of the model showed that most of the residues energy in the protein model is negative (Figure 27). In case of heavy chain, Z-score was found -5.16 and all the residues energy in the plot is negative (Figure 28).

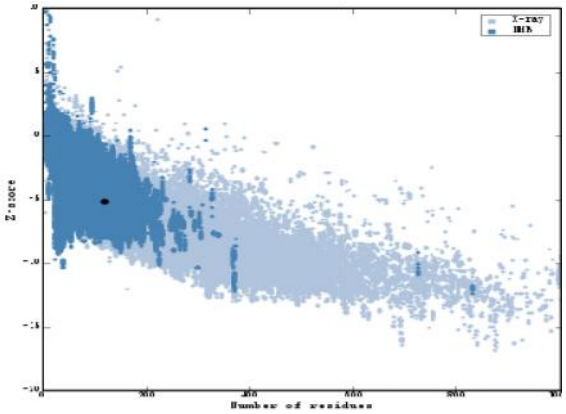
For M4-ZnM-Inaba-Fv light chain (Figure 27), Z-score was found -4.64 and the energy of the residues in plot of negative. In case of heavy chain, Z-score was found -5.51 and most of the residues energy in the plot is negative (Figure 28).

Table17: Summary of the Ramachandran Plot

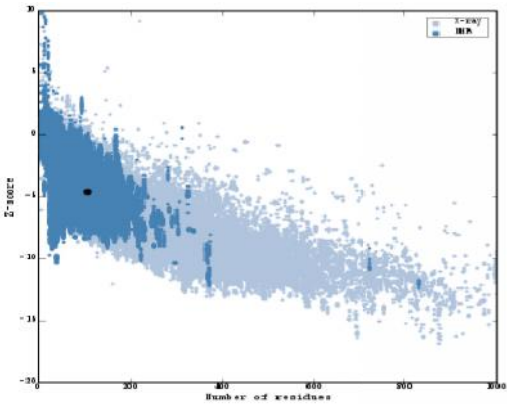
Variable Chain Seq.	Properties assessed	M2-Inaba	M4-Inaba
Light Chain (Fv)	**Core %**	81.1	81.8
	Allowed %	17.9	17
	Generous %	1.1	0
	Disallowed %	0	1 (Ala 51)
	No. of Gly	9	9
	No. of Pro	5	6
Heavy Chain (Fv)	**Core %**	81.8	89
	Allowed %	15.2	11
	Generous %	2	0
	Disallowed %	1(Arg 65)	0
	No. of Gly	13	12
	No. of Pro	3	4



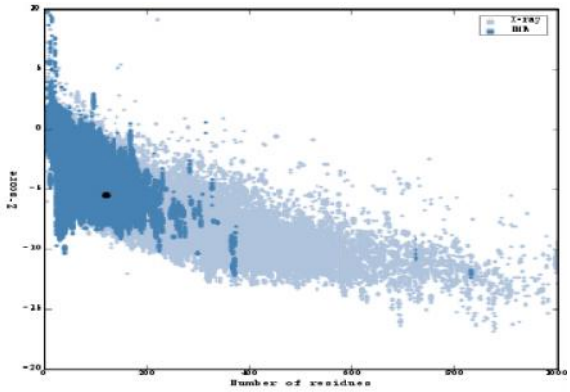
(a) Z-score (-4.41) of M2-Inaba L chain



(b) Z-score (-5.16) of M2-Inaba Heavy chain

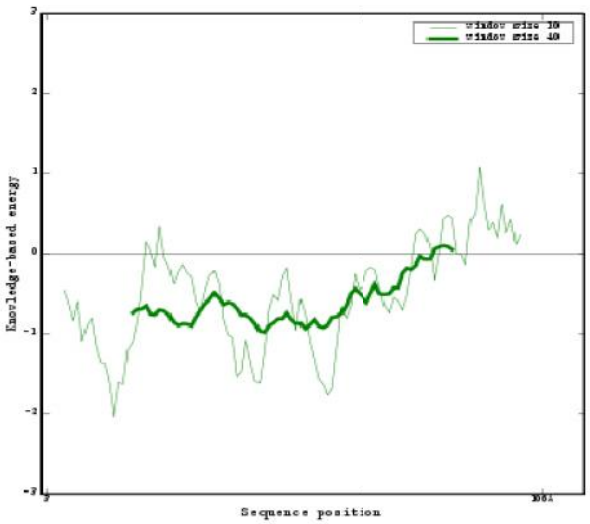


(c) Z-score (-4.64) of M4-Inaba L chain

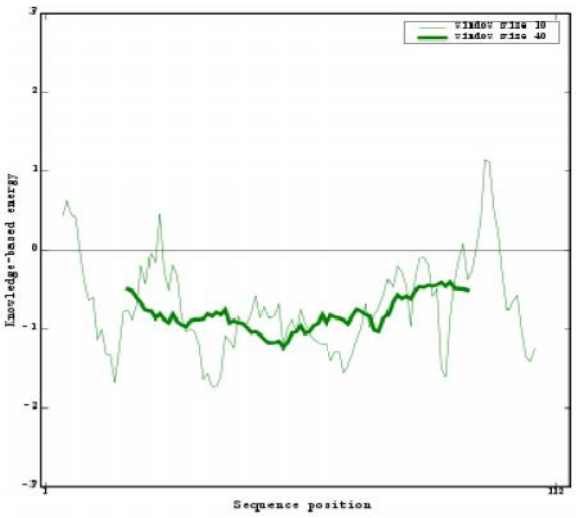


(d) Z-score (-5.51) of M4-Inaba H chain

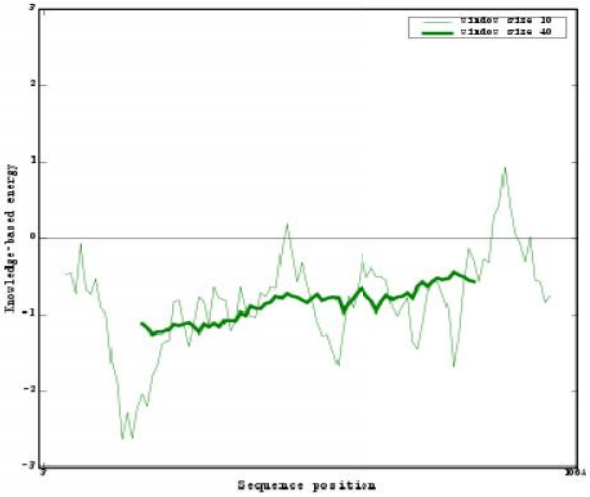
Figure27: Z-score (overall model quality, the more negative the Z-score, the better the quality of the model of both M2-Inaba-Fv and M4-Inaba-Fv. Light Blue and blue dots indicate the position of all the structure in PDB determined by X-ray crystallography and NMR respectively. Black dot indicates the position of our model which falls within the range of structures determined by X-ray crystallography and NMR.



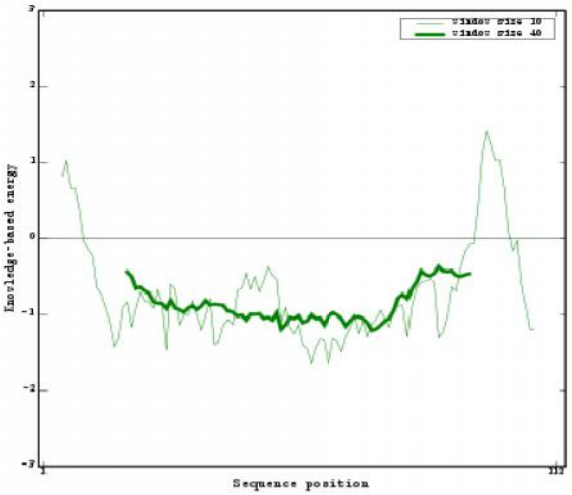
(a) M2-Inaba light chain



(b) M2-Inaba heavy chain



(c) M4- Inaba light chain



(d) M4-Inaba heavy chain

Figure28: Energy profile is shown of each residue for the M2-Inaba-Fv and M4-Inaba-Fv light and heavy chain calculated using ProSa-Web. All the graphs are smoothed using a window size of 40 residues. Note that most of the residues in the energy plot of 3D models are negative.

3.13 Interaction interface of the M2-Inaba Fv and M4-Inaba-Fv

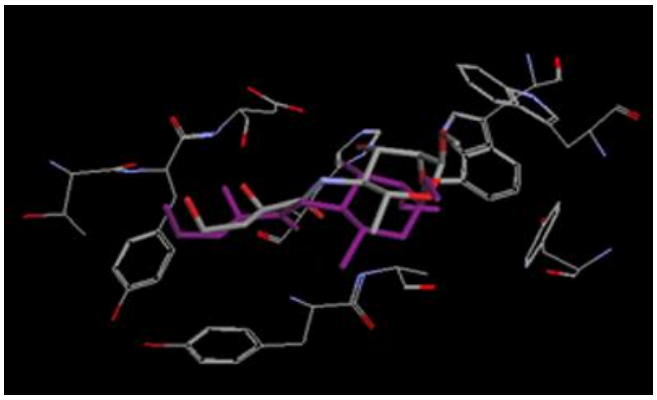
Program Promate predicted the amino acids that may act as interaction interface. Table18 shows that both heavy and light chain amino acids may be involved in interaction. It is interesting to note that most of these residues were located on the CDR region of the antibody structure. From this observation, we have designed the binding site on antibody which was used for further molecular docking of perosamine ligand.

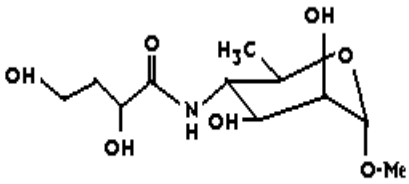
Table18: Residues predicted to be involved as interaction interface

Model	Residues Involved in interaction interface	
	Heavy Chain	**Light Chain**
M2-Inaba-Fv	33, 49, 50, 95, 96, 97, 98, 99, 100 , 101 , 103	32 , 33 , 34 , 48 , 49 , 50 , 51, 52 , 53 , 91 , 92
M4-Inaba-Fv	35 , 37 , 50 , 92 , 93 , 95 , 99, 100 , 101 , 102 , 103	32 , 34 , 50 , 87 , 89 , 90 , 91, 93 , 94 , 96 , 98

3.14 Molecular Docking of Perosamine with M2-Inaba-Fv and M4-Inaba-Fv

Before computationally docking the perosamine with the antibody models, we have tested the general ability of the docking program ArgusLab 4.0. For this test, Ogawa-specific monosaccharide (D-perosamine) was cut from the crystallographically determined structure of its complex with anti-carbohydrate antibody (S-20-4; PDB code 1F4X)(Villeneuve *et al.*, 2000) and docked back into the binding site. Comparison of the position of the perosamine in the crystal structure with the top ranked docking solution showed very good agreement, as implied by a RMSD value of 1.36 A° (Figure 29). Lowest energy conformation was found at -7.0495 Kcal/mol. After docking of perosamine with M2-Inaba-Fv, among top 150 docking solutions, the lowest energy conformation was considered as probable binding site.





(a) **(b)**

Figure29: (a) Top ranked solution of D-perosamine (Magenta color) docked into the binding site of 1F4X (13). Position of crystallographically determined D-perosamine structure is shown as grey. (b) Structure of Inaba-specific D-perosamine used in this study.

3.15 Binding mode of perosamine with M2-Inaba-Fv and M4-Inaba-Fv

In case of M2-Inaba-Fv (Figure 30), among the nine amino acids that form the ligand binding site, five of them are from the heavy chain and four from the light chain. Heavy chain amino acids taking part in interaction are Tyr 94, Gly 102, Ser 103, Ser104 and Val106. Hydrogen bonding interactions are formed between the Ser 103 H and –OH group of perosamine. Another H-bonding interaction is formed between the carbonyl group of perosamine with Ser 104 H. Light chain amino acids include Ser38, Tyr 55, Trp 56 and Glu 61. Amino acid Ser 38 is part of CDR1 (underlined) and Tyr 55, Trp 56 is part of CDR2 (Figure 33). In case of heavy chain, amino acids Gly 102, Ser 103, Ser104 and Val106 all are the part of CDR3. Another amino acid position Tyr 94 is adjacent to the CDR3 region. It's interesting to note that this amino acid position also forms the ligand binding site in case of M4-ZnM-Inaba-Fv (Figure 31). This consensus binding mode indicates that the presence of this residue at this position may have important immunological implication in carbohydrate antigen recognition (Figure 32).

In case of M4-Inaba-Fv (Figure 31), nine residues are involved in binding site formation. But six of these residues come from heavy chain. Light chain amino acids involved in interaction are Gln 38, Lys 39, Pro 40, Trp 41, Lys 42 and Ser 43. Three amino acids of heavy chain Gln 39, Pro 41 and Tyr 94 are involved in formation of binding site. First hydrogen bonding interaction is formed between the O-Me group of perosamine and the Gln 38 of light chain. This residue is

partially interacting with light chain Lys 42 and heavy chain Glu 38. Another hydrogen bonding interaction is formed between the perosamine ring and the light chain residue Pro 40. As both of these residues are conserved in three different sequences (Figure 33), these residues may be critical for antibody-carbohydrate recognition (Figure 32).

We have compared the sequence of crystal structure 2uyl (Ahmed *et al.*, 2008)(Figure 33) which represented the VH and VL sequence of monoclonal mouse antibody F-22-30 (IgG1, κ) to know the differences in binding sites. Crystal structure of a monoclonal antibody directed against an antigenic determinant common to Ogawa and Inaba serotypes predicted from molecular docking that perosamine side chain, is the only compound predicted to form a hydrogen bond with the Glu 39 of light chain (Ahmed *et al.*, 2008). In case of M4-ZnM-Inaba-Fv, (Figure 31) Gln 39 of the light chain is predicted to form several hydrogen bonding interactions with perosamine. On the other hand, the overall binding energy of perosamine with M2-Inaba-Fv (-6.35 Kcal/mol) and M4-Inaba-Fv (-7.22 Kcal/mol) is similar as predicted by the Program ArgusLab.

As we have observed the difference in the CDR and some other framework residues of 3 different Inaba-specific antibody sequences, this may be likely that the putative binding site of the 2uyl antibody may be different from two other (M2-Inaba-Fv and M4-Inaba-Fv) sequences. That's why molecular docking on the model of these antibodies shows some differences in the binding sites.

However, our preliminary molecular modeling and docking showed some consensus binding mode of perosamine with Inaba-specific antibody and some differences in the binding site residues due to some sequence variation among antibodies. Further studies on the antigen-antibody reactions particularly co-crystallization of Inaba-specific antibody with antigen is required to decipher the actual mechanism of binding.

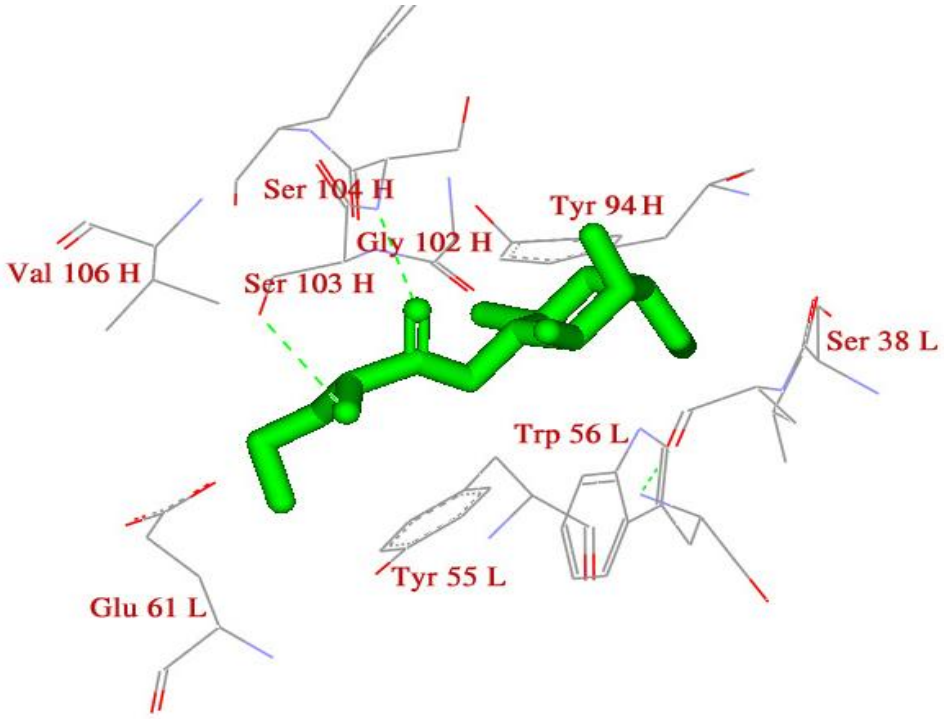


Figure30: Binding site of Perosamine on M2-Inaba-Fv.Binding energy was found -6.35 Kcal/mol.

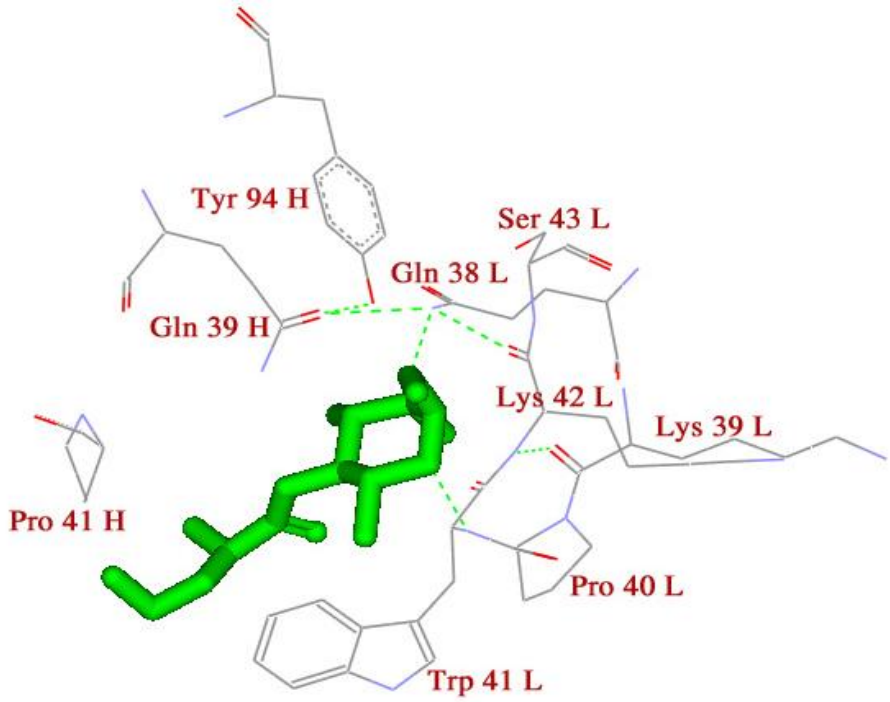
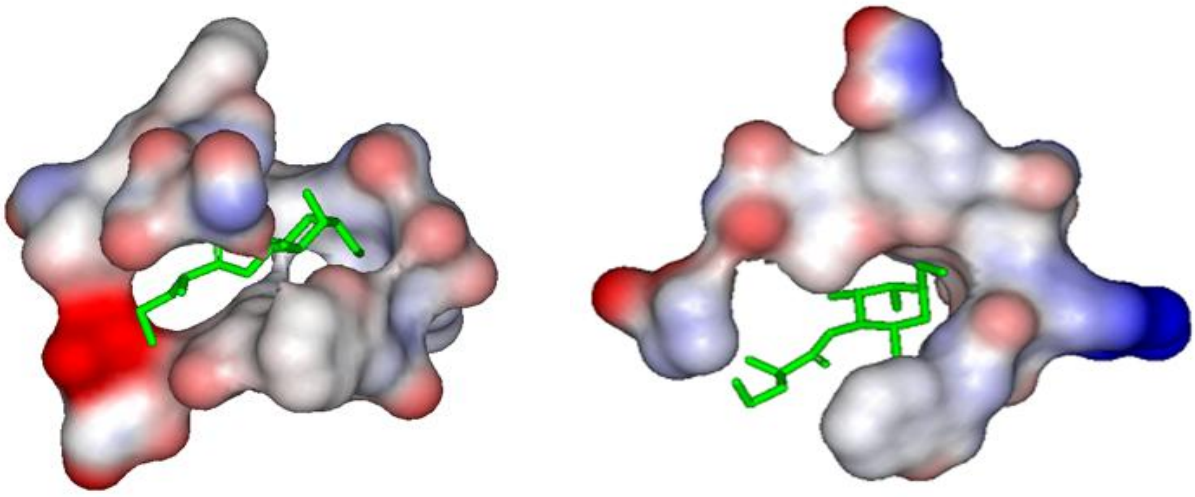


Figure31: Binding mode of perosamine with M4-Inaba-Fv. Binding energy is found -7.22 Kcal/mol.



(a) Binding pocket of M2-Inaba-Fv **(b) Binding pocket of M4-Inaba-Fv**

Figure 32: Predicted binding pocket of perosamine with M2-Inaba-Fv and M4-Inaba-Fv.

Light Chain:

```
2uyl                 ELVMTQTPPSLPVSLGDQASISCRSSQSIVHSNGD-TYLEWYLQKPGQSPKLLIYKVSNR 59
M2-Inaba-L-Chain     ELVMTQSPSSLAVSVGEKVTRNCKSSQRLLYNTNQKNSLAWYQQKPGQSPKLLIYWASXR60
M4-Inaba-L-Chain     ELVMTQSPSSMYASLGERVTITCKASQDIKS------YLSWYQQKPWKSPKTLIYYATSL 54

2uyl                 FSGVPDRFSGSGSGTDFTLEISRVEAEDLGVYYCFQGSHVPRTFGGGTKLEIK 112
M2-Inaba-L-Chain     ESGVPDRFTGSGSGTDFTLTISSVKAEDLAVYYCQQYYSYPLTLGAGTKLELK 113
M4-Inaba-L-Chain     ADGVPSRFSGSGSGSDFTLSINSVEPEDVGVYYCQNGHSFPFTFGSGTKLEIK 107
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Heavy Chain

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2uyl                  QVQLEQPGAELVKPGASVKLSCKASGYTFTSNWINWVKQRPGQGLEWIGH 50
M2-Inaba-VHV-IgG1     EVQLEQSGAELVRPGSSVKISCKASGYAFGTYWMNWVKQRPGQGLEWIGQ 50
M4-Inaba-VHV-IgG1     EVQLEQSGAELMKPGASVKISCKATGYTISSYWIEWIKQRPGHGLEWIGE 50


2uyl                  ISPGSSSTNYNEKFKSKATLTVDTSSSTAYMQLSSLTSDDSAVYYCGREE 100
M2-Inaba-VHV-IgG1     IYPGDGNTNYNGKFKDKVTLTADKSSRTAYMQLITLTSEDSAVYFCTK-E 99
M4-Inaba-VHV-IgG1     ILPGSGSTNHNEKFKGKATFTADTSSNTAYMQLSSLTSEDSAVYYCARGR 100

2uyl                  IVRASFGNWGQGTLVTVS 118
M2-Inaba-VHV-IgG1     VYGSSFWYWGRGTLVTVS 117
M4-Inaba-VHV-IgG1     VYNPWFAYWGQGTLVTVS 118
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Figure33: Multiple sequence alignment among 2uyl, M2-Inaba-Fv and M4-Inaba-Fv L and H chain.

4 Discussion

Attempts to control cholera through improvement of hygiene and socio-economic status have been sought for long but are difficult to attain. Therefore, attempts to develop an effective cholera vaccine is essential for prevention of the disease;but the role of immunity and of vaccination in the protection of man from cholera has been the subject of considerable debate(Pollitzer, 1959). As the first modern cholera vaccine, field trials (1960s-70s) of classical bivalent (Ogawa and Inaba) injectable killed whole cell cholera vaccine showed cholera-specific immune responses but later was discontinued with dispute of adverse reactions and partial protection(Kabir, 2005).

In a randomized, double-blind trial of cholera vaccine among the women and children in Bangladesh demonstrated the efficacy of B subunit whole cell vaccine (BC-WC) than whole cell vaccine only during the first eight month of observation(Clemens *et al.*, 1988a). Both of the vaccine conferred equivalent protection against less severe and severe cholera. While the protection was sustained for the children of more than five years old, it was transient for the children.The locally formulated B subunit void oral Vietnamese whole cell vaccine (ORC-Vax) showed a better performance in children (1-5 years);however, compared to the Bangladeshi trial the study was neither double-blinded nor placebo controlled with only 11% children participating of the total vaccines(Nagaraj & Babu, 1997).

Live attenuated vaccine containing the genetically modified *V. cholerae* O1 strain CVD 103-HgR gave only low levels of protection and also was poorly immunogenic among the socially less-privileged, immunity impaired people of Indonesia(Kortepeter & Parker, 1999), Peru (Gotuzzo *et al.*, 1993)and Thailand(Su-Arehawaratana *et al.*, 1992) and required an enhanced 10-fold increased dose of the vaccine for cholera endemic developing countries to induce levels of immunity similar to those observed in volunteers of USA(Richie *et al.*, 2000). Another live-attenuated oral *Vibrio cholerae* O1 vaccine candidate of the El Tor biotype and Inaba serotype CholeraGarde, also well known as Peru-15(Kenner *et al.*, 1995) displayed safety and immunogenicity profile in the USA(Cohen *et al.*, 2002) and in Bangladesh(Qadri *et al.*, 2005; 2007) but protective efficacy studies to customize into a single-dose vaccine has not yet been started. Altogether, no consensus on newer, killed (two dose) and later modified live (one dose)

WC cholera vaccines for oral delivery exists at present even after over 200 years on cholera vaccine research and there are now only two licensed and WHO prequalified whole-cell cholera vaccine in use at present.

Several other investigators have contributed to assess the efficacy of conjugate cholera vaccines which justify reevaluation of parenteral cholera vaccines, especially for young children with unresponsiveness to carbohydrate epitopes alone but respond well to carbohydrate epitopes linked to a protein(Kelly *et al.*, 2004) as observed for *Pneumococcus*spp. and *Haemophilus*spp. conjugate vaccines(Kelly *et al.*, 2005; Lausen *et al.*, 2004). With the removal of lipid A content of LPS, the antibody targets are carbohydrates which become T-dependent antigens when linked to carrier proteins as conjugates and are assumed to be immunogenic and ensure longer protection in young children(Kelly *et al.*, 2005; Lausen *et al.*, 2004). Conjugate cholera vaccines are composed of a LPS structure (carbohydrates from the O-SP or O-SP itself, the core and detoxified lipid A) with a carrier protein(Chernyak *et al.*, 2002; Gupta *et al.*, 1992). Alkali-treated LPS (Ogawa and Inaba) linked to *V. cholerae*cell surface proteins conjugate (Kabir, 1987a) followed by hydrazine or acid hydrolysis detoxified Inaba LPS coupled to CT conjugates(Gupta *et al.*, 1992) were immunogenic in animal model but inferior to native Inaba LPS. Another conjugate preparation of O-SP-core fragment of O139 LPS conjugated to tetanus toxoid although showed vibriocidal IgM and IgG titers and protection in the mouse model(Boutonnier *et al.*, 2001), the efficacy was not compared to native O139 LPS(Jertborn *et al.*, 1996). Moreover, there is ample chance of undetected toxicity of LPS to be harmful for human trial as observed for the parenteral WC-BS vaccine observed earlier.

Work with neoglyco-conjugates containing synthetic carbohydrate component of Ogawa LPS coupled to bovine serum albumin (BSA) known as Ogawa CHO-BSA conjugates demonstrated that the length of the linker component of the conjugates can influence the induction of vibriocidal antibody(Chernyak *et al.*, 2002; Saksena *et al.*, 2005; Wade *et al.*, 2006)and determined the terminal perosamine of Ogawa LPS containing a methyl group at the O-2 position binds protective antibodies(Villeneuve *et al.*, 2000). However, Inaba neoglyco-conjugates containing a terminal sugar with a hydroxyl did not function well as a protective epitope(Hisatsune *et al.*, 1993; Meeks *et al.*, 2004) and were immunogenic but did not induce

vibriocidal or protective antibody (Meeks *et al.*, 2004)necessitating further research on Inaba epitope determination.

Among the three antigenic determinants, the upstream terminal O-SP monosaccharide bearing the 2-O-methyl group has been shown to be the B antigenic determinant of the Ogawa LPS.The A and C antigenic determinants are present on both Ogawa and Inaba serotypes. However, only about half as much C antigenic determinant is present on Ogawa cells as on Inaba cells, whereas the A antigen is more abundant on Ogawa cells(Iredell *et al.*, 1998). Although Gustafsson et al. claimed on determining the C antigenic determinant to be solely in the Inaba serotype(Gustafsson *et al.*, 1982; Gustafsson & Holme, 1983; Gustafsson, 1984; Gustafsson & Holme, 1985), it remains a question of debate because the C antigenic determinant has been reported to be common to both the Ogawa and Inaba serotypes by studies of binding of the core and the O-SP of Ogawa and Inaba LPS to a monoclonal antibody (clone I-24-2) in ELISA(Villeneuve *et al.*, 1999). Moreover, two unpublished results; one with Inaba glycoconjugate formulation followed by immunization which showed vibriocidal negative Inaba specific mouse antisera and the other monoclonal antibody (clone 1G12) of NICED, India demonstrated Inaba ELISA negative, weekly vibriocidal agglutinating capability; were consistent with the existence of such an Inaba-specific antigenic determinant. Furthermore, the location and structure of the A antigenic determinant are unknown and the precise chemical structure of this putative Inaba-specific antigenic determinant could provide a rational basis for the development of Inaba glycoconjugate vaccine.

We carried out studies with ten monoclonal antibodies of IgM, IgG1 and IgG3 isotypes and one rabbit IgG sera specific to Inaba and Ogawa to select the best mAb through in vitro (agglutination titer, ELISA titer, immobilization activity, vibriocidal activity) and in vivo protection assay for crystallography and NMR studies. Based on the results of initial screening, clone F-22-30 which is of IgG1 isotype and specific to both *Vibrio cholerae* O1 Ogawa and Inaba serotypes was considered and analysed. Although homologous Fab crystal of F-22-30 was obtained,co-crystallization of Fab crystal with polysaccharide moiety (O-specific polysaccharide+core) of the LPS (pmLPS)Inaba followed by epitope mapping was not possible due to mass discrepancy (bigger size of pmLPS compared to small Fab crystal) but facilitated prediction on Inaba epitope.

The present study reports the first crystal structure of a monoclonal antibody that recognizes a common epitope present in both Ogawa and Inaba serotypes from *V. cholerae* O1. Modeling calculations and preliminary NMR results suggest that the binding pocket at the center of the antibody recombination site could bind the side-chain of a perosaminyl residue from the O-PS. This would be in agreement with the higher accessibility to antibodies of the O-PS homopolymer compared with the core-PS, and with the observation that antibodies specific for polysaccharides (such as those found in the core-PS) usually display a shallow antigen-binding site(Nguyen *et al.*, 2003; van Roon *et al.*, 2004). If the suggested model holds true, it would imply a lateral recognition of the O-PS antigen, in contrast to antibodies specific for the Ogawa serotype that recognize the terminal, 2-O-methylated, perosamine residue(Villeneuve *et al.*, 2000). However, further studies on antigen-antibody interactions are clearly required to define the actual epitope since the size and topology of the antibody surface would impose structural constraints on the LPS and must necessarily interact with other perosaminyl residues from the homopolymer and/or additional sugar moieties from the core-PS. If the tetronic acid moiety is part of the common A epitope of *V. cholerae* LPS, it is required to determine the particular stretch of perosamine residue on the O-SP polymer to be more effective at inducing protective antibody. In addition, it also explains the reason behind the better inducing effect of LPS with correct context and hierarchy of inductive perosamine epitopes compared to the existing LPS-based conjugates. Moreover, experiments with varied length conjugates need to consider the idea that subtle structural alteration may not allow binding of low affinity BCR and thus compromise B cell activation and the improper addition of a carrier might limit the accessibility of the relevant perosamine associated tetronic acid that is able to bind antibody.

Historically, cholera researchers have studied*V. cholerae* virulence factors identification and their regulation with the aim of developing an a universal and hopefully one dose cholera vaccine(Muse *et al.*, 2012). Despite of their demerits, oral and parenteral vaccines have offered some protective effect against cholera, efforts were dedicated mostly to upgrade the existing vaccine taking other confounders such as nutritional status, antigen presentation, strain selection, mode of cultivation, procedure of killing and laboratory assays in assessing protective qualities etc has been taken into account. The importance of other routes of vaccine administration in order to obtain maximum mucosal response in the gut have not been adequately studied(Finkelstein *et al.*, 1997). Recently, it has been appreciated for some time about the

expression of outer membrane associated protective antigens of *V. cholerae*, but new subunit or killed whole cell (k-WC) vaccines featuring OM structures have not been developed as alternatives to the oral cholera vaccines which are incapable to optimally express *V. cholerae* protective antigens(Ryan & Calderwood, 2000). Attempts weremadeto studytranscutaneous immunization route as an alternative vaccination route compared with subcutaneous and oral immunization routes and the probable implications of potential antigens; CT, CTB, TcpA, LPS and in-house prepared membrane extract.

When compared the three route of immunization, the subcutaneous route showed its usual elevation pattern in mice systemic compartment than other two routes as expected.IgG isotype showed superiority followed by IgM and IgA respectively in accordance with established immunological principles which was also established in the former vaccine trials when tested injectable parenteral WC vaccines(Mosley *et al.*, 1970). Immune response to TcpA is known to be generated after natural oral infection(Asaduzzaman *et al.*, 2004) or challenge with the *V. cholerae* O1(Hall *et al.*, 1991). Humoral responses can be dependent on both the route of immunization and the nature of the immunogen as it was observed that TcpA when immunized in the TCI route did not up regulate IgA isotypes. Using the TCI route also significant increases of anti-TcpA responses were seen in blood in the IgM and IgG isotypes. The response to TcpA when TCI immunizations are carried out as observed in this study and an earlier report (Scharton-Kersten *et al.*, 2000) are similar. However, IgA titer increased in the ORI route demonstrating the function of local immune system.

However, following TCI administrations, the response to CT was seen in all the three antibody isotypes including IgA. We observed a difference in kinetics in TCI route compared to the other methods where a longer immunization scheme was needed to generate the highest magnitude of response. It is interesting to note that IgA responses with oral immunization validate the GALT responses for individual immunization and co-administration. Moreover, only CTB immunization in mice with different feed supplementation showed the similar pattern or response as noted in human subject and other studies with mice(Elson & Ealding, 1984; Northrup & Fauci, 1972; Pizza *et al.*, 2001). In contrast, co-administration with CT and TCP overcomes the impact of feed supplementation and induced immune response differently compared to CTB only for the IgM isotype while secondary immune responses with IgA and IgG isotypes showed no

phenotypic changes. In addition, CTB up-regulated the optimum response over time with multiple doses and were comparable with responses through co-administered immune responses. Moreover, zinc might have influence only on CT or CTB but not on TcpA. This requires designing further studies to confirm these findings.

Comparing the three different immunogens, mice immunized with CT in the TCI and SCI route were seen with all four IgG subclasses while TcpA and LPS-specific responses were selective with only IgG1 subclass. It confirms that TCI application for all the three immunogens induced the IgG1 subtype as the highly elevated one which was also seen with SCI route of administration. Since both CT and TcpA are protein antigens, this is to be expected but membrane extract has overcome the inherent limitation of LPS. In an earlier study, we have shown that natural cholera disease also leads to responses in different antibody isotypes and subclasses but the IgG1 is the most prominent(Qadri *et al.*, 1999). On the other hand, antibody response pattern with CT and CTB indicates polyclonal stimulation of the B cell pool capturing the whole immune system which may lead to unprecedented response in relation to the aim of vaccination.

However, sera obtained from both transcutaneous and subcutaneous immunization schemes were negative for vibriocidal antibody responses and this is expected since the major antigen/antibody type is the LPS antigen(Qadri *et al.*, 2003a). Mice, when immunized with membrane extract, displayed the vibriocidal capabilities with more than 10 times elevated IgM and IgG titers than LPS-specific responses. This indicates the extreme potentials of membrane extract compared to LPS alone.

The membrane extracts immunization with both zinc supplementation and depletion showed significant elevation of Inaba-specific IgM and IgG titers in the supplemented group which could be due to the definitive influence of natural conjugate and other outer membrane proteins. Moreover, the positive effect of Zn supplementation was observed for both Inaba LPS and membrane extract specific IgM responses as also demonstrated for Ogawa specific responses. Zinc treatment also has a beneficial role in ETEC induced acute watery diarrhea(Glenn *et al.*, 2000) as well as in adults(Gustafsson & Holme, 1985) or children given oral cholera vaccine, Dukoral(Gockel *et al.*, 2000). While this is not a salient feature of LPS, there is speculation that

LPS-bound to normal protein constitutes of *V. cholerae* can act as a natural conjugate and divert the LPS to a T-dependent response during the course of infection(Provenzano *et al.*, 2006).

To establish a better correlation between the humoral immune response with in situ produced antibodies from the immunogen specific B cells, ALS antibody titers were comparable and expressed the recently formed status of the humoral response. This assay specifically validated the potential of the immunogens in stimulating the immune response purposive to the vaccination strategy. The assay has been validated for natural cholera immune responses in patients in previous studies(Qadri *et al.*, 2003b).

LPS as a protective immunogen has been established since long. Recently, new attempts have been taken in evaluation of outer membrane component demonstrated some excellent initial observation in relation to protection. These components to be added synergistically with LPS as the potential protective immunogens are alternative approaches to cholera vaccine research. Membrane extract prepared using the'in house' protocol is the crude form of immunogen which contains all outer membranes components based on the culture conditions along with LPS and can be an inexpensive representative of immunogens.

Immune responses to cholera infection as well as vaccines have revealed antibody mediated protection mechanism(Qadri *et al.*, 1999) through Th2-regulated synthesis of interleukin-4 or interleukin-5 (Losonsky *et al.*, 1996; Marinaro *et al.*, 1995) of which CD19 has been found to play a pivotal role as a response regulator for the interaction of T and B cells. Study in CD19-deficient mice suggests that CD40L-CD40-interactions are more important for Th2 compared to Th1 co-ordinated B-cell differentiation(Gardby *et al.*, 2001). Our results showed an increased expression of CD19 positive immunocytes in blood and lamina propria in both TCI and SCI immunized mice. Although, CD3 did not increase at any of the site of the immune compartment studied after the TCI as well as SCI immunization, the CD4 subpopulation increased in the lamina propria which could be the direct migration route from the skin to the gut mucosa as shown for APCs by the application of LT on the skin(Belyakov *et al.*, 2004). In spite of the strong stimulatory effects of CT, it has been shown that CT is a potent inhibitor of both mitogen and antigen-induced T cell activation both in vitro and in vivo(Elson *et al.*, 1995; Flach *et al.*, 2005).The CD8 subpopulation did not show an increase including the gut corroborating with other results obtained with CT(Elson *et al.*, 1995; Flach *et al.*, 2005). The dendritic cell

population increased only in the spleen in the subcutaneously immunized mice but not in the TCI groups. Previous studies have demonstrated a potent T cell priming capacity of dendritic cells(Banchereau & Steinman, 1998; Porgador *et al.*, 1998) although at variance with the effects observed on epidermal DCs, there were no appreciable changes in the frequency of DCs in draining lymph nodes (DLNs) after TCI with either CT or CTB at all-time points (2-24 h) examined(Anjuere *et al.*, 2003). However, the addition of immuno-stimulating agents at the site of antigen administration should provide the necessary activation signal for the DCs to mature, express high levels of co-stimulatory molecules, secrete cytokines, and become potent antigen-presenting cells (APCs) capable of priming immune responses to the co-administered antigen (Belyakov *et al.*, 2004).

Engagement of CD40 on B cells by CD40 ligand (CD154) expressed by activated T cells is a critical interaction that occurs during T-B cells collaboration and initiates many steps of B cell activation, including induction of proliferation, differentiation, and immunoglobulin production (Cerutti *et al.*, 1998; Van Kooten & Banchereau, 1996). The findings revealed increased expression of the co-stimulatory marker CD40 in the CD19 cell population in the lamina propria with increases in the spleen and blood for both TCI and SCI immunized mice. In the absence of CD154 expression, there is no information of germinal centers in secondary lymphoid organs and lack of generation of memory B cells that recognize T-dependent antigens(Agematsu *et al.*, 1998; Kawabe *et al.*, 1994). The CD154 expression was increased in CD3 and CD4 subpopulation in the spleen, lamina propria and blood in the TCI as well as the SCI mice showing an effective T-B cooperation in response to stimulation with CT and TcpA.

The CD19 positive B cells are known to upregulate the co-stimulatory molecules B7.1 (CD80) and B7.2 (CD86) which indirectly influence T- and B-cell interactions(Kozono *et al.*, 1998). These CD80 and CD86 positive cells interact with CD28 and deliver co-stimulatory signals required for T cell activation(Jaffar *et al.*, 1999). Although an upregulation was not seen in the TCI route, an increase of the B7.1 was seen in blood in the subcutaneously immunized mice. No increase of the B7.2 (CD86) expression on CD19 cells was observed in any of the two groups. Greenwald et al. (Greenwald *et al.*, 1997)have demonstrated that either CD80 or CD86 ligand interactions can provide the required co-stimulatory signals that lead to T cell effector function during a type 2 mucosal immune response in mice following nematode infection. These

responses were not seen in any compartment of the TCI group. Previous studies showed an increase in levels of CD40 in both CD11c+ (CD11c+CD40+; 1.4%) population and the CD11c-CD40+ (27%) population than control mice in Peyer's patches with unchanged expression of CD86 after TCI in mice with LT (Banchereau & Steinman, 1998).

The co-stimulatory signal mediated by CD28 is expressed on CD4+ and most CD8+ T cells and is required for the activation and production of various cytokines, including IL-2(Thompson *et al.*, 1989) which has positive influence on the production of the Th2-type cytokines IL-4, IL-5 and IL-10 and of the Th1 cytokine IFN-γ(Acuto & Michel, 2003; Kuiper *et al.*, 1994; Riley & June, 2005). In our study, CD28 expression on CD3 and CD4 cells showed significant elevation in the spleen in the TCI and SCI showing that the major virulence antigens of cholera do indeed signals which lead to production of immunomodulators for further enhancement of the responses.

Tissue-specific homing is believed to control the immune responses and accounts for the regional compartmentalization of immune system functions(Butcher & Picker, 1996). Memory T cells in inflamed skin express the cutaneous lymphocyte-associated antigen (CLA), a glycosylated epitope defined by the mAb HECA-452(Berg *et al.*, 1991). CLA occurs almost exclusively on the protein backbone of P-selectin glycoprotein ligand-1 (PSGL-1) on activated T cells(Fuhlbrigge *et al.*, 1997), circulating neutrophils and monocytes, including cultured blood dendritic cells and tether and roll on both E- and P-selectin along the vascular endothelium (Kieffer *et al.*, 2001). On the other hand, lymphocytes expressing the integrin α4β7 bind the mucosal vascular addressin MAdCAM-1(Andrew *et al.*, 1994; Berlin *et al.*, 1993)selectively expressed on HEV in mesenteric lymphnodes, intestinal lamina propria and Peyer's patches (Bargatze *et al.*, 1995) allowing the selective homing to both intestinal secondary lymphoid tissue and intestinal effector sites(Scala *et al.*, 2005). Additionally, L-selectin (CD62L) deficiency in mice or treatment of wild type animals with mAbs to L-selectin or peripheral node addressin (PNAd), the high endothelial venule (HEV)-specific ligand for L-selectin, resulted in impaired lymphocyte homing to peripheral lymph nodes(Scala *et al.*, 2005). Little is known about the development of these tissue-specific lymphocyte subsets. Expression of specific homing phenotypes may be directed by specific microenvironments within cutaneous versus intestinal secondary lymphoid organs during initial T cell activation(Picker *et al.*, 1993). In the

context of topical delivery, the APC, micro-environmental signals, and/or adjuvant may promote T cells to modulate homing receptor expression with specificity for a wide range of anatomical migratory destinations (Banchereau & Steinman, 1998). In this study, we examined the expression of homing receptors CLA, β7 and CD62L in spleen, blood and lamina propria. The CLA marker was elevated in spleen and lamina propria on CD19 positive cells as well as blood, spleen and lamina propria on CD4 positive cell populations. No increase was observed for the gut homing receptor, β7 on CD19 population in any of the three compartments. However, significant increase of CD62L expression in the CD3 and CD4 population was observed in the lamina propria in TCI and in spleen and lamina propria in SCI mice but the gut homing receptor β7 did not show increase in the any of the subpopulation in the transcutaneously and subcutaneously immunized mice. A previous study observed that after topical application of LT, a majority of T cells present in the skin DLNs express the peripheral lymphocytes homing markers β1 integrin and CD62L, while a small population of T cells express the mucosal homing marker α4β7+. CT may act as a mucosal adjuvant for immune cells after TCI as presumed for LT to provide specific signals to DCs in the DLNs to activate the expression of mucosal homing receptors on resident naïve T cells(Belyakov *et al.*, 2004). These results match with the findings that effector lymphocytes often function in nonlymphoid sites of inflammation and infection. Moreover, effector and memory lymphocytes generally display selective tropism for specific peripheral tissues such as the skin or intestinal lamina propria(Butcher *et al.*, 1999).

B cells express highly antigen-specific cell surface receptors (BCR) that bind to native, non-processed antigen. Binding of labeled antigen to B cells has been used previously as a strategy to identify these cells. Analysis of the complex B cell responses induced to pathogens has been attempted to carry out enumeration and characterization of virus-specific B cells by multicolor flow cytometry(Doucett *et al.*, 2005). Our results clearly demonstrated the presence of TcpA-specific cells in different compartments as the representative of effector cells in the transcutaneously immunized mice. Further investigations are needed including other antigens like LPS or mannose-sensitive haemagglutinin (MSHA) to test them individually or in combination with others using the transcutaneous route to get an optimal cholera vaccine.

Attempts were made to determine the percentage of immunogen specific cells at different compartments which to be involved in generating antibodies for protection. Interestingly, the

CTB-specific cells represented the major percentages of the total immune cells. This huge pool or antibody secreting cells if shows beneficence would have greater impact. However, the role of CTB and CT in cholera vaccination still remained questionable(Fujihashi *et al.*, 2002; Pizza *et al.*, 2001).

The dissertation reports the first extensive determinations of the molecular solution of the murine B-cell VH and VL chain repertoire specific for protein (CT, CTB, TcpA individually or in combination) and polysaccharide (membrane extract containing Inaba or Ogawa LPS) immunogen. Major vaccine potential cholera immunogen specific both μ and γ1 Fv sequences were obtained from immunogen-specific splenocytes using novel *in situ* positive selection procedure bypassing the conventional mAb generation technique. In depth analysis of these sequences might facilitate to explain the known *in vitro* and *in vivo* serological characteristics of antibody isotypes with protective and non-protective criteria as a means to understand why children do not respond well with the existing vaccine in cholera endemic regions.

There is evidence of difference in the antibody molecule was observed between CTB and CT in both μ and γ_1VH sequences. This might have implications in differentiating the overall immunological responses differ between vaccination and disease conditions. It was noted that majority of CTB and all TcpA immunized γ_1 and LPS immunized mice μ and γ_1 sequences were represented with VH1-VJ558 family sequences except CT immunized mice γ_1 sequences were of the VH9-VGAM3.8 family. The VH1-VJ558 gene family might have contribution in protection from cholera. Other 17 monoclonal antibodies generated against *V. cholerae* O1 showed VH5 (7183) and VH1 (J558) as the predominantly expressed families in C57BL/6 and BALB/c mice(Johnston *et al.*, 2006; Riblet *et al.*, 1986). Diversity and Junctional genes were also observed with isotypes specificity. Likewise, humans are not capable to make all possible protective Abs with equal efficiency, nor are all selected Abs protective as observed in rotavirus infection to induce infant (< 2 years old) B cells which are predominantly $CD5^+$ use VH3-23, while $CD5^-$ B cells use VH1 and VH4 with VH1-46 was associated with the immunodominant response in $CD5^-$ B cells from children and adults(Wade, 2009).

With the immunization of CTB only attempts were carried out to sequence CF, ZnM and ZnP feed supplemented and obtained CTB-specific μ and γ1 sequence. Although no distinct

difference in antibody responses were seen among the three groups, diversity in expression was observed among the sequences gel electrophoresis with specified amount of PCR product demonstrated the negative effect of Zn supplementation on CTB which expressed similar results as observed with human volunteers(Qadri *et al.*, 2004a).

In the primary sequence, three intervals of CDRs are separated from each other by four relatively conserved framework regions (FRs) (Hood & Galas, 2003). When CTB-specific μ and γ_1 chain VH sequences were aligned (result not shown), some of the amino acids in the μ chain were identical in the FRH1, CDRH1, FRH2, CDRH2 and FRH3. Although the sequences were sub-divided based on three groups upon feed supplementation, this is the first information on heavy chain variable region to define 28 positions (06, 09, 18, 20, 27, 30, 38, 42, 49, 62, 64, 67-74, 76, 77, 79, 81, 83, 85, 88, 93, 95) to be responded during class-switching during transition to γ_1 VH chain. This observations confirmed that all residues in variable domains may participate in the rigidity rather than the limited number of CDR residues as confirmed by previous thermodynamic experiments and molecular docking simulations studies (Jimenez *et al.*, 2003; Thielges *et al.*, 2008; Zimmermann *et al.*, 2010).

Moreover, feed supplementation has major impact in both μ and γ_1 chains to express amino acids in relation to the Zn depletion and supplementation. Among VH μ chain sequences, significant differences were found at various positions with the differences in CTB only and CT immunization which might explain the intrinsic difference in adjuvant effect between CTB and CT(Elson & Ealding, 1984; Northrup & Fauci, 1972; Pizza *et al.*, 2001). However, no significant changes in expression were observed between the CTB only and TcpA-CT immunization group derived sequences but zinc supplementation showed major impact on CTB only immunization derived sequences than TcpA-CT immunized sequence group. However, it was not possible to compare the expression of zinc supplemented CTB specific sequences with the similar LPS-specific sequences due to inability to obtain LPS immunized zinc supplemented μ chain sequences. Moreover, TcpA specific μ and γ1 VH chain sequences showed variability in expression of amino acids which is not similar to the positions observed for CTB only sequences. Among the TcpA only and TcpA-CT immunized sequences, individual sequence diversity was prominent in both groups, differences in expression was observed between zinc supplemented

and depleted TcpA specific sequences while disparities between TcpA only and TcpA-CT co-administration might lead to more diversity than individual immunogen.

The general shape and sequence composition of the antibody combining site do influence the class of antigen to which the antibody binds and in prediction of antigen class(Collis *et al.*, 2003). When compared, all the sequences derived from immunization of the three major immunogens, co-administration has effect in expression differently than individual immunogen specific sequences. Amino acids expressed through immunization of polysaccharide immunogens were more stable than sequences originated through immunization of protein immunogens during recombination from μ to γ1. Although LPS O1 Ogawa and Inaba specific sequences showed strong relatedness, protein immunogens, CTB and TcpA showed partial similarity in expression of amino acids at different positions. In the FR-H1, hydrophobicity increased with replacement of amino acids among the LPS specific sequences at positions 9, 16, 20 and 13 while protein immunogens were seen with either hydrophilic or charged amino acids. Sequence comparison of CDR-H1 displayed variability in the protein/ polysaccharide specific μ to γ1 transition at positions 28, 31, 33 and 35. More specifically, protein/polysaccharide specific expressions were completely different at position 30 while hydrophobicity increased in LPS and TcpA at position 34 during CSR from μ to γ1. Considering FRH2, most of the protein immunogen-specific sequences displayed changing of amino acids while LPS-specific sequences in only two positions; 43 and 50. Expression ofhistidine at position 43 in TcpA and LPS specific sequences might have significant role as noted interaction of F-22-30 with ligand in NMR assay(Ahmed *et al.*, 2008). Moreover, TcpA and LPS-specific sequences responded differently than CTB at position 50 as observed predominantly expressed negatively charged amino acids among polysaccharide-specific sequences at position 50. In the CDRH2, shifting from hydrophilic to hydrophobic and charged amino acids observed among only the LPS specific sequences at positions 52 and 60. In contrast, hydrophobic and negatively charged to hydrophilic amino acids was seen in the protein immunogen specific sequences at position 54 but hydrophilic to hydrophobic at position 56. Interestingly, TcpA only and LPS immunized sequences were stable with hydrophobic amino acids at position 54 while LPS specific μ and γ1 sequences displayed charged amino acid at position 55. Moreover, protein-polysaccharide specific sequences were different in expression is about seven of ten positions and co-administration

expressed opposite to CTB only specific sequence. However, more than 50% of the FR-H3 positions of LPS Inaba and Ogawa specific sequences displayed similar amino acids and remained unchanged in the μ and γ1 chain sequences with the predominance of hydrophilic amino acids. Co-administration has minimum or no effect in this region.

When assessed the percentage of similarities in expression among the three immunogen specific sequences considering 1-95 positions comprising the FR-H1 to FR-H3 regions of the VH chain, 62 positions were found to be considered for comparisons. About 35 of 62 (57%) positions were captured by identical amino acids in LPS-specific IgM and IgG sequences as also observed in the TcpA and CTB specific γ1 sequences while dissimilarities in expressions were observed only in 10 of 62 (16%) positions. Moreover, replacement to identical amino acids occurred only in the FR-H1 (13, 16) and FRH3 (62, 70, 95) while disparate expression between protein and polysaccharide immunogens were in 09 positions of all the five regions (FRH1- FRH3) in transition from IgM to IgG1 sequences. Absolute diversity noted only in the CDR-H2 region involving three (55, 57, 59) of the 62 positions. When compared between CTB only versus LPS-specific sequences, 10, 11 and 39 positions of the μ chain and 08, 43 and 09 positions of the γ1 chain among the evaluated 60 positions represented identical, in-between and diverse expressions respectively. Similarly, TcpA-CT immunized CTB specific sequences aligned with LPS specific sequences correspondingly showed 15, 05 and 40 of the μ chains and 09, 26 and 25 of the γ1 chain sequences as identical, in-between and diverse expressions. As a result, CTB specific IgG1 sequences of both individually and co-administered groups displayed more relatedness than IgM sequences which indicates that CTB-specific sequences expressing similar amino acids upon transition. However, when considered, the expression pattern of LPS and TcpA-specific sequences, the similarity and relatedness is more prominent between TcpA and LPS than CTB as observed LPS versus TcpA only specific γ1 sequences with 09, 40 and 11 positions along with TcpA-CT immunized TcpA-specific μ and γ1 with 12, 41 and 06 as well as 20, 22 and 18 as identical, in-between and diverse respectively with the average diversity calculated was only 19% for TcpA compared to 45% with CTB-specific sequences.

When considered VL repertories of humans and mice, a considerable difference in the relative proportion by which κ and λ type chains are present(Almagro *et al.*, 1997). In humans, roughly 60% of the VL repertoire is κ type(Klein *et al.*, 1993; Tomlinson *et al.*, 1995; Williams *et al.*,

1996) while mice sequences represent as much as 95%, κ type chain(Hood *et al.*, 1967) and hence this chainwas selected for sequence analysis. In the study, light chain gene family distribution showed diversity which entails the sequence heterogeneity with individual immunogens and co-administration. Moreover, co-administration of immunogen triggered increased variability as observed extensive distribution of sequences generated through TcpA followed by CTB immunization. However, Ogawa and Inaba LPS-specific sequences were limited to mostly V8 followed by V-9/10 and V21 respectively. In the relatedness analysis, V1 and V8 gene family sequences showed close resemblance and hence could be involved in similar type of binding pattern. Most importantly, CDR-L1 has significant role in the effector functioning for which V1 and V8 family sequence were seen with 11 and 12 amino acids respectively. In contrast, V-9B and V38c family sequences displayed relatedness but variable to V1 and V8 gene family sequences. CTB and TcpA-CT immunized CTB specific sequences showed variability in length containing 8 to 19 amino acids in the CDR-L1 region. Another study inmouse showed that CDR-L1 is longer for the more elongated antigens (peptides and nucleotides) but the trend is not clear in human antibody sequences which may be the result of the small amounts of data analyzed in the study(Collis *et al.*, 2003).

However, analysis of antibodies of known three-dimensional structure shows that the shape of the antigen-binding site is primarily determined by the canonical structures of H2 and L1 for specific classes.On the contrary, in multi-specific classes, the first level of antigen-antibody recognition would seem to be mainly determined by different H3 conformations with an effect of changes in the general geometry of the antigen-binding site(Vargas-Madrazo *et al.*, 1995). The fine adjustment for an increased affinity of the antigen-antibody interaction in both cases should be contributed by a complex combination of other factors such as: (1) the side chains of the residues at the antigen-binding site(Alzari *et al.*, 1990; Mian *et al.*, 1991; Padlan, 1994); (2) variable relative disposition of VH:VL domains (Davies & Metzger, 1983; Padlan, 1994) and (3) conformational rearrangements of the antibody in response to the ligand binding(Colman *et al.*, 1987; Padlan, 1994; Wilson & Stanfield, 1994).

Among the six CDR loops, the greatest challenge is predicting the conformation of CDR-H3, which is the most important in antigen recognition. Immunoglobulin junctional (combinatorial and somatic) diversity through the inclusion of a D gene segment and the addition of non-

germline-encoded nucleotides (N regions) is concentrated in the CDR-H3, which often plays a dominant role in antigen binding(Zemlin *et al.*, 2003). When combined with the other five complementarity determining regions, the enormous length variation of CDRH3, together with amino acid substitutions in their sequences, can provide a very large number of antibody specificities and can influence the shape of antibody combining sites. Length distributions of mouse sequences with and without known specificities are centered around a peak of 7 to 12 residues with very few contained 18 and 19 sequences while human sequences vary from 2 to 26 amino acids residues among which CDRH3 containing more than 19 sequences with known specificities of polyreactive autoantibody causing autoimmune diseases(Wu *et al.*, 1993).The length of CTB-specific CTB only and TcpA-CT sequences varied from 8 to 16 amino acids among the IgG1 sequences while those of IgM varied between 10 and 19 amino acids. Likewise CTB-specific IgM and IgG1 sequences, the length of TcpA only and TcpA-CT immunized TcpA specific sequences varied from 8 to 16 amino acids among the IgG1 sequences while those of IgM sequences showed consistent length of 10-11 amino acids although there were only two IgM sequences which could be unreliable to confirm on CDR-H3 length. In contrast, Inaba and Ogawa specific sequences presented a more consistent CDR-H3 length within 11 or 12 amino acids. As a result, unlike CTB sequences, only TcpA immunization resulted in IgG1 sequences showed restriction of CDRH3 length while TcpA-CT immunized sequences displayed longer length from 08-16 which might generate variable conformation in active form. Moreover, Inaba immunized mice VH displayed maximum similarities in length and even with the type of expressed amino acids which confers its similar binding pattern during effector function.

As observed in a number of studies, CDR-H3 plays a crucial role in repertoire selection processes(Keyna *et al.*, 1995; Kline *et al.*, 1998; Martin *et al.*, 2003). In humans, B cell development is linked with a reduction in the distribution and mean length of the expressed CDR-H3 repertoire(Shiokawa *et al.*, 1999), and loss of highly charged or hydrophobic sequences (Raaphorst *et al.*, 1997; Schroeder *et al.*, 1998; Wardemann *et al.*, 2003). However, the loss of longer sequences along with enrichment of charged amino acids to be reflected with a higher likelihood of self-reactivity in the Igs that bear them(Wardemann *et al.*, 2003).

There is evidence of close resemblance in the CDR-H3 repertoire of splenic transitional and mature follicular (FO) B cell to that of immature and mature bone marrow B cells respectively. Splenic transitional cell to mature FO development was characterized by the reduced representation of highly hydrophobic, highly polar (charged), and short CDR-H3 sequences, supporting the view that the final focusing of the repertoire occurs in the periphery, likely as a result of exposure to antigen and support the hypothesis that entry to the various peripheral B cell compartments is influenced by ligand selection(Dammers *et al.*, 2000; Gu *et al.*, 1991; Levine *et al.*, 2000; Martin & Kearney, 2000) and further suggest that selective pressure may be exerted at the level of categories of antigenic epitopes(Schelonka *et al.*, 2007). Studies of others have also indicated about the existence of self- or poly-reactive B cells in the marginal zone in both mice and rats(Chen *et al.*, 1997; Dammers & Kroese, 2005; Qian *et al.*, 2004; Witsch *et al.*, 2006). In the present study, a length of 8 amino acids sequence 'ARWDAMDC' and 'ATTSTEGY' were present in the CTB only and TcpA immunized splenic B cells. When the CTB-specific sequence displayed predominance of charged and hydrophobic residues in the CDRH3, TcpA immunization derived CDRH3 sequence showed marked difference with the presence of polar hydrophilic and neutral residues. This observation indicates that CTB might be involved in the generation of self and polyreactive antibodies as also demonstrated polyspecific recognitionof an anti-cholera toxin antibody Fab in complex with an epitope-derived D-peptide(Scheerer *et al.*, 2007). Moreover, several studies have demonstrated at least five or six unique epitopes to be present in CTB and LTB(Belisle *et al.*, 1984; Holmes & Twiddy, 1983; Ludwig *et al.*, 1985; Tamplin *et al.*, 1989) which generally induces the generation multiple antibodies with various CDRH3 composition. While changes in amino acid utilization as a function of CDR-H3 length followed similar trends in both species, murine and human CDR-H3 intervals of identical length were found to differ from each other despite the presumed need to recognize a similar range of antigen epitopes(Zemlin *et al.*, 2003). Hence, further studies with human CDRH3 repertoire can demonstrate clearly distinct amino acid composition and predicted range of structures than murine counterpart yielding various sets of paratopes that would interact preferentially with epitopes that are associated with either T-dependent or T-independent responses. These observations urge to rethink on using CTB as potential immunogen in the vaccine formulation.

The strategy of using carbohydrate immunogens from several bacteria (*Pneumococcus* spp. and *Haemophilus* spp.) to yield effective vaccine constructs which prime for protection against future infection has been established(Kelly *et al.*, 2005; Lausen *et al.*, 2004). Many sequences representing individual/separate clones were derived from individual mouse immunized after immunization with Inaba membrane extract. Although M2, M5 and M4-ZnM mice splenocytes containing immunogen specific B cells were isolated with Inaba and Ogawa-biotin-streptavidin method, it is interesting to note that some of the Ogawa and Inaba specific sequences of the μ and γ_1 variable region were identical in expression. Although there were disparities in expression, shifting of amino acids was seen through transition from IgM & IgG1. This also indicates the involvement of more than one antibody molecule to be involved in effector function which might be beneficial or detrimental in terms of competition in the B cell pool. Previous studies showed that antibody targets on LPS could incorporate O-SP, the core, the core-O-SP boundary or Lipid A as the potential source of protective epitope(Chatterjee & Chaudhuri, 2003). Both end-binders and groove-binders to be present in the Fab structure of the two general types of anti-carbohydrate antibodies(Padlan & Kabat, 1988) which comprises the structure of the antibody-antigen binding site for a particular carbohydrate array based on length and on the geometry of the sugars. End-binders Fabs display the antigen combining site as pockets that accommodate a limited number of defined sugars found at the ends of the polysaccharide as determined for anti-Ogawa antibodies to interact with the terminal sugar of Ogawa LPS(Villeneuve *et al.*, 2000). In contrast, groove-binders Fabs bind an array of sugars in an extended conformation, usually the upstream and downstream residues with an impact on the conformation of the B cell epitope. Thus, while the number of sugars bound by a groove-type antibody may be 4 to 6, the ability to induce antibodies that will bind may require additional sugars to stabilize the epitope in the correct conformation to activate B cells. Our initial study of a monoclonal antibody predicted presence of common epitope in both Ogawa and Inaba serotypes from *Vibrio cholerae* O1.

In initial study with the first crystal structure of a monoclonal antibody (F-22-30; IgG1 isotype; PDB code 2uyl) common to both Ogawa and Inaba serotypes from *Vibrio cholerae*O1 followed by modeling calculations and preliminary NMR results suggested a binding pocket at the center of the antibody recombination site which could bind the side-chain of a perosaminyl residue from the O-PS(Ahmed *et al.*, 2008). Further studies with Inaba specific sequences obtained

through transcutaneous immunization with membrane extract containing Inaba LPS from *V. cholerae* O1 El Tor Inaba applying bioinformatics approaches to assess ligand binding confirmed the presence of amino acids of similar nature to be identical groove type binding pattern. The study provides new insight in recognition of carbohydrate antigen by different antibodies against Inaba LPS and rational basis towards the development alternative vaccine strategy involving transcutaneous route with very cheaper crude membrane extract as the novel immunogenic component. It is most likely that the natural LPS protein complex to be an alternative thymus (T)-dependent antigen rather than LPS which is T-independent immunogen in nature. The immunogenic potentials of*V. cholerae* O1 cell surface proteins both in experimental animals and humans are proven(Kabir, 1983; 1987b).

To design an effective single dose cholera vaccine in future, the target population in endemic areas should be children. As the duration of immunity after cholera infection remains still controversial(Clemens *et al.*, 1991b), an effective vaccineshould be more immunogenic than natural infection(Tauxe *et al.*, 1994)considering attainment of protective immunity to be predominantly antibacterial. As there were enormous controversy followed by exaggerated blaming on side effects of former cholera vaccine formulations(Ryan & Calderwood, 2000; Sack *et al.*, 2004; Sanchez & Taylor, 1997), this problem needs to be resolved with utmost caution and preventive measures. The importance of the route of vaccine administration to be reassessed as transcutaneous route proposed as the novel one to be in compliance to ensure maximum mucosal response in the gut. Vaccination should have major impact in reducing the incidence of carriers who act as a reservoir for *V. cholerae* in the community during the inter-epidemic period in the absence of acute cases and play a very important role in the dissemination of infection(Deb *et al.*, 1986). Above all, the new generation cholera vaccine should be more cost effective than all other previous formulations.

5 Concluding Remarks

The PhD research program was initially linked with the unresolved *V. cholerae* O1 Inaba epitope determination followed by potential conjugate preparation and evaluation based on putative Inaba antigenic determinants. Secondly, characterization of the immune response to major *Vibrio cholerae* O1 antigens as the vaccine candidates and their impact on vaccine development strategies as an alternative approach is needed to find a solution to the complex problems in the field of cholera vaccine.Although initiated over 100 years ago has not resulted in an effective vaccine suitable for all age groups and different populations. In spite of all the previous efforts, improvements are needed to augment the protective efficacy of cholera vaccines especially in nutritionally immuno-compromised populations in developing countries. Mucosal adjuvants and micronutrients may improve efficacy and immunogenicity of vaccines are being tested. The findings obtained from the research work are summarized below.

First crystal structure of a monoclonal antibody that recognizes a common epitope present in both Ogawa and Inaba serotypes from *Vibrio cholerae* O1 was reported. Modeling calculations and preliminary NMR results suggest that the binding pocket at the center of the antibody recombination site could bind the side-chain of a perosaminyl residue from the O-PS.

Comparison of the three route of immunization with potential vaccine candidate immunogens; CT, CTB, TcpA, LPS Inaba and Ogawa, as well as, newly extracted membrane showed comparable increase of immune responses specific to all tested immunogens when applied through subcutaneous and transcutaneous routes as expected with superiority of IgG isotype followed by IgM and IgA respectively. Moreover, TCI application for all three immunogens induced the IgG1 subtype as the highly elevated one which was also seen with SCI route of administration. Since both CT and TcpA are protein antigens to induce elevated immune profile, this is to be expected but membrane extract has overcome the inherent limitation of LPS and showed comparable immune response to the protein immunogens.

Transcutaneous immunization with CTB only in mice with different zinc supplementation status showed the reduced CTB-specific antibodytiterin the zinc supplemented group as noted in human subject and other studies. However, this effect of zinc was overcome withco-

administration with CT and TcpA as induced immune response differently than CTB only for the IgM isotypes while secondary immune responses with IgA and IgG isotypes showed no phenotypic changes with the notion that zinc might have influence only on CT or CTB but not on TcpA. This requires designing further investigations to establish these findings.Moreover, the positive effect of Zn supplementation was observed for both Inaba LPS and membrane extract specific IgM responses as also demonstrated for Ogawa specific responses.

Immunization with membrane extract displayed the vibriocidal capabilities with more than 10 times elevated IgM and IgG titers compared to LPS-specific responses which indicate the extreme potentials of membrane extract compared to LPS alone. While CTB and TcpA immunized sera were vibriocidal negative and indicated that CT and TcpA might have either no role in protection except generating non-functional antibodies in humans or could have their role differently which to be confirmed with further studies.

Increased expression of immunocytes (CD19, CD4 and APCs) along with their co-stimulatory markers (CD40, CD154 and CD28) in different compartments (spleen, blood and lamina propria) after both TCI and SCI were found. Among the homing receptors CLA, β7 and CD62L in spleen, blood and lamina propria,elevation of CLA marker in spleen and lamina propria on immune cells establishes gut as the distant relative of skin-associated lymphoid tissue while CD62L for subcutaneous immunization.

The percentages of immunogen specific cells at different compartments were determinedto study involvement in generating antibodies for protection. Interestingly, the CTB-specific cells represented the major percentages of the total immune cells. This huge pool or antibody secreting cells showing beneficence would have greater impact. However, the role of CTB and CT in cholera vaccine still remains questionable. Alternatively, TcpA with only scanty representation might have greater role in protection as noted in mouse model of other studies.

With the novel *in situ* positive selection technique, we report here as the first murine B-cell VH and VL chain repertoire specific for protein (CT, CTB and TcpA individually or in combination) and polysaccharide (membrane extract containing Inaba or Ogawa LPS) immunogen.

When compared all the sequences derived from immunization of the three major immunogens, co-administration has effect in expression differently than individual immunogen-specific

sequences. Amino acids expressed through immunization of polysaccharide immunogens were more stable than sequences originated through immunization of protein immunogens during recombination from μ to γ1.Moreover, CDRH3 length of antibody sequences to protein immunogens varied between 8 and 19 amino acids with autoimmunity generating CTB-specific shorter sequences containing more charged and hydrophobic residues.

Further bioinformatics approaches with Inaba-specific sequences obtained through transcutaneous immunization with membrane extract confirmedligand binding with the presence of amino acids of similar nature to be identical groove-type binding pattern. The study provides new insight into recognition of carbohydrate antigen by different antibodies against Inaba LPS and rational basis towards the development of alternative vaccine strategies involving transcutaneous route with affordable crude membrane extract as the novel immunogenic component.

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Media Compositions Used in the Study

A. Alkaline Nutrient Agar

Ingredients	Amount
Peptone	5.0gm
NaCl	5.0gm
Beef extract	3.0gm
Distilled water	1000ml
Agar	15gm
pH	7.0

B. Columbia Blood Agar

Ingredients	Amount
Pancreatic Digest of Casein	12.0gm
Peptic Digest of Animal Tissue	5.0gm
Yeast Extract	3.5gm
Beef Extract	3.0gm
Corn Starch	1.0gm
NaCl	5.0gm
Blood Agar Base	13.5gm
Water	1000ml
Sheep Blood	50ml
pH	7.3±0.2

C. Hypersaline Alkaline Peptone Broth

Ingredients	Amount
Peptone	10gm
NaCl	10gm
Agar	25gm
Distilled Water	1000ml
Final pH	8.4±0.2

D. Luria Bertani Broth

Ingredients	Amount
Bactotryptone	10.0gm
Yeast Extract	5.0gm
NaCl	10.0gm
Distilled Water	11.0ml
NaOH (1N)	1.0ml
pH	7.0±0.2

E. Thiosulfate Citrate Bile Salt Sucrose Agar

Ingredients	Amount
Mixed peptone	10
Yeast extract	5
Sucrose	20
Sodium citrate	10
Ferric citrate	1
Sodium chloride	10
Sodium thiosulfate	10
Oxbile	5
Sodium cholate	3
Thymol blue	0.04
Bromothymol blue	0.04
Agar	14
pH	8.6±0.2

F. Gelatin Agar

Ingredients	Amount
Peptone	4g
Yeast extract	1g
Gelatin	15g
Agar	15g
Distilled water	1000ml
pH	7.2±0.2

Reagents and Chemicals Used

- **2, 2, 2- tribromoethanol (Avertin)**

6.2ml tert-amyl alcohol was added to the 10gm Avertin, stirred in magnetic stirrer, kept in a dark tightly capped dark bottle and stored in a dark place.

- **Acetone**

58.08gm of acetone powder was dissolved in 1000ml of deionized water and stire by a magnetic stirrer until fully dissolved and stored at 4°C until further use.

- **Agarose Gel (1%)**

1gm of Agarose powder was mixed with 100ml of TAE buffer to make the final concentration 1% (w/v), boiled at 100°C for 5minutes, brought to a temperature below 50°C and poured at the gel tank fitted with well combs.

- **Ammonia Lysing Buffer**

8.26gm of ammonium chloride (NH_4Cl), 1gm of potassium bicarbonate ($KHCO_3$) and 0.037gm of EDTA mixed together. The final volume was made 1000ml by the addition of deionized water and stored at 4°C until further use.

- **Ampicillin (1000X)**

5gm of ampicillin powder was dissolved in 25ml deionized water, 25ml of absolute alcohol was added to that solution and stored at -20°C until further use.

- **Ethylenediaminetetraacetic acid (EDTA, 0.1M)**

3.72 gm of $Na_2EDTA.2H_2O$ was resuspended in in about 100ml of deionized water and pH was adjusted with 0.2 N NaOH.

- **Ethylenediaminetetraacetic acid (EDTA , 2.5 mM)**

0.93gm of $Na_2EDTA.2H_2O$ was resuspended in in about 1000ml of deionized water and pH was adjusted with 0.02 N NaOH.

- **Ethidium Bromide (EtBr) Solution**

10µl of Ethidium Bromide was dissolved in 100ml of TAE buffer to make the final concentration of 20mg/ml and stored 4°C in the dark.

- **FACS Buffer**

5ml of 10% BSA (0.1% BSA), 1ml of 10% Azide and 5 ml EDTA mixed together and the final volume was made 500ml with 1M PBS

❖ **Gelatin (0.5%)**

5gm of gelatin sheet was added to 1000ml of deionized water, autoclaved for 30 minutes and stored at 4°C until use.

❖ **Glycin-HCl Buffer (pH 3.0)**

25ml, 0.2M glycin was mixed with 5.7 ml of 1M HCl and deionized water added to make the final volume of 100ml

❖ **Hanks Buffered Salt Solution (HBSS)**

10ml of Solution 1 (8.0gm NaCl, 0.4gm KCl and 1.0gm glucose in 100ml dH_2O) , 1ml of solution 2 (0.358gm NaH_2PO_4 and 0.6gm KH_2PO_4 in 100ml dH_2O), 1ml of solution 3 (0.72gm $CaCl_2$ in 50ml dH_2O), 1ml of Solution 4 (1.23gm of $MgSO_4.7H_2O$) and 86ml of dH_2O were mixed together to get the final volume of 100ml

❖ **Hydrochloric Acid (1N)**

36.5gm of HCl is dissolved in 1000ml of deionized water and stored at room temperature until use.

❖ **Hydrochloric Acid (3M)**

109.5gm of HCl is dissolved in 1000ml of deionized water and stored at room temperature until use.

❖ **L-cystein (10mM)**

500mg of cysteine powder has been mixed to 100ml of water yields a clear and colourless solution

❖ **NaCl (0.5M)**

29.25mg of NaCl was added to 500ml of deionized water.

❖ **$NaHCO_3$ (1M)**

35gm $Na_2CO_3$and 56gm of $NaHCO_3$ was taken together and dissolved in 1000ml of deionized water and stored at 4°C until further use.

❖ **Neotetrazolium (0.1%)**

1gm of Neotetrazolium chloride was dissolved in 1000ml of deionized water and stored at 4°C until further use.

❖ **Normal Saline**

9gm of NaCl was dissolved to 1000ml of deionized water and stored at 4°C until use

❖ **O-phenyl diamine (OPD)**

10 mg of OPD tablet (Dihydrochloride) was dissolved in 23.988 ml of 0.1 M Na-Citrate Buffer with 12.0 µl Hydrogen-Per-Oxide (H_2O_2).

❖ **O-phenyl diaminedihydrochloride (OPD-HCl)**

A 4mg OPD tablet was dissolved in 10ml of 0.05M phosphate-citrate buffer of pH 5.0, provided an OPD concentration of 0.4 mg/ml then the solution was vortex to dissolve the tablet completely.

❖ **Phosphate Buffer Saline (0.1 M, pH 7.6)**

8.00gm NaCl (0.137 M), 0.20gm KCl (2.7 mM), 0.24gm KH_2PO_4 (1.4 mM), 1.44gm Na_2HPO_4(0.01 M) was mixed together and the volume was set to 1000ml by the addition of deionized water. 10 ml of the stock solution added to 90 ml deionized water to make the final molarity 0.1 M. The final pH was set to 7.6 by the slow addition of glacial acetic acid.

❖ **Phosphate Buffer Saline (10mM)**

10ml of stock full molar concentration PBS solution was mixed with 990ml of deionized water to make the final volume 1000ml.

❖ **Phosphate Buffer Saline (40 mM, pH 8.0)**

40ml of stock full molar concentration PBS solution was mixed with 960ml of deionized water to make the final volume 1000ml.

❖ **Phosphate-gelatin-tween**

45ml of 0.01% PBS-Tween and 5ml of 5% gelatin were mixed together in magnetic stirrer and stored at 4°C further use.

❖ **Potassium Phosphate Buffer (40mM)**

87.09gm of K_2HPO_4 added to 500ml water and 68.045g of KH_2PO_4 added to 500ml water. Their combination yields 1M Potassium Phosphate Buffer. 40ml of this stock solution has been mixed with 960ml deionized water to yield the final strength 40mM.

❖ **Sodium Citrate (0.1%, pH 5.2)**

5.88gm of Tri-Sodium-Citrate (Dihydrate, 0.1M) was added to 200ml of deionized water and pH was adjusted to 5.2 by the addition of Citric Acid (Monohydrate, 0.1M, 2.10gm/ml) .

❖ **Sodium Citrate Buffer (0.1M)**

5.88gm of Tri-Sodium-Citrate (Dihydrate) was added to 200ml of deionized water and the pH was adjusted to 5.2 with Citric Acid (Monohydrate, 0.1 M, 2.10 g / ml)

❖ **Tris (1M Stock)**

121.1 g Tris base was dissolved in 800 mL of ddH_2O and the pH was adjust the with concentrated HCl and the final volume was brought to 1000ml with ddH_2O

❖ **Tris Base (2M, pH 9.0)**

6.91g Tris base (mw 121.1) was added to 300ml ddH_2O and the volume was adjusted to 400ml with dH_2O, autoclaved and stored at 4°C.

❖ **Tris-HCl (2M, pH 7.6)**

96.91g TrisHCl (mw 121.1) was added to 250ml dH2O, pH was adjusted to 7.5 and the final volume was set to 400ml with dH2O, autoclave and stored at 4°C.

❖ **Tris-NaCl Buffer/ TrisBufferrerd Saline (1X)**

6.05gm Tris and 8.76gmNaClwas dissolved in 800 mL of H_2O. The pH was adjusted to 7.5 with 1M HCl and the final volume was set to 1 L with dH_2O. TBS is stable at 4°C for 3 months.

❖ **Tween-20 (0.01%)**

1mg of Tween-20 is dissolved in 100ml dH_2O completely to make the final volume 0.01%.

Table A1: FACS Antibodies and panels for mouse

Panel 1	a). CD19 PerCP b). CD19 PerCP/CD40 FITC c). CD19 PerCP/CD80 FITC/CD86 PE
Panel 2	a). CD3 FITC b). CD3 FITC/CD28 PE c). CD3 FITC/CD154 PE e). CD3 FITC/CD8 PE/CD4 APC f). CD3 FITC/CD8 PE/CD69 PerCP/CD4 APC
Panel 3	a). CD4 APC b). CD4 APC/CD28 PE c). CD4 APC/CD154 PE
Panel 4	CD11c APC
Panel 5	Cell Only

Table A2: List of reference strains used in the study:

Strain Name	Reference No.
Vibrio cholerae O1 Inaba	**CNRVC950707**
V. cholerae O1 Inaba	**CNRVC19479**
V. cholerae O1 Ogawa	**CNRVC960250**
V. cholerae O1 El Tor	**CNRVC25049**
V. cholerae O139	**CNRVC426013**

Table A3: List of Software packages and databases used for *in-silico* analysis

Software Package	Publisher/web source
NCBI-BankIt	http://www.ncbi.nlm.nih.gov
APE Plasmid editor	http://biologylabs.utah.edu/jorgensen/wayned/ape/
VBase2-Germline V-gene database	http://www.vbase2.org/
ClustalW 2.0	http://www.ebi.ac.uk/Tools/msa/clustalw2/
GeneDoc 2.6.002	http://www.nrbsc.org/gfx/genedoc/
FlowJo	http://www.flowjo.com/
FACS Calibur	http://www.bdbiosciences.com/eu/instruments/software/
SigmaStat	http://www.sigmaplot.com/products/sigmaplot/sigmastat.php
PIGS	http://cassandra.med.uniroma1.it/pigs/
SCWRL 3.0	http://dunbrack.fccc.edu/scwrl4/
GROMOS96	http://www.gromos.net/
Swiss PDB-viewer	http://spdbv.vital-it.ch/
iMolTalk	http://www.moltalk.org/iMolTalk.html
Prosa-Web	http://prosa.services.came.sbg.ac.at/prosa.php
ProMate	http://bioportal.weizmann.ac.il/promate/6/
Argus Lab 4.0	http://www.arguslab.com/arguslab.com/Welcome.html
Accelrys Discovery Studio 2.0	http://accelrys.com/products/discovery-studio/visualization-download.php
Microsoft Excel	http://office.microsoft.com/en-us/excel/

Table 4A: List of Apparatus

Apparatus / Instrument Name	Manufacturer and Catalogue
BD-vaccutainer	BD Bioscience (Cat#367899)
DEAE Tri-sacrylIon Exchange Column	Amersham Bioscience (Cat#17-0500-01)
DNA sequencer	Chignin Genome Express (Cat#1403-019)
Emery Papers	Fisher Scientific (Cat#50-996-750)
Feeding Needle	Fuchigami, Japan (Cat#5202K)
Mesh Filter	Fisher Scientific Metrohm (Cat#TC1000-1157)
Flat Bottom Microwell	Immune Microwell, Sigma-NUNC (Cat#M9410)
Low Bottom 96 well tissue culture plate	MicrotestTM-BD-Nunc(Cat#354429)
Magnetic Particle Concentrator	Invitrogen Dynal (Cat#121.02)
Maxisorp 96 well microtiter plate	eBioscience-Nunc (44-2404-21)
Microfuge Tube	Eppendorf (Cat#22363611)
Mono QTM 5/50 GL Ion Exchange Coulmn	GE Healthcare (Cat#17-5166-01)
Mono-Q-DEAE Column	Amersham Bioscience (Cat#17-0710-01
No. 40 blade	Wahl Corp, Sterling, Illinois (Cat# 9961-900)
QIAprep Spin Miniprep DNA	Qiagen (Cat#27104)
Scalpel Blade	Fisher Scientific (Cat#S95937)
Spectrophotometer	Elson Biotek (Cat#LT-2900)
Superdex TM G75 column	Amersham Bioscience (Cat#17-1047-01)
V-bottom 96 well polystyrene plate	Nunc-Thermo (Cat#249662)

Table A5: Commercial monoclonal antibody and Other Biochemical Reagent

Chemical/Biological Agents Used	Manufacturer and Catalogue No.
Acetone	Sigma-Aldrich (Cat#320110)
Agarose	Invitrogen UltraPure™ (Cata#16500500)
Ampicillin Sodium Salt Powder	Sigma-Aldrich (Cat#A0166)
Anti-Mouse peroxidase Conjugated IgG	Sanofi diagnostic, France
APC-Conjugated hamster-anti-mouse CD11c FITC Mab	eBioscience (Cat#11-0114-82)
Biotinylatedctx/TcpA/Ogawa/LPS/Inaba LPS	Advance targeting System (Cat#IT-61-25-250)
Blood Agar Base	Thermo-Oxoid(Cat#CM0854)
Cholera Toxin	List Biological Laboratories (Cat#101B)
Citric Acid	Sigma-Aldrich (Cat#320110)
DEPC Treated Water	Invitrogen UltraPure™ (Cata#750023)
Dynabeads M-280 streptavidin	Invitrogen Dynal MPC, AS, Oslow (Cat#11206)
EDTA	Sigma-Aldrich (Cat#E9884)
EthidiumBromide	Sigma-Aldrich (Cat#E1510)
Fetal Bovine Serum	Sigma-Aldrich (Cat#12303C)
Female BalB/c	icddr,b Animal House Facility
Rat anti-Mouse B7 FITC labeled Mab	BD Pharmingen (Cat#558091)
Glycin	Sigma-Aldrich (Cat#50046)
Guinea Pig serum	Sigma-Aldrich (Cat#G9774)
Hamster-anti-mouse CD28 154Mab	BD Pharmingen (Cat#553722)
Hamster-anti-mouse CD28 PEMab	eBioscience (Cat#16-0281-81)
Hamster-anti-mouse CD80 FITCMab	BD Pharmingen (Cat#553766)
Hydrochloric Acid	Sigma-Aldrich (Cat#258148)
Horse Reddish Peroxidase Conjugate	Southern Biotech (Cat#7100-05)
Isopropanol	Sigma-Aldrich (Cat#292907)
Luria Bertani Broth Base	Miller-Oxoid (Cat#CM0996)
Lymphocyte M	Cederlane Labs (Cat#CL5030)
Mouse anti-IgGAb	Sigma-Aldrich (Cat#A9044)
Neotetrazolium	Sigma-Aldrich (Cat#72150 FLUKA)
*Ortho*PhenyleneDiamene	Sigmafast (Cat#9187)
Phosphate Buffered Saline	Sigma-Aldrich (Cat#P5493)
pCR®2.1-Topo®-TA Cloning Kit	Invitrogen (Cat#K4510-20)
Rabbit polyclonal sera	Institut Pasteur, Paris, France
Rat anti mouse CD3Ab	BD Pharmingen (Cat#555026)
Rat-anti-mouse CD19 PerCp-Cy 5.5	BD Pharmingen (Cat#553372)
Rat-anti-mouse CD86 R-PE	BD Pharmingen (Cat#553691)
M-MLV Reverse Transcriptase	Invitrogen (Cat#28025)
RNasin® Plus RNase Inhibitor	Promega (Cat#N2611)
R-PE labeled rat ant-mouse CD 62L	BD Pharmingen (Cat#560966)
R-PE labeled rat anti-mouse CD 88a	BD Pharmingen (Cat#552993)
R-PE labeled rat anti-mouse CLA	BD Pharmingen (Cat#555946)
Sodium Chloride	Sigma-Aldrich (Cat#S5886)
Taq polymerase	Invitrogen (Cat#18038240)
Tris	Sigma-Aldrich (Cat#252859)
Trizol Reagent	Invitrogen (Cat#10296-010)
Tween-20	Sigma-Aldrich (Cat#P2287)

Table A6: Genebank Accession Number of the Submitted Sequences

Submission Name	Accession No.	Type of sequence
BankIt1621952 M1-CF-CTB	KC906585	Variable Light Chain(V_L)
BankIt1621952 M2-CF-CTB	KC906586	Variable Light Chain(V_L)
BankIt1621952 M3-CF-CTB	KC906587	Variable Light Chain(V_L)
BankIt1621952 M4-CF-CTB	KC906588	Variable Light Chain(V_L)
BankIt1621952 M4-CF-CTB-A	KC906589	Variable Light Chain(V_L)
BankIt1621952 M2-ZnM-CTB	KC906590	Variable Light Chain(V_L)
BankIt1621952 M3-ZnM-CTB	KC906591	Variable Light Chain(V_L)
BankIt1621952 M3-ZnP-CTB	KC906592	Variable Light Chain(V_L)
BankIt1621952 M4-ZnP-CTB	KC906593	Variable Light Chain(V_L)
BankIt1621952 M5-ZnP-CTB	KC906594	Variable Light Chain(V_L)
BankIt1621952 M1-CF-TcpA-CT-CTB	KC906595	Variable Light Chain(V_L)
BankIt1621952 M1-CF-TcpA-CT-A-CTB	KC906596	Variable Light Chain(V_L)
BankIt1621952 M2-CF-TcpA-CT-CTB	KC906597	Variable Light Chain(V_L)
BankIt1621952 M2-CF-TcpA-CT-A-CTB	KC906598	Variable Light Chain(V_L)
BankIt1621952 M3-ZnM-TcpA-CT-CTB	KC906599	Variable Light Chain(V_L)
BankIt1621952 M3-ZnM-TcpA-CT-A-CTB	KC906600	Variable Light Chain(V_L)
BankIt1621952 M1-ZnP-TcpA-CT-CTB	KC906601	Variable Light Chain(V_L)
BankIt1621952 M1-ZnP-TcpA-CT-A-CTB	KC906602	Variable Light Chain(V_L)
BankIt1621952 M2-ZnP-TcpA-CT-CTB	KC906603	Variable Light Chain(V_L)
BankIt1621952 M2-ZnP-TcpA-CT-A-CTB	KC906604	Variable Light Chain(V_L)
BankIt1621952 M4-ZnM-TcpA	KC906605	Variable Light Chain(V_L)
BankIt1621952 M4-ZnM-TcpA-A	KC906606	Variable Light Chain(V_L)
BankIt1621952 M5-ZnM-TcpA	KC906607	Variable Light Chain(V_L)
BankIt1621952 M5-ZnM-TcpA-A	KC906608	Variable Light Chain(V_L)
BankIt1621952 M4-ZnP-TcpA	KC906609	Variable Light Chain(V_L)
BankIt1621952 M4-ZnP-TcpA-A	KC906610	Variable Light Chain(V_L)
BankIt1621952 M5-ZnP-TcpA	KC906611	Variable Light Chain(V_L)
BankIt1621952 M1-CF-TcpA-CT-TcpA	KC906612	Variable Light Chain(V_L)
BankIt1621952 M1-CF-TcpA-CT-A-TcpA	KC906613	Variable Light Chain(V_L)
BankIt1621952 M2-CF-TcpA-CT-TcpA	KC906614	Variable Light Chain(V_L)
BankIt1621952 M2-CF-TcpA-CT-A-TcpA	KC906615	Variable Light Chain(V_L)
BankIt1621952 M3-ZnM-TcpA-CT-TcpA	KC906616	Variable Light Chain(V_L)
BankIt1621952 M3-ZnM-TcpA-CT-A-TcpA	KC906617	Variable Light Chain(V_L)
BankIt1621952 M3-ZnM-TcpA-CT-AA-TcpA	KC906618	Variable Light Chain(V_L)
BankIt1621952 M1-ZnP-TcpA-CT-TcpA	KC906619	Variable Light Chain(V_L)
BankIt1621952 M2-ZnP-TcpA-CT-TcpA	KC906620	Variable Light Chain(V_L)
BankIt1621952 M2-ZnP-TcpA-CT-A-TcpA	KC906621	Variable Light Chain(V_L)
BankIt1621952 M1-CF-Ogawa	KC906622	Variable Light Chain(V_L)
BankIt1621952 M2-CF-Ogawa	KC906623	Variable Light Chain(V_L)

Submission Name	Accession No.	Type of sequence
BankIt1621952 M4-CF-Ogawa	KC906624	Variable Light Chain(V_L)
BankIt1621952 M4-CF-Ogawa-A	KC906625	Variable Light Chain(V_L)
BankIt1621952 M5-CF-Ogawa	KC906626	Variable Light Chain(V_L)
BankIt1621952 M1-CF-Inaba	KC906627	Variable Light Chain(V_L)
BankIt1621952 M1-CF-Inaba-A	KC906628	Variable Light Chain(V_L)
BankIt1621952 M2-CF-Inaba	KC906629	Variable Light Chain(V_L)
BankIt1621952 M5-CF-Inaba	KC906630	Variable Light Chain(V_L)
BankIt1621952 M4-ZnM-Inaba	KC906631	Variable Light Chain(V_L)
BankIt1621952 M1-Non-Imm	KC906632	Variable Light Chain(V_L)
BankIt1621952 M2-Non-Imm	KC906633	Variable Light Chain(V_L)
BankIt1621952 M3-Non-Imm	KC906634	Variable Light Chain(V_L)
BankIt1621952 M4-Non-Imm	KC906635	Variable Light Chain(V_L)
BankIt1621952 M5-Non-Imm	KC906636	Variable Light Chain(V_L)
BankIt1621981 M1-CF-CTB	KC906637	IgM Variable Heavy Chain (V_H-μ)
BankIt1621981 M2-CF-CTB	KC906638	IgM Variable Heavy Chain (V_H-μ)
BankIt1621981 M2-CF-CTB-A	KC906639	IgM Variable Heavy Chain (V_H-μ)
BankIt1621981 M3-CF-CTB	KC906640	IgM Variable Heavy Chain (V_H-μ)
BankIt1621981 M4-CF-CTB	KC906641	IgM Variable Heavy Chain (V_H-μ)
BankIt1621981 M2-ZnM-CTB	KC906642	IgM Variable Heavy Chain (V_H-μ)
BankIt1621981 M3-ZnM-CTB	KC906643	IgM Variable Heavy Chain (V_H-μ)
BankIt1621981 M2-ZnP-CTB	KC906644	IgM Variable Heavy Chain (V_H-μ)
BankIt1621981 M4-ZnP-CTB	KC906645	IgM Variable Heavy Chain (V_H-μ)
BankIt1621981 M5-ZnP-CTB	KC906646	IgM Variable Heavy Chain (V_H-μ)
BankIt1621981 M3-ZnP-TcpA-CT-CTB	KC906647	IgM Variable Heavy Chain (V_H-μ)
BankIt1621981 M3-ZnP-TcpA-CT-A-CTB	KC906648	IgM Variable Heavy Chain (V_H-μ)
BankIt1621981 M2-ZnP-TcpA-CT-TcpA	KC906649	IgM Variable Heavy Chain (V_H-μ)
BankIt1621981 M3-ZnP-TcpA-CT-TcpA	KC906650	IgM Variable Heavy Chain (V_H-μ)
BankIt1621981 M2-CF-Inaba-VHIIC	KC906651	IgM Variable Heavy Chain (V_H-μ)
BankIt1621981 M2-CF-Inaba-A-VHIIC	KC906652	IgM Variable Heavy Chain (V_H-μ)
BankIt1621981 M2-CF-Inaba-VHV	KC906653	IgM Variable Heavy Chain (V_H-μ)
BankIt1621981 M2-CF-Inaba-A-VHV	KC906654	IgM Variable Heavy Chain (V_H-μ)
BankIt1621981 M5-CF-Inaba	KC906655	IgM Variable Heavy Chain (V_H-μ)
BankIt1621981 M3-ZnM-Inaba-VHIIB	KC906656	IgM Variable Heavy Chain (V_H-μ)
BankIt1621981 M3-ZnM-Inaba-VHV	KC906657	IgM Variable Heavy Chain (V_H-μ)
BankIt1621981 M4-ZnM-Inaba	KC906658	IgM Variable Heavy Chain (V_H-μ)
BankIt1621981 M1-Non-Imm	KC906659	IgM Variable Heavy Chain (V_H-μ)
BankIt1621981 M2-Non-Imm	KC906660	IgM Variable Heavy Chain (V_H-μ)
BankIt1621981 M3-Non-Imm	KC906661	IgM Variable Heavy Chain (V_H-μ)
BankIt1621981 M5-Non-Imm	KC906662	IgM Variable Heavy Chain (V_H-μ)
BankIt1621996 M4-CF-CTB	KC906663	IgG Variable Heavy Chain (V_H-γ)

Submission Name	Accession No.	Type of sequence
BankIt1621996 M2-ZnM-CTB	KC906664	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M2-ZnM-CTB-A	KC906665	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M5-ZnM-CTB	KC906666	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M2-ZnP-CTB	KC906667	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M3-ZnP-CTB	KC906668	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M3-ZnP-CTB-A	KC906669	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M5-ZnP-CTB	KC906670	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M3-ZnM-TcpA-CT-CTB	KC906671	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M1-ZnP-TcpA-CT-CTB	KC906672	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M2-ZnP-TcpA-CT-CTB	KC906673	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M3-ZnP-TcpA-CT-CTB	KC906674	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M5-ZnM-TcpA	KC906675	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M5-ZnM-TcpA-A	KC906676	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M5-ZnP-TcpA	KC906677	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M5-ZnP-TcpA-A	KC906678	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M1-ZnP-TcpA-CT-TcpA	KC906679	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M1-ZnP-TcpA-CT-A-TcpA	KC906680	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M2-ZnP-TcpA-CT-TcpA	KC906681	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M1-CF-Ogawa	KC906682	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M2-CF-Ogawa	KC906683	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M2-CF-Ogawa-A	KC906684	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M4-CF-Ogawa	KC906685	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M4-CF-Ogawa-A	KC906686	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M5-CF-Ogawa	KC906687	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M5-CF-Ogawa-A	KC906688	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M2-CF-Inaba	KC906689	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M2-CF-Inaba-A	KC906690	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M5-CF-Inaba	KC906691	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M5-CF-Inaba-A	KC906692	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M4-ZnM-Inaba	KC906693	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M1-Non-Imm-VHIIA	KC906694	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M1-Non-Imm-VHV	KC906695	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M2-Non-Imm-VHIIA	KC906696	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M2-Non-Imm-A-VHIIA	KC906697	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M2-Non-Imm-VHIIIA	KC906698	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M2-Non-Imm-VHV	KC906699	IgG Variable Heavy Chain (V_H-γ)
BankIt1621996 M4-Non-Imm-VHIIA	KC906700	IgG Variable Heavy Chain (V_H-γ)