Isolation and characterization of *Vibrio cholerae* O1 from stools of diarrheal patients and studies of the methods to determine the immunological responses.

Academic Dissertation

SUBMITTTED BY
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Abstract

Cholera continues to be one of the most common causes of morbidity and mortality among children and adults in developing countries like Bangladesh. The aim of the study was to determine the percentage of cholera patients among the all rice watery diarrhoeal patients by their different microbiological, biochemical and serological assays. Ten percent (n=10) patients were confirmed with *Vibrio cholerae* O1 infected. Among *Vibrio cholerae* isolates, 7 patients were Ogawa and 3 were Inaba. Another aim was to evaluate the natural immune responses of native cholera patients infected by *Vibrio cholerae* O1. LPS specific IgA response showed an increase in convalescent sera at both day 7 and day 30 compared to the baseline, acute stage on day 2. The increase in both cases were statistically significant compared to baseline (P=0.0039). When sera were tested for LPS specific IgG responses, the response in the convalescent sera at day 7 were significantly higher than at the acute sera of day 2 (P=0.002). The day 30 IgG responses were also higher and statistically significant compared to that at day 2 (P=0.0039). CTB specific IgA response at convalescent stage at day 7 was significantly increased (P=0.002). CTB specific IgA response at day 30 compared to that of the acute stage at day 2, was statistically significantly increase (P=0.0059). When sera were tested for response to CTB specific IgG, the responses of both the convalescent sera at day 7 and day 30 were found an increase compared to the response at day 2. The responses were also statistically

significant (P=0.002). The vibriocidal antibody response was significantly increased at the convalescent stage on day 7 (P=0.002), and at day 30 (P=0.002).

Contents

Chapter 1

Introduction

1.1. Introduction

Among various infectious diseases, cholera is treated as one of the major cause of morbidity and mortality in Bangladesh due to poverty, population density, poor sanitation, malnutrition, and disease-transmitting vectors. Despite primarily affecting developing countries, it remains a serious public health problem for some developed countries (Pazzani *et al.*, 2006). Cholera is a clinical - epidemiologic syndrome caused by *Vibrio cholerae*, usually of serogroup O1. It is characterized by the passage of voluminous stools of rice water character that rapidly lead to dehydration. The rate of diarrhea may quickly reach 500 to 1,000 ml per hour (Kaper *et al.*, 1995). *Vibrio cholerae*, a noninvasive gram-negative bacterium serologically classified as belonging to the O antigenic group. Strains belonging to O group 1 (O1) and O group 139 (O139) are responsible for cholera. Strains other than O1, non-O1, can cause only sporadic infections. Strains of group O1 are divided into two biotypes, classical and El Tor. (Heidelberg *et al.*, 2000). *Vibrio cholerae*, is slightly curved rods shape whose motility depends on a single polar flagellum. Overall, the cells are 1 micron in width and 2 to 3 micron in length (Thompson and Swings, 2006).

1.2. History

Cholera has long been endemic in large parts of South Asia, but in the current pandemic, it has established endemicity throughout the African continent. The

lack of infrastructure and economic development has made many parts of Africa susceptible to cholera, a disease associated with a lack of clean water and poor sanitation (Griffith *et al.*, 2006). The spread of cholera to Sudan's Darfur region has caused international alarm, especially as it has arrived just at the start of the rainy season. Large areas are already cut off because of factional fighting, and the onset of the rains not only means that health workers can't reach many people but also heightens the risks of transmission. The World Health Organization says that 96 cases of acute watery diarrhoea and four deaths had been identified in the state of South Darfur by 14 June. A sample from one person who died, sent to Khartoum for testing, confirmed the presence of *Vibrio cholerae*, Inaba serotype (Moszynski, 2006). Outbreaks of cholera cause deaths estimated at 120,000 annually worldwide and many more cases each year, of which the vast majority occurs in children. Hallmarks of the epidemiology of cholera include (i) a high degree of clustering of cases by location and season, (ii) highest rates of infection in children 1 to 5 years of age in areas of endemic infection, (iii) antibiotic resistance patterns that frequently change from year to year, (iv) clonal diversity of epidemic strains, and (v) protection against the disease by improved sanitation and hygiene and preexisting immunity. Cholera has been categorized as one of the "emerging and reemerging infections" threatening many developing countries. Several recent events that mark the epidemiological importance of the disease include the reemergence of cholera in Latin America in 1991; the explosive outbreak of cholera among Rwandan refugees in Goma, Zaire, which

resulted in about 70,000 cases and 12,000 deaths in 1994 (Faruque *et al.*, 1998). Even as the battle was being won against the original *Vibrio cholerae*, known as serotype O1, new, hardier strains began to appear. The pandemics of the mid-20th Century were found to be due to a variant called "El Tor" and more recently serotype O139 has been implicated in an eighth pandemic that began in the 1990's. The complete DNA sequence of the genome of *V. cholerae* O1, El Tor was published in August 2000 (www.cbwInfo.com).

1.3. Biochemical Characterization

In adequate media, *Vibrio cholerae* O1 grow rapidly with a generation time of less than 30 minutes. Biochemical characterization of *Vibrio cholerae* O1 depends on several biochemical behaviours. These are ~

1 It is a sugar fermenter, producing no gas.

2 Non-lactose fermenter

3 Non-urea splitter

4 Positive cholera-red reaction, as it produces Indole and nitrate is reduced.

5 Negative Voges-Proskauer (VP) reaction

The *Vibrio cholerae* O1 are completely inhibited or grow somewhat poorly on usual enteric diagnostic media (MacConkey agar or eosin-methylene blue agar). An effective selective medium is thiosulfate-citrate-bile salts-sucrose (TCBS) agar, on which the sucrose-fermenting cholera vibrios produce a distinctive

yellow colony. However, the usefulness of this medium is limited because serologic testing of colonies grown on it occasionally proves difficult, and different lots vary in their productivity (Jean F. Mac Faddin, 1981). They can also be isolated from stool samples or rectal swabs from cholera cases on simple meat extract (nutrient) agar or bile salts agar at slightly alkaline pH. *Vibrio cholerae* can be confirmed by a rapid slide agglutination test with specific antiserum. Classical and El Tor biotypes can be differentiated at the same time by performing a direct slide hemagglutination test with chicken erythrocytes: freshly isolated agar-grown El Tor vibrios exhibit hemagglutination; whereas freshly isolated classical vibrios do not. In practice, this can be accomplished with material from patients as early as 6 hours after streaking the specimen in which the cholera vibrios usually predominate. However, to detect carriers (asymptomatically infected individuals) and to isolate cholera vibrios from food and water, enrichment procedures and selective media are recommended (Jean F. Mac Faddin, 1981). Different plates are used for *Vibrio cholerae* O1 isolation and identification.

These are ~

1 Taurocholate tellurite gelatin agar (TTGA): Selective medium for *Vibrio cholerae* O1, less expensive and more effective.

2 Thiosulfate-citrate-bile salts-sucrose (TCBS) agar: Selective medium for *Vibrio cholerae* O1, but difficult in serotyping.

3 Gelatin agar (GA) Plate (use for subculture of *Vibrio cholerae*)

Taurocholate tellurite gelatin agar (TTGA) is an inexpensive medium that produces transparent gray colonies (2 to 3 mm in diameter) of *V. cholerae* with surrounding halo; the colonies are difficult to differentiate from those of V. parahaemolyticus. To overcome this problem, a modified taurocholate tellurite gelatin agar was developed with the addition of beta-galactosidase (4-methyllumbelliferyl-beta-delta-galactosidase); *V. cholerae* produces a very strong reaction in 24h or less, showing brilliant-blue fluorescence (Gomez-Gil and Roque, 2006). Optimal pH for *Vibrio cholerae* O1 is 8.4 (Gomez-Gil and Roque, 2006). In many specimens, the concentration of *V. cholerae* is so high that enrichment is unnecessary. However, enrichment broth is commonly used to recover low levels of vibrios, particularly from formed stools. Alkaline peptone water is the most commonly used enrichment broth; the pH of this medium can range from 8.4 up to 9.2, thus taking advantage of the ability of *V. cholerae* to multiply at alkaline pH. Enrichment lasts for only 6 to 8 h, as incubation in alkaline peptone water for longer than 8h may result in overgrowth of other organisms. Less commonly used enrichment media include alkaline peptone water with tellurite, Monsur's tellurite taurocholate broth, and sodium gelatin phosphate broth (Kaper *et al.*, 1995).

1.4. Susceptibility

Recent epidemiologic research suggests that an individual's susceptibility to cholera (and other diarrhoeal infections) is affected by their blood type: Those with type O blood are the most susceptible (Swerdlow D *et al*,1994; and Harris J *et al*, 2005) while those with type AB are the most resistant. Between these two extremes are the A and B blood types, with type A being more resistant than type B (Waltz, Robert, 2007). It has also been hypothesized that the cystic fibrosis genetic mutation has been maintained in humans due to a selective advantage: heterozygous carriers of the mutation (who are thus not affected by cystic fibrosis) are more resistant to *V. cholerae* infections. The genetic deficiency in the cystic fibrosis transmembrane conductance regulator channel proteins interferes with bacteria binding to the gastrointestinal epithelium, thus reducing the effects of an infection (Bertranpetit J, Calafell F 1996).

1.5. Transmission

The mechanisms of transmission for cholera include water, unwashed contaminated food, and seafood that come from *Vibrio cholerae* O1 endemic estuaries. In West, Southern, and East Africa, water source contamination was the second most common risk factor reported, representing 32%, 30%, and 24% of the total, respectively. In Central Africa, water source contamination was the most common, accounting for 30% of the reported risk factors (Griffith *et al.*, 2006). Social phenomena such as mass migrations and burial practices may play

a greater role in transmission of cholera (Glass *et al.*, 1991).

1.6. Infectious Dose

The infective dose of *V. cholerae* O1 has been estimated as $10X10^8$ – $10X10^9$ cells (Author *et al.*, 2007). Doses of $1X10^{11}$ CFU of *V. cholerae* O1 were required to consistently cause diarrhea in healthy North American volunteers when the inoculum was given in buffered saline (pH 7.2). When stomach acidity was neutralized with 2 g of sodium bicarbonate immediately prior to administration of the inoculum, attack rates of 90% were seen with an inoculum of $1X10^6$. Food has a buffering capacity comparable to that seen with sodium bicarbonate. Ingestion of $1X10^6$ vibrios with food such as fish and rice resulted in the same high attack rate (100%) as when this inoculum is administered with buffer. Fewer data are available on inoculum size in naturally occurring infections. Studies in Bangladesh have led to the suggestion that the inoculum in nature is in the range of $1X10^2$ to $1X10^3$ (Kaper *et al.*, 1995).

1.7. Pathogenesis Mechanism

Infection due to *V. cholerae* O1 begins with the ingestion of contaminated food or water containing the organism. After passage through the acid barrier of the stomach, the vibrio colonizes the epithelium of the small intestine. Cholera enterotoxin produced by the adherent vibrios disrupts ion transport by intestinal epithelial cells. The subsequent loss of water and electrolytes leads to the severe

diarrhea characteristic of cholera (Kaper *et al.*, 1995).

1.8. Incubation Period

The incubation period of cholera can range from several hours to 5 days and is dependent in part on inoculum size (Kaper *et al.*, 1995).

1.9. Prognosis

The disease runs its course in 2 to 7 days; the outcome depends upon the extent of water and electrolyte loss and the adequacy of water and electrolyte repletion therapy (Finkelstein, 1996).

1.10. Diagnosis Overview

For rapid diagnosis, a wet mount of liquid stool is examined microscopically. The characteristic motility of vibrios is stopped by specific antiserum. Other methods are

(1) Culture of stool or rectal swab samples on TTGA and other selective and nonselective media;

(2) Slide agglutination test of colonies with specific antiserum;

(3) Fermentation tests (oxidase positive); and

(4) Enrichment in peptone broth followed by fluorescent antibody tests, culture, or retrospective serologic diagnosis.

More recently the polymerase chain reaction (PCR) and additional genetically-based rapid techniques have been recommended for use in specialized laboratories (Finkelstein, 1996). Classical and El Tor biotypes can be differentiated at the same time by performing a direct slide hemagglutination test with chicken erythrocytes. However, to detect carriers (asymptomatically infected individuals) and to isolate cholera vibrios from food and water, enrichment procedures and selective media are recommended. Enrichment can be accomplished by inoculating alkaline (pH 8.5) peptone broth with the specimen and then streaking for isolation after an approximate 6-hour incubation period; this process both enables the rapidly growing vibrios to multiply and suppresses much of the commensal microflora (Finkelstein, 1996).

1.11. Symptoms

Cholera is an acute dehydratry disease caused by *Vibrio cholerae* serogroup O1. In its severe form, cholera gravis, the clinical disease is characterized by the passage of voluminous stools of rice water character that rapidly lead to dehydration. Hypovolemic shock, acidosis, and death can ensue in adults, as well as in children, if prompt and appropriate treatment is not initiated. In cholera gravis, the rate of diarrhea may quickly reach 500 to 1,000 ml per hour, leading rapidly

to tachycardia, hypotension, and vascular collapse due to dehydration. Peripheral pulses may be absent, and blood pressure may be unobtainable. Skin turgor is poor, giving the skin a doughy consistency; the eyes are sunken; and hands and feet become wrinkled. Patients are restless and extremely thirsty (Kaper *et al.*, 1995). 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 hypoglycaemia. Electrolyte imbalance, especially hypokalaemia, can occur if the intravenous fluids are not appropriate. Miscarriage or premature delivery can occur in pregnant women as a complication of shock and poor perfusion of the placenta (Sack *et al.*, 2004).

1.12. Antigenic features:

Lipopolysaccharide (LPS) is a major component of the outer membrane of *Vibrio cholerae* O1 just like all other gram-negative bacteria. It gives the structural integrity of the bacteria and helps to give protection against certain types of chemical attack by increasing negative charge of the cell membrane of bacteria and then stabilize the over all membrane structure (Rittig MG *et al.*, 2004). Lipopolysaccharide (LPS) is a large molecule consisting of a lipid and a polysaccharide (carbohydrate) joined by a covalent bond. Basically it has three

parts. These are ~

1 Polysaccharide (O) side chain/ O-antigen

2 Core polysaccharide

3 Lipid A

Lipid A which is a disaccharide with multiple fatty acid tails is the key in the toxicity. When bacterial cells are lysed by host immune system, fragment of membrane containing Lipid A are released into the circulation, and causing fever, diarrhea and possible fatal endotoxic shock (also called septic shock). It has recently been found that a specific enzyme in the intestine (alkaline phosphatase) can detoxify LPS by removing the two phosphate groups from LPS carbohydrates. This may function as an host adaptive mechanism. O-antigen is the most variable part of LPS. It gives the antigenic specificity. Lipid A is the most conserved part of LPS (www.wikipedia.com).

1.13. Cholera toxin, the main criminal

Cholera toxin (CT), is a protein that is composed of five receptor binding B subunits surrounding one catalytic A subunit, is the main criminal for cholera that was proposed by Koch, who identified Vibrio cholerae in 1887. But until 1959, the existence of CT can not be proved. Indian scientists De (De, 1959) and Dutta (Dutta *et al*, 1959) was the first who proved the Koch's proposal. It is now established that Vibrio cholerae adhere to and colonize the small intestine and secrete cholera toxin that binds to receptors on the mucosal cells (Ouchterlony

and Holmgren, 1978). This receptor is a specific ganglioside (monosialosyl ganglioside, GM1).

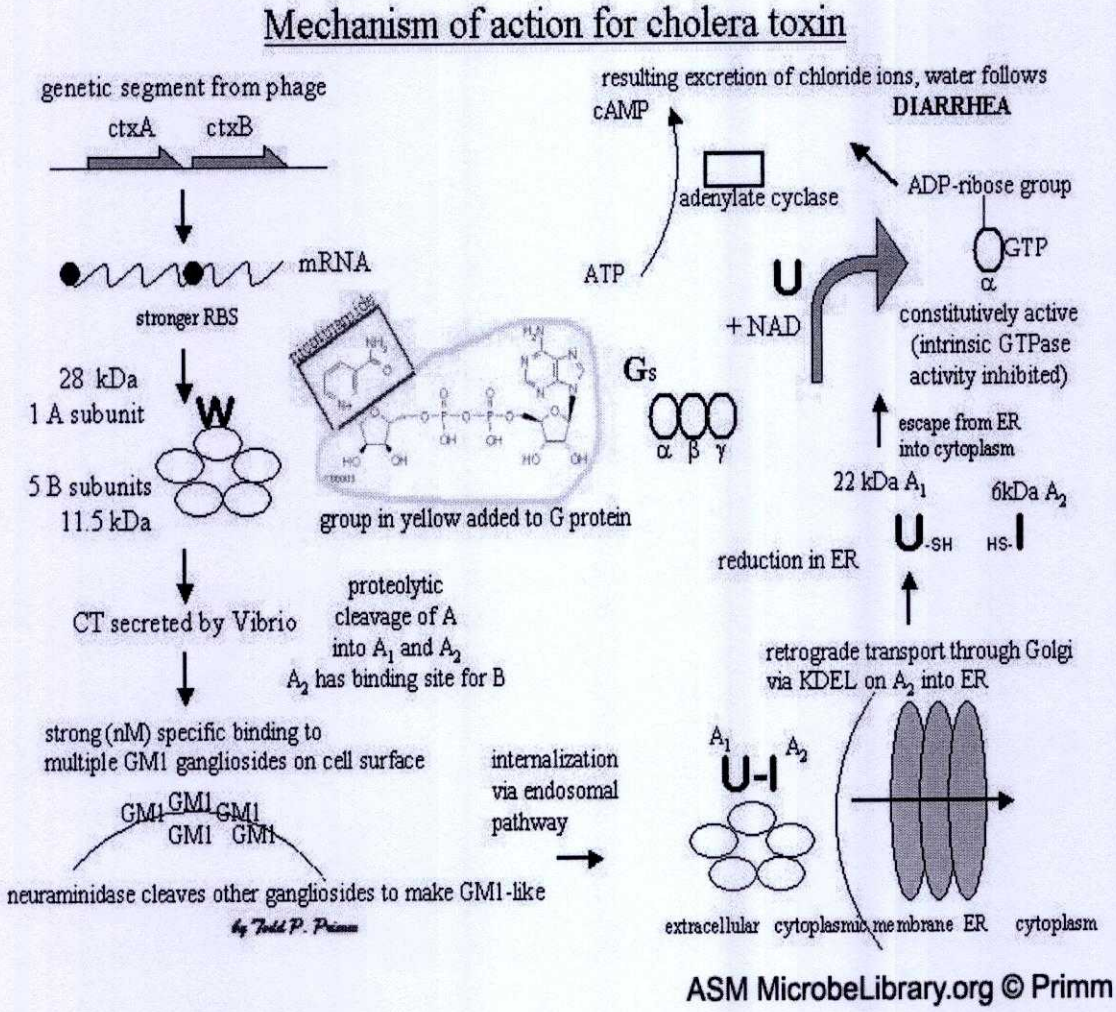

Fig-1: Mechanism of Cholera toxin (CT)

When CT is released from the bacteria in lumen of the infected intestine, it binds to the epithelial cell known as "enterocyte" through the interaction of the pentameric B subunit of the CT with GM1 receptor on the intestinal cell, triggering endocytosis of the toxin. A subunit proteolytically cleaved into A1 and A2 peptides. Once inside the enterocyte, A1 subunit enters into the cytosol where

it activates the G protein, Gsα; thereby blocking their inherent GTPase activity. This leads to constitutive activation of adenylate cylase and rapid elevation of cAMP levels. High cAMP level then activates cAMP dependent protein kinase A through phosphorylation. It after phosphorylation activates proteins involved in the secretion of chloride ions, sodium ions and water (Holmgren, 1980).

1.14. Immune response against cholera toxin

Cholera toxin B subunit (CTB) is an efficient mucosal carrier molecule for the generation of immune responses to linked antigens. The recombinant CTB (rCTB) affect human dendritic cell (DC) functions in response to LPS stimulation and induce the generation of DC with the capacity to generate CD4+ regulatory T cells (J Leukoc Biol.,2008).

Aims of the study

1. Isolation of *Vibrio cholerae* O1 from stool samples of patients with rice watery diarrhea.
2. Biochemical identification of *Vibrio cholerae* O1.
3. Serological characterization of *Vibrio cholerae* O1 to determine the serotypes.
4. Extraction and purification of lipopolysaccharide (LPS) from the structural membrane of *Vibrio cholerae* O1.
5. Studying the immune responses against different antigens (LPS and CTB) of *Vibrio cholerae* O1

Chapter 2

Materials and Methods

2.1. Sample collection

100 stool samples were collected from patients (2% systematic surveillance) enrolled in the Diarrhoeal Disease Surveillance System of the Dhaka Hospital of ICDDR,B. Studies were done in the Immunology unit, of the Laboratory Science Division of ICDDR,B.

2.2. Study Design

Patients were selected depending on their rice watery stool and dark-field microscope of their stool samples. Stool samples were cultured on the selective TTGA plates for identification and confirmation of *Vibrio cholerae* O1, followed by selective biochemical tests and serological tests. Venous blood was collected from patients on the second day of hospitalization. This was considered to be approximately 2 days after the onset of diarrhea. Blood was also collected on 7th day and 30th day.

2.3. Isolation of *Vibrio cholerae* O1

Samples from the diarrheal patients were streaked on cholera specific Taurocholate tellurite gelatin agar (TTGA) plates, and non specific *Shigella Salmonella* (SS) agar plates and MacConkey agar plates. Then all the plates were incubated at 37°C for overnight.

2.4. Biochemical identification of *Vibrio cholerae* O1

For biochemical identification of *Vibrio cholerae* O1, following tests were performed ~

1. KIA (Kligler's Iron Agar Test)
2. MIU (Motility, Indole and Urea test)
3. Citratase test

2.4.1. *Kligler's Iron Agar Test (KIA)*:

It is to determine the ability of *Vibrio cholerae* O1 to attack a specific carbohydrate (glucose and/or lactose) incorporated in a basal growth medium, without production of gas, along with the determination of number possible H_2S production.

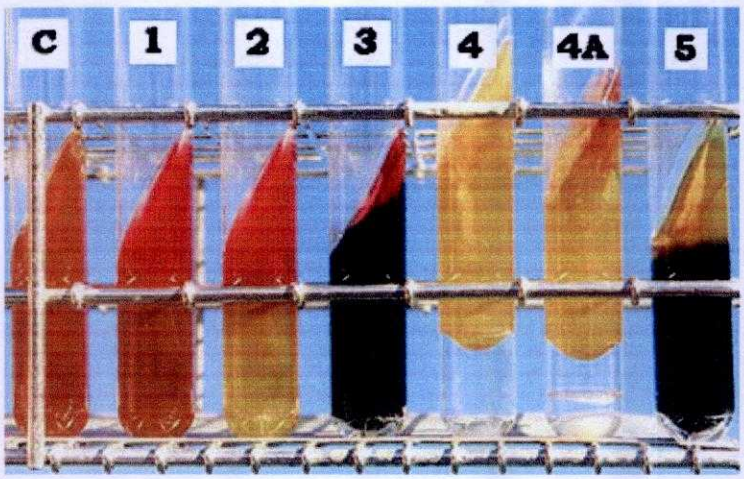

Fig-2: Biochemical characterization of different microorganisms through KIA test. Here tube C was control and tube 2 was *Vibrio cholerae* positive.

The medium was inoculated with a needle, first stabbing down the center to the bottom of the tube and then streaking up the slant. Incubation was for one day at 37°C.

2.4.2. *Motility, Indole and Urea test (MIU)*: Motility test is used to determine motility of the micro-organisms. Indole test used to determine the ability of an organism to split indole from the tryptophan. Urease test used to determine the ability of the micro-organism to split the urea. Firstly, 4 ml of cooled, sterilized tryptophan/peptone broth media containing urea was taken in a test tube. Inculation of growth was from 18 – 24 hr pure culture. Then indole reagent paper was placed and incubated at 37°C for overnight. After overnight incubation, Kovac's or Ehrlich's reagent was added to make an interpretation.

2.4.3. *Citrate test*: The citrate test was used to determine the ability of *Vibrio cholerae* O1 to utilize citrate as its only source of carbon.

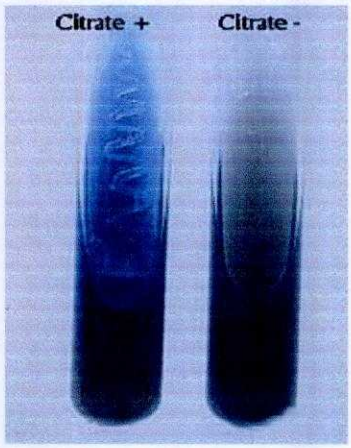

Fig-3: Positive citrate test for *Vibrio cholerae* O1

4 ml cooled, sterilized broth media containing only sodium-citrate as carbon source was taken. Then inoculated *Vibrio cholerae* O1 and incubated for overnight at 37°C with a pH indicator.

2.5. Serological identification of *Vibrio cholerae* O1

2.5.1. *Agglutination Test*: After biochemical characterization of *Vibrio cholerae* O1, the serotypes (Inaba and Ogawa) were identified through a very simple antigen-antibody agglutination reaction.

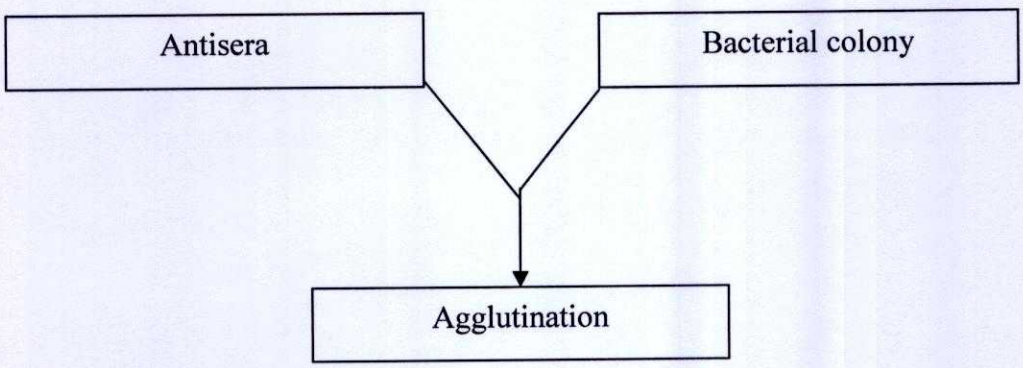

Fig-4: Agglutination test

2.6. Extraction and Purification of Lipopolysaccharide (LPS)

To extract and purify the lipopolysaccharde from *V. cholerae* O1, the *V. cholerae* O1 were streaked on GA plates and kept for over night incubation at 37°C. Two loops of inoculums were inoculated into 50 ml T1N1 broth and incubated at 37°C for 4 hr with shaking at 100rpm (Shaker: Gallenkamp). Then 200 µl of broth culture was spreaded the on GA plate. The plates then incubated at 37°C for over night. Bacterial colony were then collected in sterile Nalgene centrifuge tubes from the plates and suspended in normal saline, then centrifuged at 10,000 X g for 30 minutes. The pellets were collected in deionized water (3ml dH_2O/ plate); i.e. 600 ml for 200 plates. Preheated (at 69°C) phenol (90%) then was added to the suspension (1/2 volume of water); i.e. 300 ml for 200 plates and heat the mixer at 69°C for 15 minutes on a water bath. The mixer was then cooled to 4°C on ice for 10-15 minutes and centrifuged at 10,000 X g for 30 minutes at 4°C. Supernatant (water layer) were collected in a beaker. Supernatant was dialyzed against distilled water (dH_2O) for 24 hours at 4°C with stirring (cold room) and changed the distilled water once. The dialyzed solution was collected in a beaker and lyophilized the dialyzed solution in autoclaved lyophilized vial. Lyophilized white fluffy LPS attached to the wall of lyophilized vial was collected and weighed in falcon tube (50 ml tube) and dissolved in deionized water (3% w/v) and kept at 4°C. The content in a beaker was then mixed again with stirring for

30 minutes. After complete dissolution, ultracentrifuged the solution at 105,000 X g (~ 27,000 rpm)s for 4 hours and dissolved the pellets in 5 ml deionized water; then kept it at 4°C for over night. The solution was vortexed to completely dissolve the pellets. 10 mg of Proteinase K was then added and made the suspension to 10 ml with distilled water, mixed gently by tilting the tube and incubated for 1 hour at 37°C and over night at 4°C. Ultracentrifuged the solution at 105,000 X g for 4 hours and discarded the supernatant and collected the pellets in 5 ml distilled water and; kept it at 4°C for over night. 20 µg/ml DNase and RNase each was added (i.e. 100 µg of both DNase and RNase), and incubated the solution at 37°C for 2 hours. Ultracentrifuged the whole content at 105,000 X g for 3 - 4 hours was carried out; pellets were dissolved in 1 ml deionized water in lyophilize tube and kept at 4°C overnight. Lyophilized the solution and collected the fluffy powder LPS (very sensitive to static electricity) in drum vial. It was stored then at 4°C. Approximately 210 mg of LPS were extracted and kept the content at -20°C to use in our various immunological methods.

2.7. Immunological response of *Vibrio cholerae* O1 infected patients

Immunological response were measured through following assay procedures ~

1. CTB specific enzyme linked immunosorbant assay (ELISA)
2. LPS specific enzyme linked immunosorbant assay (ELISA)
3. Vibriocidal assay

2.7.1. LPS specific enzyme linked immunosorbant assay

To detect the antibody response through enzyme linked immunosorbant assay (ELISA) against lipopolysaccharide (LPS), plates were coated with LPS solution as a conc. of 2.5 µg/ml in PBS and coated the plates as 100µl/well. Then the plates were incubated at room temperature for overnight. After over night incubation, the LPS coated plates were washed 3 times with PBS, quenched with 0.1% BSA-PBS, 200µl/well. The plates were incubated for 30 minutes at 37°C again. Then the plates were washed 3 times with PBS-Tween (0.05%) and once with PBS. Serum samples from cholera patients were diluted 1:50 ratio in 0.1% BSA-PBS-Tween and were added onto the plate as 100 µl/well. Triple cuts were carried out for each sample. Then sample containing plates were incubated for 90 minutes at 37°C. After that, plates were washed for 3 times with PBS-Tween (0.05%) and once with PBS. Rabbit Anti-human IgA horseradish peroxidase (Jacson 309035011/1000) or Rabbit Anti-human IgG horseradish peroxidase (Jacson 309035006/1000) diluted in 0.1% BSA-PBS-Tween (0.05%) at 1:1000

dilution were added as 100 µl/well and incubated for 90 minutes at 37°C. Plates were again washed the for 3 times with PBS-Tween (0.05%) and once with PBS. 10 mg of Orthophenylenediamine (OPD) was dissolved in 10 ml of 0.1 M sodium-citrate buffer (pH 4.5), to which 4 µl of 30% H_2O_2 was added immediately before use. 100 µl/well of this solution was then added. Optical density was immediately measured by Multiskan Ascent ELISA reader instancely at 450 nm.

2.7.2. Cholera toxin specific enzyme linked immunosorbant assay

To detect the antibody response through enzyme linked immunosorbant assay (ELISA) against cholera toxin B subunit (CTB), plates were coated with gangliomonoside 1 (GM1) at a conc. of 0.3 nmol/ml in PBS and as 100µl/well ; then kept at room temperature for over night. Before use, plates were washed twice with PBS and; then blocked with 0.1% BSA-PBS as 200µl/well and kept for 60 minutes at 37°C. Plates were then washed once with PBS, followed by addition of purified recombinant cholera toxin rCTB 358 at a conc. 0.5 µg/ml of 0.1% BSA-PBS solution as 100µl/well and incubated for another 60 minutes at room temperature. Plates were then washed once with PBS-Tween 0.05%, and once with PBS. The plates were incubated for 30 minutes at 37°C. Then washed the plates 3 times with Tween and once with PBS. Collected serum samples from cholera patients were diluted 1:50 ratio in 0.1% BSA-PBS-Tween and were added

as 100 µl/well. Triple cut were carried out for each sample. Then sample containing plates were incubated for next 90 minutes at 37°C. After that plates were washed for 3 times with PBS-Tween (0.05%) and once with PBS. Rabbit Anti-human IgA horseradish peroxidase (Jacson 309035011/1000) or Rabbit Anti-human IgG horseradish peroxidase (Jacson 309035006/1000) diluted in 0.1% BSA-PBS-Tween solution at 1:1000 dilution were added as 100 µl/well and incubated for 90 minutes at 37°C. Plates were then washed the for 3 times with Tween and once with PBS. 10 mg of Orthophenylenediamine (OPD) was dissolved in 10 ml of 0.1 M sodium-citrate buffer (pH 4.5), to which 4 µl of 30% H_2O_2 was added immediately before use. 100 µl/well of this solution was then added. The readings were taken at 492 nm in a Titertek microplate reader.

2.7.3 Vibriocidal assay

To detect the antibody response using the vibriocidal assay, colones from a pure culture on blood agar plates at 37°C 18h was used. One loopful of bacteria from the each plate was inoculated in 15 ml of Brain Heart Infusion (BHI) medium in a conical flask with cotton plug. Then these were incubated on a shaker at 37°C for 3~4 hours, followed by a centrifugation of the culture for 10 minutes. The supernatant were thrown away; and re-suspended the sediments in sterile normal saline. Then the suspended content was re-centrifuged for another 10 minutes, followed by re-suspending the pellet in sterile saline. The bacterial conc. was then adjusted by spectrophotometer at 600 nm to determine the OD value at

0.3 for *V. cholerae* O1. The heat-inactivated (56°C, 30 minutes) sera were diluted into 2 fold by using sterile saline in flat-bottom microtitre plate (Nunc,F) and kept the plates at 4° C until used. Indicators were prepared immediately before use. 25 µl of the indicator was added to each well except wells in row A, B, C and D of column #1 and followed by an incubation of those plates on a shaker at 37°C for 1 hour (50rev/minute). Then 150 µl of BHI/ well were added and incubated for another 3~4 hours at 37°C without shaking. The plates were read spectrophotometically at 595 nm. The absorbance for control well had reached 0.20 to 0.28 at 595 nm. Vibriocidal antibody titer is defined as the reciprocal of the highest sera dilution resulting in greater that 50% OD reduction when compared to control wells without serum. An increase of titer by 4 folds between acute and convalescent sera is considered to be significant.

2.8. Data analysis

All data were analyzed through Wilcoxon matched pairs t-test (two-tailed) by using GraphPad Prism 4. A *P*-value of ≤0.05 was the criterion for a significant difference.

Chapter 3

Results

3.1. Isolation of *Vibrio cholerae* O1

Ten percent (n=10) patients were confirmed as *Vibrio cholera* O1 infected. There was no isolation of *Shigella* as co-pathogen, but 15% (n=15) patients also were infected in the enterotoxigenic *E. coli* (ETEC).

Table-1: Isolation of pathogens from the stools of rice watery diarrheal patients.

Stool samples	Stool culture results		
tested	***Vibrio cholera* O1**	**ETEC**	***Shigella***
100	**10% (n=10)**	**15% (n=15)**	**0% (n=0)**

3.2. Biochemical characterization of *Vibrio cholerae* O1

3.2.1. *Kligler's Iron Agar Test (KIA)*:

Vibrio cholerae O1 is only a sugar fermenter producing no gas, thus in butt and slant it showed acidic (A) and alkaline (K) type reaction respectively. The slant was alkaline and gave red or pink color, and in the butt of KIA a yellow color existed due to the anaerobic degradation of glucose.

3.2.2. *Motility, Indole and Urea test (MIU)*:

As *Vibrio cholerae* O1 has no tryptophanase activity to split tryptophan to indole and its relative derivatives, and a non-urea splitter, thus it showed negative Indole and urea test. Motility positive test showed its migration from the stab line and diffuse into medium, causing turbidity.

3.2.3. *Citrate test*:

Vibrio cholerae O1 can use citrate as a sole source of carbon in absence of fermentation. Thus it gave positive citrate reaction and turned the tube from the green medium into intense blue.

3.3. Serological identification of *Vibrio cholerae* O1

Among *Vibrio cholerae* isolates, 7 were Ogawa and 3 were Inaba serotypes.

Table-2: Serological identification of the positive isolates.

No. of *Vibrio cholerae* O1 isolates	Inaba serotype (No.)	Ogawa serotype (No.)
10	3 (30%)	7 (70%)

3.4. LPS specific IgA and IgG responses in patients

Serum samples obtained from cholera patients were tested for antibody responses to LPS.

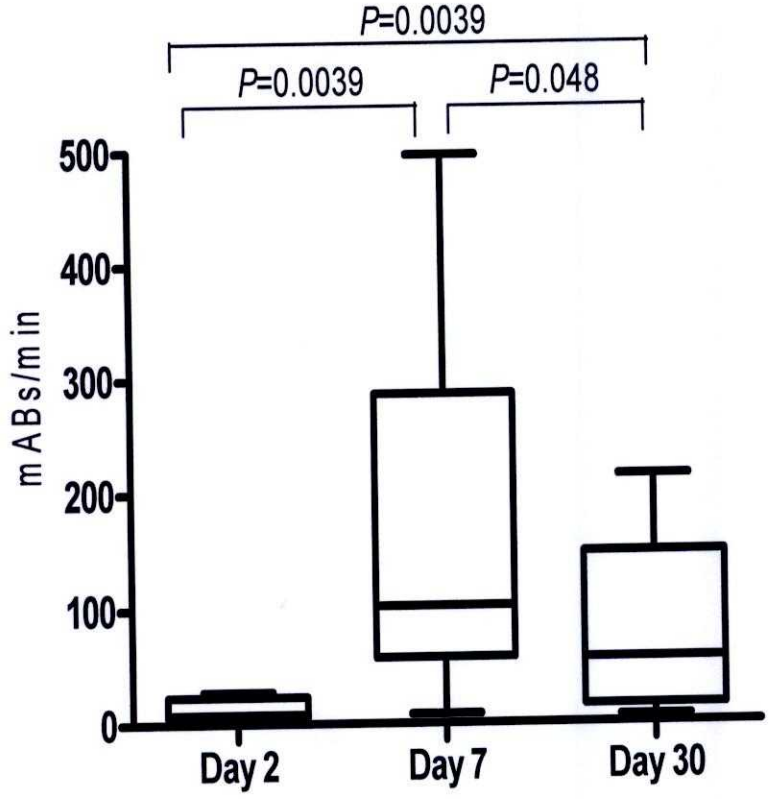

Fig-5: LPS specific IgA response

Patients showed an increase of LPS specific IgA isotype responses for convalescent sera of both day 7 and day 30 compared to the baseline, acute sera of day 2. The increase at both cases were statistically significant compared to the baseline (P=0.0039). However, the day 7 IgA antibody responses were higher than that at day 30. The difference in the IgA isotype responses between day 7 and day 30 was also statistically significant (P=0.048).

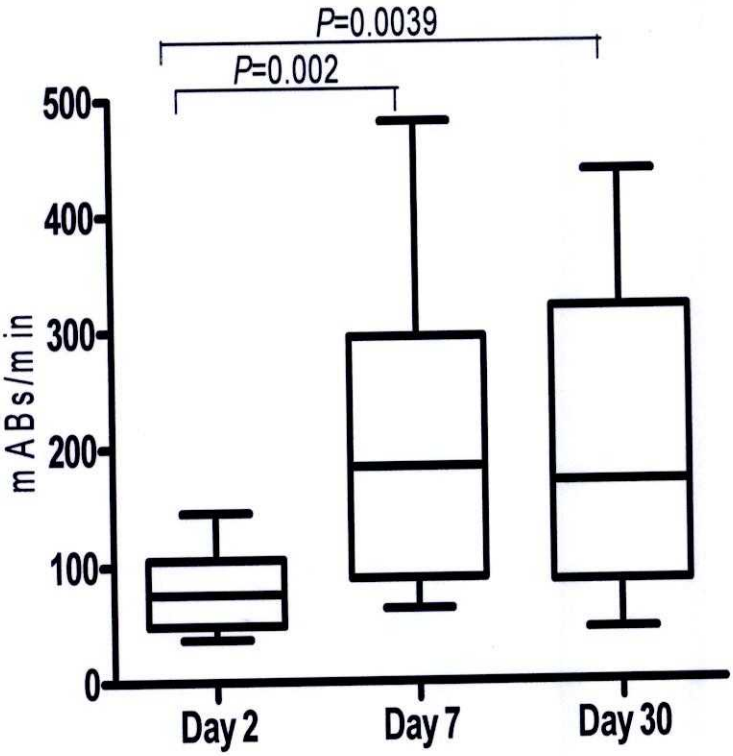

Fig-6: LPS specific IgG response

When sera were tested for IgG isotype response to LPS, the responses of convalescent sera of day 7 were significantly higher than the acute sera of day 2 (P=0.002). Day 30 IgG isotype responses were also higher and statistically significant compared to that of day 2 (P=0.0039). The variation of IgG isotype responses between day 7 and day 30 was found statistically insignificant (P=0.3323).

3.5. CTB specific IgA and IgG responses in patients

Serum samples obtained from cholera patients were also tested for antibody responses to CTB.

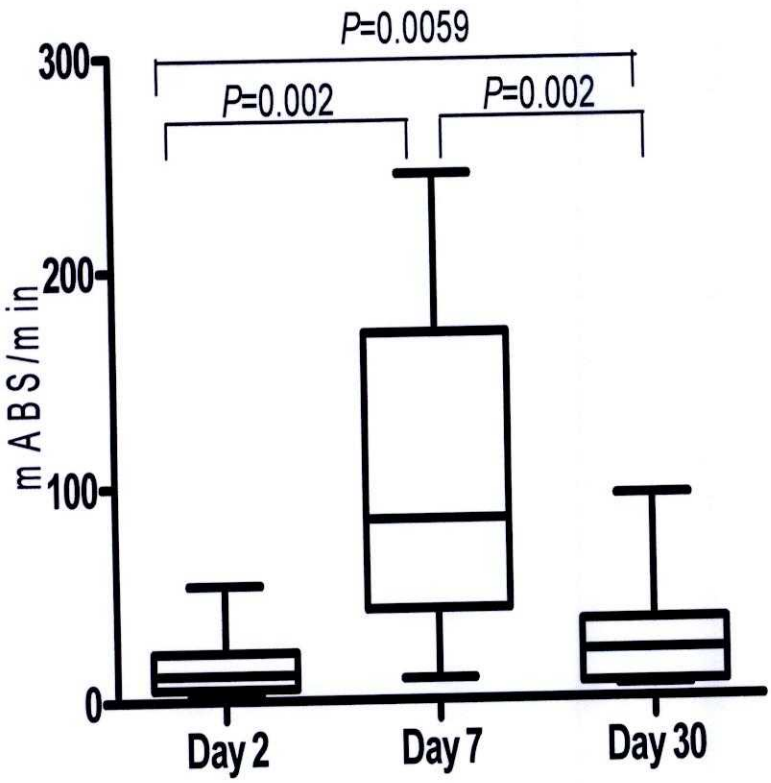

Fig-7: CTB specific IgA response

Results showed an increase of CTB specific IgA isotype response for convalescent sera of day 7 compared to baseline, sera of day 2. This increase was found statistically significant compared to baseline (P=0.002). Although there was same increase in the CTB specific IgA response of day 30 compared to that of day 2, the response was found statistically significant (P=0.0059). The difference in the IgA responses between day 7 and day 30 was also statistically significant (P=0.002).

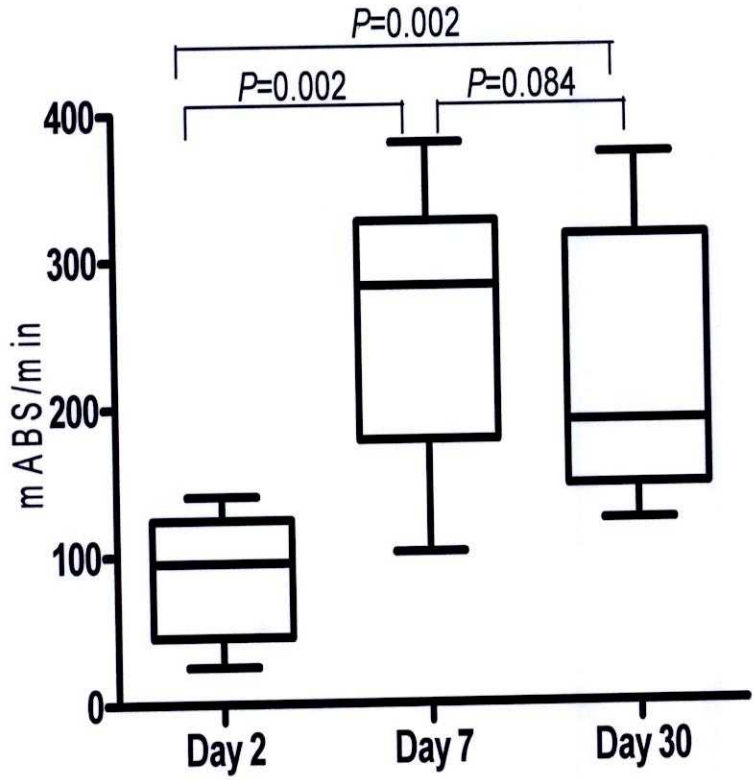

Fig-8: CTB specific IgG response

When sera were tested for IgG isotype to CTB, the responses of both the convalescent sera at day 7 and day 30 were higher than at the acute stage of day 2 (P=0.002). The difference of IgG responses between day 7 and day 30 was not different (P=0.084).

3.6. Vibriocidal responses

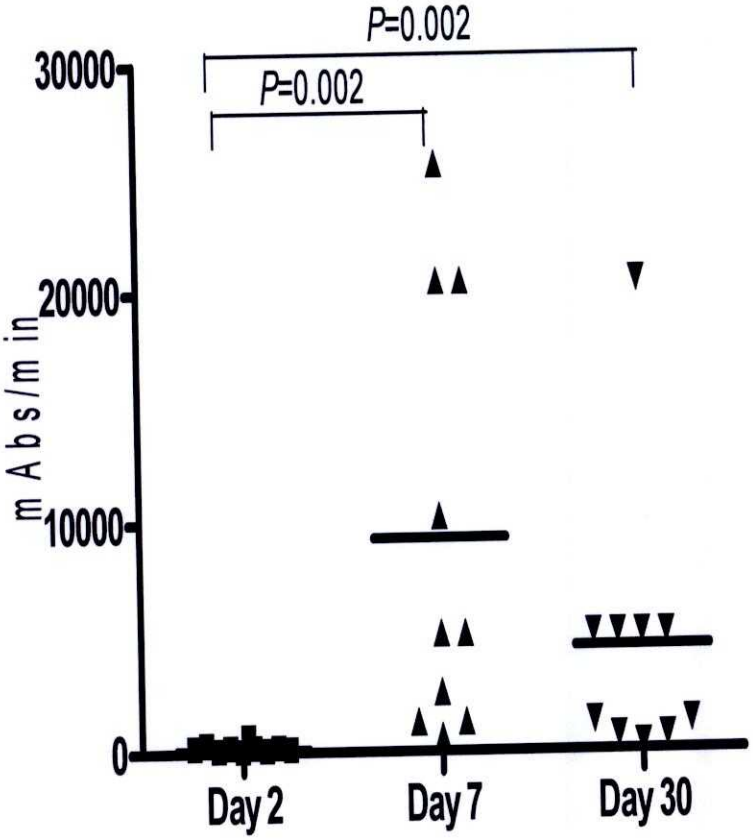

Fig-9: Vibriocidal responses

The vibriocidal antibody response were increased in the both stages at day 7 (P=0.002), and day 30 (P=0.002).

Chapter 4

Discussion

Cholera has always occurred in periodic epidemics, with most severe outbreaks limited to a few countries, namely Bangladesh, India, and countries of Africa. Cholera continues to be one of the most common causes of morbidity and mortality among infants and children in developing countries like Bangladesh (Ryan, 2000). Climate changes, natural catastrophe and large number of people crowded under poor sanitation conditions have led to the spread of cholera epidemics. Unfortunately, these conditions are still prevailing and can not be eradicated in the short term. Thus an effective cholera vaccine as cost effective public health tool appears to be a solution in the control epidemics.

During the study period, among 100 rice watery diarrheal patients only ten percent (n=10) patients had been suffering from cholera caused by *Vibrio cholerae* O1. Stool cultures of 10 cholera patients showed that 7 patients were infected with *Vibrio cholerae* O1 ogawa and the rest were with inaba serotype. Fifteen percent of the patients were infected with enterotoxigenic *E. coli* (ETEC). No Salmonella, *Shigella* was found in this period. Mixed infections were not observed with the infected patients.

LPS is known as a potent protective immunogen, able to elicit immune response in host. The principle role of both LPS and CTB specific IgA, in case of nonvasive *Vibrio cholerae* O1, is the protection of intestine from the adherence of *Vibrio cholerae* O1 to minimize the microbial load and to control inflammation (Gail A.

Hecht, 2003). The cholera patients showed an increase of LPS specific IgA responses at convalescent stage in both day 7 and day 30 compared to the baseline. The increase at both cases were statistically significant compared to baseline (P=0.0039). The day 7 IgA isotype responses were higher than that of day 30. The difference of IgA isotype responses between day 7 and day 30 was statistically significant (P=0.048). For IgG isotype response to LPS, the responses of convalescent sera of day 7 were significantly higher than the acute sera of day 2 (P=0.002). Day 30 IgG isotype responses were also higher and statistically significant compared to that of day 2 (P=0.0039).

Cholera toxin (CT), being a protein, is strictly a T-cell dependent antigen (Abbas, 1997). Antibody to CT is known to exert a synergistic protective effect against cholera in animal studies, and offer significantly higher short-term protection against cholera in humans (Qadri *et al*, 2004). CT specific IgG antibodies are predominately involved in the secondary antibody response, which exists approximately one month following antigen recognition, thus the presence of specific IgG generally corresponds to maturation of the antibody response (Meulenbroek, A.J.; Zeijlemaker, W.P. 1996). Results showed an increase of CTB specific IgA isotype response in the convalescent stage at day 7 compared to baseline, sample at day 2. This increase was significantly higher compared to baseline (P=0.002). Although there was less increase in the CTB specific IgA response of day 30 compared to that of day 2, the response was statistically

significant (P=0.0059). The difference of IgA in the responses between day 7 and day 30 was also statistically significant (P=0.002). For IgG isotype to CTB, the responses of both the convalescent sera of day 7 and day 30 were found a boost pick against that of acute sera of day 2. The resulted responses were also significant statistically (P=0.002).

An important surrogate marker for protection in cholera is the vibriocidal antibody assay, which has been used very successfully to predict the protection from natural infection in *Vibrio cholerae* O1 (Qardi *et al.*, 1995). The vibriocidal antibody responses were found significantly increase in both the convalescent sera of day 7 and day 30 compared to the acute sera of day2 (P=0.002).

Further studies should be carried out to understand the protective immune response against *Vibrio cholera* O1 which will help in vaccine development.

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Appendices

Appendix: A

The composition and method of preparation of different media, and buffers used in this study are given below

Plates

Preparation of MacConkey agar plate (1000 mL)

Pepton	20.00 g
Lactose	10.00 g
Sodium Chloride	5.00 g
Bile salts	1.50 g
Neutral red	0.05 g
Crystal violet	1.00 g
Bacto agar	15.00 g

pH=7.2

Preparation of Taurocholate-Tellerite-Gewlatin Agan (TTGA)

Sodium cholride	1%
Triptone peptone	1%
Sodium-taurocholate	0.5%
Gelatin	3%
Agar	1.5%

Preparation of Salmonella Shigella Agar (SSA)

Dehydrated media

Buffer

Preparation of phosphate buffer saline (PBS) (pH 7.2)

NaCL	80.00 g
Na_2HPO4	11.50 g
KH_2PO4	2.00 g
KCL	2.00 g
Deionized water	1000.0 ml

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

Preparation of sodium citrate beffer (pH 4.5)

Sodium citrate buffer	14.7 g
Deionized water	400 ml

When dissolved the pH of the buffer was adjusted to 4.5 with 6N HCl and more water was added to make the final volume 500 ml.

Appendix: B

Kligler Iron Agar (KIA) media:

Lab-lemco powder	3.0g
Yeast extracts	3.0g
Pepton	20.0g
Lactose	10.0g
Dextrose (glucose)	1.0g
Ferric citrate	0.1g
Sodium thiosulphate	0.3g
Phenol red	0.05g
Agar	12.0g
pH	7.2-7.6

Motility indole Urea (MIU) media

Trypton	30.0g
Potassium dihydrogen phosphate	1.0g
Sodium chloride	5.0g
Agar	5.0g
Phenol red	2ml
Distilled water	1lit
pH	6.9-7.3

Citrate Medium

Sodium ammonium sulphate	1.5 g
Potassium hydrogen phosphate	1.0g
Magnesium sulphate	0.2g
Bromothymol blue	0.016g
pH	6.6-7.0

Appendix: C

Chemical and regents

1. Recombinant Cholera Toxin B-subunit (rCTB 358)
2. Ficol, Pharmacia LKB Biotechnology AB Uppsala,
3. Rabbit Anti-human IgA HRP, Jackson Immuno Research, West Grove, P.A., USA.
4. Rabbit Anti-human IgG HRP, Jackson Immuno Research, West Grove, P.A., USA.
5. H_2O_2 (Hydrogen peroxide) 30%, Merk, Darmstadt, Germany.
6. $MgCl_2$ (Magnesium Chloride), Merk, Darmstadt, Germany.
7. Na- acetate, $NaHCO_3$ (Sodium bicarbonate), Merk, Darmstadt, Germany.
8. ELISA plate (NuncF)
9. Brain Heart Infusion (BHI) medium
10. Flat bottom microtitre plate (Nunc,F)

11. Complement(guinipique)
12. Orthophenylenediamine (OPD); Sigma chemical Co., St. Louis, MO, USA.
13. Tween 20; Sigma chemical Co., St. Louis, MO, USA.
14. Gangioside GM1; Sigma chemical Co., St. Louis, MO, USA.
15. Bovine serum albumin (BSA); Sigma chemical Co., St. Louis, MO, USA.

Appendix-D

Preparation of 1% BSA in PBS (500ml)

Phosphate buffer saline (PBS)	500ml
Bovaine Serum Albumin (BSA)	5.0 g

Preparation of 0.1% BSA in PBS –Tween (500ml)

Phosphate buffer saline (PBS)	500ml
Bovaine Serum Albumin (BSA)	0.5 g
Tween	250µl

Preparation of Orthophenylenediamine (OPD)

Orthophenylenediamine (OPD)	0.1 g
0.1 M Na-citrate buffer (pH 4.5)	10 ml
30% H_2O_2	4 µl

Appendix-E

Preparation of indicator (bacteria-complement-saline mixture)

Micro-organism	**Saline**	**Bacteria**	**Complement(guinipique)**
V. cholerae O1 (X25049 & 19479)	**2.55 ml**	**150 µl**	**300 µl**

Preparation of Brain-Heart Infusion Medium

Brain Heart Infusion	37.0 g
Yeast Extract	5.0 g
Hemin (0.1% Solution)	5.0 ml
Vitamin K1 (1% Soln)	0.05 ml
Agar	15.0 g
Distilled Water	1000.0 ml
L-Cysteine	2.0 ml

Final pH = 7.0 ± 0.2 at 25 ° C.