In vivo propagation of monoclonal antibodies in ascites fluid produced in BALB/c mice: Characterization and diagnostic application for the identification of *Vibrio cholerae* O1

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

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DECLARATION

I hereby declare that this thesis project entitled "*In vivo* propagation of monoclonal antibodies in ascites fluid produced in BALB/c mice: Characterization and diagnostic application for the identification of *Vibrio cholerae* O1" is submitted by me, Mashiat Nawar Chowdhury (ID – 16176001), to the Department of Mathematics and Natural Sciences. The work was performed under joint-supervision of Professor Naiyyum Choudhury, former Coordinator of Biotechnology and Microbiology programmes of the Department of MNS, BRAC University and Professor Firdausi Qadri, Director, Vaccine Centre, icddr,b. This thesis project was done in the Mucosal Immunology and Vaccinology Laboratory of the Infectious Diseases Division at the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) as a part of my requirement for the degree of MS in Biotechnology. I also declare that this research work is based on original results. Materials and knowledge that I have consulted from the published work accomplished by other researchers have been properly cited and acknowledged within the text of my work.

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Abstract

Monoclonal antibodies are powerful tools in biomedical sciences. High affinity of binding domains of monoclonal antibodies towards a specific antigen enables their use in diagnostic assays and kits to identify particular infectious agents. *In vitro* propagation of monoclonal antibodies is considered expensive, tedious, low yielding and difficult to purify. *In vivo* ascites methods, however, is more economic for large scale generation of monoclonal antibodies needed to meet the growing demand of diagnostic assays. In this study, *in vivo* method had been adopted to propagate monoclonal antibody (mAb) against *Vibrio cholerae* O1 responsible for majority of cholera outbreaks. Hybridoma cells were intraperitoneally injected into pristane primed BALB/c mice and ascites fluid was harvested within 10-14 days. Ascites of 2.05 ± 0.23 ml was obtained from each mouse with IP injection of $1.5\text{-}2X10^6$ hybridoma cells. Monoclonal antibody from ascites was purified by affinity chromatography and a concentration of 1.89 ± 0.62 mg/ml of purified antibody was obtained. The purified antibody was investigated for their ability to detect *V. cholerae* O1 Ogawa and Inaba serotypes by agglutination test. High specificity of the purified antibody against both *V. cholerae* Ogawa and Inaba serotypes was observed, with a titer upto 1:40-1:80 dilutions for agglutination, while no cross reactivity was found against *V. cholerae* O139 and other enteropathogenic bacteria. Dot blot immunoassay showed that the purified antibody had binding affinity against lipopolysaccharide antigen from the cell wall of *V. cholerae* O1 serotypes without possessing any cross-reactivity against lipopolysaccharide of *V. cholerae* O139. Isotype of the mAb was determined to be IgG3 by ELISA method. This mAb was then conjugated with colloidal gold nanoparticles to develop a simple lateral flow immunoassay for rapid diagnosis of cholera. For this purpose the pH and minimum concentration of mAb for conjugation were optimized as 8.5 and 24 µg/ml, respectively. This rapid diagnostic test warrants further development and evaluation.

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LIST OF ABBREVIATIONS

Abbreviations	Description
cAMP	Cyclic adenylate monophosphate
O-Ag	O-antigen polysaccharide
COAT	Coagglutination test
DLS	Dynamic light scattering
HAT	Hypoxanthine-aminopterin-thymidine
LFA	Lateral flow assay
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
RDT	Rapid diagnostic test
SMART	Sensitive Membrane Antigen Rapid Test
TTGA	Taurocholate-tellurite gelatin agar

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Chapter 1: Introduction

Chapter 1: Introduction

Cholera is an intestinal, non-invasive diarrheal disease common among developing countries and is caused mainly by *Vibrio cholerae* O1and less commonly by *V. cholerae* O139 (Clemens et al., 2014). These rod-shaped Gram negative bacteria produce several toxins, among which cholera enterotoxin causes the classic diarrheal dehydrating symptoms that functions by activating adenylate cyclase enzyme in the intestinal mucosal cells and increase cAMP. The increase in cellular cAMP causes the mucosal cells to pump out water and electrolytes (Sack et al., 1999).

Symptoms typical of cholera that rule out other diarrheal disorders include acute watery diarrhea in less than 24 hours with dehydration and vomiting (Nelson et al., 2011). Being a communicable disease, cholera spreads from person to person contact through fecal-oral route (WHO, 2010). The infectious dose of cholera is 10^5 to 10^8 cells of *V. cholerae* and a cholera patient passes about 10^{11} to 10^{12} organisms per liter in rice watery stool. This places close contacts at risk, especially in areas with poor sanitary conditions or crowded living situations that is often common in developing countries and refugee camps (WHO 2010; Nelson et al., 2011). Therefore, a single case of cholera can soon culminate in an outbreak or epidemic. More than 50 countries are harboring the disease this way, totaling 3 to 5 million cases and over 100,000 deaths from cholera every year (Zuckerman and Rombo et al. 2007).

Rapid dehydration makes cholera fatal with high mortality rate if left untreated (Lopez et al., 2014). Treatment of cholera through oral rehydration and antibiotics is important to reduce disease burden and spread (WHO, 2017). Early intervention through appropriate treatment can reduce fatality to less than 1% (WHO, 2011). This can only be possible if cholera cases are detected early with cheap and rapid diagnostic tests to instill preventative and control measures in areas with or without prior incidences.

1.1 Cholera epidemiology and disease burden

1.1.1 Cholera pandemics

Cholera is a major public health concern that is becoming increasingly important as the number of countries affected continues to increase. Seven distinct pandemics of cholera have occurred since the onset of the first pandemic (Pollitzer, 1959). Apart from the seventh pandemic, which originated on the island of Sulawesi in Indonesia (Kamal, 1974), all cholera pandemics emerged from Indian subcontinent, particularly the Ganges delta, and spread to other continents, lasting over many years (Faruque et al., 1998).

The first cholera pandemic occurred in 1817 in Ganges River delta in India and lasted through 1824, spreading from India to China, Indonesia, Japan, the Middle East, and Southern Russia before receding. The second pandemic started in India and reached Europe in 1830, claiming 22,000 lives in England. Irish immigrants carried the disease with them from Europe to North America in 1832. A second outbreak spread during 1848 across England and Wales and killed 52,000 in two years. The third pandemic that erupted in 1839 and persisted until 1856 is considered to be the most fatal as it swiped large areas of Asia, Europe, North America and Africa. The fourth pandemic commenced in the Bengal region. From there, the disease spread to the Middle East through Indian Muslim pilgrims who visited Mecca, affecting at least 30,000 of the 90,000 pilgrims, and farther migrated to Europe, Africa and North America. The fifth pandemic spread through Asia, Africa, South America and parts of France and Germany from India. The sixth had high fatality, killing 800,000 in Indiabefore moving into the Middle East, northern Africa, Russia and parts of Europe.The last and ongoing seventh pandemic to occur began in Indonesia in 1961, unlike the first six, and spread through Asia to Africa, Europe and Latin America. It emerged in South America, following 100 years of cholera free episode, killing 10,000 people in Peru. The seventh pandemic marked the emergence of a new strain called El Tor in Bangladesh that now has spread to 11 more countries (Bishop et al., 2011; Harris et al., 2012).

1.1.2 Recent outbreaks

WHO estimates that about 3-5 million cholera cases transpire each year, with more than 100,000 deaths resulting from it (Zuckerman et al. 2007). This figure is however considered to be an underestimate of the actual number of cholera cases. The actual figure is believed to be much higher due to lack of adequate surveillance and report of fewer cases to avoid trade embargo in affected country.

In recent years, there had been several major cholera epidemics in Asia and Africa, notably in Haiti (Chin et al. 2011), Cameroon (Cartwright et al. 2013), Guinea-Bissau (Luquero et al. 2011) and the Democratic Republic of the Congo (Kelvin 2011). In 2015, 42 countries reported cholera cases among which 16 countries were in Africa, 13 in Asia, 6 in Europe, 6 in the Americas, and 1 in Oceania. 80% of the total 172, 454 cases belonged to Afghanistan, the Democratic Republic of the Congo, Haiti, Kenya, and the United Republic of Tanzania. 41% of cases were reported from Africa, 37% from Asia and 21% from the Americas. These included 1304 deaths leading to an overall case fatality ratio of 0.8% (WHO, 2016). In 2014, the overall case fatality rate was higher (1.17%), and included 2231 deaths from 190, 549 cholera cases with 84% of cases belonging to the five epidemic countries (WHO, 2015).

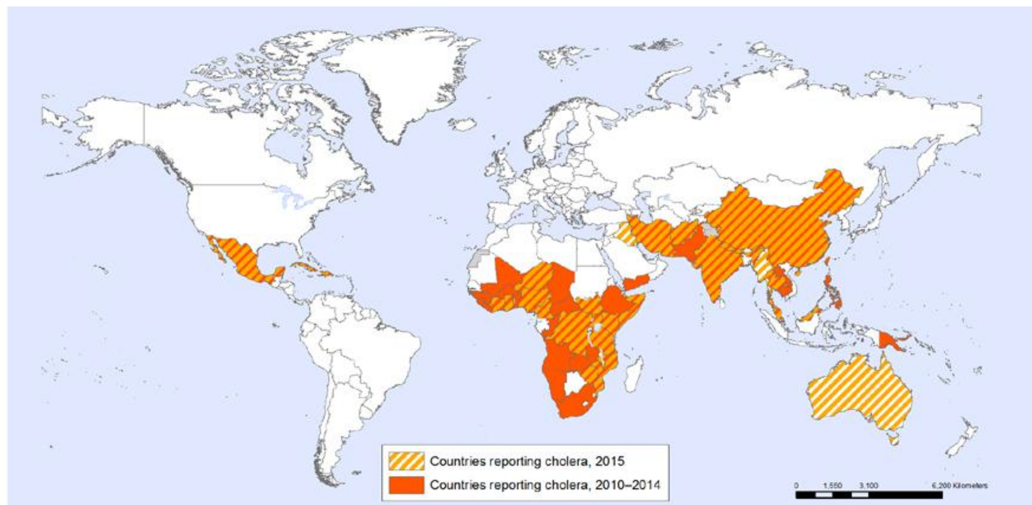

Figure 1.1.2 Countries reporting cholera between 2010-2015 (WHO, 2016)

1.1.3 Seasonality of outbreaks

Cholera cases emerge seasonally and this depends upon the geographic location of the region. In Asia two peaks are observed before and after the monsoon rains (Sack et al. 2004). These seasonal peaks can be determined by grouping countries according to latitude range. The constancy of temperature in countries near equator makes the annual peaks more evident and predictable (Emch et al., 2008). Low temperature and little rainfall during the winter reduce the incidence of cholera in these countries. Gradually, as temperature increases, number of incidence also increases until a conspicuous peak is observed at pre-monsoon season. The number of outbreaks again lowers during the actual monsoon season due to dilution of bacterial concentration in water bodies. A second peak is again observed during post-monsoon season bearing similar pattern of high temperature and mild rainfall (Emch et al., 2010).

1.2 Classification of *V. cholerae*– causative agent of cholera

Differences in the sugar composition of the heat-stable surface somatic O-antigen form the basis of the serological classification of *V. cholerae*. Currently there are about 206 O serogroups of *V. cholerae* classified based on O antigen of lipopolysaccharide (Shimada et al., 1994; Yamai et al., 1997). Of these, only O1 and O139 serogroups are known to cause cholera epidemics. *V. cholerae* O1 has been divided into two biotypes, Classical and El Tor, which are further subdivided into three serotypes: Inaba, Ogawa and Hikojima. The three serotypes have been differentiated on the basis of three antigenic determinants or epitopes, A, B and C, associated with the O-Ag of the LPS (Nair and Bartram, 2002).

Composition of O specific antigens varies among different serotypes:

- Ogawa serotype: Expresses A and B antigens and a small amount of C antigen
- Inaba serotype: Expresses only the A and C antigens
- Hikojima: Expresses A, B and C antigens (but rare and unstable)

V. cholerae O1 predominates as the causative agent of cholera. Although *V. cholerae* O139 persists in regions of South Asia, they are present at much lower levels (Harris et

al., 2008). *V. cholerae* O139 had been responsible for two epidemics till date and no major outbreak in the last decade. The O1 El Tor biotype currently prevails and had been responsible for the seventh pandemic that continues today (WHO, 2017).

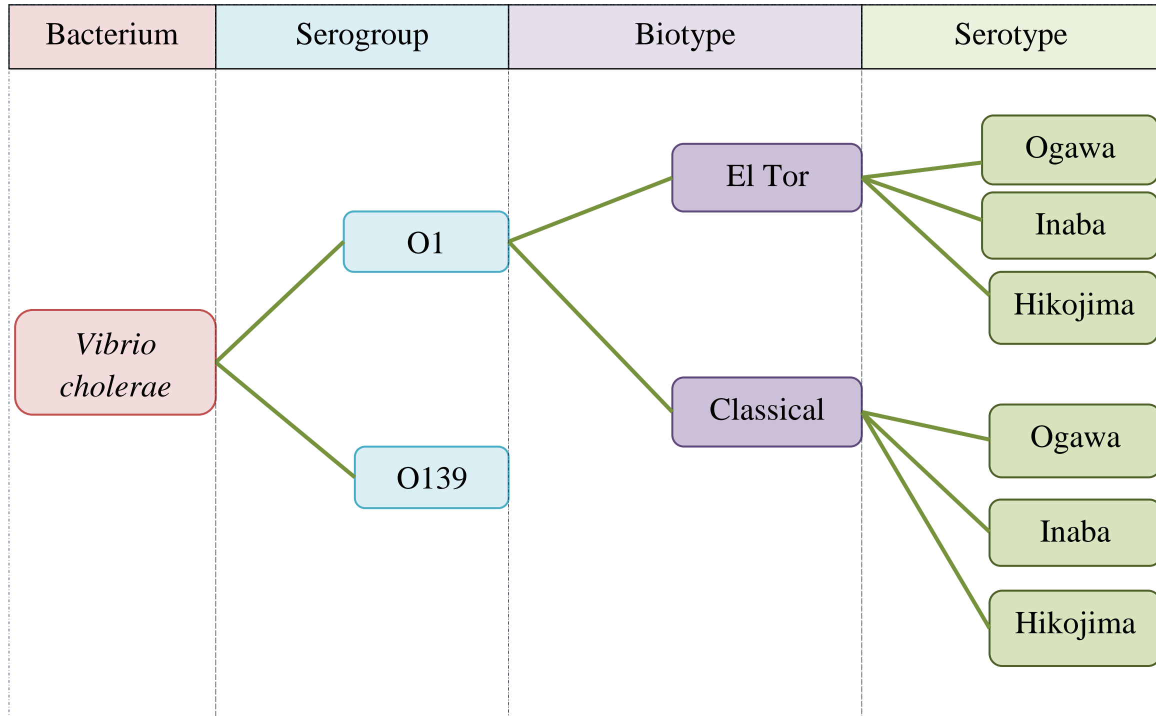

Figure 1.2: Classification of diarrheagenic *Vibrio cholerae* based on surface LPS

1.3 *V. cholerae* lipopolysaccharide

The lipopolysaccharide of all Gram-negative bacteria has two distinct regions:

1. A hydrophilic polar region, polysaccharide. This consists of:
    a. O-antigen polysaccharide (O-Ag)
    b. Core polysaccharide
2. A hydrophobic lipid portion, lipid-A (Luderitz et al., 1971)

Lipid-A is anchored to the outer leaflet of the outer membrane while the hydrophilic polar region projects outward. It is this O-antigen polysaccharide, core polysaccharide and proteins present in the outer membrane that elicit immune response (Chatterjee and Chaudhuri, 2003).

Chemical analysis of the A epitope of O-Ag present in both Ogawa and Inaba serotypes revealed the presence of homopolymer containing the amino sugar d-perosamine substituted with 3-deoxy-l-glycerotetronic acid (Kaper et al., 1995; Stroeher et al., 1992). Immunochemical studies and gel permeation chromatography of the polysaccharide fractions of the three serotypes of O1 showed that A epitope was present at multiple determinants whereas B and C epitopes were present as single determinants on each LPS (Gustaffson et al., 1985). This allows targeting both Ogawa and Inaba serotypes with a single antibody against their common A epitope while the multiple determinacy of the common epitope can form the basis of sandwich assay format of dipstick test.

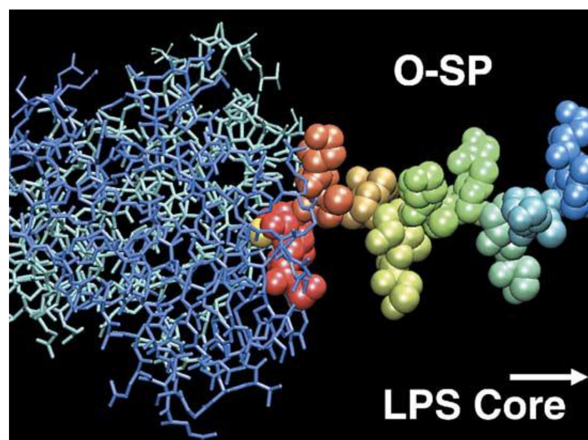

Figure 1.3: Fab fragment of monoclonal antibody complexed with methylated-α-glycoside of the disaccharide of *V. cholerae* O1 O-Ag (Villeneuve et al., 2000)

1.4 Diagnosis and detection of cholera

According to WHO, a cholera case is suspected if one of the two conditions are met:

- A patient of 5 years or over develops severe dehydration or dies from acute watery diarrhea in areas where no previous case of cholera had been reported
- A patient of 2 years or over develops acute watery diarrhea in areas where cholera is common (WHO, 2005)

Cholera is differentiated from other diarrheal causing diseases by testing stool samples or rectal swabs from patients where lab facilities are available. Samples need to be transported to labs in Cary Blair media and isolated in selective media for identification

(CDC, 2016). Although culture method is considered necessary for diagnosis of cholera, several rapid tests are being developed to improve early diagnosis.Confirmation of positive cultures is done by biochemical tests and dark-field microscopy (Qadri et al., 1997). Identification of serogroups and serotypes with specific antibodies may not be imperative for treatment of diagnosed cholera among patients; however, their tests remain important for epidemiological purpose (CDC, 2016).

1.4.1 Diagnosis of cholera in patients

Culture method: Microbiological culture of stool specimen or rectal swab is the gold standard for cholera diagnosis. Stool specimen is directly streaked on the selective TTGA (taurocholate-tellurite gelatin agar) plates and incubated for overnight at 37˚C incubator. Concurrent enrichment of fecal specimen in alkaline peptone water followed by plating on TTGA plate is also carried out to isolate *V. cholerae*. Suspicious *V. cholerae* colonies appear as gray colonies with a black center surrounded by zone of opacity on TTGA plates. These suspected colonies are finally confirmed by the slide agglutination with different monoclonal or polyclonal antibodies.

Rapid test: The current culturing method used for diagnosis of cholera requires a functional laboratory and is time-consuming. Cholera endemics are characteristic of poverty-stricken areas with deficit of microbiological facilities. Moreover, emergency situations such as war, natural disasters, refugee crisis or population displacement that commence cholera outbreaks need swift action for diagnosis and treatment, and the conventional culturing method in these scenarios may prove unbefitting. These conditions have demanded the development of cheap and rapid diagnostic tests (RDTs) for cholera as preventative and control measures (Wang et al., 2006).

To date, more than twenty RDTs have been developed and marketed to diagnose cholera (Mukharjee et al., 2010; Boncy et al., 2013; Dick et al., 2012; Sinha et al., 2012; Ley et al., 2012). Most RDTs are chromatographic-immunoassays that function by capturing a component of *V. cholerae* on a solid surface and providing a visual color change through its binding with specific reagents. Although PCR has the ability to detect the lowest amount of bacteria present in a sample, RDTs have shown to attain low detection

thresholds as well as high sensitivity and specificity (Dick et al., 2012). Several reports on their field and laboratory trials have demonstrated their variation in performance with COAT, IP cholera dipstick, SMART, IP dipstick and Medicos RDTs displaying some promise in fields (Harris et al., 2009; Wang et al., 2006; Kalluri et al., 2009; Qadri et al., 1994; Qadri et al., 1995).

1.4.2 Detection of *V. cholerae* for epidemiological purpose

Preliminary identification of colonies in TTGA agar is confirmed using serotyping and biochemical tests. This confirmation step also takes a few days and requires laboratory resources (Center for Disease Control, 2003).

Slide agglutination test: Serogroups of *V. cholerae* may be identified by simple agglutination test against O antigens. This is very simple and rapid procedure that can be accomplished with *V. cholerae* specific antisera on glass slide or petri dish.

Biochemical identification: Biochemical confirmation is only infrequently used as slide agglutination is preferred for identification of serotypes and serogroups of *V. cholerae*. These biochemical tests include oxidase test, string test, Kligler's iron agar or triple sugar iron agar test, lysine iron agar and arginine glucose slant test.

Polymerase chain reaction (PCR): Highly sensitive and specific PCR is rarely used for detection of *V. cholerae* in stool samples. PCR is more routinely used for their identification in environmental or food samples (Oyedeji et al., 2013). Drawbacks of using PCR are that it requires trained personnel and sophisticated equipment which is not always available in all parts of the world (Hasan et al., 1994).

1.5 Lateral flow immunoassays

Lateral flow immunoassays (LFA) are unidirectional point of care RDT that detect the presence or absence of an analyte. Cholera LFAs can be used to diagnose cholera quickly while confirmation of the outbreak is being carried out by culture and PCR methods. The major advantage of LFA is to improve cholera surveillance in developing countries wanting lab facilities and trained personnel (Mukharjee et al., 2010; De et al., 2013;

Kalluri et al., 2006). Benefits of LFA, from industrial perspective, include low cost and short time for development, steering the rapidly growing biotech market in RDTs. They are highly specific and sensitive for diagnosis, easy to store by obviating the need for refrigeration, and are user friendly for their one step operational ease (Innova Biosciences, 2017).

1.5.1 Principles of LFA

The most commonly used LFA for the detection of larger analytes with multiple epitopes like lipopolysaccharide uses direct or sandwich reaction format. Sample is added in less than excess amount so that microspheres that will not capture sample analyte will flow toward a control line of immobilized antibody (Innova Biosciences, 2017). The test line that recaptures the sample analyte utilizing its multiple epitope will convey presence of antigen in a sample, whereas the control line will verify the absence of experimental errors.

Lateral flow immunoassay strips include:

i. **Antibodies:** Three types of antibodies are needed for strip preparation. Capture line antibody is specific towards sample antigen and control line antibody is specific towards conjugate antibody. Conjugate antibodies are antibodies specific towards sample analyte that is attached to microspheres.
ii. **Microspheres:** Microspheres are attached to detection antibody for visualization of antigen-antibody binding. Colloidal gold nanoparticles are predominantly used microspheres due to their ability to interact with proteins. Colloidal gold NPs are negatively charged over a wide range of pH and therefore can form ionic bond with protonated proteins (Englebienne, 2000).
iii. **Membranes:** Membranes for strip preparation include nitrocellulose, cellulose acetate or glass fiber membrane. Nitrocellulose membrane is used for high protein binding.
iv. **Conjugate Pads:** Sample is applied to this area. Conjugate pads must absorb large amount of sample and steadily release them into the membrane.

v. **Membrane backing:** This provides strength to fragile membranes (Kang et al., 1996).

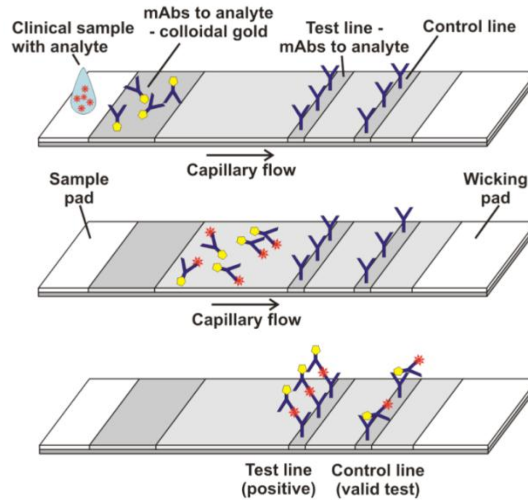

Figure 1.5.1: Lateral flow assay in direct reaction format (DxDiscovery, 2017)

1.5.2 Commercially available LFA for cholera

The most commercially successful and well evaluated LFA for cholera was developed by Institute Pasteur, France and commercialized by Span Diagnostics, India under the trade name Crystal VC. The test kit is able to detect both *V. cholerae* O1 and O139 serogroups with sensitivities of 92–97% and specificities of 71–76% (Harris et al., 2009; Mukharjee et al., 2010). This variation in performance prevents the use of Crystal VC to provide treatment decisions. World Health Organization suggests a thorough validation of such RDTs before they can be used for point of care treatment. To date, cholera RDTs can be used solely as a means for early detection of outbreak to aid in preparedness and response. All positive samples tested with RDT must be confirmed through standard laboratory tests (WHO, 2017).

1.6 Monoclonal antibody production for immunoassays

1.6.1 Generation of hybridoma cell line

Monoclonal antibodies can be produced either *in vitro* or *in vivo* or a combination of both. Before antibodies can be produced by either method, hybridoma cells need to be generated to yield a cell line capable of producing a specific type of antibody.

Before the advent of hybridoma technologies, scientists produced polyclonal antibodies from non-immortalized cells in immunized animals. This required repeated use of animals for large scale production. Development of hybridoma technology has minimized the number of animals used for large scale production of antibodies, although ascites technique still requires the use of animals for production. Hybridoma technology is required at the start of both in vitro and in vivo technique and involves the following steps:

Step 1: Mice are immunized with appropriate antigen emulsified in adjuvant or injected with whole cells, membranes and microorganisms. Mice are usually immunized every 2-3 weeks and are euthanized to remove spleen once sufficient antibody titer is obtained in serum. Antibody titer in blood can be measured by collecting small volumes of blood samples and determining the amount by ELISA and flow cytometry. If antibody titer is too low, mice are boosted until desired response is obtained, and if titer is high enough, mice are boosted with antigen devoid of adjuvants 2 weeks after previous immunization and 3 days prior to proceeding towards fusion (Loeb and Quimby, 1999).

Step 2: Myeloma cells need to be prepared for fusion with antibody-producing spleen cells obtained from mice. This is done one week prior to fusion through their culture with 8-azaguanine that force them to become sensitive to hypoxanthine-aminopterin-thymidine (HAT) medium.

Step 3: Freshly harvested spleen cells are co-centrifuged with the prepared myeloma cells in the presence of polyethylene glycol that fuses two cell membranes together. The cells are grown in HAT medium and only the myeloma-spleen cells survive. These fused hybridoma cells are next grown in 96 well plates containing feeder cells to supply growth factors from saline peritoneal washes of mice (Quinlan and O’Kennedy, 1994).

Step 4: Hybridoma cell lines are next cloned either by limiting dilution or by expansion through ascites technique (National Research Council, 1999).

1.6.2 *In vitro* propagation and its concomitant problems

The simplest method of producing mAbs in culturing hybridoma cells in batches and purifying mAbs from the medium. This method is usually employed for small-scale projects where it appears to be most practical as it entails the use of sophisticated equipment and well trained operators. The most common procedures to produce mAbs in vitro are batch tissue-culture system and semipermeable-membrane-based systems.

Batch culture: The simplest method to produce mAb is to culture hybridoma cells in batches and to purify mAb from the culture medium. Although bovine serum albumin is commonly used in tissue culture medium, it contains bovine immunoglobulin that can contaminate the final mAb products (Darby et al., 1993). To prevent this, cells are cultured for 3-4 passages in 10% FBS supplemented medium, and then transferred and grown on FBS-free medium for 10 days following which mAb can be harvested. Simplest batch culture yields about 20µg/ml mAb, although spinner flasks and roller bottles can increase the concentration a few micrograms (Reuveny et al., 1986; Tarleton and Beyer, 1991).This is one of the main disadvantages of batch culture as it produces significantly low levels of mAbs retrieved from tedious processing of large volumes of culture in the same period of time compared to *in vivo* technique. Moreover, as high as 42% mAbs are denatured during purification, lowering the final titer even further (Lulau et al., 1996; Underwood and Beans, 1985).

Semi-permeable based system: Semi-permeable based system has been developed to overcome the low yield of mAb. These systems utilize flasks or bioreactors that compartmentalize cells and mAb produced from culture media, thereby preventing loss of cells and enabling ease of purification. However, start-up costs of such units are costly, ranging from $300-$1,200 depending on the complexity of bioreactor (Jackson et al., 1996; Jaspert et al., 1995).

1.6.3 *In vivo* propagation and its advantages

In vivo production of monoclonal antibodies entails the propagation of hybridoma cells within primed mice (Hoogenraad et al., 1983). To do so, hybridoma cells must be generated through fusion of spleenocytes and myeloma cells, similar to *in vitro* methods. However, instead of culturing these cell lines in flasks, the cells are inoculated in peritoneum of primed mouse or rat for propagation. During growth, the cells start secreting large amount of antibodies within the cavity as ascites or fluid. This ascites fluid can be collected and processed to isolate monoclonal antibodies. This method of producing antibodies through ascites is considered simple and economic (Ardle et al., 1998).

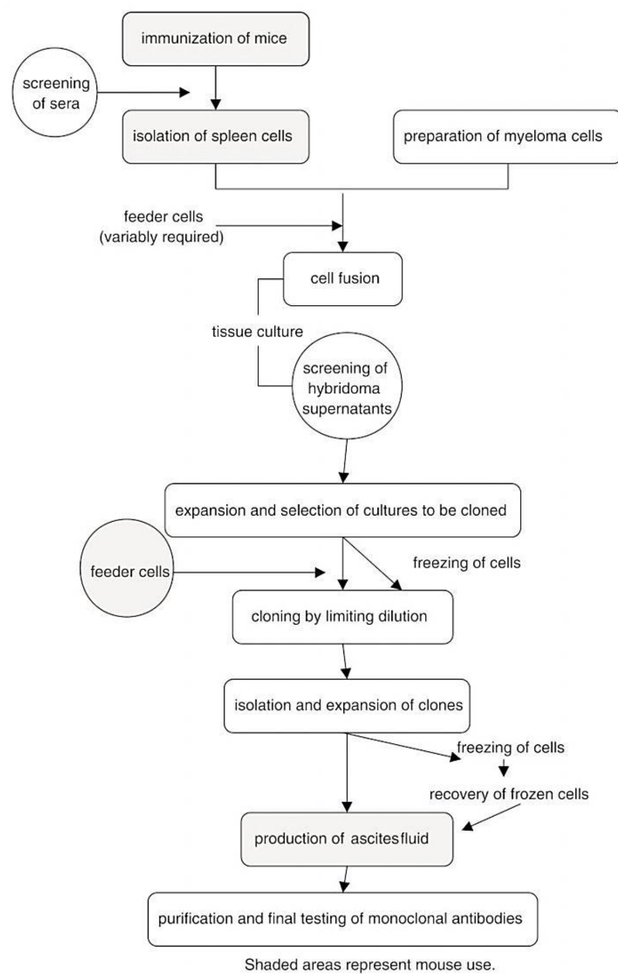

Figure 1.6.2: Steps for in vivo propagation of monoclonal antibodies (Ausubel, 1998; National Research Council, 1999)

Although more than 90% of monoclonal antibodies can be produced through *in vitro* techniques, such methods can be nugatory in certain scenarios. In many cases, large amount of monoclonal antibodies might be required in the treatment of diseases or for diagnosis, and such large production scale can prove to be an arduous task to accomplish *in vitro* (National Research Council, 1999).

Characteristics of hybridoma cell line are also important in determining whether *in vitro* or *in vivo* method should be followed. Although both production methods can be optimized, system optimization of ascites method has proved to be more economic than *in vitro* technique (Capiaumont et al., 1995; Chua et al., 1994; Shacter, 1989; Trampler et al., 1994). Growth conditions of hybridoma cells are optimal *in vivo* and therefore almost any cell line will produce mAb even if the process is not optimized for a particular cell line. Alternatively, all cell lines require prior optimization for proper growth and production of mAb for *in vitro* technique. Because of this, *in vivo* techniques are preferred where cost is involved for commercialization of a technique or product.

Table 1.1: Some mouse derived myeloma cell lines used as fusion partners for generation of hybridoma cells

Fusion cell line	Reference
X63Ag8.653	Kearney et al., 1979
Sp2/0-Ag14	Kohler and Milstein, 1976; Shulmanet al., 1978
FO	de St. Groth and Scheidegger, 1980
NSI/1-Ag4-1	Kohler et al., 1976
NSO/1	Galfre and Milstein, 1981
FOX-NY	Taggart and Samloff, 1983

Many antibodies derived from *in vitro* culture cannot be easily concentrated from culture supernatants due to substantial losses of amount and binding affinity of antibodies ensuing from standard purification procedures (Underwood and Bean 1985; Lullauet al 1996). The most commonly observed deterioration resulting from *in vitro* purification technique is the denaturation of IgG3 and IgM and concomitant loss of antigen binding affinity (Roggenbucket al 1994).

Moreover, *in vitro* derived monoclonal antibodies differ in the positioning of glycosyl moieties than those from in vivo technique. Leibiger and his colleagues (1995) reported *in vitro* derived IgG to unusually contain terminal mannose at all glycosylated sites which affected antigen-binding ability and other biological functions of the immunoglobulin. The authors deduced that the absence of such unusual positioning *in vivo* maybe be owed to their effective removal by antibodies specific for the mannose receptors of the atypical antibodies which is not possible *in vitro*. Although correct positioning of the moieties in IgG can be achieved through inducing the expression of desirable glycosylation enzymes in vitro, such a task to partake will be formidable, exorbitantly expensive and often not attainable due to limitations of current technologies (Wright and Morrison 1994, 1997, 1998; Matsuuchiet al. 1981).

Many hybridoma cell lines do not adapt well to *in vitro* conditions**.** These hybridoma cell lines demonstrate survivability at *in vitro* conditions only at initial stage during selection of desirable hybridoma cells following fusion of myeloma and spleen cells. This is contributed to the presence of normal spleen cells in culture that act as feeder cells and assist in growth of hybridomas. However, with time, as the number of normal spleen cells diminishes, growth of the hybridomas becomes restricted. Moreover, the increase in the amount of antibodies being secreted further affect hybridoma growth and secretion. One of the best ways to produce large scale antibodies from these cell lines is adaptation of ascites technique (National Research Council, 1999). Culture in tissue culture medium is also used but by far the highest concentrations of antibodies are produced in ascites fluid of pristane treated mice. Therefore, for economic production of large amount of functional monoclonal antibodies, in vivo technique needs to be adopted.

Table 1.2: Advantages of *in vivo* technique over *in vitro* technique for monoclonal antibody (mAb) propagation (National Research Council, 1999)

In vitro propagation of mAb	In vivo propagation of mAb
In vitro culture requires the use of FBS that can contaminate the final mAb and limit its use	In vivo technique involves injection of FBS free hybridoma in mice that prevents cross contamination
Loss of proper glycosylation can perturb characteristics of mAb by increasing immunogenicity, reduced binding affinity or changes in biologic functions	In vivo technique does not result in improper glycosylation of mAb
Batch culture produces very low amount of antibodies (~ 0.002–0.01 mg/ml). Although semi-permeable membrane system can yield total mAb comparable to ascites technique, they are costly.	In vivo technique produces high amount of antibody (1-2 mg/ml)
As mAb amount is low in supernatant, rigorous purification and concentration is essential which further denatures the mAb, change affinity and adds cost and time.	Purification of mAb from ascites is a straightforward technique, requiring only a few steps and limited loss in each step
Binding affinity of mAb are generally poorer	Binding affinity of mAb is high due to no loss in glycosylation
In vitro culture methods are expensive for large scale propagation of mAb	In vivo culture techniques are economically feasible for large scale mAb propagation

1.7 Factors involved in *in vivo* production of monoclonal antibodies

Several factors need to be considered for optimum production of monoclonal antibodies:

Priming: *In vivo* propagation of monoclonal antibodies requires prior priming of mice with mineral oil. Priming is commonly achieved through intraperitoneal injection of pristane at the peritoneal cavity. Pristane (2,6,10,14-tetramethyl-pentadecane) is an isoprenoid alkane that can be extracted from several different sources such as shark liver oil, petroleum crude oils, and wool wax (Budavari et al., 1989) and functions by inducing granulomatous reactions and restricting drainage of peritoneal fluid (Amyx, 1987). A time interval of 10-14 days is needed for the neoplasia to occur following several induced mutations and this time period can be shortened with the use of oncovirus along with pristane (Potter et al., 1973). Alternative agents to pristane were also evaluated in several reports, although none as effective. Freund's Incomplete Adjuvant has shown some effectiveness at doses of 0.25 or 0.5 ml.

Characteristic of hybridoma cell line: Selecting appropriate hybridoma clones is an important factor in determining the outcome of ascites technique. Subclones producing low ascites usually results in few large tumors in the peritoneal cavity whereas subclones producing high ascites level form numerous small tumors growing throughout the mesentery (Cancro and Potter, 1976). Therefore, selecting clones that produce small tumors and more ascites will increase the yield of mAbs.

Cell inoculation: Survival of mice and amount of ascites produced also depend upon the number of cell injected (Brodeur et al., 1984). Typical concentration of cell injected intraperitoneally is between 10^5 to 10^7 cells in 0.1 to 0.5 ml. In general, to high concentrations increases mice mortality, where as too less that 10^5 cells result in reduced tumor formation (NIH Guidelines, 2002).

Host: Other factors that affect *in vivo* production include age, sex and strain of host, (Hendriksen de Leeuw, 1998; Chandler, 1989) all of which can be manipulated for optimizing yield of mAb. Selecting immunodeficient host mice reduces contamination of desired mAb with mice mAb. Many substrains of BALB/c mice, known for their

characteristic plasmocytoma production, are immunodeficient and commonly used for mAb production (National Research Council, 1999).

1.8 Objectives

Cholera epidemics and pandemics are currently prevalent in about 50 countries, with new outbreaks continuing to erupt in areas with refugee displacement and environmental disasters (WHO, 2016). Rapid and early detection of cholera is imperative as preventative and control measures in such areas that are susceptible to outbreaks. Current method of diagnosis of cholera includes culturing patient stool samples or rectal swab in TTGA agar plates (Sack et al., 2004). This demand for transportation of samples, presence of functional lab, trained personnel and a minimum of 24 hrs to obtain test results. This tedious process thwarts the instillation of early preventative measure that needs to be taken to limit spread of cholera. Therefore, there is a growing need for a rapid bedside test to supply definitive results to patients at the time of testing and facilitate diagnosis of cholera.

Current predominant agent responsible for cholera outbreaks is *V. cholerae* O1. Detection of *V. cholerae* O1 serotypes either by lab-based test or by rapid diagnostic test requires monoclonal antibodies in large amount. Therefore, to meet the growing demands for lab based detection as well as rapid diagnosis of cholera, large scale production of monoclonal antibodies and its application in cholera diagnostic assays is exceedingly desirable. The objectives of this study were:

- To propagate monoclonal antibody from *Vibrio cholerae* O1 specific hybridoma cell lines in ascites fluid in large scale
- To determine sensitivity of the monoclonal antibody towards lipopolysaccharide of *Vibrio cholerae* O1 Ogawa and *Vibrio cholerae* O1 Inaba
- To observe specificity and cross-reactivity of the antibody towards other bacteria
- To determine parameters for lab-based agglutination test to detect cholera
- To develop a simple and rapid lateral flow assay in immunocromatography format for diagnosis of cholera.

Chapter 2: Materials and methods

Chapter 2: Materials and Methods

2.1 Ethical statement

The study was approved by the Research Review Committee (RRC) and the Ethical Review Committee (ERC) of the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b).

2.2 Cell line and rodent

2.2.1 Hybridoma cell line

V. cholerae specific hybridoma cell line was used in this study for the production of lipopolysaccharide specific monoclonal antibody. These hybridoma cell line was produced in icddr,b through Sp2/0-Ag14 mice myeloma cell and spleen cell fusion.

2.2.2 Host animal

BALB/c mice of 6 to 8 weeks old of both sexes were used for *in vivo* propagation of monoclonal antibody.

2.3 Hybridoma cell culture

2.3.1 Cell thawing

V. cholera specific hybridoma cell lines preserved at low temperatures of liquid nitrogen (-196°C) were revived by thawing and culturing initially in T25 flask to allow lagging of cells and entry into log phase of growth for maximum cell recovery.

Cryopreserved *V. cholerae* specific cell line was thawed in previously heated water bath at 37°C until the last ice crystal disappeared. Cells from cryovials were dispensed in 10 mL RPMI complete and centrifuged at 800-1000 rpm for 10 min. Supernatant containing cell disruptive DMSO was swiftly discarded and the pellet containing cells dispensed in few milliliter of RPMI complete. This cell suspension was transferred with Pasteur pipet into T25 culture flask containing ~20 mL RPMI complete. Flask was gently shaken to

obtain cell homogeneity, observed under inverted microscope and incubated at 37°C in 5% CO_2.

2.3.2 Cell preparation and maintenance

Cells were cultured and maintained in filter sterilized RPMI complete media at 37°C in 5% CO2, humidified incubator.

After initial culture in T25 flask following thawing of cryopreserved cells (~3X10^6 cells), cells were transferred into larger T75 flasks for culture and maintenance. Culture flasks were observed daily for change in color of media, morphology of cells and cell density.

Passaging into fresh media: Cells were passaged if the cultured media were exhausted (color of media turning yellow), but had not yet reached semi-confluent stage. Old media were discarded and replaced by fresh RPMI complete media. As hybridoma cells are adherent cell type, they remain attached to surface of vessel without being washed off during removal of media.

Harvesting of cells and splitting of culture: Once culture had reached semi-confluent stage (observed under inverted microscope), cells were harvested and split. Cells were dissociated from growth surface using the following technique:

i. About one-third of the culture media was discarded from culture vessel.
ii. The remaining media was used to detach monolayer of cells on growth surface.
iii. Maximum recovery of cells was ensured by repeating the harvesting process with few mL of fresh RPMI complete media and the cell suspensions were pooled.
iv. Harvested cell suspension was centrifuged at 800-1000 rpm for 10 min.
v. Pellet obtained was dispensed in few milliliter of fresh RPMI complete and split through transfer into two culture flasks containing media.

2.3.3 Cell viability and doubling time

Cell viability check and counting were performed with hemocytometer using trypan blue indicator dye.

To evaluate hybridoma cell doubling time, known number of cells were cultured and counted with hemocytometer after 3 days.

i. Cryopreserved hybridoma cells were thawed and suspended in 10 ml RPMI complete.
ii. Cells were centrifuged at 1500 rpm for 10 min.
iii. Pellet was resuspended in 2-3 ml RPMI complete, mixed homogeneously, and transferred to flat bottomed costar plate.
iv. Cells were incubated at 37°C in 5% CO_2 overnight.
v. Following overnight culture, cells were harvested from five different wells and counted.
vi. Remaining cells were re-incubated at 37°C in 5% CO_2 and counting repeated after 3 days.

2.4 *In vivo* monoclonal antibody production

2.4.1 Pristane priming

Pristane (2,6,10,14-tetramethyl-pentadecane) was used to induce granulomatous reaction and ascites fluid formation in mice. 6 to 8 weeks old BALB/c mice (30-50 per batch) were chosen, to which 0.5ml pristane (ACUC, 2016) was intraperitonially injected using 27G needle.

2.4.2 Hybridoma inoculation

Within 10-14 days following pristine priming, BALB/c mice were intraperitoneally injected with cultured *V. cholerae* specific hybridoma cells. A concentration of 1.5-2.0X10^6 cells per mice was used, since a higher concentration can lead to high mortality, whereas a lower concentration will give low antibody titer. Serum free RPMI complete media was used for cell injection. The following procedure was used for cell inoculation in mice:

i. Cultured *V. cholerae* specific hybridoma cells were harvested from flasks using aspirator.

ii. Following centrifugation at 800-1000 rpm for 10 min, cells were resuspended in 5 ml RPMI complete first, and final volume adjusted to 20ml.
iii. Following homogenous mixing, 0.5ml of the cell suspension was used for cell counting.
iv. Cells were centrifuged at 800-1000 rpm for 10 min to remove cell culture media. This removed FBS from cell which is antigenic in mice. Cell pellet was dissolved in appropriate amount of cell injection media consisting of RPMI, 1% sodium-pyruvate, 1% penicillin-streptomycin and 1% L-glutamine with no FBS.
v. 0.5ml of 1.5-2.0X10^6 cell suspension was intraperitonially injected in primed mice.

2.4.3 Physiological monitoring

Cell inoculated BALB/c mice were monitored twice daily for degree of abdominal distension following inoculation. The mice were monitored for anorexia, dehydration, weight loss, inactivity, difficulty in movement, tachypnea and dyspnea.

2.4.4 Harvesting ascites fluid

Mice exhibiting clinical abnormalities and maximum ascites were tapped once. The following procedure was used to collect ascites:

i. Ascites were collected by perfusion using 18G needles by slowly dripping fluid from the hub of the needles.
ii. Clots were removed from the collected fluid if present. Collected ascites were centrifuged at 3000 rpm for 10 min at 20°C and the supernatant collected. Pellet composed of red blood cells and fat debris was discarded.
iii. Ascites collected over several days from different mice were stored at -20°C to prevent oxidative or proteolytic degradation of antibodies.

2.5 Downstream processing of ascites fluid

2.5.1 Processing of ascites fluid

Ascites fluid retrieved from mice was processed to remove cells, fat debris, components of the complement system and proteases that may interfere with or inactivate antibody affinity.

i. Ascites collected over several days were thawed completely
ii. Heat inactivation of complement system components and proteases in ascites fluid were done at 56°C for 30 to 40 min.
iii. Heat treated ascites were centrifuged at 3000 rpm for 10 min at 20°C. Supernatant containing ascites were collected, while pellet with fat debris were discarded.
iv. Ascites fluid was filtered first through 0.45µm and next through 0.2µm pore size filter to remove all cellular debris.

2.5.2 Primary screening through agglutination test

Antibody specificity and sensitivity were screened using slide agglutination test. *Vibrio cholerae* O1 Ogawa, Inaba and O139 were streaked on gelatin agar plates beforehand for this test.

i. Filtered ascites were allowed to thaw in ice prior to the experiment
ii. Previously streaked *V. cholerae* O1 Ogawa and Inaba (positive control) and O139 (negative control) were serologically confirmed with commercial O1 Ogawa, O1 Inaba and O139 monoclonal antibody respectively through slide agglutination test.
iii. Ascites were 2-fold serially diluted with normal saline.
iv. Clear agglutination observed within 2 min was considered a positive reaction. Binding affinity and specificity of the monoclonal antibodies were evaluated by observing the ability of the serially diluted ascites to agglutinate *V. cholerae* O1 Ogawa, Inaba and O139 on glass slide.

2.5.3 Purification of *Vibrio cholerae* specific mAb

Monoclonal antibodies from ascites were purified using Protein G GraviTrap affinity chromatography (GE Healthcare Life Sciences). These are gravity-flow columns prepacked with 1ml bacterial G protein from Group G Streptococci that have high affinity for Fc region of IgG. This binding occurs at a wide range of pH, however, is stronger at pH 7.0. Once IgG monoclonal antibodies are sequestered from sample, low pH enables elution of IgG. Purified IgG can be collected in alkaline buffer solution to prevent acidic pH of elution buffer from disrupting antibody structure.

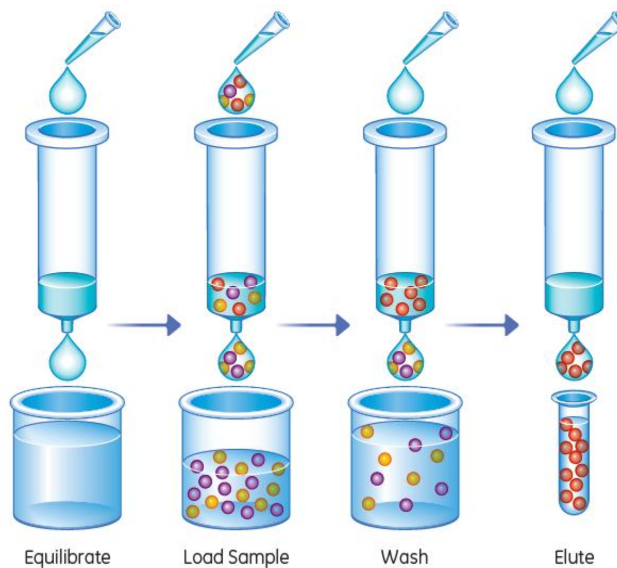

Figure 2: Steps of purification through affinity chromatography (GE Healthcare Life Sciences, 2016)

Ab buffer kit (GE Healthcare Life Sciences) with optimized binding buffer, elution buffer and neutralizing buffer was used for each step of purification. Standard procedure of Protein G GraviTrap as recommended by manufacture was used:

i. 1X binding buffer and 1X elution buffer were prepared by diluting 10X stock buffers with 0.2µm filtered autoclaved distilled water.
ii. Ascites sample was diluted in the ratio 1:2.5 using 1X binding buffer (sodium phosphate, pH 7.0) and mixed well.

iii. Tip of the column was cut, storage solution discarded and the column was mounted on Workmate stand.
iv. Column was equilibrated with 10ml of 1X binding buffer.
v. Sample was run through the column until passed completely. No more than 8ml sample was used in each run. A total volume of 20 ml sample diluted with binding buffer was used per run. Flow-through samples were collected at the bottom of the column for subsequent runs as they contain significant amount of unbound antibodies.
vi. Column was washed with 15 ml of 1X binding buffer to remove any bound impurities.
vii. Eluted sample was collected in 80µl pre-chilled neutralizing buffer (1 M Tris-HCl, pH 9.0) by passing 3.5 ml of 1X elution buffer (glycine-HCl, pH 2.7) through the column.
viii. After elution, column was regenerated with 10 ml of 1X binding buffer. Step v to vii was repeated twice using flow-through samples to retrieve unbound antibodies.
ix. pH of the eluted antibodies were measured and adjusted to pH 7.0-7.4 with elution buffer and neutralizing buffer. Samples containing purified monoclonal antibodies were stored at 4°C.

2.5.4 Buffer exchange of *Vibrio cholerae* specific mAb

Buffer exchange was carried out using size-exclusion PD-10 desalting column.

i. Storage solution was poured off the column and sealed end at notch of the column was cut.
ii. Column was equilibrated with 25 ml of 0.2 µm filtered PBS and flow-through discarded.
iii. 2.5 ml of purified mAb eluted from Protein G chromatography was run through column. Sample was allowed to enter the packed bed completely and flow-through was discarded.
iv. Purified mAb was eluted with 3.5 ml of 0.2 µm filtered PBS. Eluted sample was collected and stored at 4°C.

v. Column was regenerated by washing it with 25 ml of 0.2 µm filtered PBS and reused with new samples with for three times maximum.

2.5.5 Concentrating monoclonal antibody

Monoclonal antibody was concentrated by Vivaspin-6 concentrator.

i. Vivaspin-6 column was equilibrated with 2 ml of 0.2 µm filtered PBS centrifuged at 3500 g for 15min at 20°C.
ii. Flow-through PBS was discarded from lower part of the column.
iii. Monoclonal antibody sample of maximum 6ml was added into the column.
iv. Sample of monoclonal antibody was centrifuged at 3500 g for 30 min at 20°C.
v. Concentrated monoclonal antibodies were collected from upper part of the column.

2.6 Protein estimation

Estimation of monoclonal antibody concentration was done by Bradford protein assay.

i. 0.1% BSA in PBS (pH 7.2-7.3) stock was prepared.
ii. The following BSA standard concentrations were prepared from stock: 0, 6.25×10^{-9}, 1.25×10^{-8}, 1.875×10^{-8}, 2.5×10^{-8}, 3.125×10^{-8}, 3.75×10^{-8} and 5×10^{-8} mg/ml in PBS.
iii. Purified mAb samples were diluted to 30-200 fold using PBS as diluent.
iv. All samples and standards were vortexed for homogeneous mixing.
v. 160 µl/well standards and samples were loaded and replicates of both standards and samples were used.
vi. Following standard and sample loading, 40 µl/well Bradford reagent was added and mixed vigorously.
vii. Absorbance was taken at 595 nm with spectrophotometer.
viii. Average absorbance of the replicates was used and standard curve constructed using Instat software.

2.7 Agglutination test for specificity, sensitivity and cross-reactivity of mAb

Slide agglutination test was repeated to observe any effect of purification procedures on sensitivity of antibodies. Binding affinity and specificity of the monoclonal antibody was evaluated by observing the ability of the serially diluted monoclonal antibody to agglutinate *V. cholerae* O1 Ogawa and Inaba on glass slide.

2.8 Dot blot assay

Dot blot assay with the purified monoclonal antibody was carried out against LPS of *Vibrio cholerae* O1 Ogawa and Inaba. LPS of *Vibrio cholerae* O139 was also used to observe nonspecific binding activity of the monoclonal antibody.

i. Nitrocellulose strips were soaked in PBS and dried on blotting paper
ii. 1.2 µl antigens consisting of LPS of Ogawa (1 mg/ml and 1.5mg/ml), LPS of Inaba (1 mg/ml and 1.5mg/ml) and LPS of O139 (1 mg/ml and 1.5mg/ml) were spot on individual grids of the nitrocellulose membrane. 1.2 mg/ml affinity pure antibody was used as positive control. Coated membranes were incubated at room temperature for 5 min.
iii. Membranes were blocked with 1 ml of 1% BSA-PBS at room temperature for 20 min at 230 rpm.
iv. Membranes were washed twice with PBS.
v. 100 µl of purified monoclonal antibody diluted to 1:50 with PBS were applied to each grid coated with LPS and incubated at room temperature for 3 hrs at 230 rpm.
vi. Following incubation, membranes were washed five times with PBS-0.05% Tween, and once with PBS.
vii. Goat anti-mouse IgG, conjugated with horseradish peroxidase were diluted in 0.1% BSA-PBS-Tween at 1:500 dilutions and 100 µl was added to each grid. The strips were incubated at room temperature for 1.5 hrs at 230 rpm.
viii. Membranes were washed five times with PBS-0.05% Tween, and once with PBS.
ix. Substrate was prepared using 83% (v/v) 4-chloro-1-napthol (4CN) and 17% (v/v) Tris buffered saline (TBS). H_2O_2 was added directly before use.

x. Strips were developed with the addition of 1ml substrate to each grid.

xi. Reactions were observed within 5 min.

xii. Reaction was stopped with distilled water and the membranes dried for 10 min.

xiii. Immunoblot was considered positive if developed color could be seen with naked eye once the membranes have dried.

2.9 Isotyping

To characterize the subclass of the purified monoclonal antibody, ELISA was performed using anti-IgG conjugates.

i. Before coating, purified LPS was mixed homogeneously at 1700 rpm for 60 min.

ii. 96 well flat bottomed ELISA-plates (Nunc F) were coated with 100µL/well ofLPS of *Vibrio cholerae* O1 Ogawa or Inaba at the amount of 2.5 µg/ml in PBS (pH 7.2-7.4).

iii. The plates were incubated at room temperature overnight.

iv. The LPS-coated plates were washed three times with PBS.

v. Plates were blocked with 1% BSA-PBS, 200µL/well for 30 min at 37°C.

vi. The plates were washed three times with PBS-0.05% Tween and once with PBS.

vii. The plates were incubated with 100µL/well monoclonal antibody samples diluted to 1:50 and 1:100 in 0.1%BSA-PBS Tween at 37°C for 1.5 hrs. 0.1%BSA-PBS Tween was used as negative control.

viii. The plates were washed three times with PBS-0.05%Tween and once with PBS.

ix. Horseradish peroxidase (HRP) conjugated alpha-mouse IgG1, IgG2a, IgG2b and IgG3 were diluted separately in 0.1%BSA-PBS Tween (1:1000) and added in the amount of 100µl/well in separate wells.

x. The plates were incubated at 37°C for 1.5 hrs.

xi. The plates were washed three times with PBS-0.05%Tween and once with PBS.

xii. 30% H_2O_2 was added before use to 1mg/ml substrate orthophenylenediamine (OPD) diluted in 10 ml of 0.1 M sodium citrate buffer (pH 4.5). This was added in the amount of 100 µl/well to develop the plates.

xiii. Reading was taken kinetically for 5 min at 450nm.

2.10 Preparation of antibody conjugates

2.10.1 Preparation of colloidal gold

To prepare colloidal gold, 0.01% gold (III) chloride tihydrate and 0.024% sodium citrate were boiled with constant stirring in water for injection (WFI) until it became the color of red wine. This was filtered through 0.2µm pore size filter.

2.10.2 Aggregation test

To assess stability of conjugates and to optimize minimum amount of antibody and pH for conjugation, aggregation test was conducted.

Assessment of optimum pH for conjugation

i. pH of 1 ml colloidal gold samples were adjusted with potassium carbonate between pH 4.5-9 with 0.5 increments.
ii. To each tube, constant amount of anti-human IgG was added and allowed to conjugate by incubation for 5 min at room temperature.
iii. Following conjugation, 10% NaCl were added to each tube of the gold-protein conjugates and this was incubated for 10 min.
iv. Stability and polydispersity were assessed by measuring the absorbance of each of the aggregates at 520 nm, 580 nm, and 600 nm.

Assessment of minimum amount of antibody for conjugation

i. pH of 1 ml gold solution was adjusted to 8.5 (optimum pH for conjugation) to which a range of purified IgG was added.
i. 10% NaCl were added to the gold-protein suspension and incubated for 10 min.
ii. Stability and polydispersity were assessed by measuring the absorbance of the aggregates at 520 nm, 580 nm, and 600 nm.

2.10.3 Preparation of detection conjugates

i. 25 µl of potassium carbonate was added per ml of gold solution (pH 4.5-5.0) and properly mixed.
ii. 24 µg/ml mouse anti-LPS *V. cholerae* O1 specific IgG was added from working standard using borax as diluent.
iii. The solution was incubated for 40 min at 70 rpm at room temperature.
iv. 20% BSA (pH 8-8.5) was added to block the conjugation by incubating for 20 min at 70 rpm at room temperature.
v. The solution was centrifuged at 1000 rpm for 45 min at 4°C and the pellet resuspended in 0.02 M tris buffer (pH 8) containing 1% BSA.
vi. Following a second wash using the same buffer, the final pellet was resuspended in 0.02 M tris buffer (pH 8) containing 1% BSA.
vii. This solution was passed through a 0.2 µm-pore-size filter and used as the detection conjugate.
viii. The detection conjugate solution was used to soak conjugate pads at absorbance 3 of conjugated gold. If required, 3% maltrin was used to bring the absorbance of the solution to 3.
ix. The conjugate pads were dried at 45°C for 2 hr.

2.11 Preparation of lateral flow strip

i. Test line (anti-LPS *V. cholerae* specific monoclonal antibody) and control line (mouse anti-IgG antibody) were dispensed on nitrocellulose membrane.
ii. Dispensed membrane was dried for 90 min.
iii. Conjugate pad previously prepared was used to paste on the backing card.
iv. A glass fiber sample pad was placed to overlap conjugate pad.
v. A cellulose fiber as an absorbent pad was pasted on the backing card opposite the conjugate pad to accelerate migration of samples.
vi. Cards were cut to 3mm using guillotine cutter.

2.12 Testing the strip with stool specimens

The strip was tested using fresh and frozen stool samples collected from diarrheal patients. Presence of *V. cholerae* in samples was confirmed by culturing them in TTGA agar plate. 100µl of samples was added in reaction tube and dipstick was dipped into the tube. After 15 min, result was read by observation of lines appearing as red color. Appearance of two lines was considered positive and a single line was considered valid negative result for cholera.

2.13 Testing the strip with enriched stool specimens

The strip was tested using stool specimens enriched in alkaline peptone water. Swab stick was dipped into stool specimen and inoculated into alkaline peptone water for enrichment. This was incubated at 37°C overnight. 100µl of enriched sample was added in reaction tube and dipstick was dipped into the tube. Result was read on the strip within 15 min.

2.14 Preservation and storage

2.14.1 Cell line

Hybridoma cell line was cryopreserved in liquid nitrogen at -196°C.

i. Cultured cells were harvested from flasks and centrifuged at 800-1000 rpm for 10min.
ii. Supernatant was discarded and cell pellet was resuspended in cell freezing media composed of 10% FBS, 10% DMSO and 80% RPMI complete.
iii. 1ml cell suspension containing about $3\text{-}5X10^6$ cells were loaded into cryovials on pre-chilled freezing tray.
iv. For acclimatization, cryovials were slowly immersed into liquid nitrogen with freezing can which was rotated every 15min. This allowed cells to come to temperature of liquid nitrogen slowly.

2.14.2 Purified antibody

Purified *Vibrio cholerae* O1 specific monoclonal antibody was aliquoted and stored at -80°C.

2.14.3 Lateral flow strips

Lateral flow strips and pads were stored at room temperature in dry, non-humid conditions.

Chapter 3: Results

3.1 Hybridoma cell culture and *in vivo* mAb propagation

V. cholerae specific hybridoma cells were cultured in 25 cm^2 cell culture flasks (Corning) in RPMI-1640 media supplemented with 1% sodium pyruvate, 1% L-glutamate, 1% penicillin-streptomycin and 10% FBS at 37˚C incubator to obtain 1.5-2X10^6 cells for injection in each mice (Figure 3.1.1 A and B). Culture medium was changed periodically and cells were split based on the growth of cells on flask surface. Viability of the cells prior to IP injection in mice was observed to be 93.2 ± 2.7%, while doubling time of the hybridoma cells was approximately 24 hrs (Table 3.1).

Table 3.1: Characteristics of the hybridoma cell in RPMI complete culture medium

Cell viability	93.2 ± 2.7%
Cell doubling time (T_d) in RPMI complete	24 ± 4.2 hrs

6-8 week old BALB/c mice were used as host animal for hybridoma cell growth and antibody propagation (Figure 3.1.2 A). Pristane (2,6,10,14-tetramethyl-pentadecane) was used as priming agent in the mice to induce stimulation that results in granulomatous reaction. Following 10-14 days, 0.5ml of 1.5-2X10^6 cells was intraperitoneally injected into each of the primed mouse (Figure 3.1.2 B).

BALB/c mice (30-50) were monitored daily for change in physiological characteristics. Abdominal distension along with other physiological distress was observed in maximum number of mice within 10 days. Ascites was harvested from peritoneal cavity (Figure 3.1.3 A), heat inactivated and filtered to remove all cells (Figure 3.1.3 B). Within 14 days, all mice were tapped for ascites collection.

Following initial processing of ascites, mAb was purified from ascitic fluid by affinity chromatography (Protein G Gravitrap, GE Healthcare Bioscience). Buffer exchange was carried out using size-exclusion chromatography (PD-10 desalting, GE Healthcare Bioscience) to improve stability of the purified mAb, followed by centrifugal concentration of the mAb (Vivaspin 6, GE Healthcare Bioscience) (Figure 3.1.4 A-C).

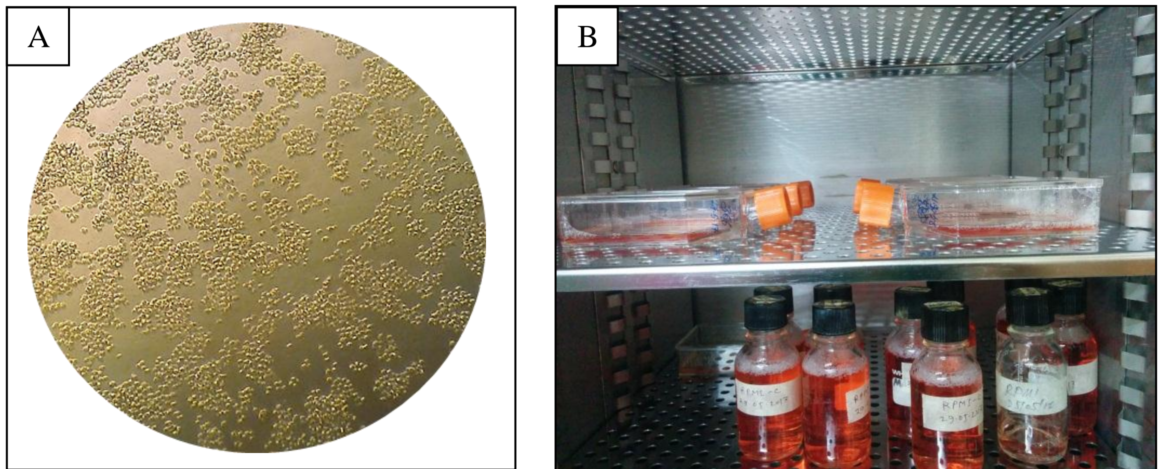

Figure 3.1.1: Hybridoma cell culture specific for generating monoclonal antibody against lipopolysaccharide (LPS) of *Vibrio cholerae* O1. A) Hybridoma cells observed under inverted microscope growing at semi-confluent state. B) Hybridoma cells cultured in RPMI complete media

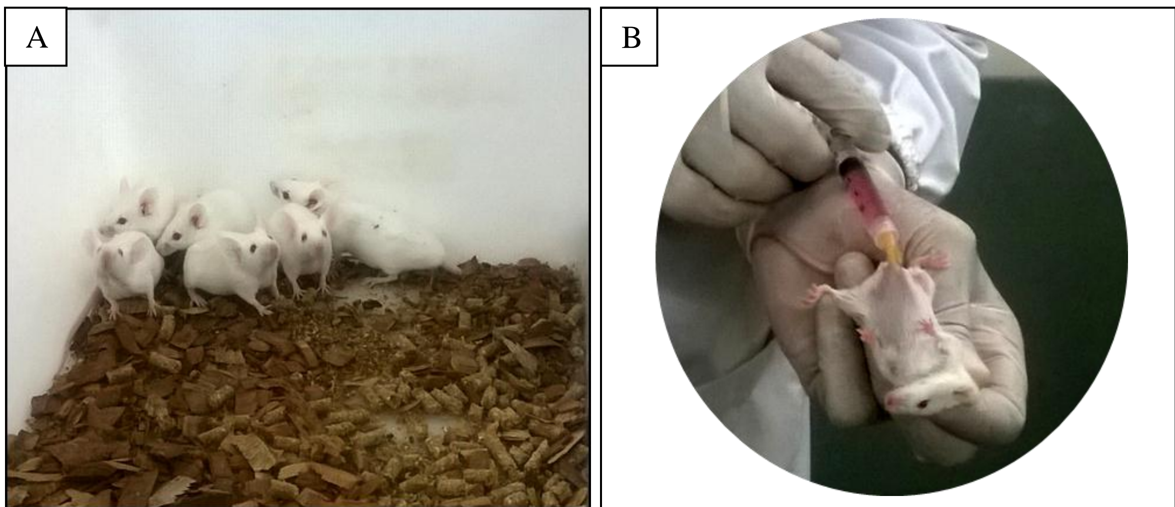

Figure 3.1.2: BALB/c mice used for in vivo propagation of monoclonal antibody. A) Male and female of 6-8 weeks old BALB/c mice were used as host animal. B) 1.5-2X10^6 hybridoma cells were intraperitonially injected using 27G needles in BALB/c mice following 10-14 days of pristine priming.

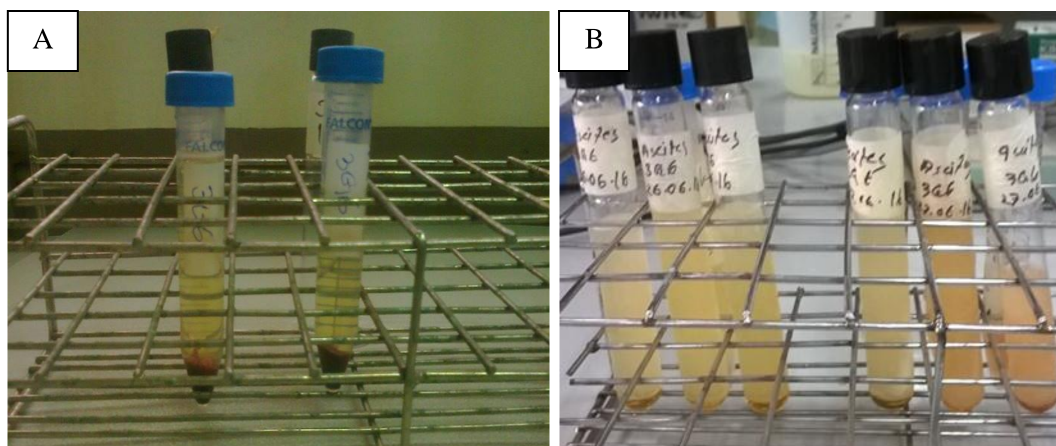

Figure 3.1.3: Ascites fluid. A) Ascites collected from intraperitoneal cavity after 10-14 days of cell injection in BALB/c mice. B) Processed ascites fluid in supernatant devoid of debris after heat inactivation and centrifugation.

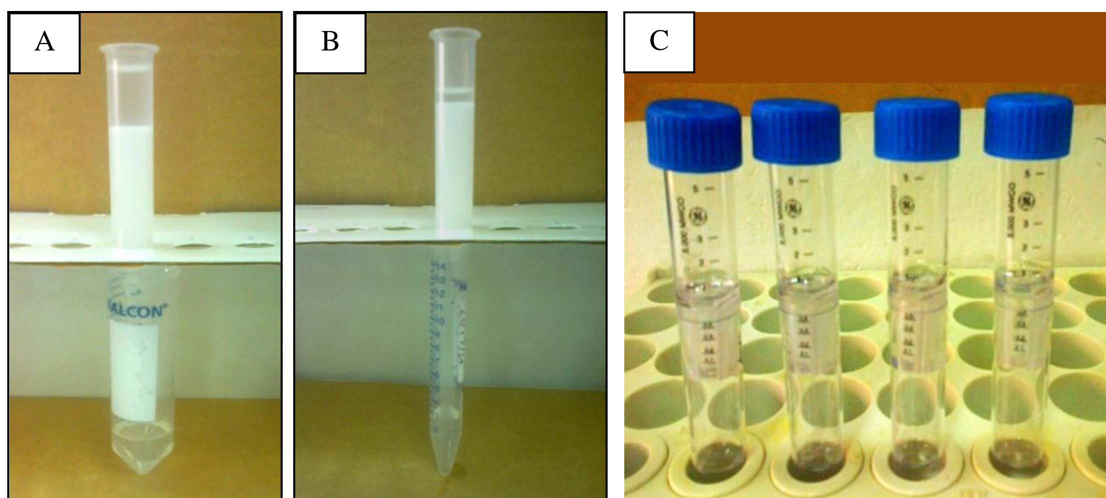

Figure 3.1.4: Purification of monoclonal antibodies by Protein G affinity chromatography (A), PD-10 desalting size-exclusion chromatography (B) and Vivaspin concentrator (C).

3.2 Volume of ascites collected per mice

About 1.5-2X10^{6} hybridoma cells that produce monoclonal antibody against LPS of *Vibrio cholerae* O1 were suspended in 0.5 ml cell inoculating media and injected into the peritoneum of 30-50 per batch BALB/c mice previously primed with 0.5 ml Pristane. Ascites harvested by single tap from mice through perfusion from peritoneum following abdominal distension ranged approximately between 1.5-2.5 ml with a mean volume of 2.05 ml.

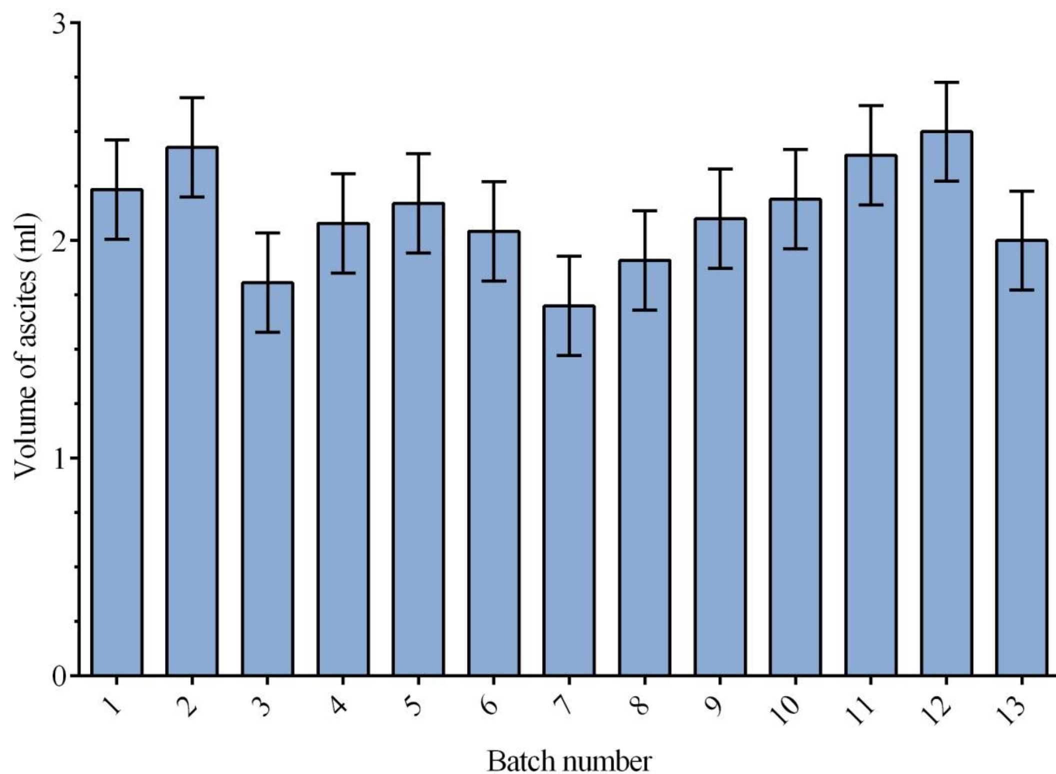

Figure 3.2: Volume of ascites collected per mice from single tap from peritoneal cavity. Bars indicate mean volume of ascites obtained from single mice in each batch of production, and error bars indicate standard errors of the mean (SEM) values. An average volume of 2.05 ml ascites were obtained per mice, with SD = 0.23.

3.3 Downstream processing of ascites and decrease in volume

Following heat inactivation and centrifugation, ascites fluid was filtered first through 0.45 µm filter, followed by 0.2 µm filter to remove fats and cell debris. There is a general trend in loss of volume of ascites due to each processing step. Percentage loss of ascites is highest during 0.45 µm filtration, accounting for 31.9% loss, whereas 0.2 µm filtration accounted for 6.55% loss of ascites fluid.

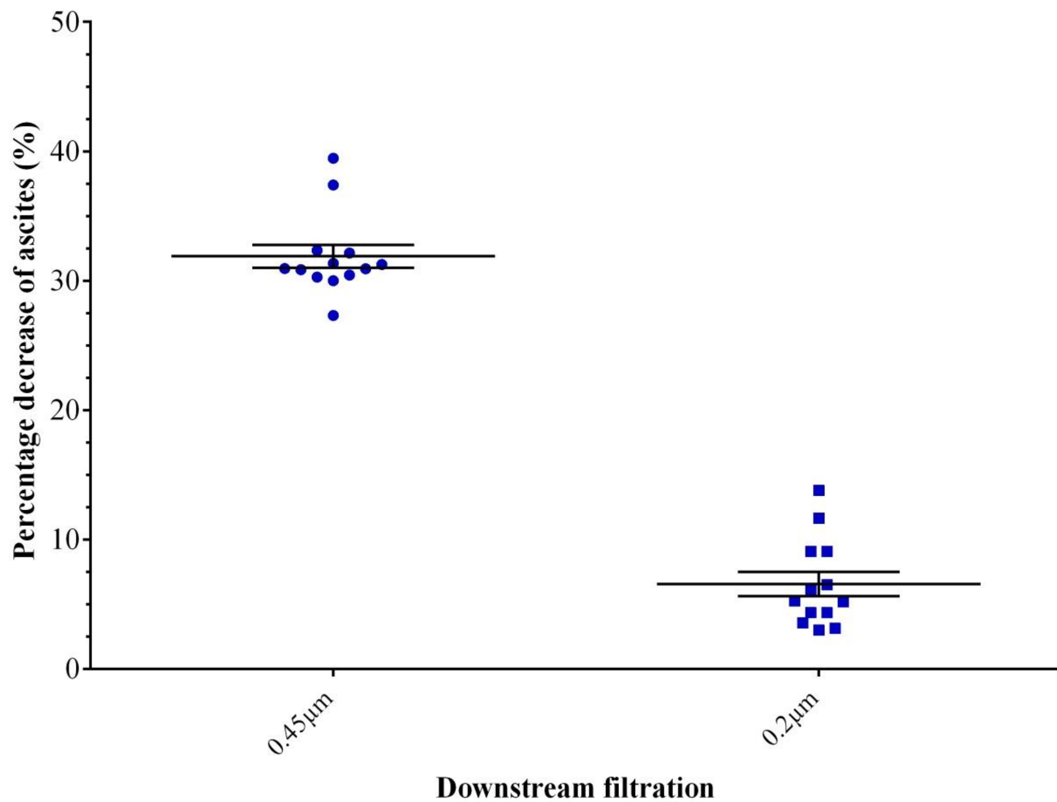

Figure 3.3: Percent loss of ascites fluid due to filtration by 0.45 µm pore size, followed by 0.2 µm pore size. Each point represents percentage loss in volume of ascites per mice per batch of production. Bar represents average percentage loss of fluid, and error bar indicates standard error of mean (SEM).

3.4 Volume of monoclonal antibody per mice obtained following purification

Following purification by affinity chromatography, size-exclusion desalting and centrifugal concentration, purified monoclonal antibody was obtained in PBS in suspension. A mean volume of 0.59 ml antibody suspension was obtained from each mice, with SD = 0.30.

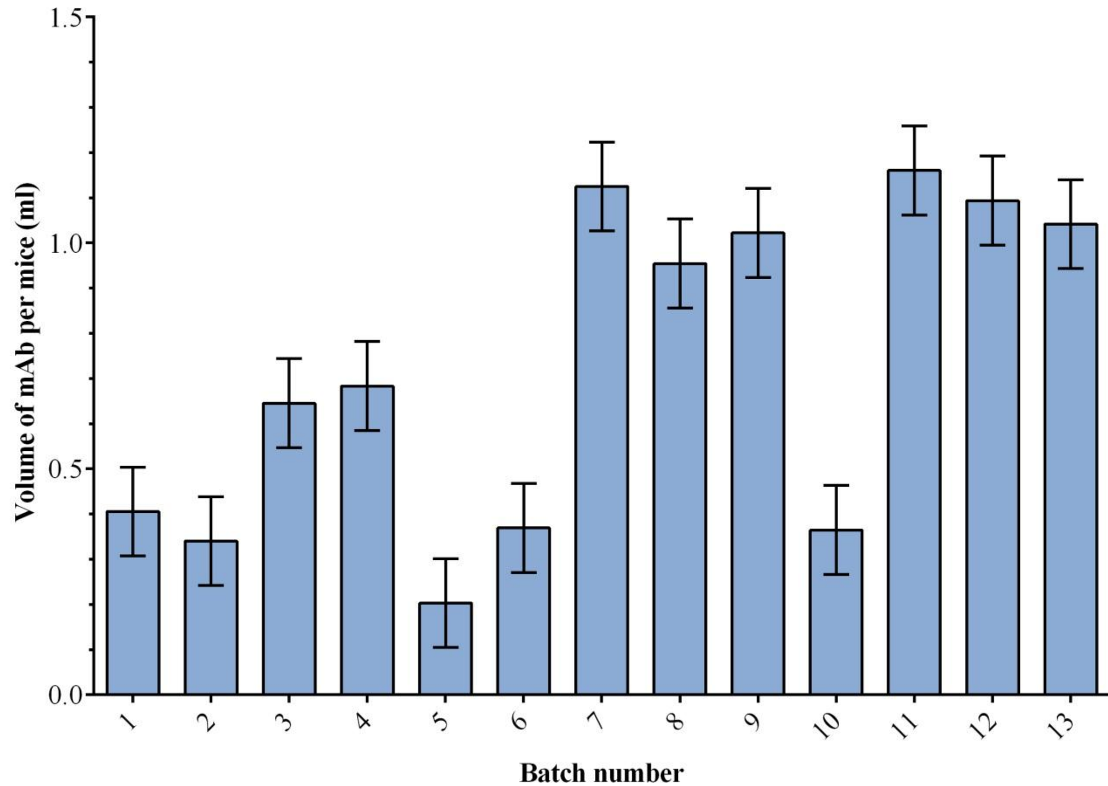

Figure 3.4: Volume of monoclonal antibody in PBS from each mouse. A mean volume of 0.59 ml mAb in PBS was obtained, with SD = 0.30.

3.5 Concentration of monoclonal antibody produced through *in vivo* technique

Purification of monoclonal antibody from ascites was done by Protein G Gravitrap affinity chromatography that specifically sequesters IgG, followed by size-exclusion chromatography to exchange buffer and centrifugal concentration using spin-column. Concentration of purified monoclonal antibody obtained was estimated using Bradford assay. Mean concentration of 1.89 ± 0.62 mg/ml monoclonal antibody was obtained from the 3 step purification process (Figure 3.5.1).

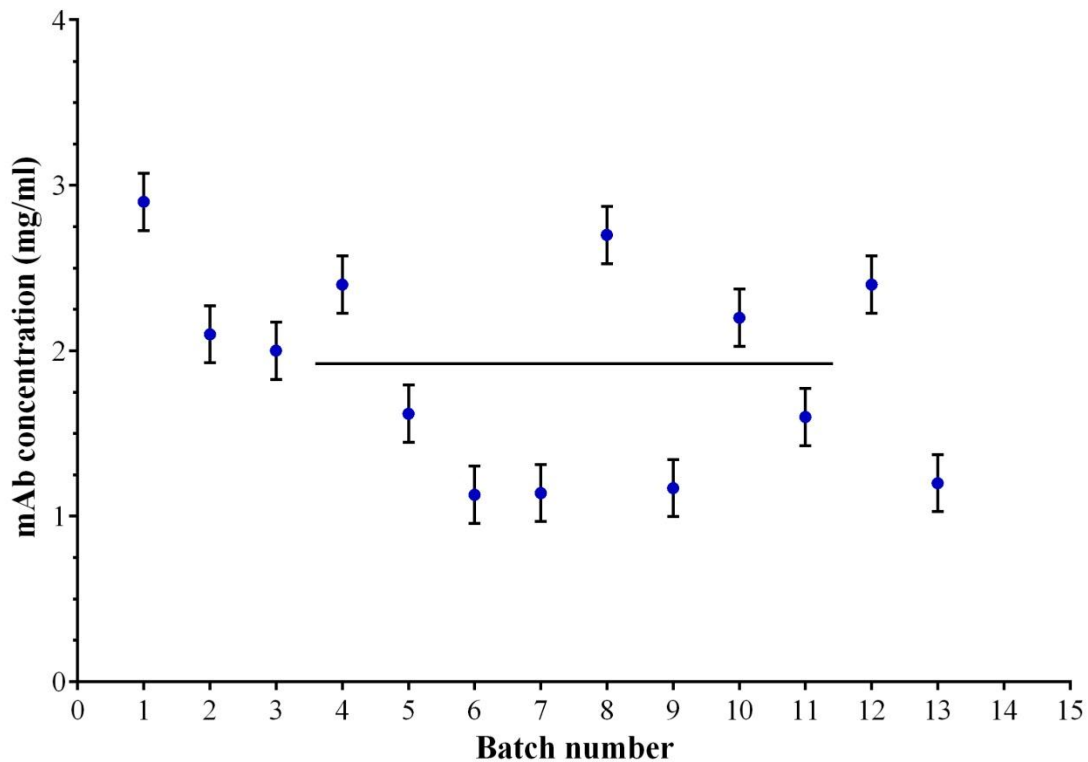

Figure 3.5.1: Concentration of monoclonal antibody per ml PBS. Concentration ranged from approximately 1-3 mg/ml, with an average value of 1.89 mg/ml (indicated by line) and SD = 0.62.

30-50 BALB/c mice were used per batch. Variation was observed in concentration of monoclonal antibody obtained from each mouse, with mean concentration being 1.247 ± 0.66 mg (Figure 3.5.3).

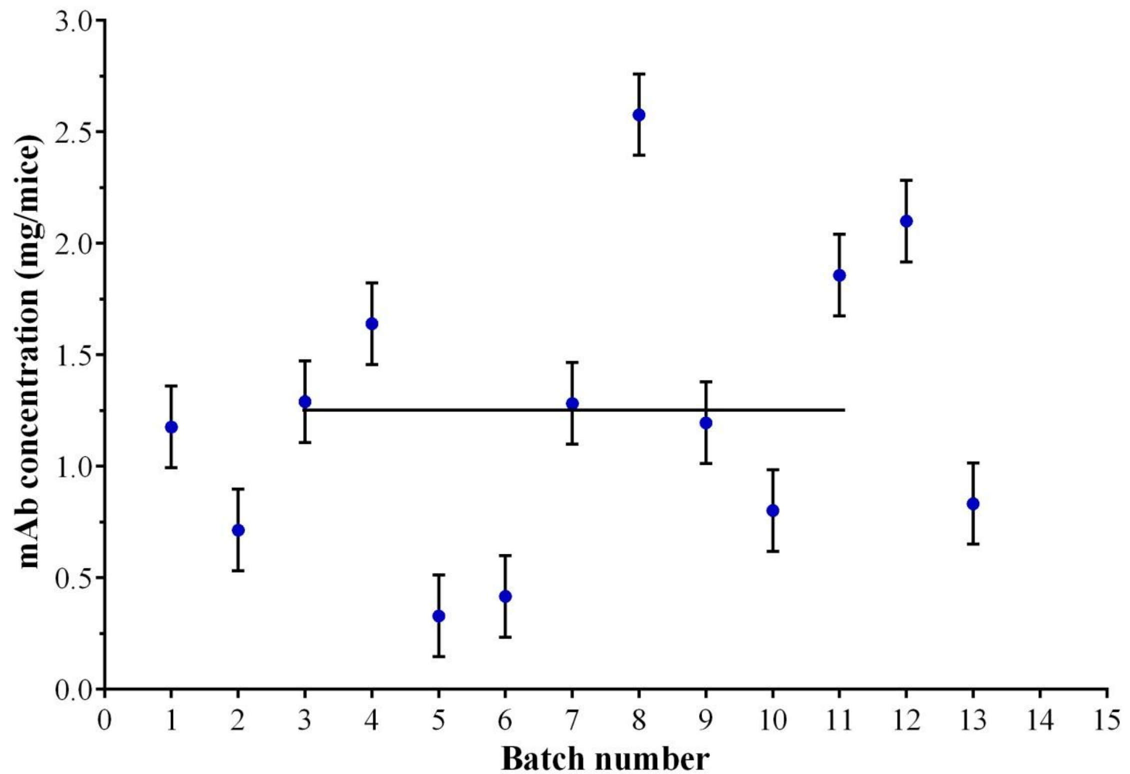

Figure 3.5.3: Concentration of monoclonal antibody obtained per mice. Average concentration of *V. cholerae* specific antibody obtained per mice, indicated by line, was 1.247 mg and SD = 0.66.

3.6 Agglutination test for specificity and sensitivity monoclonal antibody

Slide agglutination test was performed using the purified mAb to observe specificity of the mAb towards *V. cholerae* O1 Ogawa and Inaba serotypes. Within 10-15 secs, the purified mAb was able to agglutinate both serotypes (Figure 3.6.1). Sensitivity of the mAb towards *V. cholerae* O1 Ogawa and Inaba serotypes were also observed by antisera titer assay (Figure 3.6.2). Minimum titer of mAb at which agglutination was observed was between 1:40-1:80 dilutions as shown in Table 3.2.

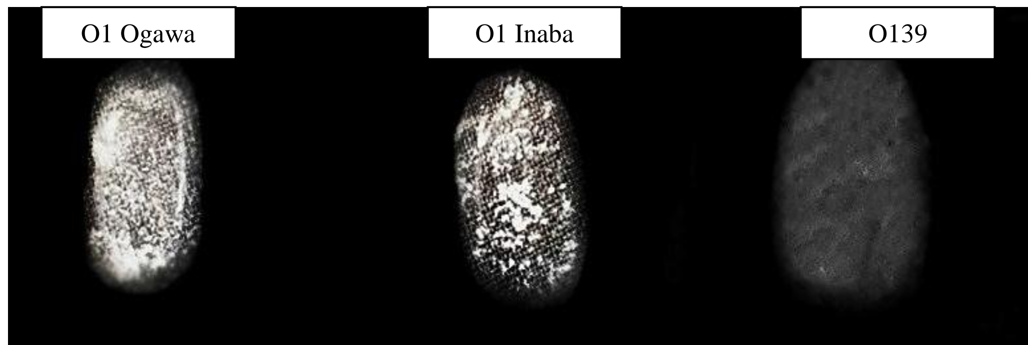

Figure 3.6.1: Agglutination test for specificity using *in vivo* produced monoclonal antibodies. Monoclonal antibodies were able to agglutinate both *Vibrio cholerae* O1 Ogawa and Inaba. No cross reactivity against *Vibrio cholerae* 0139 was observed.

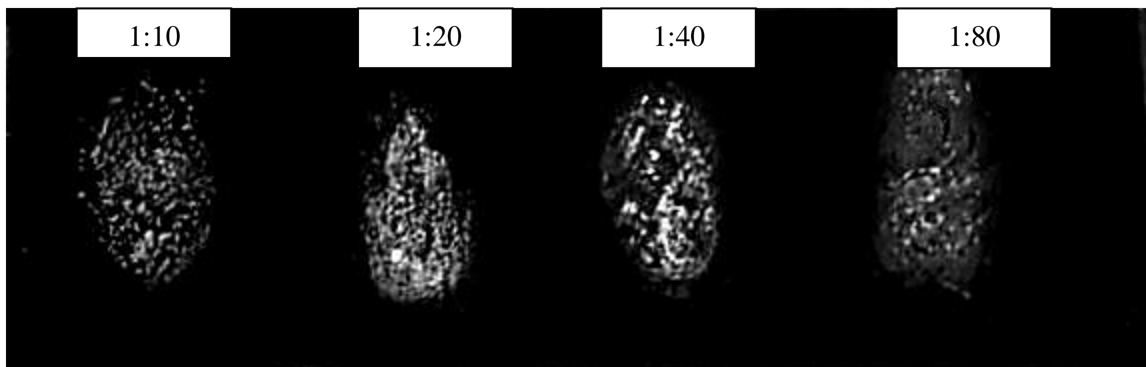

Figure 3.6.2: Agglutination test for antisera titer assay using purified monoclonal antibody. Agglutination of *V. cholerae* O1 was observed with monoclonal antibody of dilutions as high as 1:80

Table 3.2: Agglutination test using purified monoclonal antibody produced *in vivo*. Monoclonal antibody was able to agglutinate both *Vibrio cholerae* O1 Ogawa and Inaba with a minimum titer of 1:40-1:80 dilutions.

Dilution	neat		1:5		1:10		1:20		1:40		1:80		1:160	
V.cholerae sp.	Ogawa	Inaba	Ogawa	Inaba	Ogawa	Inaba	Ogawa	Inaba	Ogawa	Inaba	Ogawa	Inaba	Ogawa	Inaba
Batch 1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	-	-
Batch 2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+	-	-
Batch 3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	-	-
Batch 4	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	-	-
Batch 5	+++	+++	+++	+++	+++	+++	+++	+++	++	++	-	-	-	-
Batch 6	+++	+++	+++	+++	+++	+++	++	++	+	+	-	-	-	-
Batch 7	+++	+++	+++	+++	+++	+++	++	++	++	++	+	+	-	-
Batch 8	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	+	-	-
Batch 9	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	+	-	-
Batch 10	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	-	-
Batch 11	+++	+++	+++	+++	+++	+++	++	++	++	++	+	+	-	-
Batch 12	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	+	-	-
Batch 13	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	+	-	-

Aggutination assay performed using *in vivo* produced monoclonal antibody was agglutination positive for minimum concentrations of between 0.01 to 0.04 mg/ml (1:40 to 1:80 dilutions). An average minimum of 0.03 ± 0.009 mg/ml monoclonal antibody was required to agglutinate *Vibrio cholerae* O1 Ogawa and Inaba. This provides an estimate of the minimum concentration of monoclonal antibody required to carry out slide agglutination for diagnostic confirmation of *Vibrio cholerae* O1.

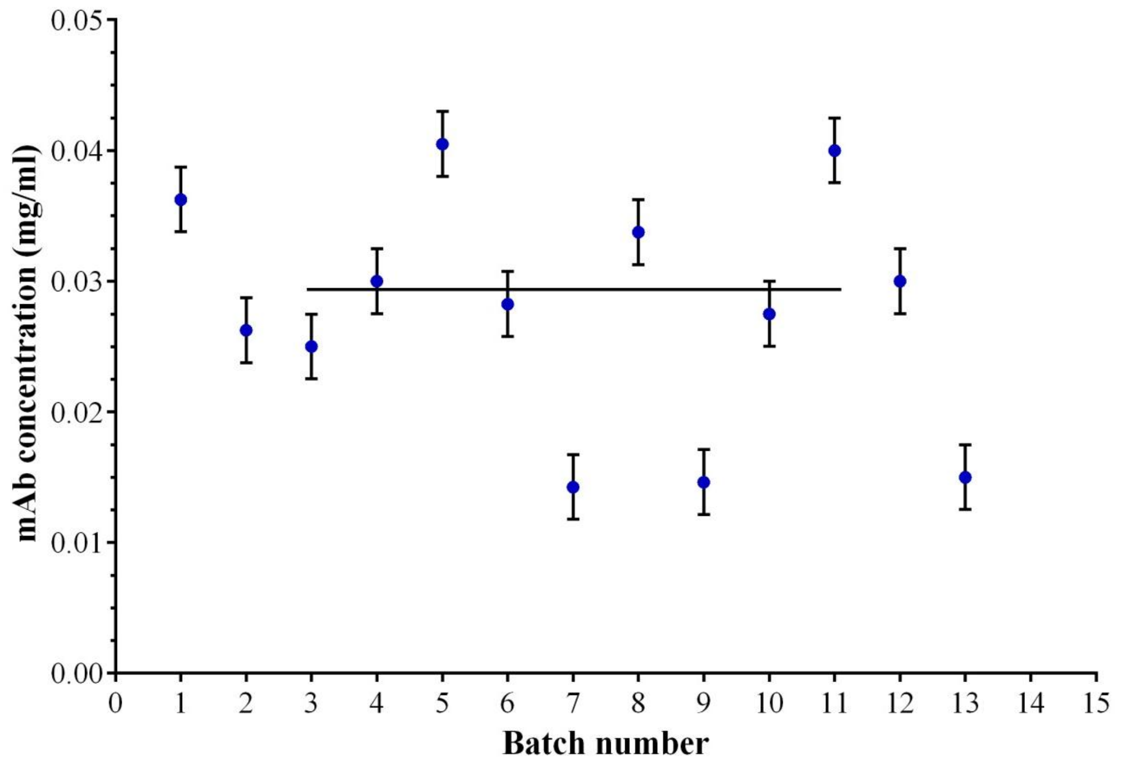

Figure 3.6.3: Minimum concentration of monoclonal antibody required to agglutinate *V. cholerae* O1 Ogawa and Inaba obtained through qualitative slide agglutination test. Mean minimum concentration of mAb, represented by line, was 0.03 mg/ml, with SD = 0.009

3.7 Cross-reactivity of the monoclonal antibodies to other bacteria

Specificity and cross-reactivity of the purified monoclonal antibody was examined with *V. cholerae* O1, O139 and other enteropathogenic bacteria using slide-agglutination test. Single colony of bacteria and undiluted mAb was used for this purpose. Agglutination test result was discerned within 2 min. The purified antibody was specific only for *V. cholerae* O1 serotypes, with no cross-reactions observed against other bacteria (Table 3.3).

Table 3.3: Slide agglutination test with monoclonal antibody with *V. cholerae* O1 and several bacteria

Organism	Agglutination with purified antibody
V. cholerae O1 Ogawa	+
V. cholerae O1 Inaba	+
V. cholerae O139	-
Salmonella spp.	-
Shigella spp.	-
Enterotoxigenic *Escherichia coli* (ETEC)	-
Escherichia coli	-
Enterobacter spp.	-
Klebsiella spp.	-
Pseudomonas spp.	-

3.8 Dot blot immunoassay

Dot blot immunoassay was carried out with purified monoclonal antibody to observe specific binding against lipopolysaccharide of *Vibrio cholerae* O1 Ogawa and O1 Inaba. Purified mAb was observed to bind to LPS of both Ogawa and Inaba as clear spot formed on the addition of 4-chloro-1-napthol substrate to HRP conjugated goat anti-mouse IgG. No cross-reactivity was observed against LPS of *Vibrio cholerae* O139 with purified mAb with either low concentrations or high concentrations of LPS.

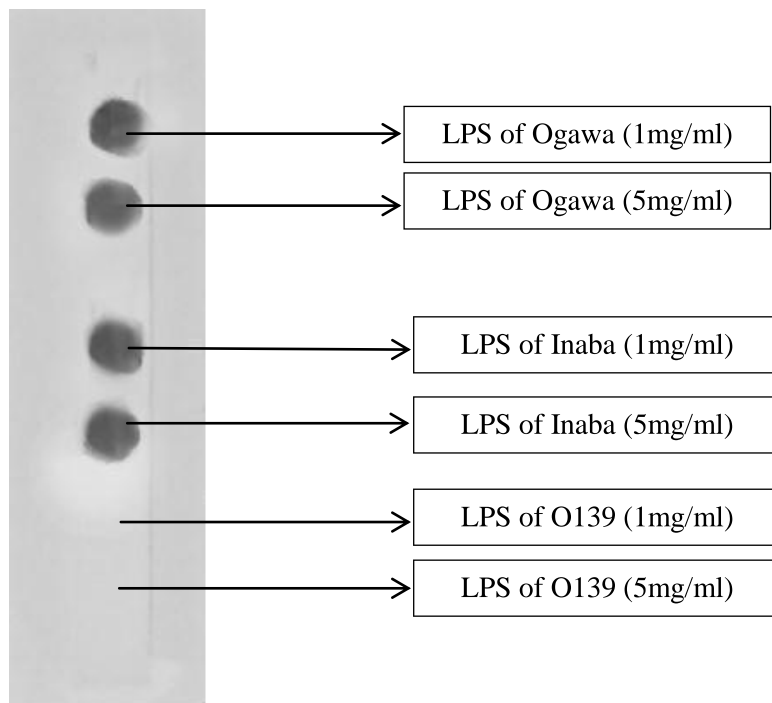

Figure 3.8 Dot blot immunoassay with purified monoclonal antibody. Positive color development was observed against lipopolysaccharide (LPS) of *Vibrio cholerae* Ogawa and Inaba at concentrations of 1 mg/ml and 5 mg/ml of LPS. No reaction observed with LPS of *Vibrio cholerae* O139 at either concentration.

3.9 Isotyping

Class and subclass of the purified monoclonal antibody was determined by ELISA method. Lipopolysaccharide of both *V. cholerae* O1 Ogawa and Inaba serotypes were used as coating antigens to bind with purified mAb of different dilutions. HRP conjugated anti-IgG1, IgG2a, IgG2b, IgG3 and IgG were used to determine specific subclass of the mAb. Sharp absorbance peak was observed against anti-IgG, suggesting that the mAb belonged to immunoglobulin class G. Sharp absorbance peak was also observed against anti-IgG3 and not against any other IgG subclass specific antibodies.

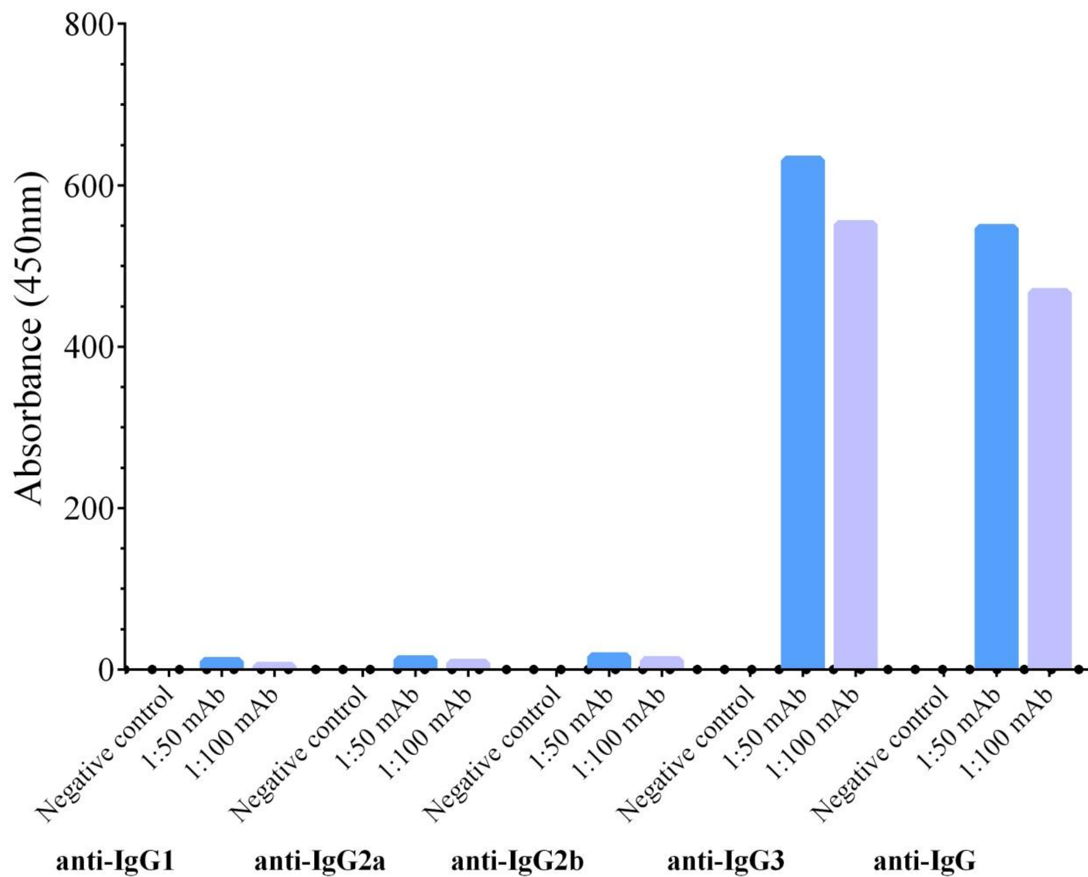

Figure 3.9.1: Isotype determination of purified monoclonal antibody using lipopolysaccharide of *V. cholerae* O1 Ogawa serotype through kinetic ELISA. Sharp absorbance was observed with anti-IgG3 and anti-IgG in both 1:50 and 1:100 dilutions of mAb.

Absorbance read at 450 nm with lipopolysaccharide of *V. cholerae* O1 Inaba was similar to that read with lipopolysaccharide of *V. cholerae* O1 Ogawa.

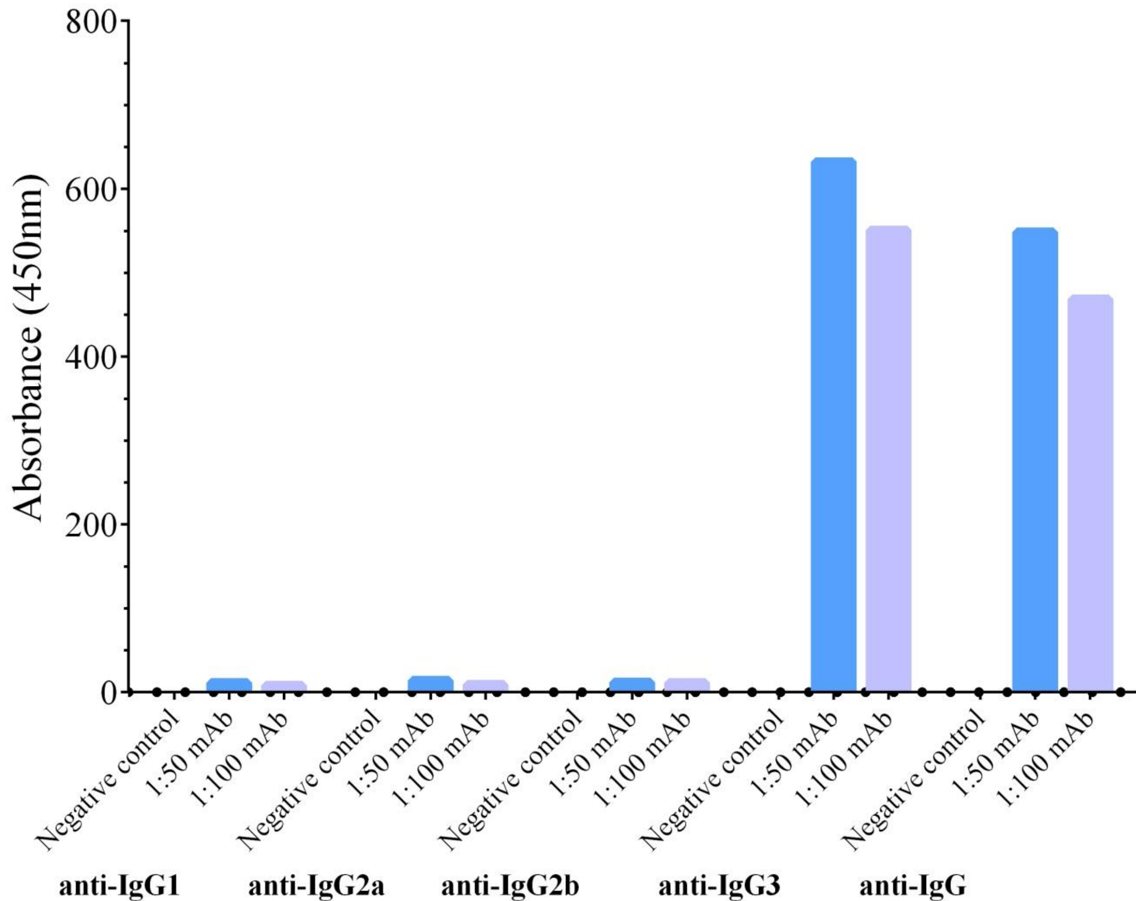

Figure 3.9.2: Isotype determination of purified monoclonal antibody using lipopolysaccharide of *V. cholerae* O1 Inaba serotype. Sharp absorbance observed with anti-IgG3 and anti-IgG.

3.10 Characterization of colloidal gold for lateral flow immunoassay

Size of the prepared colloidal gold nanoparticles was determined by differential light scatterings using a Zetasizer Nano ZS90 instrument (Malvern Instrument, Ltd.). Size of the nanoparticles, determined from the dynamic light scattering (DLS) spectrum, was 20 nm, measured with a count rate of 193.7 kcps at a scattering angle of 173° at 25°C.

3.11 Determination of optimum pH for conjugation

Aggregation testing at different pH with purified antibodies was conducted to determine optimum pH for conjugation (Nara et al., 2010). Following the addition of 10% NaCl, absorbance was taken at 520 nm, 580 nm and 600 nm. Ratio of 520 nm to 580 nm was used to measure stability of the conjugates, while the ratio of 600nm to 520nm was used to measure polydispersity of the conjugates. Stability and polydispersity was observed to increase with increase in pH. Highest stability and lowest polydispersity was observed when the monoclonal antibodies were conjugated at pH 8.5.

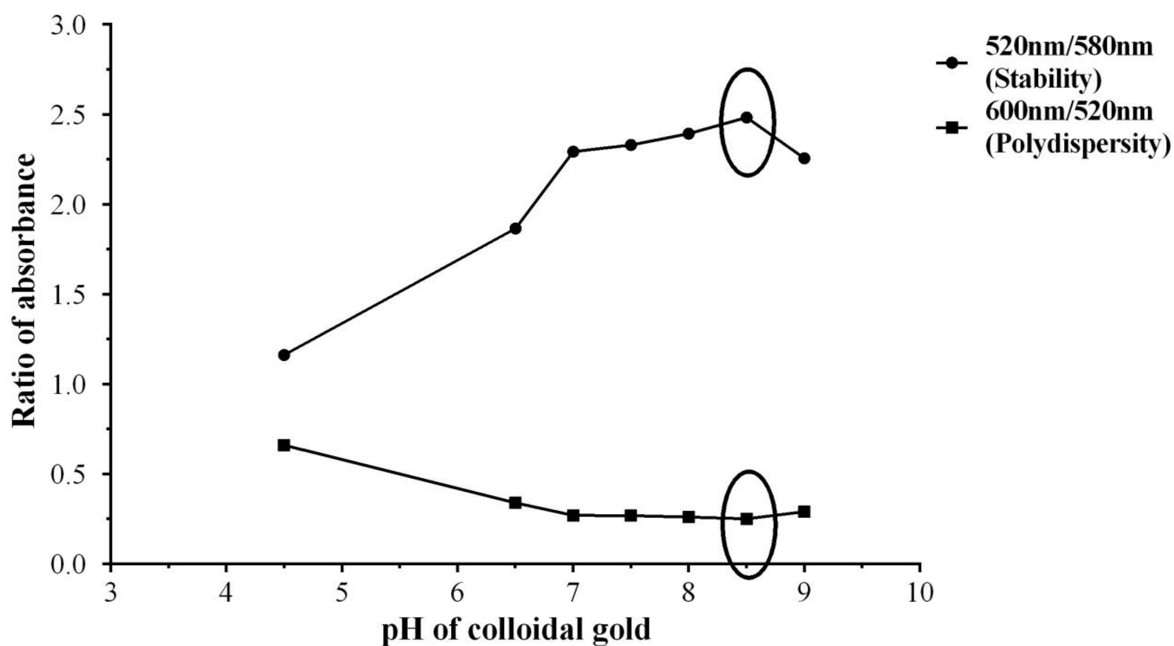

Figure 3.11: Optimization of pH for conjugation with the purified mouse anti-LPS IgG3. Ratio of absorbance at 520 nm to 580 nm and that at 600 nm to 520 nm represent stability and polydispersity, respectively. Highest stability and lowest polydispersity was observed at pH 8.5.

3.12 Determination of minimum concentration of detection antibody for conjugation

Minimum concentration of purified antibodies required for conjugation with colloidal gold was measured by aggregation testing with increasing amounts of antibodies. Ratio of 520 nm to 580 nm was used to measure stability of the conjugates, while the ratio of 600 nm to 520 nm was used to measure polydispersity of the conjugates. A minimum of 24 µg of purified antibodies was required to stabilize 1 ml of colloidal gold solution.

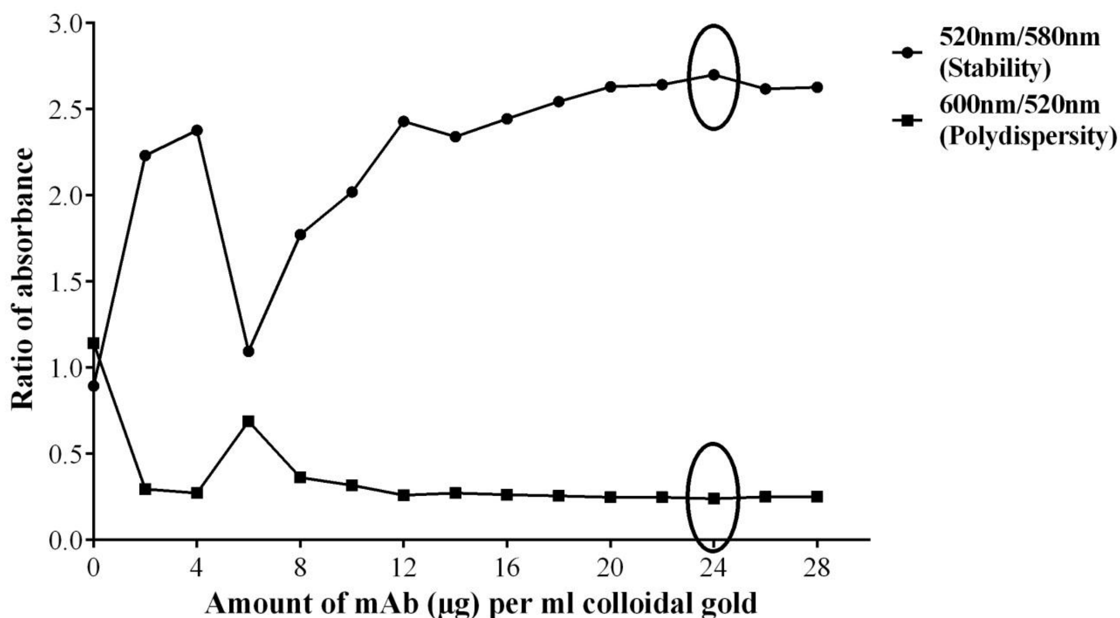

Figure 3.12: Determination of minimum concentration of purified antibody to conjugate 1 ml colloidal gold NP. Ratio of absorbance at 520 nm to 580 nm and that at 600 nm to 520 nm represent stability and polydispersity, respectively. Highest stability and lowest polydispersity was observed with 24 µg of detection antibody in 1 ml gold solution.

3.13 Testing the lateral flow assay with stool specimens

Dipstick strip was tested using fresh and frozen stool samples collected from diarrheal patients. The samples used were *V. cholerae* O1 serogroup positive and negative as confirmed through agglutination test. The test line and control lines of the dipstick appeared red during Ag-Ab binding. The presence of red lines on both the control line and the test line indicated that the sample was positive for *V. cholerae* O1. The presence of only the control line but no test line indicated the absence of *V. cholerae* O1 in sample (Figure 3.12).

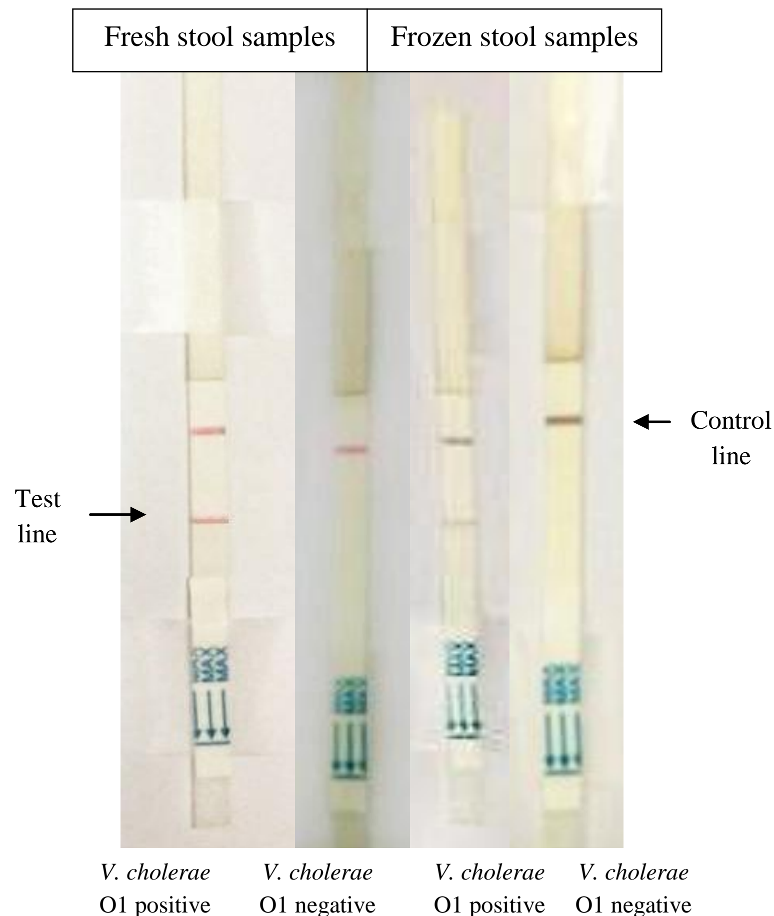

Figure 3.13: Dipsticks detecting LPS of *V. cholerae* O1 in fresh and frozen stool samples. Appearance of both control line and test line indicates a positive sample. The absence of the test line and the presence of the control line indicate a negative sample.

3.14 Testing the lateral flow assay with enriched stool specimens

Dipstick strip was tested using stool specimens enriched in alkaline peptone water. Results became visible within 15 min. A positive result appeared as two red lines while a negative result was a single red control line.

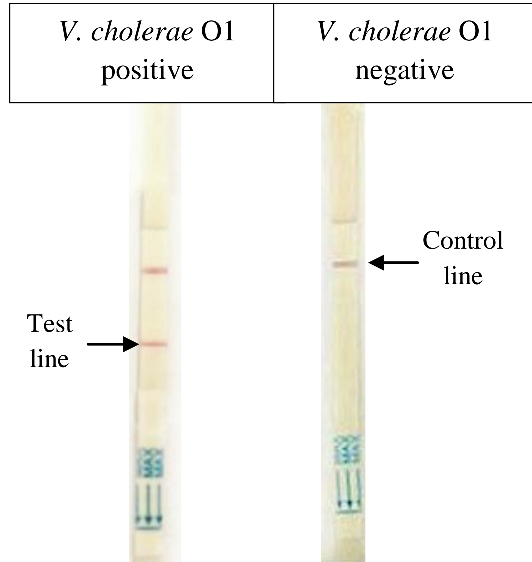

Figure 3.14: Dipsticks detecting LPS of *V. cholerae* O1 in stool specimens enriched in alkaline peptone water.

3.15 Rapid dipstick test compared to conventional culture technique

The results of dipstick test and culture methods of stool specimens (n=10) are shown in Table 3.4. All TTGA culture positive samples were dipstick positive for *V. cholerae* O1.

Table 3.4: Detection of *V. cholerae* O1 in fresh and frozen stool samples by dipstick versus culture method

Bacteriological culture	Number of samples with dipstick test results		Total number of samples
	Positive	Negative	
Positive	5	0	5
Negative	1	4	5
Total	6	4	10

Chapter 4: Discussion

Chapter 4: Discussion

Cholera is a major health problem with high disease burden and continual escalation in the number of affected countries (WHO, 2000). Cholera is endemic in both Asia and Africa, and currently affecting around 50 countries around the world. In Asia, cholera is a continuous threat that follows seasonality before and after monsoon rain, during which cholera epidemic assumes even longer cycle (Sack et al., 2004; Koelle et al., 2005).

Cholera is strongly linked to socio-economic conditions and population health. As low as 10^3 *Vibrio cholerae* O1 can cause cholera (Nelson et al., 2011). This presents close household contacts at constant risk. Harris et al. (2007) observed 21% of household contacts to be rectal swab positive for *Vibrio cholerae* within 24 hrs of positive index case. The spread can be exacerbated in areas with crowded living situation and poor sanitation, both of which are common in underdeveloped countries, refugee crisis and natural disasters. Moreover, incubation period of cholera is very short between 12 hrs and 5 days (Weil et al., 2009), and this further escalates the number of cholera cases swiftly. The fatality rate in such cases can be as high as 30-50%, unless the disease is diagnosed and treated early. Because of this, there is an urgent demand for development of diagnostic assays for early detection of cholera as preventative and control measure.

The current gold standard diagnosis of cholera is culture technique that requires transportation of samples, lab facilities, trained technician and is time-consuming. Therefore, point of care assays of cholera can enable early detection and improve the number of cases being diagnosed, limiting spread of cholera and instilling preventative measures in reported areas. Moreover, simple laboratory-based agglutination tests alongside is essential for epidemiological studies of cholera.

Monoclonal antibodies are of great value in biomedical research for their use in diagnostic assays. Production technique of monoclonal antibody has improved since they were first produced by Köhler and Milstein in 1975 through hybridoma technology (Modjtahedi et al., 2005; Gronemeyer et al., 2014). Innovations in technological development, production process and improvements in the products themselves have

ensued, leading to more efficient and economic bioprocess (Gagnon et al., 2012). Currently known processes for large-scale antibody propagation include tissue culture or *in vitro* method and mouse ascites method or *in vivo* method (Peterson et al., 1998). The tissue culture methods are more expensive and tedious and can hinder commercialization of product or process. It had been documented that the *in vitro* technique can be five times more expensive and resource intensive than the *in vivo* method to produce the same yield of monoclonal antibodies (Peterson et al., 1998). Moreover, *in vitro* culture methods can lead to poor glycosylation of antibodies, affecting its binding affinity, immunogenicity or other biological functions (Lang et al., 1991; Jackson et al., 1999). This makes mice ascites method more practical and efficient for the production of monoclonal antibodies to be used for commercial purpose.

Amount of pristane and the interval between priming and cell injection are two main criteria that determine optimum ascites production (Hoogenraad et al, 1983; Mc Ardle, 1998). In this study, 0.5 ml pristane was used for priming both male and female BALB/c mice and the interval between priming and cell inoculation was 12-14 days. Mean volume of ascites harvested after 10 days from peritoneal cavity of each mouse was 2.02 ± 0.23 ml. However, Jackson et al. (1999) observed that type of cell line determined by plasmacytoma used as fusion partner and hybridoma cell doubling time are key discriminant of ascites volume, and can produce approximately 2-3 ml ascites fluid following first tap, depending on the nature of the cell line used for cell injection. In another study, Maleki et al. (2013) obtained a similar volume of 3.5 ml ascites from first tap following 10 days after cell injection from peritoneal cavity of each mouse. In a similar study, Jafarlou et al. (2015) however harvested a higher volume of approximately 4-5 ml ascites from each mouse after 10 days of cell inoculation. Volume of ascites produced tends to decrease in subsequent taps, approximating 1-2 ml in second tap and 0.5-2 ml in third tap (Jackson et al., 1999). However, multiple taps are not permitted in all types of cell line and may increase mice distress or reduce their survivability (Jackson et al., 1999). Because of this, multiple taps were not included in this study.

Batch to batch variation in the volume of ascites produced per mice was observed for the single hybridoma cell line, with SD=0.23. Moreover, variability in the onset and

formation of significant amount of ascitic fluid was observed to differ among individual mice, ranging from 10-14 days. This makes clinical assessment of individual mice for abdominal distension and timely perfusion of ascites fluid crucial for improved mice mortality in *in vivo* production of monoclonal antibodies (Jackson et al., 1999).

The initial unit operations of downstream process were carried out to separate soluble products from larger insoluble cells and cellular debris to prevent membrane fouling in subsequent purification steps (Chandel and Harikumar, 2013). Harvested ascites fluid obtained from peritoneal cavity of mice were processed to heat denature complement system that bind to and sequester monoclonal antibodies. Primary harvest was carried out through centrifugation to remove cellular debris, followed by filtration using 0.45µm and 0.2 µm pore size filters. This processing step accounted for maximum loss in ascites volume in the overall production process. Highest loss was observed with 0.45µm filtration, accounting for a loss of 31.9% of total volume, whereas, 0.2 µm filtration accounted for 6.55% loss of volume of ascites. The final volume of ascites obtained following this processing step was 1.27 ± 0.11 ml per mice with overall mean loss accounting 0.77 ± 0.16 ml per mice. This suggests that almost half the volume of ascites harvested from each mouse were composed of cells and cellular debris and was separated in the initial downstream processing technique.

Most purification of monoclonal antibodies is carried out by chromatographic technique (Vlasek et al., 2008; Maleki et al., 2013; Jafarlou et al., 2015). In this study, Protein G affinity chromatography was performed to separate IgG antibodies from all other impurities, host cell proteins and putative viruses present in ascitic fluid. Three cycles of sample flow was performed for maximum recovery of antibodies. Purified antibody of 1.247 ± 0.66 mg from each mouse was obtained.

Vibrio cholerae O1 Ogawa and Inaba can be identified through agglutination using O1 specific antisera directed against LPS present in their cell wall (CDC, 2016). Specificity of the monoclonal antibodies towards *Vibrio cholerae* O1 Ogawa and Inaba serotypes were thus observed with slide agglutination test. Minimum titer of purified antibody required to carry out agglutination of *Vibrio cholerae* O1 serotypes was between 1:20 to 1:40. Minimum amount of monoclonal antibodies required to agglutinate *Vibrio cholerae*

Ogawa and Inaba within 15 sec was between 0.01 to 0.04 mg/ml. This provides an estimate of the amount of *in vivo* derived mAb required to carry out lab based agglutination assay with patient's rectal swab enriched on gelatin agar plates. Furthermore, it suggests that the mAb are highly sensitive for diagnosis and identification of *Vibrio cholerae* O1. In a similar study, Qadri et al. (1993) obtained a similar titer of 1:30 for monoclonal antibodies against *Vibrio cholerae* O139 through *in vivo* technique.

Cross-reactivity of the monoclonal antibodies against *Vibrio cholerae* O139 was examined. The antibodies failed to agglutinate the serogroup in both undiluted and diluted forms, suggesting their potential use for the identification of *Vibrio cholerae* O139 through the absence of agglutination in the O1 specific antisera (Finkelstein, 1996). Furthermore, no nonspecific binding of the mAb to other pathogenic and opportunistic bacteria such as *Salmonella*, *Shigella*, *Escherichia*, ETEC, *Enterobacter*, *Klebsiella,* and *Pseudomonas* was observed, supporting specificity of the antibody towards *Vibrio cholerae* O1 and its potential use for the identification of *Vibrio cholerae*.

To investigate that the monoclonal antibody is specific against lipopolysaccharide of *Vibrio cholerae* O1, dot blot immunoassay was performed. Specific binding of purified antibodies was observed against LPS of both *Vibrio cholerae* O1 Ogawa and Inaba serotypes, providing support that the Fab region of the mAb was specific against the LPS antigen of *Vibrio cholerae* O1. No cross-reactivity of the mAb against LPS of *Vibrio cholerae* O139 was observed.

To determine the class and subclass of mouse monoclonal antibody, isotyping was performed with anti-IgG1, IgG2a, IgG2b, IgG3 and IgG. Sharp absorbance was observed for anti-IgG and anti-IgG3, suggesting that the monoclonal antibody belonged to IgG class and subclass 3. ELISA against LPS of *Vibrio cholerae* O1 Ogawa and LPS of *Vibrio cholerae* O1 Inaba was separately performed for this purpose and this further provided evidence along with dot blot that the Fab of the monoclonal antibodies were specific towards LPS of *Vibrio cholerae* O1.

The purified antibody was used to develop a simple lateral flow immunoassay. The purified antibody was tested for its optimum conjugation with prepared colloidal gold

nanoparticles of 20 nm diameter. Optimum pH for highest stability and lowest polydispersity was observed at pH 8.5. At this pH, electrostatic interaction is at its least and antibody adsorption is at its maximum (Englebienne, 2000). Moreover, minimum concentration of antibody required to stabilize colloidal gold nanoparticles against 10% NaCl was 24 µg per ml of gold.

A simple lateral flow immunoassay was prepared using the optimized parameters for conjugation of detection antibodies to gold particles. Fresh and frozen stool samples that were culture positive in TTGA plates for *V. cholerae* O1 were used to test the strips. Two red lines appeared on both test line and control line within 15 min, suggesting that the strip was functional.

In this study, 10 stool specimens collected from diarrheal patients were used. All culture positive results showed positive results with rapid dipstick test. However, one culture negative sample demonstrated positive result with dipstick. This could be due to several reasons such as presence of dead *V. cholerae* in stool sample (WHO, 2012), prior treatment of patient with antibiotics (Mukherjee et al., 2010) or transformation of culturable bacilli form of *V. cholerae* to non-culturable cocci form by changes in pH or osmolarity in gut of patient (Colwell et al., 1985). To draw conclusions on sensitivity and specificity of the strips, more samples are essential.

The immunochromatography assay was simple as it does not require any sample treatment, trained staff, electricity or sophisticated equipment. It can provide results in a single operation step. Within 15 min, test results appeared as red lines, suggesting the rapid nature of the assay for detection of *V. cholerae* O1. Moreover, the dipstick strips are easy to store and requires no refrigeration, enabling their ease of transportation and use in remote areas.

4.1 Future research

In this study, monoclonal antibody against lipopolysaccharide of *V. cholerae* O1 was produced at large-scale in ascites fluid and were characterized for ease of application in cholera diagnostics. The produced mAb may be used in agglutination test for the

confirmation of *V. cholerae* O1 in patients stool sample following microbial culture. Future work should determine sensitivity, specificity, positive predictive value and negative predictive value of the lateral flow immunoassay using large number of samples. Detection threshold of the dipstick for *V. cholerae* LPS also needs to be determined. Moreover, purity of the mAb needs to be confirmed through reducing and non-reducing SDS-PAGE.

The current gold standard of bacteriological culture cannot be used for rapid diagnosis. If the lateral flow assay has high sensitivity and specificity and shows success in field evaluations, it can be used as an excellent option for early detection of cholera epidemics even in remote areas with minimum laboratory facilities.

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Appendix – I

The following media and reagents were used in the study.

1. Cell culture media (RPMI-c) for cell culture, maintenance and thawing (500ml)

Components	Amount
RPMI	500 ml
Sodium-pyruvate	1%
Penicillin-Streptomycin	1%
L-Glutamine	1%
Fetal bovine serum	10%

Filtered through 0.2 µm pore size filter

2. Cell freezing media

Components	Amount
RPMI-c	80%
Fetal bovine serum	20%
DMSO	10%

Filtered through 0.2 µm pore size filter

3. Cell injection media (500 ml)

Components	Amount
RPMI	500 ml
Sodium-pyruvate	1%
Penicillin-Streptomycin	1%
L-Glutamine	1%

Filtered through 0.2µmpore size filter

4. TTGA culture media (pH 8.5-9.0) (1000 ml)

Components	Amount
Trypticase	10 ml
NaCl	10 ml
Sodium taurocholate	5 ml
Sodium carbonate	1.2-1.5 g
Gelatin	30 g
Agar	16 g

5. Gelatin agar media (pH 7.2) (1000 ml)

Components	Amount
Gelatin	30.0 g
Casein enzymichydrolysate	10.0 g
Sodium chloride	10.0 g
Agar	15.0 g

6. Alkaline peptone water (pH 8.8) (1000 ml)

Components	Amount
Bacto Peptone	10.0 g
Sodium chloride	10.0 g

7. Phosphate buffer saline (PBS)(pH 7.2) diluted to 1X as working solution (1000 ml)

Components	Amount
NaCl	80.00 g
Na_2HPO_4	11.50 g
KH_2PO_4	2.00 g
KCL	2.00 g
Deionized water	1000.0 ml

8. Tris-buffered saline (TBS) (1000 ml)

Components	Amount
Tris	2.42 gm (20 mM)
NaCl	29.22 gm (0.5 M)

Dissolved in approximately 600 ml deionized water.pH adjusted to 7.5 with 6.0M HCl and final volume made to 1L

9. 1% BSA in PBS (500 ml)

Components	Amount
Phosphate Buffer Saline (PBS)	500 ml
Bovine Serum Albumin (BSA)	5 g

10. 0.1% BSA in PBS

Components	Amount
Phosphate Buffer Saline (PBS)	40 ml
Bovine Serum Albumin (BSA)	0.04 g

11. 0.1% BSA in PBS-Tween (500 ml)

Components	Amount
Phosphate Buffer Saline (PBS)	500 ml
Bovine Serum Albumin (BSA)	0.5 g
Tween	250 µl

12. 0.1 M Sodium citrate buffer (pH 4.5) (1000 ml)

Components	Amount
Tri-natrium citrate ($Na_3C_6H_5O_4.2H_2O$)	29.4 g
H_2O (deionized)	1000.0 ml

13. Orthophenylenediamine - H_2O_2 substrate (10 ml)

Components	Amount
OPD	0.01 g
0.1 M sodium citrate (pH 4.5)	10.0 ml
30% H_2O_2	4.0µl

14. 4-CN (200ml)

Components	Amount
Methanol	200 ml
Napthol	0.6 g

15. 4-CN Substrate (10 ml)

Components	Amount
4-Chloro-1-Napthol (4-CN)	1.7 ml
Tris Buffer Saline (TBS)	8.3 ml
H_2O_2 (Hydrogen Peroxide)	10 µl

16. Trypan Blue (100 ml)

Components	Amount
Sodium Chloride (NaCl)	0.81 g
Potassium Phosphate (K_2PO_4)	0.06 g
Trypan Blue	0.4 g
Distilled Water	100 ml

17. Colloidal gold nanoparticles

Components	Amount
Gold(III) chloride trihydrate (HAuCl4)	0.01%
Sodium citrate	0.024%
Water for injection (WFI)	100 ml

Solution was boiled until the color of red wine observed and filtered through 0.2 µm pore size filter.

Appendix II

Reagents

The following reagents were used in the study:

Reagents	Company	Catalog number
100% Absolute Ethanol	Merck Germany	1-00983-2500
AB Buffer Kit	GE Healthcare Life Sciences	28-9030-59
Bio-Rad Prottein Assay Dye Reagent Concentrate	Bio-Rad Laboratories, Inc	500-0006
Bovine Serum Albumin	Sigma	A-4503
Disposable PD-10 Desalting Columns	GE Healthcare Life Sciences	17-0851-01
DMSO	Sigma	D2650
Fetal Bovine Serum (FBS)	Gibco by Life Technologies	16140-071
Formaldehyde	Fisher Scientific	ORM-B NA1791
Hydrogen Peroxide	Fisher Scientific	H-325
L-Glutamine 200mM	Gibco by Life Technologies	25030-081
Lysol	ReckiTT BENCKISER	36241743895 (UTP Number)
Methanol	Merck KGA	603-001-00-X
Napthol	Sigma Chemical Co	A8625-25G
Na-Pyruvate 100mM 11.0mg/mL	Gibco by Life Technologies	11360-070
Penicillin 10,000 IU/mL Streptomycin 10,000 μg/mL	Gibco by Life Technologies	15140-122
Polyoxietylensorbitanmonolaurat	Sigma chemical Co.	
Pristane	Sigma	P-9622

Protein G GraviTrap	GE Healthcare Life Sciences	28-9852-55
Rabbit anti-human immunoglobulin horseradish peroxidase	Jackson Immuno Research	
RPMI Medium 1640 (1X)	Gibco by Life Technologies	11875-119
Sodium Chloride	Fisher Scientific	
Sodium Hypochloride	Fisher Scientific	ORM-A UN1198
Tris	Sigma	
Vivaspin6	GE Healthcare life Science	28-9322-94

Appendix III

Laboratory apparatus

Apparatus/Instruments	Company
Beaker (250 mL)	Pyrex
Beaker (500 mL)	Pyrex
Cell Culture Flask (25 cm^2)	Corning/BD
Cell Culture Flask (75 cm^2)	Corning/BD
Centrifuge machines	Damon IEC HN-S, Sorvall Legend
Cryo Tubes (1.0 mL)	Nunc Inter Med
Disposable Serological pipets (10 mL)	Fisher Scientific
ELISA plates (96-well)	Nunc
ELISA reader	ASCENT Multiskan
Eppendorf Tube (1.5 mL)	Eppendorf AG
Examination Gloves (Large/Medium/Small)	VWRP
CO_2 Incubator	Forma Scientific
Inverted microscope	Olympus CK2
Laminar hood (horizontal flow)	Gelman Sciences
Liquid nitrogen tank	Taylor-Wharton
Falcon 15 mL	Corning/BD
Falcon 50 mL	Corning/BD
Filter (0.2µm)	Sartorius
Filter (0.45µm)	Sartorius
Gold SEAL Micro Slides	BD
Media Bottle (Glass) (125 mL)	Life Technologies/WHEATON
Multi-channel dispenser	Lab System
Needle 18 G 1½	BD
Needle 27 G ½	BD
PD-10 Desalting Columns	GE Healthcare Life Sciences
Petri dish	ExtraGene
Pipette (0.5 to 10 µL, 2 to 20 µL, 5 to 50 µL, 20 to 200 µL, 100 to 1000 µL)	Eppendorf
Pipette Pasteur Disposable Glass(9″)	Pyrex
Protein G GraviTrap	GE Healthcare Life Sciences
Refrigerator 4°C, -20°C, -80°C	Sharp/Kelvinator
Sample Vial (4mL)/ 1drum Vial/ Bottle Glass w/s cap	WHEATON

Syringe (1 mL)	BD
Syringe (3 mL)	BD
Syringe (5 mL)	BD
Syringe (10 mL)	BD
Syringe (60 mL)	BD
Tips (200 µL)	Thermo Scientific
Tips (1.0 mL)	Thermo Scientific
Tube Culture, 13*100 mm	Pyrex
Tube Culture, 16*100 mm	Pyrex
Tube Culture, 16*125 mm(10mL)	Pyrex
Vial (drum Vial) (4ml)	WHEATON
Vivaspin6	GE Healthcare life Science
Water bath	Gallenkamp
Zetasizer Nano ZS90 instrument	Malvern Instrument, Ltd