Vaccination against ETEC diarrhea in Bangladeshi participants and the influence of age on vaccine induced immune responses

A DISSERTATION SUBMITTED TO THE UNIVERSITY OF DHAKA IN PARTIAL FULFILMENT OF THE REQUIRMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY AND MOLECULAR BIOLOGY

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January, 2019

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Dedicated
To
My Parents

ACKNOWLEDGEMENTS

All the applauses to Allah for endowing me with health, patience and mental power to accomplish this dissertation. This work has been carried out at the **icddr,b** and at the **University of Gothenburg**. I would like to give my sincerest gratitude to everyone who has contributed in making this thesis possible. I would particularly like to thank the following people for help and encouragement during my PhD studies.

I am overwhelmed to express my respect, sincere gratitude and heartfelt thanks to my supervisor **Professor Dr. Laila N. Islam**, Department of Biochemistry and Molecular Biology, University of Dhaka, for her guidance, encouragement and academic support throughout the years. Madam, I always admire you for your honesty, constructive criticism, and endearing company and especially for your scholastic guidance during my thesis writing.

I convey special thanks to my co-supervisor **Dr. Firdausi Qadri**, Senior Scientist and Head of the Mucosal Immunology and Vaccinology Unit, Infectious Disease Division, icddr,b, for letting me be a member of your research group. Apa, I gratefully acknowledge you for your advice, encouragement, and supervision. You are inspiring in so many ways and you make us all very enthusiastic. Apa, you are a brilliant scientist, full of energy and knowledge MashaAllah. You were always positive for any matters related to this PhD work, I never heard ‘no’ from you.

I would like to convey my special gratitude to my mentor and co-supervisor **Dr. Anna Lundgren**, Associate Professor, Department of Microbiology and Immunology, University of Gothenburg, Sweden. You have taught me a lot with your impressive scientific knowledge. Thanks for the endless guidance and for always being encouraging and supporting me as well as for always listening to me from another part of the world. Sorry for destroying most of your weekends in the past several years for planning experiments and reviewing manuscript and thesis. You are a very good human being. I am very grateful to you for everything especially about the support you have provided me during my stay in Sweden.

I am also thankful to **Professor Dr. Ann-Mari Svennerholm**, Department of Microbiology and Immunology, University of Gothenburg, Sweden for her crucial contribution and support throughout my research especially in data analysis and manuscript writing. I have learned many critical techniques from you on revising data especially ELISA titers.

Dr. Taufiqur Rahman, my another mentor of this PhD thesis. Bhaia, thank you for being kind and always helping me no matter what or how much I have asked you. Your gentleness is an integral part of your personality. You have always shared the experiences about your PhD and reading immunology that helped me a lot.

Thanks to **Dr. Yasmin Ara Begum** for her encouragement, love and care over my days in the icddr,b. Apa, you helped me a lot in the microbiology work especially during the screening period of the ETVAX study.

Thanks to **Dr. Farhana Khanam**, for your support and cordial help during my work especially during my critical time in pregnancy. In all these past years, we shared not only an office but also experiences and feelings related and not related to study. I always admire your passionate working style and dedication.

Special thanks to **Sarmin**. We have started together this PhD journey but life complexity could not make this for you. I trust Almighty has a better plan for you and your family. Thank you very much for helping me in lab and academic things.

I gratefully thank **Tanzeem, Sadia Isfat, Parvin Apa, Fatema Apa, Lazina and Nuder** for all help with lab work and analyses. You all were very helpful and always supported me. Thanks for always being there through the ups and downs that everyday scientific work has to offer. I would also like to thank **Rajib Da, Prodip Da, Zohir Bhai, Momo Apa, Delowar Bhai, Nabi Bhai, Shahanaz Apa and others** who have done microbiology in the ETVAX study and also co-operated with me in different works.

I would also like to thank **Dr. Sukanya Raghavan** for your nice hospitality during my stay in Sweden and for taking my immunology exam.

Thanks to **Gudrun Wiklund and Joanna Kaim** for their support in shipment, laboratory analysis and for help in the lab during my stay in Sweden. I am also grateful to **Dr. Susannah Leach** since she helped me indirectly thorough her PhD thesis.

I would like to thank our clinical team especially **Dr. Mohiul Islam Chowdhury, Dr. Tasnuva Ahmed and Dr. Arifuzzaman Khan** for their active support in the clinical trial in the field. Without your help, all these work would not have been possible to accomplish.

I also like to thank the IP formulation team in the ETVAX study especially **Aklima, Salima, Kamrul and Pinki** for their valuable contributions in preparing vaccine and dmLT.

Thanks to **Moyeen Bhai, Shahidul Bhai, Jaher Bhai, Salauddin Bhai, Lokman Bhai and Mukdhum Bhai** for their wonderful administrative work and for always being so welcoming and helpful.

Thanks to **Kamrul, Motaher, Aklima, Pinki, Salima, Isfat, Nazia, Banna and all the other juniors and seniors** in the lab for the pleasant tea time. Thank you for all the help, fun times and interesting discussions on the articles or life-related things.

I would also like to thank **Dr. Md. Saruar Bhuiyan Bhai** and **Hasan Afrad Bhai** for your technical help and good advices during writing this thesis.

I gratefully acknowledge my family especially **my parents** for supporting and inspiring me during these years. With you, I had always something to think about except the work. Thank you for taking care of Alvina when I was at work. Special thanks to my husband, **Dr. Abu Hanif**, for always supporting me and helping me with everything. Most importantly, I thank my little **Alvina** for giving constant joy and happiness as well as refreshing my days.

Finally, I would like to acknowledge the support of the **study participants and the funding sources**. The work was supported by icddr,b with funding from PATH (grant no. GR01263), the Swedish Research Council (grant number. VR 348-2013-6615 and VR 348-2014-4228), the Swedish Foundation for Strategic Research (grant number SB12-0072), European Union Seventh Framework Programme (FP7/2007–2013, grant agreement no.261472-STOPENTERICS).

ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) is one of the leading bacterial causes of diarrhea in children in low and middle income countries including Bangladesh as well as in travelers. No effective ETEC vaccine is yet available, but efforts are ongoing to develop such a vaccine since the burden of ETEC diarrhea is very high. Most oral vaccines induce lower immune responses in young children in low-resource countries than in developed countries. Several strategies are currently used to enhance ETEC vaccine immunogenicity, including addition of the mucosal adjuvant double mutant heat labile toxin (dmLT). The main objectives of this dissertation were to analyze mucosal immune responses induced by the oral inactivated ETEC candidate vaccine ETVAX in Bangladeshi adults and the influence of age on vaccine induced immune responses.

In preparations to test ETVAX in Bangladesh, optimal sampling time points for evaluation of mucosal immune responses after oral vaccination were determined. Adults, toddlers, young children and infants were given two doses of the licensed oral cholera vaccine Dukoral. Cholera toxin B subunit (CTB) and *Vibrio cholerae* membrane protein specific IgA in antibodies in lymphocyte supernatant (ALS) specimens, IgA and IgG in plasma and secretory IgA (SIgA) in fecal samples were evaluated 4/5 and 7 days after each vaccination using ELISA. After the first dose, most adults and toddlers developed high and comparable anti-CTB ALS IgA responses on day 5 and 7, while very few young children responded. After the second dose, highest ALS responses were observed on day 5 in all age groups and the responses declined on day 7. In contrast, plasma antibody responses were high on both day 5 and day 7 after the second dose. Fecal SIgA responses were increased on day 7 in young children and infants after the second dose. To evaluate the safety and immunogenicity of ETVAX in Bangladeshi adults, participants were given two doses of ETVAX±dmLT or placebo (n=15/group) and samples were collected according to the optimal sampling schedule previously identified. To facilitate the analysis of ALS responses against multiple vaccine antigens in small sample volumes for subsequent analyses in younger age groups, a highly sensitive electrochemiluminescence (ECL) assay was established. Magnitudes of IgA ALS responses determined by ECL and ELISA correlated very well and ECL was used as a primary readout method for ALS responses against colonization factors (CFs) and LTB in the trial. ETVAX±dmLT was safe and well tolerated in adults. After the first dose, IgA ALS responses against each of the primary antigens were detected in 87-100% and after the second dose in 100% of vaccinees. Plasma IgA responses against different CFs and LTB, as determined by ELISA, were observed in 62-93% and 100% of vaccinees, respectively. No statistically significant adjuvant effect of dmLT on responses was detected but the overall antigenic breadth of the plasma IgA responses tended to favor the adjuvanted vaccine. The adjuvant effect of dmLT was also studied *in vitro* in peripheral blood mononuclear cells from infants and adults. dmLT enhanced IL-17A responses in both infant and adult cells. Blocking experiments showed that the IL-17A enhancing effect may at least partly be mediated by IL-1β in both age groups. dmLT significantly enhanced IL-1β responses to low amounts of ETVAX vaccine in cells from infants but not from adults.

Collectively, the results and methodological improvements obtained from this dissertation have facilitated the design and execution of an ongoing trial of the ETVAX vaccine in young children and infants. The results demonstrate that the ETEC vaccine is safe in adults and induce strong mucosal and plasma antibody responses. The *in vitro* results show that dmLT has potential to enhance IL-17A responses in both infants and adults and may particularly promote IL-1β responses to low ETVAX doses in infants. These results are important for the clinical evaluation of ETVAX as well as other mucosal vaccines in adults and infants.

Key words: ETEC, vaccine, adjuvant, ETVAX, dmLT, antibody, T cell, ELISA, ECL

List of Papers

Based on this thesis work, the following two research papers have been published and a third one is in manuscript.

I. Kinetics of antibody-secreting cell and fecal IgA responses after oral cholera vaccination in different age groups in a cholera endemic country.
Akhtar M, Qadri F, Bhuiyan TR, Akter S, Rafique TA, Khan A, Islam LN, Saha A, Svennerholm AM, Lundgren A.
Vaccine. 2017 Jan 5;35(2):321-328.

II. Evaluation of the safety and immunogenicity of the oral inactivated multivalent enterotoxigenic *Escherichia coli* vaccine ETVAX in Bangladeshi adults in a double-blind, randomized, placebo-controlled Phase I trial using electrochemiluminescence and ELISA assays for immunogenicity analyses.
Akhtar M, Chowdhury MI, Bhuiyan TR, Kaim J, Ahmed T, Rafique TA, Khan A, Rahman SIA, Khanam F, Begum YA, Sharif MZ, Islam LN, Carlin N, Maier N, Fix A, Wierzba TF, Walker RI, Bourgeois AL, Svennerholm AM, Qadri F, Lundgren A.
Vaccine. 2018. https://doi.org/10.1016/j.vaccine.2018.11.040

III. Adjuvant activity of dmLT in immune cells from infants and adults.
Akhtar M, Bhuiyan TR, Nizam NN, Hossain L, Bashar SR, Akter S, Qadri F, Lundgren A.
Manuscript in preparation.

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ABBREVIATIONS

AEBSF	4-(2-Aminoethyl) Benzenesulfonyl Fluoride Hydrochloride
ALS	Antibody in Lymphocyte Supernatant
APC	Antigen-Presenting Cell
ASC	Antibody-Secreting Cell
BSA	Bovine Serum Albumin
CF	Colonization Factor
CFU	Colony Forming Unit
CT	Cholera Toxin
CTB	Cholera Toxin B-subunit
rCTB	Recombinant Cholera Toxin B-subunit
DALY	Disability-Adjusted Life Years
DC	Dendritic Cell
dmLT	Double-Mutant LT
ECL	Electrochemiluminiscence
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme-Linked Immunospot
ETEC	Enterotoxigenic *Escherichia coli*
FBS	Fetal Bovine Serum
FDC	Follicular Dendritic Cell
GALT	Gut-Associated Lymphoid Tissue
GC	Germinal Center
HRP	Horseradish Peroxidase
icddr,b	International Centre for Diarrhoeal Disease Research, Bangladesh
IL-1β	Interleukin-1 beta
IFN-γ	Interferon-gamma
IL-17A	Interleukin- 17A
IL-1RA	Interleukin-1 Receptor Antagonist
LPS	Lipopolysaccharide
LT	Heat-Labile Toxin
LTA	Heat-Labile Toxin A-Subunit
LTB	Heat-Labile Toxin B-Subunit
MadCAM-1	Mucosal Addressin Cell Adhesion Molecule-1
MALT	Mucosa Associated Lymphoid Tissue
MHC	Major Histocompatibility Complex
MP	Membrane Protein
MSD	Meso-Scale Discovery
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffer Saline
PHA	Phytohaemagglutinin
pIgR	Poly IgA Receptor
PKA	Protein Kinase A
SC	Secretory Component
SEB	Staphylococcal Enterotoxin B
SIgA	Secretory IgA
ST	Heat-Stable Toxin
TCR	T Cell Receptor
TGF-β	Transforming Growth Factor-β
Th	Helper T Cell
Treg	Regulatory T Cell
Tfh	Follicular Helper T cell

1. INTRODUCTION

Diarrheal disease is one of the leading causes of mortality and morbidity in children in developing countries. Recently, an estimated 688 million illnesses and 1.6–2.1 million deaths were recorded globally among children younger than five years due to diarrhea [1, 2]. Figure 1 shows the Global distribution of deaths due to diarrhea in children less than 5 years of age in year 2000. Multiple pathogens are responsible for this suffering and death, including rotavirus, *Vibrio cholerae*, enterotoxigenic *Escherichia coli* (ETEC), *Shigella* spp. and *Salmonella* spp.

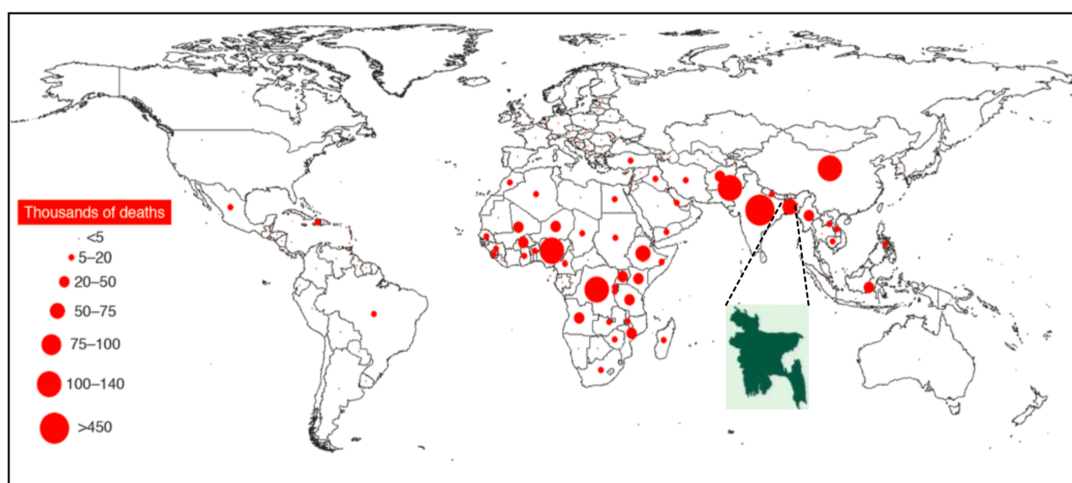

Figure 1. Global distribution of deaths caused by diarrhea in children less than 5 years of age. An estimate of 1.6–2.1 million global mortality was recorded in 2000 due to diarrhea and most of these deaths occur in children under the age of 5 years in developing countries. Adapted from [2].

ETEC is one of the most common causes of diarrhea in developing countries and its incidence is high, closely following *V. cholerae* and rotavirus. Children are mainly affected by ETEC and it is considered as a principal cause of child mortality and morbidity as well as growth retardation. No effective vaccine is yet available for ETEC, but candidates are in different development stages and some are currently in clinical trials [3]. Several efforts have also been taken to enhance vaccine specific immunogenicity, e.g. addition of mucosal adjuvants [4]. The double mutant heat-labile toxin (dmLT) derived from heat labile toxin (LT) produced by ETEC bacteria has been used as a mucosal adjuvant and has been shown to enhance host immune responses to several different vaccine antigens including an ETEC vaccine [5-8].

ETEC bacteria are non-invasive and localized in the gut, therefore intestine derived antibodies, especially secretory IgA (SIgA) antibodies are considered to confer immune protection after natural infection or vaccination [9, 10]. These antibodies can be measured directly in fecal and intestinal lavage samples. Another surrogate marker of the mucosal responses is the measurement of blood antigen-specific antibody secreting cells (ASCs) which are induced at mucosal sites after infection or vaccination and transiently migrate to the blood before rehoming to the gut [11-14]. Traditionally, ASC responses have been determined by the enzyme-linked immunospot (ELISPOT) assay. Alternatively, secretions of ASCs, known as antibodies in lymphocyte supernatant (ALS), can be determined by ELISA [15, 16]. However, both ELISPOT and ELISA assays need large volumes of blood specimens and only limited blood volumes can be collected from children and infants. The SIgA content of fecal specimens may also vary in different age groups. Hence, it is very important to establish a more optimal and sensitive method requiring small volumes of blood for measuring intestine-derived immune responses to multiple vaccine antigens.

In this dissertation, a new oral ETEC vaccine, ETVAX, developed at the University of Gothenburg, has been tested with and without the mucosal adjuvant dmLT to evaluate safety and immunogenicity in a Phase I clinical trial in Bangladeshi adults. A novel sensitive method has been established to determine vaccine specific ALS responses in small sample volumes using a novel electrochemiluminescence (ECL) assay which can be used for subsequent analyses in children and infants. To prepare for the ETVAX trial, optimal sampling time points to measure mucosal as well as systemic immune responses after oral immunization in different age groups were established in a kinetic study using the licensed oral cholera vaccine Dukoral to serve as a model vaccine for optimizing the procedure. In addition, the adjuvant effect of dmLT was also evaluated *in vitro* on antigen presenting cells and T cells in infants compared to adults and the role of IL-1β for mediating the adjuvant activity was investigated.

1.1 Literature review

1.1.1 Epidemiology of enterotoxigenic *Escherichia coli* (ETEC)

ETEC causes significant global morbidity and mortality, particularly in low-income countries. Recent global estimates implicate that, annually, ETEC causes 1 billion cases of diarrhea and more than 500,000 deaths [17-20]. In 2013, around 42,000 deaths of children under 5 years of age and 89,000 deaths over 5 years of age were reported due to diarrhea caused by ETEC [3, 21]. In addition, ETEC contributes significantly to the suffering and anticipated loss of human potential in children and accounts for around 8% of all disability-adjusted life years (DALYs) caused by diarrheal diseases [22, 23]. ETEC infection is particularly a major burden in low and middle-income countries in Africa and South Asia, where water quality and sanitation are poor [17, 24]. High prevalence of ETEC infection is also found in Bangladesh, affecting mainly children under five years of age [25]. However, adults are also commonly infected with this pathogen [25]. Studies in Dhaka, Bangladesh suggest that during the first two years of life, 20% of all diarrheal cases are due to ETEC infection with an incidence of 0.5 episode/child/year [25, 26].

ETEC is also a major contributor to traveler's diarrhea worldwide among travelers to many parts of Africa, Asia, and Latin America. It accounts for 20-60% of all traveler's diarrhea reported and causes approximately 10 million episodes each year [27, 28].

1.1.2 ETEC bacteriology

ETEC are Gram-negative, rod-shaped bacteria that are not visibly different from *E. coli* found in the normal flora of the human large intestine (Figure 2). ETEC pathogenesis involves colonization of the intestinal mucosa using a number of colonization factors (CFs) and release of enterotoxins on the epithelial surface, which causes acute watery diarrhea [29, 30].

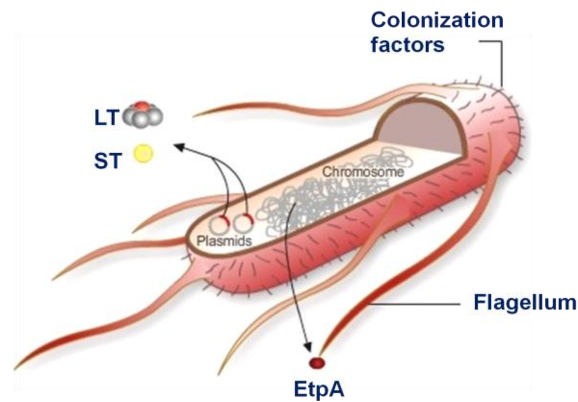

Figure 2. General morphology of ETEC bacteria. ETEC pathogenesis involves the production of colonization factors and the generation of a heat-labile enterotoxin (LT) and/or a heat-stable enterotoxin (ST). EtpA is another virulence factor which contributes to the pathogenesis of ETEC infection by acting as an intermediate adhesin in the adhesion between ETEC flagella and host cells. Adapted from *https://stopenterics.bio-med.ch/*. Accessed on January 10, 2018.

1.1.2.1 Serotypes

ETEC have two different serogroups; the O and H serogroups. The O serogroup is defined by the O-antigens, which are carbohydrates and are part of the bacterial cell wall lipopolysaccharide (LPS) and the H serogroup is determined by the flagellar antigen. Based on an extensive database analysis of ETEC from different countries, it has been reported that ETEC has 100 different O and 34 different H serogroups. The most common O groups are O6, O78, O8, O128, and O153; these accounted for over half of the ETEC strains. Considerably fewer H serogroups than O serogroups are associated with ETEC and among them, five H types accounted for over half of those strains and these are widespread [17, 31]. Hence the O and H serogroups are not used for diagnosis and identification of ETEC.

1.1.2.2 Colonization factors

ETEC colonize the small intestinal surface using different CFs, that usually, but not always, have fimbrial or fibrillar structure. CFs bind with glycoconjugate receptors on the host cell membrane, which exhibit an enormous diversity in oligosaccharide sequences [32]. Today over 25 different types of CFs have been identified, which are mostly encoded by plasmids [3, 33]. Among the wide range of CFs, the most common are CFA/I and CS1–CS6, and in certain regions, CS7, CS14 and CS17 are also common [34]. Most studies suggest that around 50-80% of the prevalent ETEC

isolates express a known CF; however, some isolates may express one, two or three different CFs. Based on significant amino acid similarity in the N-terminal region, CFs can be subdivided into three different groups; the CFA/I-like group (includes CFA/I, CS1, CS2, CS4, CS14 and CS17), the CS5-like group (includes CS5, CS7, CS18 and CS20) and those that are unique (including CS3, CS6 and CS10-12) [35]. Strains expressing CFs within these groups have been shown to induce substantial immune responses to homologous CFs with cross-reacting antigenic epitopes [36, 37].

1.1.2.3 Toxins

ETEC strains express one or both of two different enterotoxins, the heat-stable toxins (STh and STp) and LT, both of which cause fluid secretion from the small intestinal epithelial cells. LT is a holotoxin (84 KDa), composed of an A subunit (LTA) and five B subunits (LTB) and is structurally and functionally related to cholera toxin (CT) produced by *Vibrio cholerae* [38]. The LTB subunits bind irreversibly to GM1 ganglioside receptors on host epithelial cells and cause endocytosis of the LTA subunit to activate the adenylate cyclase pathway, leading to an increase in intracellular cAMP levels in host cells [39]. ST is a non-antigenic, low molecular-weight, cysteine-rich, small peptide consisting of 18 to 19 amino acids. ST binds reversibly to guanylate cyclase C at the apical membrane of host intestinal epithelial cells and activates the guanylate cyclase pathway leading to an increase of intracellular cGMP levels [40, 41]. Increased levels of cAMP/cGMP cause an imbalance in electrolyte movement in the epithelial cells and result in watery diarrhea [4, 33].

1.1.2.4 Other virulence factors

Recent investigations have identified some other novel putative ETEC virulence factors including flagellin, EtpA, EatA, EaeH, and YghJ [42-44]. EatA, a serine protease autotransporter, facilitates the delivery of ETEC toxins and EtpA, an extracellular protease, modulates adhesion and intestinal colonization by ETEC [40]. Recent studies have suggested that these antigens are relatively conserved (~70% of isolates) among a diverse range of ETEC strains from different geographic locations and are not constrained to a single CF type or toxin type [45].

1.1.3 Mechanism of ETEC infection

ETEC are transmitted by fecal-contaminated food or water. A high infectious dose (10^8 colony forming units, CFUs) of ETEC bacteria is required to establish illness in healthy subjects, though this may be lower in very young, elderly or malnourished persons. The mean incubation time of ETEC diarrhea is 2 days (range 1 to 5 days) after feeding [46]. Infection is established when the bacteria reach the small intestine where they attach to the epithelial surface via CFs but do not invade the mucosa. After attachment and colonization, the bacteria proliferate and produce LT and/or ST and affect the epithelial cells in that area.

The pathogenesis of disease caused by *V. cholerae* O1 and ETEC are similar in terms of their toxin production. CT produced by *V. cholerae* O1 and LT of ETEC share 80% identity at the nucleotide level [38]. The LTB subunit binds to GM1 ganglioside on the epithelial cell surface and causes internalization of the LTA subunit. Subsequent proteolytic cleavage and disulfide bond reduction splits the A-subunit into two domains, the enzymatically active A1 subunit, and a smaller A2 peptide. The A1 subunit exhibits both NAD-glycohydrolase activity and ADP-ribosyltransferase activity. Transport of A1 into the cytoplasm results in ADP-ribosylation of α subunit of G_s protein (Gs_α), followed by irreversible activation of adenylate cyclase and increases in intracellular levels of cAMP (Figure 3). The resulting increase in cAMP eventually causes dysregulation of cAMP-sensitive ion transport mechanisms, inhibiting intracellular salt absorption, increasing electrolyte transport into the gut lumen, creating an osmotic gradient favoring intestinal water secretion [17, 40].

ST binds to the extracellular portion of the guanylyl cyclase enzyme located on the surface of intestinal epithelial cells. Binding causes activation of the cyclase enzyme, which eventually causes accumulation of cGMP in the cell. Similar to the consequence of increasing cAMP levels in intestinal epithelial cells (by LT), an increase of cGMP levels promotes secretion of chloride but inhibits absorption of sodium chloride in host intestinal epithelial cells, which results in fluid hypersecretion [40, 41]. The fluid volume secreted far exceeds the ability of the gut to reabsorb, and the excess fluid is passed as watery diarrhea.

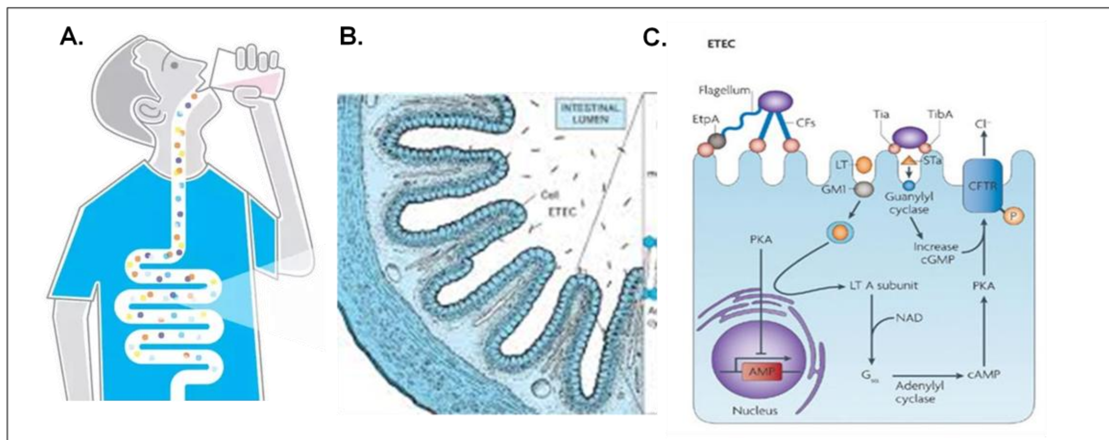

Figure 3. Pathogenesis of ETEC in diarrheal disease. (A) ETEC is transmitted by contaminated food or water and (B) colonize the mucosal surface of the small intestine. (C) The enterotoxin LT and ST activate adenylate cyclase and guanylyl cyclase, respectively, and elevates the intracellular levels of cyclic AMP which ultimately disrupt intestinal fluid homeostasis and cause hypersecretion of fluid and electrolytes. Adapted from *https://slideplayer.com/slide/5302254/17/images/20/Heatlabile+enterotoxin+%28LT%29+and+heat-stable+enterotoxin+%28ST%29.jpg*. Accessed on March 06, 2018.

1.1.4 Diagnosis of ETEC diarrhea

ETEC can grow on a variety of different media, e.g. MacConkey agar, blood agar etc. Differentiation of ETEC from other *E. coli* bacteria is achieved by identifying specific virulence factors, e.g. LT/ST and CFs [17]. Diagnosis of ETEC infection is not a regular practice in most laboratories. However, infection with ETEC can be diagnosed by culturing of stool samples of the infected individuals in specialized laboratories. Polymerase chain reaction (PCR) is a sensitive and specific method that can be used in ETEC diagnosis with a variety of primers against LT/ST and CFs genes [47]. ELISA is also used to detect enterotoxins using microtiter GM1 ganglioside methods. CFs of ETEC can also be detected by a dot-blot assay [17, 32].

1.1.5 Treatment of ETEC diarrhea

The severity of ETEC diarrhea may range from mild to very severe. ETEC infection is usually self-limiting, lasting 3-4 days. With adequate treatment, including oral rehydration and improved feeding, disease severity can be minimized [48]. Antimicrobials are useful only when the diagnosis or suspicion of ETEC related diarrhea is made [17]. Recently, azithromycin has become the preferred antibiotic for the treatment of acute watery diarrhea [49]. However, ETEC strains are frequently resistant to common antibiotics, including trimethoprim-sulfamethoxazole and

ampicillin, which limits their usefulness [48]. Eventually, a long-term solution for preventing ETEC diarrhea is improvement of water supply and sanitation systems, but until this is achieved, vaccination is the most practical approach to reduce the public health burden from ETEC.

1.1.6 Host responses against ETEC infection

Since ETEC infection is non-invasive, immune protection is most likely mediated mainly by antigen-specific antibodies rather than by immune effector cells. Among the antibodies, those that are produced locally in the intestine, mostly SIgA, are considered more critical for protection against ETEC diarrhea [9, 10]. The general mechanism of induction of mucosal immune responses against non-invasive pathogens such as ETEC is summarized in Figure 4.

1.1.7 General mechanism of mucosal immune responses

Mucosa-associated lymphoid tissue (MALT) is a highly specialized immune system which protects mucosal surfaces from a vast number of potential pathogens. The intestinal mucosa consists of epithelium and lamina propia (LP). LP is a layer of loose connective tissue just beneath the epithelium and contains innate cells and lymphocytes [50]. Gut innate immunity is mediated by the epithelial lining and the innate immune cells dispersed in the intestinal LP. The adaptive immune system in the intestinal tract includes lymphoid tissues like gut-associated lymphoid tissue (GALT) which comprises Peyer's patches, LP lymphocytes, and intra-epithelial lymphocytes. Antigen is taken up from the intestinal luminal sites by the M cells in the epithelial lining and is transported by the dendritic cells (DCs) to antigen-presenting cells (APCs) in the GALT, which eventually activate naïve T lymphocytes and subsequently B lymphocytes to proliferate and induce differentiation into effector T cells and IgA producing plasma cells, respectively (Figure 4). Effector B and T cells leave the inductive sites by the lymph, enter the blood circulation and migrate back to the intestinal LP and surface epithelium to exert their functions [50].

1.1.7.1 Gut homing of effector cells

Tropism of effector lymphocytes from circulation to intestinal LP is due to the up-regulated expression of mucosal homing receptors and chemokine receptors (integrin α4β7 and CCR9) on the lymphocytes induced by retinoic acid. Intestinal DCs and epithelial cells produce retinaldehyde dehydrogenases (RALDH), the enzyme required for retinoic acid biosynthesis [51, 52]. α4β7 binds with mucosal addressin cell adhesion molecule-1 (MadCAM-1) on the intestinal endothelium and CCR9 binds to CCL25 expressed in the intestinal environment [50]. Additionally, CCL28, a chemokine expressed in the colon but not in the small intestine, acts as a chemoattractant for circulating IgA plasmablasts expressing CCR10 [50].

1.1.7.2 Humoral immunity

Intestinal surfaces are mainly protected by SIgA antibodies [50]. Generally, plasma cells in the lamina propia secret dimeric IgA (dIgA), which contains a J-chain. The J chain regulates polymerization and transportation of polymeric IgA across the epithelial cells to the luminal site via the poly IgA receptor (pIgR). At the apical epithelial cell membrane, the external portion of pIgR, also known as the secretory component (SC), is cleaved off by a specific protease and the cleaved part remains bound covalently with the dIgA and the complexes are referred to as SIgA. The SC protects SIgA from proteolysis by luminal enzymes [50]. SIgA has several effector functions, including inhibiting bacterial adhesion, neutralizing viruses and blocking toxins [53-55]. SIgA antibodies mediate protective immunity against ETEC infection by preventing ETEC adhesion and LT toxin activity.

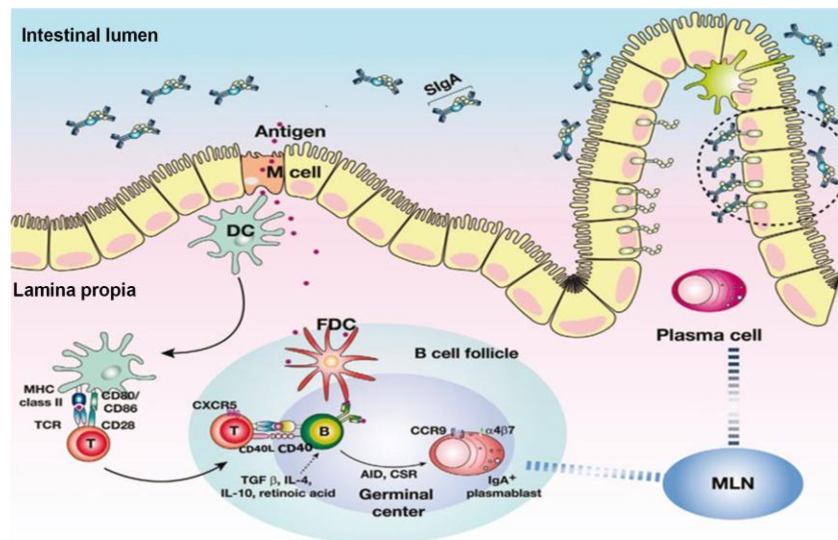

Figure 4. Mucosal immunity to non-invasive bacteria such as ETEC. In gut-associated lymphoid tissue (GALT), antigen is sampled by M cells and delivered to underlying dendritic cells (DC). Naïve lymphocytes that recognize their cognate antigen are activated to proliferate and differentiate into effector cells. B cells preferentially differentiate into IgA-secreting plasma cells. Secretory IgA (SIgA) arise from plasma cells that are located in the intestinal lamina propria and produce dimeric IgA. Dimeric IgA has the ability to bind to an epithelial cell transport receptor, called polymeric immunoglobulin receptor (pIgR), which is located in the basolateral cell surface of epithelial cells. During transport from the basolateral to the apical cell surface of epithelial cells, dimeric IgA acquires a portion of the pIgR, called the secretory piece, and thus become SIgA. SIgA antibodies give protective immunity against non-invasive pathogen like ETEC by preventing adhesion and toxin activity. Adapted from *https://www.b-cell-design.com/assets/images/7/Immunite-muqueuse-4-bf3b3cf7.jpg*. Accessed on March 15, 2018.

1.1.7.3 Tcell-mediated immunity in the gut

LP T cells are dominated by $CD4^+$ T helper (Th) cells (~65%), and most of them are Th1, Th2, Th17 and regulatory T cells (T_{regs}) [56]. Th1 cells produce several cytokines, among which IFN-γ is very important. IFN-γ activates macrophages to kill intracellular microbes, regulates the Th1/Th2 balance, induces class II major histocompatibility complex (MHC) molecule expression, promotes the cytotoxic activity of other cell types, enhances antigen presentation and stimulates the recruitment of other immune cells [57]. The Th2 cytokine IL-4 in concert with transforming growth factor (TGF-β) and IL-10 promote B cell differentiation into IgA-producing cells. Intestinal LP provides a unique site for the generation of Th17 cells [58]. The primary function of Th17 cells, which typically produce the cytokines IL-17A, IL-17F, and IL-22, appears to be the clearance of extracellular pathogens during infections and maintaining the mucosal epithelial barrier. IL-17A can also

promote the upregulation of pIgR on the epithelial cells, thus increasing IgA secretion in the lumen [59]. IL-17A is also required for IgA and IgG1 antibody production, as demonstrated by a significant impairment of mucosal IgA and systemic IgA and IgG1 production after oral immunization of IL-17A deficient mice [60]. Moreover, IL-17A can promote fibroblasts and epithelial cells to secrete proinflammatory mediators, including G-CSF, GM-CSF, and IL-8, which increase the production and release of neutrophils from the bone marrow and their recruitment to the tissue [61, 62]. Another Th17 cytokine, IL-22, can induce the production of antimicrobial peptides and promotes barrier function [61, 62]. In addition to Th1, Th2, Th17 cells, another subset of Th cells is follicular helper T cells (Tfh), which have a role in promoting long-lasting immunity by helping germinal center B cells. The Tfh cytokine IL-21 promotes class-switching and B cell differentiation into plasma cells and memory B cells [63, 64].

1.1.8 Protective immunity against ETEC infection

Epidemiological studies suggest that the incidence of ETEC causing diarrhea decreases with age [4, 26]. These observations signify the development of protective immunity after multiple natural exposures to ETEC during childhood, which ultimately protects against illness. The current knowledge of immunity against ETEC derived from the study of immune responses in humans with naturally acquired or experimentally induced ETEC disease is summarized below.

1.1.8.1 Innate immunity against ETEC infection

Although ETEC infection is commonly considered as non-inflammatory, studies found that fecal cytokine levels are associated with the duration of diarrheal illness [65]. The neutrophil chemotactic factor IL-8 significantly increase following ETEC infection and is considered to play an important role in infection resolution [65]. In contrast, increased expression of TNF-α and IL-6 are associated with increased diarrheal duration. Increased levels of lactoferrin, IL-1β, and IL-1 receptor antagonist (IL-1RA) were also found in fecal samples from travelers to India with ETEC infection [66]. Anti-inflammatory cytokines such as IL-10 were found to inhibit the clearance of ETEC infections since high fecal IL-10 levels were shown to be associated with increased duration of diarrhea [65].

1.1.8.2 Humoral responses to ETEC infection

Natural ETEC infection elicits substantial intestinal SIgA as well as systemic IgA and IgG antibody responses against the main virulence factors, i.e. CFs and LT [14, 67-69]. Multiple lines of evidence indicate that anti-CF immunity is probably the cornerstone of long-lasting immunity to ETEC [46, 70]. A birth cohort in Bangladesh studied until 2 years from birth showed that reinfections with homologous CF expressing ETEC strains are not common in children who had a primary infection with CFA/I, CS1+CS3, CS2+CS3 or CS5+CS6 expressing ETEC strains [26]. Other studies show that IgA antibodies in plasma from patients infected with CFA/I and CS5 expressing ETEC bound to both homologous as well as to heterologous cross-reacting CF antigens when analyzed by ELISA [37]. This cross-protection to heterologus antigens depends on the natural priming status in the endemic region, since Bangladeshi adults, but not North American volunteers, infected with CFA/I strains, responded to both homologous and heterologous CF antigens [71]. Anti-LT immunity with high antibody titers was observed in several studies after natural ETEC infection [67, 72, 73]. Since anti-LT immunity is giving short-term protection in humans, reinfections with LT expressing ETEC are common [26, 74]. However, animal studies as well as field studies strongly suggest that immunity against LT can be protective [75]. ETEC infected patients develop significant intestine-derived IgA antibodies and ASC responses and it has been found that locally produced SIgA have role in the clearance of ETEC bacteria [9].

1.1.8.3 Cellular immunity against ETEC infection

Very little is known about the nature and protective role of cellular immunity in response to ETEC infection. However, CD4+ Th cells promote the development of B cell memory and high avidity antibodies, thereby playing an important role as directors of the humoral response [69, 76]. LT triggers T cell responses involving both Th1 and Th2 cytokines in natural ETEC infection [77]. Additionally, studies on traveler's diarrhea showed that individuals possessing IFN-γ responses to CTB (used as a surrogate of the ETEC LT immune response) were less likely to develop diarrhea than individuals without such responses [78].

1.1.9 ETEC candidate vaccines

Development of an effective ETEC vaccine with broad protection has been identified as a key prevention strategy for children living in developing countries and for adult travelers to those regions. However, the extreme antigenic diversity of ETEC strains makes it a challenging task to develop an effective ETEC vaccine [3, 4]. Most ETEC vaccine efforts to date have focused on stimulating immunity to the most prevalent CF antigens (CFA/I and CS1-CS6) and the LT toxoid [79, 80]. Some of the more advanced ETEC candidate vaccines include an oral killed vaccine, an oral live attenuated whole-cell vaccine, and subunit vaccines [3]. Killed vaccines are safer and usually have fewer side effects in comparison to live attenuated vaccines, although the live attenuated vaccines may produce strong and long-lasting immune responses due to similarity to natural infection [81]. Most subunit vaccines usually need to be injected by parenteral routes and are not naturally stable in the gastrointestinal tract. However, parenteral vaccines normally induce poor mucosal immune responses. In addition, there are many practical advantages of oral vaccines over the parenteral vaccines, including being easy to administer and not requiring trained healthcare professionals, being painless and also having less risk of transmitting infections [81]. ETEC candidate vaccines are discussed in detail below.

1.1.9.1 First generation killed oral ETEC vaccine (rCTB-CF)

The first generation of rCTB-CF ETEC vaccine, developed at the University of Gothenburg, Sweden, contains 1 mg rCTB and formalin-killed ETEC bacteria of five strains expressing CFA/I, CFA/II (CS1 to CS3) and CFA/IV (CS4 and CS5) as well as some of the most prevalent O-antigens of ETEC. Two or three oral doses of this vaccine given at a two weeks interval were shown to be safe and immunogenic in several trials including Swedish, Bangladeshi and Egyptian adults [10, 82-84]. However, in a trial in Egypt, the vaccine was found to be not significantly protective in infants [85]. Furthermore, giving a full dose of this vaccine to 6–17-months old children resulted in vomiting in many of them [86]. In another Phase III trial in North American travelers to Mexico or Guatemala rCTB-CF ETEC provided a significant 77% protective efficacy against non-mild ETEC diarrhoeal disease although no significant protection was seen against mild to severe ETEC diarrhea [87].

The information gained from studies on the first-generation inactivated whole cell vaccine suggested that a vaccine capable of conferring broad-based protection against heterogeneous strains of ETEC should contain increased amounts of CFs per bacterium, in order to allow lower vaccine dosages to be administrated to children, and a more LT- like toxoid.

1.1.9.2 Second generation killed oral ETEC vaccine (ETVAX)

To improve the first generation ETEC vaccine, several efforts were undertaken to produce a second generation killed oral ETEC vaccine (ETVAX) with improved immunogenicity (Figure 5): (i) the amounts of protective antigens were increased by constructing recombinant *E. coli* strains over-expressing CF antigens (4-10 times higher levels of CFs than in the first generation vaccine), (ii) replacing rCTB with a more LT-like LTB/CTB hybrid toxoid (LCTB*A*) able to induce stronger anti-LT immune responses, (iii) addition of a safe and effective mucosal adjuvant to the vaccine formulation, and (iv) addition of a strain expressing CS6 in an immunogenic form even after inactivation [6, 8, 88].

A prototype of the second generation ETEC vaccine, containing only a CFA/I over-expressing *E. coli* strain and the LCTB*A* toxoid, was first tested in Swedish adults to compare the safety and immunogenicity with the corresponding CFA/I strain of the first generation rCTB-CF ETEC vaccine [89]. The vaccine was found to be safe and immunogenic, inducing significant mucosal IgA responses against CFA/I and LTB antigens. LCTB*A* also induced significantly higher systemic IgA responses than CTB [89].

Based on these results, the next step was to test the complete multivalent ETEC vaccine ETVAX, containing four different ETEC strains over-expressing prevalent CFs (CFA/I, CS3, CS5 and CS6) and the LCTB*A* toxoid in a double-blind, placebo-controlled Phase I study in 129 Swedish adults. The vaccine was given alone and in combination with two different dosages of dmLT (10 µg or 25 µg) [6]. This vaccine alone and with both dosages of dmLT was found to be safe with very few mild adverse events recorded. This vaccine also induced fecal SIgA as well as ALS IgA responses to all five primary vaccine antigens. Addition of 10 µg dmLT significantly increased anti-CS6 ALS IgA responses in comparison to vaccine alone and a similar

trend was seen for CS5, the two antigens present in lowest amounts in the vaccine. However, no significant adjuvant effect was seen on the antibody responses specific to other vaccine antigens [6]. Higher dmLT doses (25 µg) had no positive effect on the vaccine-induced responses [6]. When analyzing fecal SIgA and ALS IgA responses together, 74-83% of vaccinees responded to all five primary vaccine antigens, with little difference between groups receiving the vaccine with or without dmLT.

The capacity of ETVAX to induce mucosal immunological memory was next evaluated [90]. A single vaccine dose was administered to naïve individuals and to previously vaccinated volunteers who had participated in the Phase I clinical trial 1-2 years before. Two primary doses of ETVAX induced long-lasting IgA responses, since it was shown that significantly more frequent IgA ALS responses against all CFs and LTB were induced by the single late booster vaccine dose in the previously immunized than in non-immunized volunteers. However, priming with adjuvant did not influence memory responses [90].

The researchers also investigated if ETVAX could induce cross-reactive antibodies to related CFs in the CFA/I family (CS1, CS14 and CS17) and CS5 family (CS7) and if dmLT influenced antibody avidity development [91]. Around 65–90% of volunteers who had responded to CFA/I also developed cross-reactive antibodies to the tested CFA/I like antigens and 80% of those responding to CS5 also responded to the closely related CS7 in both ALS and fecal specimens. Moreover, the avidity of serum and ALS antibodies to the primary vaccine antigens increased after a late booster dose compared to after primary vaccinations. However, administration of ETVAX with or without dmLT during primary vaccinations had no apparent effects on antibody avidity [91].

Based on these promising safety and immunogenicity results from the Swedish clinical trials, new trials are ongoing to test this vaccine for safety and immunogenicity in young children and adults in ETEC endemic countries as well as in travelers to such countries. In this dissertation, the safety and immunogenicity of the ETVAX vaccine alone or together with dmLT adjuvant has been evaluated in Bangladeshi adults.

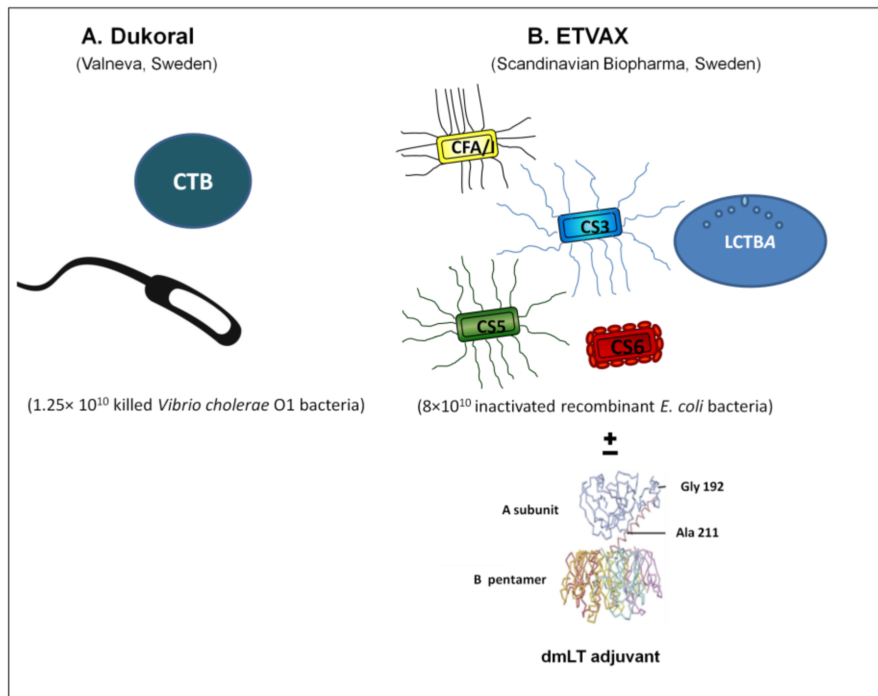

Figure 5. The composition of the Dukoral and ETVAX vaccines. (A) Dukoral is an oral cholera vaccine contains inactivated *V. cholerae* O1 bacteria and recombinant B-subunits of CT (rBS-WC) and (B) ETVAX is a candidate oral ETEC vaccine containing inactivated *E. coli* bacteria overexpressing the colonization factors CFA/I, CS3, CS5 and CS6 combined with a hybrid LTB/CTB subunit antigen (LCTB*A*), tested alone and together with dmLT adjuvant.

1.1.9.3 The oral cholera vaccine Dukoral

The Dukoral vaccine, which was primarily designed and licensed in over 60 countries for protection against cholera, has also been shown to prevent diarrhea caused by ETEC [4]. Dukoral is an oral whole-cell vaccine containing formalin-killed *V. cholerae* O1 bacteria and recombinant B-subunits of CT (rBS-WC) (Figure 5). Because of the antigenic similarity of CT and LT, Dukoral has been found to be effective in providing immunity against ETEC diarrhea [92]. This vaccine was shown to induce short-term protection against LT-ETEC in travelers to Morocco and Mexico [92]. Additionally, in a large field trial including Bangladeshi children and women, 67% fewer episodes of LT-ETEC diarrhea were noted in the group given two or three doses of Dukoral as compared to the group given the whole cell vaccine without CTB [74].

1.1.9.4 Other ETEC vaccines

Several other strategies also exist to develop an effective ETEC vaccine (Table 1). These include a live vaccine containing attenuated ETEC bacteria expressing CFs and LTB (ACE527), LT toxoid-based vaccines like dmLT, LT patch vaccine, and CF subunit vaccines. The oral live attenuated vaccine ACE527 comprises three live attenuated ETEC strains, expressing CFA/I, CS1, CS2, CS3, CS5, CS6 and LTB, from which enterotoxin and antibiotic resistance genes have been deleted and knockout mutations have been made in the *aroC*, *ompC*, and *ompF* genes. This vaccine was demonstrated to be well tolerated at doses of up to 10^{11} CFUs and also shown to induce significant immune responses to CFs in a Phase I trial in healthy adult volunteers [93]. However, in another Phase IIb study, where two doses of 10^{11} CFUs were tested, only 27% protective efficacy against moderate to severe diarrhea was observed. This vaccine also induced significantly more vomiting compared to the placebo [94].

Another approach was to develop a subunit vaccine known as LT patch, where native LT was delivered transcutaneously via a skin patch [95]. The idea was to stimulate skin APCs to take up this antigen and to generate anti-LT immune responses without inducing the enterotoxicity associated with oral administration. In a Phase II challenge study, three doses of the LT patch, given at three-week intervals, were found to be safe and immunogenic but failed to protect vaccinees against ETEC [95]. However, the LT patch was found to be protective against travelers' diarrhea caused by LT+ ETEC but not all ETEC in adult travelers to Mexico/Guatemala [96]. However, this vaccine is no longer under development. Additional approaches to develop ETEC candidate vaccines are shown in Table 1.

Table 1. Approaches for ETEC vaccine development

ETEC vaccine candidate	Status	References
Recombinant *E. coli* over-expressing CFs+LCTB*A* (ETVAX)	Phase I/II	[6]
aroC, ompF and ompC-based live attenuated (ACE527)	Phase II	[94, 97]
dmLT	Phase II	[98]
Anti-adhesin based subunit vaccine	Phase II	[99]
LT patch	Phase II	[95, 100]
CF tip subunit hybrid protein	Phase I	[101, 102]
Flagellin; EtpA; EatA; EaeH; YghJ	Preclinical	[103]
LT-ST fusion/LTB-ST conjugate	Preclinical	[104]
Recombinant *Shigellae* expressing LTB and CFs	Preclinical	[103]

1.1.10 Strategies to improve the immunogenicity of ETEC vaccines

Since most oral vaccines including ETEC vaccines have been shown to induce lower immune responses in young children in developing countries than in children in developed countries or in adults, efforts to improve vaccine immunogenicity and coverage are ongoing. Zinc supplementation is one of the approaches tested to increase vaccine-specific immune responses. It has been shown that zinc supplementation three weeks prior to vaccination causes significantly increased vaccine antibody responses [105]. Zinc treatment has also been shown to significantly increase CTB-specific CD4+ T cells in children in terms of IFN-γ production after vaccination with Dukoral [106]. Another approach was the short withdrawal of breastfeeding before vaccination in young children to prevent neutralization of the vaccine by breast milk antibodies. When breastfeeding was withheld for 3 hours before Dukoral vaccination, significantly stronger antibacterial antibody responses were observed in Bangladeshi children [105]. Another recent development is to include a mucosal adjuvant with oral vaccines [3]. Unfortunately, no licensed mucosal adjuvant is yet available. However, dmLT is one of the few candidate mucosal adjuvant currently tested with different oral vaccines [7]. Since dmLT can not only enhance immune responses to other antigens but also induce immune responses against itself it may also be used as a stand-alone ETEC vaccine (Table 1). The mode of action of dmLT as a mucosal adjuvant is summarized in Figure 6.

1.1.10.1 dmLT adjuvant

LT and CT have been shown to function as mucosal adjuvants capable of enhancing host immune responses specific to unrelated, co-administered antigens like tetanus toxoid, inactivated influenza virus, *Helicobacter pylori* and ETEC vaccine antigens in animal models [5, 6, 8, 107, 108]. However, the potent toxicity of wild-type LT and CT in humans has prevented their use as oral adjuvants for vaccines. Hence, to develop a safe but immunogenic adjuvant, Clements and coworkers have developed the dmLT molecule [88]. The mucosal adjuvant dmLT is a genetically modified form of LT (R192G/L211A) in which the arginine at amino acid position 192 has been replaced with glycine and the leucine at amino acid position 211 has been replaced with alanine. These two amino acid substitutions are located in known and putative proteolytic cleavage sites in the LTA subunit protein that are considered to be critical for activation of the secreted toxin molecules [88, 109].

Preclinical studies reported that dmLT induces significantly less intracellular cAMP production than native LT but still retains potent adjuvant activity [88]. Feeding up to 250 µg of dmLT did not cause any enterotoxicity in the mouse assay while ≥5 µg of native LT caused a lot of intestinal fluid secretion [88]. In addition, dmLT has also been shown to enhance parenteral and/or mucosal immune responses to bacterial and viral antigens after administrating dmLT adjuvanted vaccines orally, intradermally or sublingually in different animal models including vaccination with ETVAX in mice [5, 8, 110-112].

In Phase 1 and 2b challenge trials of the live-attenuated ETEC vaccine ACE527, individuals were immunized orally three times with ACE527 alone or with 25 µg of dmLT [94]. After 6-7 months of last immunization, the same individuals were challenged orally with ETEC strain H10407 to monitor for moderate to severe diarrhea (MSD). Interestingly, 66% of the individuals previously immunized with ACE527 plus dmLT were protected against MSD, which was significantly higher than the individuals receiving ACE527 alone (20%) [7, 113]. Addition of dmLT also increased the protective efficacy (58% *vs.* 3.7%) against diarrhea of any severity and also reduced the number (1 *vs.* 13) and volume of stools (30 g *vs.* 859 g) compared to individuals receiving ACE527 alone [7, 94, 113].

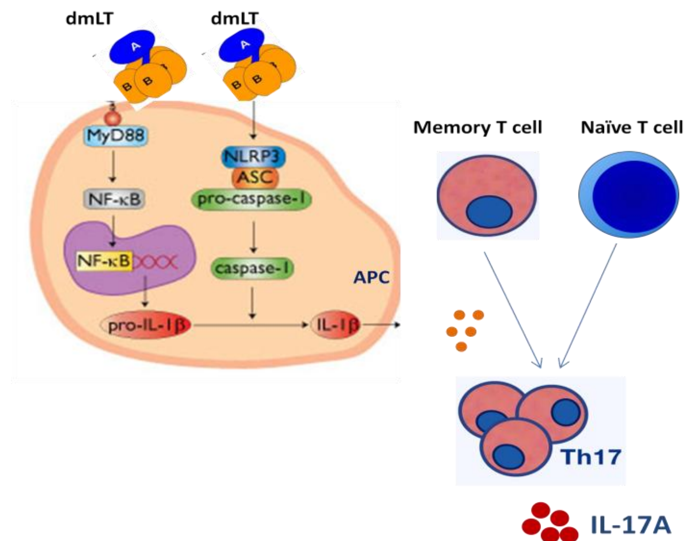

Figure 6. Proposed model for the mode of action of dmLT. The mucosal adjuvant dmLT acts on antigen presenting cells (APCs) and activates the enzyme caspase-I which is responsible for converting pro-IL-1β to mature IL-1β. IL-1β promotes Th17 differentiation and IL-17A production from both naïve and memory T cells and subsequently enhances IL-17A responses.

The mechanism by which dmLT function as a mucosal adjuvant is not yet well understood. However, most evidence suggests that the ability of LT to function as a mucosal adjuvant is related to its ability to induce cAMP [114]. DCs are the most potent APCs capable of stimulating naïve T cell differentiation and are a key target for adjuvants. LT has been shown to promote maturation of DCs and upregulation of the expression of co-stimulatory molecules such as CD80 in macrophages [115, 116]. In addition, LT synergized with LPS to induce IL-1β and IL-23 secretion by DCs, which in turn promoted antigen-specific IL-17A and IFN-γ production by CD4+ T cells in mice [117]. Several studies indicate that IL-1β, in combination with IL-6 and/or IL-23, is required for human Th17 differentiation [118]. Recently, Leach et al. investigated the effect of dmLT on adult T cell responses to different bacterial vaccine antigens [119]. dmLT enhanced the production of IL-17A by peripheral blood mononuclear cells (PBMCs) in response to the mycobacterial purified protein derivative (PPD), LTB and *Streptococcus pneumoniae* whole cell vaccine antigens in individuals previously vaccinated with Bacille Calmette Guerin (BCG), an oral ETEC vaccine or naturally exposed to pneumococci, respectively [119]. Based on these findings Larena et al. showed that dmLT acts on APCs and promotes Th17 responses via cAMP-dependent protein kinase A and caspase-1/inflammasome–dependent IL-1 signaling [120].

The immune cells including APCs and lymphocytes in children and infants are not mature and may function differently in varying degrees compared to cells from adults [121, 122]. PBMCs from infants may produce higher levels of IL-1β than cells from adults after stimulation with bacterial antigens [123]. The adjuvant effect of dmLT in the young age groups may also be different in terms of cAMP production, caspase-1/inflammasome–dependent IL-1 signaling pathway or cytokine production [124, 125]. However, no studies have been done yet to evaluate the adjuvant effect of dmLT in immune cells from children, although children are the main target group for most mucosal vaccines. Therefore, in this dissertation, the adjuvant effect of dmLT on immune cells from infants has been evaluated and the responses have been compared with those recorded in adults.

1.1.11 Available methods to measure the immunogenicity of oral vaccines

To measure vaccine specific immunogenicity, the identification of appropriate markers that can correlate with mucosal protection is crucial. Most studies of immune responses to ETEC natural infection and vaccination have also included analysis of serum/plasma antibody responses, although these do not always appropriately reflect mucosal responses. More direct evaluation of intestinal immune responses includes measurement of SIgA responses in fecal specimens or intestinal lavage samples. Since results from fecal and lavage analysis correlate significantly, fecal extracts have been considered more practical for assessing intestinal immune responses [10]. However, even fecal samples are unpractical, since they need extraction and may contain variable amounts of SIgA.

The most commonly used surrogate maker for intestine-derived responses is determination of ASCs. After infection or oral vaccination, activated intestinal lymphocytes transiently migrate to the circulation before homing back to the mucosa [13, 14, 50, 126]. Therefore, ASCs present among PBMCs about a week after mucosal vaccination are considered suitable surrogate markers of mucosal immunity. Furthermore, analyses of vaccine-specific ASCs after Dukoral vaccination show comparable responses in blood and mucosa [13]. A majority of the ASCs induced by oral vaccination has been shown to express the mucosal homing receptor integrin α4β7 [13, 14, 16]

ASC responses have traditionally been analyzed by the ELISPOT method, where PBMCs are cultured in antigen-coated wells and specific antibody secreting B-lymphocytes are detected as “spots” on the nylon membrane [11, 16, 86]. Alternatively, PBMCs can be cultured *in vitro* in plastic wells without antigen and the spontaneously secreted antibodies in the culture supernatant can be assayed for specific antibodies, commonly by an ELISA method. The analysis of antibodies in culture supernatant is known as the ALS method (antibodies in lymphocyte supernatants). Several studies have shown that results obtained from ELISPOT and ALS assays correlate very well after mucosal infection as well as vaccination [15, 89, 127, 128]. The ALS assay has many advantages over ELISPOT, especially in clinical trials, since it is much less laborious and the samples can be stored and transported, which makes it convenient to analyze pre- and post-vaccination samples in the same tests and reanalysis of stored samples is also possible. Moreover, evaluation of ASC responses by ELISPOT, either done by manual spot counting under a microscope or by using automated spot counters, are often less robust and objective than an ELISA readout. The two methods also give slightly different readouts; ELISPOT measures numbers of ASCs whereas ALS measures total levels of secreted antibodies.

In clinical trials with multivalent vaccines such as ETVAX, ALS responses to several vaccine antigens are usually evaluated, requiring relatively large volumes of ALS specimens. Hence, to allow analysis of ALS responses to multiple vaccine antigens in children, sensitive and specific procedures are required. An electrochemiluminescence (ECL) assay may be used as an alternative to conventional colorimetric ELISA methods to detect ALS responses. ECL-based techniques generally have high sensitivity, good reproducibility, and a wide dynamic analysis range reducing the need for sample titration, and often require smaller sample volumes than ELISA [129-131]. In this dissertation, an ECL assay has been established using small ALS volumes to measure IgA ALS responses against multiple antigens after ETEC vaccination.

1.1.12 Importance of measuring immune responses at optimal time points

Kinetics, magnitude, and duration of immune responses induced by vaccines or natural infection may be influenced by several factors such as age, nutritional status and previous exposure with the same or related microorganisms. As previously mentioned, most immune cells in newborn infants including APCs and lymphocytes are immature or function differently in varying degree compared to adults [121, 122]. Follicular dendritic cells (FDC) develop slowly after birth, which delays germinal center (GC) formation as well as the development of memory B cells. Since GC-derived plasma cell responses are also limited, lower antibody titers were observed in infants after several types of vaccinations [132]. However, it is still unclear how age and natural exposure to infections may influence the kinetics of ASC responses to oral vaccines.

Recent studies in adult Swedish volunteers have clearly demonstrated that the kinetics of circulating IgA as well as IgG ASC/ALS responses differs considerably after the first compared to the second dose or late booster vaccination with the oral cholera vaccine Dukoral [133]. Similar findings have also been obtained in recent studies of ETEC vaccines in Sweden [6, 90]. These results suggest that ASC responses may have been underestimated in several previous studies of oral vaccines in both endemic and nonendemic countries [12, 83, 84, 134-136]. Hence, it is very important to optimize the immunoassays and to identify optimal sampling time points to measure vaccine immunogenicity before initiating ETEC clinical vaccine trials in children and infants in ETEC endemic areas.

In this dissertation, different sampling time points have been investigated for evaluating immune responses after oral vaccination in different age groups in Bangladesh. The time kinetic study was performed with the licensed cholera vaccine Dukoral, to prepare for subsequent Phase I/II trials of ETVAX.

1.2 Aims

The overall aim of this PhD dissertation was to analyze mucosal immune responses induced by the oral inactivated ETEC vaccine ETVAX in Bangladeshi adults and the influence of age on vaccine-induced immune responses.

The specific aims were:

I. To identify optimal time points for evaluation of ALS and fecal IgA responses after oral vaccination in adults, children and infants in Bangladesh, using the licensed oral cholera vaccine Dukoral as a model vaccine.

II. To establish a sensitive ECL method for analysis of ALS IgA responses against multiple ETEC antigens in the ETVAX vaccine using small sample volumes.

III. To evaluate the safety and immunogenicity of the inactivated oral ETEC vaccine ETVAX administered with and without dmLT adjuvant in Bangladeshi adults as a preparation for further studies in children and infants.

IV. To investigate if the dmLT adjuvant can enhance IL-17A production from T cells in infants and to compare the adjuvant effects on immune cells from adults and infants.

2. MATERIALS AND METHODS

2.1 Study site

All studies in this dissertation were carried out in the Mirpur field site in Dhaka, Bangladesh. The Mirpur site was selected since this area is densely populated with a high diarrheal disease burden [25]. In addition to that, Mirpur is a very well studied field site where large numbers of surveillance studies on diarrheal diseases and vaccination trials have been carried out over the last 20 years. The studies were approved by the research review and ethical review committees of the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b).

2.2 Vaccines and adjuvant

To determine optimal sampling time points for immunogenicity measurements after oral vaccination, participants received two oral doses of the cholera vaccine Dukoral® (Valneva, Sweden) 14 days apart. The vaccine consists of 1.25×10^{11} killed *V. cholerae* bacteria and 1 mg of recombinant CTB (rCTB). A full dose of the vaccine was suspended in 150 mL (adults), 75 mL (toddlers) or 15 mL (young children and infants) carbonate buffer (Recipharm, Sweden) (Figure 7A). In this study Dukoral was termed as a 'model' vaccine since it is a licensed vaccine which is used globally and also contains the toxoid subunit CTB which is antigenically very similar to the LTB subunit of ETEC [4]. Dukoral also gives protection to LT+ ETEC infection [92].

To evaluate the safety and immunogenicity of the 2nd generation multivalent ETEC vaccine candidate in adults, two doses of ETVAX (produced for Scandinavian Biopharma by Biovian Oy, Tykistökatu 6B FI-20520 Turku, Finland, lot BX1003574) vaccine alone and together with dmLT adjuvant were given orally to study participants at a two weeks interval. The vaccine consists of 8×10^{10} inactivated *E. coli* bacteria of four different strains (ETEX 21–24, 2×10^{10} bacteria/strain), recombinantly induced to over-express the major ETEC CFs CFA/I, CS3, CS5, and CS6 respectively, and mixed with 1mg of LCTB*A* protein (Figure 7B). LCTB*A* is a recombinantly produced LTB/CTB hybrid protein [6, 8, 137]. A full vaccine dose was suspended in 150 mL bicarbonate buffer (Recipharm, Sweden) and given alone or together with 10 µg of dmLT (lot 1575, Walter Reed Army Institute, Silver Spring,

MD, USA) [88]. Participants were not allowed to eat or drink one hour before and after any of the vaccinations.

2.3 Study participants

Healthy adults aged 18-42 years (n=99), toddlers aged 2-5 years (n=19), young children aged 12-17 months (n=19) and infants aged 6-11 months (n=22) were enrolled in this study (Table 2). All participants were from similar socio-economic conditions. Informed written consent was obtained from each adult participant and for children from their parent/guardian before enrollment. Before enrollment, study physicians assessed the general health of the participants. Participants were excluded if there was any history of gastrointestinal disorders, diarrheal illness during the last two weeks, febrile illness in the preceding week or antibiotic treatment within one week prior to enrollment. In the clinical trials, participants who had been vaccinated with any cholera or ETEC vaccine previously were also excluded.

To enable measurement of optimal immune responses after oral vaccination, a time kinetic study was performed. In this study, 40 adults, 19 toddlers, 19 young children and 9 infants were enrolled and divided into 4 groups based on the sampling time points (Table 2). For the Phase I/II clinical trial of ETVAX, 135 adult participants were screened 4 to 7 days prior to enrollment for eligibility based on medical history, clinical examination and laboratory tests. Participants positive for ETEC, *Shigella*, *Vibrio cholerae* or *Salmonella* as determined by culture of a fecal sample collected during the screening period were excluded. From the 135 participants, 45 healthy volunteers were enrolled and randomized in a double blinded manner into one of the three cohorts: (A) placebo (n=15, buffer alone), (B) ETVAX vaccine (n=15) or (C) ETVAX with 10 µg dmLT adjuvant (n=15). To evaluate the *in vitro* adjuvant effect of dmLT, 14 adults and 13 infants were enrolled.

Table 2. Demographic characteristics of participants in different components of the studies

Type of study	Participants	Age		Gender[c]
		Median	Range	
Immunogenicity studies of Dukoral vaccine in different age groups: open, un-blinded	Adults (n=40)	29.5[a]	18.0-42.0[a]	29 (72%)
	Toddlers (n=19)	3.2[a]	2.0-4.4[a]	6 (32%)
	Young children (n=19)	14.0[b]	12.0-17.0[b]	8 (42%)
	Infants (n=9)	7.0[b]	6.0-11.0[b]	3 (33%)
Clinical trial of ETVAX in adults: randomized, double-blinded, placebo-controlled	Adults (n=45)	29.0[a]	19.0-44.0[a]	29 (64%)
In vitro study of the effect of dmLT adjuvant on immune responses in different age groups	Adults (n=14)	28.5[a]	18.0-35.0[a]	8 (57%)
	Infants (n=13)	10.0[b]	6.0-11.0[b]	7 (54%)

[a]Age in years
[b]Age in months
[c]Number and frequencies of females

2.4 Follow-up for adverse reactions

In the kinetics study, participants and parents/guardians were asked verbally if any adverse events that might be related to vaccination, including loose stools, nausea, vomiting and fever, had occurred during the study period. For the Phase I/II clinical trial, adverse events and serious adverse events were assessed by regular home visits, clinical chemistry and hematology tests and physical examinations. Participants were given memory aids to record solicited symptoms.

2.5 Specimen collection and processing

To determine the kinetics of immune responses after oral cholera vaccination, heparinized venous blood and fecal specimens were collected from each enrolled participant at three different time points; before immunization (day 0) and at two additional time points (day 4 or 5 and day 7), either after the first or the second vaccine dose (Figure 7A). For the clinical trial of the ETVAX vaccine, heparinized venous blood and stool specimens were obtained at 7 to 4 days prior to the first immunization and on day 7(±1) after the first dose and on day 19 (5 days after the 2nd dose, Figure 7B). To evaluate the *in vitro* effect of dmLT adjuvant, blood samples were collected once on the day of the enrollment. PBMCs and plasma were separated

from heparinized venous blood by density-gradient centrifugation using Ficoll-Isopaque (Pharmacia, Sweden). Plasma was stored at -20 °C. Cells were used for the ALS assay or different stimulation experiments.

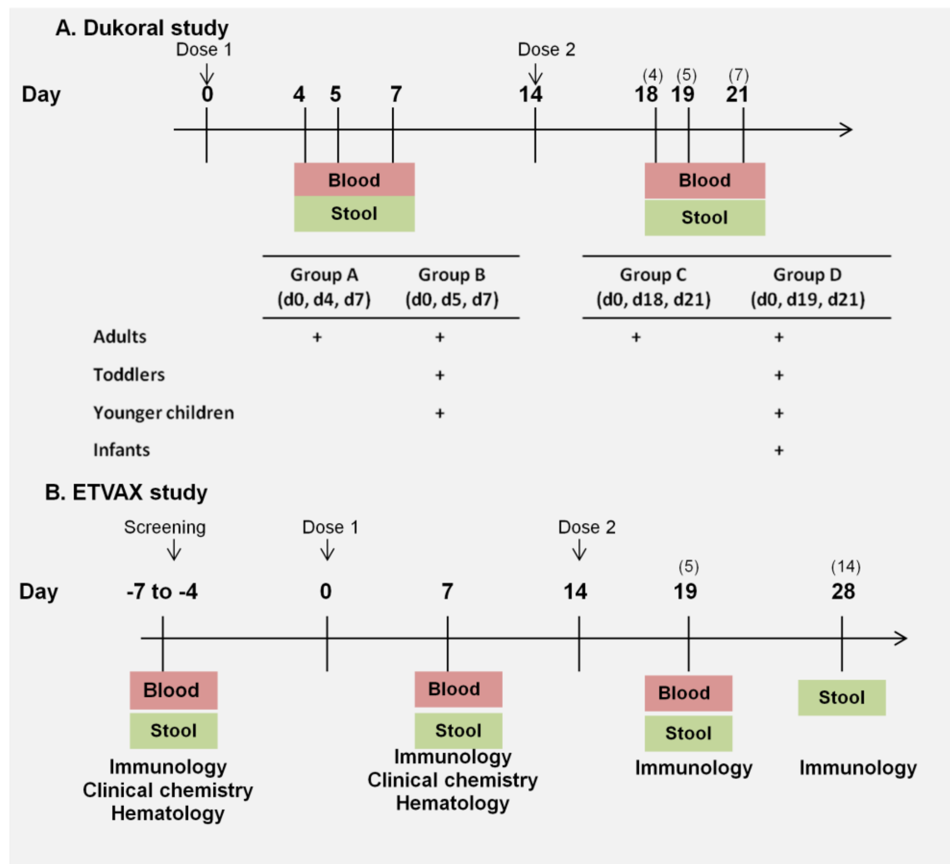

Figure 7. Vaccination and specimen collection time points. (A) Vaccination and sampling time points of participants (group A-D) receiving the Dukoral vaccine. (B) Screening, vaccination and sampling time points of participants receiving ETVAX with or without dmLT adjuvant or placebo. Numbers in brackets indicate days after the second immunization.

After collection, fecal specimens were immediately stored at -70 °C. For extractions, two gram of thawed fecal specimens were mixed with 8 mL of an extraction solution containing Soybean Trypsin Inhibitor (5 mg/mL; Sigma), EDTA (0.5 M; Sigma), Pefablock (35 mg/mL; Roche) dissolved in phosphate buffered saline (PBS) supplemented with 0.05% Tween 20. The mixture was stored at room temperature for 15 min. After centrifugation at 20,000 × *g* for 30 min, the extracts were collected and supplemented with bovine serum albumin (BSA) (final concentration, 0.1%, w/v) and sodium azide (NaN_3) (final concentration, 0.02%) and frozen in aliquots at -70 °C until they were assayed for total and specific SIgA antibody content.

2.6 Immunological analyses

2.6.1 Antigens, polyclonal stimuli and adjuvant

In this dissertation, different antigens have been used to determine specific antibody responses in plasma and ALS specimens. In the kinetics study, recombinant CTB and a membrane preparation (MP) from a *V. cholerae* O1 strain were used to measure antibody titers in plasma, ALS and fecal extracts. In the ETEC vaccine trial, immune responses were measured in plasma and ALS specimens against the different primary ETEC vaccine antigens (CFA/I, CS3, CS5, CS6 and LTB). All these antigens were generous gifts from Professor Ann-Mari Svennerholm, Dept. of Microbiology and Immunology, University of Gothenburg, Sweden and from Scandinavian Biopharma, Solna, Sweden as well as National Institute of Health, USA.

To evaluate the dmLT adjuvant effect, PBMCs were stimulated with combinations of antigens and toxins or toxin derivatives. The following polyclonal stimuli and antigens were used: Staphylococcal enterotoxin B (SEB; Sigma-Aldrich), phytohaemagglutinin (PHA, Remel, USA), LPS (Sigma) and a whole cell vaccine component corresponding to the composition of ETVAX (Scandinavian Biopharma, Etec970). The toxins/toxin derivatives used were dmLT (Scandinavian Biopharma) and LTB.

2.6.2 Mucosal immune response measurements

Mucosal immune responses were evaluated by measuring intestine-derived ASCs responses in ALS specimens and intestinal SIgA responses in fecal extracts.

2.6.2.1 ALS assay

For the ALS assay, PBMCs were resuspended at 10^7 cells/mL in RPMI complete medium supplemented with 10% fetal bovine serum (FBS), 1% Na-pyruvate (final concentration 1mM), 1% L-glutamine (final concentration 2 mM), 1% penicillin-streptomycin (final concentration 100 IU/mL pencillin and 0.1 mg/mL streptomycin) and cultured in 96-well flat-bottomed tissue culture plates. Cells were incubated at 37 °C in a 5% CO_2 incubator and supernatants were collected after 48 h of incubation. A protease inhibitor cocktail solution (0.2 μM AEBSF, 1 μg/mL aprotinin, 10 μM leupeptin hemisulfate, 1 mg/mL Na-azide) was added to the supernatant (10 μL/mL of

supernatant) and sample aliquots were immediately frozen at -70 °C for specific antibody detection using ELISA or electrochemiluminescence assays.

2.6.2.2 Determination of ALS IgA responses by ELISA

To measure CTB/LTB specific IgA antibodies in ALS specimens, 96-well ELISA plates (Nunc F) were coated with GM1 ganglioside (0.3 nmol/mL) overnight at room temperature followed by recombinant CTB/LTB subunit (1 µg/mL). To determine cholera MP specific responses, plates (Nunc F) were coated with *Vibrio cholerae* MP (3 µg/mL). To determine CF specific IgA antibody responses, high binding 96-well ELISA plates (Greiner) were coated with CFA/I (0.5 µg/mL), CS3 (0.3 µg/mL), CS5 (0.5 µg/mL) or CS6 (0.5 µg/mL). Eighty µL of ALS specimens were added to each well (initially diluted in 0.1% BSA in PBS- 0.05% Tween; 1:4 for LTB/CTB and 1:2 for cholera MP and CFs) and a serially 3-fold (LTB/CTB) or 2-fold (CFs) dilution was performed. The presence of antigen-specific antibodies was detected using horseradish peroxidase (HRP)-conjugated anti-human IgA (Jackson ImmunoResearch; 1:1500 dilution in 0.1% BSA-PBS-Tween) and ortho phenylenediamine (Sigma) in 0.1 M sodium citrate buffer (pH 4.5) and 30% hydrogen peroxide (Merck). The reactions were stopped after 20 minutes by adding 1 M H_2SO_4 and endpoint titers were determined as the reciprocal interpolated dilutions of the samples at 492 nm that were 0.2 above background.

2.6.2.3 Determination of ALS IgA responses by ECL

IgA antibody levels in ALS specimens were also analyzed by a novel ECL assay, established at the University of Gothenburg, for all five primary vaccine antigens (CFA/I, CS3, CS5, CS6 and LTB). Standard binding MULTI ARRAY 96-well plates (MSD) were coated with 0.5 µg/mL of each antigen in carbonate buffer (pH 9.8) at +4°C overnight, after 10 minutes shaking at room temperature. Plates were blocked (1% casein in PBS, Thermo Fisher, 1 h) and ALS samples (diluted 1 to 5 in 1% casein-PBS) were added (25 µL/well) for 2 h. IgA antibodies were detected using anti-human/non-human primate (NHP) IgA Sulfo Tag antibodies (1 µg/mL, MSD, 2 h). Plates were washed after each step using 0.05% Tween PBS and incubations were performed at room temperature with shaking. Finally, buffer (Read Buffer T; 1× concentration, MSD) was added and reactions were immediately analyzed on a Meso Quickplex SQ 120 reader (MSD).

2.6.2.4 Determination of fecal total and specific SIgA responses by ELISA

Total SIgA contents were determined in all fecal extracts using a human colostrum IgA reference (Sigma) as a standard in an ELISA test. Antigen specific SIgA were measured from those participants whose fecal specimens contained 10 to 1000 µg/mL of total SIgA and also when the concentration of total SIgA varied <3-fold between the different time points. In the kinetics study, ELISA plates were coated with GM1 ganglioside (0.3 nmol/mL) overnight followed by recombinant CTB subunit (1 µg/mL) or coated with MP (3 µg/mL). Fecal extracts (100 µL/well, initial 1:2 dilution, serially 3-fold diluted) were added and incubated for 90 minutes. Monoclonal anti-secretory component (100 µL/well, diluted 1:5000; Sigma) was added followed by 100 µL/well of anti-mouse IgG1-HRP (diluted 1:2000; Southern Biotech) and finally plates were developed by adding ortho phenylenediamine in 0.1 M sodium citrate buffer (pH 4.5) and hydrogen peroxide. After 20 minutes, titers were determined as the reciprocal interpolated dilutions of the samples at 450 nm that were 0.4 above background. Antibody levels were expressed as the specific SIgA titer divided by the total SIgA concentration of each sample.

2.6.3 Determination of plasma antibody responses by ELISA

Plasma IgA and IgG responses to vaccine antigens were measured to evaluate systemic immune responses after immunization with Dukoral or ETVAX vaccines. ELISA plates (Nunc F) were coated with GM1 ganglioside (0.3 nmol/mL) overnight at room temperature followed by recombinant CTB/LTB subunit (0.5 µg/mL). To determine *Vibrio cholerae* MP specific responses, plates (Nunc F) were coated with cholera MP (3 µg/mL). To determine CF specific IgA antibody responses, high binding 96-well ELISA plates (Greiner) were coated with CFA/I (0.5 µg/mL), CS3 (0.3 µg/mL), CS5 (0.5 µg/mL) or CS6 (0.5 µg/mL). An aliquot of 100 µL of plasma specimen was added to each well (initially diluted 1:10 in BSA-PBS- 0.05% Tween and a serially 3-fold dilution was performed). The presence of antigen-specific antibodies was detected using HRP-conjugated anti-human IgA (Jackson ImmunoResearch; 1:1500 dilution in 0.1% BSA-PBS-Tween) and ortho phenylenediamine in 0.1 M sodium citrate buffer (pH 4.5) and 30% hydrogen peroxide. The reactions were stopped after 20 minutes by adding 1 M H_2SO_4 and endpoint titers were determined as the reciprocal interpolated dilutions of the samples at 492 nm that were 0.4 above background.

2.6.4 Cell stimulation experiments

For analysis of adjuvant effects of dmLT in different age groups, PBMCs were cultured in DMEM F12 medium (200 µL/well) supplemented with 50 mg/mL gentamicin and 5% human AB^+ serum at 37 °C in a 5% CO_2 incubator. PBMCs (10^5 cells/well) were cultured in duplicate wells in U-bottomed 96-well plates. Cells were left untreated or stimulated alone with SEB (1 ng/mL or 10 ng/mL), PHA (1 µg/mL), or a whole cell component of ETVAX vaccine alone or in combination with increasing concentrations (1 µg/mL or 10 µg/mL) of dmLT or LTB. After 72 h of stimulation, supernatants were collected for cytokine (IL-17A) analysis by ELISA. For inhibition of IL-1 signaling, 1 µg/mL IL-1RA (R&D Systems) was added daily for the first 3 days to cell cultures. For determination of IL-1β production, PBMCs were left untreated or stimulated for 18 h with LPS (0.1-1000 ng/mL), dmLT (1 -50 µg/mL) and whole cell component of the ETVAX vaccine (2000-200,000 bacteria/mL). All supernatants were stored at -70 °C before cytokine analysis.

2.6.5 Analysis of cytokines in culture supernatants

The concentrations of IL-1β (from 18 h culture supernatants) and IL-17A (from 72 h culture supernatants) were determined using sandwich ELISA (eBioscience) following the manufacturers' instructions.

2.7 Statistical analyses

All antibody data were log10 transformed. Comparisons of pre- and post-immunization antibody levels within groups were evaluated using a paired t-test and responses between different groups using an unpaired t-test. Responder frequencies were evaluated using Fisher exact test. The magnitudes of immune responses (fold rises) were calculated as the post-immunization divided by pre-immunization antibody levels and twofold increases were regarded as responses [6]. The Pearson correlation coefficient was used to measure the correlations. Cytokine responses were evaluated using a paired t-test or unpaired t-test, as applicable. *P*-values <0.05 were considered as significant. All statistical analyses were performed with GraphPad Prism version 6.0.

3. RESULTS

3.1 Determination of optimal sampling time points

In this thesis the ETEC vaccine ETVAX has been tested in Bangladeshi adults to evaluate the safety and immunogenicity of the vaccine. After safety evaluations in adults, the study was then continued in children and subsequently in infants (Qadri et al, in manuscript). Before designing this large Phase I/II ETEC vaccine trial, the optimal sampling time points for immunogenicity analysis in different age groups were determined. In this study, the licensed cholera vaccine Dukoral, containing rCTB and killed *V. cholerae* O1 bacteria, was used as a model vaccine; two consecutive doses were given to adults, toddlers, young children and infants. ALS responses to CTB and a *V. cholerae* MP were evaluated to investigate the kinetics of IgA antibodies specific to toxin and bacterial cell antigens, including both protein and *V. cholerae* O1 LPS, respectively. The feasibility to measure vaccine specific SIgA antibody responses in fecal samples collected at different time points from the different age groups was also investigated.

3.1.1 Kinetics of ALS IgA responses against CTB

In adults, significant ALS responses to CTB were observed both after the first and the second vaccine dose (Figure 8A). After the first dose, only 10% of the participants responded on day 4, but on day 5, a majority (80%) had responded and the responses remained high on day 7. After the second dose, about half of the participants responded already on day 4 (study day 18). The highest ALS responses were detected on day 5 after the second dose (study day 19), when almost all participants had responded. Responses then declined significantly by day 7 (study day 21) after intake of the second dose. Similar kinetics of ALS IgA responses to CTB were observed in toddlers (Figure 8B). The majority (70%) of toddlers had responded on day 5–7 after the first dose and all participants responded on day 5 (study day 19) after the second dose with a significant decline on day 7 (study day 21).

In contrast to adults and toddlers, only a few young children responded in ALS specimens after the first vaccine dose (Figure 8C). However, after the second dose at day 5 (study day 19), all young children had responded. The responses then declined significantly on day 7 (study day 21). Although the ALS anti-CTB IgA response

magnitudes were relatively low in infants, a majority of infants responded significantly on day 5 after the second dose (study day 19, Figure 8D). Similar to the other age groups, responses tended to decline between day 5 (day19) and 7 (day 21) after the second dose.

3.1.2 Kinetics of plasma IgA and IgG responses

Adults, toddlers and young children developed significantly higher IgA responses in plasma 7 days after the first dose compared to on day 4 or 5 (Table 3). After the second dose, strong responses were observed already on day 4/5 and the responses were comparable or higher in magnitudes on day 7. Similar numbers of infants responded on both day 5 and 7 after the second dose though relatively higher magnitudes of responses were seen on day 7 compared to day 5 (Table 3). Similar kinetics was observed for anti-CTB IgG antibody responses in plasma (Table 4).

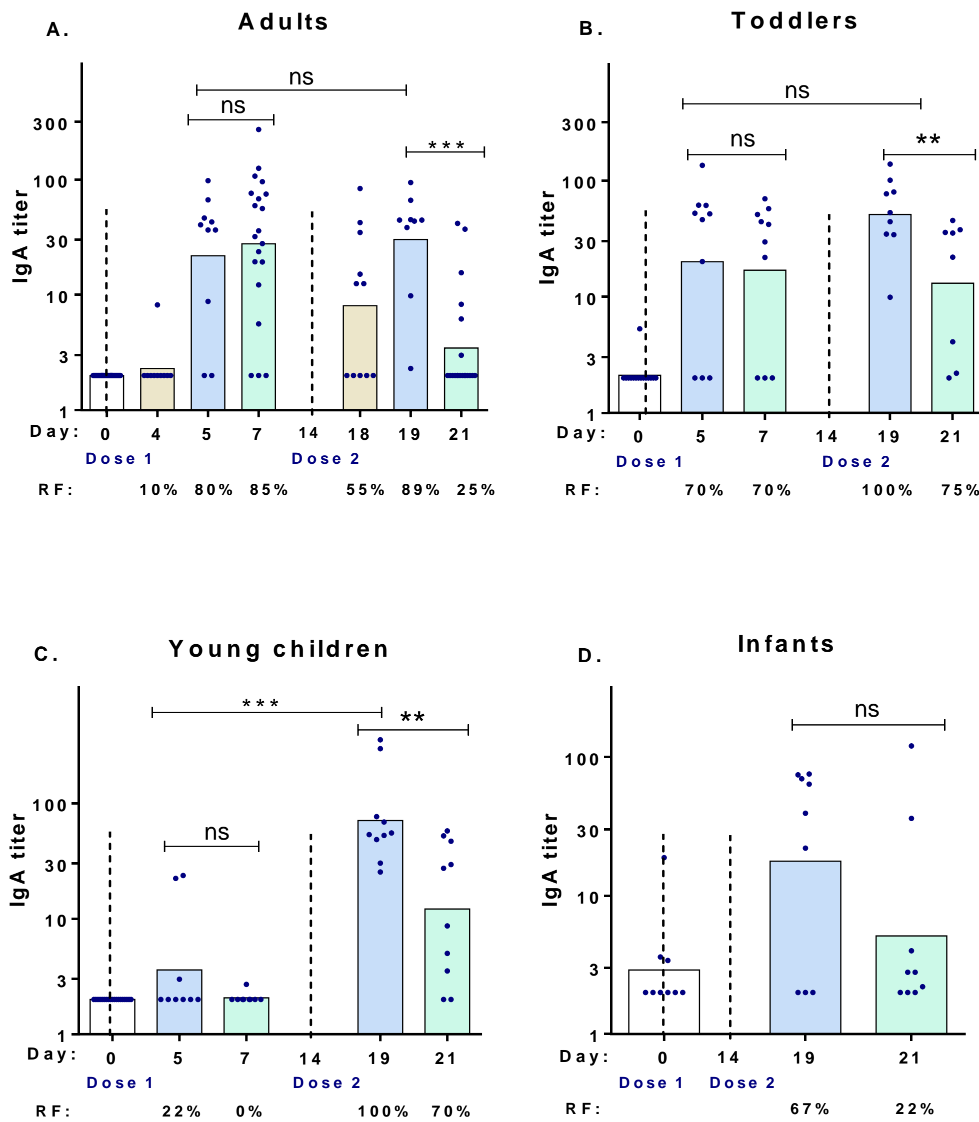

Figure 8. Kinetics of ALS IgA responses to CTB after oral Dukoral vaccination. CTB-specific IgA titers were analyzed in ALS samples from (A) adults (day 0; n=40, day 4/5; n=10, day 7; n=20, day 18; n=11, day 19; n=9, day 21; n=20), (B) toddlers (day 0; n=19, day 5; n=10, day 7; n=10, day 19; n=9, day 21; n=8), (C) young children (day 0; n=19, day 5/7; n=9, day 19/21; n=10) and (D) infants (n=9) before the first immunization (day 0) and at the indicated time points after the first and second dose. Responder frequencies (RF) on the different days are shown below the graphs. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns; not significant. Each round symbol indicates data from one participant and bars represent geometric mean titers.

Table 3. Magnitudes and responder frequencies of plasma IgA responses to CTB after vaccination with Dukoral

	Dose 1			Dose 2		
	Day 4	Day 5	Day 7	Day 4 (day 18)	Day 5 (day 19)	Day 7 (day 21)
Adults	1.2[a] (2/10, 20%)[b]	1.5 (3/10, 30%)	5.5 (15/20, 75%)	7.2 (10/11, 91%)	10.6 (8/9, 89%)	13.5 (19/20, 95%)
Toddlers	nd[c]	3.4 (8/10, 80%)	7.3 (10/10, 100%)	Nd	2.9 (9/9, 100%)	6.3 (8/8, 100%)
Young children	nd	2.9 (6/9, 67%)	6.3 (9/9, 100%)	Nd	22.0 (9/10, 90%)	40.0 (9/10, 90%)
Infants	nd	nd	nd	Nd	2.9 (7/9, 78%)	6.3 (7/9, 78%)

[a]Magnitudes of responses were expressed as geometric mean (GM) of fold rises.
[b]Response rates are shown in parentheses. Fold rises ≥2 were considered as responses.
[c]nd, not done

Table 4. Magnitudes and responder frequencies of plasma IgG responses to CTB after vaccination with Dukoral

	Dose 1			Dose 2		
	Day 4	Day 5	Day 7	Day 4 (day 18)	Day 5 (day 19)	Day 7 (day 21)
Adults	1.1[a] (1/10, 10%)[b]	1.8 (5/10, 50%)	4.1 (17/20, 85%)	3.5 (8/11, 73%)	3.9 (7/9, 78%)	4.8 (16/20, 80%)
Toddlers	nd[c]	1.8 (4/10, 40%)	3.9 (7/10, 70%)	Nd	2.9 (7/9, 78%)	4.5 (8/8, 100%)
Young children	nd	1.4 (1/9, 11%)	1.9 (3/9, 33%)	Nd	4.5 (9/10, 90%)	10.9 (9/10, 90%)
Infants	nd	nd	nd	Nd	4.5 (8/9, 89%)	4.4 (7/9, 78%)

[a]Magnitudes of responses were expressed as geometric mean (GM) of fold rises.
[b]Response rates are shown in parentheses. Fold rises ≥2 were considered as responses.
[c]nd, not done.

3.1.3 Fecal SIgA responses

The presence of vaccine antigen (CTB and *V. cholerae* MP) specific SIgA antibodies were also analyzed in fecal samples. Since the majority of stool samples from adults and toddlers contained low and variable levels of total SIgA level, specific SIgA were not analyzed further in these age groups. However, the fecal SIgA responses in young children and infants were evaluated, but only after intake of the second vaccine dose, since a majority of the total SIgA responses in young children and infants were low and infrequent after the first vaccine dose. The CTB and MP specific SIgA results from young children and infants were pooled and are shown in Figure 9.

After the second vaccine dose, anti-CTB and anti-MP SIgA responses were observed in about half of the participants on day 5 (study day 19, Figure 9). The responses increased further on day 7 (study day 21), when responses to CTB and MP were detected in 82% (9/11) and 58% (6/11) of participants, respectively, and the antibody levels in the whole group were significantly increased compared to pre-vaccination levels.

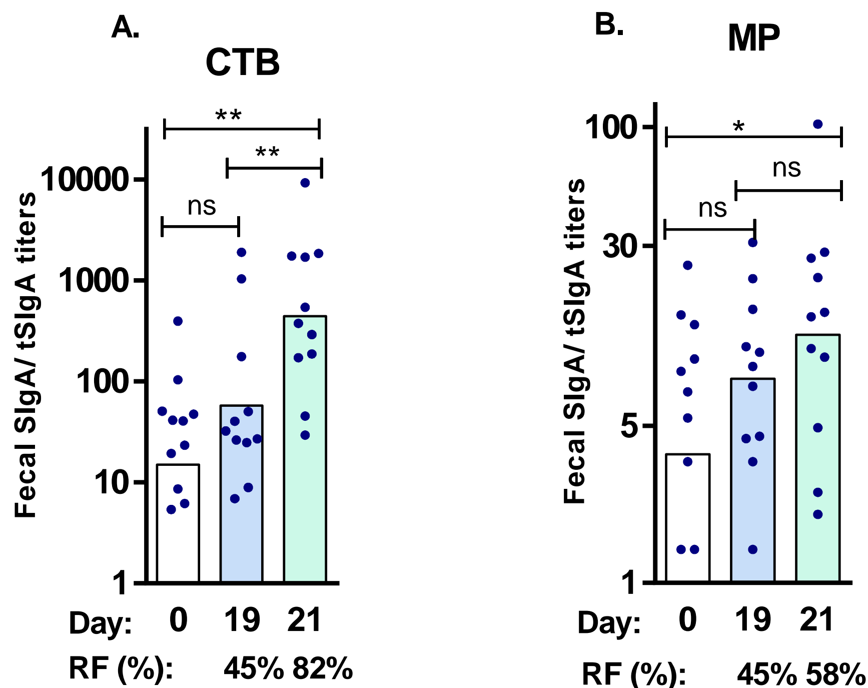

Figure 9. SIgA antibody responses in fecal extracts after oral Dukoral vaccination. SIgA responses (specific SIgA/total SIgA) against (A) CTB and (B) *Vibrio cholerae* MP were measured in young children (n=6) and infants (n=7) before immunization (day 0) and at the indicated time points after the second vaccine dose (total n=13). Responder frequencies (RF) are shown below the graphs. $^{*}P < 0.05$, $^{**}P < 0.01$, ns; not significant. Each round symbol indicates data from one participant and bars represent geometric mean titers.

Taken together, the results from the kinetic study show that the maximal ALS responses to the oral cholera vaccine can be detected 5 days after the second dose in all age groups living in a cholera and ETEC endemic country. This time point can also be used for assessment of plasma IgA and IgG responses to the vaccine. Moreover, vaccine specific responses can also be detected in fecal samples from infants and young children, but samples should be collected at least 7 days after vaccination to

detect significant responses. The identification of optimal time points for assessment of mucosal and systemic responses to oral vaccination in different age groups facilitated the design of the sampling schedule in a subsequent large Phase I/II trial of the ETEC vaccine ETVAX.

3.2 Evaluation of the safety and immunogenicity of ETVAX in adults

Based on the excellent safety and immunogenicity profile of the ETVAX vaccine observed in adult Swedes [6], the safety (primary endpoint) and immunogenicity (secondary endpoint) of ETVAX with and without dmLT adjuvant was evaluated in Bangladesh in a large Phase I/II trial in adults and subsequently in descending age groups (5 years to 6 months). The results in this dissertation describe the first part of this large clinical trial where the vaccine was tested in adults. In this initial part of the trial, a sensitive electrochemiluminiscence, ECL, method was established and optimized for sensitive analysis of ALS responses using small sample volumes of blood. Since only limited blood volumes as well as limited ALS samples can be obtained from children and infants, this sensitive ECL assay can facilitate analyses of ALS responses against multiple antigens in younger age groups in subsequent studies.

3.2.1 Safety analyses

Two oral doses of ETVAX administered alone or in combination with 10 µg dmLT were safe and well tolerated in all tested 30 Bangladeshi adults. No serious adverse events were reported at any time point during the study period. No significant differences in frequency or intensity of adverse events were observed among participants receiving placebo, vaccine or vaccine+dmLT (Table 5).

Table 5. Adverse events in study participants after receiving one or two doses of placebo, ETVAX or ETVAX plus dmLT

Adverse events	A) Placebo (n=15)	B) ETVAX (n=15)	C) ETVAX+dmLT (n=15)
Nausea	0	0	0
Vomiting	0	0	0
Diarrhea	0	0	0
Loose Stools	0	0	0
Abdominal Pain	0	0	0
Fever	1 (6.7%)[a]	0	1 (6.7%)[b]

[a]Mild fever appeared and resolved spontaneously on the same day of receiving placebo.
[b]Mild fever appeared two days after receiving the first vaccine dose and was resolved on the next day.

3.2.2 Correlations between the results from ECL and ELISA assays

Vaccine antigen specific IgA levels in ALS samples were measured in parallel by the traditional ELISA and the new ECL assay. The fold rises in antibody levels in post-compared to pre-vaccination samples were compared. High correlations (r=0.85 to 0.98, $P<0.001$) between the magnitudes of ALS responses determined by the ECL and ELISA assays were found for all five primary antigens (CFA/I, CS3, CS5, CS6 and LTB) using samples from all participants collected 5 days after the second dose (Figure 10). Magnitudes of responses to CFA/I and CS5 were 3 to 4-fold higher determined by ECL compared to ELISA, but still correlated very well, whereas the magnitudes of responses to CS3, CS6 and LTB were comparable in the two assays. The majority of placebo recipients had responses to all antigens below the 2-fold cut-off for positivity in both assays, but discrimination between vaccine and placebo recipients was slightly more distinct using the ECL assay (Figure 10).

Considering the excellent performance of the ECL assay and the relatively small volumes of ALS samples available from some participants, the ECL assay was selected as the primary readout for the ALS responses in the study.

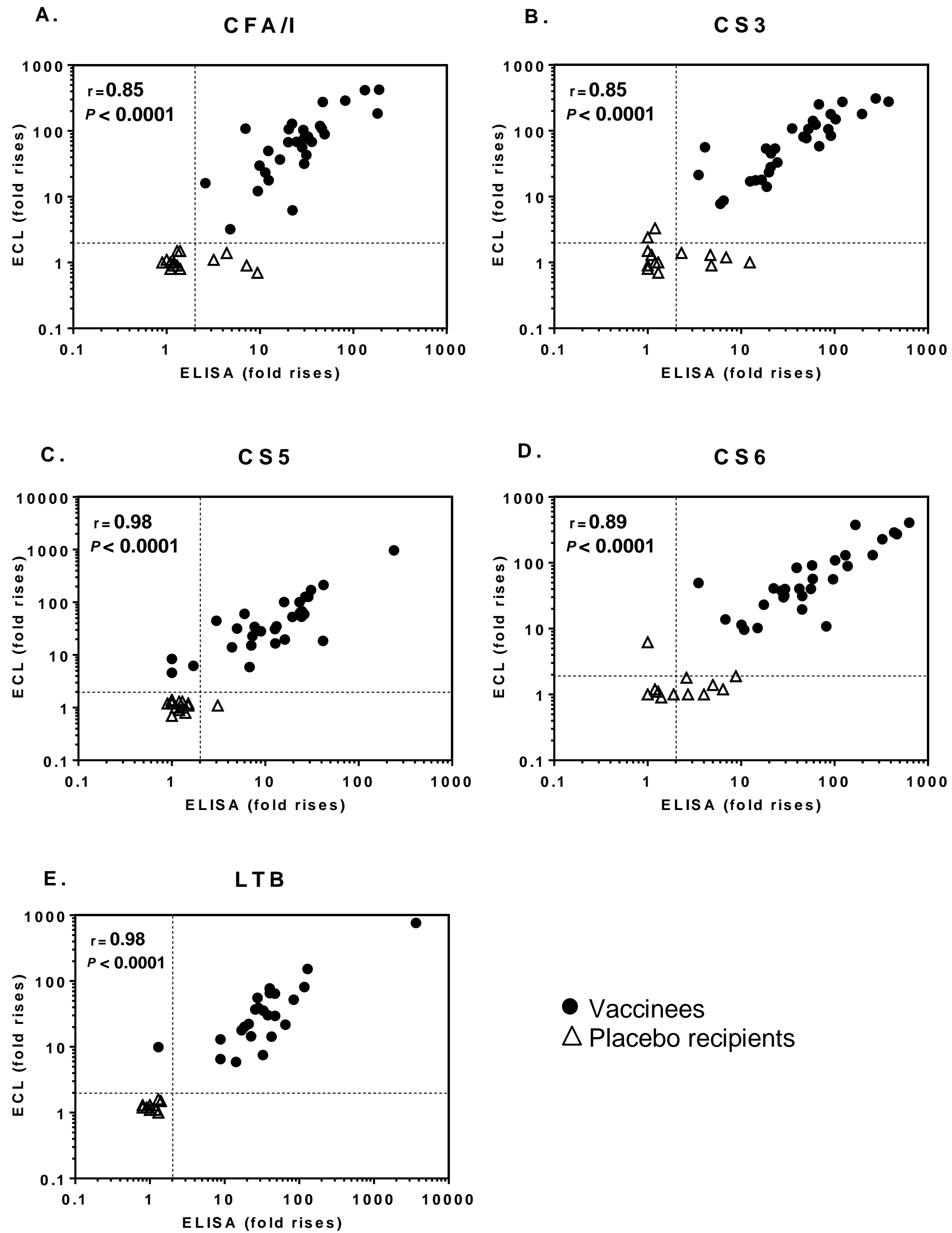

Figure 10. Comparison of ALS responses to ETVAX vaccination measured using a novel ECL and traditional ELISA assays. Magnitudes of responses (fold rises) of specific IgA antibody levels against (A) CFA/I, (B) CS3, (C) CS5, (D) CS6 and (E) LTB antigens were analyzed in day 19 ALS specimens (n=30 vaccinees and n=14 placebo recipients). Dashed lines indicate fold rises ≥2.

3.2.3 Immunogenicity analyses

To analyze the immunogenicity of the ETVAX vaccine in Bangladeshi adults, specific antibody responses were evaluated against vaccine CFs (CFA/I, CS3, CS5 and CS6) and LTB in ALS and plasma specimens using ECL and ELISA assays, respectively. Analysis of fecal samples for SIgA responses to the vaccine was carried out. However, consistent with the findings in kinetic study, a majority of stool samples from the adults contained low and variable levels of total SIgA, and did not meet the inclusion criteria for analysis. Therefore, analysis of CF and LTB specific antibody responses were not evaluated in fecal specimens in the adults.

3.2.3.1 ALS IgA responses to colonization factors

ETVAX alone or with dmLT adjuvant elicited significant increases of specific IgA responses in ALS against all four vaccine CFs (Figure 11). Analysis of ALS specimens using the ECL assay (Figure 11 A-D) showed that the magnitudes of responses were high already 7 days after the first vaccine dose compared to pre-immunization levels and responses remained at similar levels (anti-CS3, Figure 11B) or increased further 5 days (study day 19) after the second dose ($P<0.001$ when magnitudes of ALS responses on day 7 or day 19 versus pre vaccination levels were compared). Responder frequencies for antigen specific IgA ALS responses among the vaccinees were 87-100% already after the first dose and 100% after the second dose for all CFs (Table 6). In contrast, very few placebo recipients responded to any of the antigens, and then only to CS3 (4/14 subjects) and CS6 (1/14).

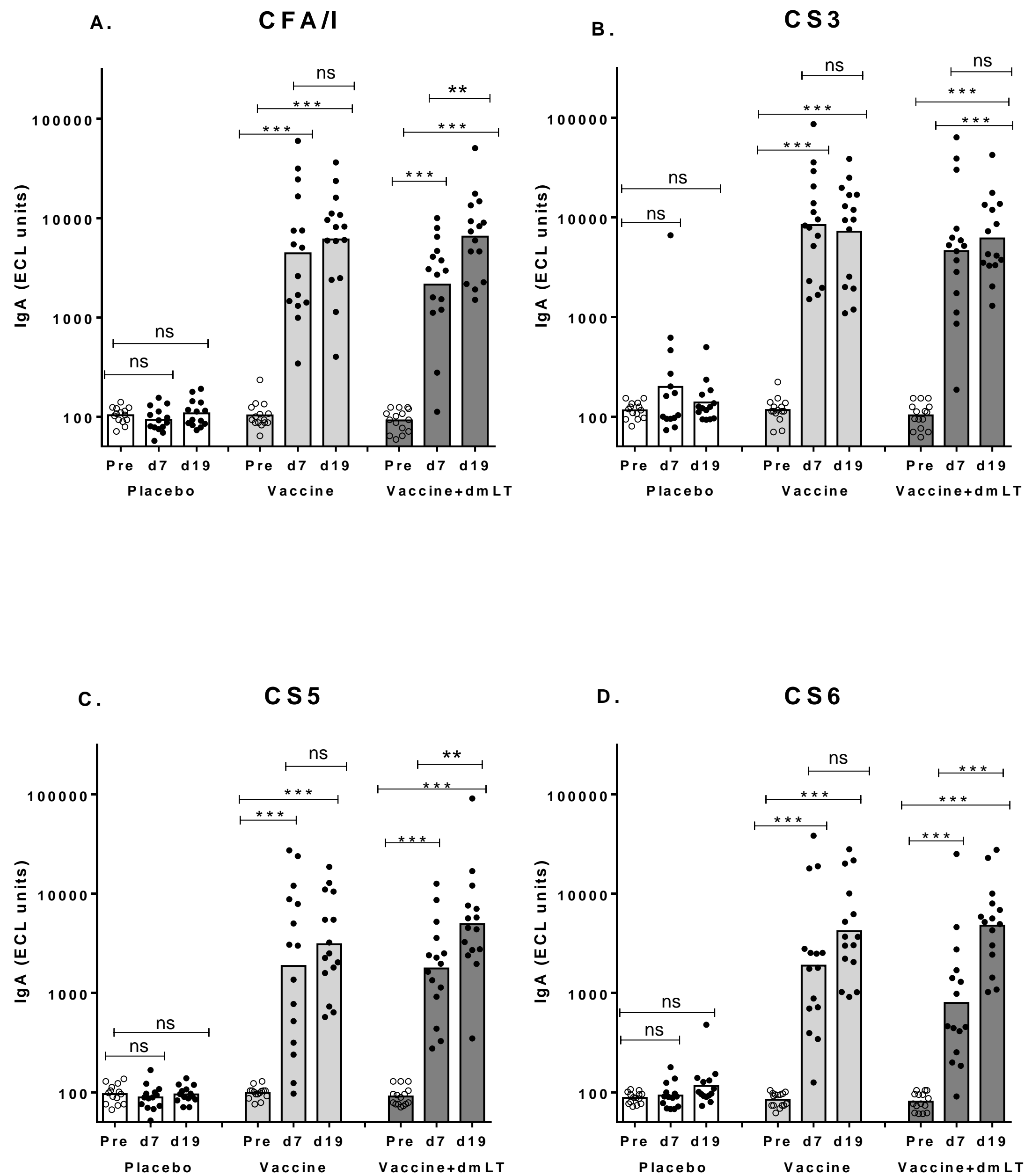

Figure 11. Colonization factors specific ALS IgA antibody responses measured by the ECL assay. IgA antibody responses against CFA/I, CS3, CS5 and CS6 antigens in (A-D) in ALS specimens in participants receiving placebo (n=13-14), ETVAX vaccine (n=13-15) and ETVAX vaccine plus dmLT (n=14-15). Each round symbol indicates data from one participant and bars represent geometric mean titers. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns; not significant.

3.2.3.2 ALS IgA responses to LTB

The LTB specific IgA responses in ALS were studied using the ECL assay (Figure 12). The vaccine alone or with dmLT adjuvant induced significantly increased levels of ALS IgA antibodies against LTB. The magnitudes of IgA responses were high (*P*<0.001) already 7 days after the first vaccine dose and responses remained at similar levels 5 days (study day 19) after the second dose (Table 6). Almost 100% of participants responded against LTB after either dose of the vaccine.

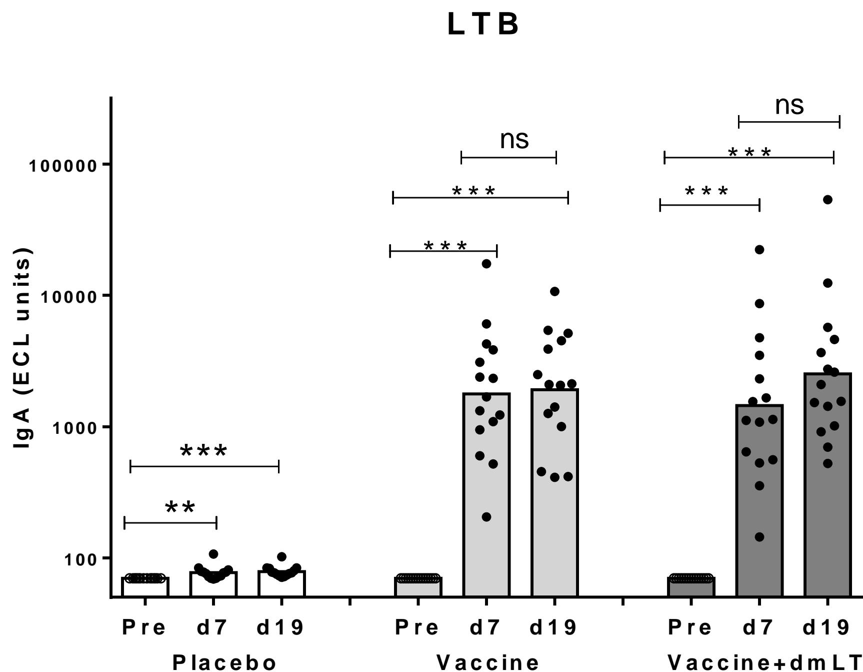

Figure 12. Toxin specific ALS IgA responses measured by ECL. IgA responses against LTB antigen in ALS specimens in participants receiving placebo (n=13-14), ETVAX vaccine (n=13-15) and ETVAX vaccine plus dmLT (n=14-15). Each round symbol indicates data from one participant and bars represent geometric mean titers. $^{**}P < 0.01$, $^{***}P < 0.001$, ns; not significant.

Table 6. Responder frequencies of ALS and plasma IgA responses against CFA/I, CS3, CS5, CS6 and LTB and plasma IgG responses against LTB determined by ECL (ALS) and ELISA (plasma) assays after administration of one and two treatment doses

	Placebo (n=14)		Vaccine[b] (n=15)		Vaccine+dmLT[b] (n=15)	
	Dose 1	**Dose 2**	**Dose 1**	**Dose 2**	**Dose 1**	**Dose 2**
ALS						
CFA/I	0[a]	0	100	100	93	100
CS3	29	14	100	100	93	100
CS5	0	0	87	100	100	100
CS6	0	7	93	100	87	100
LTB	0	0	100	100	100	100
Plasma						
CFA/I	0	0	54	73	73	80
CS3	15	21	87	93	93	86
CS5	0	0	64	50	67	80
CS6	0	0	46	62	67	73
LTB IgA	0	7	100	100	100	100
LTB IgG	7	14	73	93	87	87

[a]Fold rises ≥2 were considered as responses [6] and responder frequencies (%) using this cut-off are indicated.
[b]Responder frequencies were significantly higher ($P<0.001$) in the vaccine and vaccine+dmLT groups, respectively, compared to the placebo group, for all antigens.

3.2.3.3 Plasma IgA responses to colonization factors

The magnitudes of anti-CF IgA responses were lower in plasma than in ALS, when plasma ELISA titers were compared with ALS ECL units. Significantly increased plasma responses were found 7 days after the first dose ($P<0.001$, Figure 13) and the magnitudes of responses against all vaccine antigens remained at comparable levels 5 days after the second vaccination. Responder frequencies for plasma IgA responses among the vaccinees were 46-93% against all CFs already after the first dose and 50-93% after the second dose (Table 6). In contrast, very few placebo recipients responded to any of the antigens, and then only to CS3 (3/14 subjects).

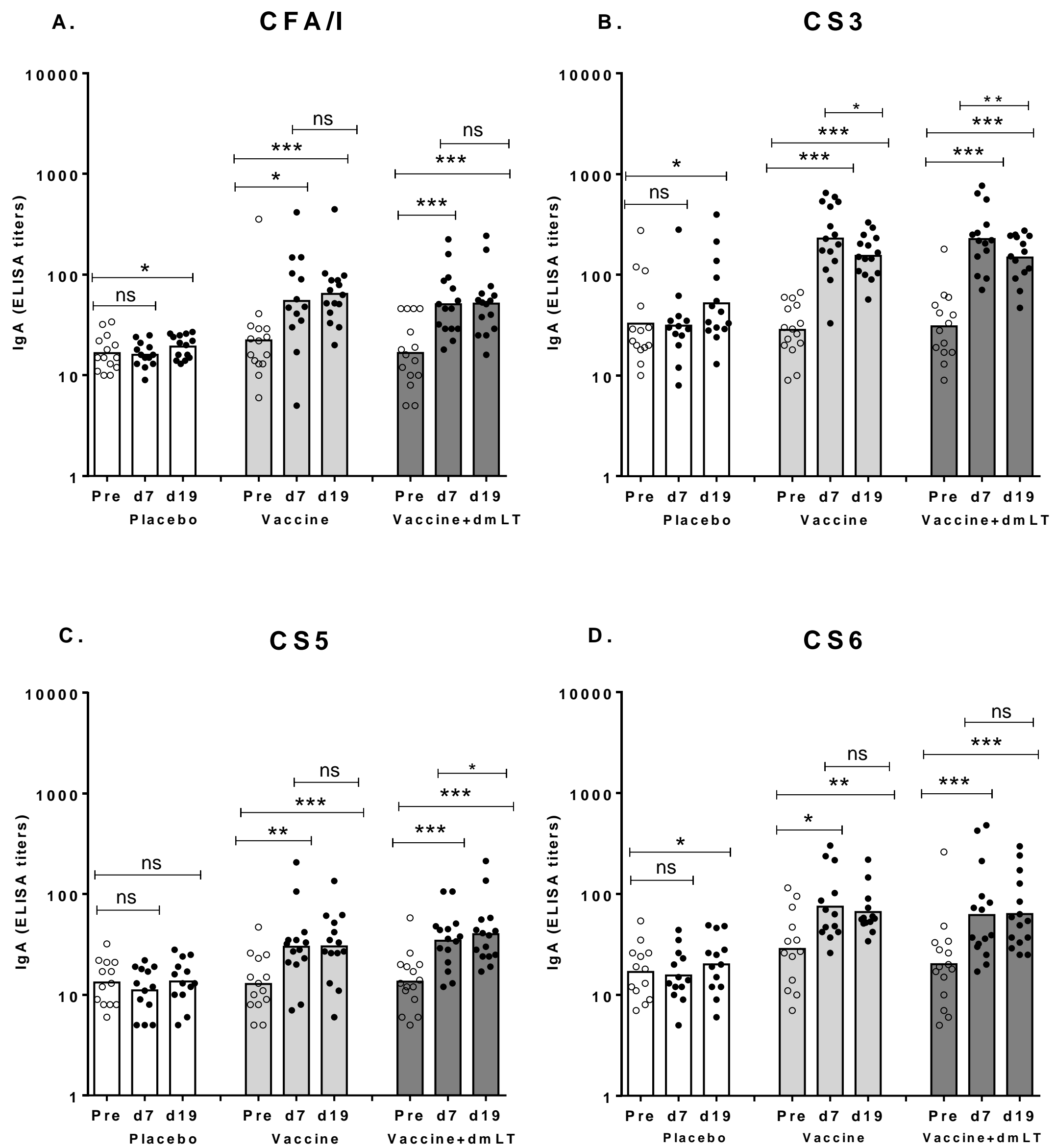

Figure 13. Colonizaton factors specific plasma IgA responses measured by ELISA. IgA antibody responses against CFA/I, CS3, CS5 and CS6 antigens (A-D) in plasma specimens in participants receiving placebo (n=13-14), ETVAX vaccine (n=13-15) and ETVAX vaccine plus dmLT (n=14-15). Each round symbol indicates data from one participant and bars represent geometric mean titers. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns; not significant.

3.2.3.4 Plasma IgA and IgG responses to LTB

The LTB specific IgA and IgG responses in plasma were measured using ELISA (Figure 14). The vaccine alone or with dmLT adjuvant induced significantly increased levels of plasma IgA and IgG antibodies against LTB. The magnitudes of IgA responses were high (P<0.001) already 7 days after the first vaccine dose and responses remained at similar levels 5 days (day 19) after the second dose.

Anti-LTB IgG responses in plasma followed the same pattern. Among the vaccinees 73-100% already responded to LTB after the first dose when both IgA and IgG responses were considered and 100% after the second dose (Table 6). In contrast, only 7-14% placebo recipients responded to LTB in plasma.

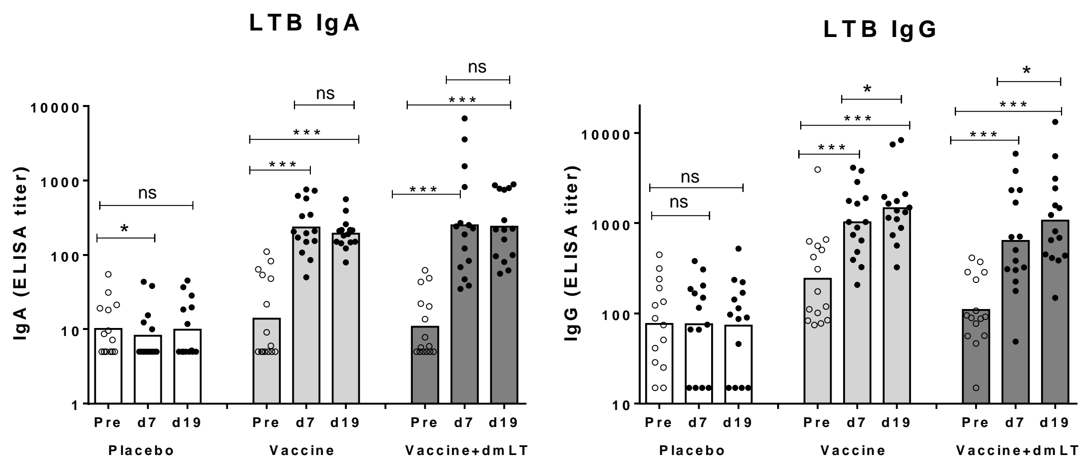

Figure 14. Toxin specific plasma IgA and IgG responses measured by ELISA. IgA and IgG responses against LTB antigen in plasma specimens in participants receiving placebo (n=14), ETVAX vaccine (n=15) and ETVAX vaccine plus dmLT (n=15). Each round symbol indicates data from one participant and bars represent geometric mean titers. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns; not significant.

3.2.3.5 Effect of dmLT adjuvant on the ETVAX immunogenicity

Addition of dmLT to the vaccine did not have any significant impact on the antibody responses in ALS. No significant differences in subjects receiving ETVAX with or without dmLT were observed in magnitudes or frequencies of IgA antibody responses against CFs (Figure 11) or LTB (Figure 12) in ALS (P>0.05). The magnitudes and frequencies of ALS responses were similar on both day 7 and day 19 in the vaccine compared to the vaccine+dmLT groups (Figure 11, 12 and Table 6).

In plasma, the responder frequencies were not significantly different (P>0.05) in the vaccine compared to the vaccine + dmLT group. However, there was a trend for higher (10-30%) responder frequencies against CFA/I (dose 1), CS5 (dose 2) and CS6 (both doses) in participants receiving ETVAX + dmLT compared to ETVAX alone (Table 6, P>0.05). A trend for higher antibody responses after addition of dmLT was also seen when plasma responses to all primary antigens were considered together; 57% of subjects immunized with ETVAX + dmLT and 38% with vaccine alone responded to all five antigens (Table 7).

Table 7. Frequencies of IgA responders against different numbers of primary vaccine antigens in plasma after one or two doses

Frequency of subjects responding to	**Placebo**	**Vaccine**[b]	**Vaccine+dmLT**[b,c]
5 antigens[a]	0/13 (0%)	5/13 (38%)	8/14 (57%)
≥4 antigens	0/13 (0%)	8/13 (62%)	13/14 (93%)
≥3 antigens	0/13 (0%)	12/13 (92%)	13/14 (93%)
≥2 antigens	1/13 (8%)	12/13 (92%)	13/14 (93%)
≥1 antigens	3/13 (20%)	13/13 (100%)	14/14 (100%)
0 antigen	10/13 (77%)	0/13 (0%)	0/14 (0%)

[a]LTB, CFA/I, CS3, CS5, CS6.
[b]Responder frequencies against ≥1 to 5 antigens were significantly higher (P<0.05) in the vaccine and vaccine+dmLT groups, respectively, compared to the placebo group.
[c]Responder frequencies were not significantly different (P>0.05) in the vaccine compared to the vaccine+dmLT group. However, including dmLT in the vaccine formulation appeared to favor a broader antigenic response than that achieved with the vaccine alone, particularly when plasma IgA response frequencies to ≥4 or 5 antigens were considered.

Overall, this Phase I trial demonstrated that the oral ETVAX vaccine is safe in Bangladeshi adults and induces strong mucosal as well as systemic immune responses against key vaccine antigens. The highly sensitive ECL assay established here allowed specific analysis of ALS responses to all key vaccine components when tested in small sample volumes. The mucosal adjuvant dmLT had no significant effect on ALS responses in adults, although a trend for improved breadth of plasma responses was observed. These findings provided a base to test this vaccine and dmLT adjuvant further in descending age groups in children and infants in Bangladesh.

3.3 Adjuvant effects of dmLT on immune cells from adults and infants

The mucosal adjuvant dmLT was tested with the oral ETVAX vaccine in adults in the Phase I study and continued studies in children and infants include different combinations of vaccine and dmLT dosages. It was therefore important to understand the potential role of the dmLT adjuvant in modulating vaccine specific responses and whether cells from infants and adults respond differently to dmLT and/or to the ETVAX vaccine. The immune enhancing effect of the dmLT adjuvant on PBMCs from both adults and infants in response to polyclonal stimuli (SEB and PHA) and a whole cell component of ETVAX were evaluated. The analysis of IL-17A responses were especially interesting, since this cytokine is known to be important for mucosal immune responses by influencing IgA antibody production and since this cytokine had previously been shown to be enhanced by dmLT via induction of IL-1β [119, 120].

3.3.1 Effect of dmLT on IL-17A production

To study how dmLT influences T cell responses in infants *vs*. adults, PBMCs were stimulated with dmLT in combination with the mitogen PHA or the superantigen SEB, and IL-17A was analyzed in culture supernatants by ELISA after 72 h of stimulation. In both adults and infants, SEB or PHA stimulation alone induced IL-17A responses, but the magnitudes of responses were lower in infants compared to adults. In contrast, stimulation with dmLT alone did not induce any detectable IL-17A production at any concentration tested (1-10 μg/mL; Figure 15A and B). Stimulation of cells with SEB in combination with increasing concentrations of dmLT resulted in

significant increases in IL-17A production compared to SEB alone in both adults and infants (Figure 15A and B). In adults, the production of IL-17A increased significantly in the presence of the lowest dmLT concentration tested (1 µg/mL, 2-fold mean increase in comparison to SEB alone) and addition of 10 µg/mL resulted in even higher IL-17A production in comparison to stimulation with SEB+dmLT 1 µg/mL (Figure 15A). PBMCs from adults also showed a similar enhanced IL-17A production when increasing concentrations of dmLT were added in combination with PHA (Figure 15A).

Significant IL-17A enhancing effects of dmLT were also observed in PBMCs obtained from infants when increasing concentrations of dmLT (1 and 10 µg/mL) was added to the cultures stimulated with SEB. However, in contrast to adults, the two dmLT concentrations induced comparable IL-17A production when combined with SEB (Figure 15B). dmLT 10 µg/mL, but not dmLT 1 µg/mL, caused significant IL-17A enhancement when combined with PHA stimulation in PBMCs from infants; although there was a trend for increased responses already at 1 µg/mL (Figure 15B). Although the PBMCs from infants produced lower levels of IL-17A compared to the adult cells, the relative increase in IL-17A production in cells stimulated with 10 µg/mL dmLT compared to without dmLT were comparable in both age groups (Figure 15C, fold rises: SEB 2.6 *vs.* 2.9, PHA 1.9 *vs.* 2.2). These results suggest that infant cells were able to respond to dmLT adjuvant but that the dose-response effect in infant cells was less robust compared to that seen in adults.

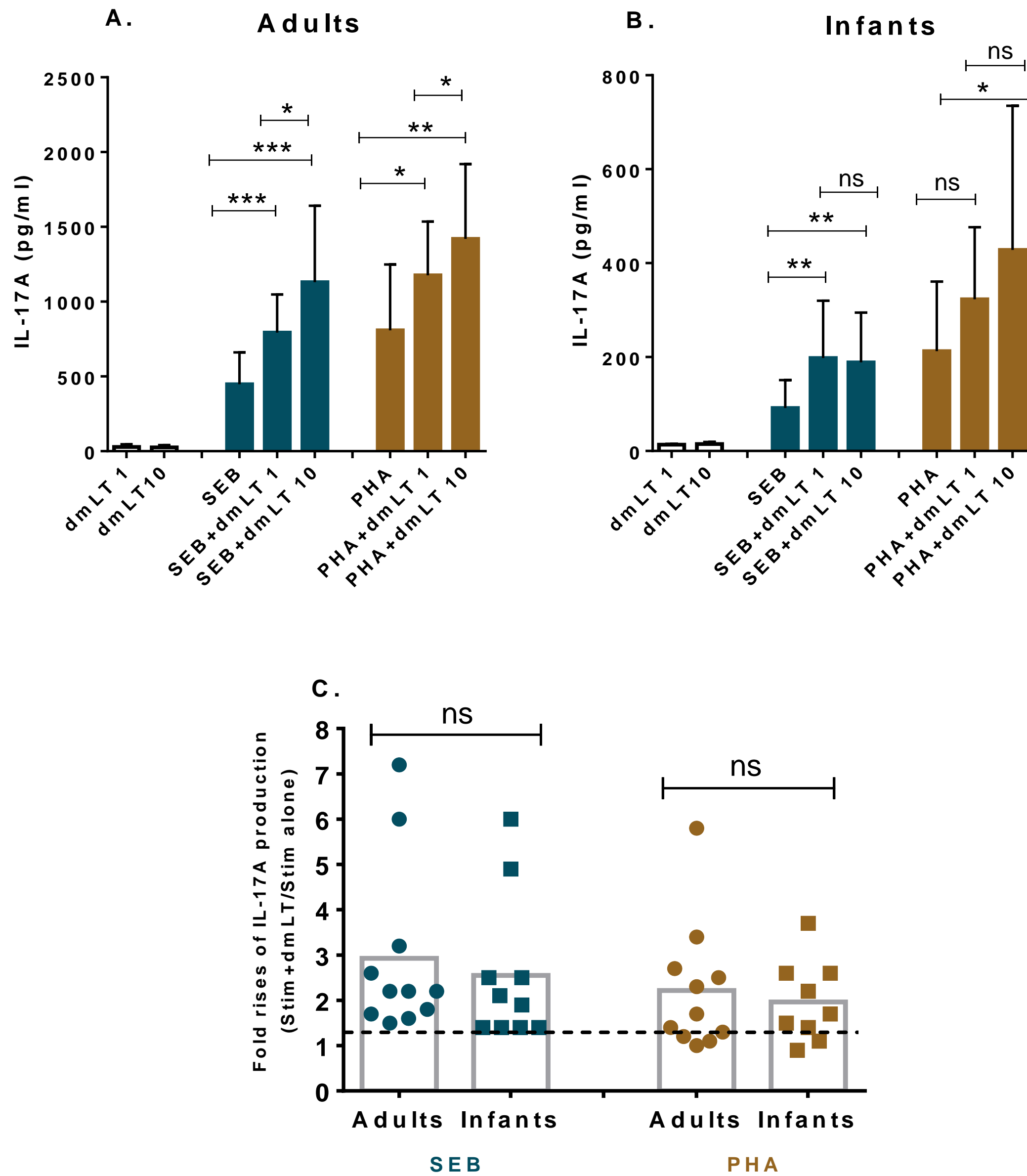

Figure 15. Effect of dmLT on IL-17A responses induced by SEB and PHA stimulation in PBMCs. IL-17A concentrations in cultures with cells from (A) adults (n=11) and (B) infants (n=10). Bars represent mean with SEM of IL-17A conc. in culture supernatants. (C) Fold changes of IL-17A responses in PBMCs treated with SEB or PHA plus dmLT (10 µg/mL) versus SEB or PHA treatment alone. Bars represent means of fold-change differences and each symbol represents data from one participant. Dashed line indicate no enhancement (fold rise=1).

3.3.2 Role of IL-1β in promotion of IL-17A responses by the dmLT adjuvant

To evaluate the role of IL-1β in mediating the dmLT adjuvant effect in both adult and infant PBMCs, IL-1RA was added to cell culture, which prevented downstream effects of IL-1β by blocking the IL-1 receptor. IL-1RA was added to the culture medium together with SEB and dmLT and concentrations of IL-17A was measured in the culture supernatants. Addition of IL-1RA caused decreased IL-17A production induced by SEB+dmLT in cells from all tested adults and infants (40% reduction for adults and 28% reduction for infants, Figure 16).

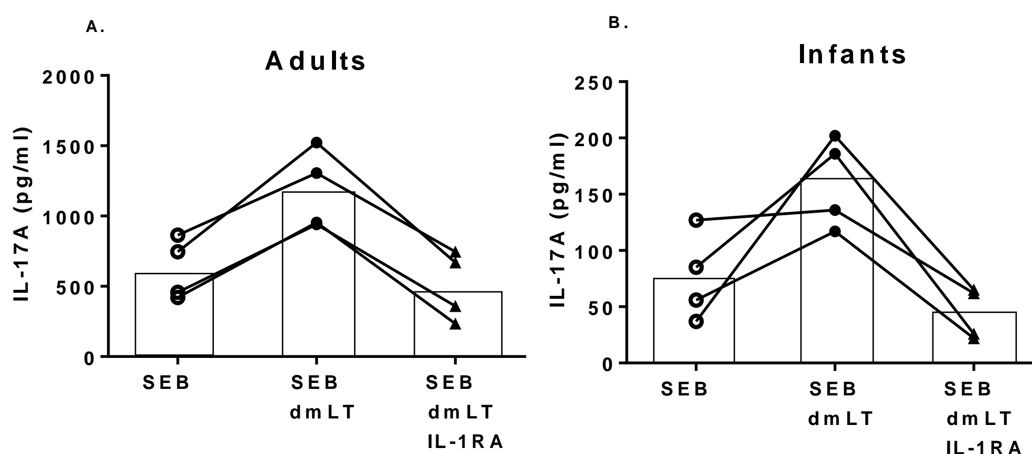

Figure 16. Role of IL-1 signaling in dmLT-induced promotion of IL-17A responses. PBMCs from (A) adults (n=4) and (B) infants (n=4) were treated for 72 h with SEB or SEB+dmLT 10 µg/mL with or without IL-1 receptor antagonist (IL-1RA) and the resulting IL-17A production was determined by ELISA.

3.3.3 Production of IL-1β from PBMCs stimulated with dmLT and ETVAX

To further investigate if dmLT alone could stimulate production of IL-1β in PBMCs from both adults and infants (Figure 17A). IL-1β responses were measured after stimulation of PBMCs with increasing concentrations of dmLT (1, 10, 20 and 50 µg/mL) for 18 hours, a time point when mainly innate cells respond to stimulation. dmLT alone could activate PBMCs from both infants and adults to produce IL-1β in a dose-dependent manner (Figure 17A). The levels of IL-1β induced by dmLT were comparable in adults and infants at all dmLT concentrations tested (Figure 17A). Increased IL-1β production (≥2 fold increase compared to stimulation with medium alone) was seen at the concentration of 10 µg/mL in PBMCs from some participants

(about 50% of subjects), but strong and consistent responses were only observed at 50 µg/mL in both adults and infants, when all volunteers except one infant showed increased (mean 11-fold) IL-1β production. Strong dose-dependent IL-1β production was also observed in PBMCs stimulated with different concentrations of *E. coli* LPS (0.1-1000 ng/mL) in both tested age groups, with similar levels produced in infants and adults (data not shown).

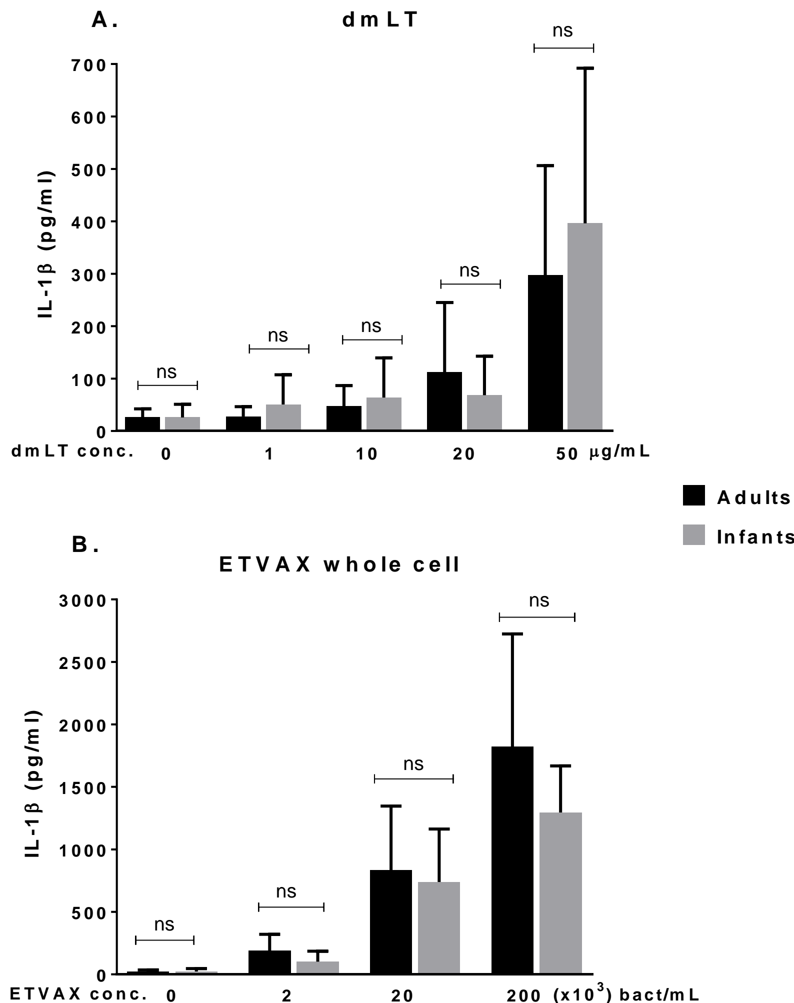

Figure 17. IL-1β production from PBMCs from adults and infants stimulated with dmLT and ETVAX whole cell component, respectively. (A) Production of IL-1β after stimulating PBMCs (adults; n=6, infants; n=5) with increasing concentrations of dmLT (1-50 µg/mL). (B) Production of IL-1β after stimulating PBMCs (adults; n=5, infants; n=6) with increasing concentrations of whole cell component of ETVAX. Bars represent means with SEM of concentrations of IL-1β.

Next, IL-1β responses were evaluated after stimulating PBMCs with the whole cell component of ETVAX. In both adult and infant cell cultures, the whole cell component induced strong and dose-dependent IL-1β responses and levels of IL-1β were comparable in the two age groups (Figure 17B).

3.3.4 IL-1β production in PBMCs stimulated with ETVAX±dmLT

To determine if dmLT could further enhance IL-1β responses induced by the whole cell component in PBMCs from adults and infants, the fold rises of IL-1β responses were measured after addition of 10 µg/mL of dmLT with a lower dose (2000 bacteria/mL) and 10-fold higher dose (20,000 bacteria/mL) of ETVAX whole cell antigen (Figure 18). In cultures with cells from adults, IL-1β responses did not increase further when dmLT was added with any of the tested vaccine doses. Interestingly, in cells from infants, addition of dmLT with the low dose of ETVAX vaccine caused significantly enhanced IL-1β responses compared to stimulation with vaccine alone. However, in combination with a higher dose of vaccine, dmLT did not enhance the IL-1β production in comparison to vaccine alone (Figure 18). The lack of enhancement at higher vaccine doses was confirmed when even higher concentrations of vaccine were tested (0.1-0.2 million bacteria/mL) in combination with dmLT in both adults and infants (data not shown).

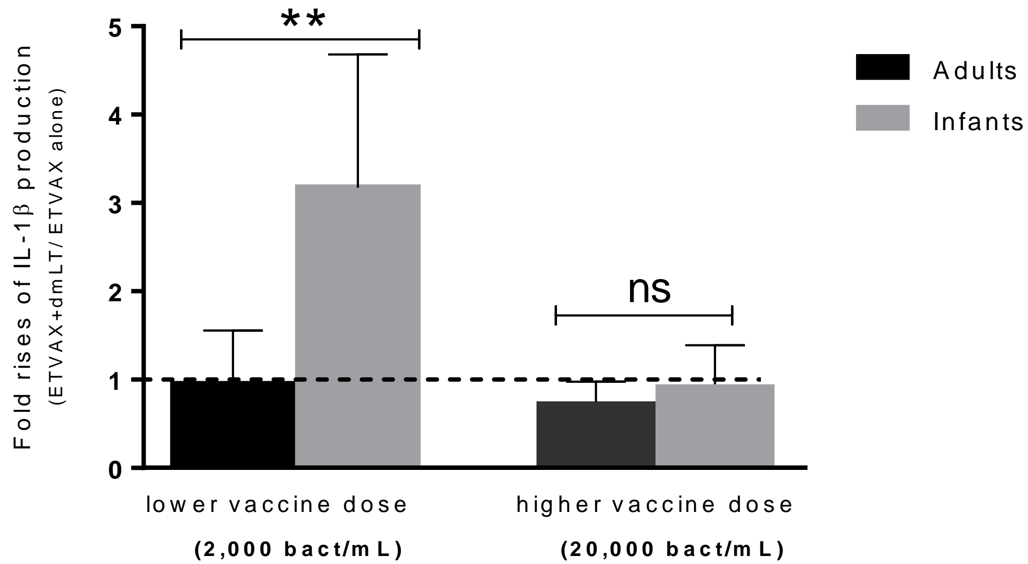

Figure 18. IL-1β production from PBMCs from adults and infants stimulated with a whole cell component of ETVAX± dmLT. Fold changes of IL-1β production after stimulating PBMCs with a lower concentration (adults; n=8, infants; n=7) and higher concentration (adults; n=6, infants; n=6) of whole cell component of ETVAX alone or with 10 µg/mL dmLT adjuvant. Bars represent means with SEM of fold-change differences between PBMCs treated with ETVAX plus dmLT versus ETVAX treatment alone.

Taken together, results from this *in vitro* study suggest that the dmLT adjuvant can promote IL-17A production from Th17 cells in both infants and adults. The adjuvant effect on T cells is at least partially mediated by IL-1β in both age-groups. In addition, preliminary results suggest that the whole cell component of ETVAX induces strong and dose-dependent IL-1β responses in both adults and infants. Furthermore, dmLT may particularly enhance IL-1β responses induced by low doses of vaccine in infant cells.

4. DISCUSSION

4.1 General discussion

In this thesis, the second generation ETEC vaccine ETVAX alone and together with dmLT adjuvant was shown to be safe and immunogenic in Bangladeshi adults. This was the first clinical trial of this ETEC vaccine, as well as the dmLT adjuvant, conducted in an ETEC endemic country. Also based on the promising results obtained from adults in this trial, the study could proceed to children and infants. ETVAX is one of the leading candidate vaccines for ETEC diarrhea, since it is an inactivated vaccine, which makes it safer than live vaccines and is easier to produce. It is also simple to administer and has a broader antigenic coverage compared to most other candidate ETEC vaccines [3]. ETVAX is produced by Scandinavian Biopharma in Sweden and has previously been tested alone and together with dmLT adjuvant in Swedish adults in a phase I trial [6]. The mucosal adjuvant dmLT is one of the most well studied non-toxic mucosal adjuvants, which can induce multifaceted immune responses to mucosally co-delivered antigens in mice [7]. This adjuvant has also shown promising effects in clinical trials [6, 94].

The clinical evaluation of ETVAX in Bangladesh was preceded by extensive efforts to improve and optimize the methods used for assessing the immunogenicity of the vaccine. Firstly, the kinetics of ASC/ALS responses in different age groups were studied after vaccination with the licensed and WHO prequalified oral cholera vaccine Dukoral to identify optimal sampling time points. Secondly, it was confirmed that fecal samples from young children and infants can be used to evaluate vaccine specific intestinal SIgA responses induced by oral vaccination. Thirdly, a sensitive chemiluminsence based ECL method was established for analysis of ALS IgA responses against multiple ETEC antigens present in the ETVAX vaccine. This was especially important since only small sample volumes of specimens are needed for the ECL analysis. This facilitated subsequent evaluation of immune responses in children and infants (in manuscript). Different *in vitro* experiments were also conducted to investigate how the dmLT adjuvant and ETVAX vaccine influence immune cells obtained from adults and infants. Collectively, the studies in this dissertation have provided both practical methodological tools and scientific insights into how oral

vaccines and mucosal adjuvants can induce and influence immune responses in individuals from an ETEC endemic area and in developing country.

In many previous studies of oral cholera and ETEC vaccines, ASC/ALS responses have traditionally been assessed 7 days after each vaccine dose, without considering the priming and endemicity or age of the participating individuals [12, 83, 84, 134-136]. In these studies, decreased levels of vaccine specific ASC responses were shown after the second compared to the first vaccine dose. For example, in the previous clinical trial of the first generation rCTB-CF-ETEC vaccine in Bangladesh, 90-100% of the adult participants responded against different CFs and CTB antigens after the first dose while only 60-80% responded after the second dose [83]. However, recently Leach et al. and Lundgren et al. reported that repeated immunization with the Dukoral as well as ETVAX vaccines induced more rapid and transient ALS responses in naïve Swedish adults than the first priming dose [6, 90, 133]. Since Sweden is not endemic for ETEC or cholera and only adult subjects were studied in Sweden, it was important to examine how age and natural exposure to cholera and ETEC infections influence the kinetics of ASC responses to oral vaccines in an endemic and developing country setting.

In the kinetics study, a clear effect of age on the ASC responses was shown after vaccination with Dukoral, particularly after the first dose. Most adults and toddlers developed significant anti-CTB ALS IgA responses after the first dose, while very few young children developed responses after the first dose. This supports the priming effect on ASC responses previously seen in Swedish adults [133, 136]. Adults and toddlers in Bangladesh are more frequently primed by symptomatic or asymptomatic infections with *V. cholerae* and/or LT ETEC than younger children; such priming may have induced CT/LT-reactive memory B cells which could be rapidly reactivated by a CTB-containing cholera vaccine, resulting in strong ALS responses already after the first vaccine dose. However, despite these differences in ASC responses after the first dose in different age groups, maximal responses were observed after the second dose on day 19 in all vaccinees independent of age. After the second dose a clear age effect on the magnitude and frequency of ALS responses was also observed; adults, toddlers and even young children responded strongly while lower responses were found in infants.

Interestingly, young children who responded very little after the first dose responded strongly after the second dose. The result of low ASC responses in infants is consistent with the recent findings from a study in India where low ASC responses among gut homing β7+ blood cells were recorded after a second dose of oral rotavirus vaccine in infants [138]. However, the most important finding of this kinetic study is that low ASC responses were observed on day 21 compared to day 19 in all age groups. These results support that in previous studies peak responses may have been missed due to suboptimal sampling time points, due to late schedule of collection of samples after the second vaccine dose [12, 83, 84, 134-136]. These results highlight the importance of collecting blood samples earlier (at day 5) than previously recommended (at day 7) after the second dose, in all age groups. Thus, identification of the same optimal time point for sample collection in all age groups greatly facilitates the planning and logistics of vaccine trials in descending age groups.

Measurements of fecal antibody responses more directly reflect mucosal responses compared to analysis of blood antibody secreting cells, ASCs. In addition, determination of fecal immune responses can often be more important in young children and infants compared to adults since limited blood volumes can be collected from these age groups and venipuncture is preferably completely avoided due to ethical reasons. However, analyses of fecal antibody responses are practically demanding because antibodies in fecal samples need to be extracted before analysis. This can be time consuming and the samples can also be unpleasant to work with. There is also a risk of getting varying quality of samples over time from the same individual. Moreover there is a possibility of non-specific binding of constituents of the complex fecal sample matrix to the solid phase used for antigen coating in the readout system. Evaluation of locally produced fecal SIgA antibody responses is advantageous compared to measurement of fecal IgA as done in previous trials [83, 134] since total IgA may contain transduced serum IgA antibodies [4].

In this dissertation, it was shown that SIgA antibody responses to CTB and *V. cholerae* membrane protein MP can be measured in fecal samples in infants and children after oral cholera vaccination, whereas it was not possible to analyze vaccine antigen specific SIgA responses in adults and toddlers due to very low and variable levels of total SIgA content in these age groups. This finding was later confirmed in

Bangladeshi adults vaccinated with ETVAX. In contrast, fecal samples from Swedish adults extracted using the same method contained higher SIgA levels and robust SIgA responses were recorded against all primary vaccine antigens after ETVAX vaccination [6]. The reason for this inconsistency may be due to several factors, including different diets and differences in the gut microbiota in people in endemic and non-endemic areas. Further studies are needed in adults and toddlers to optimize the extraction methods in order to facilitate measurement of fecal SIgA responses in endemic settings.

Kinetic analyses showed that fecal responses peaked at a later time point (day 21) compared to blood ASCs after the second dose. This delayed kinetics of antibody response in fecal extracts is consistent with mucosal homing of blood ASCs. This is because after the second vaccination plasma blasts appear transiently in blood on day 5 and then migrate to the intestinal mucosa to mature into plasma cells producing antibodies which can be detected after a few days in fecal samples. The findings of late appearance of fecal antibodies compared to ASC responses after the second dose is also consistent with previous studies in Swedish adults, where fecal responses to oral ETEC vaccines were detected 7–14 days after the booster dose [6, 89, 139].

A sensitive electrochemiluminiscence, ECL, assay was also established in this thesis as an alternate to the conventional ELISA methods to measure ALS responses against the primary ETVAX vaccine antigens using small sample volumes of specimens. The ALS/ASC responses measured by ELISA require large volumes of samples which make it difficult to evaluate responses to multivalent vaccines, particularly in pediatric studies, since only limited blood volumes can only be collected from children and infants. Furthermore, this is the first study where the ECL technology has been used to evaluate intestine-derived IgA antibody responses induced by an oral vaccine. However, ECL assays have previously been established for analysis of serum IgG antibodies against different infections and parenteral vaccines, especially pneumococcal vaccines, using both single- and multiplex analysis platforms [140-143]. The results in this dissertation show that the ALS results obtained using the novel ECL assay and traditional ELISA correlated very well for all tested antigens present in the ETVAX vaccine.

The ECL assay only requires 5 µl of ALS sample compared to a minimum of about 75 µl in ELISA for determination of responses to each antigen. Thus, the ECL assay is highly useful for assessment of intestine-derived IgA-ALS responses in pediatric studies of ETVAX, and also in studies of ASC responses to other multivalent vaccines as well as after natural ETEC infection. Furthermore, the wide dynamic range of the ECL assay made it possible to use a single sample dilution, while sample titration is normally used in ELISA, which results in significantly less work and reduced amounts of antigen used for coating the plates. The ECL assay was also found to be slightly more specific than ELISA method, since slightly higher rates of weak antibody responses were seen in the placebo group using ELISA compared to ECL. Based on these excellent results, the ECL assay was selected as the primary method for assessing intestine-derived ALS response in adults in this Phase I trial of the ETVAX vaccine.

The preliminary results from Svennerholm et al. (unpublished data, with permission) suggest that it is also possible to analyze plasma and fecal antibody responses against primary ETVAX vaccine antigens using ECL. However, further studies will also need to be conducted to establish a multiplex ECL method including several ETEC antigens in the same microtiter well to further reduce sample volumes and working time. The ECL techniques based on the MSD technology platform potentially allows analysis of 10 different antigens in separate spots in the same well and such multiplex assays have been shown to be highly useful for evaluation of plasma responses to different pneumococcal serotypes present in current multivalent pneumococcal vaccines [143]. However, the combination of ETEC antigens in a multiplex assay needs to be selected carefully, considering the cross-reactivity between antibodies binding to colonization factor antigens of ETEC belonging to the same protein families [37, 91]. Furthermore, antigen coating of multiplex assay plates must currently be performed by the company producing the ECL equipment and cannot be performed in-house.

All the results from the methodological studies in this dissertation were collectively considered when planning the immunogenicity analyses for the large Phase I/II clinical trial of the 2nd generation ETEC vaccine ETVAX in descending age groups. Thus, in this trial i) blood samples were collected day 7 after the first dose and day 5

(study day 19) after the second dose in all age groups, ii) considering the delayed kinetics of fecal SIgA responses compared to blood ASCs, fecal samples were collected at a later time point (day 28) after the second dose in addition to day 19, and iii) the ECL assay was used as the primary readout method to evaluate ALS responses against all primary vaccine antigens.

The clinical trial of ETVAX was designed to evaluate the safety and immunogenicity of the ETVAX vaccine alone and in combination with dmLT adjuvant in Bangladeshi adults and subsequently in descending age groups (5 years to 6 months). However, in this dissertation, only results from adults have been included. The results show that ETVAX vaccine alone and together with dmLT adjuvant is safe and very well tolerated in Bangladeshi adults, with almost no recorded solicited adverse events at all. These results confirm previous safety data from studies in Swedish adults who received ETVAX vaccine alone and together with 10 µg or 20 µg of dmLT [6, 89, 90]. However, in previous trials of the first generation rCTB-CF-ETEC vaccine a few adverse events were recorded in the Bangladeshi adults including mild abdominal pain, loose stools etc., which suggest ETVAX is at least as safe and possibly even better tolerated compared to the rCTB-CF ETEC vaccine [83]. After confirming the excellent safety of ETVAX in adults, children and subsequently infants have been vaccinated with different combinations of fractionated doses of ETVAX and dmLT to determine the highest safe dose of vaccine alone and in combination with dmLT (in manuscript).

Results from this dissertation further confirmed that ETVAX is highly immunogenic in Bangladeshi adults. Evaluation of ALS responses using the ECL technique showed that after the first dose 80-90% of the vaccinees responded to all primary vaccine antigens (CFs and LTB). In contrast, ALS responses after the first ETVAX dose were infrequent in Swedish adults who probably have never been exposed to ETEC previously [6, 90]. The findings of high ASC responses after the first dose further confirm the priming effect in Bangladeshi adults previously seen in the kinetics study after a single Dukoral dose. Importantly, ETVAX induced very high responses after the second dose when 100% of participants responded to all of the vaccine antigens. This is in contrast to the findings from studies of the 1st generation ETEC vaccine where lower ASC responses were found after the second compared to first dose [83].

The observation of most frequent ALS responses after the second dose confirms that the optimal time point (day 19) was selected in the ETVAX trial. However, the modified time points for specimen collection as well as the use of ALS instead of the PBMC based ELISPOT method in the ETVAX study made it less relevant to directly compare results from trials with ETVAX and the first generation ETEC vaccine.

Strong plasma antibody responses to CFs and LTB were observed after both doses of ETVAX in this endemic population. In this study, plasma antibody responses were measured using traditional ELISA assays, since larger volumes of plasma compared to ALS samples were available and ECL assays for assessment of plasma responses had not yet been established when immunogenicity analyses were initiated. Almost 70% of the Bangladeshi adults responded to the CFs in plasma which is in contrast to the Swedish study where ETVAX induced infrequent anti-CF antibody responses (3-19%) in naïve adults [6]. Similar to the findings of strong plasma anti-LTB responses in adult Swedes, almost all the Bangladeshi vaccinees responded with plasma anti-LTB responses after either of the two doses of ETVAX vaccine.

Comparatively high pre-vaccination plasma antibody titers in Bangladeshi volunteers were observed, which are consistent with the findings in previously recorded ETEC vaccine trials in Bangladesh [83]. These high pre-antibody titers suggest previous natural ETEC priming [37, 72]. Plasma antibody kinetics after oral vaccination showed increased magnitudes of responses but similar responder frequencies on day 21 compared to day 19 in younger children. On the other hand, adults, toddlers and infants showed comparable magnitudes and frequencies of antibody responses at both time points. This suggests that results using plasma would have been comparable in adults if blood samples could have been collected on day 21 instead of day 19 after ETVAX vaccination.

A few placebo recipients showed increased levels of IgA against CS3, CS6 or LTB in plasma and/or ALS samples after ETVAX vaccination. However, responses of placebo recipients were low in magnitudes and mainly below the 2-fold cut-off level for positivity against most of the antigens except CS3. However, against CS3 some responses, but not all, were probably due to LPS contamination of the antigen preparation, which was supported by control experiments using a more pure CS3

preparation. In general, some responses in placebo recipients are however not surprising in this endemic setting, since participants may acquire asymptomatic infection with ETEC, cholera or other bacteria expressing homologous or related antigens during the study period. It has been shown that during the first two years of life, 20% of all diarrheal cases at the trial site in Mirpur in urban Dhaka were due to ETEC infection, with an incidence of 0.5 episode/child/year [26] and ETEC is also an important diarrheal pathogen in Bangladeshi adults [25]. Analysis of the relative distribution of CFs in ETEC isolates from diarrhea cases at the icddr,b hospital suggest that the predominant CFs on ETEC isolated from diarrhea cases are CS5+CS6, CFA/I, CS7, CS17, CS1+CS3, CS6 and CS14 [144]. Thus, the CFs included in the ETVAX vaccine are common in Bangladesh.

The mucosal adjuvant dmLT has clear immunostimulatory properties as described in several preclinical as well as clinical studies [6, 8, 110, 112, 145]. Importantly, Holmgren et al. have shown enhanced mucosal and serum responses to all vaccine CFs and LTB in mice when the ETVAX vaccine was co-administered with dmLT [8]. In addition, in that study dmLT showed a dose-sparing effect when different dosages of vaccine were tested; the adjuvant effect of dmLT appeared especially strong for the lower vaccine dosages [8]. Interesting observations were also made regarding the adjuvant effect of dmLT on protection induced by the orally administered live, attenuated ETEC vaccine ACE527 [145]. Participants immunized with ACE527+dmLT were significantly protected against subsequent ETEC challenge, whilst those who had received vaccine alone were not. In addition, an adjuvant effect of dmLT was also observed in adult Swedes since 10 µg of dmLT with ETVAX boosted anti-CS6 IgA ALS responses compared to vaccine alone [6]. However, results from Bangladeshi adults show little or no detectable effect of dmLT on ALS responses.

The reason why dmLT did not induce a similar enhancement of the ALS responses in this trial may be explained by extensive natural priming of the Bangladeshis, limiting the ability of dmLT to further enhance the already strong ALS responses. However, a trend (not statistically significant) for an adjuvant effect was observed for plasma responses in the Bangladeshi participants against CS5 and CS6. As CS6 was the antigen present in the lowest amounts in ETVAX, this supports the previously

observed dose-sparing effect of dmLT in ETVAX vaccinated mice [8]. A trend toward an adjuvant effect was also noted when the antigenic breadth of the plasma IgA responses were evaluated, particularly when responses to 4 or more or 5 vaccine antigens were considered. All of these results support that dmLT may have important adjuvant effects in humans, particularly when co-administered with low vaccine doses, although the effects may be less evident in humans compared to mice and the complete mechanisms responsible for the effects are yet to be determined. The potential effect of dmLT when co-administered with lower doses of ETVAX evaluated in the continued trial in children and infants will give more information in this area.

Most studies of how dmLT and related LT- and CT-derived adjuvants mediate their adjuvant function have been performed in mice *in vivo,* or *in vitro* using cell lines or cultured mouse cells, and only a few studies have been done using human cells. Importantly, it was shown that dmLT and the related multiple mutant CT (mmCT) adjuvant can enhance IL-17A cytokine responses in adult T helper cells by activating cAMP-dependent protein kinase A (PKA) and caspase1/inflammasome–dependent IL-1 signaling [119, 120]. Nevertheless, immune responses in infants are different in terms of development and priming and cannot always be predicted from those observed in human adults or animal models [124, 146]. Immune cells from infants have been reported to have a higher baseline production of cAMP as well as higher production of IL-1β in response to some bacterial stimuli compared to adult cells [123, 125]. However, no studies have been carried out earlier to investigate the adjuvant function of dmLT in the younger age group. This study took advantage of previously described *in vitro* models to compare the dmLT adjuvant function in PBMCs from infants (6-11 months) and adults in terms of IL-1β and IL-17A production.

To evaluate the adjuvant effect of dmLT on T cell responses in healthy infants and adults two different polyclonal T cell activators were used: the plant lectin PHA and the *Staphylococcal* enterotoxin B (SEB). PHA can activate almost all types of T cells by binding to certain sugar residues on cell surface glycoproteins including T cell receptors (TCR) and CD3 complexes, regardless of antigen specificity [147]. The

superantigen SEB directly binds to MHC-II on APCs, bypassing the need for processing the superantigen into antigenic peptides, and activates ~20% of the T cells by binding specifically with certain types of TCR β chains [148-150]. SEB is generally considered to be a more physiological stimuli than PHA, since it crosslinks and activates both T cells and APCs, and only activates a proportion of the T cells in a culture [148]. In contrast, PHA mainly targets T cells, activating a majority of T cells in a culture, although APCs may also be activated by bystander effects [151]. Although SEB may be the preferred stimuli to investigate T cell responses in adults, age-related differences have been reported in memory T cell responses to SEB [152], whereas comparable responses to PHA have been recorded in different age groups [153]. Considering these technical difficulties and since both SEB and PHA have been used in previous *in vitro* studies with adult cells, both stimuli were used to evaluate the dmLT adjuvant effect in infants and adults in the present study.

In line with previous results from studies of PBMCs from adult Swedes [119, 120], the results of this dissertation showed that dmLT enhanced IL-17A responses in PBMCs from Bangladeshi adults when stimulated with both SEB and PHA. Consistent with the data from the Swedish studies, stimulation with dmLT alone did not give rise to any IL-17A production, which is interesting considering that the Bangladeshi volunteers are likely to have been primed with LT+ ETEC. Importantly, dmLT also significantly enhanced IL-17A responses to both SEB and PHA in PBMCs obtained from infants. However, responses in infants were of lower magnitudes and showed greater individual variation and less robust dose-dependency compared to adults. Since IL-17A is produced mainly by memory T cells [154, 155], the findings of low IL-17A responses in infants compared to adults are likely to at least partly be due to a lower frequency of memory cells present in infant compared to adult PBMCs [123].

The lower magnitude of IL-17A production in response to SEB in infants compared to adults and the relatively weak additive effect of higher doses of dmLT in combination with SEB in infants may also be related to the age-related differences in the CD4+ T cell responses to SEB shown previously [156]. In response to SEB stimulation the frequency of IFN-γ producing activated memory CD4+ T cells were lower in infants compared to adults [156], suggesting that the responsiveness to the activating signals

provided by SEB may also be partly different in infant cells. However, previous studies have shown that infant T cells respond to PHA with comparable proliferation compared to adult cells [153], suggesting that PHA may provide a more robust *in vitro* stimulation system when comparing responses in different age groups. Interestingly, when the relative increase (fold-rise) in IL-17A production were analyzed in cells stimulated with PHA or SEB plus dmLT compared to polyclonal stimulation alone, a comparable relative enhancement of IL-17A responses were observed in both infants and adults. These results suggest that cells from infants have a similar functional capacity as adult cells to be stimulated by dmLT, although the general capacity to produce IL-17A in response to polyclonal stimuli is lower in infants.

IL-17A is particularly interesting in relation to enteric infections because of its involvement in antibody mediated protection by enhancing germinal center formation, IgA and IgG class switching and SIgA formation with increased expression of the poly-Ig receptor in epithelial cells [157-160]. However, dmLT has also been shown to influence IFN-γ production to certain antigens *in vitro*, although no effects on responses to polyclonal stimuli were recorded [119, 120]. In continued studies, the adjuvant effect of dmLT on a broader range of T cell cytokines after both polyclonal and antigenic stimulation will need to be compared in both infants and adults.

Several studies demonstrated the potential role of IL-1β, in combination with IL-6 and/or IL-23 for the differentiation of human Th17 cells from naïve T cells as well as promotion of IL-17A production from already differentiated Th17 memory T cells [118, 161]. APCs, mostly monocytes and DCs, play major role in Th17 differentiation by producing IL-1β and IL-6 [118, 162, 163]. The capacity of cholera toxin (CT) to interact with monocytes or DCs was shown as a crucial step for its adjuvant mechanism [164]. Previous studies also showed that dmLT and related toxin-derived adjuvants have the potential to stimulate APCs to produce IL-1β and enhance antigen presentation through up-regulating the expression of the co-stimulatory molecule (B7-2) and MHC-II on APCs [120, 165, 166]. The role of IL-1β to mediate the adjuvant function was also evident from earlier experiments showing that inhibition of the molecules involved in IL-1β production from APCs, e.g. PKA and caspase 1, caused significantly diminished adjuvant effect of dmLT [120]. Consistently, a similar

function of IL-1β was observed in the present *in vitro* study in cells from both infants and adults; considerably lower IL-17A responses were recorded when IL-1RA was added to the PBMC cultures stimulated with SEB plus dmLT.

The role of IL-1β in mediating the adjuvant effect was further analyzed in the present study by measuring IL-1β production in both age groups after stimulating PBMCs with increasing concentrations of dmLT. A broader range of dmLT concentrations were tested than done in previous studies (1-50 µg/mL) and the results showed that immune cells from infants produced IL-1β at levels similar to adults at all concentrations of dmLT tested. However, the individual variation in responsiveness to dmLT was relatively large and while increased IL-1β secretion was seen already at 10 µg/mL in some individuals, the most consistent responses were found at higher concentrations and particularly at 50µg/mL. This is in contrast to the IL-17A enhancement which was consistently seen already at lower dmLT concentrations (1-10 µg/mL). These findings indicate the importance of TCR stimulation, which activate the T cell and in turn stimulate the APC, in concert with dmLT adjuvant stimulation of the APC, to get optimum IL-1β production and possibly also enhanced expression of costimulatory signals. The results of comparable IL-1β but low IL-17A production in infants could be due to the dominance of naïve T cells in infants, since naïve T cells express low levels of the IL-1 receptor compared to memory T cells [167]. Thus, it might be possible that IL-1β could not drive a strong IL-17A response in infants due to lack of the receptors on a majority of T cells.

Another key finding in this study is the comparable and strong dose-dependent IL-1β production in both adults and children in response to the whole cell component of ETVAX. This is partly in contrast to a previous study, where PBMCs from infants were shown to produce higher levels of IL-1β in response to stimulation by a *Helicobacter pylori* membrane preparation containing both LPS and proteins [123]. However, *H. pylori* LPS, and possibly also other toll like receptor ligands expressed by this bacterium, is known to have different immunostimulatory effects compared to corresponding substances from *E. coli* [168], which may partly explain the results. ETVAX induced production of considerably higher levels of IL-1β than stimulation with dmLT in both infants and adults. Interestingly, for the first time it was shown that dmLT significantly enhanced IL-1β responses when combined with the whole

cell component of ETVAX in infant PBMCs, but only with very low ETVAX doses; no increase was seen at higher vaccine doses. This finding is consistent with the clear dose-sparing effect of dmLT observed *in vivo* on antibody responses in mice immunized with ETVAX plus dmLT [8]. In contrast, dmLT did not influence IL-1β responses in PBMCs from adults at any of the tested vaccine doses. In future studies, higher doses of dmLT will need to be tested in combination with a range of ETVAX concentrations, to further characterize the difference in responsiveness to dmLT observed between adult and infant cells.

Although it was tried, but it was not possible to measure IL-17A responses in PBMCs stimulated with ETVAX alone or in combination with dmLT, possibly due to a lack of or a very low frequency of memory T cells specific to ETVAX antigens in healthy adults and infants. Studies are ongoing to measure the dmLT adjuvant effect on IL-17A responses specific to ETVAX antigens in individuals having a higher proportion of ETEC specific memory T cells after vaccination or natural ETEC infection.

Collectively, the results from the *in vitro* experiments suggest that dmLT may have an important function to promote immune responses to low doses of ETVAX vaccine in infants. Enhanced production of IL-1β and subsequently IL-17A may lead to increased production and secretion of SIgA, which in turn may help to protect against ETEC. Interestingly, preliminary data from the ETVAX vaccine trial in Bangladeshi infants indicate that the adjuvant function of dmLT on IgA responses may indeed be most consistently observed in the youngest age group (unpublished data), supporting the validity of the *in vitro* observations.

4.2 Conclusions

The studies presented in this dissertation have addressed several important questions related to oral vaccination against enteric infections in different age groups. In conclusion, the assessment of mucosal immune responses (ALS IgA and fecal SIgA) after oral vaccination can be carried out by analyzing potential sampling time points; also a sensitive ECL assay was evaluated for measuring vaccine specific IgA responses in ALS specimens using small sample volumes. In addition, studies in this thesis also resulted in improved knowledge about how the ETVAX vaccine and dmLT adjuvant interact with the immune system. The vaccine was found to be safe and to induce strong immune responses when tested in Bangladeshi adults. However, dmLT had no apparent effect on ALS responses in Bangladeshi adults, although a trend for improved response in plasma was observed to multiple vaccine antigens.

These results highlight the importance of ongoing clinical studies on ETVAX together with dmLT adjuvant in younger age groups. The results from the *in vitro* study suggest that dmLT can enhance IL-17A production from T cells in both infants and adults and that this effect may at least partly be mediated by increased IL-1β production. These results showing that dmLT may be more efficient at promoting IL-1β responses to low amounts of ETVAX vaccine in infants compared to adults possibly reflect the *in vivo* mechanisms. However, future studies are needed to investigate the role of the dmLT adjuvant *in vivo* in a larger number of infants and adults. Overall, the findings from this dissertation will help design trials of oral vaccines in infants and to analyze and interpret results regarding the effect of the mucosal adjuvant, dmLT in future clinical trials.

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APPENDIXES

A. Reagents

1. Sodium heparin tubes, BD vacutainer, 367874
2. Falcon tubes, Corning Science, 352096
3. Ficoll-Isopaque, Pharmacia, Uppsala, Sweden, 17-1440-03
4. Trypan blue, Sigma, T6146
5. NaCl, Sigma, 71380
6. Potassium phosphate di-basic, Sigma, 04273
7. RPMI Medium 1640 (1X), Invitrogen, 11875–119
8. Fetal Bovine Serum (FBS), Invitrogen, 16140–071
9. Na-Pyruvate, Invitrogen, 11360–070
10. L-glutamine, Invitrogen, 25030-081
11. Penicillin-Streptomycin, Invitrogen, 15140-122
12. Bovine Serum Albumin, Sigma, A4503
13. Tween 20, Sigma, P-1379
14. EDTA, Sigma, E5134
15. Soybean Trypsin Inhibitor, Sigma, T9128
16. Pefablock, Roche, 11429876001
17. Sodium azide, Sigma, S8032
18. AEBSF, Calbiochem, 101500
19. Aprotinin, USB, 11388
20. Leupeptin Hemisulfate, USB, 18413
21. 96-well tissue culture plate, Nunc, 167008
22. Anti-human IgA-HRP, Jackson Immuno Research, 309-035-011
23. Orto-phenylenediamine (OPD), Sigma, P9029
24. Hydrogen peroxide, BDH AnalaR, 101284N
25. Sulfuric Acid, Merck, 100731
26. Sodium citrate, Sigma, S4641
27. ELISA plates, Greiner, 655061
27. CFA/I_{SBL}, produced at SBL, prep. 3
28. CS3, produced at SBL, prep.6-98
29. CS5, produced at SBL, prep. 2
30. CS6, from Fred Cassel, lot 0840
31. Ganglioside GM1, from Lars Svennerholm
32. Recombinant LTB (rLTB), Etec653.tem (N Carlin), lot 2010-01-08
33. Recombinant CTB (rCTB),
34. *Vibrio cholerae* membrane protein (MP),
35. IgA reference, Sigma, I1010
36. Anti-SIgA monoclonal antibody, Sigma, I6635
37. Anti-mouse IgG1-HRP, Southern Biotech, 1070-05
38. Goat anti-human IgA, Jackson Immuno Research, 109 005 011
39. IL-17A ELISA kit, eBiosciences, 88-7176-88

40. IL-1beta ELISA kit, eBiosciences, 88-7010-22
41. DMEM-F12 medium, Gibco, 11320-074
42. ETEC mock vaccine, Scandinavian Biopharma, Etec970.tem
43. SEB, Sigma, S4881
44. PHA, Remel, HA16
45. dmLT, Lot 1575
46. *E. coli* LPS, Sigma, L8274

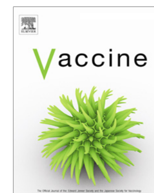
B. Published Paper I

Vaccine 35 (2017) 321–328

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine



Kinetics of antibody-secreting cell and fecal IgA responses after oral cholera vaccination in different age groups in a cholera endemic country

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ARTICLE INFO

Article history:
Received 29 July 2016
Received in revised form 10 November 2016
Accepted 13 November 2016

Keywords:
Antibody-secreting cell
Antibodies in lymphocyte supernatant
IgA
Oral vaccine
Cholera
Children

ABSTRACT

Immune responses to oral enteric vaccines in children and infants may be influenced by factors such as age, previous priming with related microorganisms and breast feeding. In this study, we aimed to determine optimal time points to assess immune responses to oral enteric vaccines in different clinical specimens. This was done by investigating antibody secreting cell (ASC) and fecal antibody responses on different days after vaccination using the licensed oral cholera vaccine Dukoral, containing cholera toxin B-subunit (rCTB) and inactivated *Vibrio cholerae* bacteria, as a model vaccine.

Two vaccine doses were given 2 weeks apart to infants (6–11 months), young children (12–18 months), toddlers (19 months–5 years) and adults in a cholera endemic country (Bangladesh). IgA ASC responses, as determined by the antibodies in lymphocyte supernatant (ALS) assay, plasma IgA and IgG responses and secretory IgA (SIgA) responses in extracts of fecal samples were evaluated 4/5 and 7 days after each vaccination.

After the first vaccine dose, anti-CTB ALS IgA responses in adults and toddlers were high and comparable on day 5 and 7, while responses were low and infrequent in young children. After the second dose, highest ALS responses were detected on day 5 among the time points studied in all age groups and the responses declined until day 7. In contrast, plasma IgA and IgG anti-CTB responses were high both on day 5 and 7 after the second dose. Fecal SIgA responses in young children and infants were highest on day 7 after the second dose.

Our results suggest that ASC/ALS responses to two doses of the oral cholera vaccine Dukoral and related oral vaccines should be analyzed earlier than previously recommended (day 7) at all ages. Fecal antibody responses should preferably be analyzed later than ASC/ALS responses to detect the highest antibody responses.

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1. Introduction

Enteric infections are important global health problems and are principal causes of morbidity and mortality in children and infants in low- and middle-income countries [1]. Since mucosal IgA antibodies are important for protection against most enteric infections it is essential to measure such responses in both adults and infants using accurate and sensitive assays suitable for clinical vaccine trials [2]. Some of the most widely used methods for evaluation of mucosal immune responses induced by oral vaccines include analysis of specific IgA antibodies in intestinal lavage or fecal specimens [3–5] as well as assessment of specific antibodies produced by intestine-derived antibody-secreting cells (ASCs), which transiently migrate through peripheral blood in response to oral vaccination [6,7] and can be detected either by ELISPOT or by the antibodies in lymphocyte supernatant (ALS) assay [8,9]. These techniques are often initially used in clinical trials of healthy adults

Abbreviations: AEs, adverse events; ALS, antibodies in lymphocyte supernatant; ASC, antibody-secreting cell; CFs, colonization factors; CT, cholera toxin; CTB, cholera toxin B-subunit; ETEC, enterotoxigenic *Escherichia coli*; LT, heat labile toxin; LTB, heat labile toxin B-subunit; MP, membrane preparation; PBMCs, peripheral blood mononuclear cells; RF, responder frequencies; SIgA, secretory IgA.

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http://dx.doi.org/10.1016/j.vaccine.2016.11.055
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in non-endemic countries, who are most likely naïve in relation to vaccine related microorganisms and antigens. However, immune responses to oral vaccines may be very different in infants and children in endemic countries due to e.g. natural exposure to infections, age-related differences in immune function, breast feeding and nutritional status [2,10]. Hence, optimization of immunoassays in these target populations should be undertaken, including identification of optimal time points for sampling of clinical specimens, before use in clinical vaccine trials.

Recent studies in adult Swedish volunteers have clearly demonstrated that repeated immunization with the licensed oral cholera vaccine Dukoral as well as a novel oral inactivated multivalent enterotoxigenic *Escherichia coli* (ETEC) vaccine (ETVAX) induce more rapid and transient ALS responses to toxin and bacterial protein antigens than previously appreciated [11–13]. These data suggest that such responses may have been underestimated in several previous studies of oral vaccines in both endemic and nonendemic countries [5,14–21]. However, it is still unclear how age and natural exposure to cholera and ETEC infections may influence the kinetics of ASC responses to oral vaccines.

Peripheral ASC responses induced by oral vaccination are known to reflect intestinal immunity, as measured by analysis of antibodies in fecal or lavage samples or intestinal tissue [3,5,21]. In clinical trials of mucosal vaccines, it is often more demanding to collect, store and/or extract intestinal compared to blood samples, and many studies of mucosal immunity are therefore mainly based on proxy markers such as ASC assessment [7]. However, we and others have shown that it is possible to use fecal samples to more directly measure the induction of vaccine specific IgA antibodies in adults and children and that such analyses may replace or complement ASC assessments [3,5,12,22–24]. Determination of fecal immune responses is particularly attractive in young children and infants since only small blood volumes can be collected from these age groups and venipuncture is preferably completely avoided. However, fecal specimens from infants in endemic areas may be contaminated by breast milk containing specific IgA antibodies against enteric pathogens which may obliterate vaccine immune responses.

The main aim of this study was to potentially improve assessment of immune responses to mucosal vaccines against enteric infections by identifying optimal sampling time points for determination of ALS responses after oral vaccination in different age groups in an endemic area. The study was performed to prepare for subsequent Phase I/II trials of oral vaccines, e.g. the ETEC vaccine ETVAX, containing a heat labile toxin B-subunit/cholera toxin B-subunit (LTB/CTB) hybrid toxoid and inactivated bacteria overexpressing common ETEC colonization factors (CFs). The licensed cholera vaccine Dukoral, containing CTB and killed *Vibrio cholerae* bacteria, was used as a model vaccine; two consecutive doses were given to adults, toddlers, young children and infants in Bangladesh. The feasibility to measure vaccine specific secretory IgA (SIgA) antibody responses in fecal samples collected at different time points from young children and infants was also investigated.

2. Materials and methods

2.1. Study design and vaccinations

The study was conducted in Mirpur, Dhaka, Bangladesh. Healthy adults (18–45 years, n = 40), toddlers (19 months to 5 years, n = 20), young children (12–18 months, n = 20) and infants (6–11 months, n = 10) were recruited to the study. The study physician assessed the general health of the subjects at enrollment. Subjects with a history of gastrointestinal disorders, diarrheal illness during the last two weeks, febrile illness in the preceding week or antibiotic treatment within one week prior to enrollment were excluded. Subjects who had been vaccinated with any cholera or ETEC vaccines previously were also excluded.

Participants received two oral doses of Dukoral® (Crucell, Sweden) 14 days apart. The vaccine consists of 1.25×10^{11} killed *V. cholerae* bacteria and 1 mg of rCTB. A full dose of the vaccine was suspended in 150 ml (adults), 75 ml (toddlers) or 15 ml (young children and infants) carbonate buffer (Recip, Sweden). Participants were not allowed to eat or drink one hour before and after vaccination.

The study was approved by the Research Review and Ethical Review Committees of the International Review Board of the icddr,b (International Centre for Diarrhoeal Disease Research, Bangladesh) and informed written consent was obtained from each participant and for children from their parent/guardian before enrollment.

2.2. Specimen collection

From each participant, heparinized venous blood and fecal specimens were collected at three different time points; before immunization (day 0) and at two additional time points (day 4 or 5 and day 7), either after the first or the second vaccine dose (Table 1).

2.3. Evaluation of immune responses

Mucosal immune responses were evaluated by measuring vaccine specific antibodies in secretions from intestine-derived ASCs using the ALS assay and by analysis of SIgA antibodies in fecal extracts. Systemic immune responses were determined as vaccine specific IgA and IgG antibodies in plasma.

Peripheral blood mononuclear cells (PBMCs) and plasma were separated by density-gradient centrifugation on Ficoll-Isopaque (Pharmacia, Sweden). Plasma was stored at −20 °C. For the ALS assay, PBMCs were cultured at 10^7 cells/ml as described [25] and supernatants were collected after 48 h of incubation and stored at −70 °C. Fecal extracts were prepared as described and stored at −70 °C [3].

IgA antibodies in ALS samples, SIgA antibodies in fecal extracts and IgA and IgG antibodies plasma samples were analyzed by ELISA using plates coated with GM1 ganglioside (0.3 nmol/ml) plus CTB (1 μg/ml for ALS and fecal samples and 0.5 μg/ml for plasma) or a membrane preparation (MP) from *V. cholerae* bacteria (3 μg/ml) [11,12,22]. The MP was purified from a *V. cholerae* O1 strain cultured in AKI medium followed by sonication and precipitation with 40% saturated ammonium sulphate [9,26]. The preparation contained 7.9 mg/ml protein and 6 mg/ml *V. cholerae* O1 LPS. Endpoint titers were determined as the reciprocal interpolated dilutions giving an absorbance of 0.2 (ALS, faeces) or 0.4 (plasma) above background at 492 nm. The total SIgA level of each fecal sample was also determined by ELISA, as described [3]. Fecal

Table 1
Sampling schedule in different age groups.

Study groups	Before vaccination	After dose 1	After dose 2
Adults			
A	Day 0	Days 4 + 7	–
B	Day 0	Days 5 + 7	–
C	Day 0	–	Days 4 + 7
D	Day 0	–	Days 5 + 7
Toddlers and young children			
B	Day 0	Days 5 + 7	–
D	Day 0	–	Days 5 + 7
Infants			
D	Day 0	–	Days 5 + 7

Table 2
Demographic characteristics of study participants (per protocol set).

	Adults n = 40	Toddlers n = 19	Young children n = 19	Infants n = 9
Age				
Mean (SD)	29.6 (6.4)[a]	3.1 (0.8)[a]	13.9 (1.4)[b]	8.0 (1.9)[b]
Range	18–42[a]	2.0–4.4[a]	12–17[b]	6–11[b]
Gender (no and freq. of participants)				
Female	29 (72%)	6 (32%)	8 (42%)	3 (33%)
Male	11 (28%)	13 (68%)	11 (58%)	6 (67%)

[a] Age in years.
[b] Age in months.

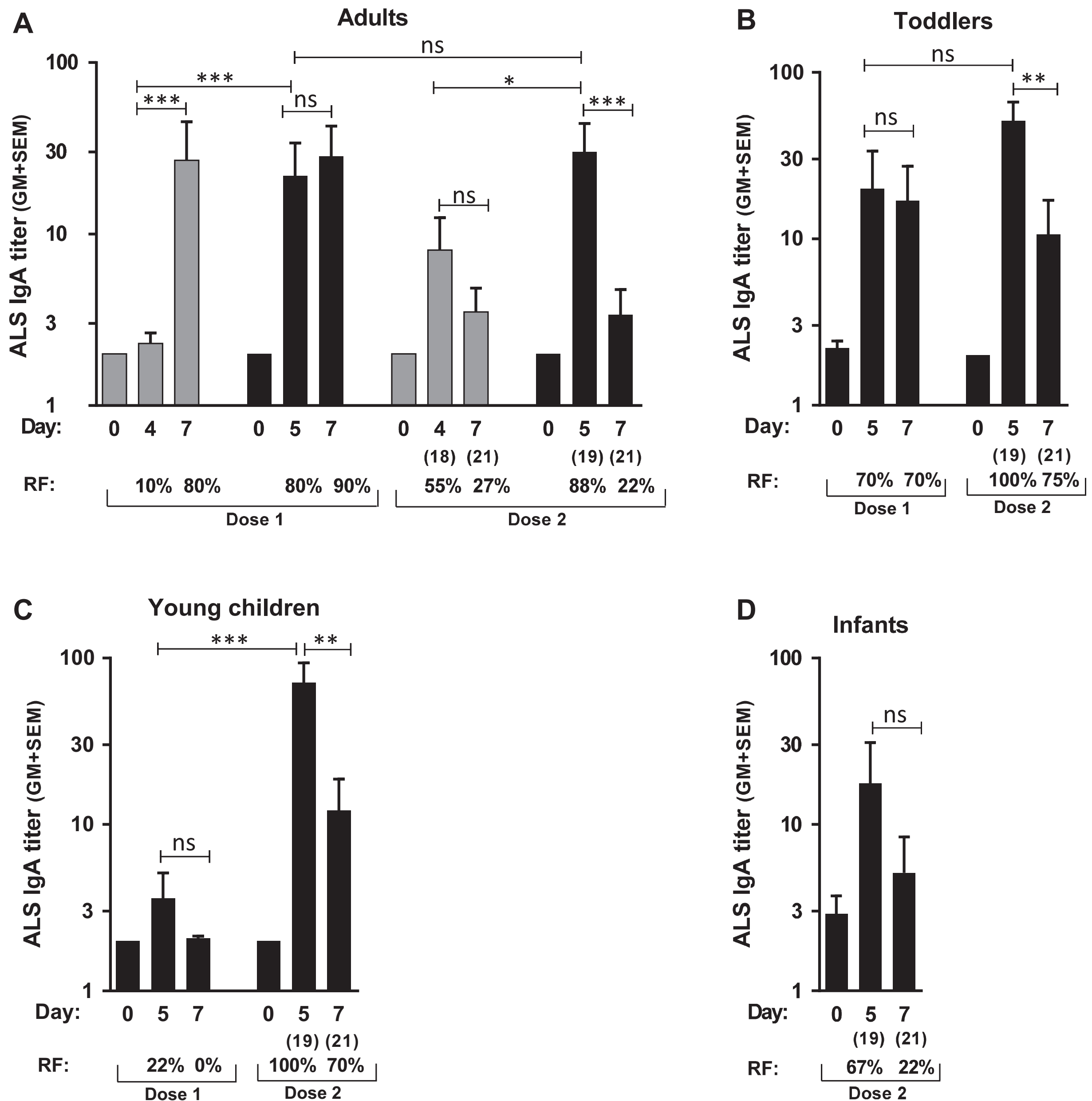

Fig. 1. Kinetics of IgA ALS responses to CTB after oral Dukoral vaccination. CTB-specific IgA titers (GM + SEM) were analyzed in ALS samples from (A) adults (sampling group A; n = 10, B; n = 10, C; n = 11 and D; n = 9), (B) toddlers (group B; n = 10 and D; n = 9), (C) young children (group B; n = 9 and D; n = 10) and (D) infants (group D; n = 9) before the first immunization (day 0) and at the indicated time points after the first and second dose. Numbers in parentheses indicate day after the first immunization. Responder frequencies (RF) on the different days are shown below the graphs. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns; not significant.

antibody levels were given as the specific SIgA titer divided by the total SIgA concentration of each sample. Specimens containing <10 μg/ml or >1000 μg/ml total SIgA were excluded from the analysis as well as samples in which the total sIgA concentration varied >3-fold between the different time points for each participant [12].

2.4. Safety

Participants and parents/guardians were asked verbally about whether any adverse events (AEs) that might be related to vaccination, including loose stools, nausea, vomiting and fever, had occurred during the study period.

2.5. Statistical analysis

All data were log transformed. Comparisons of pre- and post-immunization antibody levels within groups were evaluated using a paired *t*-test and responses between different groups using an unpaired *t*-test. Responder frequencies were evaluated using Fisher exact test. The magnitudes of immune responses (fold-rises) were calculated as the post-immunization divided by pre-immunization antibody levels and twofold increases were regarded as responses for all sample types. P-values <0.05 were considered as significant.

3. Results

3.1. Study participants

Forty healthy adults, 20 toddlers, 20 young children and 10 infants were enrolled with 9–11 participants per sampling group (groups A–D; Tables 1 and 2). All except one (one toddler receiving only one dose) of the 90 enrolled participants received two doses of the vaccine according to protocol. One infant was lost to follow up after the second dose. These two participants together with one young child, who had abnormally high preimmunization serum titers against CTB (IgA titer >4000) suggesting very recent ETEC or cholera infection, were excluded from the immunological analyses. Slightly more male than female toddlers, young children and infants were enrolled, whereas slightly more female than male adults were included (Table 2). The age and gender distributions in sampling groups A–D were comparable (not shown).

3.2. Safety

The vaccine was safe and well tolerated in all study groups. The very few AEs reported were mild in nature. No AEs were recorded in infants.

3.3. ALS IgA responses

The kinetics of mucosally-derived IgA ASC responses against CTB was evaluated by the ALS method. In adults, significant ALS responses to CTB were observed both after the first and the second vaccine dose (Fig. 1A). Very few participants responded on day 4 after the first dose, but on day 5 a majority had responded and the responses remained high on day 7. After the second dose, about half of the participants responded already on day 4. Highest ALS responses were detected on day 5 after the second dose (among the time points studied), when almost all participants had responded. Responses then declined significantly until day 7.

Similar kinetics of ALS IgA responses to CTB were observed in toddlers (day 4 responses were not evaluated in this age group). Highest responses were detected on day 5–7 after the first dose and on day 5 after the second dose with a significant decline on day 7 (Fig. 1B).

In contrast to adults and toddlers, few young children responded in ALS after the first vaccine dose (Fig. 1C). However, 5 days after the second dose all young children had responded. The responses then declined significantly on day 7.

Although the ALS anti-CTB IgA response magnitudes were relatively low in infants, a majority of infant participants responded significantly on day 5 after the second dose. Similar to the other age groups, responses declined between day 5 and 7 after the second dose.

To investigate ALS responses to bacterial cell antigens, ALS IgA responses against cholera MP antigen were also evaluated in all age groups, but significant responses were only detected in adults (Fig. 2). After the first dose, a majority of participants responded already on day 5 and responses in about half of the subjects remained high whereas the rest declined on day 7. After the second dose, highest responses to MP were detected on day 5 in all participants and responses rapidly declined in a majority of participants on day 7. ALS responses to MP were low 4 days after both the first and second dose.

3.4. Plasma IgA and IgG responses

In adults, toddlers and young children, significantly higher responses in plasma were observed 7 days after the first dose compared to on day 4 or 5 (Fig. 3A–C). After the second dose, strong responses were observed already on day 4/5 and the responses were comparable or only slightly higher in magnitude on day 7. A majority of infants responded on day 5 and 7 after the second dose with comparable magnitudes of responses on the two days (Fig. 3D). Similar kinetics was observed for anti-CTB IgG antibody responses in plasma (Fig. 4).

3.5. Fecal SIgA responses

We also analyzed if vaccine specific SIgA antibodies can be detected in fecal samples from young children and infants. We attempted to analyze fecal samples from toddlers and adults, but after extraction, a majority of stool extracts from these participants contained low levels of total SIgA. In contrast, half of the samples

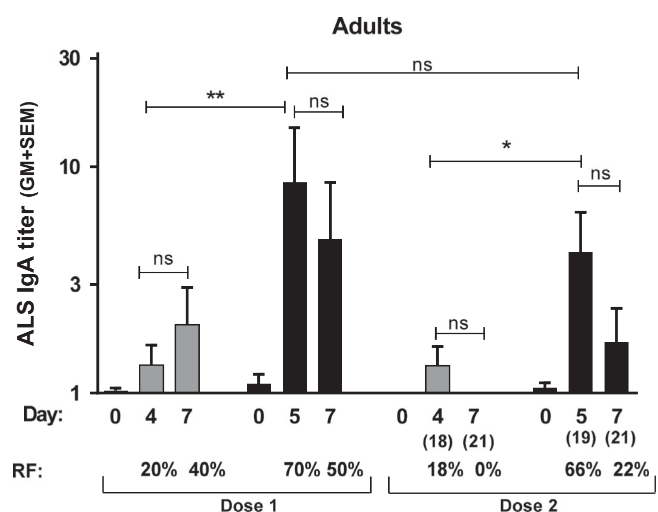

Fig. 2. Kinetics of IgA ALS responses to a cholera membrane preparation (MP) in adults after oral Dukoral vaccination. MP-specific IgA titers (GM + SEM) were analyzed in ALS samples from adults (sampling group A; n = 10, B; n = 10, C; n = 11 and D; n = 9) before the first immunization (day 0) and at the indicated time points after the first and second dose. Numbers in parentheses indicate day after the first immunization. Responder frequencies (RF) on the different days are shown below the graphs. $^{*}P < 0.05$, $^{**}P < 0.01$, ns; not significant.

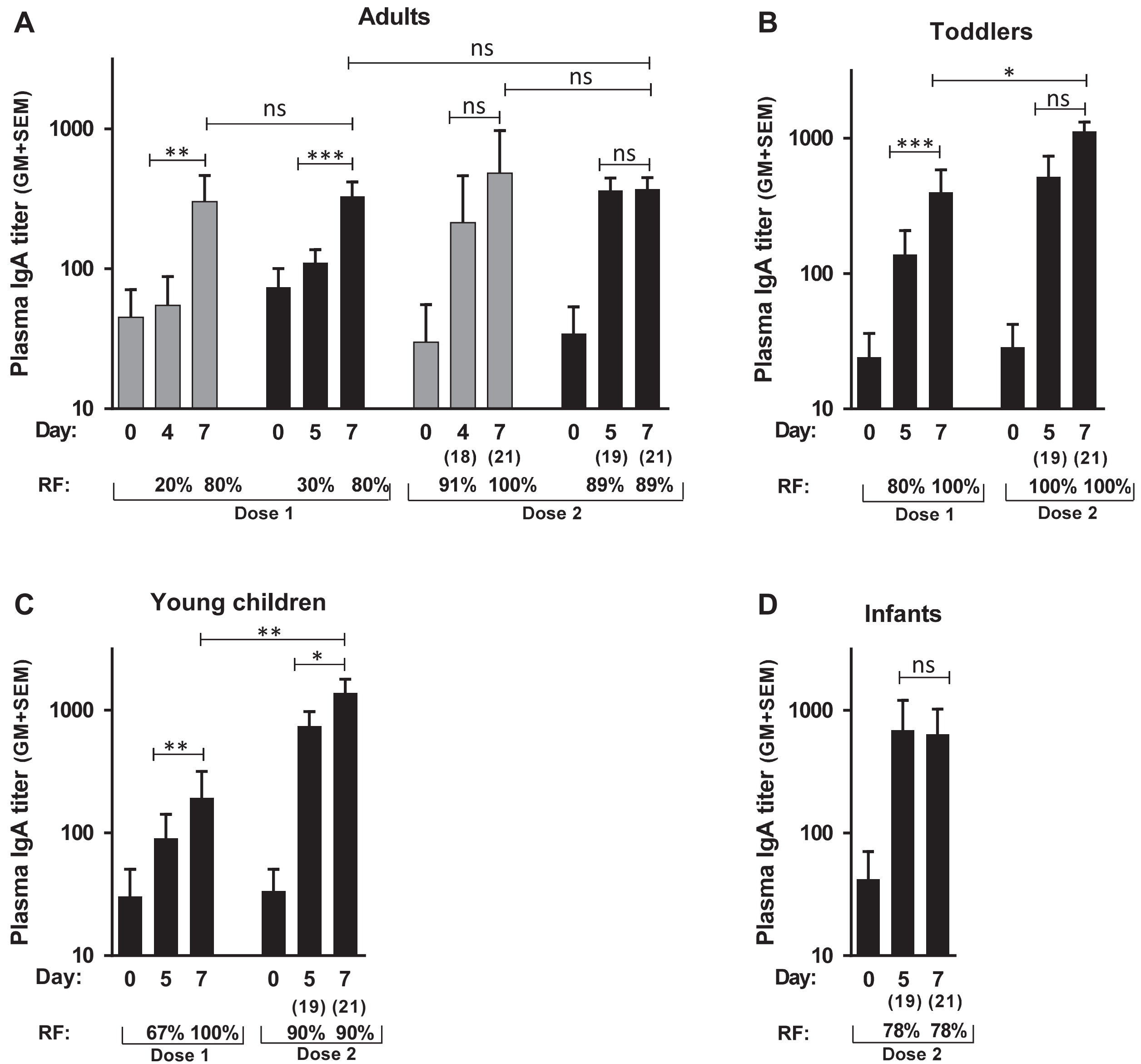

Fig. 3. Kinetics of plasma IgA antibody responses to CTB after oral Dukoral vaccination. CTB-specific IgA titers (GM + SEM) were analyzed in plasma samples from (A) adults (sampling group A; n = 10, B; n = 10, C; n = 11 and D; n = 9), (B) toddlers (group B; n = 10 and D; n = 9), (C) young children (group B; n = 9 and D; n = 10) and (D) infants (group D; n = 9) before the first immunization (day 0) and at the indicated time points after the first and second dose. Numbers in parentheses indicate day after the first immunization. Responder frequencies (RF) on the different days are shown below the graphs. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns; not significant.

from young children and infants fulfilled the inclusion criteria and could be analyzed. Since comparable responses to both CTB and MP were observed in young children and infants, we combined samples from these two age groups for this analysis. Responses to the first vaccine dose were low and infrequent (data not shown), but after the second dose, anti-CTB and anti-MP SIgA responses were observed in about half of the participants on day 5 (Fig. 5). The responses increased further on day 7, when responses to CTB and MP were detected in 80% and 60% of participants, respectively, and the antibody levels in the whole group were significantly increased compared to prevaccination levels.

4. Discussion

In this study we analyzed the kinetics of ASC responses in peripheral blood after oral cholera vaccination in adults, toddlers, young children and infants in Bangladesh. We showed that most adults and toddlers developed significant anti-CTB ALS IgA responses on day 5 after a first oral cholera vaccine dose and that the responses remained at similar levels on day 7. In contrast, very few young children developed responses to the first dose. However, the second dose induced significant anti-CTB ALS responses in all age groups with highest responses on day 5 among the time points studied and lower responses on day 7. Responses in adults on day 4 after either dose were significantly lower than on day 5. These results clearly demonstrate that in order to detect significant responses to the first dose in adults and toddlers, samples for ALS analysis can be collected on day 5 or 7. However, after the second dose, samples should be collected earlier (day 5) than previously recommended, i.e. day 7 [7], in all age groups. The poor responses detected on day 7 compared to day 5 after the second dose in all groups suggest that ASC responses to the second dose have

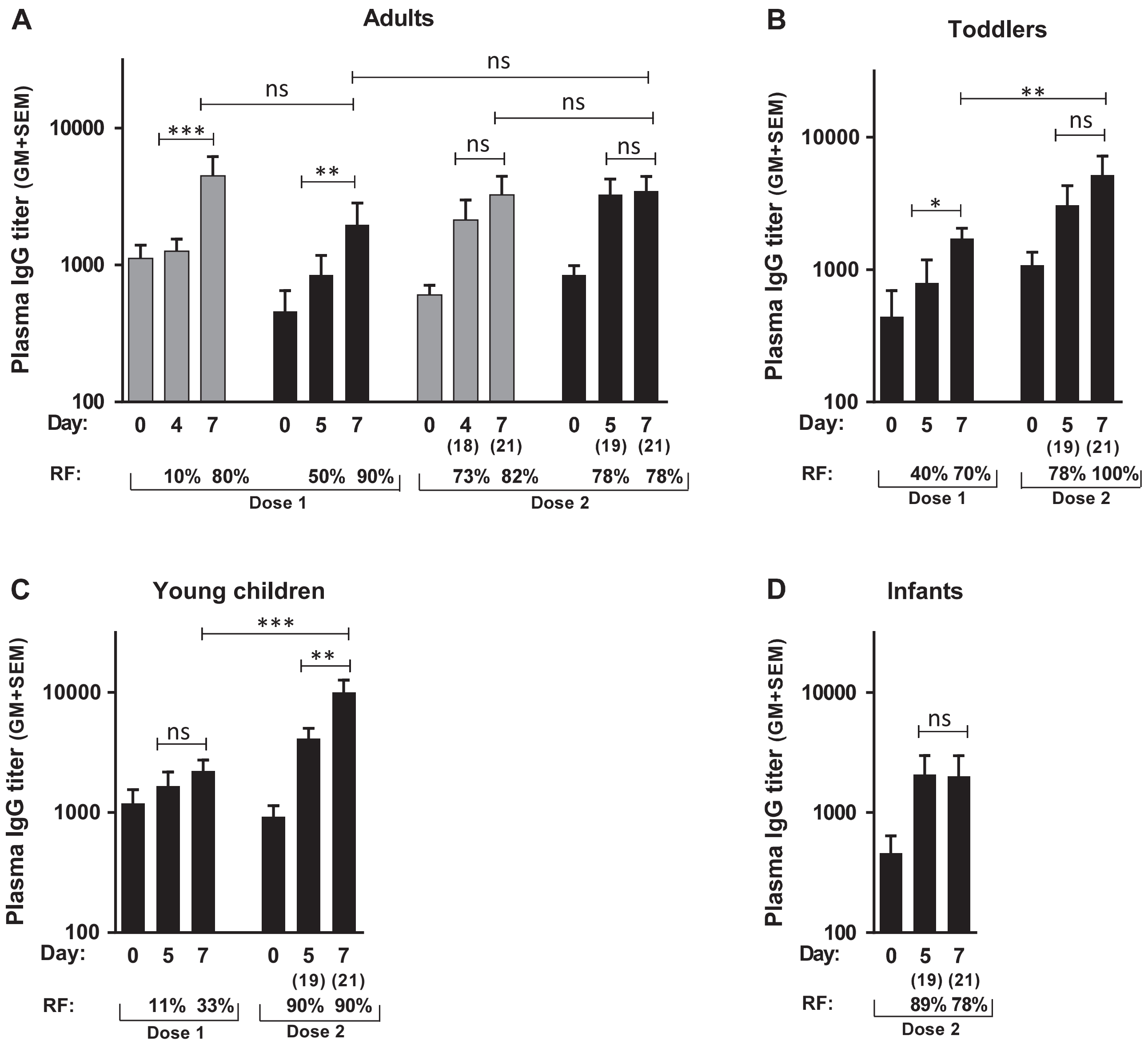

Fig. 4. Kinetics of plasma IgG antibody responses to CTB after oral Dukoral vaccination. CTB-specific IgA titers (GM + SEM) were analyzed in plasma samples from (A) adults (sampling group A; n = 10, B; n = 10, C; n = 11 and D; n = 9), (B) toddlers (group B; n = 10 and D; n = 9), (C) young children (group B; n = 9 and D; n = 10) and (D) infants (group D; n = 9) before the first immunization (day 0) and at the indicated time points after the first and second dose. Numbers in parentheses indicate day after the first immunization. Responder frequencies (RF) on the different days are shown below the graphs. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns; not significant.

probably been underestimated in many previous cholera and ETEC vaccine studies in children as well as adults, due to too late collection of samples [5,14–22]. Additional studies may be undertaken to determine if ALS responses to a second vaccine dose may increase even further from day 5 to day 6. However, since only a few samples can be collected from each infant and child due to ethical reasons, more detailed analysis of response kinetics is challenging.

The differences in responsiveness to the first dose at different ages may at least partly be explained by differences in natural priming by *V. cholerae* and ETEC producing LT, which is highly homologous to CT. Thus, adults and toddlers may have been more frequently primed by symptomatic or asymptomatic infections with *V. cholerae* and/or LT ETEC than younger children; such priming may have induced CT-reactive memory B cells which could be rapidly reactivated by a CTB-containing cholera vaccine, resulting in strong ALS responses already to the first vaccine dose. This is supported by observations in naïve Swedish adults, who similar to young Bangladeshi children also responded poorly to a first cholera vaccine dose [11,21]. However, priming by vaccination may differ from that induced by natural infection since responses developed more rapidly and were more transient after the second compared to the first vaccine dose in Bangladeshi adults and children. This difference may be a result of exposure to lower levels of CTB or LTB during natural infections than during vaccinations [27]. It is also possible that age-related differences in the mucosal immune system may explain the poor capacity of young children to respond to a single vaccine dose.

Most Bangladeshi adults also developed significant ALS responses to a *V. cholerae* membrane preparation (MP) containing both proteins and O1 LPS, but the responses to this preparation was too low in children and infants to allow analysis. The kinetics of ALS responses to the MP in adults was similar as responses to CTB with highest responses to dose 2 detected on day 5 among the time points studied. We have recently described similar

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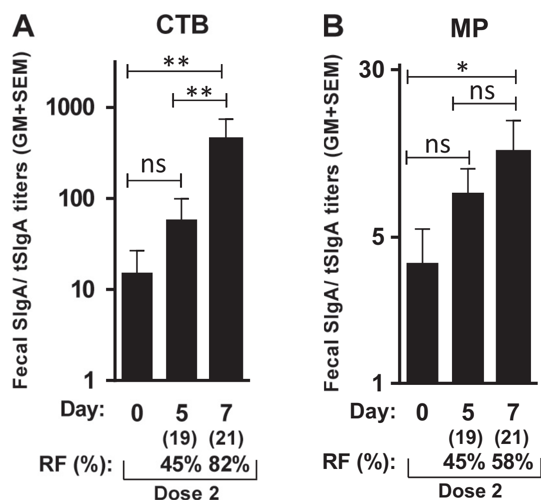

Fig. 5. SIgA antibody responses in fecal extracts after oral Dukoral vaccination. SIgA responses (specific SIgA/total SIgA) against (A) CTB and (B) a cholera membrane preparation (MP) were measured in young children (n = 6) and infants (n = 7) before immunization (day 0) and at the indicated time points after the second vaccine dose (total n = 13). Numbers in parentheses indicate day after the first immunization. Responder frequencies (RF) on the different days are shown below the graphs. $^{*}P < 0.05$, $^{**}P < 0.01$, ns; not significant.

kinetics of responses to a CTB/LTB toxoid and ETEC CFs after vaccination with the oral ETEC vaccine ETVAX, supporting similar kinetics of ALS responses to toxoid and bacterial cell antigens [12].

In most previous enteric vaccine trials, ASC responses have been evaluated by the ELISPOT method; however, the ALS method is more practical and gives comparable results with regard to frequencies and kinetics of responses [8,9,11,13,22]. Blood ASCs induced by oral cholera and typhoid vaccination express the gut homing marker $\alpha 4\beta 7$ integrin, supporting the capacity of such cells to home to intestinal mucosa [28,29]. Previous studies have also shown a relation between vaccine specific ASC responses in blood and in gastrointestinal mucosa as well as with IgA responses in fecal and lavage samples [3,5,21]. Thus, ASC responses may be used as good surrogate measures of mucosal immune responses.

In this study, we also show that fecal samples from young children and infants can be used to evaluate vaccine specific intestinal responses induced by oral vaccination. Thus, although antibodies to cholera and ETEC antigens have been observed in breast milk from women living in endemic areas [30–33] and about 90% of Bangladeshi children are breast fed for at least two years [34], breast milk antibodies do not seem to mask vaccine specific responses in fecal samples from infants and children to any significant degree. Similar to the blood ASC responses, fecal responses to the first dose of Dukoral were infrequent in young children, whereas significant responses to both CTB and MP were detected on day 7 after the second dose in a majority of children and infants. The delayed kinetics of fecal responses, compared to ALS responses which were detected already 5 days after the second dose, is consistent with mucosal homing of blood ASCs; plasma blasts appear transiently in blood on day 5 and then migrate to the intestinal mucosa to mature into plasma cells producing antibodies detectable in fecal samples a few days later. Our findings of later appearing fecal compared to ASC responses after the second dose is also consistent with previous studies in Swedish adults, where fecal responses to oral ETEC vaccines were detected 7–14 days after the booster dose [3,12,22,24]. We conclude that the fecal extraction method followed by assessment of SIgA responses to oral vaccines allow determination of fecal responses to oral vaccines in infants and young children.

We also analyzed CTB-specific responses in plasma in the different age groups. Significant IgA responses to the first dose were detected in all age groups studied, i.e. adults, toddlers and young children, with highest antibody levels detected on day 7. After the second dose, plasma IgA responses were detected already on day 5 and the responses remained high or even increased slightly on day 7 in all age-groups. Similar kinetics was observed for plasma IgG responses. These results suggest that significant plasma responses to the oral cholera vaccine can be detected on day 5 as well as 7 after the second dose in individuals of all ages.

In conclusion, the results from this study indicate that ASC/ALS responses to two doses of the oral cholera vaccine Dukoral and related oral vaccines should be analyzed earlier (day 5) in all age-groups than previously recommended (day 7), whereas fecal antibody responses should be analyzed later, i.e. ⩾7 days after the second vaccination. Significant plasma IgA and IgG responses to CTB could be detected both on day 5 and 7 after the second dose. The results from these studies are important for the interpretation of ASC results from previous studies of oral cholera and ETEC vaccines as well as for the design of ongoing and planned trials of a new oral inactivated ETEC vaccine in young children and infants in Bangladesh.

Contributors

MA, AMS, FQ, TRB, LNI and AL designed the studies. MA, SA, TRB and TAR performed the immunological analyses and AK and AS performed the clinical work. MA, AL, AMS and FQ wrote the manuscript. All authors contributed to the interpretation of results and critical review and revision of the manuscript and have approved the final version.

Disclosure

None of the authors have any conflicts of interest.

Acknowledgements

We acknowledge the support of the study participants as well as the dedicated field and laboratory workers in this study at icddr,b.

This work was supported by the European Union Seventh Framework Programme (FP7/2007–2013, grant agreement no. 261472-STOPENTERICS) and the Swedish Research Council (grant number VR 348-2013-6615). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. icddr,b is thankful to the donors for their support to its research efforts. icddr,b also gratefully acknowledges the following donors who provided unrestricted support: Government of the Peoplés Republic of Bangladesh, Global Affairs Canada (GAC), Swedish International Development Cooperation Agency (Sida) and the Department for International Development, UK (UKAid).

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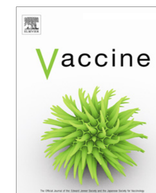

Evaluation of the safety and immunogenicity of the oral inactivated multivalent enterotoxigenic *Escherichia coli* vaccine ETVAX in Bangladeshi adults in a double-blind, randomized, placebo-controlled Phase I trial using electrochemiluminescence and ELISA assays for immunogenicity analyses

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A R T I C L E I N F O

Article history:
Received 20 August 2018
Received in revised form 25 October 2018
Accepted 14 November 2018
Available online xxxx

Keywords:
ETEC
Vaccine
Antibodies in lymphocyte supernatant
Antibody-secreting cell
IgA
Adult
ELISA
Electrochemiluminescence

A B S T R A C T

The safety and immunogenicity of the second generation oral enterotoxigenic *Escherichia coli* (ETEC) vaccine ETVAX, consisting of inactivated recombinant *E. coli* strains over-expressing the colonization factors (CFs) CFA/I, CS3, CS5 and CS6 and the heat labile toxoid LCTB*A*, were evaluated in Bangladeshi volunteers. To enable analysis of antibody responses against multiple vaccine antigens for subsequent use in small sample volumes from children, a sensitive electrochemiluminescence (ECL) assay for analysis of intestine-derived antibody-secreting cell responses using the antibodies in lymphocyte secretions (ALS) assay was established using Meso Scale Discovery technology.

Three groups of Bangladeshi adults (n = 15 per group) received two oral doses of ETVAX with or without double mutant LT (dmLT) adjuvant or placebo in the initial part of a randomized, double-blind, placebo-controlled, age-descending, dose-escalation trial. CF- and LTB-specific ALS and plasma IgA responses were analyzed by ECL and/or ELISA.

ETVAX was safe and well tolerated in the adults. Magnitudes of IgA ALS responses determined by ECL and ELISA correlated well (r = 0.85 to 0.98 for the five primary antigens, $P < 0.001$) and ECL was selected as the ALS readout method. ALS IgA responses against each of the primary antigens were detected in 87–100% of vaccinees after the first and in 100% after the second vaccine dose. Plasma IgA responses against different CFs and LTB were observed in 62–93% and 100% of vaccinees, respectively. No statistically significant adjuvant effect of dmLT on antibody responses to any antigen was detected, but the overall antigenic breadth of the plasma IgA response tended to favor the adjuvanted vaccine when responses to 4 or more or 5 vaccine antigens were considered. Responses in placebo recipients were infrequent and mainly detected against single antigens.

The promising results in adults supported testing ETVAX in descending age groups of children.

ClinicalTrials.gov Identifier: NCT02531802.

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Abbreviations: AEs, adverse events; ALS, antibodies in lymphocyte supernatant; ASC, antibody-secreting cell; CFs, colonization factors; CTB, cholera toxin B-subunit; dmLT, double mutant heat labile toxin; ECL, electrochemiluminescence; ETEC, enterotoxigenic *Escherichia coli*; LT, heat labile toxin; LCTB*A*, CTB/LTB hybrid protein; MSD, Meso Scale Discovery; PBMC, peripheral blood mononuclear cells; SAE, serious adverse event; SIgA, secretory IgA; ST, heat-stable toxin.

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https://doi.org/10.1016/j.vaccine.2018.11.040
0264-410X/© 2018 Published by Elsevier Ltd.

1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) remains one of the most common bacterial pathogens causing diarrhea in children as well as adults in developing countries [1–3]. ETEC colonize the intestinal mucosa by different colonization factors (CFs) and subsequently release heat labile (LT) and/or heat stable (ST) enterotoxins causing diarrhea [4,5]. Since ETEC do not invade intestinal epithelial cells, immune protection is most likely provided by locally produced secretory IgA (SIgA) antibodies [6]. To achieve broad protection against ETEC, immunity against both LT and CFs would be advantageous; anti-ST immunity is difficult to induce in a safe manner due to the small size of the ST peptide and potential immunological cross-reactivity with human guanylin and uroguanylin [5–9].

One approach to achieve immunity to ETEC which has been extensively investigated is to immunize orally with killed bacteria expressing common CFs and to administer the vaccine with a LT-like toxoid [6,7]. A first generation whole cell vaccine, consisting of formalin-inactivated ETEC bacteria expressing CFA/I and CS1 to CS5, combined with cholera toxin B-subunit (CTB), which is highly homologous to LT B-subunit (LTB), was shown to be immunogenic in children and adults in endemic areas and to protect against moderate/severe diarrhea in adult travellers [10–12]. However, a full dose of the vaccine caused vomiting in 6–17 months old Bangladeshi children; hence fractionated doses were tested and a quarter dose was found to be safe [13]. The vaccine did not confer protection in 6–24 months old Egyptian children, but both vaccinated and unvaccinated children experienced mainly mild disease during the study period and the impact on moderate/severe diarrhea could not be evaluated [7]. Based on these results, an improved second generation multivalent oral ETEC vaccine (ETVAX) was developed, containing inactivated *E. coli* strains over-expressing CFA/I, CS3, CS5 and CS6 antigens at significantly higher levels than the bacteria in the first generation vaccine [14]. In contrast to the first generation vaccine, ETVAX includes the common CF CS6 in an immunogenic form and is administered together with the more LT-like toxoid LCTB*A* [15,16]. To further enhance the immunogenicity of the vaccine, particularly when given at low dosages, the vaccine may be combined with the double mutant LT (dmLT) adjuvant [14,17]. When tested in Swedish adults, ETVAX with or without dmLT was found to be safe and to induce significant fecal SIgA responses as well as IgA antibody-secreting cell (ASC) responses, as determined by analysis of specific antibodies secreted into the culture supernatants using the antibodies in lymphocyte supernatants (ALS) assay, against all CFs and LTB [16]. Addition of 10 μg dmLT to the vaccine significantly enhanced ALS responses to CS6 only [16]. ETVAX also induced long-lasting immunological memory in Swedish adults [18]. Our recent results also demonstrated that ETVAX may induce IgA antibody responses that cross-react with CFs belonging to the same CF families, possibly expanding the coverage of the vaccine [19]. These successful results have led to clinical evaluation of ETVAX in a large phase I/II trial in adults and lower age groups (5 years to 6 months) in Bangladesh. Since limited blood volumes can be collected from children and infants, a new assay for analysis of ALS responses using small sample volumes needed to be established and optimized using samples from adults to allow subsequent analyses in younger age groups.

After infection or vaccination in the intestinal mucosa, activated intestinal lymphocytes transiently migrate to the circulation before homing back to the mucosa. Therefore, ASCs present among peripheral blood mononuclear cells (PBMCs) are suitable surrogate markers of mucosal immunity [13,20–24], particularly if blood samples are collected at optimal time points after lymphocyte activation [16,18,25,26]. ASC responses can be analyzed using ELISPOT or by ALS, which is commonly based on ELISA techniques for detection of secreted antibodies in the culture medium, and results from the two assays correlate very well with each other [15,24,27,28]. In clinical trials with multivalent vaccines such as ETVAX, ALS responses to several vaccine antigens are usually evaluated, requiring relatively large volumes of ALS specimens. Hence, to allow analysis of ALS responses to multiple vaccine antigens in children, sensitive and specific procedures are required.

An electrochemiluminescence (ECL) assay may be used as an alternative to conventional colorimetric ELISA methods. ECL-based techniques generally have high sensitivity, good reproducibility, and a wide dynamic analysis range reducing the need for sample titration, and allow smaller sample volumes than ELISA [29–31]. The Meso Scale Discovery (MSD) technology is an ECL method utilizing sulfo-tag-labelled detection antibodies, which emit light upon electrochemical stimulation via electrodes integrated in the bottom of carbon-based microtiter plates. Soluble antigens bind efficiently to the carbon surface of the wells, without the need for conjugation or chemical antigen modification. MSD-based ECL assays have previously been established for analysis of serum antibodies against different parenteral vaccines and infections, using both single- and multiplex analysis platforms [32–35], but have, to our knowledge, not previously been reported for analysis of serum antibody or ASC/ALS responses after mucosal vaccination.

The primary objective of the study presented here was to evaluate the safety of ETVAX alone or together with dmLT adjuvant in Bangladeshi adults; the secondary objective was to evaluate the immunogenicity of ETVAX as a basis for studies in Bangladeshi children and infants. The study also included establishment of an ECL assay based on the MSD ECL technology for detection of ALS responses against multiple ETVAX antigens in small sample volumes for subsequent use in studies in younger age groups.

2. Materials and methods

2.1. Study design

This study was planned as a randomized, double-blinded, placebo-controlled, dose-escalation and age-descending Phase I/II trial conducted in Mirpur, Dhaka, Bangladesh. Healthy adults (Phase I) and children (Phase II, including older children 24–59 months, younger children 12–23 months and infants 6–11 months) were sequentially immunized and safety was confirmed at each dosage level in each age group before the study proceeded to the next stage. The complete study design can be found at ClinicalTrials.cov (Identifier: NCT02531802). Data from the adult part were unblinded before the studies in children were completed, consistent with the study protocol. Data from the adult part of the study is reported here; results from the younger age groups will be reported later.

The study was performed in accordance with the Declaration of Helsinki and approved by the Research Review and Ethical Review Committees of the International Review Board of the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) and the Western Institutional Review Board, USA. The trial was conducted under oversight of the Federal Drug Administration, USA. Informed written consent was obtained from each participant.

2.2. Vaccine and dmLT adjuvant

Two doses of the 2nd generation multivalent ETEC vaccine (ETVAX, produced for Scandinavian Biopharma by Biovian Oy, Tykistökatu 6B FI-20520 Turku, Finland, lot BX1003574) was given orally to study participants at two weeks interval. The vaccine con-

sists of 8x10^{10} inactivated *E. coli* bacteria (strains ETEX 21–24, 2×10^{10} bacteria/strain) recombinantly induced to express high amounts of CFA/I, CS3, CS5 and CS6 antigens, respectively and mixed with 1 mg of LCTB*A* protein, a recombinantly produced LTB/CTB hybrid protein [14,16,36]. This single dose level was administered to adults. A vaccine dose was suspended in 150 ml bicarbonate buffer (Recipharm, Sweden) and given alone or together with 10 μg of the adjuvant dmLT (R192G/L211A, lot 1575, Walter Reed Army Institute, Silver Spring, MD, USA) [17]. Children and infants received lower dosages of vaccine (1/8–1/2 adult dosage) alone or combined with dmLT (2.5–10 μg) or placebo, as described in detail in the Clinical.Trials.gov registry.

2.3. Screening and randomization

Healthy adult participants (18–45 years) were screened 4–7 days prior to enrollment for eligibility based on medical history, clinical examination and laboratory tests. Key exclusion criteria included known significant systemic disorders, congenital abdominal disorders, diarrheal or febrile illnesses in the past 7 days, positive pregnancy test and use of antibiotics or immunosuppressant medications within 14 days prior to enrollment. Participants positive for ETEC, *Shigella*, *Vibrio cholerae* or *Salmonella*, as determined by culture of a fecal sample collected during the screening period, were also excluded. All participants were from similar socioeconomic backgrounds. A randomization list with treatment information for each participant was generated by the statistical and data management group at the EMMES Corporation, Rockville, MD, USA and maintained by a pharmacist, who was not otherwise involved in the study.

Enrolled participants were randomized in a double blinded manner into one of three groups (1:1:1): (A) placebo (n = 15, buffer alone), (B) ETVAX vaccine (n = 15) or (C) ETVAX with 10 μg dmLT adjuvant (n = 15). Participants were not allowed to eat or drink one hour before and after treatment.

2.4. Follow-up for adverse reactions

Study participants were given memory aids to record solicited symptoms (e.g. nausea, vomiting, diarrhea, loose stools, abdominal pain and fever). Both solicited and unsolicited adverse events (AEs) were assessed by trained study staff by carrying out active surveillance by home visits each day for 7 days after each vaccination and again on day 28 ± 2 and 182 ± 14 after the first vaccination. Clinical chemistry and hematology tests were performed at screening and on days 7 ± 1 after the first immunization, physical examinations were performed at screening and on day 19 + 1 and 42 ± 4 after the first immunization. Safety in adults was evaluated until day 3 after the second dose by an independent protocol safety team and a data safety monitoring committee before vaccinations in descending age groups of children were initiated.

2.5. Measurement of immune responses

Heparinized venous blood samples were obtained prior to the first immunization (day −7 to −4), on day 7 ± 1 after the first dose and on day 19 (5 days after the 2nd dose); i.e. optimal time points for assessment of circulating ASCs after oral vaccination [26]. Mucosal immune responses were evaluated by measuring vaccine specific IgA antibodies in ALS specimens and systemic immune responses by measuring IgA and IgG antibodies levels in plasma [14,15]. PBMCs and plasma were separated from the blood by density-gradient centrifugation on Ficoll-Isopaque (Pharmacia, Uppsala, Sweden). Plasma specimens were preserved at −20 °C. For ALS preparation, 10^7 PBMCs/ml were cultured in 96-well plates (200 μl/well) for 48 h at 37 °C with 5% CO_2, supernatants were collected and stored at −70 °C [28].

IgA antibody levels in ALS specimens were analyzed by a novel ECL assay established for all five primary vaccine antigens (CFA/I, CS3, CS5, CS6 and LTB). The optimization of the new ECL assay was performed using samples collected from previous trials of ETVAX in Sweden [16,18] and included titration of antigen coating concentrations, testing of coating buffer compositions, incubation times and temperatures. The optimized protocol was then used to measure IgA responses in ALS specimens from Bangladeshi adults. Standard binding MULTI ARRAY 96-well plates (MSD) were coated with 0.5 μg/ml of CF or LTB (without GM1) in carbonate buffer (pH 9.8) at +4 °C overnight, after 10 min shaking at room temperature. Plates were blocked (1% casein in PBS, Thermo Fisher, 1 h) and ALS samples diluted 1 to 5 in 1% casein-PBS were added (25 μl/well, single wells) for 2 h. IgA antibodies were detected using anti-Human NHP IgA Sulfo Tag antibodies (1 μg/ml, MSD, 1 h). Read Buffer T (1X concentration, MSD) was added and reactions were immediately analyzed on a Meso Quickplex SQ 120 reader (MSD). Plates were washed after each step using 0.05% Tween PBS. Incubations were performed at room temperature with shaking. Plasma and also ALS samples were analyzed by ELISA using plates coated with CFA/I, CS3, CS5, CS6 and GM1 plus LTB [15,16]. The recombinant CFA/I antigen used in the ELISA and ECL assays was prepared from a rough *E. coli* strain at the University of Gothenburg. The CS3 and CS5 antigens were prepared from O139 LPS *E. coli* strains at Scandinavian Biopharma, Sweden. The CS6 antigen was a kind gift from F. Cassels, USA. LTB was produced at Scandinavian Biopharma. The CFA/I, CS6 and LTB antigens were completely LPS free. Silver staining indicated some LPS (approximately 40 μg LPS per mg protein) in the CS3 but not in the CS5 antigen. For control experiments, a CS3 antigen provided by NIH, USA (now available through BEI Resources NR-49113), containing only trace amounts of LPS (1 EU per mg of protein, corresponding to 100 pg LPS per mg of protein), was used. Repeat analyses on about 20% of samples were conducted for control purposes.

Stool samples were collected prior to the first immunization (day −7 to −4) and day 7, 19, and 28 post immunization and extracted, stored at −70 °C and analyzed as previously described [22,26,37,38]. However, a majority of the samples contained low and variable levels of total SIgA, and did not meet the inclusion criteria for analysis [13,16,38]. Therefore, analysis of CF and LTB specific antibody responses were not evaluated in fecal specimens from adults.

2.6. Statistical analysis

The sample size was selected to detect frequent AEs. Given a sample size of 15 adults, and subsequently the same sample size in toddlers and younger children each receiving one of varying dose levels of ETVAX with and without dmLT, the study would have an approximately 80% and 90% chance of observing at least one serious AE (SAE) or AE of special interest for events that occur at a rate of 10.3% and 14.3%, respectively. Additionally, if no SAEs are observed in 15 participants, the upper bound of the one-sided 95% confidence interval on the rate of SAE occurrence is approximately 18%.

All results were log10 transformed. The magnitudes of immune responses (fold rises) were calculated as the post-immunization divided by pre-immunization antibody levels and twofold increases were regarded as responses [16]. Comparisons of pre- and post-levels within groups were evaluated using a paired *t*-test and responses between different groups using an unpaired *t*-test. Responder frequencies were evaluated using Fisher exact test. The Pearson correlation coefficient was used to measure the

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correlations. P-values < 0.05 were considered as significant. All statistical analyses were performed with GraphPad Prism version 5.0.

3. Results

3.1. Study participants

Forty five healthy adults were enrolled, from 135 participants screened, and were randomized into the three treatment groups (groups A-C; Fig. 1). The age distributions were comparable among the groups but more females than males were enrolled in all groups (Table 1).

3.2. Adverse events and safety

ETVAX administered alone or in combination with 10 µg dmLT was safe and well tolerated in all adults with only two solicited

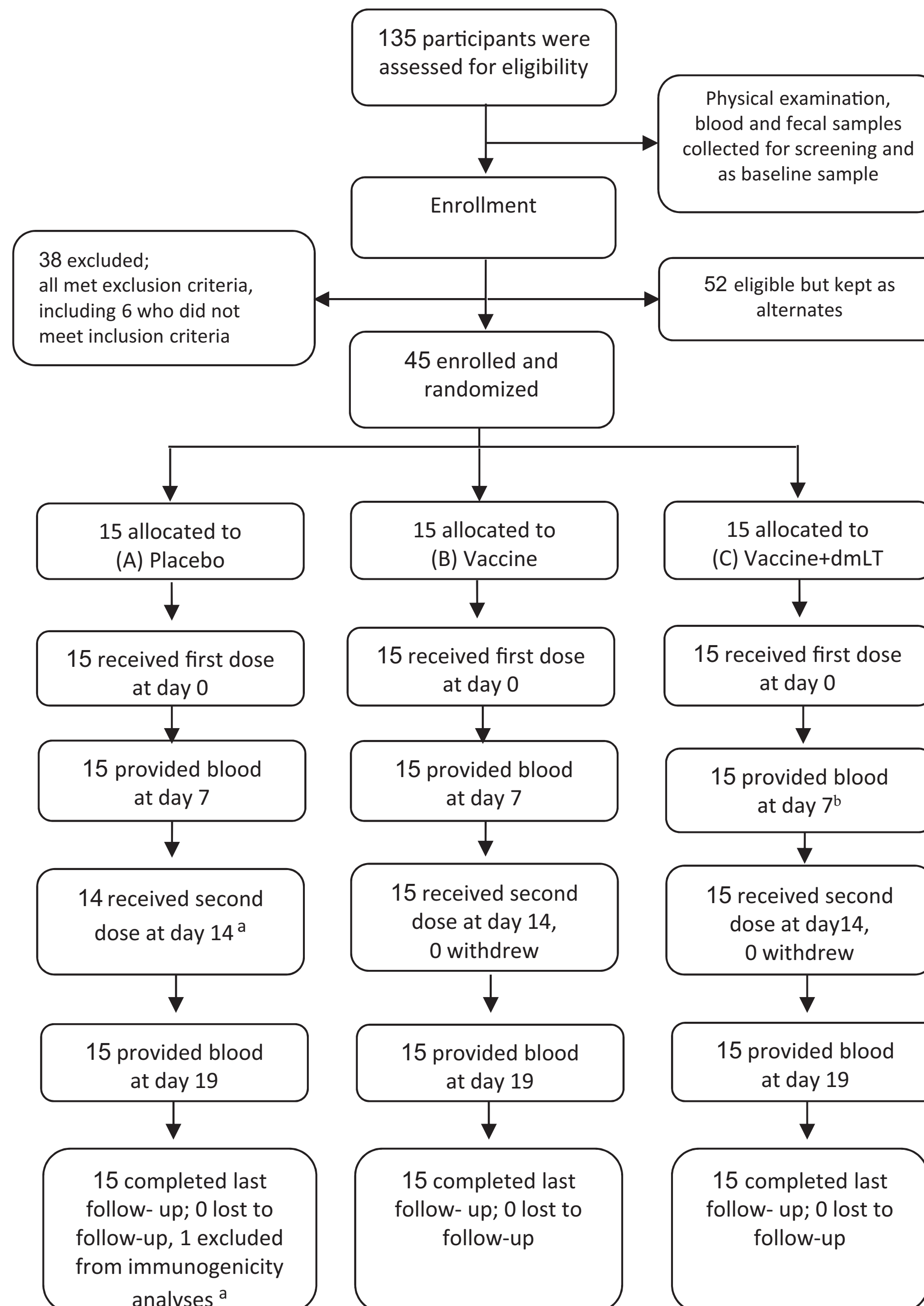

Fig. 1. Trial profile.

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Table 1
Participant demographics (safety analysis set).

	(A) Placebo (n = 15)	(B) Vaccine (n = 15)	(C) Vaccine + dmLT (n = 15)	Total (n = 45)
Age (years)				
Mean (SD)	31.5 (5.6)	30.1 (7.0)	29.4 (7.0)	30.3 (6.4)
Range	20–40	19–44	20–41	19–44
Gender (no and freq. of participants)				
Female	10 (66.7%)	8 (53.3%)	11 (73.3%)	29 (64.4%)
Male	5 (33.3%)	7 (46.7%)	4 (26.7%)	16 (35.6%)

Table 2
Solicited AEs in study participants after one or two vaccine doses (safety analysis set).

	(A) Placebo (n = 15)	(B) Vaccine (n = 15)	(C) Vaccine + dmLT (n = 15)
Nausea	0	0	0
Vomiting	0	0	0
Diarrhea	0	0	0
Loose Stools	0	0	0
Abdominal Pain	0	0	0
Fever	1 (6.7%)[a]	0	1 (6.7%)[b]
Total	1 (6.7%)	0	1 (6.7%)

[a] Mild fever appeared and resolved spontaneously on the same day of receiving placebo.
[b] Mild fever appeared two days after receiving the first vaccine dose and resolved one day later.

AEs and no SAEs reported at any time point during the study period (Table 2).

3.3. Immunogenicity

3.3.1. Correlations between results from ECL and ELISA assays

To determine if an ECL assay could be used to measure ASC responses using low antibody concentrations in small volumes of lymphocyte secretions, IgA levels in ALS samples were measured in parallel by ELISA and the novel ECL assay. High correlations between the magnitudes of ALS responses (fold rises in antibody levels in post- compared to pre-vaccination samples) determined by the optimized ECL and the traditional ELISA assays were found for all five primary antigens using samples collected 7 days after the first dose ($r = 0.88–0.96$, $P < 0.001$, data not shown) as well as 5 days after the second dose ($r = 0.85-0.98$, $P < 0.001$, Fig. 2). Magnitudes of responses to CFA/I and CS5 were 3- to 4-fold higher determined by ECL compared to ELISA, but still correlated very well, whereas the magnitudes of responses to CS3, CS6 and LTB were comparable in the two assays. Considering the excellent performance of the ECL assay and the relatively small volumes of ALS samples available from some participants, the ECL assay was selected as the primary readout for the ALS responses. Unblinding of the results revealed that the vast majority of placebo recipients had responses to all antigens below the 2-fold cut-off for positivity in both assays (Fig. 2). However, the discrimination between vaccinees and placebo recipients was more distinct using the ECL assay, since a few more placebo recipients showed weak ALS responses to CFs in ELISA (Fig. 2).

3.3.2. Intestine derived IgA ALS responses

ECL analysis of ALS specimens showed that ETVAX alone or with dmLT adjuvant elicited significant increases of specific IgA antibody responses against all four vaccine CFs and LTB (Fig. 3 and Table 3). The magnitudes of responses were high already 7 days after the first vaccine dose compared to pre-immunization levels and responses remained at similar levels (anti-CS3 and anti-LTB responses) or increased further 5 days (day 19) after the second dose ($P < 0.001$ for comparisons of magnitudes of ALS responses on day 7 or day 19 versus pre-vaccination levels for all antigens). Responder frequencies among the vaccinees were 90–100% against all antigens already after the first dose and 100% after the second dose (Table 3). In contrast, very few placebo recipients responded to any of the antigens, and then only to CS3 (4/14 subjects) or CS6 (1/14). Both the magnitudes of responses and frequencies of responders were significantly higher ($P < 0.001$) in the vaccinated groups compared to the placebo group on both day 7 and 19 (Table 3).

Addition of dmLT to the vaccine did not have any significant impact on the ALS responses. Magnitudes and frequencies of responses were similar both on day 7 and day 19 in the vaccine compared to the vaccine + dmLT groups (Fig. 3 and Table 3). The ALS responder frequencies recorded using ELISA and ECL assays were similar in the vaccine groups, but slightly higher frequencies of responders were recorded in the placebo group using ELISA (Table 4).

Since weak CF responses were detected among a few placebo recipients, all antigens used for the immunological analyses were tested for possible LPS contamination by sensitive silver staining techniques. These analyses indicated some LPS contamination in the CS3 antigen (approximately 40 μg per mg protein), but not in any of the other antigens. Therefore, control analyses using a CS3 antigen preparation that contained only trace amounts of LPS (100 pg per mg of protein) provided by NIH were performed. Only one of the four placebo recipients responding to the original CS3 preparation prepared from an *E. coli* strain expressing O139 LPS showed responses to the low LPS CS3 antigen when tested by ECL (Table 3). In contrast, when ALS samples from vaccine recipients were retested using the low LPS CS3, including samples from all weak CS3 responders and a subset of medium or strong responders, comparable ALS responses to both CS3 preparations were recorded.

3.3.3. Plasma antibody responses

CF specific IgA and LTB specific IgA and IgG responses were measured in plasma using ELISA. Significant plasma responses were found 7 days after the first dose and the magnitudes of responses against all vaccine antigens remained at comparable levels 5 days after the second vaccination ($P < 0.001$ for comparisons of plasma response magnitudes on day 7 or day 19 versus pre-vaccination IgA levels; Fig. 4 and Table 5). Highest and consistently most frequent IgA responses in all groups were observed against LTB (100% after both doses) and CS3 (about 90%), whereas 60–93% of subjects responded to CFA/I, CS5 and CS6 (Table 5). In contrast, very few participants responded in the placebo group to any of the antigens, except CS3 (3/14 subjects) and LTB (1/14 and 2/14 subjects for IgA and IgG, respectively). Both the magnitudes and frequencies of responders were significantly higher in the vaccine groups compared to the placebo group after both treatment doses ($P < 0.001$). All weak CS3 plasma responses among both placebo and vaccine recipients were confirmed using the low LPS CS3 preparation (Table 5).

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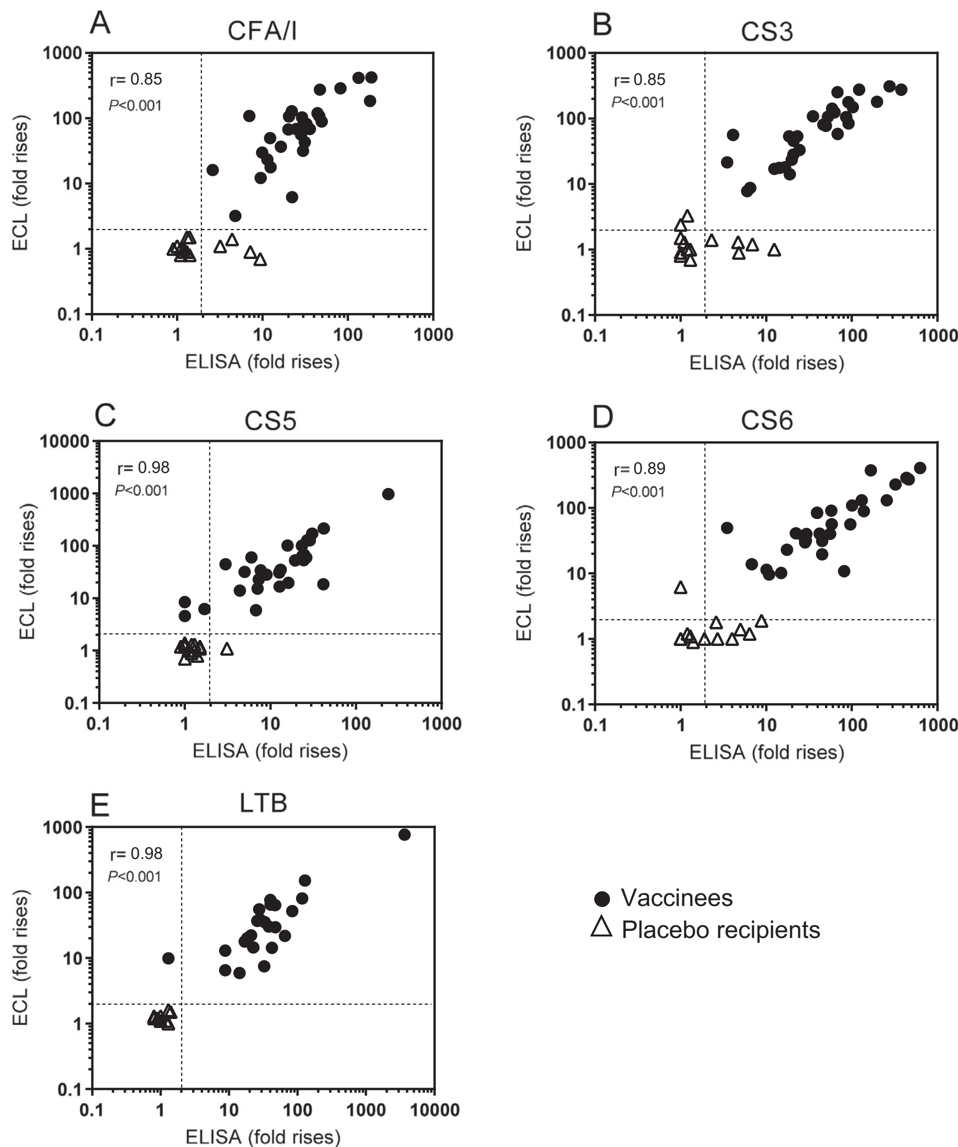

Fig. 2. Comparison of ALS results obtained using the novel ECL and the traditional ELISA assays. Magnitudes of responses (fold rises) of specific IgA antibodies against (A) CFA/I, (B) CS3, (C) CS5, (D) CS6 and (E) LTB antigens were analyzed in day 19 ALS specimens (n = 30 vaccinees and n = 14 placebo recipients). Dashed lines indicate fold rises ≥2.

Addition of dmLT to the vaccine did not cause any significant effect on plasma antibody responses. However, there was a trend for higher (10–30%) responder frequencies against CFA/I (dose 1), CS5 (dose 2) and CS6 (both doses) in participants receiving ETVAX + dmLT compared to ETVAX alone (Table 5, $P > 0.05$). A similar trend was also apparent when responses to multiple antigens were considered together; 93% of the participants immunized with ETVAX + dmLT and 62% with the vaccine alone responded to ≥4 antigens and 57% (ETVAX + dmLT) versus 38% (ETVAX) to all 5 antigens, respectively (Table 6). The breadth of the antigenic response at less stringent levels (≤ 3 antigens) were essentially identical between subjects receiving the vaccine alone or with dmLT.

4. Discussion

This is the first clinical trial of the second generation oral inactivated multivalent ETEC vaccine ETVAX conducted in an ETEC endemic country. We show that two full doses of ETVAX, both when administered alone and with 10 µg dmLT adjuvant, were safe and well tolerated in the healthy Bangladeshi adults tested, confirming previous safety data from studies in Swedish adults [15,16,18]. We further demonstrate that the vaccine is highly immunogenic in Bangladeshi adults, inducing intestine-derived ASC responses as detected by the ALS method in all, and plasma antibody responses in a majority, of the vaccinees to all primary vaccine antigens (four CFs and LTB). We also show that the ALS results obtained using a novel ECL assay and traditional ELISA correlated very well for all vaccine CFs and LTB. Since the ECL assay using the MSD platform only requires 5 µl of ALS sample compared to 75 µl in ELISA for determination of responses to each antigen, only 150 µl of sample (collected from a single ALS well on a 96-well plate, containing 2x10^6 PBMCs derived from 1 to 2 ml blood) can be used for at least 30 ECL analyses compared to two ELISA tests. Thus, the ECL assay is highly useful for future assessment of intestine-derived IgA ALS responses in pediatric studies, and also in studies of ASC responses to multivalent vaccines. Furthermore, the wide dynamic range of the ECL assay made it possible to use a single sample dilution, while sample titration is normally used in ELISA. Given the favorable results, we selected the ECL assay

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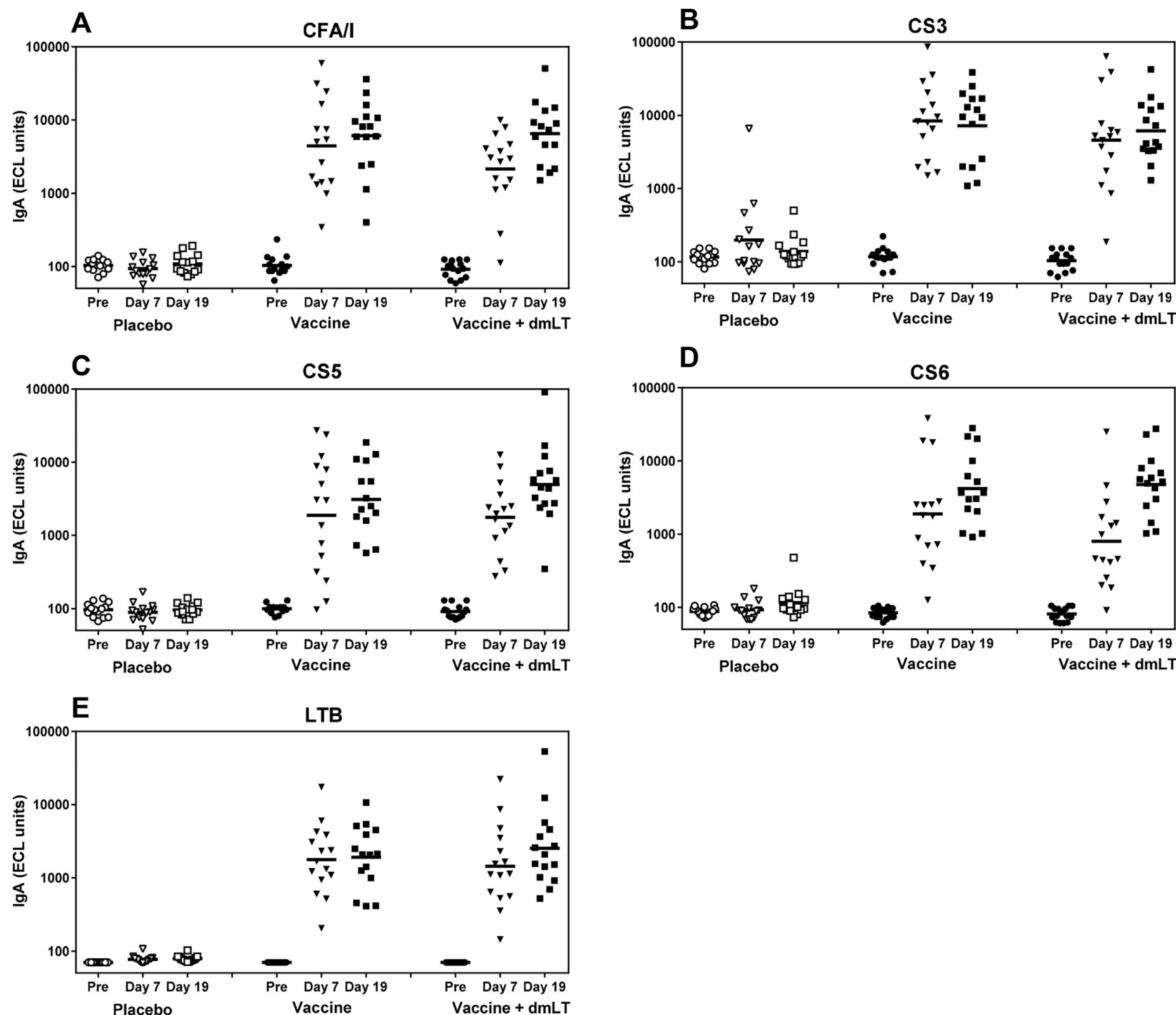

Fig. 3. ALS IgA responses in participants receiving placebo (n = 14), vaccine (n = 15) and vaccine plus dmLT (n = 15). Levels of IgA antibodies specific for (A) CFA/I, (B) CS3, (C) CS5, (D) CS6 and (E) LTB were analyzed using the ECL assay 4–7 days before administration of the first treatment dose (Pre), 7 days after the first dose (Day 7) and 5 days after the second dose (Day 19), respectively. Each symbol represents one subject and horizontal lines indicate geometric means.

as the primary method for assessing ALS responses both in adults and subsequently in children in the trial. Slightly higher rates of weak antibody responses were seen in the placebo group using ELISA compared to ECL, indicating that the ECL assay may also be more specific.

Evaluation of intestine-derived ALS responses using ECL showed that 90–100% of the vaccinees responded to all primary vaccine antigens after a single dose and 100% after the second dose, even in the absence of dmLT adjuvant. These responses were clearly more frequent than in Swedish vaccinees; ALS responses after the first dose were infrequent in the Swedes and 60–70% responded against CFA/I, CS5 and CS6 and 80–100% against LTB and CS3 after the second dose [16]. Our finding that almost all Bangladeshis responded strongly already after the first dose and that very few showed enhanced responses after the second dose support development of anamnestic responses in the Bangladeshi volunteers, since we have previously shown that only primed subjects respond strongly to a single dose of ETVAX [18]. The results are also consistent with the high prevalence of ETEC infection in Bangladesh. During the first two years of life, 20% of all diarrheal cases at the trial site in Mirpur in urban Dhaka have been shown to be due to ETEC infection, with an incidence of 0.5 episode/child/year [2] and ETEC is also an important diarrheal pathogen in Bangladeshi adults [39]. Analysis of the relative distribution of CFs in ETEC isolates from diarrhea cases at the icddr,b hospital between year 2007 and 2012 suggests that the predominant CFs on ETEC isolated from diarrhea cases are CS5 + CS6, CFA/I, CS7, CS17, CS1 + CS3, CS6 and CS14 [40]. Thus, since ETEC infection with strains expressing vaccine CFs and related CFs are common in the study population, we postulate that almost all Bangladeshi adults have been naturally primed with vaccine related ETEC strains in this study, thus explaining the high and frequent responses already after the first vaccine dose.

In this study, comparable or even slightly higher ASC/ALS responses were found against most vaccine antigens 5 days after the second compared to 7 days after the first vaccine dose. This is in contrast to previous studies of ETEC and cholera vaccines, where lower ASC responses were usually found after the second dose [10,41,42]. However, in most of these earlier studies blood samples were collected 7 days after the second immunization,

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Table 3
Magnitudes[a] and frequencies[b] of ALS IgA responses against the five primary vaccine antigens determined by ECL after administration of one and two treatment doses (per protocol analysis set).

	Dose 1[c]			Dose 2[c]		
	Placebo	Vaccine	Vaccine + dmLT	Placebo	Vaccine	Vaccine + dmLT
CFA/I						
GM	0.9	42.8	23.4	1.0	52.7	71.1
RF	0/14 (0%)	15/15 (100%)	14/15 (93.3%)	0/14 (0%)	15/15 (100%)	15/15 (100%)
CS3						
GM	1.7	71.9	44.7	1.2	61.8	59.8
RF	4/14 (28.6%)[d]	15/15 (100%)	14/15 (93.3%)	2/14 (14.3%)[d]	15/15 (100%)	15/15 (100%)
CS5						
GM	0.9	19.0	19.4	1.0	31.4	54.3
RF	0/14 (0%)	13/15 (86.7%)	15/15 (100%)	0/14 (0%)	15/15 (100%)	15/15 (100%)
CS6						
GM	1.1	22.4	9.9	1.3	49.7	59.1
RF	0/14 (0%)	14/15 (93.3%)	13/15 (86.7%)	1/14 (7.1%)	15/15 (100%)	15/15 (100%)
LTB						
GM	1.1	25.3	20.6	1.1	27.3	36.0
RF	0/14 (0%)	15/15 (100%)	15/15 (100%)	0/14 (0%)	15/15 (100%)	15/15 (100%)

[a] Magnitudes of responses were expressed as geometric mean (GM) of fold rises.
[b] Fold rises ≥2 were considered as responses [16] and responder frequencies (RF) using this cut-off are indicated.
[c] Magnitudes and responder frequencies were significantly higher ($P < 0.001$) in the vaccine and vaccine + dmLT groups, respectively, compared to the placebo group.
[d] Only 1/14 placebo recipients (7%) showed responses to CS3 after dose 1 as well as dose 2 when evaluated using a CS3 antigen preparation containing only trace amounts of LPS (100 pg per mg of protein), whereas vaccinees had comparable responses to both CS3 preparations.

Table 4
Magnitudes[a] and frequencies[b] of ALS IgA responses against the five primary vaccine antigens determined by ELISA after administration of one and two treatment doses (per protocol analysis set).

	Dose 1[c]			Dose 2[c]		
	Placebo	Vaccine	Vaccine + dmLT	Placebo	Vaccine	Vaccine + dmLT
CFA/I						
GM	1.0	17.1	10.1	1.6	27.5	23.6
RF	0/14 (0%)	14/15 (93.3%)	13/15 (86.7%)	4/14(28.6%)	15/15 (100%)	15/15 (100%)
CS3						
GM	1.5	55.0	17.8	1.9	46.9	27.3
RF	3/14 (21.4%)	15/15 (100%)	12/15 (80%)	5/14 (35.7%)	15/15 (100%)	15/15 (100%)
CS5						
GM	1.0	5.3	5.6	1.1	9.1	15.2
RF	0/14 (0%)	7/13 (53.8%)	11/15 (73.3)	1/14 (7.1%)	12/14 (85.7%)	14/15 (93.3%)
CS6						
GM	1.2	19.0	6.2	2.0	65.7	43.3
RF	1/14 (7.1%)	13/15 (86.7%)	9/15 (60%)	6/14 (42.9%)	15/15 (100%)	15/15 (100%)
LTB						
GM	1.0	21.2	23.6	1.0	30.5	38.9
RF	0/11 (0%)	11/12 (91.7%)	11/13 (84.6%)	0/11 (0%)	12/12 (100%)	11/12 (91.7%)

[a] Magnitudes of responses were expressed as geometric mean (GM) of fold rises.
[b] Fold rises ≥2 were considered as responses [16] and responder frequencies (RF) using this cut-off are indicated.
[c] Magnitudes and responder frequencies were significantly higher ($P < 0.001$) in the vaccine and vaccine + dmLT groups, respectively, compared to the placebo group.

which seems to be too late to capture maximal ASC responses, and this has most likely resulted in underestimation of ASC responses in previous trials [11,23,43,44]. We have recently shown, using a model oral cholera vaccine, Dukoral, that ASC/ALS responses peak 5 rather than 7 days after a second or late booster vaccination both in children and adults in Bangladesh [26], therefore different and more optimal time points were selected for sampling in the present study.

Plasma antibody responses against CFs and LTB were also found to be high in this endemic setting. In analogy with findings in adult Swedes almost all the Bangladeshi vaccinees responded with plasma IgA (100%) and IgG (73–93%) anti-LTB responses after either dose of ETVAX whereas anti-CF responses were considerably more frequent in the Bangladeshi than the Swedish adults (60–90% vs. 3–19%) [16]. The comparatively high pre-vaccination plasma titers recorded in several of the Bangladeshi vaccinees is consistent with previous natural ETEC priming [45,46]. Our results suggest that numerous ETEC infections may prime the systemic CF-specific B cells more efficiently than vaccination, since Swedish adults had weak serum responses to CFs also after receiving a late booster dose (third dose) of ETVAX [18].

A few placebo recipients responded against CS3 (14–28%), CS6 (0–7%) and LTB (0–14%) in plasma and/or ALS, as assessed by ECL and ELISA, respectively, but most responses were of low magnitudes and responses were mostly recorded against a single antigen and only in one type of specimen for each subject. The weak responses may be due to contamination (e.g. by LPS) in the antigen preparations. This is supported by our observation that only one of the four placebo recipients showed ALS responses to a low LPS CS3 preparation, whereas all tested vaccinees responded with comparable magnitudes to this CS3 antigen. The other antigens used in the ELISA and ECL assays were produced from LPS negative

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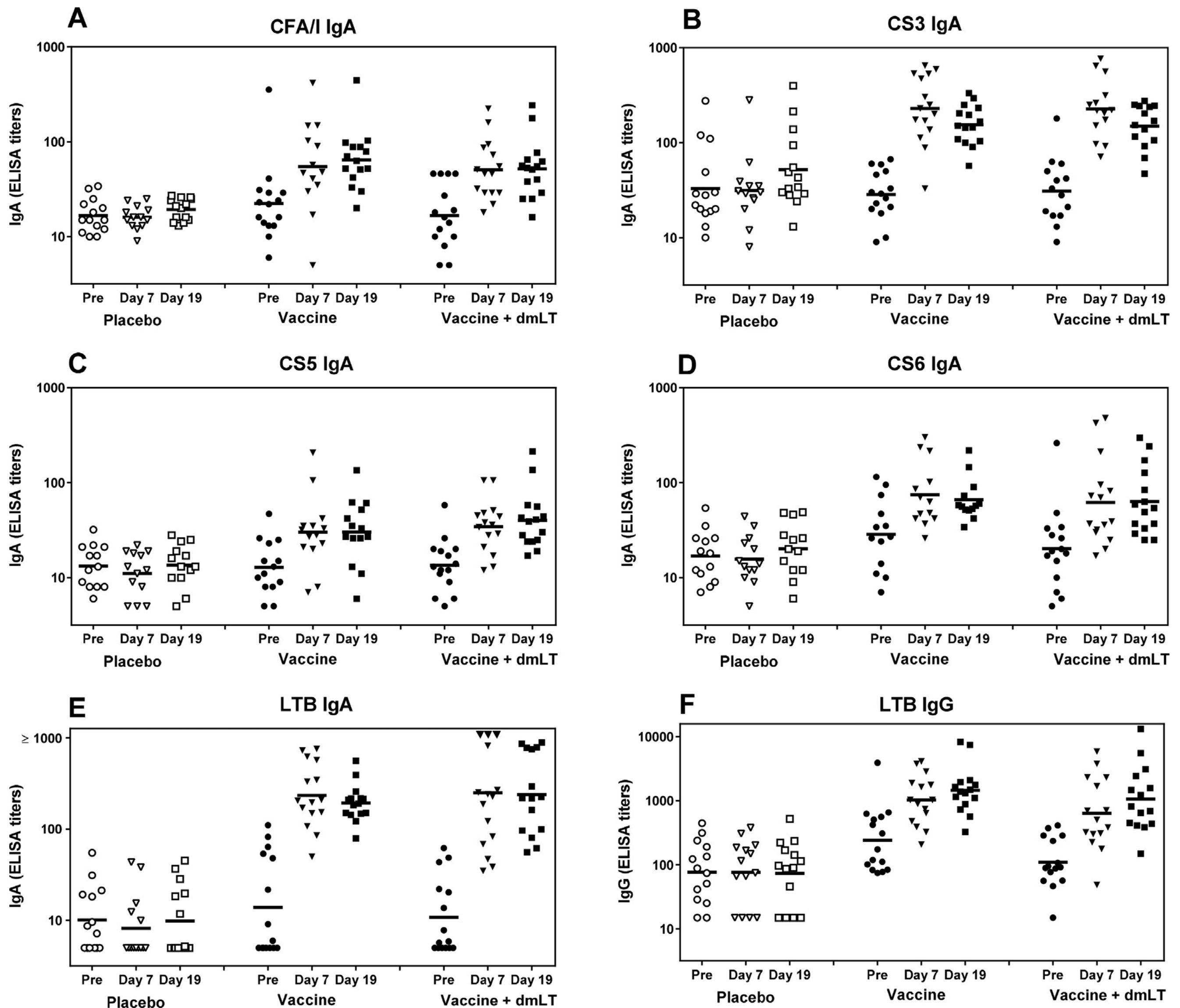

Fig. 4. Plasma IgA and IgG responses in participants receiving placebo (n = 13–14), vaccine (n = 13–15) and vaccine plus dmLT (n = 14–15). Titers of IgA antibodies specific for (A) CFA/I, (B) CS3, (C) CS5, (D) CS6, (E) LTB and (F) IgG antibodies specific for LTB were analyzed using ELISA. Samples were collected 4–7 days before administration of the first treatment dose (Pre), 7 days after the first dose (Day 7) and 5 days after the second dose (Day 19), respectively. Each symbol represents one subject and horizontal lines indicate geometric means. Limited sample volumes precluded analysis of plasma responses in a few participants (1–2 per group).

rough strains or purified to a higher degree than the CS3 antigen. These findings support the importance of using highly purified antigens for sensitive immunological analyses. However, plasma responses to CS3 were confirmed in placebo recipients using the low LPS antigen, suggesting that responses among placebo recipients may also be due to infection with ETEC, cholera or other bacteria expressing homologous or related antigens during the study period.

The first generation ETEC vaccine induced ASC and plasma responses against CFs and CTB in a majority of adult Bangladeshi vaccinees [10]. However, a direct comparison with the present study could not be done since different methods for monitoring immune responses have been used, i.e. ALS instead of ELISPOT, use of optimal time points for assessing ASC/ALS responses and more pure CF antigen preparations than were previously available. The only relevant comparison between immune responses induced by the first and the second generation vaccines is probably of plasma responses to the toxoid component of the vaccines, since the methods for evaluation of such responses have changed very little and time points for assessment of plasma responses are not as critical. The LCTB*A* toxoid, which is a hybrid between LTB and CTB [36], induced strong and frequent (100%) plasma IgA responses against LTB which were similar or even slightly more frequent than IgA responses against CTB after vaccination with the first generation rCTB-CF ETEC vaccine or the CTB-containing Dukoral vaccine [10,11,26]. Furthermore, a previous study in Sweden has shown that significantly higher systemic responses to LTB were induced by vaccination with LCTB*A* compared to vaccination with CTB in the same trial and that antibodies induced by LCTB*A* also react efficiently with CTB [15]. Thus, the LCTB*A* toxoid may induce protection against both LT-ETEC and cholera in endemic settings.

The systemic responses were measured by ELISA in the present study, since larger volumes of plasma compared to ALS samples were available. However, we have recently shown that plasma IgA and IgG responses to ETEC CFs and LTB can be efficiently evaluated by the ECL method (Svennerholm et al., unpublished data). MSD ECL assays have previously been used to evaluate serum IgG antibody responses to licensed parenteral vaccines or vaccine

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Table 5
Magnitudes[a] and frequencies[b] of plasma IgA and IgG responses against the five primary vaccine antigens determined by ELISA after administration of one and two treatment doses (per protocol analysis set).

	Dose 1[c]			Dose 2[c]		
	Placebo	Vaccine	Vaccine + dmLT	Placebo	Vaccine	Vaccine + dmLT
CFA/I IgA						
GM	1.0	2.1	3.1	1.2	2.9	3.1
RF	0/13 (0%)	7/13 (53.8%)	11/15 (73.3%)	0/14 (0%)	11/15 (73.3%)	12/15 (80.0%)
CS3 IgA						
GM	1.1	8.0	7.3	1.6	5.4	4.8
RF	2/13 (15.4%)[d]	13/15 (86.7%)	13/14 (92.9%)	3/14 (21.4%)[d]	14/15 (93.3%)	12/14 (85.7%)
CS5 IgA						
GM	0.8	2.3	2.5	1.0	2.4	3.0
RF	0/13 (0%)	9/14 (64.3%)	10/15 (66.7%)	0/13 (0%)	7/14 (50.0%)	12/15 (80.0%)
CS6 IgA						
GM	0.9	2.6	3.1	1.2	2.3	3.1
RF	0/13 (0%)	6/13 (46.2%)	10/15 (66.7%)	0/13 (0%)	8/13 (61.5%)	11/15 (73.3%)
LTB IgA						
GM	0.8	16.8	23.1	1.0	13.9	22.0
RF	0/14 (0%)	15/15 (100%)	15/15 (100%)	1/14 (7.1%)	15/15 (100%)	15/15 (100%)
LTB IgG						
GM	1.0	4.2	5.8	1.0	6.1	9.8
RF	1/14 (7.1%)	11/15 (73.3%)	13/15 (86.7%)	2/14 (14.3%)	14/15 (93.3%)	13/15 (86.7%)

[a] Magnitudes of responses were expressed as geometric mean (GM) of fold rises.
[b] Fold rises ≥2 were considered as responses [16] and responder frequencies (RF) using this cut-off are indicated.
[c] Magnitudes and responder frequencies were significantly higher ($P < 0.001$) in the vaccine and vaccine + dmLT groups, respectively, compared to the placebo group.
[d] Comparable CS3 responses were observed using a CS3 antigen preparation containing only trace amounts of LPS (100 pg per mg of protein) among both placebo and vaccine recipients.

Table 6
Frequencies of IgA responders against different numbers (1–5) of primary vaccine antigens in plasma after one or two doses.

Frequency of subjects[a] responding to	Placebo	Vaccine[c]	Vaccine + dmLT[c,d]
5 antigens[b]	0/13 (0%)	5/13 (38%)	8/14 (57%)
≥4 antigens	0/13 (0%)	8/13 (62%)	13/14 (93%)
≥3 antigens	0/13 (0%)	12/13 (92%)	13/14 (93%)
≥2 antigens	1/13 (8%)	12/13 (92%)	13/14 (93%)
≥1 antigens	3/13 (20%)	13/13 (100%)	14/14 (100%)
0 antigens	10/13 (77%)	0/13 (0%)	0/14 (0%)

[a] Subjects from whom plasma specimens were available for analysis of responses to all five antigens.
[b] LTB, CFA/I, CS3, CS5, CS6.
[c] Responder frequencies against ≥1–5 antigens were significantly higher ($P < 0.05$) in the vaccine and vaccine + dmLT groups, respectively, compared to the placebo group.
[d] Responder frequencies were not significantly different ($P > 0.05$) in the vaccine compared to the vaccine + dmLT group. However, including dmLT in the vaccine formulation appeared to favor a broader antigenic response than that achieved with the vaccine alone, particularly when plasma IgA response frequencies to ≥4 or 5 antigens were considered.

candidates, e.g. vaccines against *Streptococcus pneumoniae*, human papilloma virus, respiratory syncytial virus and rabies, utilizing carbohydrates, proteins or virus like particles for coating in both single and multiplex assays [32–35,47]. However, to our knowledge, this is the first study where the MSD ECL technology has been used to successfully evaluate intestine-derived IgA antibody responses induced by an oral vaccine. In the future, the possibility of establishing a multiplex ECL method including several ETEC antigens in the same microtiter well may be considered to further reduce sample volumes and working time.

We also attempted to measure mucosal IgA responses more directly, in fecal extracts. However, since fecal extracts from the Bangladeshi adults contained low and very variable levels of total SIgA and did not fulfill the assay criteria for specimen inclusion, such analyses were not meaningful. Similar low and variable SIgA levels were observed in adult Bangladeshis in a recent cholera vaccine study [26]. In contrast, fecal samples from Swedish adults extracted using the same method contained higher SIgA levels and robust SIgA responses were recorded against all primary vaccine antigens after ETVAX vaccination [16]. The reason for this discrepancy may be due to several factors, including different diets and microbiota. Previous analyses of vaccine induced immune responses in fecal samples from Bangladeshi adults also resulted in higher IgA levels [10,22,23], but total IgA rather than total SIgA was measured in these studies and some IgA transudated from serum may thus have been detected. Importantly, preliminary data from infants participating in the ETVAX trial in Bangladesh, as well as previous data from children vaccinated with ETEC or cholera vaccines, support that fecal samples from Bangladeshi infants contain higher and more stable levels of SIgA than adult samples and therefore are more suitable for immunogenicity analyses [10,11,26].

Addition of dmLT adjuvant to the vaccine had no apparent effect on the ALS responses in the adults in this study. In contrast, in adult Swedes, 10 μg of dmLT significantly enhanced ASC responses to CS6, which is the CF present in the lowest amount in the vaccine [16]. However, a higher dose of dmLT, 25 μg, had no significant influence on ALS responses, and hence 10 μg dmLT was tested in the Bangladeshi adults [16]. The reason why dmLT did not induce a similar enhancement of the ALS responses in this trial may be explained by extensive natural priming of the Bangladeshis, limiting the ability of dmLT to further enhance the already strong ALS responses. However, a trend (not statistically significant) for an adjuvant effect was observed for plasma responses in the Bangladeshis against the CFs present in the lowest amounts in the vaccine (CS5 and CS6), consistent with the dose-sparing effect of dmLT previously observed in ETVAX vaccinated mice [14]. The potential effect of dmLT when co-administered with a lower dose of vaccine will be evaluated in continued studies in children and infants. A strong trend toward an adjuvant effect was also noted when the antigenic breadth of the plasma IgA response was evaluated, particularly when responses to 4 or more or 5 vaccine antigens were considered. This apparent impact of dmLT on the breadth of

Please cite this article as: M. Akhtar, M. I. Chowdhury, T. R. Bhuiyan et al., Evaluation of the safety and immunogenicity of the oral inactivated multivalent enterotoxigenic *Escherichia coli* vaccine ETVAX in Bangladeshi adults in a double-blind, randomized, placebo-controlled Phase I trial using electrochemiluminescence and ELISA assays for immunogenicity analyses, Vaccine, https://doi.org/10.1016/j.vaccine.2018.11.040

the vaccine-specific antibody responses will also be evaluated more fully as lower vaccine doses are given in follow-on studies in children and infants.

Overall, this trial demonstrates that the oral ETVAX vaccine is safe in adults and induces strong mucosal as well as systemic immune responses against key vaccine antigens. These findings have provided a base for further evaluation of this vaccine in descending age groups in children and infants. The ECL assay established here allows sensitive and specific analysis of ALS responses to all key vaccine components even in young children and infants.

Acknowledgements

We acknowledge the support of the study participants as well as the dedicated field and laboratory workers in this study at icddr,b. The work was supported by icddr,b with funding from PATH through its enteric vaccine project (grant no. GR01263) from the Bill and Melinda Gates Foundation, the Swedish Research Council (grant number VR 348-2013-6615), the British Department of International Development (program number 300341-107) and the Swedish Foundation for Strategic Research (grant number SB12-0072). icddr,b is thankful to the institutional donors for their support to its research efforts. icddr,b is also grateful to the governments of Bangladesh, Canada, Sweden and the UK for providing core/unrestricted support.

Contributors

AMS, FQ, AL, TRB, NC, NM, AF, RW, LB and TW designed the studies. MIC, TA, FK, AK, and MZS performed the clinical work. MA, JK, TAR, YAB and SIAR performed the immunological analyses. MA, AL, FQ, LNI and AMS wrote the manuscript. All authors contributed to the interpretation of results and critical review and revision of the manuscript and have approved the final version.

Roles of the funding sources

The nonprofit organization PATH Vaccine Solution participated in the design of the studies, interpretation of results and reviewed the manuscript. The other funding sources only contributed financially to the study.

Conflict of interest statement

NC is employee and minority shareholder of Scandinavian Biopharma Holding AB, which holds certain commercial rights to the vaccine tested in this study. NC has patents PCT/EP2012/067598 and PCT/EP2011/065784. A-M Svennerholm is shareholder of the biotech company Gotovax AB that may receive a small royalty on sales of the ETEC vaccine if it becomes a commercial product. The other authors declare that they have no conflicts of interest.

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