Characterization of the A Subunit Epitopes in Immunogenicity and Enterotoxicity of Enterotoxigenic *Escherichia coli* (ETEC) Heat-Labile Toxin

by

Jiachen Huang

B.S., Huazhong Agricultural University, 2010

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Diagnostic Medicine/ Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2017

Approved by:

Major Professor
Weiping Zhang

Copyright

Abstract

Heat-labile enterotoxin (LT) is one of the most important toxins produced by enterotoxigenic *Escherichia coli* (ETEC). It consists of one A subunit (LTA) for intracellular enzymatic activity and five B subunits (LTB) forming a pentamer for binding to host cell receptors. In the last few decades, LT has been extensively studied as a strong immune stimulator, as well as an effective adjuvant with multiple immunomodulatory properties. To understand better the features of LT, we mapped B-cell linear epitopes of the enzymatic A subunit and explored the relationship between these epitopes and the toxicity of LT. Eleven B-cell linear (continuous) epitopes were *in silico* identified based on online software. In part one of the study, all 11 epitopes were fused into a modified ovalbumin carrier protein respectively. Each recombinant fusion protein was expressed and purified, and was characterized in ELISA and Western Blot using the anti-LT serum. Moreover, each fusion protein was used to immunize mice to determine immune response specific to LT *in vivo*. A total of eleven epitopes were identified from the LTA subunit. Results showed that anti-LT serum recognized all 11 epitopes, while the mouse immunization study indicated that antibodies derived from epitope 7 ($_{105}$SPHPYEQEVSA$_{115}$) had significantly greater anti-LT antibody titers and neutralized LT enterotoxicity more efficiently than the other epitopes. In part two of the study, to test whether individual epitope plays a role in LT toxicity, 10 epitopes in the A1 domain of LTA subunit were replaced by a foreign peptide respectively and the mutant LTs were examined for enterotoxicity. Data indicated that all these LT mutants showed enterotoxicity abolished. However, these LT mutants formed holotoxin structure and bound to GM1 *in vitro*. Results from this study indicated that replacement of these LT epitopes did not affect the forming of LT holotoxin structure and the binding to host receptors, indicating LT can serve as a safe vaccine platform to carry foreign

antigens. With the immunodominant epitope 7 being kept while other LTA epitopes replaced by epitopes from other ETEC virulence factors, this platform can be used to construct broadly protective multivalent mucosal vaccines against ETEC, and perhaps as a universal platform for vaccines against other enteric diseases.

Table of Contents

List of Figures

List of Tables

Acknowledgements

First, I would like to appreciate my major supervisor, Dr. Weiping Zhang, for providing me a chance to work his laboratory. His patience, motivation, and earnest attitude impressed me a lot. Under his mentorship during the two years, I've learned not only the knowledge, but also how to be a good researcher.

I'm very grateful to my committee members, Dr. T.G.Nagaraja, Dr. Jodi Mcgill and Dr. Brain Geisbrecht, for their guidance and insightful comments on my program and thesis. I would like to thank Dr. M.M. Chengappa and Dr. Sanjeev Narayanan for their valuable suggestions on my study. Thanks to Dr. Denver Marlow, Dr. Tracy Meisner and other faculties and staffs in CVM comparative medicine group for their assistance in the mice study.

My sincere appreciation goes to two Postdocs, Dr. Qiangde Duan, Dr. Rahul Nandre, and graduate students, Ti Lu, and Carolina Garcia as well as other lab members and friends for their kind help.

Finally, I would like to thank my parents for their support and continuous encouragement throughout my years of study. This accomplishment would not have been possible without them.

Chapter 1 - Literature Review

Enterotoxigenic *Escherichia coli*

Enterotoxigenic *Escherichia coli* (ETEC) are Gram-negative bacteria which cause severe watery diarrhea in human and animals [1]. In 1967, Gyles and Barnum reported that enterotoxins in the bacterial lysates caused fluid accumulation in ligated rabbit ileal loop [2]. Moon reported that 40% activity of enterotoxin secreted in the culture supernatant was retained after 30 minutes of incubation at 65 °C, the same percentage of activity was lost after dialysis of cell-free ETEC products [3], indicating there were two types of enterotoxin produced by the bacteria. One is relatively larger in size and could be inactivated by heat, which is called heat-labile enterotoxin (LT). The other is much smaller and stable at 65 °C, thus is called heat-stable enterotoxin (ST). Both LT and ST are encoded in plasmids which can be transferred to different *E. coli* strain; *E. coli* strains producing either one or both enterotoxins are called enterotoxigenic *E. coli* (ETEC) [4].

In addition to LT and ST enterotoxins, the proteinaceous structure on the surface of ETEC, which are called colonization factors (CFs), also play an important role in ETEC pathogenesis. CFs mediate bacterial adherence to host intestinal epithelial cells and initiate bacterial colonization [5]. To date, more than 20 colonization factors have been identified from ETEC strains associated with diarrhea in humans. These CFs were further divided into different subgroups of colonization factor antigens (CFAs) or coli surface antigens (CS). CFs differ genetically, structurally and immunologically, and presented in fimbrial, fibrillar, helical or nonfimbrial morphology [6]. CFA/I, II, and IV were the most associated with ETEC diarrhea and other CFs were observed prevalent in different regions [7]. ETEC strains causing diarrhea in

animals express a variety of CFs as well, among them K88, K99, and 987P are most prevalent [8].

After attaching to host intestinal surface, ETEC produce STa (heat-stable type I toxin) and/or LT that stimulate intestinal ion and fluid secretion through different pathways. STa is an 18 or 19-amino acid peptide and poorly immunogenic. STa shares similar structures with endogenous peptides called guanylin and uroguanylin. STa and both guanylin peptides bind to an intracellular receptor called guanylate cyclase C (GC-C), leading to an increasing level of intracellular cyclic GMP (cGMP). Elevation of cGMP level activates the ion channels on cell membrane, resulting in water hypersecretion [9; 10]. LT causes diarrhea in a similar pattern, which first binds to cell surface through ganglioside GM1 receptors and then enters into cytosol. The enzymatic A subunit activates the adenylate cyclase, thus stimulates the production of cyclic AMP (cAMP) which further cause the malfunction of ion channels [11]. Together, these two enterotoxins contribute to the excess secretion of fluid and electrolytes and osmotic diarrhea.

ETEC bacteria cause diarrhea in not only pigs, they are also responsible for lethal diarrhea in newborn calves, small ruminants, and other farm animals [12]. Moreover, ETEC are the top cause of diarrhea in humans. The first human case of ETEC infection was reported in 1956. Researchers isolated *E. coli* strains which caused diarrheal disease in a pattern similar to *Vibrio cholerae*, and found these *E. coli* strains were related with animal ETEC strains [13]. Characterized by its potent enterotoxins and the diversity of CF adhesins, ETEC is believed to be one of the leading cause of bacterial originated diarrhea, posing a great threat to people, especially children under 5 years of age in developing countries, as well as children and adults traveling from developed countries to developing countries [14]. Data showed that each year around 380,000 human deaths are related to ETEC [7]. Based on clinical data collected from

1970 to 1999, a child born in a developing country experienced 3-5 ETEC associated diarrheal episodes per year before the age of 5, and nearly 50 million children of this age group carry ETEC without showing any symptoms [15]. Therefore, ETEC remains one of the most important diarrheal pathogen among children in the developing world. Although the incidence of ETEC infection decreases with age, ETEC is still the most frequent diarrheal pathogens that affects millions of persons who travel to developing countries every year [16]. It is reported that about 30-60% cases of travelers' diarrhea are caused by ETEC, although most infections are self-eliminated and don't need special treatment, it can be risky to populations with weak or deficient immunity such as kids, pregnant women, and people with other pre-existing diseases [17].

ETEC is transmitted through contaminated food and water in areas lack of hygiene practice and supply of safe drinking water. The outbreak of ETEC can also happen in non-endemic countries through food importation [18]. In some areas in South America, Africa, and Southeast Asia, ETEC infections manifest seasonality, with most frequent during warm periods of the year, suggesting travelers are more susceptible at these times. Malnutrition, which is common in children from developing countries, is another factor that contributes to more severe ETEC infection [19]. The loss of fluids and electrolytes lead to severe dehydration, therefore, rehydration intravenously or orally is crucial when treating ETEC diarrhea [20]. When bacterial infection is diagnosed in adult cases, antimicrobial treatment is another option because bacterial load can be effectively controlled and the duration of illness can be shortened by some antimicrobials [21]. However, antimicrobial treatment is not advised to treat diarrhea in children, since these diseases are often caused by multiple bacterial and viral agents [22]. Due to over use of antibiotics, increasing numbers of antibiotic-resistant diarrheagenic *E. coli* including ETEC have rapidly spread worldwide, causing serious problems to the control of diarrheal

diseases in developing countries. It is reported that 86.4% of *E. coli* isolated from diarrheal children were resistant to at least three different classes of antibiotics [23]. Studies from Bangladesh and India have also found multiple antimicrobial resistance in ETEC isolates [24; 25]. Rapid buildup of antibiotic resistance in ETEC bacteria alarms public health communities to seek other approaches to prevent and treat ETEC infection. While community- or country-wide sanitation system and supply of safe drinking water and good practice off food preparation and distribution can block the transmission of the diseases effectively [26], development of effective ETEC vaccines is surfaced as the leading prevention approach for ETEC prevention.

Heat-labile enterotoxins

Heat-labile enterotoxin (LT) one of the most important virulence factors of ETEC. A review paper which summarized 17,205 ETEC isolates in 136 studies from 1961 to 2009 and showed that 60% of ETEC isolates expressed LT either alone (27%) or in combination with ST (33%) [7]. There are two major groups of LT. Type I LT (LT-I) has over 80% homology in amino acid sequence with cholera toxin (CT) expressed by *Vibrio cholerae*. Type II LT includes LT-IIa, LT-IIb, and LT-IIc, which are genetically different from LT-I and do not cross react with LT-I immunologically [27]. LT-I is found in both human and animal ETEC strains, while type II LT is mainly reported in animal associated ETEC. In this study, we will only focus on human LT-I, the word LT in this thesis refers to LT-I unless distinguished by Roman numbers.

LT is a typical AB_5 type toxin which is composed of one A subunit (28kDa) and five B subunits (11.5kDa each). The enzymatically active A subunit is composed of two domains (A1 and A2) which are combined through a disulfide bond. The A1 domain has an ADP-ribosyltransferase activity, while the A2 domain connects A1 to the ring-like pentameric B5 in

the center. Each LT-B subunit binds specifically to ganglioside (GM1) which is a group of glycolipids present on the surface of most eukaryotic cells [1]. After binding to the surface of host cells, LT is subsequently endocytosed and retrogradely transported through Golgi apparatus to the endoplasmic reticulum (ER). The catalytic A1 domain is then cleaved and secreted through the vacuolar membrane. A1 acts on the α subunit of the GTP-binding protein (Gs-α) located on the basolateral membrane of intestinal epithelial cells by transferring an ADP-ribosyl moiety from NAD to Gs-α. The activation of Gs protein stimulates its adenylate cyclase activity, thereby increases the level of intracellular cAMP which is an important second messenger. High cAMP level modulates protein kinase A and results in abnormal activation of membrane ion transporters. The secretion of Cl^- is increased while the absorption of Na^+ and Cl^- are decreased, the abnormal ion distribution in the intestine lumen, finally result in osmotic diarrhea [11].

Since the 1980s, the mucosal adjuvant effect of CT has been verified. When orally co-administered with an antigen, the adjuvant activity of CT was proved to be dose dependent [28]. CT acts as an adjuvant by inducing multiple Th2 cells secreting cytokines which stimulate IgG and secretory IgA response in mice [29]. Similar with CT, LT induces the activation of both Th1 and Th2 cells, provides more comprehensive immune responses compared to CT [30]. However, the toxicity of LT and CT became an obstacle to further use these toxins in vaccine development. To solve this problem, a group of reduced- or non-toxic LT and CT derivations were generated through mutating one or several amino acids [31]. Some of them such as mutant LT (L192G, LTK63) [32; 33], and double mutant LT (dmLT L192G/L211A) [34] have been demonstrated to be less toxic and the adjuvant properties were retained. Recent research found that, even without the holotoxin structure, the A subunit or B subunits alone also have some immune modulatory abilities [35; 36].

To date, a variety of vaccines in combination with LT and its derivations as adjuvants through novel immunization routes have been reported. Lee found dmLT helped a subunit vaccine targeting the tip protein of *Shigella flexneri* type III secretion system (T3SS) to elicit strong immunity in mice model [37]. Marchioro immunized mice with LTB-fused antigens and LTB co-administered antigens respectively and proved that LTB effectively enhanced the host immune response in both ways, but the fusion protein induced significantly higher IgG level than co-administered antigens [36]. The addition of dmLT to inactivated *Helicobacter pylori* vaccine enhanced *H. pylori* specific $CD4^+$ T cell response through the activation of DCs [38]. A live attenuated vaccine generated by introducing a dmLT encoded plasmid into *Salmonella enterica* serovar Enteritidis strain SE JOL1087 significantly enhanced the production of plasma IgG and intestinal secretory IgA in orally immunized chicken [39]. Using patch delivery system (PDS) to deliver recombinant birch pollen allergen Bet v1 with LT epicutaneously induced allergen-specific IgG antibodies blocking allergic IgE in guinea pig, suggesting a promising role LT plays in immunotherapy against hypersensitivity and autoimmune diseases [40].

Compared to traditional parenteral immunization routes, LT shows better immune-stimulating abilities when administered at mucosal sites. Oral, intranasal, sublingual, and intradermal routes could be more feasible and effective options for LT related immunization. A combination of mucosal and systemic immunization further enhance the immune responses [41].

Vaccines against ETEC

Vaccination is believed to be the best way to prevent ETEC other than maintaining a sanitary environment. By now, there is one licensed cholera vaccine (Dukoral®) that provides only short-term protection against ETEC, which can stimulate anti-LT immunity due to the high

homology between LT and CT [42]. A variety of more protective ETEC vaccines are still in development.

Inactivated whole cell vaccines

In 1988, an ETEC prototype strain H10407 which contains LT, STa, and CFA/I inactivated with colicin 2 was used as a vaccine candidate, and showed good protection in a small scale human challenge study [43]. A killed whole-cell ETEC that expressing the most common colonization factor antigens (CFAs), i.e., CFA/I, CFA/II, and CFA/IV, co-administered with 1 mg of recombinant cholera toxin B subunit (rCTB) induced IgA against both CFAs and CTB in the intestine after two doses of vaccine [44]. However, this vaccine was not efficacious in clinical trials, the protection rate was not significant and vaccination caused side effects like vomiting or diarrhea in children under two years old. To modify the vaccine, researchers tried to overexpress CFA antigens in ETEC strains so that less bacteria would be needed to induce immune response and thus reduce the adverse effects. Meanwhile, a non-toxic double mutant LT (dmLT; LTR192G/L211A) was added as an adjuvant to enhance host immune response [45]. Further evaluation of this vaccine candidate is still in process.

Live attenuated vaccines

An ETEC mutant strain E1392/75-2A that losing LT and STa genes was the first ETEC strain used for live-attenuated vaccine [46]. It provided good protection in a rabbit challenge model, but some vaccinated human volunteers showed side effect indicated that this vaccine candidate had some safety issues [47]. To further attenuate the bacteria, some mutant strains were subsequently generated based on E1392/75-2A. PTL-002 (*ΔaroCΔompCΔompF*) and PTL-003 (*ΔompRΔaroC*) were proved to be safe and effective in both mice and human trials [48]. One disadvantage of these vaccine candidates is that they only contained limited virulence factors of

ETEC. To obtain more broadly protection, a new live attenuated vaccine that contained three different ETEC strains ACAM 2025 (CFA/I, LTB), ACAM 2022 (CS5/CS6, LTB) and ACAM 2027 (CS1/CS2/CS3, LTB) was generated which showed great potential in preventing ETEC that have multiple CFAs and LT [49].

Instead of directly using ETEC strains as the vaccine candidate, there are several vectored live attenuated ETEC vaccines using bacteria other than ETEC as host strains. For example, an attenuated *Shigella flexneri* 2a strain was engineered to express ETEC CFAs and toxoid LT, which induced the immune response against both *Shigella* and ETEC [50-52]. Besides, ETEC vaccines using *Vibrio cholerae* [53], and *Salmonella typhi* [54] as vectors were also promising candidates if confirmed in further clinical trials.

Subunit vaccines

ETEC subunit vaccines usually contain single or multiple purified antigens such as toxoid or surface molecules which are easily recognized by immune system. In most cases, adjuvant plays an indispensable role in helping subunit vaccines inducing immune responses effectively.

Back in 1980s, LT was used as a vaccine through a needless transcutaneous route [55]. However, as a potent bacterial toxin, wild type LT causes diarrhea and it is neurotoxic when administered intranasally [56]. Several detoxified LT were then created by mutating one or two amino acids. Among them, dmLT (LTR192G/L211A) shows the best potential which maintains its antigenicity and abandoned toxicity. It has been successfully used as either antigen or adjuvant in ETEC vaccine development recently [34; 57-59]. To get a more broadly protection against ETEC, another enterotoxin STa should be also included in subunit vaccines. The combination of STa and LT by genetic fusion or chemical conjugation solved the problem of lack of immunogenicity when STa served as an antigen alone [60]. Substitution of some amino

acids in the native STa toxin reduced its toxicity while its conformational structure was retained. These LT-STa toxoid fusions was able to elicit antibodies to neutralize both LT and STa [61].

CFAs are also important candidates for ETEC subunit vaccines. The major structural subunits, minor structural subunits, and tip adhesins of CFA are potential candidates for vaccine development. To include as much antigen as possible, a new multiepitope fusion antigen (MEFA) strategy was introduced [62]. A MEFA contains epitopes from major subunits of CFA/I/II/IV could induce specific antibody that inhibit the adherence of *E. coli* strains expressing CFA/I, and CS1-6 [57; 58]. Mice serum derived from another similar MEFA using tip protein epitopes prevented the adherence of 9 ETEC adhesins [63]. These studies provided a new approach for ETEC subunit vaccine development in the future.

Some novel antigens of ETEC other than LT, ST, and CFAs were discovered recently. For example, EtpA and EaeH are surface adhesins required for efficient colonization of the small intestine in murine models [64; 65]. EatA is a protease that degrades mucin in the intestinal lumen, thus facilitates bacterial adhesion [66]. Another important protein YghJ, an effector molecule secreted by the same type II secretion system (T2SS), is responsible for LT delivery. Toxin secretion could be inhibited by anti-YghJ antibody *in vitro* [67]. These proteins could be possibly new targets to generate more broadly protective ETEC vaccines.

Purpose of study

Enterotoxigenic *Escherichia coli* (ETEC) is one of the leading causes of diarrhea disease in both human and animals, which is a great threat to people especially children under five years old in developing countries, as well as foreigners travel to prevalent areas [7; 15]. Except for improving sanitary conditions, vaccination is another important way to prevent and control

ETEC infection. Due to the heterogeneity of virulence factors among ETEC strains, it is challenging to develop a broadly protective ETEC vaccine. Recently, a new multiepitope fusion antigen (MEFA) strategy showed a promising approach for the development of ETEC subunit vaccine [62]. Briefly, epitopes of different vaccine candidate antigens were selected and combined to generate a fusion antigen which is expected to provide effective protection against all of these antigens. Selecting an appropriate carrier or backbone is pivotal for the construction of MEFA protein. Ideal carrier protein should be safe, stable, highly immunogenic and more importantly, capable of displaying multiple epitopes efficiently. According to previous studies, the major subunit CfaB of ETEC CFA/I has been proved to be a good carrier [57; 58]. However, only limited epitopes from CFAs were included in this MEFA, and two important enterotoxins, LT and ST, are missing.

Heat-labile enterotoxin has been proved to have potent immunogenicity as well as adjuvanticity, the property of its intracellular retrograde transportation can be exploited to deliver antigen through the cell membrane. These features make LT a combination of antigen, adjuvant, and even a delivery vehicle, which shows a great potential for vaccine development not only for ETEC, but also applicable for other pathogens. Many attempts have been made to reform LT to become an antigen carrier. Epitope mapping of LTB subunit has been completed by several researchers [68; 69]. But it is inappropriate to put foreign epitopes into LTB because LTB is crucial for cell binding and alteration of B subunit will affect its GM1 binding ability as well as its adjuvanticity and immunogenicity [70]. There are some researchers reported that native LTB alone could be used as vaccine carrier [71; 72], suggested that the adjuvanticity of LTB is independent of A subunit. However, intranasal administration of LTB alone only induced a moderate systemic and a restricted low mucosal antibody response, while the addition of LT

holotoxin to LTB strongly stimulated both serum antibody and systemic mucosal S-IgA response [73], indicating that A subunit also plays important roles in adjuvanticity and stimulation of mucosal immunity. The immune modulating ability of LTA is not related to its ADP-ribosylation activity, since non-toxic dmLT is still a potent adjuvant. Besides, a potential endoplasmic reticulum (ER) retention sequence (RDEL) on the C-terminal of A2 domain is believed to be crucial for movement of LT from the Golgi to ER [74]. Therefore, keeping an intact structure of LTA subunit helps to introduce antigens into the cell which is a benefit for some vaccines against virus and intracellular bacteria.

The purpose of this study is to evaluate LT enterotoxin as a carrier protein to develop new epitope-based subunit vaccines. To keep the immunogenicity and adjuvanticity of LT to the utmost, the holotoxin structure of LT is expected to be retained. Foreign epitopes will be added to the A1 domain of LTA subunit to minimize their effect on the structure and function of LT. To achieve the objective, there are two important things to figure out. The first thing is to find out the number of epitopes in LTA and which epitopes are the most immunogenic. The immunodominant epitopes will be kept in the MEFA protein so that it can still induce immune response against LT toxin, while the less immunogenic epitopes will be replaced by epitopes from other antigens. Secondly, we try to determine whether changing epitopes will influence the structure as well as other biological functions like toxicity and GM1 binding ability of LT.

Chapter 2 - Identification of immunodominant epitopes of enterotoxigenic *Escherichia coli* (ETEC) heat-labile toxin (LT) A subunit

Introduction

Heat-labile enterotoxin is (LT) one of the toxins produced by ETEC that causing diarrhea, making LT an indispensable candidate for ETEC vaccines. Except for its strong immunogenicity, the immune-modulatory properties of LT make it an excellent adjuvant. Multiple studies on LTB subunit provided intensive understanding of its antigenic properties [68; 69]. Meanwhile, LTB has been well applied as an important component in various vaccine candidates [71; 75]. LTA has been proved to be a protective antigen against ETEC [76] as well as a mucosal adjuvant [35; 73; 77]. However, there is a lack of study of roles LTA plays in activating immune response. To evaluate the immunogenic characteristic of LTA subunit, a preliminary epitope mapping was finished in this study. A total of 11 B-cell linear epitopes were predicted and each of them was fused into an ovalbumin carrier protein for further analysis. The immune-dominant epitopes of LT were determined by a series of immunoassays and a mice immunization.

Materials and Methods

Epitope prediction and protein modeling

B-cell linear epitopes of LTA subunit were predicted in silico using an online software [78]. Peptides that have highest scores were selected as epitopes. Protein models were generated by Phyre2 online server [79] based on their amino acid sequences and viewed through PyMOL.

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study were shown in Table 2.1. The epitope carrier protein, chicken ovalbumin, was obtained from *E. coli* DH5α strain 9511 which is constructed in a previous study [80]. Vector pET28α (Novagen, WI) and *E. coli* strain BL21 (GE Healthcare, NJ) was used to clone and express recombinant fusion proteins. Recombinant *E. coli* strains were cultured in Luria Broth (LB) medium supplemented with kanamycin (30μg/ml).

Table 2.1 Bacterial strains used in study 1

Strains	Properties	Reference
BL21	*F⁻ ompT hsdS* (r_B^-, m_B^-), *gal dcm*	GE Healthcare
9511	3×STa fusion with chicken ovalbumin in pET28α/DH5α Kan^+	[80]
9529	Ovalbumin fused LT epitope1 in pET28α/BL21 Kan^+	This study
9546	Ovalbumin fused LT epitope2 in pET28α/BL21 Kan^+	This study
9550	Ovalbumin fused LT epitope3 in pET28α/BL21 Kan^+	This study
9547	Ovalbumin fused LT epitope4 in pET28α/BL21 Kan^+	This study
9551	Ovalbumin fused LT epitope5 in pET28α/BL21 Kan^+	This study
9552	Ovalbumin fused LT epitope6 in pET28α/BL21 Kan^+	This study
9548	Ovalbumin fused LT epitope7 in pET28α/BL21 Kan^+	This study
9543	Ovalbumin fused LT epitope8 in pET28α/BL21 Kan^+	This study
9553	Ovalbumin fused LT epitope9 in pET28α/BL21 Kan^+	This study
9544	Ovalbumin fused LT epitope10 in pET28α/BL21 Kan^+	This study
9549	Ovalbumin fused LT epitope11 in pET28α/BL21 Kan^+	This study

Construction of plasmids

To identify the most immunogenic epitopes of LTA subunit, each predicted epitope was fused to chicken ovalbumin, which has been proved to be an effective vaccine carrier protein. Splicing overlap extension (SOE) PCR was used to replace epitopes into the carrier protein (Figure 2.1). Briefly, PCR products of upstream and downstream fragments were overlapped through a pair of reverse complement primers which encode the epitope peptide. The overlapped fragments were amplified and double-digested by EagI and NcoI (New England Biolabs, MA), then cloned into pET28α to get pET28α-LTe1~e11. All constructed plasmids were first transformed into DH5α competent cells with heat shock. After PCR screening and gene sequencing (Genscript, NJ), positive clones were selected for following experiment. The primers used in this study are listed in Table 2.2. The sequences of ovalbumin carrier are shown in uppercase, the sequences of LT epitopes are shown in lowercase, the sequences of restriction sites are shown in bold.

Figure 2.1 Construction of ovalbumin-LT epitope fusion genes

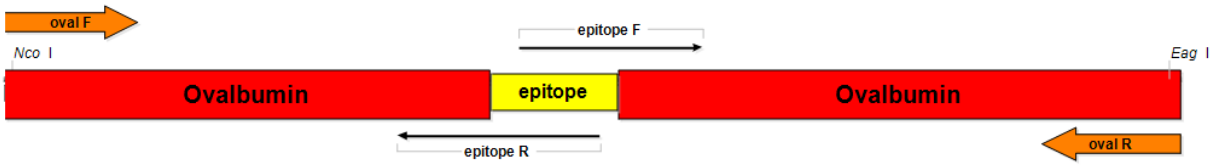


Table 2.2 Primers used in study 1

Primers	DNA sequences (5'-3')
Oval-upF	CATG**CCATGG**GCGGACCCGGACCTGGTA
Oval-downR	TAT**CGGCCG**TTAGGTCCGGGTCCTTCGT
LT-e1F	ggtgatacttgtaatgaggagacccagGATGAAGATACCCAGGCGATGCCGTTTCGC
LT-e1R	ctcattacaagtatcacctgtaattgtTTTAAACGCTTTTTCCCACAGGCCTTTAAA
LT-e2F	ggcgacaaattataccgtgctgactctagaGATGAAGATACCCAGGCGATG
LT-e2R	acggtataatttgtcgccatttgcataTTTAAACGCTTTTTCCCACAG
LT-e3F	agatgaaataaaacgttccggaggtGATGAAGATACCCAGGCGATG
LT-e3R	gaacgttttatttcatctgggggtctagagtcTTTAAACGCTTTTTCCCACAG
LT-e4F	aatgagtacttcgatagaggaactcaaGATGAAGATACCCAGGCGATG
LT-e4R	tctatcgaagtactcattatgccctctTTTAAACGCTTTTTCCCACAG
LT-e5F	agaggaacacaaaccggcGATGAAGATACCCAGGCGATG
LT-e5R	gccggtttgtgttcctctcgcgtgatcataTTTAAACGCTTTTTCCCACAG
LT-e6F	gatgacggatatgtttccacttctGATGAAGATACCCAGGCGATG
LT-e6R	ggaaacatatccgtcatcatatctTTTAAACGCTTTTTCCCACAG
LT-e7F	ccatatgaacaggaggtttctgcgGATGAAGATACCCAGGCGATG
LT-e7R	aacctcctgttcatatgggtgagggctTTTAAACGCTTTTTCCCACAG
LT-e8F	aacagggaatatagagaccggtatGATGAAGATACCCAGGCGATG
LT-e8R	gtctctatattccctgttacgatgTTTAAACGCTTTTTCCCACAG
LT-e9F	gcagaggatggttacagattagcaggtGATGAAGATACCCAGGCGATG
LT-e9R	tctgtaaccatcctctgccggagcTTTAAACGCTTTTTCCCACAG
LT-e10F	accggatcaccaagcttggagagaagaaGATGAAGATACCCAGGCGATG
LT-e10R	caagcttggtgatccggtgggaaacctgcTTTAAACGCTTTTTCCCACAG
LT-e11F	ccacaaggttgtggaaattcatcaagaacaGATGAAGATACCCAGGCGATG
LT-e11R	atttccacaaccttgtggtgcatgatgTTTAAACGCTTTTTCCCACAG
T7-F	TAATACGACTCACTATAGGG
T7-R	GCTAGTTATTGCTCAGCGG

Expression of recombinant ovalbumin-LT epitope fusion protein

E. coli BL21 was used to express recombinant proteins following a previous protocol [63]. A single colony from each recombinant strain was cultured in 5 ml LB medium supplemented with kanamycin (30 μg/ml) shaking at 37 °C overnight. Then, 2 ml of overnight bacterial culture was added to 200 ml 2×Yeast Extract Tryptone (YT) medium (Fisher Scientific, MA) supplemented with kanamycin (30 μg/ml) and shaking at 37 °C. After optical density (OD) value of the culture reached 0.5~ 0.7, Isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma, MO) was added into the culture to a final concentration of 30 μM and grew for four more hours. Centrifuge the culture at 12,000 rpm for 15 min to collect bacteria. Pellets were resuspended in 10 ml bacterial protein extraction reagent (B-PER; Thermo Fisher Scientific, MA) and shaking for 30 min at room temperature, then the mixture was centrifuged at 12,000 rpm for 15 min at 4 °C. Pellet was resuspended in 10 ml B-PER with vortex and pipetting. Add freshly prepared lysozyme to the final concentration of 200 μg/ml, and shaking at room temperature for 40 min to completely lyse the bacteria. Suspensions were centrifuged again and resuspended in B-PER supplemented with lysozyme to make sure all bacterial cell were lysed. After centrifugation, pellets were suspended in 100 ml 1:10 diluted B-PER, vortexed, and centrifuged. Pellets were washed by resuspending and vortexing with 100 ml PBS, centrifuged at 12,000 rpm for 15 min at 4 °C. The washing step was repeated for 3 to 5 times. Final pellets were proceeded to refolding process. Briefly, extracted proteins were mixed with 1xIB solubilization buffer (50 mM CAPS, pH 11.0) supplemented with 0.3% N-lauroylsarcosine and 1 mM DTT. After incubation at room temperature for 40 min, suspension was centrifuged at 12,000 rpm for 20 min at room temperature. Solubilized proteins in supernatant were subsequently transferred to molecular porous membrane tubing (Spectrum Laboratories, Inc., CA) and refolded using Dialysis Buffer

(20 mM Tris-HCl, pH 8.5) supplemented with 0.1 mM DTT at 4 °C. After 3–4 h, protein samples (in tubing) were moved to Dialysis Buffer without DTT, and followed by two more buffer changes. Refolded recombinant proteins were collected with centrifugation at 12,000 rpm for 10 min at 4 °C, measured for protein concentration, aliquoted, and stored at −80 °C. Ten micrograms of each refolded fusion protein were analyzed in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot assay.

Screening epitopes with anti-CT and anti-LT serum

The recombinant epitope fusion proteins were screened by anti-CT (Sigma, MO) rabbit polyclonal antibody and anti-dmLT (made in our lab) mice polyclonal antibody through two assays.

For Enzyme-Linked ImmunoSorbent Assay (ELISA), each recombinant ovalbumin-LT epitope fusion protein (500 ng per well in 100 μl coating antigen buffer) was coated in Immulon 2HB 96-well plates (Thermo Fisher Scientific, MA). Plates incubated at 37°C for 1 h and then 4°C overnight were washed three times with PBS-0.05% Tween 20 (PBST). After incubation with 10% skim milk at 37 °C for 1 h to block uncoated sites, wells were washed three times with PBST and incubated with anti-dmLT or anti-CT serum at 37 °C for 1 h. Two-fold dilutions of primary antibodies start from 1:200 to 1:12800. Then the plates were again washed with PBST 3 times. One hundred microliters of horseradish peroxidase (HRP) conjugated goat-anti-mouse IgG (for dmLT) or goat-anti-rabbit IgG (for CT) second antibodies (Sigma, MO) were diluted 3000 times and added in each well and incubated at 37°C for 1 h. Finally, after three washes with PBST each well was incubated with 100 μl 3,3’,5,5’-tetramethylbezidine (TMB) Microwell Peroxidase Substrate (KPL, MD) at room temperature for 30 min and measured for OD_{650}. The highest dilution that gave an OD reading above 0.3 after subtraction of background readings was

calculated (OD × dilution) for antibody titer. Antibody titers were presented in $\log_{10}$ value [57; 58]. Each recombinant epitope fusion protein was tested in duplicate wells repeated for three times.

For Western Blot, recombinant fusion proteins separated in SDS-PAGE gels were transferred to nitrocellulose membrane (GE Healthcare, NJ) and blocked with 10% skim milk overnight. After three washes with PBST, the membrane was then incubated with anti-dmLT or anti-CT serum (1:3000) at room temperature for 1 h. Again, wash the membrane with PBST 3 times and add IRDye-labeled second antibodies goat-anti-mouse IgG (for dmLT) or goat-anti-rabbit IgG (for CT) (1:10000; LI-COR, NE). Proteins were detected with a LI-COR Odyssey premium infrared gel imaging system (LI-COR) at the wavelength of 700 nm [57].

Mouse immunization with ovalbumin-LT epitope fusion protein

For each epitope, five eight-week-old female BALB/c mice (Charles River Laboratories International, Inc., MA) were used for immunization. Besides, five mice immunized with ovalbumin carrier without LT epitope and five mice immunized with sterile PBS were used as two control groups. Each mouse was immunized subcutaneously with 40μg (in 25μl PBS) refolded ovalbumin-LT epitope fusion protein and 25 μl Montanide ISA 51 VG (SEPPIC, NJ) adjuvant (50μl in total). Antigen and adjuvant were emulsified in a water in oil formula following manufacturer's protocol. Two weeks after prime immunization, two booster injections at the same dose of the primary were followed in an interval of two weeks. All the mice were euthanized two weeks after the second booster, 0.5~ 1.0 ml blood sample was collected from each mouse. Mouse immunization study complied with the Animal Welfare Act by following the 1996 National Research Council guidance and was approved by the Kansas State University's Institutional Animal Care and Use Committee (IACUC #3800).

Anti-toxin antibody titration

Anti-toxin antibody titration was determined through ELISA following a previous procedure [57; 58; 63]. Recombinant LT (gifted by Dr. John Clements) was coated (200 ng per well) with 100 µl coating antigen buffer in wells of Immulon 2HB 96-well plates (Thermo Fisher Scientific, MA). After incubation with 10% skim milk at 37 °C for 1 h to block uncoated sites, wells were washed three times with PBST and incubated with mouse serum two-fold dilutions at 37 °C for 1 h. Serum samples collected from immunized mice were initially diluted in 1:400 and then two-folded diluted till to 1: 25,600. For serum from the PBS control group, dilutions of 1:200 were also included in ELISAs as negative control. Then the plates were again washed with PBST 3 times. One hundred microliters of horseradish peroxidase (HRP) conjugated goat-anti-mouse IgG second antibodies (1:3000; Sigma) was added in each well and incubated at 37°C for 1 h. Finally, after three washes with PBST each well was incubated with 100 µl 3,3',5,5'-tetramethylbezidine (TMB) Microwell Peroxidase Substrate (KPL, MD) at room temperature for 30 min and measured for OD_{650}. The highest dilution that gave an OD reading above 0.3 after subtraction of background readings was calculated (OD × dilution) for antibody titer. Antibody titers were presented in $\log_{10}$ value [57; 58]. Serum sample of each mice was tested in duplicate wells repeated for three times.

Anti-toxin antibody neutralization assay

Serum samples pooled from five mice in each group were used for antibody neutralization assay with EIA cAMP kits (ENZO Life Sciences Inc., NY) in vitro. T-84 cells, a human colon carcinoma cell line cultured in DMEM: F12 medium (ATCC, VA) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, GA) were used in this assay. When incubated with LT enterotoxin, T-84 cell will produce high level of intracellular cAMP, however,

anti-LT antibodies can neutralize LT toxin specifically to prevent LT from stimulating intracellular cAMP. Therefore, it is possible to quantify neutralization activities of anti-LT antibodies and compare which epitope can induce neutralizing antibody most effectively. First, 30 μl of pooled serum sample of each immunization group or the control group was incubated with 20 ng LT for 30 min at room temperature. Then the serum/toxin mixture was added to each well of a 24-microplate (Corning Inc., NY) ($1x10^5$ T-84 cells per well) and incubated in a CO_2 incubator for 3 h. Finally, T-84 cells were lysed and the supernatant was used to measure intracellular cAMP levels (pmol/ml) with EIA cAMP kit according to manufacturer's instructions. LT alone (without serum) was used as the positive control to show enterotoxicity in stimulation of cAMP in T-84 cells, and culture medium only (without toxin or serum) was used as blank control to show a baseline of intracellular cAMP level in T-84 cells [57; 61]. Serum sample of each epitope was tested in duplicate wells repeated for three times.

Statistical analysis

Data were analyzed using GraphPad Prism 5. Results were presented as means with standard deviations. Differences between groups were calculated by one-way analysis of variance with a confidential interval of 95%. Calculated p values of less than 0.05 were considered as significant difference between groups.

Results

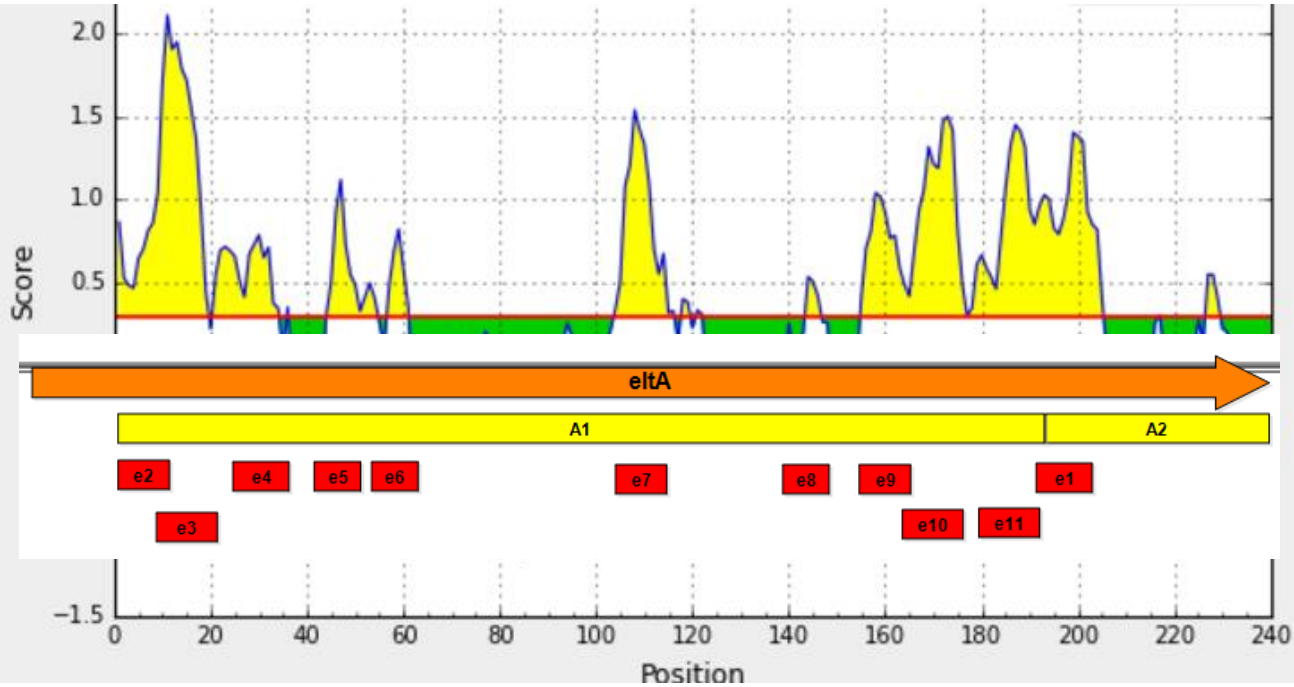
Eleven B-cell linear epitopes were predicted in LTA subunit

The amino acid sequence of LTA subunit was analyzed through BepiPred with a window size of 13 amino acids and threshold of 0.3. A total of 11 B-cell linear epitopes that have highest scores were predicted and listed in Table 2.3. Among them epitope 3 has the highest score, followed by epitope 1, 7, 9, 10, and 11. The positions and scores of these epitopes were shown in Figure 2.2. Epitope 1 is on the A2 domain of LTA, while the rest 10 epitopes are on A1 domain.

Table 2.3 B-cell linear epitopes predicted in LTA subunit

	aa sequences	Position	Length
Epitope1	TITGDTCNEETQ	193-204	12
Epitope2	NGDKLYRADSR	1-11	11
Epitope3	DSRPPDEIKRSGG	9-21	13
Epitope4	RGHNEYFDRGTQ	25-36	12
Epitope5	YDHARGTQTG	42-51	10
Epitope6	RYDDGYVSTS	54-63	10
Epitope7	SPHPYEQEVSA	105-115	11
Epitope8	HRNREYRDRY	140-149	10
Epitope9	APAEDGYRLAG	156-166	11
Epitope10	AGFPPDHQAWREE	165-177	13
Epitope11	HHAPQGCGNSSRT	181-193	13

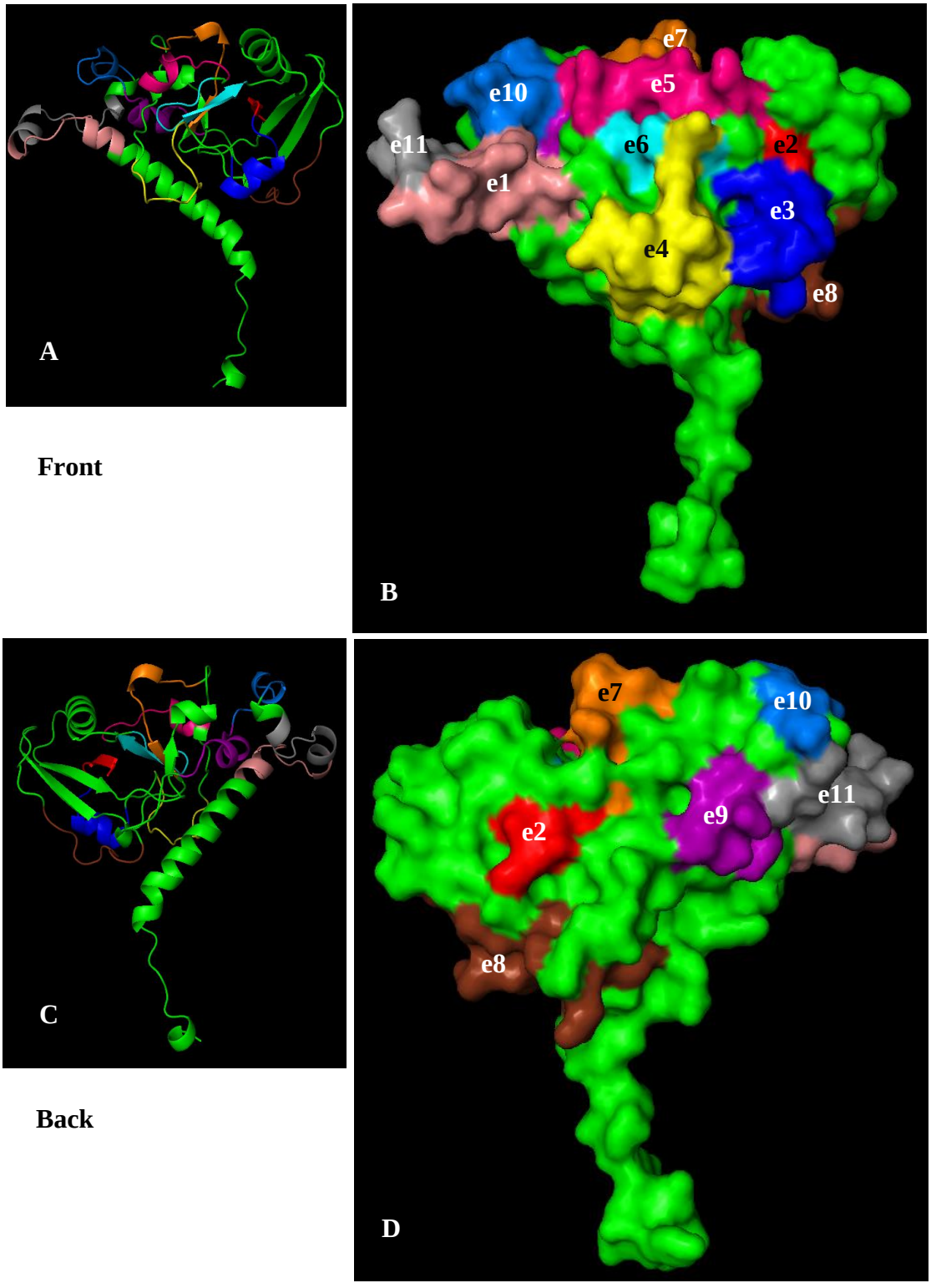
Figure 2.2 Positions and scores of predicted epitopes



Structural modeling of ovalbumin-LT epitope fusion proteins

The locations of predicted epitopes were shown in a 3D model of LTA (PDB ID:1LTA) (Figure 2.3) in different colors. Figure A and C display the secondary structure of LTA while B and D show the surface accessibility of predicted epitopes. A and B are front views while C and D are back views of the model. Except for epitope 2 and 6, all predicted epitopes are exposed on surface of LTA protein, indicating that these epitopes are more easily interacting with specific antibodies.

Figure 2.3 Locations of predicted epitopes on LTA subunit



Different epitopes were recognized by anti-LT and anti-CT serum

Recombinant ovalbumin-LT epitope fusion proteins were expressed and purified, SDS-PAGE gel showed that the size of fusion proteins is around 34kDa, which is similar to the size of carrier protein (Figure 2.4). To determine whether these epitopes can be recognized by anti-dmLT or anti-CT antibodies, ELISA and Western Blot were performed.

Epitope 3 and 4 showed the strongest bands in Western Blot using anti-CT prime antibody. Although it is not quite obvious, there was a slight band against epitope 7 at around 34kDa, while other epitope fusion proteins can not be detected by anti-CT antibody (Figure 2.5A). All 11 epitopes showed specific bands against anti-dmLT antibody (Figure 2.5B), and neither antibodies recognize the carrier protein.

All 11 epitope fusion proteins can be recognized by both anti-CT and anti-dmLT antibodies by ELISA. In consistent with Western Blot, anti-CT antibody preferred epitope 3 and 4, whose titers are significantly higher than the rest of epitopes (Figure 2.6A). Epitope 7, 8, and 9 have significant higher titers than the other epitopes when using anti-dmLT serum as prime antibody (Figure 2.6B). The ovalbumin carrier protein had no cross reaction with neither anti-CT nor anti-dmLT antibody.

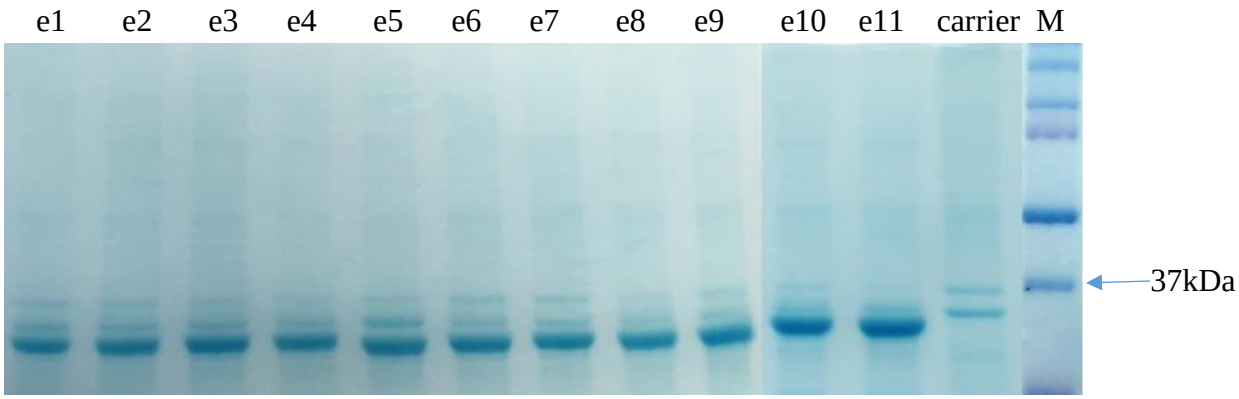


Figure 2.4 SDS-PAGE detection of recombinant ovalbumin-LT epitope fusion proteins

Figure 2.5 Western Blot detection of fusion protein with anti-CT and anti-dmLT

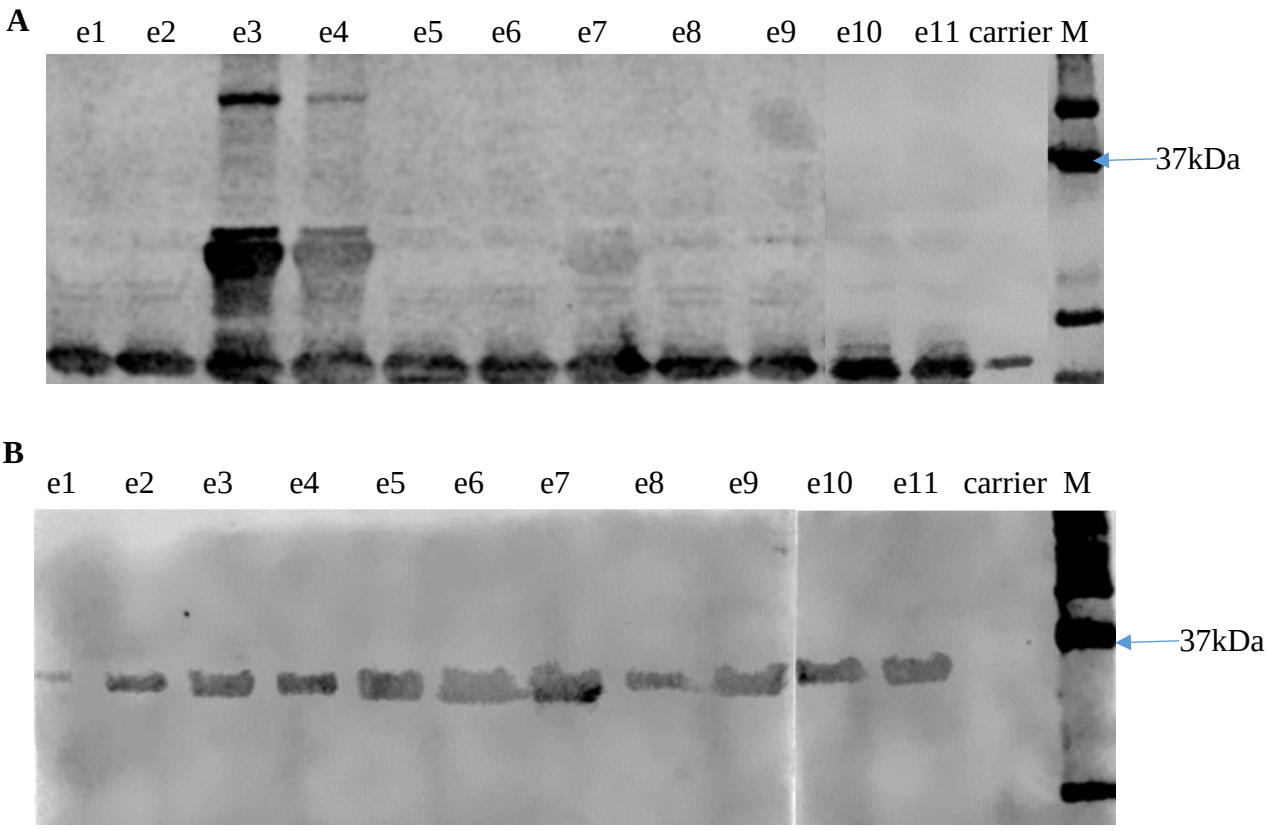
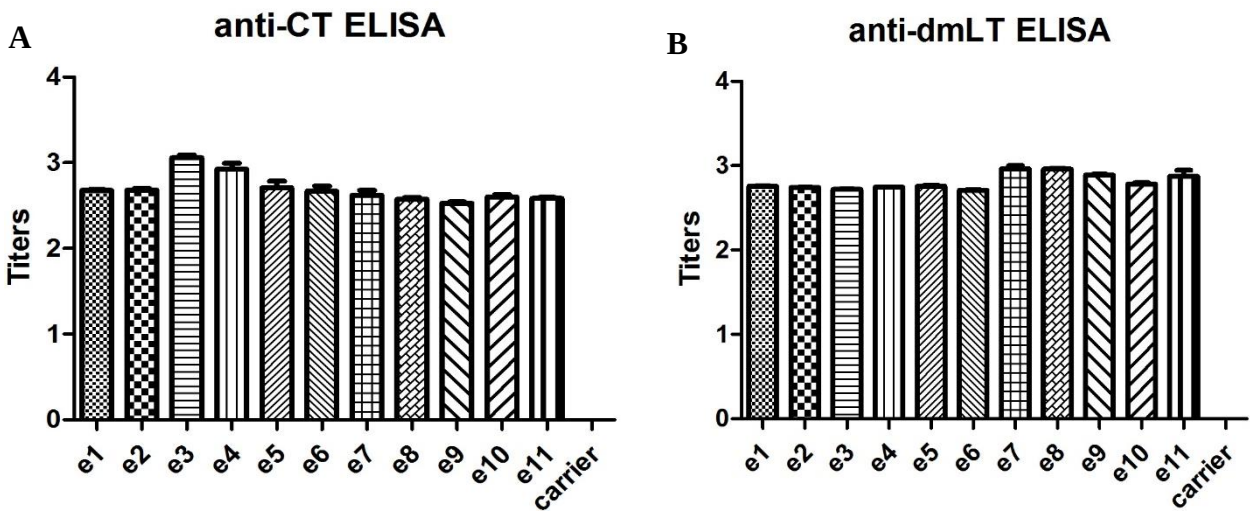


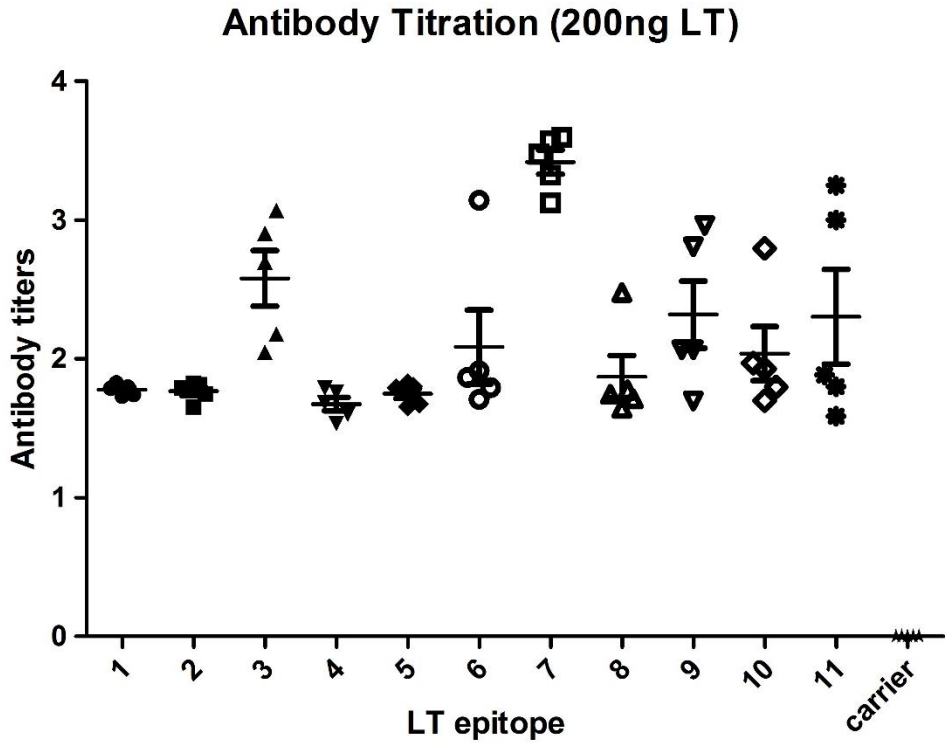
Figure 2.6 ELISA detection of fusion protein with anti-CT and anti-dmLT



Ovalbumin-LT epitope fusion proteins induced anti-LT antibody in mice

Mice were immunized with ovalbumin-LT epitope fusion proteins and Montanide emulsion subcutaneously. Two weeks after second booster, mice were euthanized and blood was collected. Anti-LT antibody titers of serum samples from each mouse was tested by ELISA. Among all 11 epitopes, epitope 7 showed the highest antibody titer, which is significantly higher than all other epitopes beside epitope 3, which indicates that epitope 7 is the immune dominant epitope in LT. Medium antibody titers were observed in epitope 3, 9, 11, 6, and 10, only 1 to 3 mice out of 5 have good immune responses against these epitopes. Epitope 1, 2, 4, and 5 showed poor immunogenicity because only low levels of antibody detected. The ovalbumin carrier protein failed to induce antibody against LT.

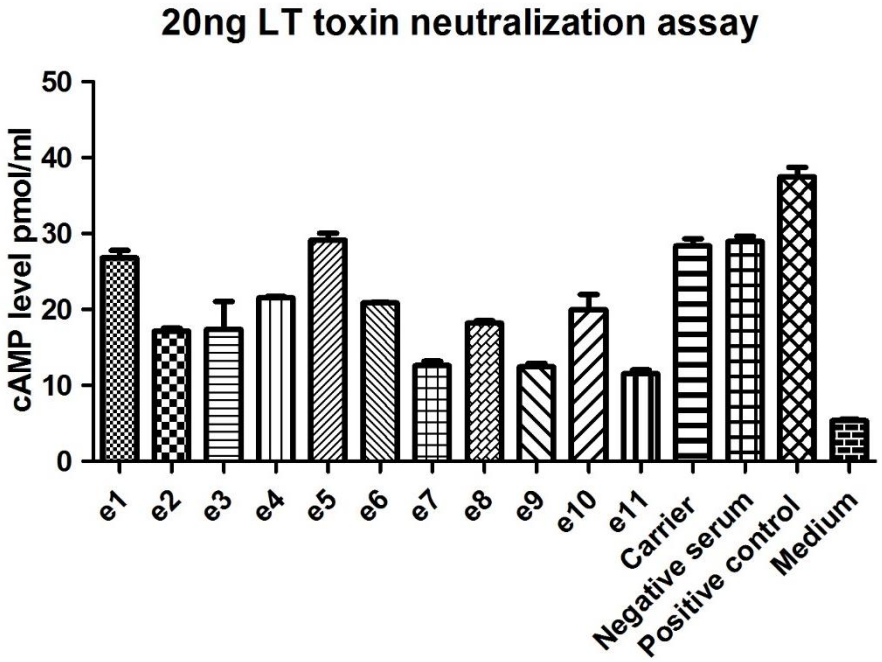
Figure 2.7 Anti-LT antibody titration



LT could be neutralized by immunized mice serum

To determine whether antibody induced by ovalbumin-LT epitope fusion proteins have biological function, i.e. neutralizing LT toxin, a toxin neutralization assay was conducted. As shown in Figure 7, mice serum from LT epitope 7, 9, and 11 had the best toxin neutralizing ability, intracellular cAMP levels were significantly reduced compared to control groups. Serum of epitope 2, 3, 4, 6, 8, and 10 had moderate toxin neutralizing activities, while epitope 1 and 5 can not induce neutralizing antibody in mice model, whose cAMP levels had no significant difference with that in carrier protein and negative control group. Positive control group without serum showed that 20ng LT toxin is enough to induce a significant increase of cAMP level compared to blank control group which had only DMEM:F12 medium.

Figure 2.8 Anti-LT antibody neutralization assay



Discussion

In this study, we determined that LT epitope 7 is the most immunogenic epitope. It has relative high score given by the epitope predicting software. Epitopes completely exposed at the surface of 3D model suggesting that they can be easily detected by host immune system. Epitope screening using anti-dmLT polyclonal serum indicated that the amount of antibody specific to epitope 7 is significantly higher than antibodies specific to other epitopes, thus this epitope might be the main target of the immune responses. Additionally, mice immunized with epitope 7 fusion protein produced the highest anti-LT serum IgG level, and these anti-epitope 7 antibodies were functional which neutralized LT toxin most effectively *in vitro*. These results proved the concept that single epitope of LTA is capable of inducing anti-LT neutralizing antibody in mice model. Except for epitope 7, epitope 3, 9, and 11 could also induce moderate immune response, while antibody titers of epitope 1, 2, 5, 6, 8, 10 were relatively lower. Therefore, these six epitopes can be replaced by more immunogenic foreign epitopes and epitope 7 should be retained when using LT as a multi-epitope fusion antigen carrier.

As we know, LT and CT are similar in amino acid sequences, structures and immunomodulatory properties. There is immunogenic cross reaction between LT and CT, since human immunized with CT could produce immune response against both CT and LT. However, this cross protection seems mainly rely on antibodies targeting specific shared conformational structures between LT and CT which explains why anti-CT and anti-dmLT antibodies recognized different linear epitopes in this study. Although for each epitope, LT and CT still shared most part of their sequences, difference in 2-3 amino acids might completely block the cross reaction between LT and CT. Epitope 3, 4, and 7 can be recognized by both anti-CT and anti-dmLT antibodies suggested that LT and CT are highly similar in both sequences and

conformational structures at these regions. These shared epitopes, especially epitope 3, could be used to induce protection against both LT and CT. In addition, all of these conclusions were made based on experiments in mice model. Other models include pigs, primates, and human should be included in future study to confirm whether this pattern works for other different immune systems.

Normally, dmLT is the best adjuvant for ETEC related subunit vaccines. But in this study, dmLT is not applicable because it would be hard to distinguish the anti-LT immune responses between dmLT and the LT epitope. Montanide ISA 51 VG, a modified mineral oil adjuvant that enhances humoral immune response was used as adjuvant in mice experiment. One thing need to be mentioned is that an inconsistency of immune responses was observed in group 6, 8, 10, and 11. In these groups, only 1 or 2 mice showed good antibody titers while the rest of mice did not. The differences within these groups might be caused by individual variances of mouse reacting with the Montanide adjuvant. Anyway, result from epitope 7 is still quite convincing since all 5 mice in this group showed good immune responses.

Chapter 3 - Significance of the A subunit epitopes in enterotoxicity of enterotoxigenic *Escherichia coli* (ETEC) heat-labile toxin (LT)

Introduction

The ideal LT as a vaccine component should be non-toxic while the AB_5 holotoxin structure remains unharmed, so that antigens can be carried through cell membrane and delivered into the cell without causing side effects. To utilize LT as an antigen carrier, it is necessary to figure out the method to detoxify LT and the location to insert foreign antigens without disrupting other properties of LT. Both A and B subunits are required for LT toxicity. Alteration the structure of either LTA or LTB will dramatically influence the toxicity of LT. LTB subunits indirectly contribute to LT toxicity because A subunit alone can't get inside of the cell without binding to cell surface through LTB. Besides, the A2 domain of LTA plays an important role in intracellular transportation of LT [74]. The cell binding and intracellular transportation is important for a delivery platform, suggesting that the sequence of LTB and LTA2 can't be altered. Therefore, LTA1 is the only option to be manipulated as an antigen carrier.

In Chapter 2, 11 B-cell linear epitopes on LTA subunit have been predicted, and their immunogenic properties were analyzed. But the relationships between these epitopes with the structure and functions of LT are still not clear. This chapter will further explore the relationships between these epitopes and the toxicity of LT. Theoretically, the enzymatic function of LTA1 is so delicate that substitution of one or two amino acid would weaken the toxicity. Mutant LT derivations including LT L192G, and LTK63 have been proved to be less toxic than native LT. To testify whether replacing epitopes on LTA1 with foreign epitopes will influence the toxicity, receptor binding ability, and holotoxin structure of LT, 10 epitopes on LTA1, which were

predicted in Chapter 2, were substitute with a foreign epitope to construct mutant LT proteins. Since epitope 1 is on A2 domain, it will be not included in this part of study.

Materials and Methods

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study were shown in Table 3.1. An *E. coli* TOP10 (Invitrogen, CA) strain 8460 [81] which contains a complete open reading frame of *eltAB* gene originated from human ETEC strains was used for plasmid construction. Vector pBR322 (Promega, WI) and pMAL-c5X (New England Biolabs, MA) which encodes a maltose-binding protein (MBP) were used to construct epitope-mutated LT toxins. Recombinant *E. coli* strains were cultured in Luria Broth (LB) medium supplemented with ampicillin (100μg/ml).

Table 3.1 Strains and plasmids used in study 2

Strains	**Properties**	**Reference**
8460	Native LT in pBR322/TOP10	[81]
9646	LT epitope 2 mutant in pBR322/DH5α Amp^+	This study
9647	LT epitope 3 mutant in pBR322/DH5α Amp^+	This study
9648	LT epitope 4 mutant in pBR322/DH5α Amp^+	This study
9649	LT epitope 5 mutant in pBR322/DH5α Amp^+	This study
9650	LT epitope 6 mutant in pBR322/DH5α Amp^+	This study
9651	LT epitope 7 mutant in pBR322/DH5α Amp^+	This study
9652	LT epitope 8 mutant in pBR322/DH5α Amp^+	This study
9653	LT epitope 9 mutant in pBR322/DH5α Amp^+	This study
9654	LT epitope 10 mutant in pBR322/DH5α Amp^+	This study
9655	LT epitope 11 mutant in pBR322/DH5α Amp^+	This study
Plasmids		
pBR322		Promega
pMAL-c5X		NEB

Construction of plasmids

To determine whether epitope alteration influence the toxicity and structure of the toxin, each epitope was substituted by an epitope (KDAQTNSSS) of maltose binding protein (MBP). Splicing overlap extension (SOE) PCR was used to replace or insert epitopes into the carrier protein (Figure 3.1). Briefly, PCR products of upstream and downstream fragments were overlapped through a pair of reverse complement primers which encode the epitope peptide. The primers used in this study are listed in Table 5. Then the overlapped fragment was amplified and subsequently used for enzyme digestion and ligation. For LT epitope mutant protein, NheI and EagI (New England Biolabs, MA) were used for digestion and vector pBR322 was used to express these LT mutant proteins. All constructed plasmids were first transformed into DH5α competent cells with heat shock. After PCR screening and gene sequencing (Genscript, Piscataway, NJ), positive clones were selected for following experiment.

Figure 3.1 Construction of LT mutants

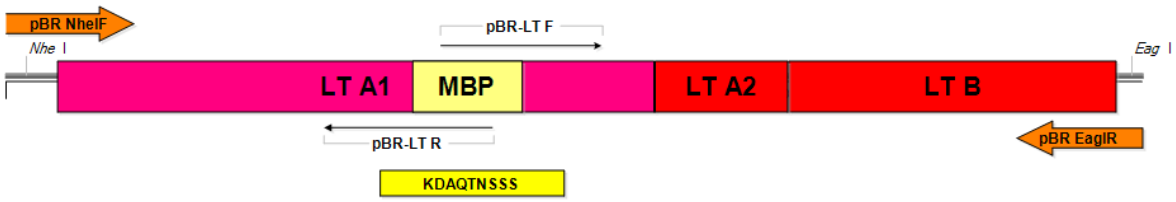


Table 3.2 Primers used in study 2

Primers	DNA sequences (5'-3')
pBR-NheIF	TGCGGTAGTTTATCACAG
pBR-EagIR	GTCCCTGATGGTCGTCATCT
pBR-LTe2F	ACGCGCAGACTAATTCGAGCTCGagacccccagatgaaata
pBR-LTe2R	TCGAATTAGTCTGCGCGTCTTTatataatggcgatgctaa
pBR-LTe3F	ACGCGCAGACTAATTCGAGCTCGggtcttatgcccagaggg
pBR-LTe3R	TCGAATTAGTCTGCGCGTCTTTgtcagcacggtataattt
pBR-LTe4F	CGCGCAGACTAATTCGAGCTCGcaaatgaatattaatctt
pBR-LTe4R	CTCGAATTAGTCTGCGCGTCTTTtctgggcataagacctcc
pBR-LTe5F	ACGCGCAGACTAATTCGAGCTCGggctttgtcagatatgat
pBR-LTe5R	TCGAATTAGTCTGCGCGTCTTTataaagattaatattcat
pBR-LTe6F	CGCGCAGACTAATTCGAGCTCGtctcttagtttgagaagt
pBR-LTe6R	CTCGAATTAGTCTGCGCGTCTTTtctgacaaagccggtttg
pBR-LTe7F	CGCGCAGACTAATTCGAGCTCGgcgttaggtggaatacca
pBR-LTe7R	CTCGAATTAGTCTGCGCGTCTTTgctgtatacgcctaatac
pBR-LTe8F	CGCGCAGACTAATTCGAGCTCGtattacagaaatctgaat
pBR-LTe8R	CTCGAATTAGTCTGCGCGTCTTTatgtaatcgttcatcaat
pBR-LTe9F	ACGCGCAGACTAATTCGAGCTCGggtttcccaccggatcac
pBR-LTe9R	TCGAATTAGTCTGCGCGTCTTTagctatattcagatttct
pBR-LTe10F	CGCGCAGACTAATTCGAGCTCGgaaccctggattcatcat
pBR-LTe10R	CTCGAATTAGTCTGCGCGTCTTTtgctaatctgtaaccatc
pBR-LTe11F	CGCGCAGACTAATTCGAGCTCGacaattacaggtgatact
pBR-LTe11R	CTCGAATTAGTCTGCGCGTCTTTatgaatccagggttcttc

Detection of mutant LT

To testify whether LT is still normally expressed after changing one of its epitope, Western Blot was used to detect toxin secreted in the supernatant of bacterial culture. Strains

containing 10 mutant LT plasmids and 8460 which expresses native LT toxin as positive control were cultured in 10 ml LB supplemented with 100μg/ml ampicillin overnight. A native DH5α was also cultured as negative control. Then the culture was centrifuged at 12,000 rpm for 10 minutes to remove bacteria cells. Around 9 ml culture supernatant was subsequently filled in dialysis membrane tubing (Spectrum Laboratories, Inc., CA) and concentrated with Aquacide II (EMD Millipore, MA) for 8 h to a final volume of about 1 ml. Concentrated samples were then used for Western Blot. Similar to the procedure in Chapter 2, protein samples were transferred from SDS-PAGE gels to nitrocellulose membrane and blocked with 10% skim milk overnight. The membrane was then incubated with anti-LTA serum (1:3000) at room temperature for 1 h and IRDye-labeled goat-anti-mouse IgG second antibodies (1:10000; LI-COR, NE). Proteins were detected with a LI-COR Odyssey premium infrared gel imaging system (LI-COR) at the wavelength of 700 nm.

GM1 binding of mutant LT

To assess the ability of mutated LT proteins binding to GM1 receptor, the GM1-ELISA was conducted [82]. 96-well Plates were coated at 4 °C overnight with 100 μl monosialo ganglioside GM1 (4μg/ml) in each well and then washed by PBST 3 times. Overnight bacterial culture of each LT epitope mutant strain was adjusted to same OD value (OD_{600}=1.0) and then centrifuge at 12,000 rpm for 10 minutes. 100 μl supernatant which contains mutated LT protein was added to each well and incubated at 37 °C for 1 h. The supernatant of strain 8460 which has wild type LT was used as positive control, while supernatant of native DH5α was used as negative control. After washed with PBST 3 times, the GM1- LT complex was blocked at 37 °C for 1 h with 10% skim milk in PBST and washing with PBST 3 times again. Serial dilutions of anti-LTA mouse polyclonal serum (Sigma, MO) were prepared and added to the wells. Plates

were incubated at 37 °C for 1 h and after washing, 100 µl 1:3000 dilutions of HRP goat anti-mouse IgG second antibody (Sigma, MO) was added to each well. Following steps are same with ELISA protocols described in epitope screening and antibody titration. Each LT mutant was tested in duplicate wells and repeated for three times.

Mutant LT toxicity assay

Toxicity of mutated LT proteins was tested by cAMP ELISA with a direct cyclic AMP enzyme immunoassay (EIA) kit according to the manufacturer's instruction [57; 61]. Briefly, 1×10^5 T-84 cells were seeded and cultured in each well of a 24-well plate. After removing the Dulbecco's modified Eagle medium (DMEM:F12; ATCC, VA), 75 µl overnight culture growth supernatant from each strain was added to each well (in duplicate). Cells were lysed with 200 µl (per well) 0.1 M HCl after 3 h of incubation. One hundred microliters of lysis product were mixed with conjugates and antibody reagents supplied in the kit, and the mixture was added to each well of a supplied EIA plate. After incubation on a shaker (500 rpm) at room temperature for 2 h, plates were washed and then incubated with p-nitrophenyl phosphate (pNpp) (disodium salt) substrate solution (100 µl per well) for 1 h. The OD was measured at 405 nm after adding stop solution. Each LT mutant was tested in duplicate wells and repeated for three times.

Statistical analysis

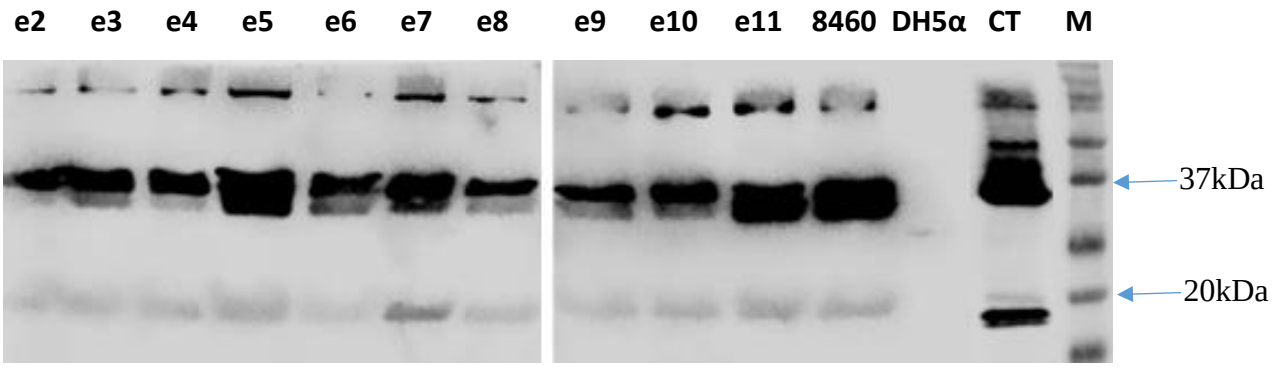
Data were analyzed using GraphPad Prism 5. Results were presented as means with standard deviations. Differences between groups were calculated by one-way analysis of variance with a confidential interval of 95%. Calculated p values of less than 0.05 were considered as significant difference between groups.

Results

Mutant LT proteins were expressed and secreted

In Figure 3.2, Western Blot using anti-LTA antibody showed that 10 LT mutants can be normally expressed and secreted in DH5α. The bands at around 37kDa indicate the holotoxin structure of LT is not influenced by replacing one of its epitope on LTA subunit. Bands below 20kDa confirmed the normal expression of LTA subunit in these mutants. The culture supernatant of 8460 which secrets native LT and commercialized CT toxin (Sigma, MO) were used for positive controls.

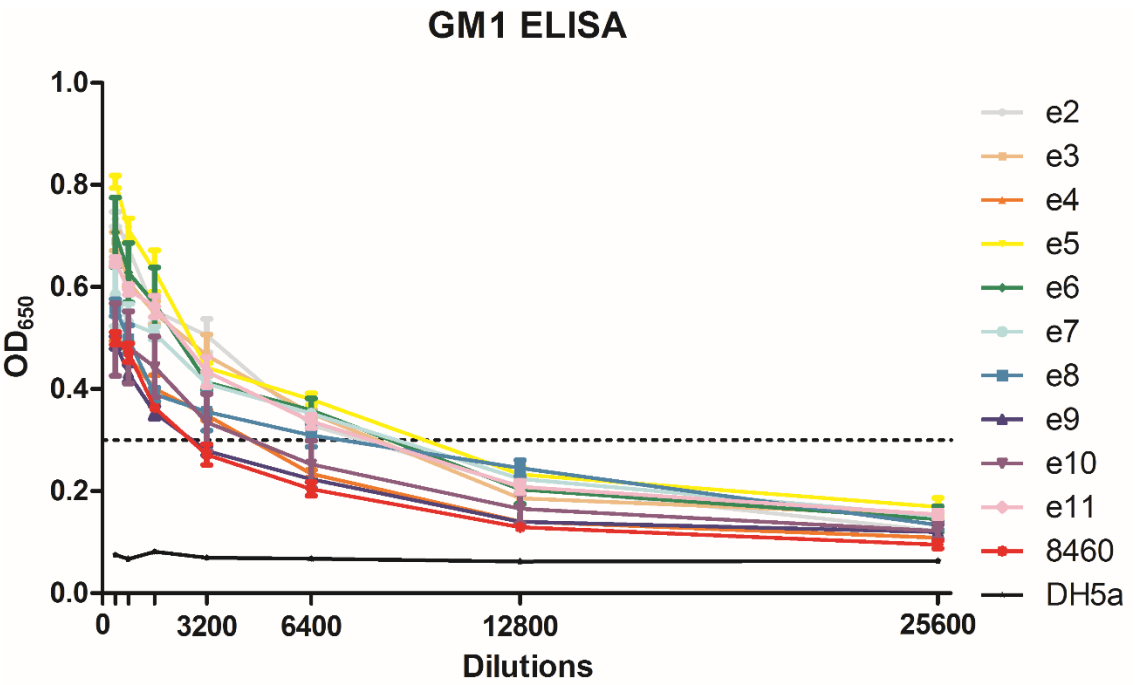
Figure 3.2 Western Blot detection of secreted mutated LT proteins



LT mutants bind to GM1

The culture supernatant containing secreted LT mutants was used to determine the GM1 binding abilities of these LT mutants by GM1 ELISA. All LT mutants showed strong GM1 binding abilities that had no significant difference with native LT expressed by 8460 (Figure 3.3), indicated that replacing any epitope on LTA subunit does not affect the receptor binding ability of LT. The supernatant of DH5α used as a negative control here showed no GM1 binding at all.

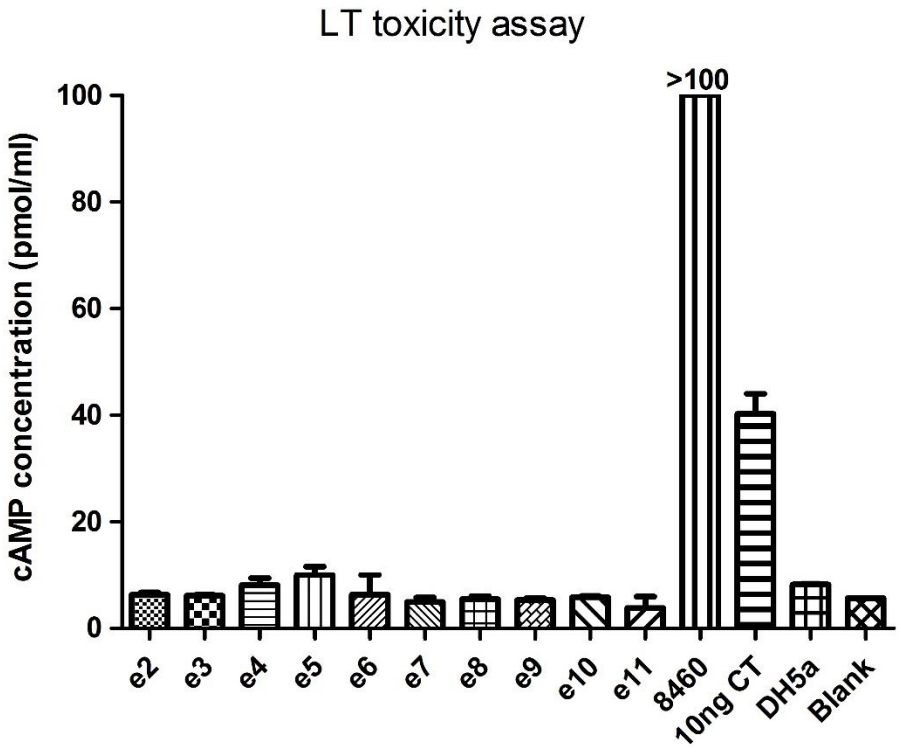
Figure 3.3 GM1 binding of LT mutant proteins



LT mutants lose enterotoxicity

To test whether LT mutants have reduced toxicity, a toxicity assay using T84 cells was conducted. It is clear that intracellular cAMP concentration indicates LT toxicity, a direct cAMP enzyme immunoassay (EIA) kit was used to quantify intracellular cAMP level of T84 cells after treating with bacterial supernatant which contain secreted mutant LT toxins. All 10 mutant toxins failed to induce high levels of cAMP, while same volume of culture supernatant of 8460 induced extremely high concentration of cAMP which is out of upper limit that the kit can detect. Cells treated with 10ng of cholera toxin had a significantly increased cAMP concentration of about 40 pmol/ml, while cells treated with supernatant of DH5α and cell culture medium still have normal cAMP levels. The results suggest that changing a single epitope on LTA subunit eliminates almost all the toxicity of LT.

Figure 3.4 Enterotoxicity assay of mutated LT proteins



Discussion

In this study, we complete a preliminary evaluation of the characteristics of mutant LT proteins. After replacing an epitope on LTA1, the enzymatic activity of LTA subunit was seriously damaged, but the holotoxin structure and the cell binding ability of B subunits were unaffected. Hence, LT could be used as a safe platform carrying foreign antigens to provide broadly protection against multiple virulence factors or even different pathogens.

Interestingly, using pBR322 vector, LT can be secreted in DH5α with complete holotoxin structure. Although the expression level is low, it is still detectable by immunoassays and enough to induce a significant toxicity response in T84 cells. These findings might indicate an easier way to evaluate LT based vaccines which purified protein is not required. The secretion of LT suggested that the LT is not only suitable for subunit vaccine, but also applicable for live or vectored vaccines. When combined with the MEFA strategy, epitopes of STa and CFAs can be introduced into this platform to generate a powerful ETEC vaccine. Foreign antigens of interest from one specific or several different pathogens can be also included to develop vaccine candidates against other diseases. The chimeric LT can be either purified as recombinant subunit antigen, or expressed in a live vector strain. All of these possibilities might bring more innovations to vaccine development in future.

However, there are some problems need to be solved. Replacing only one epitope of LTA1 at a time did not affect the protein structure of LT. But LT structure might be unstable when multiple epitopes are introduced, because there might be a certain limit for alteration. It is necessary to figure out how these epitopes influence the structure of LT. The relationship between LT structure and its adjuvanticity and the mechanisms of LT adjuvanticity are still unknown. The adjuvanticity of these mutant LT are yet to be further determined in animal

experiments. Future studies on LT adjuvanticity may help explain the mechanisms of how LT stimulate host immune system.

Refernces

1. Nataro, J. P., & Kaper, J. B. (1998). Diarrheagenic *Escherichia coli*. *Clinical microbiology reviews, 11*(1), 142-201.
2. Gyles, C., & Barnum, D. (1967). *Escherichia coli* in ligated segments of pig intestine. *The Journal of pathology and bacteriology*, *94*(1), 189-194.
3. Moon, H., Whipp, S., Engstrom, G., & Baetz, A. (1970). Response of the rabbit ileal loop to cell-free products from *Escherichia coli* enteropathogenic for swine. *Journal of Infectious Diseases*, *121*(2), 182-187.
4. Smith, H. W., & Halls, S. (1967). Studies on *Escherichia coli* enterotoxin. *The Journal of pathology and bacteriology*, *93*(2), 531-543.
5. Turner, S. M., Scott-Tucker, A., Cooper, L. M., & Henderson, I. R. (2006). Weapons of mass destruction: virulence factors of the global killer enterotoxigenic *Escherichia coli*. *FEMS Microbiology Letters, 263*(1), 10-20.
6. Gaastra, W., & Svennerholm, A.-M. (1996). Colonization factors of human enterotoxigenic *Escherichia coli* (ETEC). *Trends in microbiology, 4*(11), 444-452.
7. Isidean, S., Riddle, M., Savarino, S., & Porter, C. (2011). A systematic review of ETEC epidemiology focusing on colonization factor and toxin expression. *Vaccine, 29*(37), 6167-6178.
8. Gaastra, W., & De Graaf, F. K. (1982). Host-specific fimbrial adhesins of noninvasive enterotoxigenic *Escherichia coli* strains. *Microbiological reviews, 46*(2), 129.
9. Schulz, S., Green, C. K., Yuen, P. S., & Garbers, D. L. (1990). Guanylyl cyclase is a heat-stable enterotoxin receptor. *Cell, 63*(5), 941-948.
10. Chao, A. C., de Sauvage, F. J., Dong, Y., Wagner, J., Goeddel, D., & Gardner, P. (1994). Activation of intestinal CFTR Cl-channel by heat-stable enterotoxin and guanylin via cAMP-dependent protein kinase. *The EMBO Journal, 13*(5), 1065.
11. Spangler, B. D. (1992). Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. *Microbiological reviews, 56*(4), 622-647.
12. Nagy, B., & Fekete, P. Z. (1999). Enterotoxigenic *Escherichia coli* (ETEC) in farm animals. *Veterinary research, 30*(2-3), 259-284.

13. De, S., Bhattacharya, K., & Sarkar, J. (1956). A study of the pathogenicity of strains of Bacterium coli from acute and chronic enteritis. *The Journal of pathology and bacteriology, 71*(1), 201-209.
14. Lanata, C. F., Fischer-Walker, C. L., Olascoaga, A. C., Torres, C. X., Aryee, M. J., & Black, R. E. (2013). Global causes of diarrheal disease mortality in children< 5 years of age: a systematic review. *PloS one, 8*(9), e72788.
15. Wennerås, C., & Erling, V. (2004). Prevalence of enterotoxigenic *Escherichia coli*-associated diarrhoea and carrier state in the developing world. *Journal of Health, Population and Nutrition*, 370-382.
16. Steffen, R., Hill, D. R., & DuPont, H. L. (2015). Traveler's diarrhea: a clinical review. *Jama, 313*(1), 71-80.
17. Jiang, Z.-D., Lowe, B., Verenkar, M., Ashley, D., Steffen, R., Tornieporth, N., DuPont, H. L. (2002). Prevalence of enteric pathogens among international travelers with diarrhea acquired in Kenya (Mombasa), India (Goa), or Jamaica (Montego Bay). *Journal of Infectious Diseases, 185*(4), 497-502.
18. MacDonald, E., Møller, K., Wester, A., Dahle, U., Hermansen, N., Jenum, P., Vold, L. (2015). An outbreak of enterotoxigenic *Escherichia coli* (ETEC) infection in Norway, 2012: a reminder to consider uncommon pathogens in outbreaks involving imported products. *Epidemiology and infection, 143*(03), 486-493.
19. Abu-Elyazeed, R., Wierzba, T. F., Mourad, A. S., Peruski, L. F., Kay, B. A., Rao, M., Kamal, S. M. (1999). Epidemiology of enterotoxigenic *Escherichia coli* diarrhea in a pediatric cohort in a periurban area of lower Egypt. *Journal of Infectious Diseases, 179*(2), 382-389.
20. King, C. K., Glass, R., Bresee, J. S., Duggan, C., Control, C. f. D., & Prevention. (2003). Managing acute gastroenteritis among children. *MMWR Recomm Rep, 52*(1), 16.
21. Black, R. E., Levine, M. M., Clements, M. L., Cisneros, L., & Daya, V. (1982). Treatment of experimentally induced enterotoxigenic *Escherichia coli* diarrhea with trimethoprim, trimethoprim-sulfamethoxazole, or placebo. *Review of Infectious Diseases, 4*(2), 540-545.
22. Carpenter, C., Barua, D., Wallace, C., Sack, R. B., Mitra, P., Werner, A., Lewis, G. (1965). Clinical and physiological observations during an epidemic outbreak of non-

vibrio cholera-like disease in Calcutta. *Bulletin of the World Health Organization, 33*(5), 665.

23. Zeighami, H., Haghi, F., Hajiahmadi, F., Kashefiyeh, M., & Memariani, M. (2015). Multi-drug-resistant enterotoxigenic and enterohemorrhagic *Escherichia coli* isolated from children with diarrhea. *Journal of Chemotherapy, 27*(3), 152-155.
24. Rodas, C., Mamani, R., Blanco, J., Blanco, J. E., Wiklund, G., Svennerholm, A.-M., Iniguez, V. (2011). Enterotoxins, colonization factors, serotypes and antimicrobial resistance of enterotoxigenic *Escherichia coli* (ETEC) strains isolated from hospitalized children with diarrhea in Bolivia. *The Brazilian Journal of Infectious Diseases, 15*(2), 132-137.
25. Pazhani, G., Chakraborty, S., Fujihara, K., Yamasaki, S., Ghosh, A., Nair, G., & Ramamurthy, T. (2011). QRDR mutations, efflux system & antimicrobial resistance genes in enterotoxigenic *Escherichia coli* isolated from an outbreak of diarrhoea in Ahmedabad, India.
26. Qadri, F., Svennerholm, A.-M., Faruque, A., & Sack, R. B. (2005). Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. *Clinical microbiology reviews, 18*(3), 465-483.
27. Nawar, H. F., Greene, C. J., Lee, C. H., Mandell, L. M., Hajishengallis, G., & Connell, T. D. (2011). LT-IIc, a new member of the type II heat-labile enterotoxin family, exhibits potent immunomodulatory properties that are different from those induced by LT-IIa or LT-IIb. *Vaccine, 29*(4), 721-727.
28. Lycke, N., & Holmgren, J. (1986). Strong adjuvant properties of cholera toxin on gut mucosal immune responses to orally presented antigens. *Immunology, 59*(2), 301.
29. Xu-Amano, J., Kiyono, H., Jackson, R., Staats, H., Fujihashi, K., Burrows, P., McGhee, J. (1993). Helper T cell subsets for immunoglobulin A responses: oral immunization with tetanus toxoid and cholera toxin as adjuvant selectively induces Th2 cells in mucosa associated tissues. *Journal of Experimental Medicine, 178*(4), 1309-1320.
30. Pizza, M., Giuliani, M., Fontana, M., Monaci, E., Douce, G., Dougan, G., Del Giudice, G. (2001). Mucosal vaccines: non toxic derivatives of LT and CT as mucosal adjuvants. *Vaccine, 19*(17), 2534-2541.

31. Da Hora, V. P., Conceição, F. R., Dellagostin, O. A., & Doolan, D. L. (2011). Non-toxic derivatives of LT as potent adjuvants. *Vaccine*, *29*(8), 1538-1544.
32. Baudner, B., Balland, O., Giuliani, M., Von Hoegen, P., Rappuoli, R., Betbeder, D., & Del Giudice, G. (2002). Enhancement of protective efficacy following intranasal immunization with vaccine plus a nontoxic LTK63 mutant delivered with nanoparticles. *Infection and immunity*, *70*(9), 4785-4790.
33. Dickinson, B. L., & Clements, J. D. (1995). Dissociation of *Escherichia coli* heat-labile enterotoxin adjuvanticity from ADP-ribosyltransferase activity. *Infection and immunity*, *63*(5), 1617-1623.
34. Norton, E. B., Lawson, L. B., Freytag, L. C., & Clements, J. D. (2011). Characterization of a mutant *Escherichia coli* heat-labile toxin, LT (R192G/L211A), as a safe and effective oral adjuvant. *Clinical and Vaccine Immunology*, *18*(4), 546-551.
35. Norton, E. B., Lawson, L. B., Mahdi, Z., Freytag, L. C., & Clements, J. D. (2012). The A subunit of *Escherichia coli* heat-labile enterotoxin functions as a mucosal adjuvant and promotes IgG2a, IgA, and Th17 responses to vaccine antigens. *Infection and immunity*, *80*(7), 2426-2435.
36. Marchioro, S. B., Fisch, A., Gomes, C. K., Jorge, S., Galli, V., Haesebrouck, F., Conceição, F. R. (2014). Local and systemic immune responses induced by a recombinant chimeric protein containing Mycoplasma hyopneumoniae antigens fused to the B subunit of *Escherichia coli* heat-labile enterotoxin LTB. *Veterinary microbiology*, *173*(1), 166-171.
37. Lee, S., Picking, W. L., & Tzipori, S. (2014). The immune response of two microbial antigens delivered intradermally, sublingually, or the combination thereof. *Microbes and infection*, *16*(9), 796-803.
38. Sjökvist Ottsjö, L., Jeverstam, F., Yrlid, L., Wenzel, A. U., Walduck, A. K., & Raghavan, S. (2017). Induction of mucosal immune responses against Helicobacter pylori infection after sublingual and intragastric route of immunization. *Immunology*, *150*(2), 172-183.
39. Lalsiamthara, J., Kamble, N. M., & Lee, J. H. (2016). A live attenuated Salmonella Enteritidis secreting detoxified heat labile toxin enhances mucosal immunity and confers protection against wild-type challenge in chickens. *Veterinary research*, *47*(1), 60.

40. Cabauatan, C., Campana, R., Niespodziana, K., Reinisch, C., Lundberg, U., Meinke, A., Valenta, R. (2017). Heat‐labile *Escherichia coli* toxin enhances the induction of allergen‐specific IgG antibodies in epicutaneous patch vaccination. *Allergy, 72*(1), 164-168.
41. Vajdy, M., Singh, M., Ugozzoli, M., Briones, M., Soenawan, E., Cuadra, L., Norelli, F. (2003). Enhanced mucosal and systemic immune responses to Helicobacter pylori antigens through mucosal priming followed by systemic boosting immunizations. *Immunology, 110*(1), 86-94.
42. Jelinek, T., & Kollaritsch, H. (2008). Vaccination with Dukoral® against travelers' diarrhea (ETEC) and cholera. *Expert review of vaccines, 7*(5), 561-567.
43. Evans, D. J., Evans, D. G., Opekun, A. R., & Graham, D. Y. (1988). Immunoprotective oral whole cell vaccine for enterotoxigenic *Escherichia coli* diarrhea prepared by in situ destruction of chromosomal and plasmid DNA with colicin E2. *FEMS microbiology immunology, 1*(1), 9-18.
44. Åhrén, C., Jertborn, M., & Svennerholm, A.-M. (1998). Intestinal immune responses to an inactivated oral enterotoxigenic *Escherichia coli* vaccine and associated immunoglobulin A responses in blood. *Infection and immunity, 66*(7), 3311-3316.
45. Holmgren, J., Bourgeois, L., Carlin, N., Clements, J., Gustafsson, B., Lundgren, A., Svennerholm, A.-M. (2013). Development and preclinical evaluation of safety and immunogenicity of an oral ETEC vaccine containing inactivated *E. coli* bacteria overexpressing colonization factors CFA/I, CS3, CS5 and CS6 combined with a hybrid LT/CT B subunit antigen, administered alone and together with dmLT adjuvant. *Vaccine, 31*(20), 2457-2464.
46. Sack, R. B., Kline, R., & Spira, W. (1988). Oral immunization of rabbits with enterotoxigenic *Escherichia coli* protects against intraintestinal challenge. *Infection and immunity, 56*(2), 387-394.
47. Zhang, W., & Sack, D. A. (2012). Progress and hurdles in the development of vaccines against enterotoxigenic *Escherichia coli* in humans. *Expert review of vaccines, 11*(6), 677-694.
48. McKenzie, R., Bourgeois, A. L., Engstrom, F., Hall, E., Chang, H. S., Gomes, J. G., Randall, R. (2006). Comparative safety and immunogenicity of two attenuated

enterotoxigenic *Escherichia coli* vaccine strains in healthy adults. *Infection and immunity*, *74*(2), 994-1000.

49. McKenzie, R., Darsley, M., Thomas, N., Randall, R., Carpenter, C., Forbes, E., Bourgeois, A. L. (2008). A double-blind, placebo-controlled trial to evaluate the efficacy of PTL-003, an attenuated enterotoxigenic *E. coli* (ETEC) vaccine strain, in protecting against challenge with virulent ETEC. *Vaccine*, *26*(36), 4731-4739.
50. Koprowski, H., Levine, M. M., Anderson, R. J., Losonsky, G., Pizza, M., & Barry, E. M. (2000). Attenuated Shigella flexneri 2a vaccine strain CVD 1204 expressing colonization factor antigen I and mutant heat-labile enterotoxin of enterotoxigenic *Escherichia coli*. *Infection and immunity*, *68*(9), 4884-4892.
51. Altboum, Z., Barry, E. M., Losonsky, G., Galen, J. E., & Levine, M. M. (2001). Attenuated Shigella flexneri 2a ΔguaBA Strain CVD 1204 Expressing Enterotoxigenic*Escherichia coli* (ETEC) CS2 and CS3 Fimbriae as a Live Mucosal Vaccine against Shigella and ETEC Infection. *Infection and immunity*, *69*(5), 3150-3158.
52. Ranallo, R. T., Fonseka, C. P., Cassels, F., Srinivasan, J., & Venkatesan, M. M. (2005). Construction and characterization of bivalent Shigella flexneri 2a vaccine strains SC608 (pCFAI) and SC608 (pCFAI/LTB) that express antigens from enterotoxigenic *Escherichia coli*. *Infection and immunity*, *73*(1), 258-267.
53. Favre, D., Lüdi, S., Stoffel, M., Frey, J., Horn, M. P., Dietrich, G., Viret, J.-F. (2006). Expression of enterotoxigenic *Escherichia coli* colonization factors in Vibrio cholerae. *Vaccine*, *24*(20), 4354-4368.
54. Khan, S. A., Stratford, R., Wu, T., Mckelvie, N., Bellaby, T., Hindle, Z., Pickard, D. (2003). Salmonella typhi and S. typhimurium derivatives harbouring deletions in aromatic biosynthesis and Salmonella Pathogenicity Island-2 (SPI-2) genes as vaccines and vectors. *Vaccine*, *21*(5), 538-548.
55. Frantz, J. C., & Robertson, D. C. (1981). Immunological properties of *Escherichia coli* heat-stable enterotoxins: development of a radioimmunoassay specific for heat-stable enterotoxins with suckling mouse activity. *Infection and immunity*, *33*(1), 193-198.
56. Fujihashi, K., Koga, T., van Ginkel, F. W., Hagiwara, Y., & McGhee, J. R. (2002). A dilemma for mucosal vaccination: efficacy versus toxicity using enterotoxin-based adjuvants. *Vaccine*, *20*(19), 2431-2438.

57. Ruan, X., Sack, D. A., & Zhang, W. (2015). Genetic fusions of a CFA/I/II/IV MEFA (multiepitope fusion antigen) and a toxoid fusion of heat-stable toxin (STa) and heat-labile toxin (LT) of enterotoxigenic *Escherichia coli* (ETEC) retain broad anti-CFA and antitoxin antigenicity. *PloS one, 10*(3), e0121623.

58. Ruan, X., Knudsen, D. E., Wollenberg, K. M., Sack, D. A., & Zhang, W. (2014). Multiepitope fusion antigen induces broadly protective antibodies that prevent adherence of *Escherichia coli* strains expressing colonization factor antigen I (CFA/I), CFA/II, and CFA/IV. *Clinical and Vaccine Immunology, 21*(2), 243-249.

59. Norton, E. B., Bauer, D. L., Weldon, W. C., Oberste, M. S., Lawson, L. B., & Clements, J. D. (2015). The novel adjuvant dmLT promotes dose sparing, mucosal immunity and longevity of antibody responses to the inactivated polio vaccine in a murine model. *Vaccine, 33*(16), 1909-1915.

60. Taxt, A., Aasland, R., Sommerfelt, H., Nataro, J., & Puntervoll, P. (2010). Heat-stable enterotoxin of enterotoxigenic *Escherichia coli* as a vaccine target. *Infection and immunity, 78*(5), 1824-1831.

61. Zhang, W., Zhang, C., Francis, D. H., Fang, Y., Knudsen, D., Nataro, J. P., & Robertson, D. C. (2010). Genetic fusions of heat-labile (LT) and heat-stable (ST) toxoids of porcine enterotoxigenic *Escherichia coli* elicit neutralizing anti-LT and anti-STa antibodies. *Infection and immunity*, *78*(1), 316-325.

62. Zhang, W., & Sack, D. A. (2015). Current progress in developing subunit vaccines against enterotoxigenic *Escherichia coli*-associated diarrhea. *Clinical and Vaccine Immunology*, *22*(9), 983-991.

63. Nandre, R. M., Ruan, X., Duan, Q., Sack, D. A., & Zhang, W. (2016). Antibodies derived from an enterotoxigenic *Escherichia coli* (ETEC) adhesin tip MEFA (multiepitope fusion antigen) against adherence of nine ETEC adhesins: CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS21 and EtpA. *Vaccine, 34*(31), 3620-3625.

64. Roy, K., Hamilton, D., Allen, K. P., Randolph, M. P., & Fleckenstein, J. M. (2008). The EtpA exoprotein of enterotoxigenic *Escherichia coli* promotes intestinal colonization and is a protective antigen in an experimental model of murine infection. *Infection and immunity, 76*(5), 2106-2112.

65. Fleckenstein, J., Sheikh, A., & Qadri, F. (2014). Novel antigens for enterotoxigenic *Escherichia coli* vaccines. *Expert review of vaccines, 13*(5), 631-639.
66. Kumar, P., Luo, Q., Vickers, T. J., Sheikh, A., Lewis, W. G., & Fleckenstein, J. M. (2014). EatA, an immunogenic protective antigen of enterotoxigenic *Escherichia coli*, degrades intestinal mucin. *Infection and immunity, 82*(2), 500-508.
67. Luo, Q., Kumar, P., Vickers, T. J., Sheikh, A., Lewis, W. G., Rasko, D. A., Fleckenstein, J. M. (2014). Enterotoxigenic *Escherichia coli* secretes a highly conserved mucin-degrading metalloprotease to effectively engage intestinal epithelial cells. *Infection and immunity, 82*(2), 509-521.
68. Takahashi, I., Kiyono, H., Jackson, R. J., Fujihashi, K., Staats, H. F., Hamada, S., McGhee, J. R. (1996). Epitope maps of the *Escherichia coli* heat-labile toxin B subunit for development of a synthetic oral vaccine. *Infection and immunity, 64*(4), 1290-1298.
69. Guan, W., Liu, W., Bao, J., Li, J., Yuan, C., Tang, J., & Shi, D. (2015). Analysis and application of a neutralizing linear epitope on liable toxin B of enterotoxin *Escherichia coli*. *Applied microbiology and biotechnology, 99*(14), 5985-5996.
70. De Haan, L., Verweij, W., Feil, I., Holtrop, M., Hol, W. G., Agsteribbe, E., & Wilschut, J. (1998). Role of GM1 binding in the mucosal immunogenicity and adjuvant activity of the *Escherichia coli* heat‐labile enterotoxin and its B subunit. *Immunology, 94*(3), 424-430.
71. Nashar, T. O., Amin, T., Marcello, A., & Hirst, T. R. (1993). Current progress in the development of the B subunits of cholera toxin and *Escherichia coli* heat-labile enterotoxin as carriers for the oral delivery of heterologous antigens and epitopes. *Vaccine, 11*(2), 235-240.
72. Oszvald, M., Kang, T.-J., Tomoskozi, S., Tamas, C., Tamas, L., Kim, T.-G., & Yang, M.-S. (2007). Expression of a synthetic neutralizing epitope of porcine epidemic diarrhea virus fused with synthetic B subunit of *Escherichia coli* heat labile enterotoxin in rice endosperm. *Molecular biotechnology, 35*(3), 215-224.
73. de Haan, L., Holtrop, M., Verweij, W. R., Agsteribbe, E., & Wilschut, J. (1996). Mucosal immunogenicity of the *Escherichia coli* heat-labile enterotoxin: role of the A subunit. *Vaccine, 14*(4), 260-266.

74. Lencer, W., Constable, C., Moe, S., Jobling, M., Webb, H. M., Ruston, S., Holmes, R. (1995). Targeting of cholera toxin and *Escherichia coli* heat labile toxin in polarized epithelia: role of COOH-terminal KDEL. *The Journal of Cell Biology, 131*(4), 951-962.

75. Fingerut, E., Gutter, B., Goldway, M., Eliahoo, D., & Pitcovski, J. (2006). B subunit of *E. coli* enterotoxin as adjuvant and carrier in oral and skin vaccination. *Veterinary immunology and immunopathology, 112*(3), 253-263.

76. Norton, E. B., Branco, L. M., & Clements, J. D. (2015). Evaluating the A-subunit of the heat-labile toxin (LT) as an immunogen and a protective antigen against enterotoxigenic *Escherichia coli* (ETEC). *PloS one, 10*(8), e0136302.

77. Haan, D. (1999). Mucosal immunogenicity and adjuvant activity of the recombinant A subunit of the *Escherichia coli* heat‐labile enterotoxin. *Immunology, 97*(4), 706-713.

78. Larsen, J. E., Lund, O., & Nielsen, M. (2006). Improved method for predicting linear B-cell epitopes. *Immunome research, 2*(1), 2.

79. Kelley, L. A., Mezulis, S., Yates, C. M., Wass, M. N., & Sternberg, M. J. (2015). The Phyre2 web portal for protein modeling, prediction and analysis. *Nature protocols, 10*(6), 845-858.

80. Duan, Q., & Zhang, W. (2016). Structure, Enterotoxicity, and Immunogenicity of Enterotoxigenic *Escherichia coli* Heat-Stable Type I Toxin (STa) and Derivatives.

81. Liu, M., Ruan, X., Zhang, C., Lawson, S. R., Knudsen, D. E., Nataro, J. P., Zhang, W. (2011). Heat-labile-and heat-stable-toxoid fusions (LTR192G-STaP13F) of human enterotoxigenic *Escherichia coli* elicit neutralizing antitoxin antibodies. *Infection and immunity, 79*(10), 4002-4009.

82. Hayat, S.-M. G., Gargari, S.-L. M., & Nazarian, S. (2016). Construction and immunogenic properties of a chimeric protein comprising CfaE, CfaB and LTB against Enterotoxigenic *Escherichia coli*. *Biologicals, 44*(6), 503-510.

Appendix A - Your Appendix Title