


REVIEW



Development of an enterotoxigenic *Escherichia coli* vaccine based on the heat-stable toxin

Ephrem Debebe Zegeye^a, Morten Larsen Govasli^a, Halvor Sommerfelt^{b,c}, and Pål Puntervoll ^a

^aCentre for Applied Biotechnology, Uni Research AS, Bergen, Norway; ^bCentre for Intervention Science in Maternal and Child Health, Centre for International Health, Department of Global Public Health and Primary Care, University of Bergen, Bergen, Norway; ^cNorwegian Institute of Public Health, Oslo, Norway

ABSTRACT

Infection with enterotoxigenic *Escherichia coli* (ETEC) is an important cause of diarrhea-related illness and death among children under 5 years of age in low- and middle-income countries (LMIC). Recent studies have found that it is the ETEC subtypes that produce the heat-stable enterotoxin (ST), irrespective of whether they also secrete the heat-labile enterotoxin (LT), which contribute most importantly to the disease burden in children from LMIC. Therefore, adding an ST toxoid would importantly complement ongoing ETEC vaccine development efforts. The ST's potent toxicity, its structural similarity to the endogenous peptides guanylin and uroguanylin, and its poor immunogenicity have all complicated the advancement of ST-based vaccine development. Recent remarkable progress, however, including the unprecedented screening for optimal ST mutants, mapping of cross-reacting ST epitopes and improved ST-carrier coupling strategies (bioconjugation and genetic fusion), enables the rational design of safe, immunogenic, and well-defined ST-based vaccine candidates.

ARTICLE HISTORY

Received 1 April 2018
Revised 8 June 2018
Accepted 24 June 2018

KEYWORDS

enterotoxigenic *Escherichia coli*; diarrhea; vaccine; heat-stable toxin; toxoid; toxicity; immunological cross-reaction

Introduction

Infection with enterotoxigenic *Escherichia coli* (ETEC) is one of the major causes of diarrhea-related illness and death among children under 5 years of age in low- and middle-income countries (LMIC).^{1–3} These very common infections are estimated to cause some 25,000 child deaths annually and contribute to child malnutrition.¹ Besides, ETEC is the leading cause of diarrhea among tourists^{4,5} and military personnel^{6,7} staying in ETEC endemic regions. Deaths associated with ETEC infection are declining worldwide; nevertheless, morbidity remains high.¹ Notably, children who survive repeated diarrheal episodes have a higher risk of long-term sequelae, including impaired cognitive development, growth faltering, and obesity.⁸ Collectively, these short and long-term negative impacts of ETEC infection underline the need for effective interventions.

Protection against ETEC colonization and diarrhea through the use of effective vaccines appears to be the most promising way forward.² There are, however, no effective vaccines against ETEC at present. Currently, multiple efforts, including the development of whole-cell and subunit vaccines, are underway to develop a broadly protective ETEC vaccine.⁹ The more advanced candidates include the inactivated whole-cell (ETVAX)¹⁰ and the live-attenuated ACE527¹¹ vaccines. Recent studies confirm our earlier work¹² that ETEC strains producing the heat-stable enterotoxin (ST), with or without the heat-labile enterotoxin (LT), are strongly associated with diarrhea among children under 5 years of age,^{3,13} while the strains that only produce LT contribute only marginally. This underpins the importance of including an ST toxoid in

vaccine formulations to achieve a broad protection against ETEC.⁹ Herein, we review the recent advances in ETEC subunit vaccine development, with an emphasis on the approaches that target ST.

Mechanisms of human ETEC-mediated diarrhea

Human ETEC is comprised of genetically diverse stains,¹⁴ but they share the ability to colonize the small intestine where they secrete the plasmid-encoded ST and/or LT. Either of these potent toxins can cause diarrhea individually, but when present together, they appear to act synergistically.¹⁵ Following an infection through the fecal-oral route, ETEC utilizes surface exposed adhesins, called colonization factors (CFs), to colonize the small intestine and elaborate the toxins. The CFs consist of repeating protein subunits and can be fibrial, helical or fibrillary structures.¹⁶

LT is a heterohexameric protein containing one A subunit (LTA) and five identical B subunits (LTB), and it is structurally and functionally related to the cholera toxin.¹⁷ ST exist in two nearly identical variants, STa I and STa II, which are commonly called STp (18 amino acids) and STh (19 amino acids), where p and h refer to their initial discovery in ETEC isolated from pigs and humans, respectively.¹⁸ While ETEC producing either STh or STp can induce diarrhea in humans,^{12,19,20} the STh-producers contribute with the bulk of the diarrheal disease burden in LMIC children.^{12,21} ST and LT induce diarrhea by binding to their cognate receptors, the transmembrane guanylate cyclase C (GC-C) and GM1-ganglioside on the epithelial cell surface, respectively,

thereby triggering a cascade of signaling pathways that disrupt the electrolyte and fluid homeostasis, ultimately leading to secretion of water and electrolytes into the intestinal lumen. While GM1 is the primary receptor for LT, secondary receptors, including blood group antigens, Toll-like receptor 2 and additional gangliosides or ganglioside derivatives have been reported.^{22,23} Guanylin and uroguanylin are endogenous ligands that act on GC-C to regulate fluid and electrolyte secretion into the intestinal lumen. Interestingly, ST also appears to modulate the immune system of the host, thereby preventing a strong immune response against the infection.¹⁵ Likewise, exposure to LT has been shown to downregulate expression of human antimicrobial peptides.²⁴

Structure and function of the GC-C receptor ligands

The ETEC heat-stable toxins were discovered already in the 1970s²⁵ and shown to cause increased levels of cGMP in intestinal cells.²⁶ In 1990, the GC-C receptor was shown to be responsible for the ST-induced increased levels of intracellular cGMP,²⁷ and a few years later the endogenous peptides guanylin²⁸ and uroguanylin²⁹ were discovered.

GC-C is a heavily glycosylated transmembrane protein consisting of an extracellular receptor domain (ECD), a single transmembrane helix, a kinase homology domain, and a catalytic GC domain.^{18,30} Although the structure of GC-C has

not been solved, homology modeling suggests that the receptor may form oligomers and that the membrane-proximal subdomain of ECD appears to contain the binding site for ST and the endogenous ligands.^{18,31,32} An additional receptor for guanylin and uroguanylin has been identified, namely GC-D, which is exclusively expressed in olfactory neurons.^{33,34} In kidneys, however, guanylin and uroguanylin act primarily through GC-C independent receptors that are not yet identified.³⁵

The structure of a fully toxic truncated synthetic STp analog, where C5 was substituted with β -mercaptopropionic acid, was solved by X-ray crystallography in 1991.³⁶ The STp analog covers the 13-amino acid *toxic domain*, from the first (C5) to the last (C17) cysteine residues. The solution structures of guanylin³⁷ and uroguanylin³⁸ were solved later by NMR, demonstrating striking structural similarities to each other and to STp (Figure 1A). These three experimental structures were used as templates to build structural models for the full-length STp and STh peptides³⁹ (Figure 1A), which are useful when comparing the structural properties of the four GC-C ligand peptides. The guanylin and uroguanylin peptides have two disulfide bridges that define their structures (1–3/2–4 pattern, Figure 1B). Interestingly, these peptides can form two topological isomers that can dynamically interconvert.^{37,38} Isomer A of guanylin and uroguanylin are biologically active and their structures resemble those of the

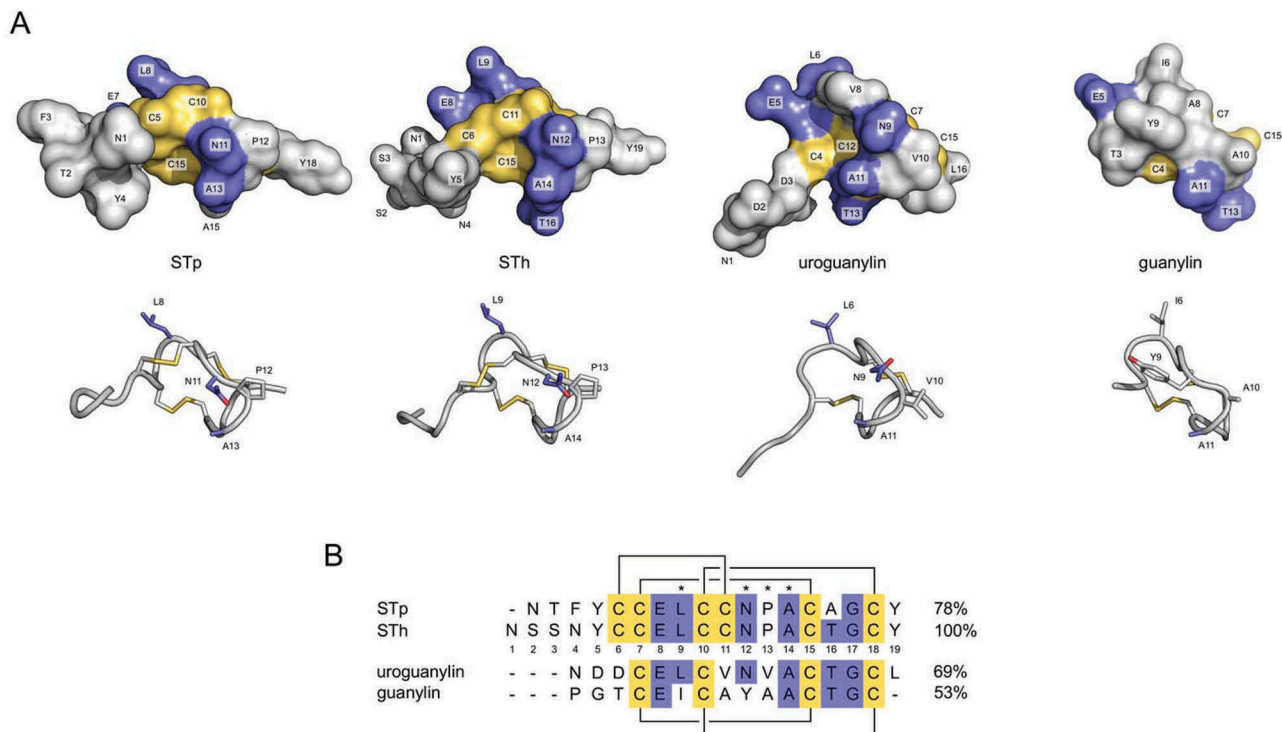


Figure 1. Structural models and sequence alignment of STp, STh, uroguanylin and guanylin. A) Surface (top) and cartoon (bottom) representation of structural models of STp³⁹ and STh³⁹ and NMR structures of uroguanylin (PDB accession no. 1UYA) and guanylin (PDB accession no. 1GNA). Each residue is named using the one-letter amino acid code and numbered according to the individual peptide sequence. The first two N-terminal residues in guanylin are largely unstructured in solution, and thus only the structure of the 13 C-terminal residues was solved by NMR.³⁷ The disulfide bridge-forming cysteines are shown in yellow, and other residues shared between at least three of the peptides are shown in blue. In the cartoon representation, the disulfide bridges are shown as sticks with sulfur atoms in yellow. The receptor-interacting residues of the ST peptides, and the equivalent residues of uroguanylin and guanylin, are also shown as sticks with oxygen atoms in red and nitrogen atoms in dark blue. B) Sequence alignment of STp, STh, uroguanylin, and guanylin. The residues are colored as described for the structures: cysteines in yellow and residues shared between at least three peptides in blue. The disulfide bridge connectivity for STp and STh is shown above the alignment, and that of uroguanylin and guanylin is shown below. Note that all peptides share two of the disulfide bridges. The receptor-interacting residues are marked with asterisks, and the residue numbers of STh are shown below the STh sequence. Sequence identity (percentage) relative to STh is shown to the right of the alignment.

ST peptides (Figure 1A). Isomer B, on the other hand, are unable to activate the GC-C receptor. The ST peptides share the two disulfide bridges of the endogenous peptides (2–5/3–6 pattern, Figure 1B), but has an additional disulfide bridge (1–4 pattern, Figure 1B) which apparently locks the ST structures in the active topological form and may explain why ST has a higher affinity to the GC-C receptor than the endogenous peptides.^{18,40,41}

The structural similarities between the four peptides are also reflected in their sequences (Figure 1B). STh and STp are very similar and share 13 of their 19 and 18 residues, respectively (78% sequence identity), and only one residue is different inside the toxic domain (STh: T16; STp: A15). The endogenous peptide which is most similar to the ST peptides is uroguanylin, which shares 11 residues with STh (69% sequence identity). Guanylin is less similar but shares 8 residues with STh (53% sequence similarity).

There is no direct structural evidence of the interaction between GC-C peptides and the GC-C receptor, but several mutational studies performed in the 1990s and 2000s on STh and STp have resulted in the identification of residues important for function.^{42–44} These studies mainly targeted the residues N12, P13, and A14 (STh residue numbering is used here and throughout the review), all of which appeared to be important for the biological activity of the ST peptides, and were labeled *receptor-interacting residues*. We recently published the most comprehensive mutational study of STh to date, where all possible 361 single amino acid substitutions of STh were screened for effects on toxicity and antigenicity.⁴⁵ This study confirmed the functional importance of N12, P13, and A14, but additionally identified L9 as a novel receptor-interacting residue (Figure 1A). The A14 residue has the most compelling evidence for being directly involved in the interaction with the receptor, as 17 of 19 possible substitutions in this position leads to dramatically reduced biological activity.⁴⁵ Even the two least dramatic substitutions lead to around 10-fold reduction in biological activity,^{43,45} despite entailing the minor changes of either removing the methyl side chain of alanine (glycine mutation), or the addition of a hydroxyl group to the alanine side chain (serine). The importance of the A14 residue for a biological function is further corroborated by the fact that A14 is the only receptor-interacting residue that is conserved in all four GC-C peptides (Figure 1).

With the exception of P13, all the receptor-interacting residues identified in the ST peptides are shared with uroguanylin (Figure 1). Other residues that are shared between STh and uroguanylin are E8, T16, G17, and the four cysteine residues C7, C10, C15, and C18. The extent of shared residues explains the observed immunological cross-reaction between anti-STh antibodies and uroguanylin,^{39,45} and underpins the importance of addressing unwanted immunological cross-reaction when constructing and evaluating vaccine candidate ST toxoids.

Recent updates on CF-based ETEC subunit vaccine development

Subunit vaccines have the potential of allowing the development of safe and well-defined vaccines containing only

the desired antigens leading to targeted immune responses towards specific epitopes.⁴⁶ They further allow exclusion of parts of antigens that may cause unwanted immunological cross-reactions or allergenic and reactogenic responses.^{46,47}

In a response to infection with ETEC, the human body normally elicits antibody responses against the CFs and LT.^{48,49} Therefore, canonical approaches for ETEC vaccine development have primarily been focused on engendering protection against the CFs and LT.^{11,50,51} Unfortunately, CF- and LT-based vaccines have not yet been shown to confer adequate protection, even in travelers, in whom ETEC that express only LT is indeed an important contributor to diarrhea.^{51–53} As mentioned above, because such strains do not contribute importantly to the diarrheal disease burden in LMIC children, it is inappropriate to only aim for anti-LT immunity. This challenge could be circumvented by engendering immune responses to important somatic antigens on ETEC strains that express STh. Thus, despite there being no evidence that CFs induce anticolonizing immunity after natural ETEC infections,⁵⁴ important efforts are being made to develop a vaccine which induces immune responses against the major CFs, i.e. those on ETEC that account for the bulk of ETEC diarrheal disease burden. However, this vaccine development strategy is complicated by the fact that human ETEC may produce one or more of over 25 immunologically distinct CFs,¹⁶ and it is likely that new CFs will be discovered.¹⁴ Recently, some level of cross-protection was reported.¹⁰

To overcome the challenges posed by the heterogeneity of CF, recent subunit vaccine approaches have employed multi-epitope fusion antigen (MEFA) technology to integrate epitopes from CFs that are produced by strains most commonly found associated with moderate and severe diarrhea.⁵⁵ Accordingly, *in silico* predicted B-cell epitopes from CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS21, and EtpA were inserted into the backbone of the colonization factor antigen I (CFA/I) tip subunit CfaE where they replaced surface exposed, but less antigenic peptides.⁵⁶ Notably, mice immunized with the chimeric antigen elicited antibodies against all the nine epitopes, and the sera prevented the attachment of ETEC strains to Caco-2 cells *in vitro*.⁵⁶ MEFA CFs were further genetically fused to an ST toxoid and LT antigen to afford even broader protection.^{57,58} Immunized mice that were challenged with lethal doses of ETEC (CFA/I⁺, LT⁺, ST⁺) were protected.⁵⁸ Collectively, these results are encouraging, and future ETEC challenge studies will determine whether multi-epitope fusions may provide broad protection against ETEC diarrhea in humans.

Generally, a multivalent subunit vaccine that targets different stages of the ETEC infection process, including mucin degradation, adhesion and toxin neutralization, may lead to a broadly protecting ETEC vaccine. In this regard, the recently identified novel and fairly conserved protective antigens, including YghJ, EtpA, EatA, EaeH, and flagellin⁵⁹ could be exploited for vaccine development, whilst evaluating the risk that YghJ, EaeH and flagellin-based vaccines may pose to the *E. coli* commensals, since these antigens are often also produced by commensal *E. coli*.

Strategies for designing ST-based subunit vaccines

The ST amino acid sequences are highly conserved (Figure 1B)⁶⁰ and STh-producing ETEC, whether they also produce LT or not, contribute to the bulk of diarrhea in LMIC children.^{3,13,21} In addition, ETEC that produce STp and/or LT contribute to diarrhea in travelers and may cause outbreaks.¹⁹ Taken together, this makes it important to also target ST when developing a broadly protective ETEC vaccine. Nonetheless, the *poor immunogenicity* of ST, its *potent toxicity*, and its potential for *immunological cross-reactivity* with the human gastrointestinal peptides (guanylin and uroguanylin) have so far hampered the advancement of an ST-based vaccine.^{39,42} The development of an ST-toxoid for inclusion in a broadly protective ETEC vaccine thus needs to overcome these three challenges, while ensuring that the ST toxoid engenders antibodies that effectively neutralize STh, and preferably also STp.

Making ST immunogenic

Natural ETEC infections do not appear to induce an immune response against ST. This is presumably due to the small size of ST (~ 2 kDa) as small molecules called haptens (incomplete antigens) are generally poor immunogens.⁶¹ To elicit anti-ST antibodies, ST has commonly been coupled to an immunogenic protein (carrier protein) through chemical crosslinking (bioconjugation)^{62,63} or genetic fusion.⁶⁴ Both approaches have been effective in eliciting antibodies against ST. Efforts have also been made to develop ST-based vaccines without using carrier proteins. Encouraging results have emerged from experiments with ST polymerization using glutaraldehyde,^{65,66} ST coupled to a poly lactic-co-glycolic acid (PLGA) nanoparticle,⁶⁷ and ST coupled to a lipopeptide module (T-helper cell epitope and S-[2,3-bis (palmitoyloxy) propyl] cysteine).⁶⁸

Abolishing ST toxicity

ST's toxicity is significantly reduced or abolished when conjugated⁶³ or genetically fused⁶⁹ to carrier proteins. However, the reversibility of some conjugation reactions⁷⁰ or proteolytic activity⁷¹ in the vaccine recipients after oral delivery could potentially release the native toxin. Therefore, detoxifying mutations are required to ensure a safe ST-based vaccine. But due to its small size, ST has a limited repertoire of epitopes, and mutations must be carefully chosen to avoid disrupting protective epitopes or forming neo-epitopes.

Minimizing immunological cross-reactivity with guanylin and uroguanylin

The endogenous peptides guanylin and uroguanylin play key roles in regulating electrolytes and fluid homeostasis in the human intestine via GC-C, which is the primary receptor for guanylin and uroguanylin.^{27,72} Although GC-C is predominantly expressed in the intestines,⁷³ transcripts have also been detected in kidneys, adrenal gland, brain, lung and reproductive organs,^{33,34,74,75} suggesting crucial roles played by the

GC-C ligands in the human physiology. Recently, there is a renewed interest in these peptide hormones as potential therapeutic targets for obesity, ulcerative colitis, and metabolic diseases.^{75–77}

Given their key roles in human physiology, their resemblance to ST (Figure 1A) raises a safety concern that anti-ST antibodies elicited by ST-based vaccines may cross-react with these endogenous peptides. Indeed, it has already been shown that anti-STh antibodies can cross-react with uroguanylin, albeit only partially and with lower affinity.³⁹ Hence, one or more mutations may also be needed to minimize the risk of engendering antibodies that cross-react with the endogenous ligands.

In the following, we review recent efforts to address the three challenges, focusing on two strategies for making ST immunogenic by coupling it to a protein carrier, and on abolishing toxicity and reducing the risk for unwanted immunological cross-reaction by mutation.

Making ST immunogenic by coupling it to a protein carrier

Protein carriers provide the epitopes needed for recognition by T-helper cells in genetic fusions or chemical conjugates, thereby enabling the induction of high-affinity antibodies and immune memory also to peptide haptens.^{46,78} Carrier proteins to which ST has been coupled include the porcine immunoglobulin G, bovine serum albumin (BSA), cholera toxin B (CT-B), outer membrane OmpC, major subunit ClpG of *E. coli* CS31A fimbriae, *Salmonella* flagellin, and green fluorescent protein.^{42,79,80} In addition, LT and LT derivatives have been widely used with the rationale that broad protection can be achieved against ETEC strains producing either or both enterotoxins.^{62,63,69,81,82} To provide a more comprehensive protection, an ST toxoid-LTB fusion has further been genetically fused to ETEC CF antigens or epitopes that elicit anti-adhesin antibodies.^{57,58} The non-toxic double mutant LT (dmLT; LT_{R192G/L211A}) has also been used as a carrier, offering the additional benefit of engendering neutralizing anti-LT antibodies.^{83–85} Additionally, an LT-STh_{P13F} fusion was incorporated into a live attenuated oral *E. coli* vaccine (ZCR533) that constitutively express the antigen on its surface or as a secretion.⁸⁶

ST-carrier genetic fusion

The finding that native ST genetically fused with a carrier protein such as LT could elicit anti-ST neutralizing antibodies was first made in the 1980s.⁸⁷ A genetic fusion of an ST toxoid and LTb coupled to a gelatin nanoparticle was shown to protect mice when experimentally infected with ETEC.⁸⁸ Genetic fusion approaches to ST-based vaccine development has, therefore, been an important strategy for developing ST toxoid-based ETEC vaccines.^{57,81,89–92} Three copies of an STh-N12S mutant genetically fused with dmLT, 3xSTh_{N12S}-dmLT, was identified as a promising vaccine candidate that may engender neutralizing antibodies against both ST and LT.^{84,85,92} Notably, piglets born from 3xSTh_{N12S}-dmLT immunized pregnant gilts seemed to be passively protected

from ETEC diarrhea during challenge.⁸⁵ The 3xSTh_{N12S}-dmLT construct was further genetically fused to seven putative immunodominant colonization factor epitopes from prevalent ETEC strains (CFA/I/II/IV).⁵⁷ Interestingly, the expressed single polypeptide immunogen elicited anti-adhesin antibodies against all the seven CFA in addition to anti-ST and anti-LT antibodies in mice.^{57,93} The related adhesin-toxoid MEFA (CFA/I/II/IV-3xSTh_{N12S}-mnLT_{G192G/L211A}) containing epitopes of seven CF adhesins, three copies of STh toxoid and a monomeric LT mutant is currently among the leading ETEC subunit candidates in the preclinical phases,⁹³ and it remains to be seen whether it can elicit an active protective response in animal and human challenge studies.

The abovementioned recently developed ST fusion constructs were expressed as inclusion bodies in the cytoplasm of *E. coli* and subjected to a simple refolding protocol (Novagen). To our knowledge, the refolded immunogens have not been characterized structurally, and hence, it remains unclear to what extent the individual ST copies have correctly formed disulfide bridges and intact structures. Despite the fact that these fusion immunogens seem to elicit neutralizing anti-ST antibodies, one concern is to what extent that different immunogen batches will have consistent biophysical properties.

ST-carrier bioconjugation

Making ST immunogenic by chemically conjugating it to a protein carrier has two advantages over genetic fusion. The first is that the ST peptides (both native and mutants) can be made either recombinantly or by chemical synthesis,⁹⁴ and subsequently be subjected to thorough biophysical characterizations to ensure that protective epitopes are intact. This is important because ST contains conformational epitopes.^{95,96} The second advantage is that one can achieve much higher hapten-to-carrier ratios with bioconjugation than genetic fusions. This may be essential for eliciting strong and consistent immune responses. The drawback compared to genetic fusion is that the production procedure is more elaborate.

Chemical conjugation offers a plethora of options for making conjugates. The choice of coupling chemistry will govern the overall structure of the conjugate vaccine as well as how ST epitopes are presented to the immune system. It is important that the chosen coupling method leads to a well-defined conjugate that elicits ST-specific neutralizing antibodies with a high and consistent titer.

The most commonly used chemical crosslinkers for coupling ST to carrier proteins have been the carbodiimides^{62,79,97} and glutaraldehyde.^{39,63,65} However, these crosslinkers promote polymerization, leading to poorly defined and higher molecular weight conjugates that may eventually precipitate.⁹⁸ An improved protocol that involves introducing carboxyl groups on BSA and coupling ST through its N-terminus using dicyclohexylcarbodiimide (DCC) crosslinker has been reported.⁸⁰ Alternatively, heterobifunctional crosslinkers (containing different reactive groups on either end) used in a two-step conjugation will allow one to tailor the molecular orientation of ST on the carrier.⁹⁸ Additional factors that need to be considered for optimization include the solubility of the

resulting conjugate, spacer arm (affects flexibility and steric hindrance), the potential for immunogenicity of the cross-linker, and *in vivo* toxicity.⁹⁹

The way ST is oriented on the carrier may have a profound effect on the immune response to the conjugate. For example, ST coupled to a lipopeptide module via N-terminus elicited better neutralizing antibodies than when coupled via the C-terminus.⁶⁸ Likewise, a 19-amino acid peptide (verotoxin 2e) conjugated to ovalbumin through a central amino acid gave higher antibody titers than when the peptide was coupled via terminal residues.¹⁰⁰ ST has three reactive groups that can be used for conventional conjugation: the amino group of the N-terminus, the carboxyl groups of the C-terminus and the glutamic acid residue (E8), and the thiol groups of the cysteines. However, the thiol groups should be excluded to avoid disruption of the disulfide bridges. When producing ST by chemical synthesis it is possible to block certain reactive groups or to introduce non-natural amino acids to enable conjugation through a targeted residue.¹⁰¹ Interestingly, linking ST with a lipopeptide module via an oxime linkage generated antibodies with a better neutralizing capacity than when thioether linkage was used⁶⁸ suggesting that the chemical nature of the linkage could also affect the quality of the immune response to the toxoid.

As mentioned, bioconjugation allows for higher hapten-to-carrier ratios than genetic fusion. However, although higher ratios usually lead to higher antibody titers, it does not guarantee that the antibodies produced will have a higher affinity to ST or be more capable of neutralizing ST. For instance, an increasing ratio of verotoxin 2e to ovalbumin was found to correlate with an increased antibody titer, but the affinity of the antibodies became poorer.¹⁰⁰ Therefore, the ST-to-carrier ratio will require optimization for each vaccine design.

Mutating ST to abolish toxicity and reduce the risk of unwanted immunological cross-reactions

Two safety concerns require that the native ST toxin is modified by mutation, namely to abolish toxicity and to reduce the risk of eliciting antibodies that cross-react with the endogenous peptides. As mentioned, the small size of ST implies that it has a limited repertoire of epitopes, and that mutations must be carefully chosen to avoid disrupting protective epitopes.

In a recent screen of 14 single amino acid ST mutants fused to a dmLT, STh-N12S, STh-N12T and STh-A14H were identified as promising ST toxoids.⁹² The STh-N12S seems to be the most promising one, and is the current lead candidate mutation for the LT-based genetic fusions reviewed above.^{84,85}

In our mutational study of all possible 361 single-amino acid substitutions of STh, where we screened for effects on toxicity and antigenicity, the top 30 toxoid candidates (based on their antigenicity-toxicity ratios) had mutations either in L9, N12 or A14.⁴⁵ Notably, 17 A14 mutation toxoids had undetectable toxicities in the T84 cell assay,⁴⁵ suggesting that A14 is the prime residue to target for abolishing toxicity.

The screen further demonstrated that mutations of the structurally important cysteine residues had a profound

impact on both toxicity and antigenicity,^{45,102} and that mutations of P13 also, but to a lesser extent, affected both toxicity and antigenicity.⁴⁵ Hence, to ensure the formation of ST mutants with a correct structure and intact epitopes, these residues should not be targeted for mutation.

The STh mutant library was also used to map the epitopes of three neutralizing monoclonal antibodies.⁴⁵ This led to two key observations: that the L9 residue which is shared with uroguanylin is the main epitope residue of a cross-reacting anti-STh monoclonal antibody and that the main epitope residue of two non-cross-reacting anti-STp monoclonal antibodies was Y19. This implies that L9 is an attractive residue to target for mutation, resulting in mutant variants with both reduced toxicity and risk for cross-reactions to the endogenous peptides. It also implies that Y19 constitutes an ST-specific and safe epitope, which should be left intact and exposed in a vaccine candidate toxoid. This may also explain why ST coupled to a lipopeptide module via the N-terminus elicited better neutralizing antibodies than when coupled via the C-terminus.⁶⁸

The single STh mutant, N12S, and two double mutants, L9A/N12S, and N12S/A14T, were recently reported to elicit neutralizing antibodies in mice that showed little immunological cross-reactions to the endogenous peptides, when genetically fused in triplicate to monomeric-LT_{R192G/L211A}.⁹¹ However, when the authors assessed the immunological cross-reactions using a competitive ST ELISA, the ratio between free and bound peptide competing for binding to antibody was kept constant, in contrast to our previously published approach where we varied the ratio between free and bound peptides.³⁹ In our opinion, a proper dose-response experiment must be conducted in order to convincingly describe possible immunological cross-reactions or the lack thereof. In addition, sera were pooled from six individual mice prior to testing for both neutralization and cross-reactions, which is unfortunate, as it may mask inter-individual variations, which we have recently observed (Diaz et al., manuscript in preparation).

In conclusion, over the recent years considerable progress has been made on making ST immunogenic, and on identifying mutations that will reduce or abolish toxicity. But the important challenge of demonstrating the reduction of unwanted immunological cross-reactions to guanylin and uroguanylin remains.

Concluding remarks and future perspectives

The lack of well-conserved antigens to formulate a broadly protective vaccine has been the main challenge in ETEC vaccine development. The heterogeneity of the CFs has so far posed a practical challenge in formulating a CF-based ETEC vaccine that targets the ETEC strains responsible for the bulk of ETEC diarrhea. Recent ST toxoid-based ETEC subunit strategies outlined here are showing encouraging results that we believe may importantly complement the CF- and LT-based approaches. We expect that these efforts will soon result in ST toxoid candidates that can be evaluated in clinical trials.

Meanwhile, the lack of a suitable animal model that recapitulates ETEC mediated infection and diarrhea hinders the screening of potential vaccine candidates at an early stage of

vaccine development. So far, mouse models are restricted to studying the protective efficacy of antigens in ETEC colonization^{103,104} and protection against lethal ETEC doses.⁵⁸ Furthermore, human challenge models for evaluating the protective efficacy of ETEC vaccines have so far focused on strains that also produce LT.^{105,106} There is a need to develop a human challenge model for ETEC that only express STh, and important progress towards establishing such a model has recently been made.¹⁰⁷

Finally, since ETEC is an enteric pathogen, the ETEC vaccine needs to primarily induce mucosal IgA responses. In this respect, dmLT has shown promise as an adjuvant that redirects parenterally administered vaccines to an immune response in the gut.¹⁰⁸

It is our hope that ongoing efforts to develop a safe and immunogenic ST toxoid capable of inducing strong mucosal immune responses in the human gut can importantly complement ETEC vaccine candidates currently in the pipeline to produce a broadly protective ETEC vaccine that can substantially reduce the diarrheal disease burden in LMIC children.

Disclosure of potential conflicts of interest

The research team has submitted a patent application (application no. 61/766,958) for ST toxoid which is intended for use in ETEC vaccines.

Acknowledgments

We thank Dr. Hans Steinsland and Dr. Gro Elin Kjørang Bjerga for their valuable comments on the manuscript, and Dr. Kurt Hanevik for sharing unpublished result on ETEC human challenge studies.

Funding

Research Council of Norway through GLOBVAC (grant # 234364) and FORNY (grant # 260686), and PATH (grant #102290-002).

ORCID

Pål Puntervoll  <http://orcid.org/0000-0003-0093-7816>

References

1. Wang H, Naghavi M, Allen C, Barber R, Bhutta ZA, Carter C, Casey C, Charlson F, Chen C, Coates M, et al. Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980–2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet*. 2016;388:1459–1544. <http://www.ncbi.nlm.nih.gov/pubmed/27733281>
2. Kotloff KL, Platts-Mills JA, Nasrin D, Roose A, Blackwelder WC, Levine MM. Global burden of diarrheal diseases among children in developing countries: incidence, etiology, and insights from new molecular diagnostic techniques. *Vaccine* [Internet]. 2017;35:6783–6789. <http://www.ncbi.nlm.nih.gov/pubmed/28765005>.
3. Platts-Mills JA, Babji S, Bodhidatta L, Gratz J, Haque R, Havt A, McCormick BJJ, McGrath M, Olortegui MP, Samie A, et al. Pathogen-specific burdens of community diarrhoea in developing countries: A multisite birth cohort study (MAL-ED). *Lancet Glob Heal* [internet]. 2015;3:e564–75. doi:10.1016/S2214-109X(15)00151-5.

4. WHO. Future directions for research on enterotoxigenic *Escherichia coli* vaccines for developing countries. *Relev Epidemiol Hebd* [Internet]. 2006;81:97–104. <http://www.ncbi.nlm.nih.gov/pubmed/16671213>.
5. Torres OR, González W, Lemus O, Pradesaba RA, Matute JA, Wiklund G, Sack DA, Bourgeois AL, Svennerholm A-M. Toxins and virulence factors of enterotoxigenic *Escherichia coli* associated with strains isolated from indigenous children and international visitors to a rural community in Guatemala. *Epidemiol Infect* [Internet]. 2015;143:1662–1671. <http://www.ncbi.nlm.nih.gov/pubmed/25233938>.
6. Nada RA, Armstrong A, Shaheen HI, Nakhla I, Sanders JW, Riddle MS, Young S, Sebeny P. Phenotypic and genotypic characterization of enterotoxigenic *Escherichia coli* isolated from U.S. military personnel participating in Operation Bright Star, Egypt, from 2005 to 2009. *Diagn Microbiol Infect Dis* [Internet]. 2013;76:272–277. <http://www.ncbi.nlm.nih.gov/pubmed/23639795>.
7. Monteville MR, Riddle MS, Baht U, Putnam SD, Frenck RW, Brooks K, Moustafa M, Bland J, Sanders JW. Incidence, etiology, and impact of diarrhea among deployed US military personnel in support of Operation Iraqi Freedom and Operation Enduring Freedom. *Am J Trop Med Hyg* [Internet]. 2006;75:762–767. <http://www.ncbi.nlm.nih.gov/pubmed/17038708>.
8. Guerrant RL, DeBoer MD, Moore SR, Scharf RJ, Lima AAM. The impoverished gut—a triple burden of diarrhoea, stunting and chronic disease. *Nat Rev Gastroenterol Hepatol* [Internet]. 2013;10:220–229. <http://www.ncbi.nlm.nih.gov/pubmed/23229327>.
9. Bourgeois AL, Wierzbica TF, Walker RI. Status of vaccine research and development for enterotoxigenic *Escherichia coli*. *Vaccine* [Internet]. 2016;34:2880–2886. <http://www.ncbi.nlm.nih.gov/pubmed/26988259>.
10. Leach S, Lundgren A, Carlin N, Löfstrand M, Svennerholm A-M. Cross-reactivity and avidity of antibody responses induced in humans by the oral inactivated multivalent enterotoxigenic *Escherichia coli* (ETEC) vaccine ETVAX. *Vaccine* [Internet]. 2017;35:3966–3973. <http://www.ncbi.nlm.nih.gov/pubmed/28625524>.
11. Darsley MJ, Chakraborty S, DeNearing B, Sack DA, Feller A, Buchwaldt C, Bourgeois AL, Walker R, Harro CD. The oral, live attenuated enterotoxigenic *Escherichia coli* vaccine ACE527 reduces the incidence and severity of diarrhea in a human challenge model of diarrheal disease. *Clin Vaccine Immunol* [Internet]. 2012;19:1921–1931. <http://www.ncbi.nlm.nih.gov/pubmed/23035175>.
12. Steinsland H, Valentiner-Branth P, Perch M, Dias F, Fischer TK, Aaby P, Mølbak K, Sommerfelt H. Enterotoxigenic *Escherichia coli* infections and diarrhea in a cohort of young children in Guinea-Bissau. *J Infect Dis* [Internet]. 2002;186:1740–1747. <http://www.ncbi.nlm.nih.gov/pubmed/12447759>.
13. Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, Wu Y, Sow SO, Sur D, Breiman RF, et al. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. *Lancet*. 2013;382:209–222. doi:10.1016/S0140-6736(13)60844-2.
14. Sahl JW, Sistrunk JR, Baby NI, Begum Y, Luo Q, Sheikh A, Qadri F, Fleckenstein JM, Rasko DA. Insights into enterotoxigenic *Escherichia coli* diversity in Bangladesh utilizing genomic epidemiology. *Sci Rep* [Internet]. 2017;7:3402. <https://www.nature.com/articles/s41598-017-03631-x>.
15. Read LT, Hahn RW, Thompson CC, Bauer DL, Norton EB, Clements JD. Simultaneous exposure to *Escherichia coli* heat-labile and heat-stable enterotoxins increases fluid secretion and alters cyclic nucleotide and cytokine production by intestinal epithelial cells. *Infect Immun* [Internet]. 2014;82:5308–5316. <http://www.ncbi.nlm.nih.gov/pubmed/25287923>.
16. Madhavan TPV, Sakellaris H. Colonization factors of enterotoxigenic *Escherichia coli*. *Adv Appl Microbiol* [Internet]. 2015;90:155–197. <http://www.ncbi.nlm.nih.gov/pubmed/25596032>.
17. Spangler BD. Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. *Microbiol Rev* [Internet]. 1992;56:622–647. <http://www.ncbi.nlm.nih.gov/pubmed/1480112%5Chttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC372891>.
18. Weiglmeier PR, Rösch P, Berkner H. Cure and curse: *E. coli* heat-stable enterotoxin and its receptor guanylyl cyclase C. *Toxins (Basel)* [Internet]. 2010;2:2213–2229. <http://www.ncbi.nlm.nih.gov/pubmed/22069681>.
19. Konishi N, Obata H, Monma C, Nakama A, Kai A, Tsuji T. Bacteriological and epidemiological characteristics of enterotoxigenic *Escherichia coli* isolated in Tokyo, Japan, between 1966 and 2009. *J Clin Microbiol* [Internet]. 2011;49:3348–3351. <http://www.ncbi.nlm.nih.gov/pubmed/21752981>.
20. McKenzie R, Porter CK, Cantrell JA, Denearing B, O'Dowd A, Grahek SL, Sincok SA, Woods C, Sebeny P, Sack DA, et al. Volunteer challenge with enterotoxigenic *Escherichia coli* that express intestinal colonization factor fimbriae CS17 and CS19. *J Infect Dis* [Internet]. 2011;204:60–64. <http://www.ncbi.nlm.nih.gov/pubmed/21628659>.
21. Liu J, Platts-Mills JA, Juma J, Kabir F, Nkeze J, Okoi C, Operario DJ, Uddin J, Ahmed S, Alonso PL, et al. Use of quantitative molecular diagnostic methods to identify causes of diarrhoea in children: a reanalysis of the GEMS case-control study. *Lancet* [Internet]. 2016;388:1291–1301. <http://linkinghub.elsevier.com/retrieve/pii/S014067361631529X>.
22. Ma Y. Recent advances in nontoxic *Escherichia coli* heat-labile toxin and its derivative adjuvants. *Expert Rev Vaccines* [Internet]. 2016;15:1361–1371. <http://www.ncbi.nlm.nih.gov/pubmed/27118519>.
23. Heggelund JE, Bjørnstad VA, Kregel U. *Vibrio cholerae* and *Escherichia coli* heat-labile enterotoxins and beyond. In: Alouf J, Ladant D, Popoff MR, editors. *The comprehensive sourcebook of bacterial protein toxins* (4th ed). Amsterdam, Netherlands: Elsevier Ltd; 2015. p. 195–229.
24. Chakraborty K, Ghosh S, Koley H, Mukhopadhyay AK, Ramamurthy T, Saha DR, Mukhopadhyay D, Roychowdhury S, Hamabata T, Takeda Y, et al. Bacterial exotoxins downregulate cathelicidin (hCAP-18/LL-37) and human beta-defensin 1 (HBD-1) expression in the intestinal epithelial cells. *Cell Microbiol* [Internet]. 2008;10:2520–2537. <http://www.ncbi.nlm.nih.gov/pubmed/18717821>.
25. Sack DA, Merson MH, Wells JG, Sack RB, Morris GK. Diarrhoea associated with heat-stable enterotoxin-producing strains of *Escherichia coli*. *Lancet* (London, England) [Internet]. 1975;2:239–241. <http://www.ncbi.nlm.nih.gov/pubmed/49793>.
26. Hughes JM, Murad F, Chang B, Guerrant RL. Role of cyclic GMP in the action of heat-stable enterotoxin of *Escherichia coli*. *Nature* [Internet]. 1978;271:755–756. <http://www.ncbi.nlm.nih.gov/pubmed/203862>.
27. Schulz S, Green CK, Yuen PS, Garbers DL. Guanylyl cyclase is a heat-stable enterotoxin receptor. *Cell* [Internet]. 1990;63:941–948. <http://www.ncbi.nlm.nih.gov/pubmed/1701694>.
28. Currie MG, Fok KF, Kato J, Moore RJ, Hamra FK, Duffin KL, Smith CE. Guanylin: an endogenous activator of intestinal guanylate cyclase. *Proc Natl Acad Sci U S A* [Internet]. 1992;89:947–951. <http://www.ncbi.nlm.nih.gov/pubmed/1346555>.
29. Hamra FK, Forte LR, Eber SL, Pidhorodeckyj NV, Krause WJ, Freeman RH, Chin DT, Tompkins JA, Fok KF, Smith CE. Uroguanylin: structure and activity of a second endogenous peptide that stimulates intestinal guanylate cyclase. *Proc Natl Acad Sci U S A* [Internet]. 1993;90:10464–10468. <http://www.ncbi.nlm.nih.gov/pubmed/7902563>.
30. Vaandrager AB. Structure and function of the heat-stable enterotoxin receptor/guanylyl cyclase C. *Mol Cell Biochem* [Internet]. 2002;230:73–83. <http://www.ncbi.nlm.nih.gov/pubmed/11952098>.
31. van den Akker F, Zhang X, Miyagi M, Huo X, Misono KS, Yee VC. Structure of the dimerized hormone-binding domain of a

- guanylyl-cyclase-coupled receptor. *Nature* [Internet]. 2000;406:101–104. <http://www.ncbi.nlm.nih.gov/pubmed/10894551>.
32. Arshad N, Ballal S, Visweswariah SS. Site-specific N-linked glycosylation of receptor guanylyl cyclase C regulates ligand binding, ligand-mediated activation and interaction with vesicular integral membrane protein 36, VIP36. *J Biol Chem* [Internet]. 2013;288:3907–3917. <http://www.ncbi.nlm.nih.gov/pubmed/23269669>.
 33. Sindic A. Current understanding of guanylin peptides actions. *ISRN Nephrol*, 2013:813648. <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4045495&tool=pmcentrez&rendertype>.
 34. Basu N, Visweswariah SS. Defying the stereotype: non-canonical roles of the Peptide hormones guanylin and uroguanylin. *Front Endocrinol (Lausanne)* [Internet]. 2011;2:14. <http://www.ncbi.nlm.nih.gov/pubmed/22654795>.
 35. Carrithers SL, Ott CE, Hill MJ, Johnson BR, Cai W, Chang JJ, Shah RG, Sun C, Mann EA, Fonteles MC, et al. Guanylin and uroguanylin induce natriuresis in mice lacking guanylyl cyclase-C receptor. *Kidney Int* [Internet]. 2004;65:40–53. <http://www.ncbi.nlm.nih.gov/pubmed/14675035>.
 36. Ozaki H, Sato T, Kubota H, Hata Y, Katsube Y, Shimonishi Y. Molecular structure of the toxin domain of heat-stable enterotoxin produced by a pathogenic strain of *Escherichia coli*. A putative binding site for a binding protein on rat intestinal epithelial cell membranes. *J Biol Chem* [Internet]. 1991;266:5934–5941. <http://www.ncbi.nlm.nih.gov/pubmed/2005130>.
 37. Skelton NJ, Garcia KC, Goeddel DV, Quan C, Burnier JP. Determination of the solution structure of the peptide hormone guanylin: observation of a novel form of topological stereoisomerism. *Biochemistry* [Internet]. 1994;33:13581–13592. <http://www.ncbi.nlm.nih.gov/pubmed/7947768>.
 38. Marx UC, Klodt J, Meyer M, Gerlach H, Rösch P, Forssmann WG, Adermann K. One peptide, two topologies: structure and interconversion dynamics of human uroguanylin isomers. *J Pept Res* [Internet]. 1998;52:229–240. <http://www.ncbi.nlm.nih.gov/pubmed/9774236>.
 39. Taxt AM, Diaz Y, Bacle A, Grauffel C, Reuter N, Aasland R, Sommerfelt H, Puntervoll P. Characterization of immunological cross-reactivity between enterotoxigenic *Escherichia coli* heat-stable toxin and human guanylin and uroguanylin. *Infect Immun* [Internet]. 2014;82:2913–2922. <http://www.ncbi.nlm.nih.gov/pubmed/24778111>.
 40. Brierley SM. Guanylate cyclase-C receptor activation: unexpected biology. *Curr Opin Pharmacol* [Internet]. 2012;12:632–640. <http://www.ncbi.nlm.nih.gov/pubmed/23131468>.
 41. Potter LR. Regulation and therapeutic targeting of peptide-activated receptor guanylyl cyclases. *Pharmacol Ther* [Internet]. 2011;130:71–82. <http://www.ncbi.nlm.nih.gov/pubmed/21185863>.
 42. Taxt A, Aasland R, Sommerfelt H, Nataro J, Puntervoll P. Heat-stable enterotoxin of enterotoxigenic *Escherichia coli* as a vaccine target. *Infect Immun* [Internet]. 2010;78:1824–1831. <http://www.ncbi.nlm.nih.gov/pubmed/20231404>.
 43. Yamasaki S, Sato T, Hidaka Y, Ozaki H, Ito H, Hirayama T, Takeda Y, Sugimura T, Tai A, Shimonishi Y. 1990. Structure-activity relationship of *Escherichia coli* heat-stable enterotoxin: role of Ala residue at position 14 in toxin-receptor interaction. *Bull Chem Soc Jpn*. 63:2063–2070. doi:10.1246/bcsj.63.2063.
 44. Okamoto K, Okamoto K, Yukitake J, Miyama A. Reduction of enterotoxic activity of *Escherichia coli* heat-stable enterotoxin by substitution for an asparagine residue. *Infect Immun* [Internet]. 1988;56:2144–2148. <http://www.ncbi.nlm.nih.gov/pubmed/3294186>.
 45. Taxt AM, Diaz Y, Aasland R, Clements JD, Nataro JP, Sommerfelt H, Puntervoll P. Towards rational design of a toxoid vaccine against the heat-stable toxin of *Escherichia coli*. *Infect Immun* [Internet]. 2016;84:1239–1249. <http://www.ncbi.nlm.nih.gov/pubmed/26883587>.
 46. Moyle PM, Toth I. Modern subunit vaccines: development, components, and research opportunities. *ChemMedChem* [Internet]. 2013;8:360–376. <http://www.ncbi.nlm.nih.gov/pubmed/23316023>.
 47. Li W, Joshi MD, Singhanian S, Ramsey KH, Murthy AK. Peptide vaccine: progress and challenges. *Vaccines* [Internet]. 2014;2:515–536. <http://www.mdpi.com/2076-393X/2/3/515/>.
 48. Roy K, Bartels S, Qadri F, Fleckenstein JM. Enterotoxigenic *Escherichia coli* elicits immune responses to multiple surface proteins. *Infect Immun* [Internet]. 2010;78:3027–3035. <http://www.ncbi.nlm.nih.gov/pubmed/20457787>.
 49. Alam MM, Aktar A, Afrin S, Rahman MA, Aktar S, Uddin T, Rahman MA, Al Mahbuba D, Chowdhury F, Khan AI, et al. Antigen-specific memory B-cell responses to enterotoxigenic *Escherichia coli* infection in Bangladeshi adults. *PLoS Negl Trop Dis* [Internet]. 2014;8:e2822. <http://www.ncbi.nlm.nih.gov/pubmed/24762744>.
 50. Lundgren A, Bourgeois L, Carlin N, Clements J, Gustafsson B, Hartford M, Holmgren J, Petzold M, Walker R, Svennerholm A-M. Safety and immunogenicity of an improved oral inactivated multivalent enterotoxigenic *Escherichia coli* (ETEC) vaccine administered alone and together with dmlT adjuvant in a double-blind, randomized, placebo-controlled Phase I study. *Vaccine* [Internet]. 2014;32:7077–7084. <http://www.ncbi.nlm.nih.gov/pubmed/25444830>.
 51. Behrens RH, Cramer JP, Jelinek T, Shaw H, Von Sonnenburg F, Wilbraham D, Weinke T, Bell DJ, Asturias E, Pauwells HLE, et al. Efficacy and safety of a patch vaccine containing heat-labile toxin from *Escherichia coli* against travellers' diarrhoea: a phase 3, randomised, double-blind, placebo-controlled field trial in travellers from Europe to Mexico and Guatemala. *Lancet Infect Dis* [Internet]. 2014;14:197–204. <http://www.ncbi.nlm.nih.gov/pubmed/24291168>.
 52. Sack DA, Shimko J, Torres O, Bourgeois AL, Francia DS, Gustafsson B, Kärnell A, Nyquist I, Svennerholm A-M. Randomised, double-blind, safety and efficacy of a killed oral vaccine for enterotoxigenic *E. coli* diarrhoea of travellers to Guatemala and Mexico. *Vaccine* [Internet]. 2007;25:4392–4400. <http://www.ncbi.nlm.nih.gov/pubmed/17448578>.
 53. Walker RI, Steele D, Aguado T; Ad Hoc ETEC Technical Expert Committee. Analysis of strategies to successfully vaccinate infants in developing countries against enterotoxigenic *E. coli* (ETEC) disease. *Vaccine* [Internet]. 2007;25:2545–2566. <http://www.ncbi.nlm.nih.gov/pubmed/17224212>.
 54. Steinsland H, Valentiner-Branth P, Gjessing HK, Aaby P, Mølbak K, Sommerfelt H. Protection from natural infections with enterotoxigenic *Escherichia coli*: longitudinal study. *Lancet (London, England)* [Internet]. 2003;362:286–291. <http://www.ncbi.nlm.nih.gov/pubmed/12892959>.
 55. Duan Q, Lee KH, Nandre RM, Garcia C, Chen J, Zhang W. MEFA (multi-epitope fusion antigen)-novel technology for structural vaccinology, proof from computational and empirical immunogenicity characterization of an enterotoxigenic *Escherichia coli* (ETEC) adhesin MEFA. *J Vaccines & Vaccin* [Internet]. 2017;8(4):367. <http://www.ncbi.nlm.nih.gov/pubmed/28944092>.
 56. Nandre RM, Ruan X, Duan Q, Sack DA, Zhang W. Antibodies derived from an enterotoxigenic *Escherichia coli* (ETEC) adhesin tip MEFA (multi-epitope fusion antigen) against adherence of nine ETEC adhesins: CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS21 and EtpA. *Vaccine* [Internet]. 2016;34:3620–3625. <http://www.ncbi.nlm.nih.gov/pubmed/27228947>.
 57. Ruan X, Sack DA, Zhang W. Genetic fusions of a CFA/I/II/IV MEFA (multi-epitope 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* [Internet]. 2015;10:e0121623. <http://dx.plos.org/10.1371/journal.pone.0121623>.
 58. Zeinalzadeh N, Salmanian AH, Goujani G, Amani J, Ahangari G, Akhavian A, Jafari M. A Chimeric protein of CFA/I, CS6 subunits and LTb/STa toxoid protects immunized mice against enterotoxigenic *Escherichia coli*. *Microbiol Immunol* [Internet]. 2017;61:272–279. <http://www.ncbi.nlm.nih.gov/pubmed/28543534>.

59. Fleckenstein J, Sheikh A, Qadri F. Novel antigens for enterotoxigenic *Escherichia coli* vaccines. *Expert Rev Vaccines* [Internet]. 2014;13:631–639. <http://www.ncbi.nlm.nih.gov/pubmed/24702311>.
60. Joffré E, von Mentzer A, Svennerholm A-M, Sjöling Å. Identification of new heat-stable (STa) enterotoxin allele variants produced by human enterotoxigenic *Escherichia coli* (EPEC). *Int J Med Microbiol* [Internet]. 2016;306:586–594. <http://www.ncbi.nlm.nih.gov/pubmed/27350142>.
61. Flaherty DK. Immunogenicity and Antigenicity [Internet]. Immunology for Pharmacy. Elsevier Inc.; 2012. p.23–30. doi:10.1016/B978-0-323-06947-2.10049-5
62. Klipstein FA, Engert RF, Clements JD. Development of a vaccine of cross-linked heat-stable and heat-labile enterotoxins that protects against *Escherichia coli* producing either enterotoxin. *Infect Immun* [Internet]. 1982;37:550–557. <http://www.ncbi.nlm.nih.gov/pubmed/6749682>.
63. Klipstein FA, Engert RF, Houghton RA. Mucosal antitoxin response in volunteers to immunization with a synthetic peptide of *Escherichia coli* heat-stable enterotoxin. *Infect Immun* [Internet]. 1985;50:328–332. <http://www.ncbi.nlm.nih.gov/pubmed/3899936>.
64. Zhang W, Zhang C, Francis DH, Fang Y, Knudsen D, Nataro JP, Robertson DC. Genetic fusions of LTAB and STa toxoids of porcine enterotoxigenic *Escherichia coli* (EPEC) elicited neutralizing anti-LT and anti-STa antibodies. *Infect Immun* [Internet]. 2010;78(1):316–325. <http://www.ncbi.nlm.nih.gov/pubmed/19858307>.
65. De Mol P, Hemelhof W, Retoré P, Takeda T, Miwatani T, Takeda Y, Butzler JP. A competitive immunosorbent assay for the detection of heat-stable enterotoxin of *Escherichia coli*. *J Med Microbiol* [Internet]. 1985;20:69–74. <http://www.ncbi.nlm.nih.gov/pubmed/3894668>.
66. van Wijnendaele F, Dobrescu L, Boon B. Induction of immunity against *E. coli* ST-enterotoxin. *Zentralbl Veterinarmed B* [Internet]. 1982;29:441–450. <http://www.ncbi.nlm.nih.gov/pubmed/6890747>.
67. Aref N-EM, Nasr M, Osman R. Construction and immunogenicity analysis of nanoparticulated conjugate of heat-stable enterotoxin (STa) of enterotoxigenic *Escherichia coli*. *Int J Biol Macromol* [Internet]. 2018;106:730–738. <http://www.ncbi.nlm.nih.gov/pubmed/28823704>.
68. Zeng W, Azzopardi K, Hocking D, Wong CY, Robevska G, Tauschek M, Robins-Browne RM, Jackson DC. A totally synthetic lipopeptide-based self-adjuncting vaccine induces neutralizing antibodies against heat-stable enterotoxin from enterotoxigenic *Escherichia coli*. *Vaccine* [Internet]. 2012;30:4800–4806. <http://www.ncbi.nlm.nih.gov/pubmed/22634295>.
69. Liu M, Zhang C, Mateo K, Nataro JP, Robertson DC, Zhang W. Modified heat-stable toxins (hSTa) of enterotoxigenic *Escherichia coli* lose toxicity but display antigenicity after being genetically fused to heat-labile toxoid LT(R192G). *Toxins (Basel)* [Internet]. 2011;3:1146–1162. <http://www.ncbi.nlm.nih.gov/pubmed/22069760>.
70. Ross PL, Wolfe JL. Physical and chemical stability of antibody drug conjugates: current status. *J Pharm Sci* [Internet]. 2016;105:391–397. <http://www.ncbi.nlm.nih.gov/pubmed/26869406>.
71. Wang J, Yadav V, Smart AL, Tajiri S, Basit AW. Toward oral delivery of biopharmaceuticals: an assessment of the gastrointestinal stability of 17 peptide drugs. *Mol Pharm* [Internet]. 2015;12:966–973. <http://www.ncbi.nlm.nih.gov/pubmed/25612507>.
72. Forte LR. Uroguanylin and guanylin peptides: pharmacology and experimental therapeutics. *Pharmacol Ther* [Internet]. 2004;104:137–162. <http://www.ncbi.nlm.nih.gov/pubmed/15518884>.
73. Waldman SA, Camilleri M. Guanylate cyclase-C as a therapeutic target in gastrointestinal disorders. *Gut*, 2018;316029. <http://www.ncbi.nlm.nih.gov/pubmed/29563144>.
74. Jaleel M, London RM, Eber SL, Forte LR, Visweswariah SS. Expression of the receptor guanylyl cyclase C and its ligands in reproductive tissues of the rat: a potential role for a novel signaling pathway in the epididymis. *Biol Reprod* [Internet]. 2002;67:1975–1980. <http://www.ncbi.nlm.nih.gov/pubmed/12444076>.
75. Hodes A, Lichtstein D. Natriuretic hormones in brain function. *Front Endocrinol (Lausanne)* [Internet]. 2014;5:201. <http://www.ncbi.nlm.nih.gov/pubmed/25506340>.
76. Rodríguez A, Gómez-Ambrosi J, Catalán V, Ezquerro S, Méndez-Giménez L, Becerril S, Ibáñez P, Vila N, Margall MA, Moncada R, et al. Guanylin and uroguanylin stimulate lipolysis in human visceral adipocytes. *Int J Obes (Lond)* [Internet]. 2016;40:1405–1415. <http://www.ncbi.nlm.nih.gov/pubmed/27108812>.
77. Lan D, Niu J, Miao J, Dong X, Wang H, Yang G, Wang K, Miao Y. Expression of guanylate cyclase-C, guanylin, and uroguanylin is downregulated proportionally to the ulcerative colitis disease activity index. *Sci Rep* [Internet]. 2016;6:25034. <http://www.ncbi.nlm.nih.gov/pubmed/27125248>.
78. Siegrist C. Vaccine Immunology [Internet]. In: Plotkin SA, Orenstein W, Offit PA, Edwards KM, editors. Plotkin's Vaccines. Elsevier, Inc.; 2013. p. 16–34. <https://www.elsevier.com/books/vaccines/9781455700905#>.
79. Kauffman PE. Production and evaluation of antibody to the heat-stable enterotoxin from a human strain of enterotoxigenic *Escherichia coli*. *Appl Environ Microbiol* [Internet]. 1981;42:611–614. <http://www.ncbi.nlm.nih.gov/pubmed/7039508>.
80. Aref N-EM, Saeed AM. Design and characterization of highly immunogenic heat-stable enterotoxin of enterotoxigenic *Escherichia coli* K99+. *J Immunol Methods* [Internet]. 2011;366:100–105. <http://linkinghub.elsevier.com/retrieve/pii/S0022175911000159>.
81. Liu M, Ruan X, Zhang C, Lawson SR, Knudsen DE, Nataro JP, Robertson DC, Zhang W. Heat-labile (LT) and heat-stable (STa) toxoid fusions (LTR192G-STaP13F) of human enterotoxigenic *Escherichia coli* elicited neutralizing antitoxin antibodies. *Infect Immun* [Internet]. 2011;79(10):4002–4009. <http://www.ncbi.nlm.nih.gov/pubmed/21788385>.
82. Zhang C, Knudsen DE, Liu M, Robertson DC, Zhang W. Toxicity and immunogenicity of enterotoxigenic *Escherichia coli* heat-labile and heat-stable toxoid fusion 3xSTaA14Q-LTS63K/R192G/L211A in a murine model. *PLoS One* [Internet]. 2013;8:e77386. <http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0077386>.
83. Ruan X, Clements JD, Robertson DC, Nataro JP, Puntervoll P, Sommerfelt H, Barry EM, Francis DH, Zhang W. Heat-stable (STa) toxoids of enterotoxigenic *E. coli* for toxoid fusions with double mutant heat-labile toxin (dmLT) elicit neutralizing anti-STa antibodies. Presented at Vaccines for Enteric Dis; 2013 Nov 6–8, Bangkok (Thailand)
84. Nandre R, Ruan X, Duan Q, Zhang W. Enterotoxigenic *Escherichia coli* heat-stable toxin and heat-labile toxin toxoid fusion 3xSTaN12S-dmLT induces neutralizing anti-STa antibodies in subcutaneously immunized mice. *FEMS Microbiol Lett* [Internet]. 2016;363:fnw246. <https://academic.oup.com/femsle/article-lookup/doi/10.1093/femsle/fnw246>.
85. Nandre RM, Duan Q, Wang Y, Zhang W. Passive antibodies derived from intramuscularly immunized enterotoxin fusion 3xSTaN12S-dmLT protect against STa+ enterotoxigenic *Escherichia coli* (EPEC) diarrhea in a pig model. *Vaccine* [Internet]. 2017;35:552–556. <http://linkinghub.elsevier.com/retrieve/pii/S0264410X16312282>.
86. Zhu C, Setty P, Boedeker EC. Development of live attenuated bacterial vaccines targeting *Escherichia coli* heat-labile and heat-stable enterotoxins. *Vet Microbiol* [Internet]. 2017;202:72–78. <http://linkinghub.elsevier.com/retrieve/pii/S0378113517302055>.
87. Sanchez J, Uhlin BE, Grundström T, Holmgren J, Hirst TR. Immunoactive chimeric ST-LT enterotoxins of *Escherichia coli* generated by in vitro gene fusion. *FEBS Lett* [Internet].

- 1986;208:194–198. <http://www.ncbi.nlm.nih.gov/pubmed/2430831>.
88. Deng G, Zeng J, Jian M, Liu W, Zhang Z, Liu X, Wang Y. Nanoparticulated heat-stable (STa) and heat-labile B subunit (LTB) recombinant toxin improves vaccine protection against enterotoxigenic *Escherichia coli* challenge in mouse. *J Biosci Bioeng* [Internet]. 2013;115:147–153. <http://www.ncbi.nlm.nih.gov/pubmed/23040995>.
 89. Zhang W, Francis DH. Genetic fusions of heat-labile toxoid (LT) and heat-stable toxin b (STb) of porcine enterotoxigenic *Escherichia coli* elicit protective anti-LT and anti-STb antibodies. *Clin Vaccine Immunol* [Internet]. 2010;17:1223–1231. <http://www.ncbi.nlm.nih.gov/pubmed/20505006>.
 90. Liu M, Ruan X, Zhang C, Lawson SR, Knudsen DE, Nataro JP, Robertson DC, Zhang W. Heat-labile- and heat-stable-toxoid fusions (LTR₁₉₂G-STaP₁₃F) of human enterotoxigenic *Escherichia coli* elicit neutralizing antitoxin antibodies. *Infect Immun* [Internet]. 2011;79:4002–4009. <http://www.ncbi.nlm.nih.gov/pubmed/21788385>.
 91. Duan Q, Huang J, Xiao N, Seo H, Zhang W. Neutralizing anti-STa antibodies derived from enterotoxigenic *Escherichia coli* (ETEC) toxoid fusions with heat-stable toxin (STa) mutant STaN12S, STaL9A/N12S or STaN12S/A14T show little cross-reactivity with guanlylin or uroguanlylin. *Appl Environ Microbiol* [Internet]. 2018;84(2):e01737–17. <http://www.ncbi.nlm.nih.gov/pubmed/29079628>.
 92. Ruan X, Robertson DC, Nataro JP, Clements JD, Zhang W; STa Toxoid Vaccine Consortium Group. Characterization of heat-stable (STa) toxoids of enterotoxigenic *Escherichia coli* fused to double mutant heat-labile toxin peptide in inducing neutralizing Anti-STa antibodies. *Infect Immun* [Internet]. 2014;82:1823–1832. <http://www.ncbi.nlm.nih.gov/pubmed/24549325>.
 93. Nandre R, Ruan X, Lu T, Duan Q, Sack D, Zhang W. Enterotoxigenic *Escherichia coli* adhesin-toxoid multiepitope fusion antigen CFA/I/II/IV-3xSTa N12S -mnLT G192G/L211A -derived antibodies inhibit adherence of seven adhesins, neutralize enterotoxicity of LT and STa toxins, and protect piglets against diarr. *Infect Immun* [Internet]. 2017;86:e00550–17. <http://iaa.asm.org/content/86/3/e00550-17>.
 94. Matecko I, Burmann BM, Schweimer K, Kalbacher H, Einsiedel J, Gmeiner P, Rosch P. Structural characterisation of the *E. coli* heat stable enterotoxin STh. *Open Spectrosc J* [Internet]. 2009;2:34–39. <http://benthamopen.com/ABSTRACT/TOSPECJ-2-34>.
 95. Garrett BM, Visweswariah SS. A conformational epitope in the N-terminus of the *Escherichia coli* heat-stable enterotoxins is involved in receptor-ligand interactions. *Biochim Biophys Acta* [Internet]. 1996;1317:149–154. <http://www.ncbi.nlm.nih.gov/pubmed/8950201>.
 96. Löwenadler B, Lake M, Elmlad A, Holmgren E, Holmgren J, Karlström A, Svennerholm AM. A recombinant *Escherichia coli* heat-stable enterotoxin (STa) fusion protein eliciting anti-STa neutralizing antibodies. *FEMS Microbiol Lett* [Internet]. 1991;66:271–277. <http://www.ncbi.nlm.nih.gov/pubmed/1769524>.
 97. Lockwood DE, Robertson DC. Development of a competitive enzyme-linked immunosorbent assay (ELISA) for *Escherichia coli* heat-stable enterotoxin (STa). *J Immunol Methods* [Internet]. 1984;75:295–307. <http://www.ncbi.nlm.nih.gov/pubmed/6890747>.
 98. Hermanson GT Vaccines and immunogen conjugates [Internet]. *Bioconjugate Techniques*. Elsevier Inc.; 2013. p. 839–865. <http://linkinghub.elsevier.com/retrieve/pii/B9780123822390000194>
 99. Lemus R, Karol MH. Conjugation of haptens. *Methods Mol Med* [Internet]. 2008;138:167–182. <http://www.ncbi.nlm.nih.gov/pubmed/18612607>.
 100. Pedersen MK, Sorensen NS, Heegaard PMH, Beyer NH, Bruun L. Effect of different hapten-carrier conjugation ratios and molecular orientations on antibody affinity against a peptide antigen. *J Immunol Methods* [Internet]. 2006;311:198–206. <http://www.ncbi.nlm.nih.gov/pubmed/16574142>.
 101. Hu Q-Y, Berti F, Adamo R. Towards the next generation of biomedicines by site-selective conjugation. *Chem Soc Rev* [Internet]. 2016;45:1691–1719. <http://xlink.rsc.org/?DOI=C4CS00388H>.
 102. Okamoto K, Okamoto K, Yukitake J, Kawamoto Y, Miyama A. Substitutions of cysteine residues of *Escherichia coli* heat-stable enterotoxin by oligonucleotide-directed mutagenesis. *Infect Immun* [Internet]. 1987;55:2121–2125. <http://www.ncbi.nlm.nih.gov/pubmed/3305364>.
 103. Roy K, Hamilton D, Allen KP, Randolph MP, Fleckenstein JM. The EtpA exoprotein of enterotoxigenic *Escherichia coli* promotes intestinal colonization and is a protective antigen in an experimental model of murine infection. *Infect Immun* [Internet]. 2008;76:2106–2112. <http://www.ncbi.nlm.nih.gov/pubmed/18285493>.
 104. Bernal-Reynaga R, Thompson-Bonilla R, Lopez-Saucedo C, Pech-Armenta M, Estrada-Parra S, Estrada-García T. C57-CD40 ligand deficient mice: a potential model for enterotoxigenic *Escherichia coli* (H10407) colonization. *Vet Immunol Immunopathol* [Internet]. 2013;152:50–56. <http://www.ncbi.nlm.nih.gov/pubmed/23098671>.
 105. Skrede S, Steinsland H, Sommerfelt H, Aase A, Brandtzaeg P, Langeland N, Cox RJ, Saevik M, Wallevik M, Skutlaberg DH, et al. Experimental infection of healthy volunteers with enterotoxigenic *Escherichia coli* wild-type strain TW10598 in a hospital ward. *BMC Infect Dis* [Internet]. 2014;14:482. <http://www.ncbi.nlm.nih.gov/pubmed/25190096>.
 106. Porter CK, Riddle MS, Tribble DR, Louis Bougeois A, McKenzie R, Isidean SD, Sebeny P, Savarino SJ. A systematic review of experimental infections with enterotoxigenic *Escherichia coli* (ETEC). *Vaccine* [Internet]. 2011;29:5869–5885. <http://www.ncbi.nlm.nih.gov/pubmed/21616116>.
 107. Sakkestad ST, Steinsland H, Skrede S, Lillebø K, Skutlaberg DH, Guttormsen AB, Søyland H, Sævik M, Heien AR, Tellevik MG, et al. ETEC strain TW10722 may be suitable for use in vaccine challenge studies for testing heat-stable toxoid based vaccine candidates. Presented at 2nd International Vaccines Against Shigella and ETEC (VASE) Conference, 2018 June 12 to 14, Mexico City (Mexico).
 108. Frederick DR, Goggins JA, Sabbagh LM, Freytag LC, Clements JD, McLachlan JB. Adjuvant selection regulates gut migration and phenotypic diversity of antigen-specific CD4+ T cells following parenteral immunization. *Mucosal Immunol* [Internet]. 2017;11(2):549–561. <http://www.nature.com/doi/10.1038/mi.2017.70>