Review article

Title: An assessment of enterotoxigenic *Escherichia coli* and *Shigella* vaccine candidates for infants and children

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Abstract: Despite improvements to water quality, sanitation, and the implementation of current prevention and treatment interventions, diarrhea remains a major cause of illness and death, especially among children less than five years of age in the developing world. Rotavirus vaccines have already begun making a real impact on diarrhea, but several more enteric vaccines will be necessary to achieve broader reductions of illness and death. Among the many causes of diarrheal disease, enterotoxigenic *Escherichia coli* (ETEC) and *Shigella* are the two most important bacterial pathogens for which there are no currently licensed vaccines. Vaccines against these two pathogens could greatly reduce the impact of disease caused by these infections. This review describes the approaches to ETEC and *Shigella* vaccines that are currently under development, including a range of both cellular and subunit approaches for each pathogen. In addition, the review discusses strategies for maximizing the potential benefit of these vaccines, which includes the feasibility of co-administration, consolidation, and combination of vaccine candidates, as well as issues related to effective administration of enteric

vaccines to infants. Recent impact studies indicate that ETEC and *Shigella* vaccines could significantly benefit global public health. Either vaccine, particularly if they could be combined together or with another enteric vaccine, would be an extremely valuable tool for saving lives and promoting the health of infants and children in the developing world, as well as potentially providing protection to travelers and military personnel visiting endemic areas.

Keywords: enterotoxigenic *Escherichia coli*, *Shigella*, diarrhea, vaccine, combined vaccine

1. Introduction

Enteric pathogens pose one of the largest threats to human health and productivity in the world [1-5]. The consequences of infection with these pathogens are felt at the individual, family, community, and national levels in many countries. Although interventions to reduce the mortality from enteric diseases are available, little progress has been made in reducing the staggering number of infections associated with these pathogens [3;6]. In spite of significant gains in improving access to clean water and sanitation, there are still approximately 1.7 billion cases of diarrhea that occur annually [7]. In addition, it is estimated that each year nearly 600,000 children less than five years of age die from severe dehydrating diarrhea, mostly in the developing world [8]. Beyond these startling mortality rates, many more children suffer from diarrheal disease-associated intestinal enteropathy and malnutrition, and their consequent toll on physical and cognitive development [9-12]. Diarrhea in young children in the developing world can also be a triggering event for death from other causes, particularly pneumonia [13].

Management of diarrheal diseases in the face of an exploding population will best be accomplished through multiple interventions. Many treatment and prevention tools are currently available, but the full potential of adding vaccines to the control process is yet to be realized. Certainly rotavirus vaccines are already making a real impact, but several more enteric vaccines will be necessary to achieve broader reductions of illness and death. To ensure that these potential new vaccines are implemented in national health programs, a combination of some of these agents is likely to be necessary.

Among the many causes of diarrheal disease, enterotoxigenic *Escherichia coli* (ETEC) and *Shigella* are the two most important bacterial pathogens for which there are no currently licensed vaccines. The importance of these two pathogens was recently confirmed by the results of the Global Enteric Multicenter Study (GEMS), which identified the etiology and population-based burden of pediatric diarrheal disease in sub-Saharan Africa and South Asia [14]. When stool samples from children less than five years of age with moderate-to-severe diarrhea were tested for almost 40 pathogens, ETEC and *Shigella* were among the top four enteric pathogens identified across different locations and age ranges. Vaccines against these two pathogens could greatly reduce the impact of disease caused by these infections. This review describes the approaches to ETEC and *Shigella* vaccines that are currently under development, and discusses strategies for maximizing the potential benefits of these vaccines.

2. Approaches to ETEC vaccines

Background on ETEC

Disease caused by ETEC is usually acquired by ingestion of contaminated food or water and is characterized by watery diarrhea. ETEC strains attach to epithelial cells of the small intestine via binding proteins called colonization factor (CF) antigens. ETEC causes diarrhea as a result of the production of a heat-stable toxin (ST), a heat-labile toxin (LT), or both. Importantly, the incidence of ETEC-mediated diarrheal disease in children decreases with age, suggesting an acquired immunity to disease, but not necessarily to infection. Indeed, epidemiological and human challenge studies indicate that antibody responses to certain CF antigens are involved in

protective immunity. Data from GEMS [14] clearly demonstrate that the most significant ETEC disease burden in children less than five years old is due to ST-expressing strains.

An ETEC vaccine will need to contain CF antigens from the most prevalent strains in order to provide broad coverage. Antibodies to CF antigens elicited as a result of either infection or immunization can prevent diarrhea by inhibiting binding of ETEC to the intestinal mucosa. LT is highly immunogenic and has adjuvant properties when administered in combination with other antigens. Surveillance studies have shown, however, that LT is produced by only 40 to 50% of ETEC strains, and thus other antigens are needed to broaden vaccine strain coverage [15]. Moreover, antibodies to LT only provide short-term immunity. As a result, ETEC vaccine strategies often include both CF and LT antigenic components, and studies have suggested that these antigens synergize to offer stronger protective immunity than either component alone [16]. A vaccine containing the CF antigens CFA/I, CS1, CS3, CS5, and CS6 combined with LT may provide protection against 80% of ETEC strains worldwide [16;17].

Strategies for the development of cellular ETEC vaccines

Live, genetically attenuated ETEC vaccine. ACE527 is a three-component, live attenuated ETEC vaccine candidate currently under development. It expresses the CF antigens (CFAs) most frequently found on ETEC strains associated with diarrheal disease occurring among children in the developing world (CFA/I, CS1, CS2, CS3, CS5, and CS6). In addition, each of the three strains also express the B subunit of the ETEC heat-labile toxin (LTB), so the vaccine is capable of inducing both anti-toxin and anti-CFA immunity. A Phase 1 trial with frozen cells showed a significant ($\geq 4X$ rise) immune response to key antigens by antibody in lymphocyte supernatant

(ALS; reflecting mucosal IgA responses) in volunteers given two doses of 3×10^{10} cfu of each strain [18]. A subsequent Phase 2b vaccination and challenge study of ACE527, using virulent ETEC strain H10407, demonstrated that the vaccine had only a modest impact on the overall incidence of moderate-to-severe ETEC diarrhea as determined by the number or volume of diarrhea stools passed in a 24-hour period post-challenge [19].

Based on the potential shown in the challenge study, a subsequent trial was undertaken to evaluate the use of three doses administered four weeks apart of a lyophilized formulation of ACE527 at a ten-fold lower dose than the previous trial [20]. Half of the vaccine recipients received 25 μ g of double-mutant LT (dmLT) with the vaccine, in order to investigate the potential for adjuvant activity. The vaccine was well tolerated and immune responses induced against LTB and CFA/I by the three-dose regimen with or without 25 μ g dmLT were comparable in magnitude and frequency to those induced by two doses of the frozen vaccine at 10^{11} cfu total dose and were significantly better than those induced by the frozen vaccine when given at 10^{10} cfu. There was no evidence of adjuvant activity from the dmLT on immune responses when given as a 25 μ g dose. The frequencies of peripheral memory B-cell responses to LTB and CF (CFA/I, CS3, CS6) in subjects immunized with ACE527 were equivalent to those seen in naïve subjects experimentally infected with virulent ETEC strain H10407. A number of subjects from this trial, along with a new cohort of naïve individuals were subsequently challenged with H10407 and the results from this challenge trial will be reported in late 2014.

Live hybrid vectors expressing ETEC antigens. These vaccine candidates offer the presentation of enteric antigens for more than one pathogen in an effort to minimize the number of vaccine

components. Although living vaccines are generally targeted for single-dose administration in travelers, it is probable that they would have to be given as multiple doses to the target population of children living in endemic regions.

- *V. cholerae hybrids*. The attenuated cholera vaccine CVD103-HgR has been reported as a vector to express CFA/I, CS3, and CS6, but the immunogenicity of an early CFA/I-expressing prototype was not confirmed in early Phase 1 trials. This indicated that further process development work was needed to optimize CF and CS antigen expression in the CVD103-HgR background [21;22]. CVD103-HgR also expresses the B-subunit of cholera toxin (CTB) which is highly homologous to the B-subunit of LT. This vector has a strong safety record. Based on experience in Indonesia, the vaccine may be made more effective in the target population by increasing the dose ten-fold over what is necessary in developed countries [23]. The attenuated cholera vaccine Peru-15 has also been modified to be a strong producer of CTB and in the future could be constructed to also deliver key colonization antigens. Peru-15 has already been shown to be safe and immunogenic in older infants in a low-resource country [22].

- *Shigella hybrid*. A combined ETEC-*Shigella* vaccine composed of five attenuated *Shigella* strains (*S. sonnei*; *S. flexneri* 2a, 3a, and 6; and *S. dysenteriae* 1) with each expressing critical ETEC antigens represented on the most prevalent clinical isolates is under development [24-27]. Other recent efforts have focused on the development of efficient approaches to the stable expression of ETEC antigens in *Shigella* live vectors [28]. Fimbrial and toxoid antigens have been stably expressed from chromosomal loci in

Shigella vaccine strains. These induced robust immune responses to both ETEC and *Shigella* antigens in preclinical animal studies. Recent advances using autotransporter technology to efficiently expose antigens on the surface of *Shigella* live vectors have demonstrated unprecedented levels of antibody responses. For example, the LTB subunit was expressed via fusion to the Sat autotransporter in several configurations in the *Shigella* vaccine CVD1208S and induced significant anti-LTB responses in 100% of immunized animals after a single dose of the optimal construct [28].

Similar work has been undertaken to use *Shigella* with VirG mutations to express ETEC antigens [29]. *S. flexneri* 2a SC602 was modified by *asd*-based plasmid vectors to express a structural subunit of CFA/I alone or in combination with the B subunit of heat-labile enterotoxin. This construct, termed SC608, was used to immunize guinea pigs with protection against *Shigella* and expression of conformational epitopes required for the generation of antibodies that have functional activities.

- *S. typhi* hybrid. This hybrid approach for the generation of antibodies specific for CFA, LT, and ST by a poly-antigen fusion protein involves its expression as a heterologous antigen construct in the clinically proven ZH9 attenuated *S. typhi* vaccine strain. ZH9 is being developed to produce an oral combination vaccine with the potential to protect against both typhoid fever and ETEC. This construct has been shown to induce antibodies against both toxins and CFs after oral immunization of mice [30].

Inactivated whole cell ETEC vaccine. Although an early version of a vaccine candidate made from killed ETEC induced significant protection among travelers against moderate-to-severe diarrhea in two Phase 3 field trials [31], it lacked sufficient immunogenicity to be protective in Egyptian children [32; 33]. Unfortunately, the active surveillance design of this pediatric trial made it difficult to assess the impact of vaccination on the risk of moderate-to-severe ETEC diarrhea, so it was impossible to determine whether the vaccine may have shown efficacy against moderate-to-severe illness in these children in a way similar to that achieved in the earlier studies with travelers. An in-depth analysis of the trial results suggested higher levels of CF/CS and a mucosal adjuvant may be needed in the second-generation vaccine to improve protection in younger age-groups [34]. This vaccine approach has since been reformulated to be made from recombinant *E.coli* K12 engineered to express high quantities of key CF antigens [35;36]. In addition, the new formulation includes in intact form the antigen CS6, whose clinical importance was not recognized when the first-generation vaccine was made. The new vaccine, called ETVAX, also contains a CTB-LTB (LCTBA) hybrid to provide anti-toxin immunity against LT, which may be superior to that obtained with the CTB component provided alone in the earlier vaccine.

The results of a placebo-controlled Phase 1 study of ETVAX administered in two oral doses two weeks apart alone or with 10µg or 25µg of the dmLT adjuvant to healthy adult subjects indicated that the vaccine was well tolerated and the majority of vaccinated subjects developed broad immune responses to the majority of key antigens [37]. The prospectively defined immunological endpoint to trigger further development was surpassed by a wide margin and additional clinical trials with ETVAX are currently being planned.

Strategies for the development of subunit ETEC vaccines

ETEC toxin-based vaccines. These vaccine candidates may be given orally, but could also be amenable to parenteral administration. Although protection against ETEC infections can be obtained through immunity against the CFs only [38;39], toxin components in combination with CFs may contribute to protection. The protection seen in travelers given the B subunit of cholera toxin, which induces cross-reacting antibodies that recognize the B subunit of LT or immunized with the LT holotoxin by skin patch, is short-lived, lasting only a few months and is restricted to ETEC strains only expressing the LT toxin [40]. Several approaches have been reported to provide anti-toxin immunity against ETEC.

- *dmLT protection.* In a Phase 1 dose-escalating trial, dmLT was given orally to adults in single doses of up to 100µg. This induced strong immune responses at the higher doses with no unacceptable side effects and an apparent optimum in the level of immune response reached at 50µg [41], which could support its inclusion as a component of an ETEC vaccine.
- *Labile-toxin patch.* A challenge trial supported the idea that delivery of LT holotoxin via a patch for transcutaneous immunization (TCI) could reduce severity of disease and at least be an important ETEC antigen and potential adjuvant for use with other vaccine components [42]. A skin patch was used to deliver complete LT by TCI to travelers [43]. Some protection was seen against LT-only strains of ETEC and the severity of ETEC

diarrhea cases, in general, were less severe. However, it did not provide the level of protection needed to be used as a vaccine alone.

- *Cholera whole cell/B subunit*. The antigenic similarity between the B subunit of cholera toxin and the LT of ETEC led to studies showing that this cholera vaccine could provide short-term protection in travelers against severe LT-ETEC diarrhea. The vaccine is licensed for ETEC in some countries as Dukoral[®] [44]. The cost of the vaccine, along with its short-term protective effect and large cold-chain storage footprint, are problematic for broad use in ETEC-endemic settings.

ST constructs. Given that 75% of ETEC strains produce ST, if this antigen could be delivered in a safe and immunogenic format, it could contribute to the coverage that could be provided by a vaccine consisting of LT and CFAs [17]. Like dmLT, it has the potential to be administered orally or parenterally. ST's small size makes it poorly immunogenic, with no immune response seen after natural infection. In contrast to LT, until recently, ST has evaded all attempts to produce a non-toxic yet immunogenic vaccine. However, some newer approaches offer a potential promise of success. Preclinical studies on ST mutants made by site-directed mutagenesis show encouraging results toward developing a safe and immunogenic ST-containing vaccine [45]. Systematic replacement of each of 18 residues in ST by all possible amino acids resulted in a library of 360 mutants which were analysed. The results showed that the regions responsible for enterotoxicity and induction of immune responses were clustered in two non-overlapping epitopes. Four of the mutants were able to elicit neutralizing antibodies in mice when coupled to bovine serum albumin (BSA) as a carrier protein [45]. In an attempt to

generate an immunogen capable of protecting against both toxins, some of these ST mutants were genetically fused to dmLT, expressed in *E.coli*, purified, and used to immunize mice [46;47]. Of 14 ST mutant fusions tested, three generated high levels of anti-ST antibody and one, Sta(N12S), induced significant levels of ST-neutralizing activity. ST shares epitopes with guanylin and uroguanylin, and that cross-reactivity is a real challenge to ST vaccine development. However, the risk of cross-reactivity may be reduced by basing an ST-immunogen on STp (porcine ST) or a selectively mutated variant of STh (human ST); thus providing a potentially safer path forward for vaccine development [48].

In an alternative approach, another group has expressed a mutant ST toxoid as a fusion protein along with LT and elements from seven CF antigens, which was then used to immunize mice. The results showed that antibodies were generated that neutralized the activity of both toxins and recognized each of the CFs [49].

Fimbrial tip adhesin. A vaccine approach has been developed using the fimbrial tip proteins from CF antigens to block adhesion of the bacteria to the intestinal epithelium [50]. A prototype adhesin, CfaE, was immunogenic when given by intradermal (ID) administration in mice. Subsequent non-human primate studies showed that ID immunization with the adhesin protected against an antigenically homologous ETEC challenge delivered orally [51].

The CfaE molecule, which is the tip adhesin for CFA/I, was delivered by ID injection to volunteers either alone or as a chimeric molecule in which it was fused with the A2 domain of cholera toxin and non-covalently complexed with LTB; CfaE alone was also administered by

TCI patch delivery [52;53]. In both cases, the vaccine was given with LT attenuated by a single amino acid substitution (mLT). The ID route was the more robust of the two, and was subsequently evaluated in a Phase 2 challenge study where subjects immunized with CfaE and mLT were challenged with the CFA/I⁺ ETEC strain H10407. Data from this study may be available in late 2014.

Novel ETEC proteins. The potential for alternative virulence factors to play a role in ETEC vaccines is being explored. Two specific novel targets have been identified to date: YghJ, a type 2 secretion system effector molecule, secreted by the same pathway as LT; and EatA, a serine protease, which function as secondary virulence factors by degrading mucin and promoting access to the mucosal surface [54]. Both proteins contribute to effective toxin delivery *in vitro*, are highly conserved, and are recognized by convalescent sera obtained following natural infection or experimental human volunteer challenge studies with ETEC. In a new collaborative initiative led by PATH, whole proteome arrays representing multiple ETEC strains are being constructed and will be probed with sera from vaccinated and naturally and experimentally infected subjects with known clinical histories to identify additional target antigens and possibly immunological markers which correlate with protection.

3. Approaches to *Shigella* vaccines

Background on *Shigella*

Shigellosis is often characterized by a prodrome of watery diarrhea before the onset of overt dysentery. Active fluid secretion has been detected in the small intestine, presumably caused by

Shigella enterotoxins ShET1 [55] and ShET2 [56] expressed as the organisms traverse the upper intestinal track on the way to the colon. These enterotoxins are involved in the entire spectrum of shigellosis symptoms since deletion of the *sen* (ShET1) and *set* (ShET2) genes ameliorated diarrhea, fever, and dysentery in a Phase 1 volunteer trial of experimental *S. flexneri* 2a vaccines [57]. Although enterotoxins apparently play a role in shigellosis, translocation of the bacteria from the intestinal lumen into the subepithelial lamina propria mucosae of the colon is the primary *Shigella* virulence mechanism [58].

Three species of *Shigella* are responsible for the majority of enteric disease [59]. *S. flexneri* is the main cause of endemic shigellosis in many low-resource countries. *S. sonnei* is also highly prevalent in these areas and is the serotype most commonly associated with travelers' diarrhea caused by *Shigella*. It is also the most prevalent *Shigella* serotype in developed countries. Finally, though infrequently seen, *S. dysenteriae* type 1 can cause severe diarrheal disease and life-threatening kidney damage (due to the production of Shiga toxin) and is responsible for epidemics in low-resource countries. The fourth *Shigella* species, *S. boydii*, is not normally considered a major cause of diarrhea, though recent studies suggest it may be more prevalent than previously reported.

There are 15 different *S. flexneri* serotypes, thus complicating vaccine development for *Shigella*, although only a few species predominate. Furthermore, studies in animals have suggested cross-protective immunity between some of the *S. flexneri* serotypes, so that a vaccine comprising a combination of *S. sonnei*, *S. dysenteriae*, *S. flexneri* 2a, *S. flexneri* 3a, and *S. flexneri* 6 might provide broad coverage against most of the endemic and epidemic *Shigella* species, though this

remains to be confirmed in humans [60]. If so, it is possible that even a trivalent vaccine would confer protection against 80% of infections with *Shigella*.

Strategies for the development of cellular *Shigella* vaccines

Live, genetically attenuated Shigella vaccines. Molecular engineering has allowed construction of live oral *Shigella* vaccine candidates with defined deletion mutations in selected virulence or metabolic genes.

An *S. flexneri* 2a vaccine prototype, CVD1208S [61], is a reconstructed version of CVD1208 [57] grown in media free of animal products. The vaccine has deletion mutations in *guaBA* (purine biosynthesis) [62], *sen* (ShET1), and *set* (ShET2 enterotoxin) [57]. The Δ *guaBA* mutation in the *de novo* purine biosynthetic pathway results in guanine auxotrophy. A Phase 1 trial was conducted of lyophilized CVD1208S prepared under cGMP using three-dose regimens of 10^7 or 10^8 cfu [63]. The vaccine was well tolerated by adult North American volunteers with 75% vaccine excretion in the 10^8 cfu cohort with no requirement for antibiotic treatment after vaccination. IgA anti-lipopolysaccharide (LPS) antibody secreting cells (ASC) responses were observed in 83% of subjects receiving the 10^8 cfu dose of CVD1208S. The magnitude of this ASC response correlates with clinical protection based on previous *S. flexneri* challenge trials indicating that further clinical development of CVD1208S is warranted.

In contrast to the Δ *guaBA* auxotroph strategy, another strategy has been developed based on the Δ *virG* (*icsA*) virulence gene mutation [64;65]. In Phase 1 trials, the attenuated *S. flexneri* 2a, SC602, was highly immunogenic with 10 to 15% short-term reactogenicity (diarrhea or fever)

when ingested as a single dose of 10^4 or 10^5 cfu by adult North American or Israeli volunteers. A single dose of 10^4 cfu SC602 also demonstrated efficacy against an experimental challenge with wild-type *S. flexneri* 2a [66]. However, residual reactogenicity in North American volunteers and lack of immunogenicity in Bangladeshi children [67] have discouraged further trials of this candidate. The disappointing results in individuals living in a developing country may have been due to a low availability of iron in the intestine. SC602 included the $\Delta icsA$ mutation which would put it at a disadvantage in an iron-poor environment. *S. flexneri* and *S. sonnei* mutants that do not have the iron-binding mutation of SC602 are in clinical development and the most advanced of these, the attenuated *S. sonnei* mutant, WRSS1, is currently being evaluated in a descending-age trial in Bangladesh to determine the safety and immunogenicity of the new series of VirG-based mutants in a setting similar to that in which SC602 was tested.

Building on the demonstrated attenuation of CVD1204 by *sen* and *set* enterotoxin deletions described above [57], a series of “second-generation” $\Delta virG$ mutants has been developed with additional deletions in the enterotoxin genes to ameliorate the residual diarrheal reactions seen in some volunteers ingesting SC602 and WRSS1 [68]. In addition, a deletion in the *msbB2* gene, with one copy of the *Shigella msbB* genes encoding fatty acyl transferases necessary for synthesis of the fully pyrogenic form of LPS lipid A has been engineered [69]. Deletion of *msbB2* from the *Shigella* virulence plasmid attenuates the mutant in the mouse lung model of *Shigella* infection, resulting in decreased levels of pro-inflammatory cytokines in the lungs and decreased TNF- α production in infected mouse macrophages [70]. Deletion of either *msbB1* or *msbB2* (or both copies of the gene) also dramatically attenuates *S. flexneri* in the ligated rabbit ileal loop model of *Shigella* infection, preventing both TNF- α production and destructive

inflammation in the infected rabbit intestine [69]. The $\Delta virG$ attenuation in combination with $\Delta senA$, $\Delta senB$ (a paralog), and Δset enterotoxin mutations along with $\Delta msbB2$ (under acylated lipid A) could yield fully invasive, but highly attenuated, second-generation live vaccine candidates. Early clinical studies have begun to evaluate these newer candidates [71].

At present, the safety issues associated with using attenuated *Shigella* candidates in infants in low-resource countries are far from resolved. It will be necessary to carefully monitor progress with these constructs to determine whether they offer advantages in efficacy with sufficient safety to support their use over non-living or vectored *Shigella* vaccine candidates. Another issue to consider is that attenuated vaccines need to be stored in a dry state and rehydrated for use, which can be an undesirable characteristic for use in less-developed countries.

Salmonella typhi Ty21a-vectored *Shigella* vaccines. Ty21a was attenuated in the mid-1970s by random mutagenesis of *S. typhi* with nitrosoguanidine [72]. The attenuating feature was thought to be a *galE* mutation, but recent sequencing of the Ty21a genome has revealed 679 single nucleotide polymorphisms, some of which may also contribute to the remarkable attenuation of this licensed typhoid vaccine [73]. Ty21a has been safely ingested by more than 200 million individuals, and large-scale clinical trials have demonstrated its clinical efficacy against typhoid fever. In the early 1980s, Ty21a was investigated as a carrier of heterologous O-specific polysaccharide (O-SP) expressed by the conjugally transferred form 1 plasmid from *S. sonnei* [74]. These studies led to inconsistent results. However, a current recombinant version of the construct incorporates the *S. sonnei* LPS genes into the Ty21a chromosome, resulting in a stable hybrid that expresses both homologous typhi and heterologous *S. sonnei* O-SP [75]. This hybrid

vaccine elicits strong anti-LPS serum immune responses and solid protection in mice against *S. sonnei* challenge. Hybrid strains expressing *S. sonnei*, *S. flexneri* 2a, *S. flexneri* 3a, and *S. dysenteriae* 1 O-SP in a Ty21a carrier have also been constructed.

Recent advances in the formulation of Ty21a have produced dried preparations capable of survival for 12 months at 25°C, more than one month at 37°C, and a predicted shelf-life of five to ten years at 4°C. In addition, further genetic modification to increase acid resistance may allow for delivery of the vaccine as a rapidly dissolving oral wafer without the need for buffering [76].

Inactivated whole-cell Shigella vaccines. Results of a Phase 1 trial were reported in 2006 with a prototype formalin-killed *S. sonnei* whole-cell (SsWC) vaccine candidate in a three- or five-dose oral regimen of 2×10^{10} killed whole cell [77]. This preliminary trial confirmed the safety of the killed whole-cell approach in adult North American volunteers and showed that multiple doses of SsWC could elicit significant IgG seroconversion against *Shigella* proteins (e.g., IpaC component of the type three secretion system [TTSS]) and *S. sonnei* LPS in more than half of the vaccine recipients [77].

Subsequent investigations with inactivated *Shigella* whole cells have found that they interact effectively with key antigen-presenting cells [78]. In contrast to live *Shigella*, the inactivated cells were not cytotoxic for human dendritic cells, although they were able to induce cytokine secretion. Further, in addition to protecting mice from live challenge with *Shigella* strains of the same O-SP, the inactivated vaccine was also able to induce immune responses to cloned ETEC

antigens expressed on the bacterial surface, suggesting potential for use in a combined vaccine [78].

More recently, a Phase 1 trial of a prototype, formalin-killed *S. flexneri* 2a whole-cell (Sf2aWC) vaccine candidate was conducted in adult North American volunteers [79]. The vaccine was given in three doses ranging in ten-fold regimens from 2.6×10^8 to 2.6×10^{11} whole cells. The vaccine was found to be safe, and there were no significant anti-LPS responses in the two lower-dose groups. Only about 50% of the volunteers in the cohort given the 10^{10} whole cell mounted a significant immune response; however, more than four-fold ALS responses were detected in more than 80% of the volunteers in the cohort given the 10^{11} whole cell. Indeed, the latter group had a 24-fold geometric mean fold rise in anti-LPS ALS IgA. ALS data were confirmed by anti-LPS serum responses that were detected in 70% of vaccine recipients. These results support further development of killed *Shigella* whole cell vaccines and encourages future vaccine trials in children in low-resource settings using doses compatible with their smaller body mass.

Strategies for the development of subunit *Shigella* vaccines

Conjugate vaccines. The concept of *Shigella* O-SP-protein conjugate vaccine candidates for intramuscular injection has received much attention [80]. Since purified O-SP is poorly immunogenic, O-SP purified from *Shigella* LPS was covalently coupled to protein carriers in order to induce stronger and longer-lasting T cell-dependent immune responses [81]. Although this led to strong IgG responses, it is possible that to be maximally effective, conjugate vaccines need to be given to individuals previously primed by the mucosal route or some other strategy to elicit mucosal as well as systemic responses. This may help explain the protection found with

conjugate vaccines in adults and older children from endemic areas, but much less effectiveness seen in children less than three years of age [82]. Innovative approaches are being taken to improve the immunogenicity and production of *Shigella* conjugate vaccine candidates. During the last decade, a glycoconjugate vaccine candidate has been developed consisting of synthetically produced *S. flexneri* 2a oligosaccharide “mimics” conjugated to protein carriers (i.e., neoglycopeptides) [83;84].

In contrast to O-SP-protein conjugates and neoglycoproteins manufactured *in vitro*, *in vivo* production of glycoconjugates in genetically modified *E. coli* K12 is being actively developed [85]. This approach takes advantage of the general protein glycosylation activity of the *Campylobacter jejuni* gene product PglB, which transfers O-SP from an inner membrane lipid carrier (undecaprenyl pyrophosphate) to N-glycosylation receptor sites on proteins in the periplasm [86]. Purification of glycoconjugate vaccines from *E. coli* is a simpler process than manufacturing O-SP-protein conjugates or neoglycoproteins. Since the glycoconjugate is transported to the periplasm by *E. coli*, the product can easily be extracted by osmotic shock or lysozyme treatment, filter sterilized, and purified by standard immobilized metal affinity chromatography (IMAC) using the affinity of the genetically engineered histidine tag on the carrier protein for nickel on the chromatography column [87]. A prototype *S. dysenteriae* 1 glycoconjugate vaccine candidate (GVXN SD133), employing the well-studied recombinant *Pseudomonas aeruginosa* exoprotein A as a protein carrier, has been evaluated in Phase 1 trials [88]. Adult volunteers received two doses of GVXN SD133 injected intramuscularly two months apart. The vaccine was formulated to contain 10µg of O-SP per dose. The overall seroconversion rate (at least a four-fold increase in IgG and IgA) was 70% after the first dose and 80% after the

second dose. The IgG geometric mean immunoglobulin titers rose approximately 16-fold, suggesting that *in vivo* production of *Shigella* glycoconjugates is a promising vaccine technology.

Invaplex. A naturally occurring complex of invasion plasmid antigens (Ipas) IpaB, IpaC, IpaD, and LPS known as Invaplex has been developed as a candidate vaccine for *Shigella* [89]. This TTSS-LPS complex has been extracted from virulent shigellae by hypotonic shock (incubation in distilled water) and was partially purified by centrifugation and ion-exchange chromatography under cGMP. Invaplex is a biologically active product that binds to mucosal cell receptors (including M cells) resulting in endocytic uptake and delivery of *Shigella* antigens to underlying antigen presenting cells [90;91]. Since Invaplex induces immune responses against LPS as well as TTSS proteins, it is considered a safe, subcellular surrogate of natural *Shigella* infection when administered by the mucosal route [90]. Consistent with experience with natural *Shigella* infections, cross-protection by Invaplex to heterologous LPS serotypes has not been proposed [92].

A prototype *S. flexneri* 2a Invaplex vaccine candidate has been safely evaluated in Phase 1 clinical trials using three intranasal doses of up to 690µg [90;93]. IgA and IgG ASC and fecal IgA recognizing 2a LPS and/or Invaplex were detected in more than half of the vaccine recipients, suggesting that intranasal immunization can stimulate an intestinal immune response in humans [90]. However, a follow-on Phase 2b challenge trial with the same intranasal regimen yielded disappointing immunogenicity and showed no efficacy against intestinal challenge with virulent *S. flexneri* 2a [94].

466
467 The development of a potentially more immunogenic second-generation TTSS-LPS complex
468 vaccine candidate is now underway, constituting artificial Invaplex (Invaplex_{AR}) from
469 recombinant IpaB and IpaC purified by IMAC technology and LPS purified by standard hot-
470 phenol extraction [90]. Unlike the naturally occurring complex, Invaplex_{AR} is a chemically
471 defined product that can be constituted with any LPS in the manufacture of multivalent *Shigella*
472 vaccines. Moreover, preliminary studies in mice and guinea pigs suggest that Invaplex_{AR} is ten-
473 fold more protective than Invaplex extracted from *Shigella* cultures [90]. Although the second-
474 generation Invaplex_{AR} represents substantial technical progress in TTSS-LPS vaccine
475 development, the fact remains that the immunogenicity of Invaplex administered to humans by
476 the intranasal route has been unpredictable in adult North American volunteers and is deemed by
477 most experts to be impractical for children in less-developed countries if administered by this
478 route. Invaplex would probably be too expensive or impractical for use as an oral vaccine, and
479 the presence of pyrogenic LPS seems to preclude intramuscular or ID routes of administration.
480 The future use of *msbB* mutants of LPS may help avoid toxicity that could be associated by using
481 parenteral routes.

482
483 *Generalized Modules for Membrane Antigens*. Development of outer membrane vesicles
484 (OMVs) as *Shigella* vaccine candidates has been reported [95]. These vesicles have been
485 designated as Generalized Modules for Membrane Antigens (GMMA) because they are
486 produced by a genetically modified $\Delta tolR$ *S. sonnei* strain that overproduces GMMA vesicles. *S.*
487 *sonnei* prototype GMMA have been purified from $\Delta msbB$ $\Delta galU$ mutants which negate both O-
488 SP expression and Lipid A/LPS pyrogenicity [96]. An industrially scalable, high-yielding

manufacturing process for GMMA using tangential flow filtration for purification and micro-filtration for sterilization has been identified [95].

Three intranasal immunizations using GMMA derived from *S. sonnei* without O-SP/LipidA protected against either *S. sonnei* or *S. flexneri* in the intranasal challenge model, but only GMMA with homologous LPS were protective by the ID route. As with OMV-NP (see below) and Invaplex_{AR}, ID inoculation with GMMA expressing O-SP present reactogenicity issues; however, the non-pyrogenic $\Delta galU$, $\Delta msbB$ GMMA are being further investigated as an OMP-based approach for ID, intramuscular, or intranasal vaccination in Phase 1 clinical trials [97].

Purified IpaB and IpaD. This approach represents a new paradigm for *Shigella* immunization because it is independent of serotype-specific immunity. An Ipa-based vaccine alone may present conserved antigens for cross-strain protection, but it remains to be seen whether Ipa alone can avoid the need for inclusion of serotype-specific antigens to achieve protection. Antibody against Ipa proteins is invariably present in the serum of healthy children or adults living in areas of endemic shigellosis [98;99]. Since these immunogenic proteins play a critical role in pathogenesis and are conserved in all *Shigella* species, they could be ideal targets for the development of a broad-spectrum, serotype-independent vaccine [100].

The potential of purified TTSS components IpaB and/or IpaD as broad-coverage vaccine antigens admixed with 2.5 μ g of dmLT adjuvant has been reported [101]. After purification by standard IMAC technology, recombinant IpaB and/or IpaD were administered intranasally (2.5 μ g and 10 μ g, respectively) three times to mice [100]. High-titered IgG and IgA immune

responses against the Ipa antigens were achieved after only two intranasal immunizations. Both mucosal antibody and T cell responses were induced. Intranasal challenge with multiple LD₅₀ doses of virulent *S. flexneri* 2a or *S. sonnei* demonstrated highly significant protective efficacy against both species in this animal model of *Shigella* infection [100]. Intranasal immunization provided proof-of-concept for an experimental vaccine, but it is probably not a practical route of delivery for children in the developing world. Up to 100-fold lower doses of IpaB/IpaD/dmLT administered to mice by the ID route using microneedle injection have been evaluated. Immunofluorescent staining demonstrated IpaB/IpaD uptake by macrophages, Langerhans cells, and dendritic cells in the dermal compartment [102]. Serum IgG against all vaccine components was elevated, but mucosal IgA responses were not as robust as those elicited by intranasal immunization. Nonetheless, the protective efficacy of TTSS proteins given by the ID route was 70% against a lethal intranasal challenge with *S. flexneri* and 50% against *S. sonnei*.

The IpaB/IpaD proteins have recently been expressed as a single recombinant polypeptide chain, known as the DB Fusion [103]. The DB Fusion protein is co-expressed and purified using the IpgC cognate chaperone as a carrier. The IpgC is released by mild detergent treatment, and the resulting DB Fusion is properly folded and stable. When co-administered with the dmLT adjuvant, the recombinant protein is comparable to individually purified IpaB/IpaD protecting against lethal *S. flexneri* 2a or *S. sonnei* challenge in the intranasal mouse challenge model. These preliminary studies of broadly protective TTSS proteins as a serotype-independent *Shigella* vaccine candidate support further investigation of this strategy in clinical trials.

OMVs. Acellular *Shigella* vaccine candidates based on OMVs that are naturally secreted into the bacterial culture medium during the stationary phase of growth of wild-type strains are under development [104]. OMVs are isolated from killed whole-cell cultures by differential centrifugation and tangential filtration with a yield of approximately 2%. *S. flexneri* 2a OMVs are 40% LPS, and they also contain major outer membrane protein antigens such as OmpA, OmpC/OmpF, IpaB, IpaC, and IpaD. OMVs can be loaded onto poly(anhydride) nanoparticles (OMV-NP), a Toll-like receptor agonist and Th1 adjuvant [105;106]. Preclinical studies in mice showed that both OMVs and OMV-NPs protected mice from intranasal challenge with homologous *S. flexneri* 2a after a single immunization by the intranasal, ocular, or oral routes (i.e., mucosal immunization). However, only the mice immunized with OMV-NPs by the ID route were protected [104]. As expected, mice injected with OMV-NP showed signs of endotoxic shock, so this highly antigenic material could only be considered for development as a mucosal vaccine.

Pan-Shigella surface protein (PSSP-1). This protein represents another serotype-independent approach. PSSP-1 has been found on the surface of several hundred different shigellae and has provided cross-species and cross-serotype protection in animal studies [107]. Problems with process development have limited clinical development of this vaccine candidate.

OmpA. A 34kDa outer membrane protein antigen has been identified as a major protective antigen against experimental challenges with *Shigella* [108]. This protein is cross-reactive and antigenically conserved among *Shigella* species, and the epitope is surface-exposed on the intact

bacterium [109]. The OmpA evokes protective type 1 cell-mediated immunity, further suggesting its vaccine potential [110].

4. Strategies for maximizing the potential benefit of ETEC and *Shigella* vaccines

Co-administration, consolidation, and combination of vaccine candidates

Recent studies suggest that the commercial market for both ETEC and *Shigella* vaccines would be improved if they could be developed as combined vaccine formulations or even be considered for co-administration with each other or other enteric vaccines [111]. PATH hosted a workshop in October 2013 to solicit expert opinions on the potential merits and challenges of developing combined ETEC and *Shigella* vaccines to benefit children in developing countries. Another article to be published separately summarizes the key issues raised during the workshop and provides an analysis of the recommendations regarding the strategic, clinical and regulatory, and manufacturing considerations for the development of a combined enteric vaccine, which aim to guide future vaccine development efforts and donor investment strategies in this area.

In addition, as described in another article to be published elsewhere, PATH and the Johns Hopkins Bloomberg School for Public Health recently conducted a modeling exercise using the Lives Saved Tool (LiST) to examine the potential impact of singular or combined ETEC and *Shigella* vaccines over the timeframe of 2020 to 2040 in 90 low and lower-middle income countries, even when coverage of certain public health interventions are scaled up (i.e., improvements in water and sanitation, use of oral rehydration therapy, and access to rotavirus vaccine). They estimated that more than 850,000 deaths of children less than five years of age

could be averted in that timeframe with the introduction of an effective combined ETEC-*Shigella* vaccine. Looking at the example of one steady year post-introduction of a combined ETEC-*Shigella* vaccine (2040), they estimated that the vaccine would avert 106,000 diarrhea-associated deaths among children less than five years of age (compared to approximately 160,000 deaths being averted by rotavirus vaccine), in addition to averting nearly 82 million cases of diarrhea [111].

Many of the enteric vaccines currently in use and under development have the potential for some level of combination. Co-administration could involve two separate vaccines, such as a rotavirus vaccine and an ETEC vaccine that could be given as separate products on the same Expanded Programme on Immunization schedule, and it could be useful even without the vaccines being co-formulated. If both vaccines are administered orally, one product could, for example, benefit from a buffer included in one vaccine and a possible adjuvant included in the other vaccine. A specific example of products that could be used in this type of regimen might be a rotavirus vaccine and a killed ETEC vaccine that contains dmLT.

The possibility of antigen consolidation is facilitated by the discovery of conserved antigens and the production of fusion proteins, thereby reducing the need to prepare multiple serotype-specific or species-specific antigens to be combined in a subunit vaccine or expressed in vectors in a cellular vaccine. Vectored vaccines without conserved antigens are certainly also a means of achieving possible antigen consolidation, as would be indicated by the development efforts using Ty21a to deliver *Shigella* antigens [75] and the attenuated CVD strains of *Shigella* to deliver ETEC antigens [24-26]. It should be pointed out that a vectored vaccine does not have to require

the use of a live attenuated vaccine. It has been observed that oral or intranasal immunization of mice with an inactivated *S. flexneri* 2a strain (CVD1203) expressing the CFA/I and CS3 antigens of ETEC induced IgG responses to both heterologous antigens [78].

Some useful combinations may also exist naturally. For example, antibody to cholera toxin (CT) cross-reacts with the major outer membrane proteins of all *Campylobacter jejuni* strains tested [112]. Based on this finding, it has been investigated whether immunization with CT protects against intestinal colonization with *C. jejuni* in an adult mouse model. Orally immunizing mice with CT protected them against colonization upon challenge with different serotypes of *C. jejuni* [113]. Similar results had been obtained previously with recombinant PorA, a 53-kDA protein of *C. jejuni* [114]. It was subsequently shown that the CTB is the component mediating cross-reaction. These data may help explain the protection seen against *Campylobacter* in ETEC vaccine trials in Central America where the vaccine contained CTB [115]. When successful vaccination was achieved with an oral killed ETEC vaccine candidate, infections with *Campylobacter* were seen in only 0.6% of subjects compared to 5.0% of placebo recipients. Going forward, it will be important to determine whether vaccine candidates containing CTB, such as the inactivated whole-cell vaccine ETVAX [35;36] which contains a hybrid of CTB/LTB, can generate protective levels of *Campylobacter* surface antigen.

To make practical combinations of two or more enteric vaccines, whether cellular or subunit candidates, will require considerable attention to formulation and packaging for effective delivery to infants and children in developing countries. Some antigens may be put together in the same container, such as the enterotoxin subunit and the whole cells that make up ETVAX

and Dukoral[®] [116]. For some types of vaccine components, such as an inactivated whole-cell vaccine and a live attenuated vaccine, it may be more effective to package them separately in a delivery system that will facilitate their co-administration. This may even become a necessity as the number and types of antigens in the combination are increased. Finally, technical and quality assessment factors may impose limitations on the complexity of a potential combined vaccine, and it may be productive to consider frangible-seal type devices for co-administration.

Effective administration of enteric vaccines to infants

The childhood vaccination schedule for the first few years of life is already complex, and it is critical to introduce new vaccines in a way that does not overly complicate the existing schedule. The orally administered cellular ETEC and *Shigella* vaccine candidates are most likely to be the first to reach trials for licensure and probably will be given in a multi-dose regimen. This delivery route is not often used in existing vaccination schedules. Recent data show that, at least with non-living antigens, oral vaccination may not be maximally effective unless multiple doses are given [117]. Apparently the gut IgA system requires multiple exposures to antigen to select and expand high-affinity B-cell clones. With this in mind, most oral vaccines should be given in two or more doses. It may also be beneficial to administer a two- to three-dose prime to children less than six months of age, followed by a single-dose booster at the age of 9 to 18 months. This approach is consistent with a boosting of anti-CTB and vibriocidal responses and an earlier peaking of serologic responses following Dukoral[®] [116] re-immunization months after primary oral immunization [118;119]. Therefore, combining various enteric pathogens into a single vaccine will make a multiple-dose regimen more practical.

A multiple-dose regimen for a combined enteric vaccine could also help address the challenges of maximizing the immune response to oral vaccines among children living in endemic areas. Induction of mucosal immunity has proven to be a challenge even for immunizing well-nourished individuals in developed countries. However, this presents an even greater hurdle when attempting to immunize children in low-resource countries with oral vaccines that have previously been shown to be highly immunogenic and protective in developed countries [120;121]. In endemic settings, orally administered vaccines have generally been found to be less immunogenic and protective, possibly because the poor sanitation and malnutrition in these areas has led to conditions in the intestine that significantly diminish oral vaccine “take.” As a result, considerable effort has been expended to develop new approaches to maximizing the immune responses of young children living in these areas to enteric vaccines. One aspect of the strategy to overcome poor intestinal immune responses has been to use higher doses or multiple doses of vaccine. A two- to three-dose regimen before weaning followed by a booster dose at a later time, as suggested above, could improve the level of immune response. Another approach that could benefit intestinal immune responses in children is the use of a mucosal adjuvant. Recent research with the dmLT adjuvant [122] indicates that this vaccine component may be able to improve the immune response to orally administered vaccines in children. Studies to confirm this are expected to be initiated in the near future.

In addition to maximizing mucosal responses through the use of sufficient doses and adjuvants, a third possibility for overcoming poor intestinal immune responses to administered antigens is to use alternate routes of administration that may circumvent conditions in the intestine. It has recently been shown in humans and animals that, in the presence of an enterotoxin adjuvant,

antigen delivered by the ID route can evoke an immune response in the intestine [52]. This may also be achievable with antigen and adjuvant delivered sublingually [123;124]. Intramuscular administration of a vaccine antigen and an LT mutant may be another possible way to achieve mucosal immunity (John Clements, personal communication). In addition, the use of these routes could facilitate the potential use of conserved subunit antigens, which may not be sufficiently immunogenic at practical doses if given orally. All of these routes, if successful, would also eliminate the need for buffers, which may be needed for many oral vaccines.

Enteric vaccines are usually delivered orally, often accompanied with a significant amount of buffer to neutralize gastric acidity. A few enteric vaccines, such as Shanchol[®] [125] for cholera, are based on antigens such as LPS that are acid-resistant and do not need a co-administered buffer. More frequently, 150 to 200 ml of a bicarbonate or other buffer is used in adults, with proportionally smaller amounts used in children. Research is still needed to determine the amount of buffering, if any, that will be needed for ETEC and *Shigella* vaccines used in children during the first year of life. To be truly practical, oral administration of any vaccine to small children in endemic settings should be accomplished with only very small amounts of liquid.

5. Conclusions

Market studies suggest that the private sector in low-middle-income countries represents one of the largest untapped potential markets for ETEC and *Shigella* vaccines. One example is a market assessment for ETEC vaccine development conducted by PATH and BIO Ventures for Global Health [126], which estimated a modest US\$400 to 600 million market for ETEC vaccines, with

the public and private sectors in emerging middle-income economies projected to represent approximately 38% of anticipated revenues ten years after global launch. This type of research demonstrating the significant potential return on investment may help to attract more vaccine manufacturers in emerging economies, such as India, Brazil, and China, to participate in the development of new enteric vaccines.

In addition, as described earlier, recent impact studies indicate that ETEC and *Shigella* vaccines could significantly benefit global public health. Either vaccine, particularly if they could be combined together or with another enteric vaccine, would be an extremely valuable tool for saving lives and promoting the health of infants and children in the developing world, as well as potentially providing protection to travelers and military personnel visiting endemic areas.

In short, the market and the need for vaccines against ETEC and *Shigella* exist. New funding has encouraged much valuable development of promising near-term, as well as more upstream, vaccine candidates and supportive technologies, as described in this review, making success seem ever more likely. The challenge now is to sustain these efforts so that nearer-term vaccine candidates can be licensed quickly in order to expedite getting them to the populations who need them the most desperately.

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1100

Table 1: Current landscape of ETEC and *Shigella* vaccine candidates

		Nature of vaccine	Candidate	Current stage	Developer
Cellular	ETEC	Attenuated	ACE527	Phase 2b	PATH
			ZH9, second generation expressing ETEC antigens	Preclinical	Prokarium
		Vectored	<i>S. flexneri</i> 2a, second generation expressing CF/CS antigens	Preclinical	Center for Vaccine Development (CVD), University of Maryland, Baltimore (UMB)
			ETEC whole cell	Phase 1	Scandinavian Biopharma
	<i>Shigella</i>		CVD1208S	Phase 1	CVD, UMB
		Attenuated	WRSS1	Phase 2b	Walter Reed Army Institute of Research (WRAIR)
		Vectored	Ty21a expressing LPS	Preclinical	Protein Potential
		Inactivated	<i>Shigella</i> whole cell	Phase 1	PATH; WRAIR
Subunit	ETEC		Dukoral® (CTB)	Licensed	Crucell
		Anti-toxin	dmLT	Phase 1	Tulane University; PATH
			ST	Preclinical	EntVac Consortium

	LT patch	Phase 3	Intercell
Fimbriae	Fimbrial tip adhesin	Phase 2/2b	US Naval Medical Research Center
Adhesin-toxoid fusion	CF/CS consensus peptide fused to dmLT-ST mutants	Preclinical	Johns Hopkins University; Kansas State University
Novel proteins	YghJ, EatA	Preclinical	Washington University School of Medicine
<i>Shigella</i>	Chemical glycoconjugate	Phase 3	US National Institutes of Health's National Institute of Child Health and Human Development Laboratory of Developmental and Molecular Immunology
	Recombinant glycoconjugate	Phase 1	GlycoVaxyn
	Synthetic glycoconjugate	Preclinical	Institut Pasteur
	Invaplex _{AR}	Preclinical	WRAIR
	GMMA mixture	Phase 1	Novartis Vaccines Institute for Global Health

Serotype-independent protein	OMV	Preclinical	University of Navarra, Spain
	DB Fusion	Preclinical	PATH
	PSSP-1	Preclinical	International Vaccine Initiative
	34kDa OMP	Preclinical	National Institute of Cholera and Enteric Diseases, India