Human Vaccines & Immunotherapeutics 11:3, 601–619; March 2015; © 2015 Taylor & Francis Group, LLC

Vaccines for viral and bacterial pathogens causing acute gastroenteritis: Part II: Vaccines for *Shigella*, *Salmonella,* enterotoxigenic *E. coli* (ETEC) enterohemorragic *E. coli* (EHEC) and *Campylobacter jejuni*

Miguel O'Ryan, Roberto Vidal*, Felipe del Canto, Juan Carlos Salazar, and David Montero

Microbiology and Mycology Program; Institute of Biomedical Sciences; Faculty of Medicine; Universidad de Chile; Santiago, Chile

Keywords: acute diarrhea, *Campylobacter*, *E. coli*, ETEC, enteric pathogens, gastroenteritis, norovirus, rotavirus, *Shigella*, *Salmonella*, STEC, *V. cholera*, vaccines

Abbreviations: WHO, World Health Organization; GEMS, Global enterics multicenter study; rEPA, recombinant exoprotein A of *Pseudomonas aeruginosa*; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; LPS, lipopolysaccharide; CFU, colony-forming units; TTSS, type III secretion system; LT, heat labile toxin; dmLT, double mutant heat labile toxin; LT-B; heat labile toxin B subunit; OMV, outer membrane vesicles; ETEC, enterotoxigenic *E. coli*; CT, cholera toxin; CT-B cholera toxin B subunit; CFs, colonization factors; ST, heat stable toxin; STh, human heat stable toxin; STp, porcine heat stable toxin; EHEC; enterohemorrhagic *E. coli*; EPEC, enteropathogenic *E. coli*; STEC, shigatoxin producing *E. coli*; Stx, shigatoxin; LEE, locus of enterocyte effacement; HUS, hemolytic uremic syndrome; CtdB, cytolethal distending toxin subunit B; IM, intramuscular

In Part II we discuss the following bacterial pathogens: *Shigella*, *Salmonella* (non-typhoidal), diarrheogenic *E. coli* (enterotoxigenic and enterohemorragic) and *Campylobacter jejuni*. In contrast to the enteric viruses and *Vibrio cholerae* discussed in Part I of this series, for the bacterial pathogens described here there is only one licensed vaccine, developed primarily for *Vibrio cholerae* and which provides moderate protection against enterotoxigenic *E. coli* (ETEC) (*Dukoral*®), as well as a few additional candidates in advanced stages of development for ETEC and one candidate for *Shigella* spp. Numerous vaccine candidates in earlier stages of development are discussed.

Introduction

In Part I we introduced the rationale behind the development of vaccines for enteric pathogens. It is important to add that the Centers for Disease Control and Prevention (CDC) published on its website in *Global Diarrhea Burden* that diarrhea is the second-leading cause of death in children under 5 years of age, accounting for 11% of deaths in this age group. Clinically, acute childhood diarrhea is usually classified into 3 clinical presentations, watery, bloody and persistent diarrhea. However, for some bacterial infections, these clinical manifestations overlap. The major bacterial pathogens associated with diarrheal illness in humans include: *Vibrio cholera* (discussed in the previous section), enterotoxigenic *Escherichia coli* (ETEC), Shigatoxin producing *E. coli* / enterohemorrhagic *E. coli* (STEC / EHEC), *Campylobacter* spp, *Shigella* spp. and *Salmonella* spp Innumerable clinical studies have established that ETEC produces non-inflammatory watery diarrhea; however, other pathogens, such as STEC, *Campylobacter*, *Shigella* and non-typhoidal *Salmonella*, may produce overlapping clinical symptoms. It is important to consider that some of the abovementioned enteropathogens have exclusively human reservoirs (ETEC and *Shigella* spp.), while others are zoonotic pathogens with known animal reservoirs (cattle and chickens, among others). Furthermore, it is widely accepted that the use of antimicrobials is not the best strategy to control of enteric infection; furthermore, controversy exists regarding the use of antimicrobials for treatment, particularly for STEC. In the case of ETEC, *Shigella*, *Salmonella* and *Campylobacter* an increase in antimicrobial resistance has been observed, due to selection of resistant or multiresistant strains as a consequence of unregulated antimicrobial use in human health and animal production. Faced with this epidemiological panorama, the development of vaccines seems like the best option. The second part of this review aims to give an overview of existing vaccines and vaccine candidates for *Shigella*, *Salmonella* (non-typhoidal), diarrheogenic *E. coli* (enterotoxigenic and enterohemorrhagic), and *Campylobacter jejuni* (**Table 1**). As in the previous section, for each pathogen the flow is as follows: i) a discussion of the main epidemiological and pathogenic features; and ii) a discussion of vaccines based on their stage of development, moving from current licensed vaccines to vaccines in advanced stage of development (in phase IIb or III trials) to

*Correspondence to: Roberto Vidal; Email: rvidal@med.uchile.cl
Submitted: 07/29/2014; Revised: 11/13/2014; Accepted: 11/24/2014
http://dx.doi.org/10.1080/21645515.2015.1011578

Table 1. Human vaccines for individual enteric pathogens including status of development, main feature(s) and selected references

Pathogen	Vaccine (s)	Status*	Comment	Selected references
Shigella	Intramuscularly administrated, conjugates from LPS with protein carriers such as rEPA	Advanced clinical development	Phase III was well tolerated by children under 4 years of age. Coupling different LPS from the species allows for the development of multivalent vaccines.	Passwell et al. 2001[20]; 2003[19], 2010[22]
	Orally administrated *Shigella flexneri* 2a SC602	Early clinical development	Phase I and phase II in volunteers. However, this candidate does not show cross-reaction against other species of *Shigella*.	Rahman et al., 2011[26]
	Orally administrated *Shigella dysenteriae* 1 SC599	Early clinical development	Phase I, was immunogenic and well tolerated by volunteers.	Sadorge et al., 2008[28]; Launay et al., 2009[27,28]
	Killed whole-cell vaccine, intranasally administered	Early clinical development	An increased level of antibodies against LPS from the species used were present in animal experiments.	Barman et al., 2011[13]
	Orally administered *Shigella sonnei* WRSS1	Early clinical development	Phase I, developed antibodies against *S. sonnei* LPS, but reactogenic when high doses are used.	Kotloff et al., 2002[30]; Orr et al., 2005[31]
	Orally administered *Shigella dysenteriae* 1 WRSd1	Early clinical development	Phase I, vaccinated volunteers do not developed Shigellosis symptoms.	McKenzie et al., 2008[33]
	Intranasally administered *INVAPLEX*®	Early clinical development	Phase I, this candidate contains the Ipa proteins plus LPS and has been shown to develop a good intestinal immune response.	Tribble et al., 2010[43]; Riddle et al., 2011[42]
	Intranasally or ocularly administered *Shigella flexneri 2a WRSf2G11, WRSf2G12 and WRSf2G15*	Preclinical development	Second generation of vaccines, shown in animal model to protect against *S. flexneri* and to induce an immune response.	Ranallo et al., 2012[51]; 2014[52]
	Nasogastrically administered *Shigella sonnei WRSs2 and WRSs3*	Preclinical development	Second generation of vaccines from WRSS1. Shown by animals models to be safety and immunogenic.	Barnoy et al., 2010[53]; 2011[54]
	Proteins and conjugates vaccines (subcutaneous, intraperitoneal and intramuscular injections)	Preclinical development	Using synthetic carbohydrates derived from the LPS, it is possible to generate a multivalent vaccine diminishing the reactogenicity of LPS.	Pozsgay et al., 1999[38]; Phalipon et al., 2006[36]; 2009[37]; Robbins et al., 2009[39]
Nontyphoidal *Salmonella*	Orally administered live attenuated *S. Typhimurium* WT05	Early clinical development	Phase I, immune response observed in volunteers receiving a high dose (10^9 CFU).	Hindle et at, 2002[63]
	O antigen conjugated to tetanus toxoid, administered by subcutaneous injection	Preclinical development	This vaccine candidate showed protection in animal models by challenged an passive immunization	Watson et al 1992[65]
	O antigen conjugated with porins or BSA, antibodies injected intravenously	Preclinical development	This candidate confer protection thought passive immunization	Svenson et al 1979[66], 1981[67]
	O antigen conjugated with CRM_{197}, administered by subcutaneous injection	Preclinical development	This vaccine candidate produce antibodies against LPS	Stefanetti et al 2014[68]

(*continued on next page*)

Table 1. Human vaccines for individual enteric pathogens including status of development, main feature(s) and selected references *(Continued)*

Pathogen	Vaccine (s)	Status*	Comment	Selected references
	Core O LPS conjugated to H antigen, administered by intramuscular injection.	Preclinical development	This vaccine candidate showed protection in animal models.	Simon et al., 2011[69]; 2013[70]
	Orally administered *S.* Enteritidis: CVD1941 and CVD1943	Preclinical development	These live attenuated strains induce antibodies against LPS and flagellin in mice.	Tennant et al., 2011[75]
ETEC	*Dukoral*® (Oral administration)	Worldwide License	Licensed to prevent cholera. Cross protection by similitude between CT and LT. Recommended for travelers to endemic regions.	Clemens et al., 1988[84]
	LT patch	Advanced clinical development	Limited protection evidenced after phase III trial.	Behrens et al., 2014[89]
	Orally administered live attenuated ETEC expressing CFA/I, CS1, CS2, CS3, CS5 and CS6 (ACE257)	Advanced clinical development	No protection evidenced after phase IIb trial.	Darsley et al., 2012[90]
	Orally administered killed whole cells + CTB	Advanced clinical development	Limited protection evidenced after phase III trial. Reduction in the number of severe diarrhea cases noted.	Wiedermann et al., 2000[95]
	Orally administered double mutant LT (dmLT)	Early clinical development	Safe and immunogenic after phase I trial. Currently used as an adjuvant in newer formulations.	El-Kamary et al., 2013[102]
	Orally administrated live attenuated ETEC expressing CS1 and CS3 (PTL-003)	Early clinical development	Safe and immunogenic after phase I trial. No significant protection in phase II trial.	McKenzie et al., 2008[99]
	Orally administered CS6 coated microspheres	Early clinical development	Limited immunogenicity after phase I trial.	Lapa et al., 2008[100]
	CS6 patch	Early clinical development	Limited immunogenicity after phase I trial.	Güereña-Buegueño et al., 2002[101]
	Intragastrically administered killed whole cells + CTB/LTB + dmLT	Preclinical development	Safe and immunogenic in mice.	Holmgren et al., 2013[103]
	Intranasally administered *Shigella* carrying ETEC CFs	Preclinical development	Safe and immunogenic in guinea pigs.	Barry et al., 2006[104]
	Intranasally administered *Vibrio cholerae* Peru-15-pCTB	Preclinical development	Stimulates production of anti-LT antibodies in mice.	Roland et al., 2007[105]
	STh/dmLT administered by intraperitoneal injection	Preclinical development	Safe and immunogenic. Stimulates production of anti-LT and anti-STh antibodies.	Ruan et al., 2014[106]
	Intranasally administered type V-secreted proteins (EtpA, Ag43, pAT)	Preclinical development	Reduce intestinal colonization in mice.	Harris et al., 2011[110]; Roy et al., 2008[111]
Shiga-toxin producing *Escherichia coli*	O157-rEPA Vaccine based on *E. coli* O157: H7 O-specific polysaccharide conjugated to recombinant exotoxin A of *Pseudomonas aeruginosa* . Intramuscular injection (administered into the deltoid muscle)	Early clinical development	Phase I trial in adults, vaccine was safe and immunogenic; phase II trial in children, no significant adverse reactions were observed, concluding that the vaccine was safe and immunogenic.	Konadu et al., 1998[176]; Ahmed et al., 2006[176,177]
Campylobacter jejuni	Capsular polysaccharide conjugates administered by subcutaneous injection.	Preclinical development	Conferred significant protection to an oral challenge in monkeys (*Aotus nancymaae*).	Monteiro et al., 2009[190]

vaccines in early stages of clinical development (in phase I/II) or preclinical development in animal models. Although this review is focused on vaccines for use in humans, we also briefly discuss vaccines aimed at reducing the burden of zoonotic pathogens in their main animal reservoirs, with the final goal of reducing disease in humans.

Shigella spp

Pathogen and disease overview

Shigellosis only affects humans, particularly children under 5 y of age. Infection is caused by bacterial species from the *Shigella* genus (*S. dysenteriae*, *S. boydii*, *S. sonnei* and *S. flexneri*).[1] *Shigella* are Gram-negative bacteria from the family Enterobacteriaceae; they are non-motile and rod-shaped, and were discovered by Dr. Kiyoshi Shiga in 1936.[2] *Shigella* infections are endemic worldwide; however, the primary disease burden falls on developing countries, where it is reported that over 160 million individuals are infected annually, 60% of whom are children, making a vaccine against this pathogen a priority for the WHO.[3] The significant impact of Shigellosis in children from resource-deprived countries was documented in the recent GEMS study, as highlighted further in part I of this review.[4] Shigellosis is characterized by an acute intestinal infection, with a range of symptoms, from mild, watery diarrhea to severe inflammatory bacillary dysentery, with intense abdominal pain, fever and the presence of blood and mucus in the feces. *Shigella* spp can invade and disseminate through the colonic epithelium, inducing the recruitment of polymorphonuclear cells and generating an inflammatory response that causes associated symptoms. The molecular pathogenesis of *Shigella* is currently well characterized.[5,6]

Severe *Shigella* infections benefit from antimicrobial treatment, which reduces the duration of symptoms and bacterial shedding in stools; however, treatment has been hampered by the significant increase in antimicrobial resistance.[7-10] *Shigella* infection in humans results from the acquisition of a low number of bacteria, as low as 10-100 colony-forming units (CFU).[11] Since humans are the only reservoir for these bacteria, there are no animal models that have successfully replicated Shigellosis, making vaccine development difficult. Nevertheless, researchers have been using animal models and human volunteers to study the 2 types of *Shigella* vaccine candidates, whole-cell and conjugate vaccines.[12] One recent model is a guinea pig model, capable of developing Shigellosis within one day post-infection with *S. dysenteriae* or *S. flexneri*; however, this model also required a cecal ligation.[13] Two nonhuman primate models have been described:, the rhesus monkey model capable of developing Shigellosis symptoms after a high dose of bacteria,[14] and the *Aotus nancymaae* model, used in a protection study for the vaccine candidate SC602 with an efficacy of 80%.[15] Recently a mouse model that reproduces human-like Shigellosis symptoms after intraperitoneal inoculation has been developed.[16]

To date, there are no commercially licensed vaccines for this pathogen, despite significant research efforts. Two main strategies have been pursued: i) whole-cell vaccines, including killed and live attenuated strains; and ii) proteins or conjugated antigens. Killed whole-cell candidates are simple to produce and relatively inexpensive, although they require controlled temperature during storage to preserve antigens for recognition by the human-immune system. These vaccines could be stored at room temperature, which is advantageous for distribution in resource-deprived countries.[12] Live *Shigella* vaccines are based on genetically modified bacteria leading to attenuation, while different proteins and lipopolysaccharides (LPS) present in *Shigella* spp have been used as potential immunogens.

Vaccines in advanced stages of clinical development

Conjugate vaccines S. flexneri O-SP-rEPA and S. sonnei O-SP-rEPA

These vaccine candidates are based on O antigen from *S. flexneri* 2a and/or *S. sonnei* conjugated to the recombinant exoprotein A of *Pseudomonas aeruginosa* (rEPA) as a carrier protein (O-SP-rEPA). In a phase I study in army recruit volunteers receiving 2 intramuscular doses of either one of these antigens (or a third antigen of *Shigella dysenteriae* O-SP-TT, discussed below), only 1/116 subjects had fever, indicating that these antigen preparations were generally safe while proving immunogenic (IgG, IgM and IgA) against the LPS of each microorganism.[17] Several methods have been used to couple the O antigen to the carrier protein, in order to increase the immune response, including binding the O antigen to a succinylated mutant form of rEPA ($rEPA_{succ}$) or to a native or succinylated form of *Corynebacterium diphtheriae* toxin (CRM9 or $CRM9_{succ}$). These preparations have been successful in terms of safety and immunogenicity in volunteers, including adults and children in Israel.[18-20] Moreover, antibodies obtained from children vaccinated with either *S. sonnei* or *S. flexneri* O antigen-conjugate prevented the invasion of *Shigella* in an in vitro assay.[21] Additionally, in a recent phase III study in children 1 to 4 years of age, O-SP-$rEPA_{succ}$ conjugates from *S. sonnei* or *S. flexneri* were shown to be safe, as less than 5% of vaccinated children developed fever or localized pain. The protective efficacy of these candidates (disease rate of controls minus disease rate of vaccinees, divided by disease rate of controls, result expressed as percentage) was 27.5% (95% CI: -16.9-54.0%) for *S. sonnei* (including all age groups), reaching 71% (95% CI: -4.43-92.0%) in children older than 3 years of age, who also presented fewer diarrhea episodes per year post vaccination. Although, the efficacy for *S. flexneri* was only 7.9% (95% CI: -153.2-66.5).[22] The main challenge to this vaccine strategy is to provide broad protection against the most common *Shigella* strains with only one vaccine, as immunogenicity tends to be strain and serotype specific. Given this, synthetic O antigen-derived carbohydrates seem to be promising candidates, first, to reduce the LPS-associated toxicity and second, as a strategy for developing a multivalent conjugate vaccine, which is still required to confer broad protection against different *Shigella* serotypes (see below).

Vaccine candidates in early stages of clinical development

Live attenuated Shigella flexneri 2a SC602

This strain has been constructed by deletion of the *icsA* gene, which encodes a protein involved in the intra- and inter-cellular

dissemination of *Shigella*. This strain also includes a deletion of both *iuc-iut* genes, which encode proteins involved in synthesis of siderophores, compounds required for iron capture from the medium.[23] The first vaccination protocol involved a single dose with 10^4 cells from this strain and challenge with the virulent strain *S. flexneri* 2a (2×10^3 CFU) 8 days after vaccination in 7 volunteers and 7 placebo recipients. Six individuals who did not receive the vaccine, developed Shigellosis, while none of the vaccinated individuals developed Shigellosis and 3 reported mild diarrhea.[24] Another study evaluating the safety and immunogenicity of this vaccine in 34 North American volunteers showed that the vaccine was safe with only 4 vaccinated volunteers developing mild diarrhea, while inducing an immune response against LPS from *Shigella flexneri*.[25] However, in Bangladeshi volunteers, this vaccine strain did not induce production of IgG against LPS from *S. flexneri*.[26] More recently, this vaccine candidate provided protection in a mouse model for Shigellosis, as described by Yang et al.[16] This is the most extensively studied vaccine candidate and may prove promising. However, it may not be protective worldwide, as it does not show cross-reaction against other species of *Shigella*, indicating that immune response may be LPS specific.[25,26] Further studies are required, specifically those focused on including additional strains or antigens, deleting other genes and on using different concentrations of the various vaccine candidates to obtain a broad range of vaccination.

Live attenuated Shigella dysenteriae 1 SC599

This strain was constructed by deletion of 4 genes: 1) *icsA*; 2 and 3) *ent* and *fep*, both of which encode for synthesis of the siderophore enterobactin; and 4) *stxA*, which encodes the A subunit of the Shiga toxin. In phase I and phase II trials, this candidate was immunogenic and well tolerated at 10^8 CFU, albeit protective only against homologous strains; [27,28] further research is required.

Live attenuated Shigella sonnei WRSS1

This candidate was generated by deletion of the *icsA* gene, similar to the SC602 strain, and proved immunogenic and protective in a Sereny assay using a guinea pig model.[29] In North American (27) and Israeli (15) volunteers, the candidate was safe at low doses (10^3–10^4 CFU) and stimulated the production of antibodies against *S. sonnei* LPS. The bacteria did not seem to be transmissible from volunteers to other people living in their household.[30,31]

Live attenuated Shigella dysenteriae 1 WRSd1

The *S. dysenteriae* Δ*icsA*Δ*stxA*Δ*stxB* was developed using a similar strategy to the 2 *S. sonnei* vaccines. This strain did not cause damage to guinea pigs' eyes in the Sereny test, while protecting against challenge with the pathogenic wild-type *S. dysenteriae* strain in the same test. A combination vaccine, using the same proportion of this strain (WRSd1) together with SC602 and WRSS1, conferred protection against homologous virulent *Shigella* species used in this vaccination protocol.[32] McKenzie et al.[33] tested the WRSd1 vaccine candidate in a phase I trial, inoculating 40 volunteers (8 people per study-group) with a single dose of 10^3 to 10^7 CFU. The vaccine was safe, as none of the vaccinees developed fever or shigellosis, while IgA against *S. dysenteriae* LPS was present in almost 2-thirds of the vaccinees. Further study is required to determine this vaccine candidate's potential for conferring protection against future bacterial infections.

Shigella dysenteriae O-SP-TT and other conjugate vaccines

This candidate, based on the conjugated O antigen of lipid A of *S. dysenteriae* 1 to the tetanus toxoid protein, induced an immune response against the *S. dysenteriae* LPS in subcutaneously immunized mice.[34] It has been proposed that, for several pathogens, the use of synthetic carbohydrates based on LPS structure may be promising in creating vaccine antigens (reviewed by Pozsgay).[35] Using a mouse model, researchers have tested synthetic carbohydrates, whose repeated units have been derived from the specific *Shigella* spp. LPS O-antigen. In studies performed with these molecules at NIH and Institute Pasteur in France, vaccinated animals produced antibodies against the corresponding bacterial LPS.[36-39] However, these vaccine candidates have a narrow spectrum of protection, producing an immune response only directed at the bacterial strain from which the synthetic conjugate was obtained, it is for this reason that they remain in the development stage. The most advanced conjugate vaccine candidates, *S. flexneri* O-SP-rEPA and *S. sonnei* O-SP-rEPA, were discussed above.

Type III-secreted proteins

Among the molecules that are shared by all 4 species of *Shigella* are those secreted by the type III secretion system (TTSS), known as Ipa proteins.[5,6] Invaplex was developed as a possible vaccine and includes the secreted antigens IpaB, IpaC and IpaD, as well as LPS. In preclinical studies using monovalent or bivalent complexes, these candidates produced an immune response in guinea pigs and mice after intranasal administration. They were also shown to provide protection against *Shigella* in studies of keratoconjunctivitis or lethal lung infection.[40,41] In a phase I trial, intranasal application of the Invaplex vaccine candidate in adult volunteers was safe and well tolerated, while generating a strong intestinal immune response against the antigens included in the vaccine.[42,43] Furthermore, recent studies using the antigens IpaB and IpaD, conjugated to the *E.coli* heat-labile enterotoxin (dmLT), or both conjugated recombinant antigens in mouse studies, stimulated immunogenicity against both proteins regardless of preparation. Interestingly, in preclinical lethal pulmonary challenge studies, the fused protein IpaB-IpaD preparation confered protection against *S. flexneri* and *S. sonnei*, but was less protective against *S. dysenteriae*.[44,45]

Killed whole-cell vaccine

This strategy aims to develop a safe vaccine that includes all bacterial components of the various diarrheal causing *Shigella* species. Heat-killed *S. dysenteriae* type 1 and *S. flexneri* induced IgG in serum and IgA in mucosa in guinea pigs, against the LPS of each microorganism, in addition to protection against homologous bacteria in a challenge study.[13] McKenzie et al.[46] obtained

a whole-cell vaccine by treatment of *S. sonnei* (grown in rich containing deoxycholate) with formalin, in order to boost the surface antigens. Two weeks after intranasal vaccination of the guinea pigs, using 2×10^7 inactivated cells, the animals were challenged with pathogenic *S. sonnei* in a Sereny test. None of the 20 animals showed inflammation of the eye. A phase I trial of this vaccine candidate demonstrated that it is well tolerated by human volunteers, inducing an immunogenic response, as all individuals had immunoglobulins reacting to at least 1 of the antigens studied.[46] Moreover, a recent study adjusted the formalin concentrations and incubation temperatures to obtain a trivalent inactivated *Shigella* whole-cell vaccine, which includes *S. flexneri* 2a, 3a and *S. sonnei*. Intranasal immunization of guinea pigs protected against the corresponding pathogenic bacterium in a Sereny test.[47] More recently, pregnant mice were inoculated with a cocktail of several heat-killed *Shigella* strains, including *S. dysenteriae* 1, *S. flexneri* 2a, *S. flexneri* 3a, *S. flexneri* 6, *S. boydii* 4 and *S. sonnei*, which induced high titers of IgG and IgA against LPS from homologous strains. Furthermore, vertical protection was observed in neonatal mice when challenged with each homologous strain.[48]

Vaccine candidates in preclinical development

Live Attenuated Shigella flexneri 2a WRSf2G11, WRSf2G12 and WRSf2G15

These three strains represent a second generation of vaccine candidates, in which additional genes have been deleted. WRSf2G11, *icsA*, *set* and *senA* (the later 2 of which encode 2 enterotoxins, ShET1 and ShET2 respectively) were deleted. In experimental animals, this attenuated strain showed 88–100% protection, similar to the SC602 strain however with decreased levels of reactogenicity.[49] The WRSf2G12 strain differs from WRSf2G11 in the deletion of the *senB* gene. The WRSf2G15 strain also differs from WRSf2G12, because in addition to the previously deleted gene, it contains the mutated *msbB2* gene, which encodes an enzyme that modifies the lipid A.[50] These strains also demonstrated protection against *S. flexneri* and induced an immune response in the Sereny assay and in a monkey model.[51,52]

Live attenuated Shigella sonnei WRSs2 and WRSs3

Because WRSS1 was reactogenic when a high bacterial dose was used, the next generation of vaccine candidates required additional mutations. The WRSs2 strain has deleted the *icsA*, *senA* and *senB* genes, while the WRSs3 strain also includes the deletion of the *msbB2* gene. Both strains were shown to be safe, generated an immune response in the guinea pig Sereny test and were also immunogenic in rhesus monkeys.[53,54]

Outer membrane vesicles (OMVs)

OMVs are normally formed by Gram-negative bacteria and have proven immunogenic for several pathogens, including *Vibrio cholerae*.[55] Recently, Camacho et al.[56] obtained OMVs after treatment of *S. flexneri* with binary ethylenimine plus formaldehyde. The vesicles harbored several *Shigella* antigens, such as the proteins IpaB and IpaC. Nearly 90% of intranasally immunized mice were protected against challenge with virulent *S. flexneri*.

Nontyphoidal *Salmonella enterica* Subspecies *enterica* Serotype Enteritidis and Typhimurium

Pathogen and disease overview

The genus *Salmonella* is divided into 2 species, *S. bongori* and *S. enterica*, which include more than 2,400 serovars. Warm-blooded animals, including humans, are primarily affected by *S. enterica* subspecies *enterica*, which includes nearly 1,500 different serovars. The species *S. enterica* also contains another 5 subspecies, which are responsible for colonization of cold-blooded animals and are present in the environment.[57] *S. enterica* subspecies *enterica* includes: serotype Typhimurium (*S*. Typhimurium), and serotype Enteritidis (*S*. Enteritidis), both responsible for the infection of animals and humans; and serotype Typhi (*S*. Typhi), which infects only humans.[58] In this review, we will focus only on nontyphoidal *Salmonella*: *S*. Typhimurium and *S*. Enteritidis.

Nontyphoidal *Salmonella* are foodborne enteropathogens capable of causing serious gastroenteritis and fatal invasive disease in children under 2 years of age and HIV infected individuals.[59] The global burden of nontyphoidal *Salmonella* illness was recently estimated at nearly 93.8 million cases and 155,000 deaths per year.[60] The primary sources of infection are contaminated drinking water and food, including poultry products.[61] *Salmonella* invades intestinal cells, aided by the secretion of several effector proteins, through a TTSS encoded in the *Salmonella* pathogenicity island-1 (SPI-1). In addition, *Salmonella* survives in the intracellular environment of phagocytic and nonphagocytic cells in the so-called *Salmonella*-containing vacuoles, a step that requires a second TTSS encoded in the *Salmonella* pathogenicity island 2 (SPI-2).[62] Vaccine strategies against *Salmonella* have focused on the prevention of systemic infection and to a lesser degree on the prevention of gastroenteritis. There are currently no licensed vaccines for nontyphoidal *Salmonella* and currently all candidates are in the early stages of development.

Vaccines in early stages of clinical development

Live attenuated S. Typhimurium WT05

This candidate has deletions in 2 loci: the *ssaV* gene, which encodes for a structural protein of the SPI-2 TTSS; and the *aroC* gene, encoding chorismate synthase involved in the pathway of aromatic amino acids synthesis required for bacterial growth in vivo. A total of 9 healthy volunteers tolerated a single dose of 10^7–10^9 CFU of bacteria, reporting no diarrheal episodes; however, the induction of an immune response against the pathogenic bacteria was observed only in volunteers who received 10^9 CFU. Moreover, the vaccinees suffered prolonged shedding of *Salmonella* in stools.[63] To date, mutation of these genes has proven to be safe for human use. Further studies, and possibly additional mutations, will be required to determine the potential efficacy of these candidates.

Vaccines in preclinical stages of development

Conjugate vaccines candidates

Several research groups have developed vaccines candidates using the O antigen linked to carrier proteins.[64] One of these vaccine candidates is a tetanus toxoid (TT) bound to the O antigen.[65] This candidate showed an increased immune response against the corresponding LPS in mice, after subcutaneous inoculation. Moreover, the immunization conferred protection against an intraperitoneal challenge with *S*. Typhimurium, by increasing by 160-fold the lethal dose, 50% (LD_{50}), compared to controls. Sera obtained from immunized mice provided protection through passive immunization.[65] Similar results had been obtained previously by Svenson et al. with conjugation of the O antigen with porins or BSA.[66,67] More recently, conjugation of the O antigen to the carrier protein CRM_{197}, also produced antibodies against LPS in subcutaneously immunized mice.[68] In *S*. Enteritidis, the same strategy was used to obtain a conjugate between the O antigen and the homologous H antigen from flagellin. This antigen induced the synthesis of antibodies against *S.* Enteritidis LPS and its flagellin antigen in mice that were antigen specific in an opsonophagocytic assay. In this model, conjugates were 100% protective against an intraperitoneal challenge of 5×10^5 CFU of the *S.* Enteritidis strain, decreasing to 91.7% when the challenge was increased by 1 $\log_{10}$.[69] In a recent study, a dose of 0.025 μg of this conjugate formulation protected up to 90% of vaccinated mice. Importantly, protection was observed in naïve mice, passively immunized with antibodies obtained from mice immunized with the conjugate.[70]

Live attenuated vaccine candidates

S. Typhimurium SA186. This attenuated strain involved the deletion of the Zinc transporter genes *znuABC*. Oral inoculation with this strain, protected mice against a challenge with 2×10^8 CFU of pathogenic *S,* Typhimurium, reducing colonization in the spleen and cecum and the number of virulent bacteria in the gut, as well as decreasing intestinal inflammation.[71,72]

S. Typhimurium Δhfq STMΔhfq. This attenuated strain was obtained by the deletion of the *hfq* gene, encoding a small RNA chaperone involved in post-transcriptional regulation of gene expression in enterobacteria. Mice, orally inoculated mice with 10^7 CFU, had 100-1,000 fold fewer bacteria in the spleen, mesenteric lymph nodes and liver. Additionally, mice vaccinated with 3 doses of 10^3-10^5 CFU were protected against oral and intraperitoneal challenge with 10^8 CFU of wild-type *S*. Typhimurium. A single dose of 10^8 CFU of STM Δ*hfq* proved to be effective, providing long-term protection against challenge with the wild-type strain.[73]

S. Typhimurium ΔssaVΔfur MT13. This attenuated strain was obtained by the deletion of 2 genes: *ssaV,* which encodes for a structural protein necessary for the efficient functioning of the SPI-2 TTSS; and *fur,* encoding for the ferric uptake regulator, responsible for iron homeostasis. MT13 was safe in immunocompromised mice and induced an immune response against the O-antigen. In addition, vaccinated mice (4) were challenged with 200 CFU of wild-type *S*. Typhimurium after ampicillin-treatment in order to eliminate the residual MT13 strain. Under these conditions, the wild-type strain was unable to colonize the liver, spleen and mesenteric lymph nodes, indicating protection against *S. typhimurium* infections.[74]

S. Typhimurium CVD1921, CVD1923 and S. Enteritidis CVD1941 and CVD1943. Several chromosomal genes of *S.* Typhimurium and *S.* Enteritidis have been deleted in an attempt to obtain live attenuated immunogenic vaccine candidates. The target genes for deletion have been: i) *guaBA*, which participates in guanine synthesis; ii) *clpPX*, which encodes for a protease responsible for degradation of the flagellum's synthesis regulator, providing mutants producing abundant flagellum; and iii) *fliD*, which encodes for a protein located at the tip of the flagellum; absence of this protein allows the bacterium to secrete flagellin subunits to the supernatant. The following candidates have been designed based on these chromosomal gene deletions, for *S*. Typhimurium strains CVD1921 (Δ*guaBA*, Δ*clpP*) and CVD1923 (Δ*guaBA*, Δ*clpP,* Δ*fliD*) and for *S*. Enteritidis strains CVD1941 (Δ*guaBA*, Δ*clpP*) and CVD1943 (Δ*guaBA*, Δ*clpP,* Δ*fliD*). These strains have proven attenuated and capable of inducing antibodies against *Salmonella* LPS and flagellin in mice immunized with 10^9 CFU. In a challenge assay, using 2×10^6 CFU of *S*. Typhimurium I77 or *S*. Enteritidis R11 (both pathogenic clinical isolates), more than 80% of mice survived after vaccination with CVD1921 and CVD1923. For *S*. Enteritidis, protection was 79% with CVD1941 and 33% with CVD1943.[75]

Proteins associated with the membrane as vaccine candidates. An alternative strategy to using purified proteins has been the use of outer membrane antigens. Surface antigens were obtained from LPS-defective strains of *S*. Enteriditis obtained from clinical isolates treated at 100°C for 15 min in a saline buffer. Mice were then inoculated intraperitoneally with 1 dose; controls were injected, in parallel, with extracts of the isogenic wild-type strain. Both extracts induced an immunogenic response, and in a challenge experiment showed 80% protection for the wild-type strain and 60% protection for the LPS mutant strain. However, immunizations with the LPS deficient strain (*wcaI* mutant) did not provoke distress in mice. In this experiment, immunized mice were challenged with 1.4×10^2 CFU of pathogenic bacteria. There was nearly a 60% survival rate in vaccinated mice, while all controls died 19 days after challenge.[76] Recently, chickens intramuscularly immunized with a *S*. Enteritidis ghost, as an alternative strategy to outer membrane antigens, had an increase in specific IgG and IgA and a decrease in bacterial colonization of the liver, spleen and cecum after challenge with a virulent strain, suggesting that immunization of chickens might be another vaccine strategy to indirectly protect humans.[77]

The WT05 vaccine is the only nontyphoidal *Salmonella* vaccine in clinical trials to date, but it was discontinued after demonstration of prolonged shedding of the vaccine strain. Significant future research is required in order to advance a vaccine for use in humans, while less effort may be necessary to develop vaccines for use in animal reservoirs of *S*. Enteritidis.

Enterotoxigenic *Escherichia coli* (ETEC)

Pathogen and disease overview

Among the different *E. coli* pathotypes, Enterotoxigenic *Escherichia coli* (ETEC) is the most common cause of diarrhea, primarily affecting children in developing countries and travelers who visit endemic regions. A 2005 review attributed close to 1 billion diarrheal episodes per year to ETEC, resulting in an estimated 300,000 to 400,000 deaths.[78]

ETEC are *E. coli* strains producing heat-labile (LT) and/or heat-stable (ST) enterotoxins.[79] A high number of *E. coli* serotypes are considered ETECs according to classification based on structural antigenic determinants (the somatic O antigen, the flagellar H antigen and the capsular K antigen).[79] Recently, ETEC strains producing ST alone or in combination with LT, but not those that produce only LT, were found to be one of the top 4 causes of acute infectious diarrhea in the GEMS trial, which included children residing in resource-deprived countries.[4]

ETEC strains colonize the small bowel epithelium and secrete LT and/or ST, causing an increase in intracellular levels of cAMP and cGMP (cyclic nucleotides that act as second messengers), followed by an opening of membrane integrated channel proteins, and finally leading to massive efflux of water and electrolytes.[79]

ETEC adheres to the gut epithelium using a diverse repertoire of adhesins, including more than 20 multimeric structures known as colonization factors (CFs) (also called colonization factor antigens [CFAs] or coli surface-associated antigens [CS]).[80] ETEC strains commonly carry up to 3, and occasionally up to 5, CFs simultaneously. The current nomenclature denominates CFs as "CS" plus a number that differentiates the various types. This is true for all the CFs except for CFA/I, the first CF discovered, which maintains its original acronym.[80] The most common adhesins among strains evaluated in epidemiological surveys worldwide are CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS12, CS17 and CS21.[81]

ETEC vaccine candidates have been developed based on whole cell formulations and predominant virulence determinants,[82] none of which are currently licensed. The main obstacles to designing a vaccine are: i) the great diversity of ETEC strains and their virulence repertoire, and ii) the poor immunogenicity of ST. Given that cholera toxin (CT) is highly similar to LT in sequence and structure, *Dukoral*® has been recommended for the prevention of ETEC associated travelers' diarrhea. Therefore it is worth noting, 2 earlier studies of a precursor to *Dukoral*®, licensed to prevent cholera and prequalified by the WHO[83] that contained killed whole *V. cholerae* cells and purified non-recombinant CT-subunit B (CT-B), evaluated protection against ETEC associated diarrhea. The first study was performed in nearly 50,000 individuals, including adults and children in Bangladesh, and reported a protective efficacy of 67% (95% CI LB: 30%) against diarrhea caused by LT-producing ETEC.[84] In the second study, 612 Finnish travelers to Morocco were evaluated, and the formulation was found to confer 60% (95% CI: 52-68%) protection against LT-producing ETEC.[85]

Only one study has evaluated the currently licensed *Dukoral*® formulation containing the recombinant CT-B for prevention of ETEC associated diarrhea in a group of 502 college students from the USA traveling to Mexico.[86] Upon arrival 252 subjects received 2 oral doses of *Dukoral*®, 7 days apart, and 250 received buffer as a placebo. Among all travelers, 75 suffered an ETEC associated diarrhea episode, of which 36 received the vaccine and 39 placebo. No differences in enterotoxin repertoires between ETEC strains were reported. These results suggest that the formulation does not protect against ETEC associated diarrhea in young adult travelers. However, the authors reported that most of the diarrhea cases attributed to ETEC (55 cases, 30 vaccinated subjects versus 25 placebos) occurred before administration of the second dose, i.e., before day 7. When only cases occurring after day 6 are considered, the authors reported a protective efficacy of about 50% (12 diarrhea cases in vaccinated subjects and 7 in placebo recipients). In order to prevent travelers' diarrhea, administration of 2 doses of *Dukoral*® is recommended 1-6 weeks apart, with the first dose administered at least 2 weeks prior to travel.[87] However, more evidence is required in order to support its use for protection against ETEC caused travelers' diarrhea.[88] Certainly, inclusion of live or killed whole ETEC cells, or specific ETEC antigens, is needed to develop an effective vaccine useful in endemic or outbreak scenarios.

Vaccines in advanced stages of clinical development

Despite the fact that several ETEC vaccine candidates have undergone clinical protection trials without fully successful results, these trials have provided important information on the strategies and antigens to consider in future formulations.

LT patch

This transcutaneous patch releasing LT was proven to be safe and immunogenic, but not efficacious in a phase III trial.[89] In a recent report, it provided 34% (95% CI: -2.2-58.9%) protection against ETEC diarrhea in US travelers to Mexico. Consistent with the conclusions derived from *Dukoral*® trials, authors concluded that additional ETEC antigens may be needed in order to improve protection levels.[89]

ACE257

This formulation, based on 3 non-toxigenic ETEC strains (lacking genes encoding chorismate synthase [*aroC*] and 2 outer membrane proteins [*ompC* and *ompF*]) carrying the CS CFA/I, CS1, CS2, CS3, CS5 and CS6, plus recombinant LTB), was evaluated in 70 adult volunteers (36 vaccinees and 34 placebos) in Baltimore (Maryland, USA).[90] Two doses of 10^{11} CFU were administered 21 days apart and were generally well tolerated, even as a few adverse effects, such as vomiting, were noted (7 vaccinees vs. 0 placebos). Humoral response against LT-B, CFA/I and CS3 was detected; however, humoral response against CS6 was lower. Protective efficacy was 27% (95% CI: -12.8-52.1%), and the number of mild and severe diarrhea cases after challenge with ETEC H10407 was not reduced. However, this candidate reduced fecal shedding of H10407 on day 2 post-challenge. Different vaccine dosages and buffer composition, as well as the use of adjuvants, are currently being evaluated to reduce adverse effects and stimulate a better immune response. If a higher

protective efficacy is reached this formulation may be a promising candidate, as it contains widely distributed ETEC antigens, particularly CFs.

Hyperimmune bovine colostrum//passive immunization

Antibodies contained in hyperimmune colostrum confer passive protection to infants of diverse mammal species. A study evaluating the capacity of colostrum, obtained from pregnant cows vaccinated with ETEC proteins, to confer protection against ETEC caused diarrhea was performed in 90 adult volunteers in Warsaw, Poland.[91] Extracts obtained from ETEC strains belonging to 14 different serogroups, commonly associated with diarrhea, and expressing CFs CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS12, CS14 and CS17, were injected into pregnant cows in 5 doses over a period of 10 weeks. Colostrum was obtained from the first milk after calving. Lyophilized colostrum mixed with adjuvant was orally administered to volunteers in 2 200 mg tablets, 3 times a day for 1 week. Alternatively, various combinations of lower doses, with or without bicarbonate buffer, were also assessed. Regardless of the dose, the colostrum tablets conferred significant protection against diarrhea after challenge with the prototype ETEC H10407 strain. It has been suggested that this formulation and its regimen of administration may potentially be useful in the prevention of travelers' diarrhea. No information regarding patents or ongoing studies was found.

Killed whole cells/CTB

First designed at the University of Gothenburg, Sweden, this candidate includes a recombinant purified CT-B and 5 different formalin-killed ETEC strains carrying 7 CFs: CFA/I, CS1, CS2, CS3, CS4, CS5 and CS6. Despite carriage of CS6 by 2 of the strains, immunogenicity against this CF was not induced because formalin treatment destroyed its non-fimbrial structure. Two of the strains were originally ST producing, but none produced LT. Studies including 158 children and adults performed in Sweden, Egypt and Bangladesh concluded that the vaccine was safe and that immunogenicity was conferred after the administration of 2 doses containing 1mg of CT-B and approximately 5×10^{10} killed bacteria.[92-94] A preliminary evaluation of efficacy was performed in 187 Austrian volunteers traveling to various tropical locations. Five cases of diarrhea attributed to ETEC were observed in placebo recipients compared to 1 case in vaccinated subjects.[95] However, further evaluation of efficacy in 1,357 volunteers from the USA traveling to Mexico or Guatemala, 2 vaccine doses prior to travel marginally reduced the overall number of diarrhea cases attributed to ETEC strains carrying homologous CFs and LT (24%, not significant protective efficacy).[96] Importantly, a significant reduction in the number of moderate diarrhea episodes (77%, 95% CI: -6-95%) and episodes affecting normal activities (85%, 95% CI: -20-98%) was reported. Higher efficacy rates are expected if expression levels of CFs are increased, a theory that is currently being tested, as development of new formulations continues.[97]

Vaccine candidates in early stages of clinical development

Several ETEC vaccine candidates have been evaluated in phase I or II trials, including improved versions of previously designed formulations.

Killed whole cells vaccines

A new formulation including formalin-killed ETEC overexpressing CFA/I plus a hybrid CT/LT-B subunit, capable of inducing a high cross-immune response against LT-B, was recently evaluated in a phase I clinical trial.[98] Approximately 600 μg of CFA/I per dose were detected in this formulation, compared to 200 μg per dose contained in the previously tested candidate. Safety and immunogenicity were evaluated in 60 Swedish adult volunteers receiving 2 doses 2-weeks apart. Compared to its precursor, which in this case included only CFA/I-expressing killed ETEC plus the hybrid, the new vaccine candidate induced an equivalent immune response.[98] Only the serum anti-LT IgA was significantly higher after administration of the new design. A higher mucosal immune response against LT and CFA/I was achieved only when the vaccine was administered in a 4-fold increased dose. Therefore, even when a higher amount of CFA/I was included in the formulation, it seems that a stronger immune response against this CF is still needed.

Live attenuated strains

The non-toxigenic ETEC strain PTL 003 Δ*aroC*, Δ*ompC*, Δ*ompF*, carrying CS1 and CS3, was evaluated in its capacity to protect against infection in 33 adult volunteers (17 vaccinees and 16 placebos). Two doses of the formulation were administered 10 days apart, but they failed in reducing the number of total and severe cases of diarrhea after challenge with a homologous $CS1^{+}CS3^{+}$ toxigenic ETEC strain at day 28 post-vaccination, despite inducing an immune response against these CFs.[99] A different schedule including a later dose was suggested by the authors for further improvement of the protective potential.

Purified CS6

Purified CF subunits have been proposed as potential vaccine candidates against ETEC. Safety and immunogenicity of biodegradable polymer microspheres coated with CS6 were evaluated in a clinical trial of 60 adult volunteers. However, a few subjects developed a significant response against CS6, regardless of whether mutant LT (R192G) was added as an adjuvant.[100] In another study, a similar combination was administered (the only difference being that it contained native LT) to 26 volunteers by means of a transcutaneous patch. Skin rash and inflammation occurred in a few cases, but no other adverse effects were reported. Vaccination with CS6-LT induced significant increases in anti-CS6 serum IgG and IgA in 68% and 53% of vaccinated individuals, respectively. No significant response was noted when CS6 was administered alone, indicating that the adjuvant is needed to stimulate production of anti-CF antibodies.[101] As mentioned previously, for some ETEC vaccine candidates stimulating a good response against CS6 seems to be a challenge, as it is

one of the most common CFs in ETEC diarrhea causing strains worldwide.

Double mutant LT

Safety and immunogenicity of a double mutant LT (dmLT) was evaluated in 36 adult volunteers from Baltimore (Maryland, USA) and Cincinnati (Ohio, USA). For this toxin variant, 2-point substitutions were introduced in order to disrupt ADP-ribosylation activity, i.e., the toxic effect, and a putative pepsin cleavage site. After single administration of doses containing 10, 25, 50 or 100 μg of dmLT, diarrhea cases were not reported, and the formulation was well tolerated.[102] Systemic and mucosal humoral immune responses against LT were detected, particularly in the group receiving the 50 μg dose. Thus, dmLT is a suitable candidate for inclusion in multiple-antigen formulations or as an adjuvant.

Vaccine candidates in preclinical stages of development

Killed whole cells/hybrid CTB-LTB/double mutant LT (dmLT)

An improved version of the previously designed killed whole cell formulations was generated, containing 4 formalin-killed ETEC strains expressing recombinant CFA/I, CS3, CS5 and CS6, the hybrid CT-B/LT-B and dmLT as adjuvant.[103] This new version includes 1×10^9 inactivated bacteria, containing 10 g CFA/I, 38 μg CS3, 6.4 μg CS5 and 1.5 μg CS6, plus 13 μg LT/CT and 25 μg dmLT. Vaccination of BALB/c and C57/BL6 mice elicited immune responses to all components included in the formulation. Addition of dmLT significantly increased serum anti-LT IgA and anti-IgG, as well as anti-CS3 and anti-CS6 IgA. Additionally, mucosal antibodies, IgG and IgA, against the 4 CFs and LT were significantly increased by the addition of dmLT. The adjuvant effect of dmLT was particularly notable when 3 doses, rather than 2 doses, were administered to mice. These observations support the use of dmLT to improve immune response against CFs.

Shigella/V. cholerae strains

Attenuated *Shigella* strains have been tested as vectors to deliver ETEC antigens. Strains attenuated by deletion of the *sen* and *guaBA* loci (responsible for directing toxin production and synthesis of guanine nucleotides, respectively) and transformed with loci encoding for CFA/I, CS2, CS3 and CS4, elicit humoral immune response against these factors after intranasal administration to guinea pigs. This is a promising strategy that could be useful in the design of a unique formulation conferring protection against 2 of the most prevalent enteric pathogens in the developing world, ETEC and *Shigella*.[104] Peru-15-pCTB, a variant of the Peru-15 *V. cholerae* strain, secreting higher levels of CT-B (30-fold higher than Peru-15) was tested for its capacity to stimulate anti-LT immunogenicity. Single administration of 1×10^9 CFU to mice and 2×10^{10} CFU to rabbits resulted in, at least, a 30-fold increase in anti-CT antibodies. The humoral response induced by Peru-15-pCTB was able to block the toxigenic effect of LT according to in vitro assays.[105] This formulation seems to be another alternative to obtain a vaccine against 2 of the most important diarrheal pathogens in the developing world. Improving its efficacy against ETEC would certainly imply the inclusion of ETEC antigens.

STh-dmLT

ST has not been considered for inclusion in ETEC vaccine candidates because it is a short and poorly immunogenic peptide and because of its toxic effect. However, single amino acid substitutions in ST have been shown to reduce its toxigenic activity, and conjugation to LT has been promising for increasing immunogenicity. This is valid for both genetic variants of ST, human ST (STh) and porcine ST (STp), produced by human ETEC strains. Recently, screening of a mini-library of STh toxoids (non-toxigenic mutants) conjugated to the dmLT was performed to identify immunogenic, potentially non-toxigenic formulations.[106] Three copies of each non-toxigenic STh mutant were conjugated to 1 dmLT molecule and intraperitoneally injected into BALB/6 mice in 3 doses of 200 μg, separated by 7 days. All formulations induced anti-LT antibodies in serum and feces, and one in particular (3xSTaN12S-dmLT) was effective in stimulating significant increases in serum anti-STh IgA and IgG and mucosal IgA. Other conjugations were capable of inducing some of the responses with variable results. Serum and feces obtained from vaccinated animals displaying significant humoral responses blocked the effect of purified STh and CT over polarized T84 cells in vitro, thus proving the induction of anti-toxin antibodies.[106] Therefore, it now appears to be feasible to include ST toxoids in the development of future vaccine candidates targeting ETEC.

Non-CF surface proteins

Three proteins exported by type V secretion systems and involved in adherence mechanisms of ETEC or other *E. coli* strains, have been evaluated for their ability to reduce colonization of the mouse intestine. They include the non-classical adhesin EtpA, and the autotransporters Ag43 and pAT; all of which are recognized by sera obtained from patients who suffered diarrhea caused by ETEC.[107] EtpA attaches the tip of the flagellum and, acting as a molecular bridge, it directs adherence to intestinal epithelial cells.[108] Based on detection of the *etpA* gene, it is widely distributed among diverse ETEC strains, similar to or even more so than the most common CFs.[109] On the other hand, Ag43 and pAT are autotransporters, known to be carried by at least 3 prototype ETEC strains that are also conserved in non-ETEC pathogenic *E. coli* strains.[110] Separate intranasal administration of the purified proteins, significantly reduced colonization of the intestines after challenge with wild-type ETEC in mice.[110,111] Therefore, non-CF adhesins may be suitable candidates for future vaccine development.

Enterohemorragic *Escherichia coli*

Pathogen and disease overview

EHEC was first described in 1983 and has been associated with 2 outbreaks of hemorrhagic diarrhea in 2 cities in the USA; the commonality between these 2 outbreaks was poorly cooked hamburger meat in fast food restaurants. The *E. coli* strain isolated from these outbreaks was of the O157:H7 serotype,[112] a strain that, that same year, had been associated with sporadic cases of hemolytic uremic syndrome (HUS). This strain produced cytotoxins (verotoxins or Shiga toxins) that were present in patients with HUS.[113] These cytotoxins were originally identified by Konawalchuk et al.,[114] who described their cytopathic activity on Vero and HeLa cells.

EHEC is characterized by the production of 2 Shiga toxins (Stx1 and Stx2), as well as by the presence of the *eae* gene, located in the locus enterocyte effacement (LEE) locus, which codes for the protein intimin. This protein is a non-fimbrial adhesion and considered the main factor involved in the adherence of EHEC to epithelial intestinal cells.[115] A lesion, called the attaching/effacing or A/E phenotype, is produced as a consequence of this adhesion, characterized by a re-ordering of the cytoskeleton of the enterocytes, which generates pedestals on the intimin adherence sites of the bacteria and later loss of the microvellosity of the surface of the intestinal epithelium.[116] Due to the production of Stx, EHEC are considered a subgroup of Shiga toxin-producing *E. coli* (STEC), although some of them may not contain the LEE locus (LEE-negative STEC).

Intestinal infections caused by different pathogenic strains of *E. coli* are one of the principal public health problems worldwide.[117] In particular, infections by STEC are the most serious and are associated with development of HUS,[118] mainly in children under 5 years of age.[119] Serotype O157:H7 is the serotype most frequently associated with sporadic outbreaks and severe illness; however other serogroups such as O26, O103, O111 and O113 have also been implicated in these outbreaks.[120] Current clinical treatment for infection includes only support, since the use of antimicrobial drugs to combat STEC infection increases the risk of developing HUS.[121] In spite of the low incidence of STEC infections, the economic losses caused by outbreaks, the hospital costs, the possible consequences and mortality in the infant population, all underscore the need to develop prevention and therapeutic strategies to reduce their public health impacts.[119]

Vaccination has been promoted as one of the most efficient means to decrease the incidence and prevalence of STEC outbreaks.[119,122-126] Two main intervention strategies have been proposed: i) vaccination of the infant population; and ii) vaccination of cattle, the main animal reservoir and the primary means of human contamination, due to the consumption of poorly-cooked meat.[127] It should be noted that cattle colonized by STEC are generally asymptomatic, thus subsidies for farmers may be necessary to promote animal vaccination. Hurd and Malladi[122] predicted a 60% decrease in human cases associated with O157:H7 assuming a bovine vaccination effectiveness of 80% and an adoption rate of 100%. The study by Matthews et al.,[123] utilizing a different mathematical model, predicted an 85% decrease in the incidence of human cases associated with O157:H7 in Scotland, assuming a decrease of 50% in the bovine/human transmission risk. However, the impossibility of experimentation in humans, due to the risk of developing HUS, and the lack of an animal model that reproduces the entire clinical profile caused by infection in humans have been important barriers for the development of an effective vaccine.[128] Notably, experimental animal models that partially reproduce the infection have been developed in mice, rats and rabbits, and have been important in the evaluation of different therapeutic strategies (reviewed in [129]).

The characterization of STEC antigens using human serum, naturally and artificially infected cattle and other animal models, has allowed the identification of different immunogenic proteins that have been proposed as targets for the development of vaccines.[130-135] The antigens best characterized immunologically, and that have been used systematically in different vaccine candidates, include: the Shiga toxins Stx1 and Stx2, LPS, flagellin (FliC – H7) and virulence factors encoded in the LEE and secreted by a TTSS, such as Tir, intimin, EspA, EspB and EspD. The use of effectors coded by the LEE has been particularly attractive, given their importance in STEC pathogenesis and due to the possibility of generating cross-protection against enteropathogenic *Escherichia coli* (EPEC) [reviewed in [136]]; this is another important pathotype associated with infantile diarrhea that presents an LEE locus with a high degree of homology.[117] Interestingly, cross-protection against STEC has been reported in mouse models infected with EPEC,[137] and moderate protection against virulent strains of EPEC has been reported in rabbits immunized with intimin.[138] However, more studies are necessary to identify specific antigens that confer cross protection and to evaluate their efficacy and clinical viability.

Different strategies of active immunization against STEC have been evaluated in animal models. These include the use of Stx toxoids,[139,140] hybrid Stx toxoids,[141,142] proteins secreted by TTSS,[143-148] chimeric proteins,[148-153] DNA vaccines,[154,155] live attenuated vaccines[156-159] and phantom strains of O157:H7.[160-162] These vaccine candidates have had variable success, and their results are discussed extensively in 2 recent reviews.[129,163] However, proteins secreted by the TTSS, whether used individually, in combination or as conjugated proteins, are those that have been most commonly evaluated in trials.

These vaccines are fundamentally based on a limited set of antigens and results have differed considerably; some are promising candidates, while others did not decrease colonization and/or STEC loads in stools. Consequently, it is evident that there are gaps in our understanding of immune response against STEC, which is relevant in the type of formulation, use of adjuvants, administration route and the quantity and number of doses utilized. In particular, the possible immunogenic consequences of polymorphisms and antigen variations among strains must be evaluated.[164] In fact, polymorphisms have been reported for almost all the effectors coded in LEE and secreted by TTSS.[165-168] Currently only 2 effective control strategies for livestock have been described. These 2 vaccines against *E. coli* O157:

H7 target TTSS-secreted proteins (*Econiche*® Bioniche Life Sciences Inc., Belleville, Ontario, Canada), a siderophore receptor and porin proteins (SRPs) (*Epitopix*®, Epitopix LLC, Wilmar, MN, USA).[126]

Econiche® (Bioniche Life Sciences Inc., Belleville, Ontario, Canada) was announced as the world's first vaccine designed to reduce cattle contamination by EHEC. This vaccine, administered subcutaneously, was approved and fully licensed by the Canadian Food Inspection Agency (CFIA) in 2008. The vaccine has the potential to significantly reduce the amount of *E. coli* O157:H7 released into the environment by cattle and, therefore, to reduce the risk to human health. Vaccines for bovine immunization are based on TTSS secreted proteins. Several articles summarize clinical vaccine trials including different number of animals. In 2009, Smith et al.[169] described a large-scale clinical vaccine trial to test the efficacy of a 2-dose regimen of *Econiche*® against TTSS secreted proteins of *Escherichia coli* O157:H7 and its effect on the detection of the same organism colonizing the terminal rectal mucosa (TRM) of cattle. Several studies concluded that vaccination with TTSS-secreted proteins showed efficacy in reducing STEC O157 detection in cattle feces and in hiding contamination probably associated with lower TRM colonization.[112,148,169,170]

On the other hand, ***Epitopix***® (Epitopix LLC, Willmar Poultry Company (WPC), Minnesota, USA), was the first vaccine against *E. coli* O157 licensed for use in beef cattle in the USA. This vaccine was developed by veterinary researchers at Kansas State University in collaboration with Epitopix LLC. *Escherichia coli Bacterial Extract* became the first federally licensed vaccine against *E. coli* O157 in February 2009, and the current USDA license is conditional; at this time sales are limited to licensed veterinarians. Also intended for bovine immunization, this vaccine is administered subcutaneously and uses an alternative therapeutic target, which triggers the generation of antibodies against bacterial wall antigens, called SRPs, of O157:H7. As a consequence, the capture of iron is blocked, inducing a disadvantageous competitive state of O157:H7 compared to other bacteria that normally colonize the bovine intestine.[171] Several research trials conducted to quantify the efficacy of SRP protein-based vaccines in feedlot cattle, demonstrate that the prevalence of *E. coli* O157 was lower, with a significant reduction of *E. coli* O157 concentration in fecal samples (98.2%). Furthermore, the number of days that cattle were culture positive for *E. coli* O157 and that they were identified as high shedders, was reduced. Finally, the results showed that the timing of vaccination in calves and the dose regimen of the SRP vaccine reduced the prevalence of *E. coli* O157:H7.[171-174]

Three systematic reviews[124-126] have analyzed the available literature on trials performed in domestic ruminants infected naturally or artificially, and have concluded that both *Econiche*® and *Epitopix*® significantly decrease the colonization and stool load of O157:H7. However, these vaccines do not eliminate O157:H7 completely, and it is not known if they generate cross-protection against other STEC serogroups that represent a highest risk of severe illness and even death in young children and the elderly (O26, O103, O45, O111, O121 and O145; named "top 6").[175]

Vaccines in early stages of clinical development for humans

O157-rEPA vaccine

The most promising vaccine candidate for humans to-date is based on the covalent *E. coli* O157:H7 O-specific polysaccharide conjugated to recombinant exotoxin A of *Pseudomonas aeruginosa* (O157-rEPA). A 1997 phase I trial in adults showed that this formulation was safe and immunogenic.[176] A phase II trial was conducted in 49 children 2-5 years of age, divided randomly into groups receiving 1 or 2 doses of the vaccine.[177] One week after the first dose, most of the children (81%) showed a > 4-fold increase in IgG-LPS antibody titers in serum, and 6 weeks after the first dose, all children responded with a > 8-fold increase in antibody titers. A second dose did not elicit a booster response. Twenty-six weeks after the first dose, the geometric mean titer of IgG-LPS antibodies in serum was approximately 20 times the titer observed prior to vaccination. High titers of bactericidal activity correlated with the titers of IgG-LPS antibodies in serum ($r = 0.78$). No significant adverse reactions were observed, leading to the conclusion that the O157-rEPA vaccine was safe and immunogenic in young children. To our knowledge phase III trials were planned but have not been initiated.

It must be emphasized that the development of candidate vaccines has been limited to a reduced number of antigens, focusing solely on O157:H7. Consequently, these candidates provide incomplete coverage against STEC strains, especially against strains that lack LEE, that cause illness in humans. Thus it is necessary to continue the search for antigens and immunogenic proteins that may improve existing vaccines, or to develop new vaccines that provide complete protection against this pathogen. Recently, García-Angulo et al.,[178] using comparative genomics and immunoinformatic analysis, identified 25 DNA sequences coded in the genome of O157:H7, which are absent in the commensal *E. coli* strains K12 and *E. coli* HS. Evaluation of these proteins in a mouse model suggested that they are promising antigens for vaccine development.

Our own group has used an immunoproteomic focus to identify antigens of the outer membrane present in the strains STEC O26:H11, O103, O113:H21 and O157:H7. We have identified 7 immunoreactive antigens to serum from patients with HUS. These antigens are absent in *E. coli* HS, and their immunogenic profile suggests that they are potential candidates for the development of a vaccine with broad strain coverage.[179] The next steps include evaluation of these candidates in mouse models.

Campylobacter jejuni

Pathogen and disease overview

Campylobacter jejuni, a spiral-shaped Gram-negative bacteria, is currently one of the most common causes of foodborne enteric disease in industrialized countries.[180] *C. jejuni* infections are estimated to occur in 1.3 million Americans (USA) and 190,000 Europeans per year.[181,182] *C. jejuni* was recently identified as an important cause of moderate-to-

severe diarrhea in children from resource-deprived countries, specifically in western Asia (Bangladesh, India and Pakistan).[4] Additionally, it is the second leading cause of travelers' diarrhea, after ETEC. *C. jejuni* infection can potentially lead to Guillian-Barre syndrome, an auto-immune neurodegenerative disease characterized by damage to the myelin sheath surrounding nerves, with the consequent loss of signal transmission.[183] Poultry are the main animal reservoir for *C. jejuni*, and the majority of infections are caused by consumption of chicken meat and derived products.[184]

C. jejuni colonizes the lower digestive tract (jejunum, ileum and colon), and an infectious dose in humans of approximately 500 bacteria is believed to be enough to cause enteritis.[184] Colonization is facilitated by a set of adhesins and by the flagellum, whereas the only toxin that has been identified to date is the cytolethal distending toxin (CdtB).[180] Following attachment to the mucosa, the bacteria can invade and damage the epithelial barrier, leading in some cases to bloody diarrhea.

Controlling *C. jejuni* colonization in chickens is believed to be the best strategy for reducing illness rates in humans. *C. jejuni* colonizes a bird's digestive tract during the first weeks of life, and appears to become part of the commensal microbiota within the intestinal lumen, but it does not attach to or penetrate the epithelial barriers.[184] Bacteria are constantly shed in chicken feces, therefore it is easily spread between birds within a poultry yard. Thus, isolation of colonized birds, improvements in hygienic conditions and elimination of *C. jejuni* by competitive exclusion (bacterial interference or bacterial antagonism) are some of the possible strategies to avoid transmission to humans. However, as these steps have not yet proven effective, development of a safe and effective vaccine continues to be an important goal. Currently, there are no licensed vaccines to prevent illness caused by *C. jejuni* and all current candidates are in the preclinical stages of development.

Vaccine candidates in preclinical stages of development mostly for animal use

Natural proinflammatory immune responses induced by *C. jejuni* occur in chickens,[185] and colonization could be prevented by inducing a protective immune response during the first days after hatching or by *in ovo* vaccination.[184] Examples of candidate formulations evaluated for prevention of *C. jejuni* colonization in chicks, in addition to a candidate tested in primates, will be discussed briefly.

Inactivated C. jejuni

Heat and formalin-inactivated strains administered orally to 2-day-old chicks have reduced the number of *C. jejuni* in the digestive tract of birds by about 1 $\log_{10}$, results that could certainly be improved if shedding and spreading could be avoided. Inclusion of known adjuvants, such as ETEC labile toxin (LT), did not improve antibody production or increase protection against colonization.[186]

In ovo injection of whole-cell heat-inactivated *C. jejuni* has been reported.[187] Injection of 10^8 bacteria into amniotic fluid was performed on day 16 of incubation. Anti-*C. jejuni* flagellin specific IgA, IgM and IgG were detected in significantly higher concentrations in vaccinated compared to non-vaccinated 14-day-old chicks. A booster dose given 7 d post-hatching did not improve results. This strategy seems promising and if proven effective could become a feasible mode of massive and automated vaccine administration in hatcheries, similar to strategies currently in place to prevent transmission of viral illnesses such as infectious bursal disease, Marek's disease, and Newcastle disease.[184]

Flagellin and capsule

As for most bacterial pathogens, flagellin and capsular polysaccharides are 2 of the most immunogenic structures of *C. jejuni*.[188] Administration of 2 doses of flagellin conjugated to the ETEC LT-B subunit demonstrated a moderate capacity to protect chickens against subsequent challenge.[189] Forty of the 145 vaccinated chicks (27.6%) were positive for *C. jejuni* vs. 70 of 142 in the control group (49.3%). As a higher protective efficacy is necessary, the inclusion of other types of adjuvants should be evaluated. In addition, an analysis to identify the flagellin epitopes recognized by the induced anti-flagellin antibodies has been suggested, as they seem to recognize non-surface exposed or hypervariable regions.

It has been demonstrated that capsular polysaccharides confer protection in a non-human primate model, *Aotus nancymaae* (also known as the new world monkey), when administered chemically conjugated to CRM_{197}, using alum as adjuvant.[190] The monkeys were orally challenged with *C. jejuni* in a single dose containing 3×10^9 CFU; diarrhea occurred in 7 of 10 unvaccinated monkeys and in none of the 14 vaccinees. These results are promising, as it might be possible to use this type of formulation to develop a vaccine suitable for human use. In order to develop such a vaccine, determination of the most frequent capsular polysaccharides carried by *C. jejuni* strains from different geographical regions would be an important first step.

Attenuated Salmonella carrying CjaA

To our knowledge, this is the most promising vaccine formulation against *C. jejuni* for use in chicks reported to date. CjaA was identified as one of the most immunogenic antigens within genomic libraries based on 3 *C. jejuni* strains. It is an N-glycosylated inner membrane protein, part of an ABC cysteine transport system[191] detected in a high proportion of clinical *Campylobacter* isolates.[192] A previously characterized, attenuated *Salmonella enterica* serovar *Typhimurium* χ3987 strain was selected to carry the antigen, which was administered orally in 2 doses of 10^9 CFU, 2 weeks apart, to 1-day-old chicks.[193] After challenge with 2×10^8 CFU of a *C. jejuni* strain, a 6-$\log_{10}$ reduction in bacterial counts recovered from fecal contents was observed in vaccinated compared to non-vaccinated chicks. Other vectors expressing CjaA, specifically attenuated *Salmonella enterica* serovar *Typhimurium* Δ*aroA* and an attenuated parasite *Eimera tenella*, did not show the same efficacy in preventing colonization, with a 1-$\log_{10}$ reduction detected for both formulations.[194,195]

Conclusions

In this 2 part series we have reviewed the full spectrum of vaccine development against viral and bacterial pathogens causing acute gastroenteritis. The main challenges are to increase vaccine usage in endemic areas, accepting that these protective efficacy rates are important in reducing cholera morbidity and mortality, and advancing from the current concept of considering vaccination only for travelers to endemic areas or for outbreak control. Although research efforts to produce improved vaccines are quite intense, at this time a new more effective vaccine is unlikely in the near future. Vaccines for *Shigella*, one of the "big 5" causes of childhood diarrhea and mortality, have proven a monumental challenge. The main hurdle has been the strain specificity of the various antigens under evaluation, and the significant number of candidates currently being evaluated reflects the lack of success in advancing a broad-spectrum, efficacious vaccine. The search for more broadly immunogenic antigens that may provide protection against epidemiologically significant *S. flexnerii* and *S. sonnei* strains continues. A vaccine for ETEC is highly desirable, as it is the main bacterial cause of childhood watery diarrhea, but the difficulty in obtaining a highly-immunogenic broadly-reactive antigen has been unsuccessful to date. New generation vaccines, combining different strategies such as killed strains, live mutated strains and LT-B in one formulation, may be the way to achieve this goal in the future. For *Salmonella*, EHEC and *Campylobacter*, vaccines for human use will not be available for some time. The alternative strategy of vaccinating animals, cattle in the case of EHEC and chicken in the case of *Campylobacter*, to indirectly decrease transmission to humans is an attractive approach and is providing promising preliminary results. The epidemiological impact of this strategy in reducing disease at the community-level will require large field trials monitoring animals for pathogen transmission and humans for disease occurrence.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest are disclosed.

Acknowledgments

We thank Anne J. Lagomarcino for her invaluable support in manuscript writing and editing.

Funding

The authors were supported by Chilean Fondecyt grants 1110260 (RV), 3130555 (FdC), and 1130561 (MO).

References

1. Hale TL. Genetic basis of virulence in *Shigella* species. Microbiol Rev 1991; 55:206-24; PMID:1886518.
2. Shiga K. The trend of prevention, therapy and epidemiology of dysentery since the discovery of its causative organism. New Engl J Med 1936; 215:1205-11; http://dx.doi.org/10.1056/NEJM193612242152602
3. Kotloff KL, Winickoff JP, Ivanoff B, Clemens JD, Swerdlow DL, Sansonetti PJ, Adak GK, Levine MM. Global burden of *Shigella* infections: implications for vaccine development and implementation of control strategies. Bull World Health Organ 1999; 77: 651-66; PMID:10516787
4. 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; 382:209-22; PMID:23680352; http://dx.doi.org/10.1016/S0140-6736(13)60844-2
5. Schroeder GN, Hilbi H. Molecular pathogenesis of *Shigella* spp.: controlling host cell signaling, invasion, and death by type III secretion. Clin Microbiol Rev 2008; 21:134-56; PMID:18202440; http://dx.doi.org/10.1128/CMR.00032-07
6. Parsot C. *Shigella* spp. and enteroinvasive *Escherichia coli* pathogenicity factors. FEMS Microbiol Lett 2005; 252:11-8; PMID:16182469; http://dx.doi.org/10.1016/j.femsle.2005.08.046
7. Bhattacharya D, Bhattacharya H, Sayi DS, Bharadwaj AP, Singhania M, Sugunan AP, Roy S. Changing patterns and widening of antibiotic resistance in *Shigella spp*. over a decade (2000-2011), Andaman Islands, India. Epidemiol Infect 2014:1-8; PMID:24763083; http://dx.doi.org/10.1017/S0950268814000958
8. Gu B, Ke X, Pan S, Cao Y, Zhuang L, Yu R, Qian H, Liu G, Tong M. Prevalence and trends of aminoglycoside resistance in *Shigella* worldwide, 1999-2010. J Biomed Res 2013; 27:103-15; PMID:23554801; http://dx.doi.org/10.7555/JBR.27.20120125
9. Kosek M, Yori PP, Olortegui MP. Shigellosis update: advancing antibiotic resistance, investment empowered vaccine development, and green bananas. Curr Opin Infect Dis 2010; 23:475-80; PMID:20689423; http://dx.doi.org/10.1097/QCO.0b013e32833da204
10. Marcoleta A, Toro C, Prado V, Serrano M, Fernandez P, Benadof D, Camponovo R, Campos V, Porte L, Zamorano J, et al. Antibiotic susceptibility patterns among *Shigella sonnei*, isolated during three different periods in Region Metropolitana, Chile. Rev Chilena Infectol 2013; 30:616-21; PMID:24522304; http://dx.doi.org/10.4067/S0716-10182013000600007
11. DuPont HL, Levine MM, Hornick RB, Formal SB. Inoculum size in shigellosis and implications for expected mode of transmission. J Infect Dis 1989; 159:1126-8; PMID:2656880; http://dx.doi.org/10.1093/infdis/159.6.1126
12. Kim YJ, Yeo SG, Park JH, Ko HJ. *Shigella* vaccine development: prospective animal models and current status. Curr Pharm Biotechnol 2014; 14:903-12; PMID:24372251; http://dx.doi.org/10.2174/1389201014666131226123900
13. Barman S, Saha DR, Ramamurthy T, Koley H. Development of a new guinea-pig model of shigellosis. FEMS Immunol Med Microbiol 2011; 62:304-14; PMID:21539623; http://dx.doi.org/10.1111/j.1574-695X.2011.00810.x
14. Islam D, Ruamsap N, Khantapura P, Aksomboon A, Srijan A, Wongstitwilairoong B, Bodhidatta L, Gettayacamin M, Venkatesan MM, Mason CJ. Evaluation of an intragastric challenge model for *Shigella dysenteriae* 1 in rhesus monkeys (*Macaca mulatta*) for the pre-clinical assessment of *Shigella* vaccine formulations. APMIS 2014; 122:463-75; PMID:24028276; http://dx.doi.org/10.1111/apm.12168
15. Gregory M KR, Lugo-Roman LA, Galvez Carrillo H, Tilley DH, Baldeviano C, Simons MP, Reynolds ND, Ranallo RT, Suvarnapunya AE, Venkatesan MM, et al. Development of an *Aotus nancymaae* model for *Shigella* Vaccine immunogenicity and efficacy studies. Infect Immun 2014; 82:2027-36; PMID:24595138; http://dx.doi.org/10.1128/IAI.01665-13
16. Yang JY, Lee SN, Chang SY, Ko HJ, Ryu S, Kweon MN. A mouse model of shigellosis by intraperitoneal infection. J Infect Dis 2014; 209:203-15; PMID:23904297; http://dx.doi.org/10.1093/infdis/jit399
17. Taylor DN, Trofa AC, Sadoff J, Chu C, Bryla D, Shiloach J, Cohen D, Ashkenazi S, Lerman Y, Egan W. Synthesis, characterization, and clinical evaluation of conjugate vaccines composed of the O-specific polysaccharides of *Shigella dysenteriae* type 1, *Shigella flexneri* type 2a, and *Shigella sonnei* (*Plesiomonas shigelloides*) bound to bacterial toxoids. Infect Immun 1993; 61:3678-87; PMID:8359890
18. Ashkenazi S, Passwell JH, Harlev E, Miron D, Dagan R, Farzan N, Ramon R, Majadly F, Bryla DA, Karpas AB, et al. Safety and immunogenicity of *Shigella sonnei* and *Shigella flexneri* 2a O-specific polysaccharide conjugates in children. J Infect Dis 1999; 179:1565-8; PMID:10228084; http://dx.doi.org/10.1086/314759
19. Passwell JH, Ashkenazi S, Harlev E, Miron D, Ramon R, Farzam N, Lerner-Geva L, Levi Y, Chu C, Shiloach J, et al. Israel *Shigella* Study G. Safety and immunogenicity of *Shigella sonnei*-CRM_9 and *Shigella flexneri* type 2a-$rEPA_{succ}$ conjugate vaccines in one- to four-year-old children. Pediatr Infect Dis J 2003; 22:701-6; PMID:12913770; http://dx.doi.org/10.1097/01.inf.0000078156.03697.a5
20. Passwell J, Harlev E, Ashkenazi S, Chu C, Miron D, Ramon R, Farzan N, Shiloach J, Bryla D, Majadly F, et al. Safety and immunogenicity of improved *Shigella* O-specific polysaccharide-protein conjugate vaccines in adults in Israel. Infect Immun 2001; 69:1351-7; PMID:11179298; http://dx.doi.org/10.1128/IAI.69.3.1351-1357.2001
21. Chowers Y, Kirschner J, Keller N, Barshack I, Bar-Meir S, Ashkenazi S, Schneerson R, Robbins J, Passwell JH. O-specific polysaccharide conjugate vaccine-induced antibodies prevent invasion of *Shigella* into

Caco-2 cells and may be curative. Proc Natl Acad Sci USA 2007; 104:2396-401; PMID:17287349
22. Passwell JH, Ashkenzi S, Banet-Levi Y, Ramon-Saraf R, Farzam N, Lerner-Geva L, Even-Nir H, Yerushalmi B, Chu C, Shiloach J, et al. Age-related efficacy of *Shigella* O-specific polysaccharide conjugates in 1-4-year-old Israeli children. Vaccine 2010; 28:2231-5; PMID:20056180; http://dx.doi.org/10.1016/j.vaccine.2009.12.050
23. Barzu S, Fontaine A, Sansonetti P, Phalipon A. Induction of a local anti-IpaC antibody response in mice by use of a *Shigella flexneri* 2a vaccine candidate: implications for use of IpaC as a protein carrier. Infect Immun 1996; 64:1190-6; PMID:8606077
24. Coster TS, Hoge CW, VanDeVerg LL, Hartman AB, Oaks EV, Venkatesan MM, Cohen D, Robin G, Fontaine-Thompson A, Sansonetti PJ, et al. Vaccination against shigellosis with attenuated *Shigella flexneri* 2a strain SC602. Infect Immun 1999; 67:3437-43; PMID:10377124
25. Katz DE, Coster TS, Wolf MK, Trespalacios FC, Cohen D, Robins G, Hartman AB, Venkatesan MM, Taylor DN, Hale TL. Two studies evaluating the safety and immunogenicity of a live, attenuated *Shigella flexneri* 2a vaccine (SC602) and excretion of vaccine organisms in North American volunteers. Infect Immun 2004; 72:923-30; PMID:14742537; http://dx.doi.org/10.1128/IAI.72.2.923-930.2004
26. Rahman KM, Arifeen SE, Zaman K, Rahman M, Raqib R, Yunus M, Begum N, Islam MS, Sohel BM, Rahman M, et al. Safety, dose, immunogenicity, and transmissibility of an oral live attenuated *Shigella flexneri* 2a vaccine candidate (SC602) among healthy adults and school children in Matlab, Bangladesh. Vaccine 2011; 29:1347-54; PMID:21040694; http://dx.doi.org/10.1016/j.vaccine.2010.10.035
27. Launay O, Sadorge C, Jolly N, Poirier B, Bechet S, Van der Vliet D, Seffer V, Fenner N, Dowling K, Giemza R, et al. Safety and immunogenicity of SC599, an oral live attenuated *Shigella dysenteriae* type-1 vaccine in healthy volunteers: results of a Phase 2, randomized, double-blind placebo-controlled trial. Vaccine 2009; 27:1184-91; PMID:19135496; http://dx.doi.org/10.1016/j.vaccine.2008.12.021
28. Sadorge C, Ndiaye A, Beveridge N, Frazer S, Giemza R, Jolly N, Johnson J, Liddy H, Cosgrove CA, Allavena P, et al. Phase 1 clinical trial of live attenuated *Shigella dysenteriae* type-1 D*icsA* D*ent* D*fep* D*stxA*: HgR oral vaccine SC599 in healthy human adult volunteers. Vaccine 2008; 26:978-87; PMID:18207287; http://dx.doi.org/10.1016/j.vaccine.2007.11.024
29. Hartman AB, Venkatesan MM. Construction of a stable attenuated *Shigella sonnei* D*virG* vaccine strain, WRSS1, and protective efficacy and immunogenicity in the guinea pig keratoconjunctivitis model. Infect Immun 1998; 66:4572-6; PMID:9712824
30. Kotloff KLTD, Sztein MB, Wasserman SS, Losonsky GA, Nataro JP, Venkatesan M, Hartman A, Picking WD, Katz DE, Campbell JD, et al. Phase I evaluation of delta virG *Shigella sonnei* live, attenuated, oral vaccine strain WRSS1 in healthy adults. Infect Immun 2002; 70:2016-21; PMID:11895966; http://dx.doi.org/10.1128/IAI.70.4.2016-2021.2002
31. Orr N, Katz D, Atsmon J, Radu P, Yavzori M, Halperin T, Sela T, Kayouf R, Klein Z, Ambar R, et al. Community-based safety, immunogenicity, and transmissibility study of the *Shigella sonnei* WRSS1 vaccine in Israeli volunteers. Infect Immun 2005; 73:8027-32; PMID:16299296; http://dx.doi.org/10.1128/IAI.73.12.8027-8032.2005
32. Venkatesan M. Construction, characterization, and animal testing of WRSd1, a *Shigella dysenteriae* 1 vaccine. Infect Immun 2002; 70:2950-8; PMID:12010984; http://dx.doi.org/10.1128/IAI.70.6.2950-2958.2002
33. McKenzie R, Venkatesan M, Wolf M, Islam D, Grahek S, Jones A, Bloom A, Taylor D, Hale T, Bourgeois A. Safety and immunogenicity of WRSd1, a live attenuated *Shigella dysenteriae* type 1 vaccine candidate. Vaccine 2008; 26:3291-6; PMID:18468742; http://dx.doi.org/10.1016/j.vaccine.2008.03.079
34. Chu C, Liu B, Watson D, Szu S, Bryla D, Shiloach J, Schneerson R, Robbins JB. Preparation, characterization, and immunogenicity of conjugates composed of the O-specific polysaccharide of *Shigella dysenteriae* type 1 (Shiga's bacillus) bound to tetanus toxoid. Infect Immun 1991; 59:4450-8; PMID:1937803
35. Pozsgay V. Recent developments in synthetic oligosaccharide-based bacterial vaccines. Curr Top Med Chem 2008; 8:126-40; PMID:18289082; http://dx.doi.org/10.2174/156802608783378864
36. Phalipon A, Costachel C, Grandjean C, Thuizat A, Guerreiro C, Tanguy M, Nato F, Vulliez-Le Normand B, Bélot F, Wright K, et al. Characterization of functional oligosaccharide mimics of the *Shigella flexneri* serotype 2a O-antigen: Implications for the development of a chemically defined glycoconjugate vaccine. J Immunol 2006; 176:1686-94; PMID:16424198; http://dx.doi.org/10.4049/jimmunol.176.3.1686
37. Phalipon A, Tanguy M, Grandjean C, Guerreiro C, Bélot F, Cohen D, Sansonetti P, Mulard L. A synthetic carbohydrate-protein conjugate vaccine candidate against *Shigella flexneri* 2a infection. J Immunol 2009; 182:2241-7; PMID:19201878; http://dx.doi.org/10.4049/jimmunol.0803141
38. Pozsgay V, Chu C, Pannell L, Wolfe J, Robbins JB, Schneerson R. Protein conjugates of synthetic saccharides elicit higher levels of serum IgG lipopolysaccharide antibodies in mice than do those of the O-specific polysaccharide from *Shigella dysenteriae* type 1. Proc Natl Acad Sci USA 1999; 96:5194-7; PMID:10220442
39. Robbins J, Kubler-Kielb J, Vinogradov E, Mocca C, Pozsgay V, Shiloach J, Schneerson R. Synthesis, characterization, and immunogenicity in mice of *Shigella sonnei* O-specific oligosaccharide-core-protein conjugates. Proc Natl Acad Sci USA 2009; 106:7974-8; PMID:19346477; http://dx.doi.org/10.1073/pnas.0900891106
40. Turbyfill K, Hartman A, Oaks E. Isolation and characterization of a *Shigella flexneri* invasin complex subunit vaccine. Infect Immun 2000; 68:6624-32; PMID:11083774; http://dx.doi.org/10.1128/IAI.68.12.6624-6632.2000
41. Oaks E, Turbyfill K. Development and evaluation of a *Shigella flexneri* 2a and *S. sonnei* bivalent invasin complex (Invaplex) vaccine. Vaccine 2006; 24:2290-301; PMID:16364513; http://dx.doi.org/10.1016/j.vaccine.2005.11.040
42. Riddle M, Kaminski R, Williams C, Porter C, Baqar S, Kordis A, Gilliland T, Lapa J, Coughlin M, Soltis C, et al. Safety and immunogenicity of an intranasal *Shigella flexneri* 2a Invaplex 50 vaccine. Vaccine 2011; 29:7009-19; PMID:21787825; http://dx.doi.org/10.1016/j.vaccine.2011.07.033
43. Tribble D, Kaminski R, Cantrell J, Nelson M, Porter C, Baqar S, Williams C, Arora R, Saunders J, Anantha krishnan M, et al. Safety and immunogenicity of a *Shigella flexneri* 2a Invaplex 50 intranasal vaccine in adult volunteers. Vaccine 2010; 28:6076-85; PMID:20619378; http://dx.doi.org/10.1016/j.vaccine.2010.06.086
44. Heine SJ, Diaz-McNair J, Andar AU, Drachenberg CB, Van De Verg LL, Walker R, Picking WL, Pasetti MF. Intradermal delivery of *Shigella* IpaB and IpaD type III secretion proteins: kinetics of cell recruitment and antigen uptake, mucosal and systemic immunity, and protection across serotypes. J Immunol 2014; 192:1630-40; PMID:24453241; http://dx.doi.org/10.4049/jimmunol.1302743
45. Martinez-Becerra FJ, Chen X, Dickenson NE, Choudhari SP, Harrison K, Clements JD, Picking WD, Van De Verg LL, Walker RI, Picking WL. Characterization of a novel fusion protein from IpaB and IpaD of *Shigella* spp. and its potential as a pan-*Shigella* vaccine. Infect Immun 2013; 81:4470-7; PMID:24060976; http://dx.doi.org/10.1128/IAI.00859-13
46. McKenzie R, Walker RI, Nabors GS, Van De Verg LL, Carpenter C, Gomes G, Forbes E, Tian JH, Yang HH, Pace JL, et al. Safety and immunogenicity of an oral, inactivated, whole-cell vaccine for *Shigella sonnei*: preclinical studies and a Phase I trial. Vaccine 2006; 24:3735-45; PMID:16095766; http://dx.doi.org/10.1016/j.vaccine.2005.07.014
47. Kaminski RW, Wu M, Turbyfill KR, Clarkson K, Tai B, Bourgeois AL, Van De Verg LL, Walker RI, Oaks EV. Development and preclinical evaluation of a trivalent, formalin-inactivated *Shigella* whole-cell vaccine. Clin Vaccine Immunol 2014; 21:366-82; PMID:24403527; http://dx.doi.org/10.1128/CVI.00683-13
48. Barman S, Koley H, Nag D, Shinoda S, Nair GB, Takeda Y. Passive immunity with multi-serotype heat-killed *Shigella*e in neonatal mice. Microbiol Immunol 2014; 58:463-6; PMID:24909404; http://dx.doi.org/10.1111/1348-0421.12164
49. Ranallo RT, Thakkar S, Chen Q, Venkatesan MM. Immunogenicity and characterization of WRSF2G11: a second generation live attenuated *Shigella flexneri* 2a vaccine strain. Vaccine 2007; 25:2269-78; PMID:17229494; http://dx.doi.org/10.1016/j.vaccine.2006.11.067
50. D'Hauteville H, Khan S, Maskell DJ, Kussak A, Weintraub A, Mathison J, Ulevitch RJ, Wuscher N, Parsot C, Sansonetti PJ. Two *msbB* genes encoding maximal acylation of lipid A are required for invasive *Shigella flexneri* to mediate inflammatory rupture and destruction of the intestinal epithelium. J Immunol 2002; 168:5240-51; PMID:11994481; http://dx.doi.org/10.4049/jimmunol.168.10.5240
51. Ranallo R, Fonseka S, Boren T, Bedford L, Kaminski R, Thakkar S, Venkatesan M. Two live attenuated *Shigella flexneri* 2a strains WRSf2G12 and WRSf2G15: a new combination of gene deletions for 2nd generation live attenuated vaccine candidates. Vaccine 2012; 30:5159-71; PMID:22658966; http://dx.doi.org/10.1016/j.vaccine.2012.05.003
52. Ranallo RT, Kaminski R, Baqar S, Dutta M, Lugo-Roman LA, Boren T, Barnoy S, Venkatesan MM. Oral administration of live *Shigella* vaccine candidates in rhesus monkeys show no evidence of competition for colonization and immunogenicity between different serotypes. Vaccine 2014; 32:1754-60; PMID:24522159; http://dx.doi.org/10.1016/j.vaccine.2013.12.068
53. Barnoy S, Jeong KI, Helm RF, Suvarnapunya AE, Ranallo RT, Tzipori S, Venkatesan MM. Characterization of WRSs2 and WRSs3, new second-generation *virG* (*icsA*)-based *Shigella sonnei* vaccine candidates with the potential for reduced reactogenicity. Vaccine 2010; 28:1642-54; PMID:19932216; http://dx.doi.org/10.1016/j.vaccine.2009.11.001
54. Barnoy S, Baqar S, Kaminski RW, Collins T, Nemelka K, Hale TL, Ranallo RT, Venkatesan MM. *Shigella sonnei* vaccine candidates WRSs2 and WRSs3 are as immunogenic as WRSS1, a clinically tested vaccine candidate, in a primate model of infection. Vaccine 2011; 29:6371-8; PMID:21596086; http://dx.doi.org/10.1016/j.vaccine.2011.04.115
55. Acevedo R, Fernandez S, Zayas C, Acosta A, Sarmiento ME, Ferro VA, Rosenqvist E, Campa C, Cardoso D, Garcia L, et al. Bacterial outer membrane vesicles and vaccine applications. Front Immunol 2014; 5:121; PMID:24715891; http://dx.doi.org/10.3389/fimmu.2014.00121
56. Camacho AI, Souza-Rebouças J, Irache JM, Gamazo C. Towards a non-living vaccine against *Shigella flexneri*: from the inactivation procedure to protection studies. Methods 2013; 60:264-8; PMID:23046911; http://dx.doi.org/10.1016/j.ymeth.2012.09.008

57. Brenner FW, Villar RG, Angulo FJ, Tauxe R, Swaminathan B. *Salmonella* nomenclature. J Clin Microbiol 2000; 38:2465-7; PMID:10878026
58. Canals R, McClelland M, Santiviago C, Andrews-Polymenis H. Genomics of *Salmonella* species. In: Wiedmann M, Zhang W, eds. Genomics of Foodborne Bacterial Pathogens, New York: Springer, 2011, 171-235.
59. MacLennan CA, Martin LB, Micoli F. Vaccines against invasive *Salmonella* disease. Hum Vaccin Immunother 2014; 10:1-16; PMID:24832715; http://dx.doi.org/10.4161/hv.29054
60. Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, O'Brien SJ, Jones TF, Fazil A, Hoekstra RM. The global burden of nontyphoidal *Salmonella* gastroenteritis. Clin Infect Dis 2010; 50:882-9; PMID:20158401; http://dx.doi.org/10.1086/650733
61. Christenson JC. *Salmonella* infections. Pediatr Rev 2013; 34:375-83; PMID:24000341; http://dx.doi.org/10.1542/pir.34-9-375
62. Santos RL. Pathobiology of salmonella, intestinal microbiota, and the host innate immune response. Front Immunol 2014; 5:252-; PMID:24904595; http://dx.doi.org/10.3389/fimmu.2014.00252
63. Hindle ZCS, Phillimore J, Bentley M, Johnson J, Cosgrove CA, Ghaem-Maghami M, Sexton A, Khan M, Brennan FR, Everest P, et al. Characterization of *Salmonella enterica* derivatives harboring defined *aroC* and *Salmonella* pathogenicity island 2 type III secretion system (*ssaV*) mutations by immunization of healthy volunteers. Infect Immun 2002; 70:3457-67; PMID:12065485; http://dx.doi.org/10.1128/IAI.70.7.3457-3467.2002
64. Simon R, Levine M. Glycoconjugate vaccine strategies for protection against invasive *Salmonella* infections. Hum Vaccines Immunother 2012; 8:494-8; PMID:22370510; http://dx.doi.org/10.4161/hv.19158
65. Watson DC, Robbins JB, Szu SC. Protection of mice against *Salmonella typhimurium* with an O-specific polysaccharide-protein conjugate vaccine. Infect Immun 1992; 60:4679-86; PMID:1383154
66. Svenson SB, Nurminen M, Lindberg AC. Artificial *Salmonella* vaccines: O-antigenic oligosaccharide-protein conjugates induce protection against infection with *Salmonella typhimurium*. Infect Immun 1979; 25:863-72; PMID:387597
67. Svenson SB, Lindberg AC. Artificial *Salmonella* vaccines: *Salmonella typhimurium* O-antigen-specific oligosaccharide-protein conjugates elicit protective antibodies in rabbits and mice. Infect Immun 1981; 32:490-6; PMID:6166555
68. Stefanetti G, Rondini S, Lanzilao L, Saul A, MacLennan C, Micoli F. Impact of conjugation chemistry on the immunogenicity of *S.* Typhimurium conjugate vaccines. Vaccine 2014; 32:6122-9; PMID:25192974; http://dx.doi.org/10.1016/j.vaccine.2014.08.056
69. Simon R, Tennant SM, Wang JY, Schmidlein PJ, Lees A, Ernst RK, Pasetti MF, Galen JE, Levine MM. *Salmonella enterica* serovar Enteritidis core O polysaccharide conjugated to H:g,m flagellin as a candidate vaccine for protection against invasive infection with *S.* Enteritidis. Infect Immun 2011; 79:4240-9; PMID:21807909; http://dx.doi.org/10.1128/IAI.05484-11
70. Simon R, Wang J, Boyd M, Tulapurkar M, Ramachandran G, Tennant S, Pasetti M, Galen J, Levine M. Sustained protection in mice immunized with fractional doses of *Salmonella* Enteritidis core and O polysaccharide-flagellin glycoconjugates. PLoS One 2013; 8:e64680; PMID:23741368; http://dx.doi.org/10.1371/journal.pone.0064680
71. Pasquali P, Ammendola S, Pistoia C, Petrucci P, Tarantino M, Valente C, Marenzoni M, Rotilio G, Battistoni A. Attenuated *Salmonella enterica* serovar Typhimurium lacking the ZnuABC transporter confers immune-based protection against challenge infections in mice. Vaccine 2008; 26:3421-6; PMID:18499306; http://dx.doi.org/10.1016/j.vaccine.2008.04.036
72. Pesciaroli M, Aloisio F, Ammendola S, Pistoia C, Petrucci P, Tarantino M, Francia M, Battistoni A, Pasquali P. An attenuated *Salmonella enterica* serovar Typhimurium strain lacking the ZnuABC transporter induces protection in a mouse intestinal model of *Salmonella* infection. Vaccine 2011; 29:1783-90; PMID:21219981; http://dx.doi.org/10.1016/j.vaccine.2010.12.111
73. Allam U, Krishna M, Lahiri A, Joy O, Chakravortty D. *Salmonella enterica* Serovar Typhimurium lacking *hfq* gene confers protective immunity against murine typhoid. PLoS One 2011; 6:e16667; PMID:21347426; http://dx.doi.org/10.1371/journal.pone.0016667
74. Vishwakarma V, Pati N, Chandel H, Sahoo S, Saha B, Suar M. Evaluation of *Salmonella enterica* serovar Typhimurium TTSS-2 deficient *fur* mutant as safe live-attenuated vaccine candidate for immunocompromised mice. PLoS One 2012; 7:e52043; PMID:23284865; http://dx.doi.org/10.1371/journal.pone.0052043
75. Tennant SM, Wang J-Y, Galen JE, Simon R, Pasetti MF, Gat O, Levine MM. Engineering and preclinical evaluation of attenuated nontyphoidal *Salmonella* strains serving as live oral vaccines and as reagent strains. Infect Immun 2011; 79:4175-85; PMID:21807911; http://dx.doi.org/10.1128/IAI.05278-11
76. Ochoa-Repáraz J, García B, Solano C, Lasa I, Irache J, Gamazo C. Protective ability of subcellular extracts from *Salmonella* Enteritidis and from a rough isogenic mutant against salmonellosis in mice. Vaccine 2005; 23:1491-501; PMID:15670885; http://dx.doi.org/10.1016/j.vaccine.2004.09.016
77. Jawale CV, Lee JH. Characterization of adaptive immune responses induced by a new genetically inactivated *Salmonella* Enteritidis vaccine. Comp Immunol Microbiol Infect Dis 2014; 37:159-67; PMID:24878070; http://dx.doi.org/10.1016/j.cimid.2014.05.001
78. Sanchez J, Holmgren J. Virulence factors, pathogenesis and vaccine protection in cholera and ETEC diarrhea. Curr Opin Immunol 2005; 17:388-98; PMID:15963708; http://dx.doi.org/10.1016/j.coi.2005.06.007
79. Croxen MA, Law RJ, Scholz R, Keeney KM, Wlodarska M, Finlay BB. Recent advances in understanding enteric pathogenic *Escherichia coli*. Clin Microbiol Rev 2013; 26:822-80; PMID:24092857; http://dx.doi.org/10.1128/CMR.00022-13
80. Gaastra W, Svennerholm AM. Colonization factors of human enterotoxigenic *Escherichia coli* (ETEC). Trends Microbiol 1996; 4:444-52; PMID:8950814; http://dx.doi.org/10.1016/0966-842X(96)10068-8
81. Isidean SD, Riddle MS, Savarino SJ, Porter CK. A systematic review of ETEC epidemiology focusing on colonization factor and toxin expression. Vaccine 2011; 29:6167-78; PMID:21723899; http://dx.doi.org/10.1016/j.vaccine.2011.06.084
82. Svennerholm AM, Lundgren A. Recent progress toward an enterotoxigenic *Escherichia coli* vaccine. Expert Rev Vaccines 2012; 11:495-507; PMID:22551034; http://dx.doi.org/10.1586/erv.12.12
83. Organization WH. Oral cholera vaccine stockpile for cholera emergency response. [Internet] 09/16/2013. Available from http://www.who.int/cholera/vaccines/Briefing_OCV_stockpile.pdf?ua=1
84. Clemens JD, Sack DA, Harris JR, Chakraborty J, Neogy PK, Stanton B, Huda N, Khan MU, Kay BA, Khan MR. Cross-protection by B subunit-whole cell cholera vaccine against diarrhea associated with heat-labile toxin-producing enterotoxigenic *Escherichia coli*: results of a large-scale field trial. J Infect Dis 1988; 158:372-7; PMID:3042876; http://dx.doi.org/10.1093/infdis/158.2.372
85. Peltola H, Siitonen A, Kyronseppa H, Simula I, Mattila L, Oksanen P, Kataja MJ, Cadoz M. Prevention of travellers' diarrhoea by oral B-subunit/whole-cell cholera vaccine. Lancet 1991; 338:1285-9; PMID:1682684; http://dx.doi.org/10.1016/0140-6736(91)92590-X
86. Scerpella EG, Sanchez JL, Mathewson, III, Torres-Cordero JV, Sadoff JC, Svennerholm AM, DuPont HL, Taylor DN, Ericsson CD. Safety, Immunogenicity, and Protective Efficacy of the Whole-Cell/Recombinant B Subunit (WC/rBS) Oral Cholera Vaccine Against Travelers' Diarrhea. J Travel Med 1995; 2:22-7; PMID:9815355; http://dx.doi.org/10.1111/j.1708-8305.1995.tb00615.x
87. Crucell. Dukoral. When to take Dukoral® for traveller's diarrhea [Internet] 2014 [Last Updated October 2014]. Available from http://www.dukoralcanada.com/when-to-take-dukoral
88. Ahmed T BT, Zaman K, Sinclair D, Qadri F. Vaccines for preventing enterotoxigenic *Escherichia coli* (ETEC) diarrhoea. Cochrane Database Syst Rev 2013; 7:CD009029; PMID:23828581
89. 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 2014; 14:197-204; PMID:24291168; http://dx.doi.org/10.1016/S1473-3099(13)70297-4
90. 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 2012; 19:1921-31; PMID:23035175; http://dx.doi.org/10.1128/CVI.00364-12
91. Otto W, Najnigier B, Stelmasiak T, Robins-Browne RM. Randomized control trials using a tablet formulation of hyperimmune bovine colostrum to prevent diarrhea caused by enterotoxigenic *Escherichia coli* in volunteers. Scand J Gastroenterol 2011; 46:862-8; PMID:21526980; http://dx.doi.org/10.3109/00365521.2011.574726
92. Jertborn M, Ahren C, Holmgren J, Svennerholm AM. Safety and immunogenicity of an oral inactivated enterotoxigenic *Escherichia coli* vaccine. Vaccine 1998; 16:255-60; PMID:9607039; http://dx.doi.org/10.1016/S0264-410X(97)00169-2
93. Qadri F, Wenneras C, Ahmed F, Asaduzzaman M, Saha D, Albert MJ, Sack RB, Svennerholm A. Safety and immunogenicity of an oral, inactivated enterotoxigenic *Escherichia coli* plus cholera toxin B subunit vaccine in Bangladeshi adults and children. Vaccine 2000; 18:2704-12; PMID:10781858; http://dx.doi.org/10.1016/S0264-410X(00)00056-6
94. Savarino SJ, Brown FM, Hall E, Bassily S, Youssef F, Wierzba T, Peruski L, El-Masry NA, Safwat M, Rao M, et al. Safety and immunogenicity of an oral, killed enterotoxigenic *Escherichia coli*-cholera toxin B subunit vaccine in Egyptian adults. J Infect Dis 1998; 177:796-9; PMID:9498468; http://dx.doi.org/10.1086/517812
95. Wiedermann G, Kollaritsch H, Kundi M, Svennerholm AM, Bjare U. Double-blind, randomized, placebo controlled pilot study evaluating efficacy and reactogenicity of an oral ETEC B-subunit-inactivated whole cell vaccine against travelers' diarrhea (preliminary report). J Travel Med 2000; 7:27-9; PMID:10689236; http://dx.doi.org/10.2310/7060.2000.00007
96. Sack DA, Shimko J, Torres O, Bourgeois AL, Francia DS, Gustafsson B, Karnell 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 2007; 25:4392-400; PMID:17448578; http://dx.doi.org/10.1016/j.vaccine.2007.03.034

97. Tobias J, Svennerholm A-M. Strategies to overexpress enterotoxigenic *Escherichia coli* (ETEC) colonization factors for the construction of oral whole-cell inactivated ETEC vaccine candidates. Appl Microbiol Biotechnol 2012; 93:2291-300; PMID:22350259; http://dx.doi.org/10.1007/s00253-012-3930-6
98. Lundgren A, Leach S, Tobias J, Carlin N, Gustafsson B, Jertborn M, Bourgeois L, Walker R, Holmgren J, Svennerholm AM. Clinical trial to evaluate safety and immunogenicity of an oral inactivated enterotoxigenic *Escherichia coli* prototype vaccine containing CFA/I overexpressing bacteria and recombinantly produced LTB/CTB hybrid protein. Vaccine 2013; 31:1163-70; PMID:23306362; http://dx.doi.org/10.1016/j.vaccine.2012.12.063
99. McKenzie R, Darsley M, Thomas N, Randall R, Carpenter C, Forbes E, Finucane M, Sack RB, Hall E, Bourgeois AL. A double-blind, placebo-controlled trial to evaluate the efficacy of PTL-003, an attenuated enterotoxigenic *E. coli* (ETEC) vaccine strain, in protecting against challenge with virulent ETEC. Vaccine 2008; 26:4731-9; PMID:18602960; http://dx.doi.org/10.1016/j.vaccine.2008.06.064
100. Lapa JA, Sincock SA, Ananthakrishnan M, Porter CK, Cassels FJ, Brinkley C, Hall ER, Van Hamont J, Gramling JD, Carpenter CM, et al. Randomized clinical trial assessing the safety and immunogenicity of oral microencapsulated enterotoxigenic *Escherichia coli* surface antigen 6 with or without heat-labile enterotoxin with mutation R192G. Clin Vaccine Immunol 2008; 15:1222-8; PMID:18579693; http://dx.doi.org/10.1128/CVI.00491-07
101. Guerena-Burgueño F, Hall ER, Taylor DN, Cassels FJ, Scott DA, Wolf MK, Roberts ZJ, Nesterova GV, Alving CR, Glenn GM. Safety and immunogenicity of a prototype enterotoxigenic *Escherichia coli* vaccine administered transcutaneously. Infect Immun 2002; 70:1874-80; PMID:Can't; http://dx.doi.org/10.1128/IAI.70.4.1874-1880.2002
102. El-Kamary SS, Cohen MB, Bourgeois AL, Van De Verg L, Bauers N, Reymann M, Pasetti MF, Chen WH. Safety and immunogenicity of a single oral dose of recombinant double mutant heat-labile toxin derived from enterotoxigenic *Escherichia coli*. Clin Vaccine Immunol 2013; 20:1764-70; PMID:24049109; http://dx.doi.org/10.1128/CVI.00464-13
103. Holmgren J, Bourgeois L, Carlin N, Clements J, Gustafsson B, Lundgren A, Nygren E, Tobias J, Walker R, Svennerholm AM. Development and preclinical evaluation of safety and immunogenicity of an oral ETEC vaccine containing inactivated *E. coli* bacteria overexpressing colonization factors CFA/I, CS3, CS5 and CS6 combined with a hybrid LT/CT B subunit antigen, administered alone and together with dmLT adjuvant. Vaccine 2013; 31:2457-64; PMID:23541621; http://dx.doi.org/10.1016/j.vaccine.2013.03.027
104. Barry EM, Wang J, Wu T, Davis T, Levine MM. Immunogenicity of multivalent *Shigella*-ETEC candidate vaccine strains in a guinea pig model. Vaccine 2006; 24:3727-34; PMID:16169130; http://dx.doi.org/10.1016/j.vaccine.2005.07.013
105. Roland KL, Cloninger C, Kochi SK, Thomas LJ, Tinge SA, Rouskey C, Killeen KP. Construction and preclinical evaluation of recombinant Peru-15 expressing high levels of the cholera toxin B subunit as a vaccine against enterotoxigenic *Escherichia coli*. Vaccine 2007; 25:8574-84; PMID:18045752; http://dx.doi.org/10.1016/j.vaccine.2007.09.074
106. Ruan X, Robertson DC, Nataro JP, Clements JD, Zhang W. 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 2014; 82:1823-32; PMID:24549325; http://dx.doi.org/10.1128/IAI.01394-13
107. Roy K BS, Qadri F, Fleckenstein JM. Enterotoxigenic *Escherichia coli* elicits immune responses to multiple surface proteins. Infect Immun 2010; 78:3027-35; PMID:20457787; http://dx.doi.org/10.1128/IAI.00264-10
108. Roy K HG, Hamilton DJ, Luo J, Ostmann MM, Fleckenstein JM. Enterotoxigenic *Escherichia coli* EtpA mediates adhesion between flagella and host cells. Infect Immun 2009; 457:594-8
109. Del Canto F, Valenzuela P, Cantero L, Bronstein J, Blanco JE, Blanco J, Prado V, Levine M, Nataro J, Sommerfelt H, et al. Distribution of classical and nonclassical virulence genes in enterotoxigenic *Escherichia coli* isolates from Chilean children and tRNA gene screening for putative insertion sites for genomic islands. J Clin Microbiol 2011; 49:3198-203; PMID:21775541; http://dx.doi.org/10.1128/JCM.02473-10
110. Harris JA RK, Woo-Rasberry V, Hamilton DJ, Kansal R, Qadri F, Fleckenstein JM. Directed evaluation of enterotoxigenic *Escherichia coli* autotransporter proteins as putative vaccine candidates. PLoS Negl Trop Dis 2011; 5:e1428; PMID:22163060; http://dx.doi.org/10.1371/journal.pntd.0001428
111. Roy K HD, 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 2008; 76:2106-12; PMID:18285493; http://dx.doi.org/10.1128/IAI.01304-07
112. Riley LW, Remis RS, Helgerson SD, McGee HB, Wells JG, Davis BR, Hebert RJ, Olcott ES, Johnson LM, Hargrett NT, et al. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N Engl J Med 1983; 308:681-5; PMID:6338386; http://dx.doi.org/10.1056/NEJM198303243081203
113. Karmali MA, Petric M, Lim C, Fleming PC, Steele BT. *Escherichia coli* cytotoxin, haemolytic-uraemic syndrome, and haemorrhagic colitis. Lancet 1983; 2:1299-300; PMID:6139632; http://dx.doi.org/10.1016/S0140-6736(83)91167-4
114. Konowalchuk J, Speirs JI, Stavric S. Vero response to a cytotoxin of *Escherichia coli*. Infect Immun 1977; 18:775-9; PMID:338490
115. McKee ML, Melton-Celsa AR, Moxley RA, Francis DH, O'Brien AD. Enterohemorrhagic *Escherichia coli* O157:H7 requires intimin to colonize the gnotobiotic pig intestine and to adhere to HEp-2 cells. Infect Immun 1995; 63:3739-44; PMID:7642319
116. Moon HW, Whipp SC, Argenzio RA, Levine MM, Giannella RA. Attaching and effacing activities of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. Infect Immun 1983; 41:1340-51; PMID:6350186
117. Nataro JP, Kaper JB. Diarrheagenic *Escherichia coli*. Clin Microbiol Rev 1998; 11:142-201; PMID:9457432
118. Consensus conference statement: *Escherichia coli* O157:H7 infections–an emerging national health crisis, July 11-13, 1994. Gastroenterology 1995; 108:1923-34; PMID:7768399; http://dx.doi.org/10.1016/0016-5085(95)90159-0
119. Prado JV, Cavagnaro SMF. Hemolytic uremic syndrome associated to shigatoxin producing *Escherichia coli* in Chilean children: clinical and epidemiological aspects. Rev Chilena Infectol 2008; 25:435-44; PMID:19194606
120. WHO. Scientific Working Group Meeting on Zoonotic Non-0157 Shiga Toxin-Producing *Escherichia coli* (1998: Berlin G, Unit WHOVPH. Zoonotic Non-0157 Shiga Toxin-Producing *Escherichia coli* (STEC): report of a WHO scientific working group meeting, Berlin, Germany 23-26 June 1998. Geneva: World Health Organization, 1998.
121. Wong CS, Jelacic S, Habeeb RL, Watkins SL, Tarr PI. The risk of the hemolytic-uremic syndrome after antibiotic treatment of *Escherichia coli* O157:H7 infections. N Engl J Med 2000; 342:1930-6; PMID:10874060; http://dx.doi.org/10.1056/NEJM200006293422601
122. Hurd HS, Malladi S. An outcomes model to evaluate risks and benefits of *Escherichia coli* vaccination in beef cattle. Foodborne Pathog Dis 2012; 9:952-61; PMID:22989170; http://dx.doi.org/10.1089/fpd.2012.1150
123. Matthews L, Reeve R, Gally DL, Low JC, Woolhouse MEJ, McAteer SP, Locking ME, Chase-Topping ME, Haydon DT, Allison LJ, et al. Predicting the public health benefit of vaccinating cattle against *Escherichia coli* O157. Proc Natl Acad Sci USA 2013; 110:16265-70; PMID:24043803; http://dx.doi.org/10.1073/pnas.1304978110
124. Snedeker KG, Campbell M, Sargeant JM. A systematic review of vaccinations to reduce the shedding of *Escherichia coli* O157 in the faeces of domestic ruminants. Zoonoses Public Health 2012; 59:126-38; PMID:21824378; http://dx.doi.org/10.1111/j.1863-2378.2011.01426.x
125. Thomas DE, Elliott EJ. Interventions for preventing diarrhea-associated hemolytic uremic syndrome: systematic review. BMC Public Health 2013; 13:799-; PMID:24007265; http://dx.doi.org/10.1186/1471-2458-13-799
126. Varela NP, Dick P, Wilson J. Assessing the existing information on the efficacy of bovine vaccination against *Escherichia coli* O157:H7–a systematic review and meta-analysis. Zoonoses Public Health 2013; 60:253-68; PMID:22856462; http://dx.doi.org/10.1111/j.1863-2378.2012.01523.x
127. Renwick SA, Wilson JB, Clarke RC, Lior H, Borczyk AA, Spika J, Rahn K, McFadden K, Brouwer A, Copps A. Evidence of direct transmission of *Escherichia coli* O157:H7 infection between calves and a human. J Infect Dis 1993; 168:792-3; PMID:8354927; http://dx.doi.org/10.1093/infdis/168.3.792
128. Mohawk KL, O'Brien AD. Mouse models of *Escherichia coli* O157:H7 infection and shiga toxin injection. J Biomed Biotechnol 2011; 2011:258185; PMID:21274267; http://dx.doi.org/10.1155/2011/258185
129. Garcia-Angulo VA, Kalita A, Torres AG. Advances in the development of enterohemorrhagic *Escherichia coli* vaccines using murine models of infection. Vaccine 2013; 31:3229-35; PMID:23707170; http://dx.doi.org/10.1016/j.vaccine.2013.05.013
130. Asper DJ, Karmali MA, Townsend H, Rogan D, Potter AA. Serological response of Shiga toxin-producing *Escherichia coli* type III secreted proteins in sera from vaccinated rabbits, naturally infected cattle, and humans. Clin Vaccine Immunol 2011; 18:1052-7; PMID:21593239; http://dx.doi.org/10.1128/CVI.00068-11
131. Greatorex JS, Thorne GM. Humoral immune responses to Shiga-like toxins and *Escherichia coli* O157 lipopolysaccharide in hemolytic-uremic syndrome patients and healthy subjects. J Clin Microbiol 1994; 32:1172-8; PMID:8051241
132. Jenkins C, Chart H, Smith HR, Hartland EL, Batchelor M, Delahay RM, Dougan G, Frankel G. Antibody response of patients infected with verocytotoxin-producing *Escherichia coli* to protein antigens encoded on the LEE locus. J Med Microbiol 2000; 49:97-9101; PMID:10628831
133. John M, Kudva IT, Griffin RW, Dodson AW, McManus B, Krastins B, Sarracino D, Progulske-Fox A, Hillman JD, Handfield M, et al. Use of in vivo-induced antigen technology for identification of *Escherichia coli* O157:H7 proteins expressed during human infection. Infect Immun 2005; 73:2665-79; PMID:15845468; http://dx.doi.org/10.1128/IAI.73.5.2665-2679.2005
134. Li Y, Frey E, Mackenzie AM, Finlay BB. Human response to *Escherichia coli* O157:H7 infection:

antibodies to secreted virulence factors. Infect Immun 2000; 68:5090-5; PMID:10948130; http://dx.doi.org/10.1128/IAI.68.9.5090-5095.2000
135. Sherman P, Soni R, Yeger H. Characterization of flagella purified from enterohemorrhagic, vero-cytotoxin-producing *Escherichia coli* serotype O157:H7. J Clin Microbiol 1988; 26:1367-72; PMID:3045153
136. Horne C, Vallance BA, Deng W, Finlay BB. Current progress in enteropathogenic and enterohemorrhagic *Escherichia coli* vaccines. Expert Rev Vaccines 2002; 1:483-93; PMID:12901587; http://dx.doi.org/10.1586/14760584.1.4.483
137. Calderon Toledo C, Arvidsson I, Karpman D. Cross-reactive protection against enterohemorrhagic *Escherichia coli* infection by enteropathogenic *E. coli* in a mouse model. Infect Immun 2011; 79:2224-33; PMID:21402761; http://dx.doi.org/10.1128/IAI.01024-10
138. Keller R, Hilton TD, Rios H, Boedeker EC, Kaper JB. Development of a live oral attaching and effacing *Escherichia coli* vaccine candidate using *Vibrio cholerae* CVD 103-HgR as antigen vector. Microb Pathog 2010; 48:1-8; PMID:19878715; http://dx.doi.org/10.1016/j.micpath.2009.10.005
139. Ishikawa S, Kawahara K, Kagami Y, Isshiki Y, Kaneko A, Matsui H, Okada N, Danbara H. Protection against Shiga toxin 1 challenge by immunization of mice with purified mutant Shiga toxin 1. Infect Immun 2003; 71:3235-9; PMID:12761104; http://dx.doi.org/10.1128/IAI.71.6.3235-3239.2003
140. Marcato P, Mulvey G, Read RJ, Vander Helm K, Nation PN, Armstrong GD. Immunoprophylactic potential of cloned Shiga toxin 2 B subunit. J Infect Dis 2001; 183:435-43; PMID:11133375; http://dx.doi.org/10.1086/318080
141. Marcato P, Griener TP, Mulvey GL, Armstrong GD. Recombinant Shiga toxin B-subunit-keyhole limpet hemocyanin conjugate vaccine protects mice from Shigatoxemia. Infect Immun 2005; 73:6523-9; PMID:16177326; http://dx.doi.org/10.1128/IAI.73.10.6523-6529.2005
142. Smith MJ, Teel LD, Carvalho HM, Melton-Celsa AR, O'Brien AD. Development of a hybrid Shiga holotoxoid vaccine to elicit heterologous protection against Shiga toxins types 1 and 2. Vaccine 2006; 24:4122-9; PMID:16551486; http://dx.doi.org/10.1016/j.vaccine.2006.02.035
143. Babiuk S, Asper DJ, Rogan D, Mutwiri GK, Potter AA. Subcutaneous and intranasal immunization with type III secreted proteins can prevent colonization and shedding of *Escherichia coli* O157:H7 in mice. Microb Pathog 2008; 45:7-11; PMID:18487034; http://dx.doi.org/10.1016/j.micpath.2008.01.005
144. Cataldi A, Yevsa T, Vilte DA, Schulze K, Castro-Parodi M, Larzabal M, Ibarra C, Mercado EC, Guzman CA. Efficient immune responses against Intimin and EspB of enterohaemorragic *Escherichia coli* after intranasal vaccination using the TLR2/6 agonist MALP-2 as adjuvant. Vaccine 2008; 26:5662-7; PMID:18675866; http://dx.doi.org/10.1016/j.vaccine.2008.07.027
145. Fan H-Y, Wang L, Luo J, Long B-G. Protection against *Escherichia coli* O157:H7 challenge by immunization of mice with purified Tir proteins. Mol Biol Rep 2012; 39:989-97; PMID:21567195; http://dx.doi.org/10.1007/s11033-011-0824-0
146. McNeilly TN, Mitchell MC, Rosser T, McAteer S, Low JC, Smith DGE, Huntley JF, Mahajan A, Gally DL. Immunization of cattle with a combination of purified intimin-531, EspA and Tir significantly reduces shedding of *Escherichia coli* O157:H7 following oral challenge. Vaccine 2010; 28:1422-8; PMID:19903545; http://dx.doi.org/10.1016/j.vaccine.2009.10.076
147. Potter AA, Klashinsky S, Li Y, Frey E, Townsend H, Rogan D, Erickson G, Hinkley S, Klopfenstein T, Moxley RA, et al. Decreased shedding of *Escherichia coli* O157:H7 by cattle following vaccination with type III secreted proteins. Vaccine 2004; 22:362-9; PMID:14670317; http://dx.doi.org/10.1016/j.vaccine.2003.08.007
148. Stanford K, Hannon S, Booker CW, Jim GK. Variable efficacy of a vaccine and direct-fed microbial for controlling *Escherichia coli* O157:H7 in feces and on hides of feedlot cattle. Foodborne Pathog Dis 2014; 11:379-87; PMID:24673729; http://dx.doi.org/10.1089/fpd.2013.1693
149. Cheng Y, Feng Y, Luo P, Gu J, Yu S, Zhang W-J, Liu Y-Q, Wang Q-X, Zou Q-M, Mao X-H. Fusion expression and immunogenicity of EHEC EspA-Stx2Al protein: implications for the vaccine development. J Microbiol 2009; 47:498-505; PMID:19763426; http://dx.doi.org/10.1007/s12275-009-0116-8
150. Gu J, Liu Y, Yu S, Wang H, Wang Q, Yi Y, Zhu F, Yu X-J, Zou Q, Mao X. Enterohemorrhagic *Escherichia coli* trivalent recombinant vaccine containing EspA, intimin and Stx2 induces strong humoral immune response and confers protection in mice. Microbes Infect 2009; 11:835-41; PMID:19427397; http://dx.doi.org/10.1016/j.micinf.2009.04.024
151. Amani J, Salmanian AH, Rafati S, Mousavi SL. Immunogenic properties of chimeric protein from espA, eae and tir genes of *Escherichia coli* O157:H7. Vaccine 2010; 28:6923-9; PMID:20709010; http://dx.doi.org/10.1016/j.vaccine.2010.07.061
152. Zhang X-H, He K-W, Zhang S-X, Lu W-C, Zhao P-D, Luan X-T, Ye Q, Wen L-B, Li B, et al. Subcutaneous and intranasal immunization with Stx2B-Tir-Stx1B-Zot reduces colonization and shedding of *Escherichia coli* O157:H7 in mice. Vaccine 2011; 29:3923-9; PMID:21338683; http://dx.doi.org/10.1016/j.vaccine.2011.02.007
153. Cai K, Gao X, Li T, Wang Q, Hou X, Tu W, Xiao L, Tian M, Liu Y, Wang H. Enhanced immunogenicity of a novel Stx2Am-Stx1B fusion protein in a mice model of enterohemorrhagic *Escherichia coli* O157:H7 infection. Vaccine 2011; 29:946-52; PMID:21134452; http://dx.doi.org/10.1016/j.vaccine.2010.11.035
154. Bentancor LV, Bilen M, Brando RJF, Ramos MV, Ferreira LCS, Ghiringhelli PD, Palermo MS. A DNA vaccine encoding the enterohemorragic *Escherichia coli* Shiga-like toxin 2 A2 and B subunits confers protective immunity to Shiga toxin challenge in the murine model. Clin Vaccine Immunol 2009; 16:712-8; PMID:19176691; http://dx.doi.org/10.1128/CVI.00328-08
155. Shariati Mehr K, Mousavi SL, Rasooli I, Amani J, Rajabi M. A DNA vaccine against *Escherichia coli* O157:H7. Iran Biomed J 2012; 16:133-9; PMID:23023214
156. Fujii J, Naito M, Yutsudo T, Matsumoto S, Heatherly DP, Yamada T, Kobayashi H, Yoshida S-I, Obrig T. Protection by a recombinant Mycobacterium bovis Bacillus Calmette-Guerin vaccine expressing Shiga toxin 2 B subunit against Shiga toxin-producing *Escherichia coli* in mice. Clin Vaccine Immunol 2012; 19:1932-7; PMID:23035176; http://dx.doi.org/10.1128/CVI.00473-12
157. Gu J, Ning Y, Wang H, Xiao D, Tang B, Luo P, Cheng Y, Jiang M, Li N, Zou Q, et al. Vaccination of attenuated EIS-producing *Salmonella* induces protective immunity against enterohemorrhagic *Escherichia coli* in mice. Vaccine 2011; 29:7395-403; PMID:21807051; http://dx.doi.org/10.1016/j.vaccine.2011.07.069
158. Oliveira AF, Cardoso SA, Almeida FBdR, de Oliveira LL, Pitondo-Silva A, Soares SG, Hanna ES. Oral immunization with attenuated *Salmonella* vaccine expressing *Escherichia coli* O157:H7 intimin gamma triggers both systemic and mucosal humoral immunity in mice. Microbiol Immunol 2012; 56:513-22; PMID:22671942; http://dx.doi.org/10.1111/j.1348-0421.2012.00477.x
159. Rojas RLG, Gomes PADP, Bentancor LV, Sbrogio-Almeida ME, Costa SOP, Massis LM, Ferreira RCC, Palermo MS, Ferreira LCS. *Salmonella enterica* serovar Typhimurium vaccine strains expressing a nontoxic Shiga-like toxin 2 derivative induce partial protective immunity to the toxin expressed by enterohemorrhagic *Escherichia coli*. Clin Vaccine Immunol 2010; 17:529-36; PMID:20147499; http://dx.doi.org/10.1128/CVI.00495-09
160. Cai K, Gao X, Li T, Hou X, Wang Q, Liu H, Xiao L, Tu W, Liu Y, Shi J, et al. Intragastric immunization of mice with enterohemorrhagic *Escherichia coli* O157:H7 bacterial ghosts reduces mortality and shedding and induces a Th2-type dominated mixed immune response. Can J Microbiol 2010; 56:389-98; PMID:20555401; http://dx.doi.org/10.1139/W10-025
161. Mayr UB, Kudela P, Atrasheuskaya A, Bukin E, Ignatyev G, Lubitz W. Rectal single dose immunization of mice with *Escherichia coli* O157:H7 bacterial ghosts induces efficient humoral and cellular immune responses and protects against the lethal heterologous challenge. Microb Biotechnol 2012; 5:283-94; PMID:22103353; http://dx.doi.org/10.1111/j.1751-7915.2011.00316.x
162. Vilte DA, Larzabal M, Mayr UB, Garbaccio S, Gammella M, Rabinovitz BC, Delgado F, Meikle V, Cantet RJC, Lubitz P, et al. A systemic vaccine based on *Escherichia coli* O157:H7 bacterial ghosts (BGs) reduces the excretion of *E. coli* O157:H7 in calves. Vet Immunol Immunopathol 2012; 146:169-76; PMID:22460171; http://dx.doi.org/10.1016/j.vetimm.2012.03.002
163. Vande Walle K, Vanrompay D, Cox E. Bovine innate and adaptive immune responses against *Escherichia coli* O157:H7 and vaccination strategies to reduce faecal shedding in ruminants. Vet Immunol Immunopathol 2013; 152:109-20; PMID:23084625; http://dx.doi.org/10.1016/j.vetimm.2012.09.028
164. Van der Woude MW, Baumler AJ. Phase and antigenic variation in bacteria. Clin Microbiol Rev 2004; 17:581-611; PMID:15258095; http://dx.doi.org/10.1128/CMR.17.3.581-611.2004
165. Adu-Bobie J, Frankel G, Bain C, Goncalves AG, Trabulsi LR, Douce G, Knutton S, Dougan G. Detection of intimins alpha, beta, gamma, and delta, four intimin derivatives expressed by attaching and effacing microbial pathogens. J Clin Microbiol 1998; 36:662-8; PMID:9508292
166. Bono JL, Keen JE, Clawson ML, Durso LM, Heaton MP, Laegreid WW. Association of *Escherichia coli* O157:H7 *tir* polymorphisms with human infection. BMC Infect Dis 2007; 7:98; PMID:17718910; http://dx.doi.org/10.1186/1471-2334-7-98
167. China B, Goffaux F, Pirson V, Mainil J. Comparison of eae, tir, espA and espB genes of bovine and human attaching and effacing *Escherichia coli* by multiplex polymerase chain reaction. FEMS Microbiol Lett 1999; 178:177-82; PMID:10483737; http://dx.doi.org/10.1111/j.1574-6968.1999.tb13775.x
168. Crepin VF, Shaw R, Knutton S, Frankel G. Molecular basis of antigenic polymorphism of EspA filaments: development of a peptide display technology. J Mol Biol 2005; 350:42-52; PMID:15921692; http://dx.doi.org/10.1016/j.jmb.2005.04.060
169. Smith DR, Moxley RA, Peterson RE, Klopfenstein TJ, Erickson GE, Bretschneider G, Berberov EM, Clowser S. A two-dose regimen of a vaccine against type III secreted proteins reduced *Escherichia coli* O157:H7 colonization of the terminal rectum in beef cattle in commercial feedlots. Foodborne Pathog Dis 2009; 6:155-61; PMID:19105625; http://dx.doi.org/10.1089/fpd.2008.0136
170. Vogstad AR, Moxley RA, Erickson GE, Klopfenstein TJ, Smith DR. Assessment of heterogeneity of efficacy of a three-dose regimen of a type III secreted protein vaccine for reducing STEC O157 in feces of feedlot cattle. Foodborne Pathog Dis 2013; 10:678-83;

PMID:23692077; http://dx.doi.org/10.1089/fpd.2012.1374
171. Thomson DU, Loneragan GH, Thornton AB, Lechtenberg KF, Emery DA, Burkhardt DT, Nagaraja TG. Use of a siderophore receptor and porin proteins-based vaccine to control the burden of *Escherichia coli* O157:H7 in feedlot cattle. Foodborne Pathog Dis 2009; 6:871-7; PMID:19737063; http://dx.doi.org/10.1089/fpd.2009.0290
172. Fox JT TD, Drouillard JS, Thornton AB, Burkhardt DT, Emery DA, Nagaraja TG. Efficacy of *Escherichia coli* O157:H7 siderophore receptor/porin proteins-based vaccine in feedlot cattle naturally shedding *E. coli* O157. Foodborne Pathog Dis 2009; 6:893-9; PMID:19737065; http://dx.doi.org/10.1089/fpd.2009.0336
173. Wileman BW TD, Olson KC, Jaeger JR, Pacheco LA, Bolte J, Burkhardt DT, Emery DA, Straub D. *Escherichia coli* O157:H7 shedding in vaccinated beef calves born to cows vaccinated prepartum with *Escherichia coli* O157:H7 SRP vaccine. J Food Prot 2011; 74:1599-604; PMID:22004804; http://dx.doi.org/10.4315/0362-028X.JFP-11-034
174. Cull CA PZ, Nagaraja TG, Bello NM, Babcock AH, Renter DG. Efficacy of a vaccine and a direct-fed microbial against fecal shedding of *Escherichia coli* O157:H7 in a randomized pen-level field trial of commercial feedlot cattle. Vaccine 2012; 30:6210-5; PMID:22704925; http://dx.doi.org/10.1016/j.vaccine.2012.05.080
175. Wang F YQ, Kase JA, Meng J, Clotilde LM, Lin A, Ge B. Current trends in detecting non-O157 Shiga toxin-producing *Escherichia coli* in food. Foodborne Pathog Dis 2013; 10:665-77; PMID:23755895; http://dx.doi.org/10.1089/fpd.2012.1448
176. Konadu EY, Parke JC, Tran HT, Bryla DA, Robbins JB, Szu SC. Investigational vaccine for *Escherichia coli* O157: phase 1 study of O157 O-specific polysaccharide-Pseudomonas aeruginosa recombinant exoprotein A conjugates in adults. J Infect Dis 1998; 177:383-7; PMID:9466525; http://dx.doi.org/10.1086/514203
177. Ahmed A, Li J, Shiloach Y, Robbins JB, Szu SC. Safety and immunogenicity of *Escherichia coli* O157 O-specific polysaccharide conjugate vaccine in 2-5-year-old children. J Infect Dis 2006; 193:515-21; PMID:16425130; http://dx.doi.org/10.1086/499821
178. Garcia-Angulo VA, Kalita A, Kalita M, Lozano L, Torres AG. Comparative genomics and immunoinformatics approach for the identification of vaccine candidates for enterohemorrhagic *Escherichia coli* O157:H7. Infect Immun 2014; 82:2016-26; PMID:24595137; http://dx.doi.org/10.1128/IAI.01437-13
179. Montero D OP, Gutiérrez D, Araya D, Salazar JC, Prado V, Oñate A, Del Canto F, Vidal R. Immunoproteomic analysis to identify Shiga toxin-producing *Escherichia coli* outer membrane proteins expressed during human infection. Infect Immun 2014; 82:4767-77; PMID:25156722; http://dx.doi.org/10.1128/IAI.02030-14
180. Dasti JI, Tareen AM, Lugert R, Zautner AE, Gross U. *Campylobacter jejuni*: a brief overview on pathogenicity-associated factors and disease-mediating mechanisms. Int J Med Microbiol 2010; 300:205-11; PMID:19665925; http://dx.doi.org/10.1016/j.ijmm.2009.07.002
181. Centers for Disease Control and Prevention U. *Campylobacter*, general information. [Internet] 2014 [Last updated 06/03/2014]. Available from http://www.cdc.gov/nczved/divisions/dfbmd/diseases/Campylobacter/
182. European Food Safety Authority. *Campylobacter* [Internet]. 2014 [Last updated 07/14/2014]. Available from http://www.efsa.europa.eu/en/topics/topic/campylobacter.htm
183. Guerry P, Poly F, Riddle M, Maue AC, Chen Y-H, Monteiro MA. *Campylobacter* polysaccharide capsules: virulence and vaccines. Front Cell Infect Microbiol 2012; 2:7; PMID:22919599; http://dx.doi.org/10.3389/fcimb.2012.00007
184. De Zoete MR, Van Putten JPM, Wagenaar JA. Vaccination of chickens against *Campylobacter*. Vaccine 2007; 25:5548-57; PMID:17224215; http://dx.doi.org/10.1016/j.vaccine.2006.12.002
185. Smith CK, Abuoun M, Cawthraw SA, Humphrey TJ, Rothwell L, Kaiser P, Barrow PA, Jones MA. Campylobacter colonization of the chicken induces a proinflammatory response in mucosal tissues. FEMS Immunol Med Microbiol 2008; 54:114-21; PMID:18647351; http://dx.doi.org/10.1111/j.1574-695X.2008.00458.x
186. Rice BE, Rollins DM, Mallinson ET, Carr L, Joseph SW. *Campylobacter jejuni* in broiler chickens: colonization and humoral immunity following oral vaccination and experimental infection. Vaccine 1997; 15:1922-32; PMID:9413103; http://dx.doi.org/10.1016/S0264-410X(97)00126-6
187. Noor SM, Husband AJ, Widders PR. *In ovo* oral vaccination with *Campylobacter jejuni* establishes early development of intestinal immunity in chickens. Br Poult Sci 1995; 36:563-73; PMID:8590089; http://dx.doi.org/10.1080/00071669508417802
188. Widders PR, Thomas LM, Long KA, Tokhi MA, Panaccio M, Apos E. The specificity of antibody in chickens immunised to reduce intestinal colonisation with *Campylobacter jejuni*. Vet Microbiol 1998; 64:39-50; PMID:9874102; http://dx.doi.org/10.1016/S0378-1135(98)00251-X
189. Khoury CA, Meinersmann RJ. A genetic hybrid of the *Campylobacter jejuni* flaA gene with LT-B of *Escherichia coli* and assessment of the efficacy of the hybrid protein as an oral chicken vaccine. Avian Dis 1995; 39:812-20; PMID:8719215; http://dx.doi.org/10.2307/1592418
190. Monteiro MA, Baqar S, Hall ER, Chen Y-H, Porter CK, Bentzel DE, Applebee L, Guerry P. Capsule polysaccharide conjugate vaccine against diarrheal disease caused by *Campylobacter jejuni*. Infect Immun 2009; 77:1128-36; PMID:19114545; http://dx.doi.org/10.1128/IAI.01056-08
191. Pawelec D, Rozynek E, Popowski J, Jagusztyn-Krynicka EK. Cloning and characterization of a *Campylobacter jejuni* 72Dz/92 gene encoding a 30 kDa immunopositive protein, component of the ABC transport system; expression of the gene in avirulent *Salmonella typhimurium*. FEMS Immunol Med Microbiol 1997; 19:137-50; PMID:9395059; http://dx.doi.org/10.1111/j.1574-695X.1997.tb01083.x
192. Pawelec DP, Korsak D, Wyszynska AK, Rozynek E, Popowski J, Jagusztyn-Krynicka EK. Genetic diversity of the *Campylobacter* genes coding immunodominant proteins. FEMS Microbiol Lett 2000; 185:43-9; PMID:10731605; http://dx.doi.org/10.1111/j.1574-6968.2000.tb09038.x
193. Wyszynska A, Raczko A, Lis M, Jagusztyn-Krynicka EK. Oral immunization of chickens with avirulent *Salmonella* vaccine strain carrying *C. jejuni* 72Dz/92 *cjaA* gene elicits specific humoral immune response associated with protection against challenge with wild-type *Campylobacter*. Vaccine 2004; 22:1379-89; PMID:15063560; http://dx.doi.org/10.1016/j.vaccine.2003.11.001
194. Buckley AM, Wang J, Hudson DL, Grant AJ, Jones MA, Maskell DJ, Stevens MP. Evaluation of live-attenuated *Salmonella* vaccines expressing *Campylobacter* antigens for control of *C. jejuni* in poultry. Vaccine 2010; 28:1094-105; PMID:19853682; http://dx.doi.org/10.1016/j.vaccine.2009.10.018
195. Clark JD, Oakes RD, Redhead K, Crouch CF, Francis MJ, Tomley FM, Blake DP. *Eimeria* species parasites as novel vaccine delivery vectors: anti-*Campylobacter jejuni* protective immunity induced by *Eimeria tenella*-delivered CjaA. Vaccine 2012; 30:2683-8; PMID:22342500; http://dx.doi.org/10.1016/j.vaccine.2012.02.002