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Advances in the development of enterohemorrhagic *Escherichia coli* vaccines using murine models of infection

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Abstract

Enterohemorrhagic *Escherichia coli* (EHEC) strains are food borne pathogens with importance in public health. EHEC colonizes the large intestine and causes diarrhea, hemorrhagic colitis and in some cases, life-threatening hemolytic-uremic syndrome (HUS) due to the production of Shiga toxins (Stx). The lack of effective clinical treatment, sequelae after infection and mortality rate in humans supports the urgent need of prophylactic approaches, such as development of vaccines. Shedding from cattle, the main EHEC reservoir and considered the principal food contamination source, has prompted the development of licensed vaccines that reduce EHEC colonization in ruminants. Although murine models do not fully recapitulate human infection, they are commonly used to evaluate EHEC vaccines and the immune/protective responses elicited in the host. Mice susceptibility differs depending of the EHEC inoculums; therefore, displaying different mortality rates and Stx-mediated renal damage. Therefore, several experimental protocols have being pursued in this model to develop EHEC-specific vaccines. Recent candidate vaccines evaluated include those composed of virulence factors alone or as fused-subunits, DNA-based, attenuated bacteria and bacterial ghosts. In this review, we summarize progress in the design and testing of EHEC vaccines and the use of different strategies for the evaluation of novel EHEC vaccines in the murine model.

Keywords

enterohemorrhagic *E. coli*; diarrhea; vaccine; murine model; bacterial ghost; EHEC; shiga toxin

1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) are intestinal zoonotic pathogens causing sporadic outbreaks worldwide. EHEC is a type of Shiga toxin-producing *E. coli* (STEC) that colonize the human intestine and cause diarrheal illness that can progress to hemorrhagic colitis and in several cases, life-threatening hemolytic uremic syndrome (HUS) (reviewed in [1-2]). Children less than 5 years of age and the elderly are most susceptible to severe HUS complications. Around 450 serotypes of STEC have been isolated from humans with disease

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[3]; out of which 10 serogroups are responsible for the majority of cases. In most countries, EHEC O157:H7 is the predominant serotype associated with outbreaks [4]. Developed countries have been particularly affected by EHEC infections and it is calculated that in the USA, O157:H7 caused 0.9 illnesses per 100,000, still leading to a significant number of deaths a year [5].

The majority of EHEC O157:H7 outbreaks in the USA are food borne and they are linked to the consumption of contaminated bovine-derived products, fresh produce such as lettuce, spinach and sprouts [4]. Cattle and other ruminants serve as a reservoir for this pathogen and fecal shedding is linked to food contamination. The principal site of colonization of EHEC O157:H7 in cattle is the lymphoid follicle-dense mucosal region at the terminal rectum, called recto-anal junction mucosa [6]. Survey studies in cattle from high prevalence countries demonstrated carriage ranging from <1% to more than 30% [7]. Because of this, a large amount of EHEC control studies are focused on the eradication of this bacterium from the gastrointestinal tract of ruminants, whether by improved breeding practices or by vaccination.

2. EHEC virulence factors as targets for vaccine development

In a simplistic way, EHEC's major virulence factors can be classified in 3 major groups, including those encoded or associated with the Locus of Enterocyte Effacement (LEE), toxins such as Stx, and surface fimbrial and afimbrial adhesins [2]. EHEC is a member of those intestinal pathogens that the ability to form attaching and effacing (A/E) lesions in host intestinal epithelium [8]. A/E lesions are characterized by the bacterial attachment with the formation of an actin pedestal-like structure and destruction (effacement) of the enterocyte microvilli [9]. A/E pathogens possess a pathogenicity island termed the LEE, which encodes the proteins required for the assembly of a type III secretion system (T3SS) [8-9], a molecular syringe able to inject proteins directly into the cytosol of eukaryotic cells [10-11]. EHEC's and all other T3SS found in A/E-pathogens are composed of a basal structure spanning both bacterial membranes and a needle structure, comprised by polymers of the EscF and EspA proteins. In the tip of the structure, the translocon proteins EspD and EspB form a pore in the epithelial cell, through which translocated proteins are delivered [10]. Type III-translocated proteins are called "effectors" and it has been proposed that EHEC genome encode up to 50 of such proteins, whose genes are located within the LEE island or scattered thorough the chromosome [12]. Tir, one of the first translocated proteins during infection, localizes in the enterocyte membrane functioning as a receptor for the EHEC outer membrane protein intimin [13-14]. Tir-intimin interaction docks the bacteria into the surface of the host cell and trigger cytoskeletal rearrangements leading to the formation of the actin pedestal underneath the adherent bacteria [15]. Thus, Tir and intimin, as well as proteins required for the assembly and function of the T3SS, are essential for EHEC virulence [6, 16]. As such, it has been shown that strong human immune response to Tir, and to a lesser extent to intimin, EspA and EspB, is observed following EHEC infection [17-18], which indicates that these proteins are potential vaccine candidates.

One of the main differences between EHEC pathogenesis and that of the other A/E pathogens is the production of Shiga toxins (Stx). The development of HUS is associated with the expression of *stx* genes encoded in lamboid prophages [19-20]. The family of Stx toxins is divided into the immunologically distinct Stx1 and Stx2 [20] and although EHEC strains may produce one or both Stx types, Stx2 is associated with more severe disease in humans [21-22]. Stxs are members of the AB₅ family of toxins, formed by an enzymatically active A subunit non-covalently bound to a homopentamer of B subunits. The B subunit is responsible for binding to the globotriaosylceramide (Gb3) receptor on the host cell surface [23]. Upon receptor binding, Stx is internalized by endocytosis and the A subunit is cleaved

by furin into A1 and A2 fragments. A1, which possess N-glycosidase activity, reaches the large ribosomal subunit where it cleaves a specific adenine residue of the 28S rRNA, halting protein synthesis and leading to cell death. Given the importance of Stx2 in EHEC disease and that anti-Stx1 B subunit antibodies do not confer protection against a Stx2 holotoxin challenge [24], it is accepted that any potential EHEC vaccine must generate a protective Stx2 immune responses.

Further, EHEC virulence is enhanced by the presence of wide variety surface-exposed or secreted factors, implicated in initial adhesion or subsequent colonization of the intestine. Some of these factors include outer membrane proteins (Iha, OmpA) [25-27], fimbrial proteins (curli, Lpf1, Lpf2, F9, HCP) [2, 28-30], autotransporters (EhaA, EhaB, Saa and EhaG) [2, 27] and flagella [2]. In addition, EHEC possess a pO157 plasmid that encodes numerous virulent genes associated with the disease, particularly the EHEC hemolysin [31]. The information available about the human immune responses mounted against these factors is less than that obtained in cattle experimental models; however, it is well accepted that host immune responses to the initial stages of colonization as well as the later stages during infection have to be stimulated to produce a fully protective vaccine.

3. Intestinal inflammatory responses to EHEC infection in humans and cattle

There is evidence that chemokine and pro-inflammatory mediators, such as neutrophils and dendritic cells, produce and initiate the host acute mucosal inflammatory response during EHEC infection in humans [32-33]. In epithelial cells, EHEC infection activates p38 and ERK MAP kinases, promote nuclear translocation of NF- κ B and increase pro-inflammatory interleukin-8 (IL-8). These pro-inflammatory effects are mainly mediated by EHEC flagellin [34], which is detected by TLR-5, signaling the activation of NF- κ B to cause inflammation [33, 35]. Interestingly, HUS patients showed elevated urinary levels of monocyte chemoattractant protein 1 and IL-8 [36], which are associated with macrophages activation and recruitment of polymorphonuclear leukocytes (PMNs). PMNs act as toxin carriers, transporting Stx to renal endothelial cells that express Gb3 and therefore, contributing to the pathogenesis of HUS.

On the other hand, EspB and non-LEE-encoded effectors (Nle's) have been shown to suppress host NF- κ B activation and inflammatory responses, counteracting the host immune response observed in response to EHEC flagellin [37]. Stx also contributes to the host intestinal response because it induces multiple chemokines that stimulate a pro-inflammatory response, resulting in influx of acute inflammatory cells and thus contributing to the intestinal tissue damage seen in STEC infection [32]. Recent work showed that Stxs are capable of activating and deactivating MAPK-dependent responses for cytokine/chemokine production in human macrophage-like cells [38]. The finding indicating that specific chemokines, such as GRO- α , MIP-1 β , and MCP-1 are produced during *E. coli* O157:H7 enteritis in children, whether or not they developed hemorrhagic colitis or HUS [39], supports the role of leukocyte recruitment during infection and provides useful clues in how to enhance intestinal immune responses during vaccination.

EHEC colonization in cattle remains generally asymptomatic and animals do not develop intestinal damage as seen in humans. However, cattle can develop a discrete inflammatory response and small mucosal lesions as a result of EHEC colonization [40]. The flagella of EHEC acts as an adhesin to bovine intestinal epithelium, initiating colonization at the mucosal surface [41]. During infection of cattle, EHEC induces flagellin-specific immunoglobulin (Ig) IgG, but also inhibit flagellin-mediated TLR5 activation, thereby impairing innate immune response [42-43]. Therefore, it is believed that the balance

between pro-inflammatory flagellin, and the anti-inflammatory Nle's effector proteins, is responsible for shaping the outcome of the host immune response.

LPS is another important component recognized by bovine innate immune cells. Bovine macrophage cells exposed to *E. coli* O157 LPS induces the expression of TNF- and interferon-induced genes and inhibitors of apoptosis [44]. Although the signaling pathway induced by O157 LPS is not known, few reports have suggested a TLR4-mediated signaling occurred during infection [45-46]. Stx does not play a key role in the onset of the early immune response in cattle. However, it has been shown that Stx2 has a suppressive effect in the development of specific cellular immune responses due to its interaction with bovine lymphocytes expressing Gb3/CD77 binding receptor [47]. Therefore, induction of effective bovine systemic and mucosal humoral responses to *E. coli* O157:H7 infection will result in a significant reduction in colonization. EHEC Stx1, Stx2, O157 LPS and T3SS proteins (intimin, Tir, EspA, EspB) serum antibodies are detected in naturally infected cattle [40] and antibodies against Stx1, Stx2, O157 LPS, OmpC, H7 flagellin, intimin, Tir, EspA, EspB, EspD and the EhaB autotransporter have been detected after experimental EHEC infection of cattle (reviewed in [40]). These data strongly suggest that prophylactic induction of such EHEC-specific antibodies contributes in reducing EHEC colonization.

4. Small animal models

Due to the risk associated with antibiotic treatment against EHEC infections, it has been accepted that vaccination, both in cattle and humans, could represent the best way to reduce EHEC incidence and prevent outbreaks [48]. It will be ideal to test therapeutics and vaccines in humans, however this type of experiments are discouraged due to the possibility for HUS development. Because large animal models, such as ruminants or non-human primates are costly and require specialized facilities for experimentation, several small animal models have been extensively employed to study EHEC infection [20, 49]. The list includes mice [50], rats [51] and rabbits [52] that have been used to evaluate different therapeutic strategies. They are affordable, ease of care and handling, not requiring substantial veterinary skills and space for maintenance, and it is feasible to use enough animals in a single study to perform statistical analysis [20, 53]. Although mice do not developed the symptoms associated with diarrheal disease observed in humans, development of murine models of *E. coli* O157:H7 infection, including axenic (no indigenous intestinal flora) or streptomycin-treated Balb/c (reduced normal flora) mice have proved to be useful for EHEC colonization and candidate vaccine testing [20]. Because the lack of a naturally EHEC infected mouse model is a major obstacle, the ability of *Citrobacter rodentium* to naturally colonize and form A/E lesions in mice has been explored as a alternative for A/E pathogens-mediated disease and could be used for vaccine evaluation [54]. As such, a new Stx-expressing *C. rodentium* strain has been developed to resemble EHEC infection in mice [55]. Next, we summarize recent progress in the development of EHEC vaccine candidates assayed in different murine models. This next sections focus on the effect of the type of immunogen and delivery routes on the outcome of immune response and disease after challenge. The magnitude of disease may vary depending on mouse strain and challenge method, as reviewed by others [56]

5. Immunization with subunit vaccines

5.1 Shiga Toxin

Because Stx is the main virulence factor of EHEC, vaccination studies are aimed to induce immunity responses to prevent intoxication. Early work demonstrated that vaccination with inactive Stx-derivatives effectively induced the production of neutralizing antibodies and in some cases, protection against toxemia in mice and other animal models [24, 57-61].

Further, passive immunization of mice with human or murine antibodies raised against Stx2 inhibited toxin RNA glycosylase activity, protecting against Stx2 challenge and reducing EHEC colonization [62-64]. Stx2-based vaccine candidates are preferred over Stx1 due to Stx2-association with HUS [21-22]. Nonetheless, the ability to induce immune responses against both Stx types is an important feature of a viable candidate.

To achieve this, recent studies assayed hybrid toxins and different delivery routes (Table 1). A hybrid toxoid, consisting of a point mutation-inactivated StxA2 subunit and a native StxB1 subunit induced the production of neutralizing sera antibodies against both Stx types when immunized by the intraperitoneally (i.p.) route and also protected mice from challenge with the combination of Stx1 and Stx2 [65]. Similarly, purified Stx2B subunit administered intranasally (i.n.) induced systemic IgG and mucosal IgA antibodies, which were able to neutralize Stx1 and Stx2 cytotoxicity *in vitro* and protect mice against heterologous toxemia. In the same study, mice immunized with Stx1B elicited only anti-Stx1 protective response [66]. A novel fusion protein composed of the B subunits of the two types of Stx (named S2 protein) generated antibodies able to neutralize the cytotoxic activity of both Shiga toxins *in vitro* and to increase the survival of mice challenged with a lysate of *E. coli* O157:H7 [67] (Table 1). Interestingly, the antibodies titers and protective effects elicited by S2 were higher than when using a mixture of non-fused Stx1B and Stx2B, probably due to the adjuvanticity of the fused Stx1B [67]. Further, it has been shown that Stx1B has the ability to induce the proliferation of immune cells [68]. A S2 derivative fusion containing an enzymatically inactive Stx2A subunit instead of the Stx2B (Stx2Am-Stx1B) displayed enhanced immunogenicity compared to the S2 fusion. Stx2Am-Stx1B generated higher levels of Stx2 neutralizing antibodies and significantly higher level of protection against a lethal dose of an O157:H7 lysate [69]. Efforts to improve the immunogenicity of Stx-based candidates include the generation of mammalian expression vector-based DNA vaccines that could improve antigen presentation. A DNA vaccine designed to express a mutated non-toxic Stx2 form, consisting of the B subunit and a partially deleted A subunit (Stx2ΔAB) induced systemic neutralizing Stx-specific antibodies against both subunits and partially protected mice against Stx2 toxemia [70].

As EHEC colonization site is the intestinal mucosa, it is reasonable to propose that oral immunization is a more efficient way to trigger mucosal responses interfering with EHEC pathogenesis. As such, genetically engineered non-pathogenic bacteria able to colonize the mice intestine have been used to optimize mucosal antigen delivery. *Salmonella enterica* strains expressing the Stx2ΔAB toxoid colonized the mice gut and induced anti-Stx2B IgG. The anti-toxoid antibodies neutralized Stx2 toxic activity *in vitro*, but conferred only limited protection against the Stx2 challenge *in vivo* [71]. While the generation of neutralizing antibodies against Stx-derivatives has been well documented, the immune pathways triggered during vaccination with these candidates are poorly understood. S2-vaccinated mice elicited high titers of IgG1 subtype, which suggested that this fusion biased immune response towards Th2 [67]. The S2-derivative Stx2Am-Stx1B fusion also induces a Th2-mediated humoral response, characterized by production of IgG1 and cytokines IL-4 and IL-10 [69]. Although promising vaccine candidates have been produced using Stx-derivatives, further research assessing the type of immune responses induced is required to establish the adequate surrogates of protection.

5.2 Type 3 Secretion System-related protein vaccines

It is known that naturally-occurring human EHEC O157:H7 infections induce antibodies against T3SS-related proteins, such as Tir, intimin, EspB, NleA and EspA [17, 72]. This has led to the use of bacterial surface located and secreted T3SS proteins (TTSPs) as vaccine candidates. For example, the formulation known as Econiche™, one of the two licensed vaccines designed to reduce EHEC shedding in cattle, is based on TTSPs [40, 73]. The mice

model contributed to the development of this vaccine by allowing comparison of different delivery routes and the assessment of immune protective responses. Intranasal (i.n.) immunization of mice with TTSPs induced serum and fecal specific anti-Tir and anti-EspA antibodies and reduced shedding when co-delivered with adjuvant [74]. Intramuscular (i.m.) TTSPs immunization eliminated bacterial shedding without producing fecal anti-Tir or anti-EspA IgA, which resembled results previously obtained in cattle [73-74]. Data suggest that with these vaccines, secretory IgA may be dispensable to protect against *E. coli* O157:H7 [74]. However, a recent report using purified Tir as a vaccine found that i.n. immunization increased total IgG and IgA in serum and feces, reducing shedding and increasing mice survival when challenged with a lethal dose of lysed *E. coli* O157:H7 [75]. In the same study, subcutaneous (s.c.) immunization increased serum IgG but failed to significantly increase IgA in feces and to protect mice against challenge.

Similar results were obtained when comparing different administration routes of the KT-12 peptide, a predicted B-cell epitope from intimin. Immunization with KT-12 conjugated to adjuvant increased sIgA levels and mice survival rate to a lethal dose of O157:H7 only when i.n. administration was used [76]. The i.n. administration of EspB and the carboxy-terminus of intimin (C280 γ -intimin) induced serum specific IgG and fecal specific IgA when administered with adjuvant [77]. In the same work, antigen-specific proliferative responses were observed in IFN γ -, IL-2- and IL-4-expressing lymphocytes obtained from EspB- or C280 γ -intimin-vaccinated mice, with MALP-2 adjuvant boosting these cellular responses.

Intestinal mucosa immune responses have also been targeted by intimin-, EspB-, and EspA-derived vaccines using non-pathogenic carrier strains. Orally inoculated *Salmonella enterica* serovar Typhimurium x3987 expressing intimin was able to colonize the mice Peyer's patches and spleen, producing specific serum IgG and fecal IgA antibodies and reducing EHEC shedding after challenge [78]. *Lactobacillus lactis* expressing EspA or EspB induced antigen-specific humoral IgG responses. Polyclonal anti-EspA from immunized mice was able to block EHEC-induced actin rearrangements *in vitro* [79]. In mice inoculated with EspB-expressing *L. lactis*, seroconversion required an intraperitoneal boost and, in addition to IgG, mice also developed fecal IgA [80]. Later, it was shown that the monoclonal antibody 1H10, which recognizes the conserved E07 epitope from EspA, is able to block EHEC-induced actin polymerization *in vitro*. The 1H10 antibodies mixed with bacteria prior to challenge reduced mice colonization [81]. Finally, a recent study using CoilA and CoilB, two synthetic peptides designed to interact with EspA, were shown to block colon damage in mice infected with *C. rodentium* [82]. Overall, these studies made evident a lack of consensus regarding the role of sIgA during EHEC vaccination with T3SS proteins, which suggests that the protective mechanisms elicited in mice may vary depending on the type or subset of antigens used, the delivery route, and the adjuvant selected.

5.3 Subunit fusion vaccines

To generate multivalent protective effects of subunit-based vaccines, fusion proteins that include Stx subunits and T3SS proteins have been constructed and evaluated. The EIS fusion, comprised by EspA, the C-terminal 300 residues of intimin and the B subunit of Stx2, was shown to induce specific humoral antibodies against the three antigens (Table 1). EIS protected mice against EHEC challenge more efficiently than the 3 antigens independently administered [83]. Further, oral inoculation of mice with a live attenuated *Salmonella* strain expressing EIS produced specific sIgA against EspA, intimin and Stx2B and antigen-induced proliferation of splenocytes. These effects were enhanced by s.c. boost with purified EIS fusion vaccine [84]. Long lasting immunity is a key requirement for any feasible vaccine candidate. In this regard, the immune responses triggered by the EIS-expressing *Salmonella* can be subcutaneously boosted even after 70 days and produce significant protection against a lethal challenge [84]. Long lasting immunity was also

achieved with Stx2B fused to the alpha antigen expressed and secreted in *Mycobacterium bovis* bacillus Calmette-Guerin vaccine. This fusion induced specific mucosal IgA that lasted for two months and partially protected against the purified Stx2 or heterologous challenge with the Stx2-expressing *E. coli* [85].

The s.c. immunization with a fusion consisting of the C-terminal 120 residues of EspA, the C-terminal 282 amino acids of intimin and the Tir site for intimin interaction (the EIT fusion), reduced the duration of EHEC shedding. This effect correlated with the generation of specific IgG antibodies able to block EHEC cell adherence *in vitro*, without the induction of specific sIgA [86]. However, serum specific IgG and both serum and fecal specific IgA are induced when a plant-derived EIT fusion protein is orally delivered. This induction correlated with reduction in EHEC shedding [87]. Moreover, i.m. vaccination with a plasmid DNA vaccine coding the EIT fusion induced EIT-specific IgG, reducing shedding in mice and these effects were enhanced by a boost with purified EIT [88].

Other fused antigens evaluated in mice include a fusion composed of the B subunits of the two types of Stx, Tir and the Zonula occludens toxin (Zot) from the CTX ϕ bacteriophage of *Vibrio cholerae*, which improves antigen delivery by increasing epithelial permeability [89]. The s.c. immunization of Stx2B-Tir-Stx1B-Zot fusion produced antigen-specific systemic IgG, while i.n. immunization generated both serum IgG and sIgA. Both immunization routes decreased bacterial shedding after challenge. In a different study, the SSI fusion (consisting of Stx2B, Stx1B and a fragment of intimin) was shown to be protective against large doses of EHEC [90]. As other Stx-based candidates, SSI induced a Th2-mediated humoral response, characterized by predominance on IgG1 subtype and expression of IL-4 and IL-10. Finally, a different fusion containing the A1 peptide of Stx2 fused to EspA was shown to increase mice survival to a lethal dose of Stx2 [91].

5.4 Bacterial cell-based candidates

Traditional vaccine approaches using attenuated or killed bacteria are still evaluated for protection against EHEC infection. EHEC strain 86-24 *ler/stx* deletion mutant (F25), as well as its derivative expressing inactive Stx1 and Stx2 (F105), was shown to reduce the bacterial shedding in mice [92]. F25 cytotoxicity *in vitro* was abolished and F105 cytotoxicity was highly diminished, resulting in both strains being non-pathogenic to mice. Noteworthy, immunized pregnant mice were able to passively immunize their suckling offspring. In contrast to this traditional method of vaccination, other novel approaches have been developed that are producing promising candidates.

For example, a recently evaluated technology in search for an effective EHEC vaccine is the generation of bacterial ghosts (BG) [93-96]. BG consist of empty bacterial cell envelopes devoid of cytoplasm by the controlled expression of the viral protein E, which forms a tunnel structure across the bacterial cell wall, resulting in the release of the cytosolic content [93]. The BGs maintain intact cellular morphology and bacterial surface structures and are highly immunogenic, possessing natural adjuvant activity. Because of this, BG has been assayed as a vaccine in several Gram-negative pathogens [93]. EHEC N^oCIP 105282 BG has been produced with the combination of E protein and the staphylococcal nuclease A, in order to ensure degradation of pathogenic DNA [95-96]. Oral [95] and rectal [96] single dose immunization of these BG were able to stimulate humoral and cellular immune responses and significantly protect against a lethal challenge with a heterologous EHEC strain. Oral immunization protection was significantly increased after a boost, while single rectal inoculation was sufficient to fully protect against one LD₅₀. Oral inoculation of EHEC EDL933 BG in mice also showed similar results [94]. Protection increases from 56% to 84% after a boost. BG vaccination induced specific IgG and IgA in serum and colon. In this case, humoral and cellular immune responses showed increases in both Th1- and Th2-markers,

which indicated a mixed immune response. However, the detection of a high IgG1/IgG2 ratio and the high concentration of IL-4 in peripheral blood as well as the lack of BG-induced proliferation of CD8⁺ T cells indicated that the immune response was dominated by a Th2-mediated humoral response. Accordingly, experiments with systemic vaccination using EHEC BGs in calves significantly reduced total bacterial shedding and induced the production of BG-specific IgG in serum together with low levels of specific IgA and IgG in saliva [97].

6. Concluding remarks and future directions

Using mice as a model for EHEC-mediated disease, several vaccine candidates have shown promising results. Although the immune response developed varies according to the delivery route, all protective candidates seem to induce a Th2-biased humoral immune response. As expected when mucosal sites are primed, sIgA is generally involved in protection when i.n., oral or rectal routes are used, but serum IgG-mediated protection without the induction of IgA are also reported, particularly when systemic routes are used. Cellular immune responses are involved, but lack of assessment in this regard on most studies may mask their importance during protection. Finally, the development of vaccine candidates has been limited to a reduced number of virulence factors and; therefore, the likelihood to develop a human vaccine is restricted to those antigens. As such, our group has used the murine model to identify EHEC-specific antigens that can be used as potential vaccine candidates by screening whole genome *E. coli* sequences. Using bioinformatics analysis, we have identified 25 DNA sequences as those encoding potential vaccine targets against EHEC infection and evaluation of these candidates as DNA vaccines in mice has rendered lead candidates that provide different levels of protection (data not shown). The murine model is and remains a useful and reliable screening tool necessary to identify additional candidates that can be incorporated into the pipeline for development of an optimal human vaccine.

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Highlights

- Review of different murine model approaches to evaluate EHEC-specific vaccines.
- Summarize recent progress in the development of enterohemorrhagic *Escherichia coli* vaccines.
- Compare murine immune responses to those required for protection in human infections.
- Summarize limitations and advantages to use murine models to test EHEC vaccine immunogenicity

Table 1
Comparative of recent studies for EHEC vaccines using active immunization and *in vivo* challenge in the murine model.

Antigen	Immunization Doses /Route ^a	Immune responses	Challenge	Outcome	Reference
Stx2A/StxB1	4 / i.p.	Neutralizing anti-Stx1 and anti-Stx serum IgG	10 LD ₅₀ of Stx1, Stx2 or both	100% survival to single toxin and 90% survival to mixed toxin challenge	[65]
Stx1B or Stx2B	3 / i.n.	Cross neutralizing anti-Stx1 and anti-Stx2 serum IgG and bronchial alveolar IgA when Stx2B was used	200 µg/kg Stx1 or 1 µg/kg Stx2	50% survival to Stx1 or Stx2 with the stx2B vaccine	[66]
Stx2B-Stx1B (2S)	3 / i.p.	Serum IgG. Th2-biased	2 and 4 LD ₅₀ of EHEC lysate	90% survival to low dose, 30% survival to high dose	[67]
Stx2Am-Stx1B (SAmB)	3 / i.p.	Specific serum IgG and IgA. Th2-biased	10 LD ₅₀ of EHEC lysate or purified Stxs	93% survival to lysate 80-100% survival to Stxs	[69]
Stx2ΔAB	3 / i.m. plasmid DNA	Systemic specific IgG	1.6 × 10 ⁴ µg of Stx2	50%-70% survival	[70]
	3 / i.g. antigen-expressing <i>Salmonella</i>	Specific serum IgG and fecal IgA	53 ng of purified Stx2	12.5%-25% survival	[71]
K-alpha-Stx2B	3 / i.p. antigen-expressing BCG	Specific serum IgG and long-lasting specific sIgA	2.9 ng/mouse (2 LD ₅₀) of Stx2 or 10 ³ of Stx2-producing <i>E. coli</i> B2F1	Partial survival to purified Stx2 and B2F1 strain (57-63 %).	[85]
Type III-secreted proteins (TTSPs)	2 / i.n. or s.c.	i.n.: serum specific IgG and fecal IgA s.c.: serum specific IgG	10 ¹⁰ CFU <i>E. coli</i> O157:H7	i.n.: Reduction of shedding s.c.: Elimination of shedding	[74]
Tir	2 / i.n. or s.c.	i.n.: serum IgG and IgA. Fecal IgA s.c.: serum IgG	1 LD ₅₀ of lysed EHEC	i.n.: 92.9% survival s.c.: 64.3% survival	[75]
KT-12 intimin B-cell epitope	3 / i.n. or s.c.	i.n.: Serum IgG and IgA s.c.: Serum IgG	1 × 10 ¹⁰ CFU O157:H7	i.n.: 60% survival s.c.: 26.6% survival	[76]
Intimin	2 / i.g. antigen-expressing <i>Salmonella</i>	Specific serum IgG and sIgA	10 ⁸ CFU O157:H7	Reduced bacterial shedding	[78]
EspA-Intimin ₃₀₀ -Stx2B (EIS)	3 / s.c.	Serum specific IgG	0.05 mg of lysate of O157:H7 or 1 × 10 ⁹ CFU of O157:H7	2/3 survival to toxemia. 93% survival and reduced shedding to live EHEC challenge	[83]
	3 / i.g. antigen expressing <i>Salmonella</i> . s.c. boost with protein	Serum specific IgG and fecal IgA. Antigen-induced splenocyte proliferation	1 × 10 ⁹ CFU of O157:H7	83.3 % protection ratio with short term boost. 66% with long term boost.	[84]
EspA _{C120} -intimin _{C282} -Tir ₂₅₈₋₃₆₁ (EIT)	3 / s.c.	Serum specific IgG. No fecal IgA	10 ¹⁰ CFU O157:H7	Reduced bacterial shedding	[86]
	5 / s.c. purified and/or i.g. with transgenic canola extract	Serum specific IgG and IgA. Fecal IgA	10 ¹⁰ CFU O157:H7	Reduced the duration of shedding	[87]

Antigen	Immunization Doses /Route ^a	Immune responses	Challenge	Outcome	Reference
	4 / s.c. protein and/or i.m. plasmid DNA	Serum specific IgG	10 ¹⁰ CFU O157:H7	Reduced bacterial shedding	[88].
EspA-Stx2A1	3 / s.c.	Serum specific IgG	50 µg of Stx2	95% survival	[91]
Stx2-Tir-Stx1B-Zot	2 / s.c. or i.n.	s.c.: serum specific IgG i.n.: serum specific IgG and fecal IgA	10 ¹⁰ CFU of O157:H7	Increased mice survival, reduced shedding duration.	[89]
Stx2B-Stx1B-Int281	2 or 3 / i.p.	Serum specific IgG, predominant IgG1. Induction of IL-4 and IL-10	10, 50 or 100 LD ₅₀ of O157:H7	Increased survival of mice to large doses of EHEC.	[90]
EHEC <i>ler/stx</i> deletion mutant expressing inactive Stx1 and Stx2	2 / i.p.	ND	2 × 10 ⁷ CFU O157:H7	Reduction of the duration of shedding. 60 % survival.	[92]
EHEC bacterial ghosts	1 or 2 / rectal	Specific serum IgG and IgA and intestinal IgA. Proliferation of IFN-γ splenocytes.	1 LD ₅₀ of O157:H7	Reduction of shedding. 100% survival	[96]
	1 or 2 / i.g.	Specific IgG and IgA serum. Colonic IgA. Th2-dominated Th1/Th2 mixed response. Induction of IL-4 but also INF-γ and TNF-α	10 ⁹ O157:H7	Reduction in shedding. 56%-84% survival.	[94]

^a Abbreviations: i.p., intraperitoneal; i.n., intranasal; s.c., subcutaneous; i.g., intragastrically