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SHORT REPORT

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OMV-based vaccine formulations against Shiga toxin producing *Escherichia coli* strains are both protective in mice and immunogenic in calves

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ABSTRACT

Strains of Shiga toxin-producing *Escherichia coli* (STEC) can cause the severe Hemolytic Uremic Syndrome (HUS). Shiga toxins are protein toxins that bind and kill microvascular cells, damaging vital organs. No specific therapeutics or vaccines have been licensed for use in humans yet. The most common route of infection is by consumption of dairy or farm products contaminated with STEC. Domestic cattle colonized by STEC strains represent the main reservoir, and thus a source of contamination. Outer Membrane Vesicles (OMV) obtained after detergent treatment of gram-negative bacteria have been used over the past decades for producing many licensed vaccines. These nanoparticles are not only multi-antigenic in nature but also potent immunopotentiators and immunomodulators. Formulations based on chemical-inactivated OMV (OMVi) obtained from a virulent STEC strain (O157:H7 serotype) were found to protect against pathogenicity in a murine model and to be immunogenic in calves. These initial studies suggest that STEC-derived OMV has a potential for the formulation of both human and veterinary vaccines.

Hemolytic Uremic Syndrome (HUS) is a serious human disease of the microvasculature frequently affecting children below the age of five. Its acute phase can cause 3–5% deaths and in the long term up to 30% of the patients suffer different degrees of kidney damage, which eventually leads to chronic kidney failure.[1,2] Shiga toxins producing *Escherichia coli* (STEC) strains are the main etiological agent of the infectious form of HUS.[3,4] Shiga toxins (Stx) are a group of AB_5 protein toxins that exert their pathogenicity through binding and killing microvascular cells.[3] To date, no specific treatment is available for HUS, although some therapeutic candidates are in advanced stages of development.[5,6] Out of the hundreds of STEC serotypes detected in HUS patients, O157:H7 serotype is by far the most frequently isolated.[7,8] Typical STEC infection in humans is linked to consumption of meat and dairy or farm products contaminated with ruminant feces. STEC strains are able to attach to and colonize the gastrointestinal tract of a wide array of hosts, including humans.[3,9] Healthy cattle is considered the main zoonotic reservoir of STEC strains. The Stx produced after colonizing the human gut go through the epithelia by a complex mechanism involving neutrophil transmigration.[10] Having been granted access to circulation, Stx are able to exert their detrimental action onto target tissues. Although Stx are responsible for most of the pathogenicity of STEC strains, they do not play a key role in gastrointestinal tract colonization. The molecular mechanisms of STEC colonization have been extensively studied (for reviews, see ref. [3,11–13]). Flagella, LPS, and long polar fimbriae act during initial contact of bacteria with the epithelia. Proteins associated with or secreted by a type three secretion system (TTSS) display a critical role after this initial interaction, originating the so-called attaching and effacing (A/E) lesions on intestinal epithelia. Other relevant virulence factors during gastrointestinal tract colonization are intimin and enterohemorrhagic *E. coli* factor for adherence 1 (efa-1). Vaccine candidate's design for prevention of HUS has been a large field of research through the past three decades. The main strategies that are followed can be divided in two groups, according to their specific objectives: (1) the generation of systemic responses able to bind and neutralize Stx, thus abrogating their detrimental effect on target tissues; (2) inhibition of STEC attachment and colonization of the gastrointestinal tract through mucosal immune defenses. The first strategy is aimed at direct prevention of the disease by human vaccination. Although vaccine candidates based on Stx toxoids, recombinant Stx, heterologous expression, and outer membrane vesicles (OMV) have proven successful in animal models of lethal Stx challenge, none of them has been licensed to date.[14–18] The second strategy could also be divided in two, whether it is destined at direct protecting humans through vaccination or indirect protecting humans by vaccinating cattle. Indeed, massive cattle vaccination is proposed as one of the interventions with the highest potential for lowering HUS incidence in humans.[19–21] Extensive research has been conducted on such vaccine candidates, in both murine and bovine models. These models include formulations based on recombinant expression of virulence factors,[22–26] culture supernatants from

virulent strains grown under conditions promoting virulence factor secretion,[27,28] subunits or components directly extracted from STEC strains,[29–31] and heterologous expression of STEC virulence factors in both attenuated and unrelated bacteria.[32] Due to the complex mechanism responsible for colonization, it is not surprising that protection has only been observed for multi-antigenic formulations. Noteworthy, as anti-STEC vaccination in domestic cattle will not bring any economic benefit to cattle breeders, keeping the cost per dose of candidates as low as possible is mandatory.[19] This might explain why despite the proven efficacy of various vaccine candidates, only two have been commercialized: Econiche® from Vetòquinol and Epitopix® from Pfizer. Both commercial vaccines are relatively inexpensive to produce from virulent STEC cultures, thus avoiding more expensive technologies such as recombinant protein expression. Econiche® vaccine is based on TTSS proteins obtained from culture supernatants, while Epitopix® is composed mainly of two kinds of proteins (porins and siderophores) extracted from the culture biomass. They have shown effectiveness in reducing O157 serotype prevalence in cattle under conditions of natural exposure.[33] Unfortunately, an increasing number of outbreaks are linked to STEC strains that do not carry the locus of enterocyte effacement (LEE), where the TTSS is coded. This fact, combined with the variability observed in STEC strains responsible for recent important outbreaks, highlights the need for a broader antigenic-range vaccine. Moreover, it would be of great value if this candidate could also be applied to direct HUS prevention in humans.

The OMV are proteolipidic nanoparticles purified from the external membrane of gram-negative bacteria. They are usually obtained by two alternative methodologies, one based on detergent extraction and the other by inducing the release of blebs during bacterial growth.[34] Lipopolysaccharide (LPS) is a major constituent of the outer membrane that gives OMV relevant adjuvant properties but it also poses a threat due to its inherent toxicity. In this sense, native OMV produced during growth of STEC strains harbor enough Stx and LPS to induce HUS-like symptoms and signs when administered intraperitoneally to mice.[35] One key advantage of OMV production based on detergent extraction is that during this process the vesicles are submitted to simple steps of purification that drive their LPS content within limits compatible with vaccine formulation. Although OMV consist of components of the outer membrane associated with detergent molecules and filled with periplasmic content, traces of material present in the external milieu, including extracellular virulence factors, are also expected to be present. Moreover, since detergent addition during OMV production causes some degree of cell lysis, intracellular components are also expected to be incorporated into the vesicles up to a certain level. In brief, OMV are multi-antigenic, the protective response that they induce is obtained by a cooperative effect of the specific responses to multiple antigens and Molecular Patterns Associated Pathogens (PAMPs), each of which may be insufficient to provide immunity if administered independently.[34] These properties, combined with low-cost production processes at the industrial level, make them promising vaccine candidates.[36]

Most murine models that evaluate toxicity induced by STEC strains rely on injection of crude or pure Stx preparations and often measure mortality as the endpoint.[37] The two types of Stx (Stx1 and Stx2) are coded in lysogenized phages integrated into the bacterial chromosome. Their expression is induced under conditions that initiate the phage lytic cycle, causing Stx release to the external milieu. Other relevant extracellular virulence factors are also produced by STEC strains, such as enterohemolysin (EHEC-Hly), subtilase (SubAB), and serine protease (EspP).[38–40] We therefore decided to evaluate the immunoprotective potential of the OMV-based vaccine formulations against preparations comprising also extracellular toxins other than Stx. To address this objective, a concentrated culture supernatant (CS) was prepared as follows: A virulent E. coli. O157:H7 strain (EO1570110, kindly provided by Dr. Marta Rivas, Servicio de Fisiopatogenia, ANLIS "Dr. Carlos G. Malbrán", Buenos Aires, Argentina) was cultured at laboratory scale under Stx expression-inducing conditions, i.e., addition of 0.08 μg/ml ciprofloxacin to a late log phase culture of the EO1570110 strain. After 12–16 h induction, the cultures were centrifuged, the supernatants concentrated 10X and diafiltered against 15 volumes of saline using a Sartocon Slice® with a 10,000Da MWCO filtration membrane. The protein concentration in the CS was 7.5 mg/ml, as determined by the Lowry method. Preliminary experiments in Balb/c mice showed that intraperitoneal injection of a CS dose containing 0.08 μg protein was sufficient to cause 90% mortality 7 days after challenge (results not shown).

Two formulations were then prepared for evaluating their immunoprotective potential in this murine challenge model. One formulation was based exclusively on OMV obtained by detergent extraction, as in ref. 41, with minor modifications. Briefly, an inoculum of the EO1570110 strain was grown at laboratory scale in trypticase soy broth (TSB) supplemented with 44 mM sodium bicarbonate, until late log-phase. The biomass was then isolated by centrifugation and homogenized in a 30 mM tris buffer, containing 2mM EDTA, pH 8.5 at a ratio of 100–200 mg/mL. Sodium deoxycholate (Fluka, Switzerland) was then added at a ratio of 0.1-1 mL/g of biomass, incubated for 1 hour and centrifuged for 15 min at 33,000 g. All supernatants were collected, subjected to a sequence of diafiltration processes against saline, and filter-sterilized using a Sartorius Minisart-plus unit of 0.2 μm pore size. General features of the OMV were characterized by routine techniques: protein concentration in stock solution (>0.5 mg/ml), DNA content (<0.035 μg DNA/μg protein), LPS content (0.02–0.12 μg LPS/ μg protein), endotoxicity (<20,000 EU/ml, LAL test), particle size and polydispersion (99 nm and 0.28, respectively). These physicochemical properties are similar to those of licensed OMV vaccines or formulations currently in advanced phases of development.[34,41] As protein toxins including Stx are expected to be present in the OMV, a glutaraldehyde inactivation step was introduced after OMV extraction from the biomass.[42] The inactivated OMV (OMVi) were finally adsorbed onto aluminum hydroxide adjuvant (Alhydrogel®), at a relation 1 mg adjuvant to 25 μg protein, to obtain a single-bulk. Protein adsorption to adjuvant in this single-bulk was determined and found to be above 90% level. Vaccine formulations at the time of inoculation were prepared by taking samples under sterile conditions from this single-bulk and achieving the desired final protein concentration by dilution with saline. The other vaccine

formulation was also based on OMVi antigen, but in combination with glutaraldehyde-inactivated CS preparation (CSi). This combined antigen was also adsorbed to aluminum adjuvant following the same single-bulk strategy as before, also formulating the vaccines at the time of inoculation by sampling and dilution with saline aseptic conditions (OMVi/CSi formulation).

Three groups of 10 Balb/c mice were allocated to their cages, 10 μg protein doses of the OMVi formulation were assigned to group 1, 10 μg protein doses of the OMVi/CSi formulation to group 2, and aluminum adjuvant in saline to group 3 (control). All three groups were vaccinated by the subcutaneous route on days 0 and 21 and intraperitoneally challenged 2 weeks after the second dose with the CS preparation. Mice were kept under observation during the following seven days and signs of distress and mortality were recorded on a daily basis (Fig. 1). All animals in the control group started showing signs of distress 24 hours after challenge, with lethal outcome in 90% of the cases by day 7. By contrast, no signs of distress were observed in mice from the two vaccinated groups, showing 100% protection from lethal outcome by day 7. Since equal protection was observed in both vaccinated groups, it can be concluded that the effect of the OMVi component was dominant. To further characterize the immune response elicited by this component, mice sera from group 1 were used as the primary antibody in the following western blot experiment. As OMVi are produced by chemical inactivation of OMV, this process generates aggregates of different size that are unable to be analyzed by SDS-PAGE. This situation precluded direct analysis of the immune response generated by the antigens in the OMVi in a western blot. To overcome this impediment the immunological analysis was organized as follows: a bacterial lysate of the O157:H7
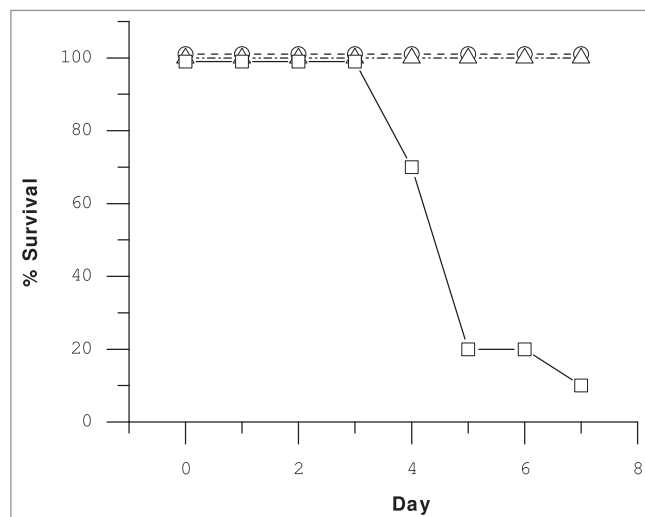
strain, representative of biomass constituents, was run on one lane of an SDS-PAGE gel. The CS preparation used for the challenge, representative of extracellular components, was run in a second lane of the same SDS-PAGE gel. After resolution, the proteins were transferred to a nitrocellulose membrane, and pooled sera from group 1 mice were used as the primary antibodies and anti-mouse IgG conjugated to horseradish peroxidase as the secondary antibody in a western blot (Fig. 2). Intense recognition was observed in immunized mice sera on three molecular weight regions present in both protein extracts (below 14 kDa region, 33 kDa band, and 50–65 kDa region). The identity of the bands has not been elucidated yet, although proteomic analyses of OMV composition are being performed. Also, some minor protein bands were exclusively present or recognized in one or the other protein fraction (i.e., 22 kDa, 30 kDa). Therefore, CSi addition to vaccine formulations has no clear effects or benefits. As maintaining formulation complexity low helps reduce production costs, formulations containing only the OMVi component were considered for further experiments.

Since domestic cattle are the main reservoir of zoonotic STEC strains, it would be important to assess whether OMVi formulations are able to prevent bovine colonization. As a first step, immunogenicity testing was performed. Fifteen calves from a beef producing brand (Aberdeen Angus) between six and eight months old were allocated to a single pen and randomly divided into three groups of five. Group 1 was assigned a 50 μg OMVi plus aluminum adjuvant per dose, group 2 a 100 μg OMVi plus aluminum adjuvant per dose, and group 3 was treated with aluminum adjuvant in saline (control). Each group was vaccinated subcutaneously on days 0, 21, and 42. Blood was extracted periodically for 99 days and the humoral response for each day and animal was assessed by an indirect ELISA using OMVi as the capture antigen in the solid phase and anti-bovine IgG conjugated to horseradish peroxidase as the secondary antibody. The optical density at 450 nm (OD_{450nm}) registered for each well was normalized to the OD_{450nm} of an in-house positive control serum (response). Fig. 3 shows the kinetics of the specific humoral response observed on each group. The error correlation structure was modeled based on linear mixed model statistical analysis and maximum-likelihood criteria. After a significant interaction was detected between the time and treatment factors ($p<0.05$), a contrast analysis of the response was performed between each pair time*treatment. As a result, both OMVi doses are immunogenic at similar levels, showing non-significant differences between them at peak (day 49) and long-term times ($p<0.05$). This is important in terms of productivity since a comparable response was achieved in group 1 with half the antigen dose employed for group 2. Noteworthy, both vaccinated groups showed a rapid decay in their humoral response 50 days after the peak, with response levels at day 99 being approximately half of those at day 49. Similar kinetics has been reported in cattle vaccinated with commercial vaccines. Allen et al. showed that for the commercial Econiche® vaccine, antibodies peak 14 days after the last immunization dose and fall approximately to half during the following two weeks.[27] However, these results should be analyzed with caution. The levels of circulating antibodies are not expected to correlate with protection at the

Figure 1. Protection against lethal Stx challenge: Thirty Balb/c mice (18-22 g weight) were divided into three equal groups and allocated in their cages. Group 1 (open circles) was treated with 10 μg protein per dose of a formulation based on OMVi immunogen adsorbed to aluminum adjuvant. Group 2 (open triangles) was treated with 10 μg protein per dose of a formulation based on the combined OMVi / CSi immunogen adsorbed to aluminum adjuvant. Group 3 (open squares) received aluminum adjuvant in saline (control). All three groups were vaccinated subcutaneously on days 0 and 21 and intraperitoneally challenged 2 weeks after the last dose with the CS preparation. Mice were maintained under observation during the following seven days and signs of distress and mortality were recorded on a daily basis. In this Figure, the probability of survival at each time point (survival) is plotted against the days of observation after challenge.

effective against infection in humans, a critical aspect for the control of outbreaks. Studying the inhibition of bacterial adhesion to cell lines or tissue explants by sera from immunized animals could add complementary information to the functional analysis of immunized sera. Although mice are not naturally colonized by STEC strains and do not show the typical A/E lesions, murine models of infection have been developed for evaluating protection by anti-STEC vaccine formulations.[37] One of these models is currently used for studying OMVi-based vaccine formulations with different adjuvants (oleic vs mineral adjuvants), routes of administration (subcutaneous vs intrarectal), and changes on immunogenic composition (production of OMV from the biomass of bacteria grown under different conditions). These experiments will help in the design of an optimized formulation to be directly tested for protection against cattle colonization. Two types of studies are usually performed
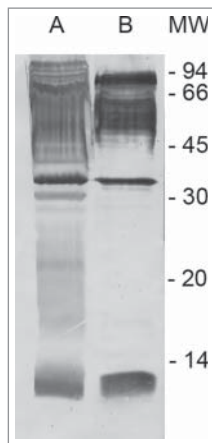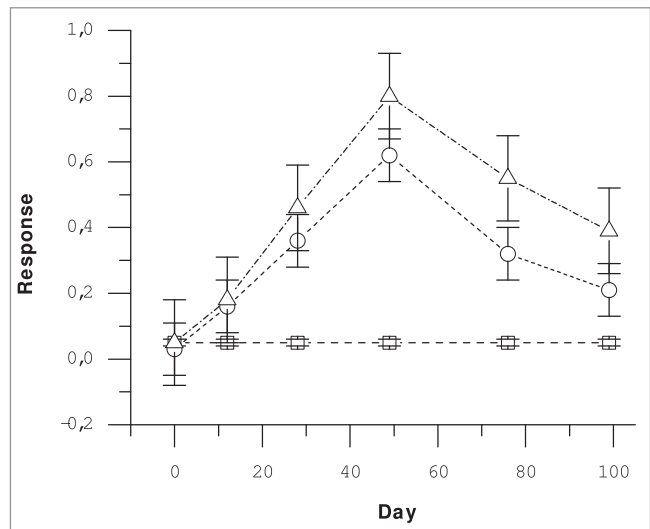

Figure 2. Humoral response characterization: Two protein extracts derived from the virulent O157:H7 serotype strain used in these experiments were separated on 15% SDS-PAGE and analyzed by the western blot technique. Sera from mice immunized with the OMVi formulation (group 1) were used as the primary antibody (1:300 dilution in saline). Anti-mouse IgG conjugated to horseradish peroxidase (Sigma Aldrich, San Luis, USA) was the secondary antibody (1:1000 dilution in saline). Lane A was loaded with the CS used in the lethal challenge and lane B with a bacterial lysate of the O157:H7 strain on. Molecular weight marker (MW) positions are indicated in kDa on the right of the image.

colonization site, the recto-anal junction. First of all, locally produced IgA and not circulating IgG are the dominant immunoglobulins at this site. Also, it has been recently shown a Th1 skew to the immunological response at the rectal mucosa after *E. coli* O157:H7 infection, suggesting a relevant role for cellular immunity effectors on bacterial clearance.[43] Nevertheless, the aim of our work was to make an initial assessment on the immunogenicity of OMVi formulations. Other relevant immunologic parameters, such as specific IgA levels in feces or cellular immune response assessment at the rectal mucosa, will be addressed in future studies.

To conclude, OMVi-based formulations are protective against STEC pathogenicity in a murine model and immunogenic in calves. The protection observed against STEC pathogenicity is based on the immune neutralization of the activity of several toxins present in the SC. In terms of a human vaccine, it would be important to evaluate how much of this effect was originated on a specific anti-Stx response. Toxicity to Vero cell monolayers is the gold standard technique for specific Stx detection and quantification.[3] Then, the anti-Stx response in immunized animals' sera could be analyzed by evaluating their neutralization potential against SC verocytoxicity. As stated before, cross-linking of OMV during glutaraldehyde inactivation precluded OMVi analysis by means of SDS-PAGE and immunoblot. OMV samples obtained before inactivation will then be used in future experiments to provide the capture antigens in the western blot for assessing the immunogenic properties of OMVi. Moreover, this non-cross-linked material is currently been analyzed based on mass spectrometry.

Human infection and cattle colonization are initiated by bacterial attachment to the distal portions of the rectum. Moreover, the sera from HUS patients and infected cattle cross-recognize most of the virulence factors known to have a critical role in colonization.[44-46] Although our work is focused on preventing infection in cattle, it could be expected that an effective vaccine candidate against infection in cattle could also be

Figure 3. Immunogenicity testing in calves: Fifteen calves from a beef-producing brand (Aberdeen Angus) between six and eight months old were allocated in a single pen and randomly divided into three groups of five. Colonization status by O157 serotype strains was analyzed before and during the experiments for each animal by immune-specific enrichment and detection techniques. All animals were free from O157 from the beginning to the end of experiments (data not shown). Group 1 (open circles) was treated with 50 μg OMVi plus aluminum adjuvant per dose, group 2 (open triangles) with 100 μg OMVi plus aluminum adjuvant per dose and group 3 (open squares) was treated with aluminum adjuvant in saline (control). Each group was vaccinated subcutaneously on days 0, 21, and 42. Jugular vein blood was extracted periodically during the 99 days of the experiment and clotted to obtain sera for further immunogenicity testing. The humoral response for each day and animal was assessed by an indirect ELISA using OMVi as the capture antigen in the solid phase. All sera were diluted 1:100 with saline and added, in triplicate, to each well of a 96 positions ELISA plate (Polysorp®, Nunc, Denmark). An in-house positive control was constructed by pooling the sera obtained from Group 2 animals on day 49 of the experiments. This positive control was diluted 1:100 in saline and added in triplicate to the wells of every ELISA plate. A 1:10,000 dilution of a rabbit anti-bovine IgG conjugated to horseradish peroxidase (Sigma Aldrich, San Luis, USA) was used as the secondary antibody. After stopping peroxidase reaction with 2 N sulfuric acid, the optical density at 450 nm (OD_{450nm}) was determined. In order to account for plate to plate variability, an arbitrary variable was defined (Response) as the quotient between each well's OD_{450nm} and the mean OD_{450nm} from the positive controls in the same plate. In this figure, mean Response values for each group of animals are shown, from three independent plates, with their corresponding standard error bars. The error correlation structure was modeled based on linear mixed model statistical analysis and maximum-likelihood criteria. Statistical analysis revealed a significant interaction between the factors time and treatment ($p < 0.05$). Contrast analysis was performed on the response observed for each pair time*treatment ($p < 0.05$).

with such an aim: protection from natural exposure in the field and protection from an experimental challenge by oral administration of virulent bacteria. The latter, although requiring more complex assets (ABSL-2 for large animals) and only indirectly representing a situation of natural exposure to the bacteria, allows for testing and comparing different variables (antigenic composition, doses, vaccination schedules, different adjuvants, routes of administration) with lesser animals. It is then adequate for initial assessment of vaccine efficacy against colonization. Noteworthy, several STEC serotypes other than O157:H7 (e.g., O26:H11, O121:H19, O145:H-, O103:H2, etc.) are responsible for an increasing number of outbreaks.[47] While most lines of research on veterinary vaccines are aimed exclusively at the O157:H7 serotype, available commercial vaccines have not proven effective against non-O157 strains, in the field.[48,49] The multi-antigenicity of OMV-based formulations vaccine could bring an opportunity for cross-protection against different serotypes. This important aspect will also be addressed in future experiments.

Disclosure of potential conflicts of interest

The authors report no conflict of interest.

Funding

References

1. Repetto HA. Long-term course and mechanisms of progression of renal disease in hemolytic uremic syndrome. Kidney Int Suppl. 2005;68:S102–6. doi:10.1111/j.1523-1755.2005.09717.x.

2. Cobeñas CJ, Alconcher LF, Spizzirri AP, Rahman RC. Long-term follow-up of Argentinean patients with hemolytic uremic syndrome who had not undergone dialysis. Pediatr Nephrol. 2007;22:1343–7. doi:10.1007/s00467-007-0522-2. PMID:17564728.

3. Nataro JP, Kaper JB. Diarrheagenic Escherichia coli. Clin Microbiol Rev. 1998;11:142–201. PMID:9457432.

4. Karmali MA, Mascarenhas M, Shen S, Ziebell K, Johnson S, Reid-Smith R, Isaac-Renton J, Clark C, Rahn K, Kaper JB. Association of genomic O island 122 of Escherichia coli EDL 933 with verocytotoxin-producing Escherichia coli seropathotypes that are linked to epidemic and/or serious disease. J Clin Microbiol. 2003;41:4930–40. doi:10.1128/JCM.41.11.4930-4940.2003. PMID:14605120.

5. Melton-Celsa AR, O'Brien AD. 2014. New therapeutic developments against Shiga toxin-producing Escherichia coli. Microbiol Spectrum 2014;2(5):EHEC-0013-2013. doi:10.1128/microbiolspec.EHEC-0013-2013.

6. Mejías MP, Hiriart Y, Lauché C, Fernández-Brando RJ, Pardo R, Bruballa A, Ramos M V., Goldbaum FA, Palermo MS, Zylberman V. Development of camelid single chain antibodies against Shiga toxin type 2 (Stx2) with therapeutic potential against Hemolytic Uremic Syndrome (HUS). Sci Rep. 2016;6:24913. doi:10.1038/srep24913. PMID:27118524.

7. Ibarra C, Goldstein J, Silberstein C, Zotta E, Belardo M, Repetto HA. [Hemolytic uremic syndrome caused by enterohaemorrhagic Escherichia coli]. Arch Argent Pediatr. 2008;106:435–42. PMID:19030644.

8. Rivas M, Miliwebsky E, Chinen I, Deza N, Leotta GA. [The epidemiology of hemolytic uremic syndrome in Argentina. Diagnosis of the etiologic agent, reservoirs and routes of transmission]. Medicina (B Aires). 2006;66 (Suppl) 3:27–32.

9. Naylor SW, Low JC, Besser TE, Mahajan A, Gunn GJ, Pearce MC, McKendrick IJ, Smith DGE, Gally DL. Lymphoid follicle-dense mucosa at the terminal rectum is the principal site of colonization of enterohemorrhagic Escherichia coli O157:H7 in the bovine host. Infect Immun. 2003;71:1505–12. doi:10.1128/IAI.71.3.1505-1512.2003. PMID:12595469.

10. Hurley BP, Thorpe CM, Acheson DWK. Shiga Toxin Translocation across Intestinal Epithelial Cells Is Enhanced by Neutrophil Transmigration. Infect Immun. 2001;69:6148–55. doi:10.1128/IAI.69.10.6148-6155.2001. PMID:11553554.

11. Farfan MJ, Torres AG. Molecular Mechanisms That Mediate Colonization of Shiga Toxin-Producing Escherichia coli Strains STEC O157:H7 AND NON-O157 STEC. Infect Immun 2012; 80:903–13.

12. Tesh VL, O'Brien AD. Adherence and colonization mechanisms of enteropathogenic and enterohemorrhagic Escherichia coli. Microb Pathog. 1992;12:245–54. doi:10.1016/0882-4010(92)90043-N. PMID:1630295.

13. Etcheverría AI, Padola NL. Shiga toxin-producing Escherichia coli: factors involved in virulence and cattle colonization. Virulence. 2013;4:366–72. doi:10.4161/viru.24642. PMID:23624795.

14. Choi KS, Kim S-H, Kim E-D, Lee S-H, Han SJ, Yoon S, Chang K-T, Seo KY. Protection from Hemolytic Uremic Syndrome by Eyedrop Vaccination with Modified Enterohemorrhagic E. coli Outer Membrane Vesicles. PLoS One. 2014;9:e100229. doi:10.1371/journal.pone.0100229. PMID:25032703.

15. 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. doi:10.1016/j.vaccine.2010.11.035. PMID:21134452.

16. 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. doi:10.1128/IAI.73.10.6523-6529.2005. PMID:16177326.

17. Smith M, Tee L, Carvalho H, Melton-Celsa A, O'Brien A. D1. Smith M, Tee L, Carvalho H, Meltoncelsa A, O'brien A. Development of a hybrid Shiga holotoxoid vaccine to elicit heterologous protection against Shiga toxins types 1 and 2★. Vaccine. 2006;24:4122–9. evelopment of a hybrid Shiga holotoxoid vaccine to e. Vaccine. 2006;24:4122–9. doi:10.1016/j.vaccine.2006.02.035. PMID:16551486.

18. Gao X, Cai K, Li T, Wang Q, Hou X, Tian R, Liu H, Tu W, Xiao L, Fang L, et al. Novel fusion protein protects against adherence and toxicity of enterohemorrhagic Escherichia coli O157:H7 in mice. Vaccine. 2011;29:6656–63. doi:10.1016/j.vaccine.2011.06.106. PMID:21742003.

19. Smith DR. Vaccination of Cattle against Escherichia coli O157:H7. Microbiol Spectr. 2014;2. PMID:26104456.

20. 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.

21. 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. doi:10.1016/j.vetimm.2012.09.028. PMID:23084625.

22. Dean-Nystrom EA, Gansheroff LJ, Mills M, Moon HW, O'Brien AD. Vaccination of pregnant dams with intimin(O157) protects suckling piglets from Escherichia coli O157:H7 infection. Infect Immun. 2002;70:2414–8. doi:10.1128/IAI.70.5.2414-2418.2002. PMID:11953378.

23. Dziva F, Vlisidou I, Crepin VF, Wallis TS, Frankel G, Stevens MP. Vaccination of calves with EspA, a key colonisation factor of Escherichia coli O157:H7, induces antigen-specific humoral responses but does not confer protection against intestinal colonisation. Vet Microbiol. 2007;123:254–61. doi:10.1016/j.vetmic.2007.02.016. PMID:17374460.

24. 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. doi:10.1016/j.vaccine.2009.10.076. PMID:19903545.

25. McNeilly TN, Mitchell MC, Corbishley A, Nath M, Simmonds H, McAteer SP, Mahajan A, Low JC, Smith DGE, Huntley JF, et al. Optimizing the Protection of Cattle against Escherichia coli O157:H7 Colonization through Immunization with Different Combinations of H7 Flagellin, Tir, Intimin-531 or EspA. PLoS One. 2015;10:e0128391. doi:10.1371/journal.pone.0128391. PMID:26020530.

26. Vilte DA, Larzábal M, Garbaccio S, Gammella M, Rabinovitz BC, Elizondo AM, Cantet RJC, Delgado F, Meikle V, Cataldi A, et al. Reduced faecal shedding of Escherichia coli O157:H7 in cattle following systemic vaccination with γ-intimin C_{280} and EspB proteins. Vaccine. 2011;29:3962–8. doi:10.1016/j.vaccine.2011.03.079. PMID:21477674.

27. Allen KJ, Rogan D, Finlay BB, Potter AA, Asper DJ. Vaccination with type III secreted proteins leads to decreased shedding in calves after experimental infection with Escherichia coli O157. Can J Vet Res. 2011;75:98–105. PMID:21731179.

28. 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. doi:10.1016/j.vaccine.2003.08.007. PMID:14670317.

29. Vilte DA, Larzábal 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. doi:10.1016/j.vetimm.2012.03.002. PMID:22460171.

30. Cull CA, Paddock ZD, 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. doi:10.1016/j.vaccine.2012.05.080. PMID:22704925.

31. Thornton AB, Thomson DU, Loneragan GH, Fox JT, Burkhardt DT, Emery DA, Nagaraja TG. Effects of a siderophore receptor and porin proteins-based vaccination on fecal shedding of Escherichia coli O157:H7 in experimentally inoculated cattle. J Food Prot. 2009;72:866–9. doi:10.4315/0362-028X-72.4.866. PMID:19435240.

32. Khare S, Alali W, Zhang S, Hunter D, Pugh R, Fang FC, Libby SJ, Adams LG. Vaccination with attenuated Salmonella enterica Dublin expressing E coli O157:H7 outer membrane protein Intimin induces transient reduction of fecal shedding of E coli O157:H7 in cattle. BMC Vet Res. 2010;6:35. doi:10.1186/1746-6148-6-35. PMID:20609252.

33. 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. doi:10.1111/j.1863-2378.2011.01426.x. PMID:21824378.

34. Acevedo R, Fernández 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. doi:10.3389/fimmu.2014.00121. PMID:24715891.

35. Kim S-H, Lee Y-H, Lee S-H, Lee S-R, Huh J-W, Kim S-U, Chang K-T, Oswald E. Mouse model for hemolytic uremic syndrome induced by outer membrane vesicles of Escherichia coli O157:H7. FEMS Immunol Med Microbiol 2011; 63:427–34.

36. Perez O, Bracho G, Lastre M, Mora N, Del Campo J, Gil D, Zayas C, Acevedo R, Gonzalez D, Lopez JA, et al. Novel adjuvant based on a proteoliposome-derived cochleate structure containing native lipopolysaccharide as a pathogen-associated molecular pattern. Immunol

37. Mohawk KL, O'Brien AD. Mouse models of Escherichia coli O157:H7 infection and shiga toxin injection. J Biomed Biotechnol. 2011;2011:258185. doi:10.1155/2011/258185. PMID:21274267.

38. Bielaszewska M, Aldick T, Bauwens A, Karch H. Hemolysin of enterohemorrhagic Escherichia coli: Structure, transport, biological activity and putative role in virulence. Int J Med Microbiol. 2014;304:521–9. doi:10.1016/j.ijmm.2014.05.005. PMID:24933303.

39. Paton AW, Paton JC. Escherichia coli Subtilase Cytotoxin. Toxins (Basel). 2010;2:215–28. doi:10.3390/toxins2020215. PMID:20871837.

40. Weiss A, Brockmeyer J. Prevalence, biogenesis, and functionality of the serine protease autotransporter EspP. Toxins (Basel). 2012;5: 25–48. doi:10.3390/toxins5010025. PMID:23274272.

41. Fernández S, Fajardo EM, Mandiarote A, Año G, Padrón MA, Acosta M, Cabrera RA, Riverón LA, Álvarez M, Blaín K, et al. A proteoliposome formulation derived from Bordetella pertussis induces protection in two murine challenge models. BMC Immunol 2013 141. 2013;14:S8.

42. Brown JE, Griffin DE, Rothman SW, Doctor BP. Purification and Biological Characterization of Shiga Toxin from Shigella dysenteriae 1. Infect Immun. 1982;36:996–1005. PMID:7047403.

43. Corbishley A, Ahmad NI, Hughes K, Hutchings MR, McAteer SP, Connelley TK, Brown H, Gally DL, McNeilly TN. Strain-Dependent Cellular Immune Responses in Cattle following Escherichia coli O157: H7 Colonization. Infect Immun. 2014;82:5117–31. doi:10.1128/IAI.02462-14. PMID:25267838.

44. 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. doi:10.1128/IAI.68.9.5090-5095.2000. PMID:10948130.

45. Nart P, Holden N, McAteer SP, Wang D, Flockhart AF, Naylor SW, Low JC, Gally DL, Huntley JF. Mucosal antibody responses of colonized cattle to Escherichia coli O157-secreted proteins, flagellin, outer membrane proteins and lipopolysaccharide. FEMS Immunol Med Microbiol. 2008;52:59–68. doi:10.1111/j.1574-695X.2007.00341.x. PMID:17995963.

46. 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. doi:10.1128/CVI.00068-11. PMID:21593239.

47. Bettelheim KA. Non-O157 verotoxin-producing Escherichia coli: a problem, paradox, and paradigm. Exp Biol Med (Maywood). 2003;228:333–44. doi:10.1177/153537020322800402. PMID:12671176.

48. Paddock ZD, Renter DG, Cull CA, Shi X, Bai J, Nagaraja TG. Escherichia coli O26 in Feedlot Cattle: Fecal Prevalence, Isolation, Characterization, and Effects of an E. coli O157 Vaccine and a Direct-Fed Microbial. Foodborne Pathog Dis. 2014;11:186–93. doi:10.1089/fpd.2013.1659. PMID:24286301.

49. Cernicchiaro N, Renter DG, Cull CA, Paddock ZD, Shi X, Nagaraja TG. Fecal Shedding of Non-O157 Serogroups of Shiga Toxin–Producing Escherichia coli in Feedlot Cattle Vaccinated with an Escherichia coli O157:H7 SRP Vaccine or Fed a Lactobacillus-Based Direct-Fed Microbial. J Food Prot. 2014;77:732–7. doi:10.4315/0362-028X.JFP-13-358. PMID:24780326.

Cell Biol. 2004;82:603–10. doi:10.1111/j.1440-1711.2004.01293.x. PMID:15550118.