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Vaccines Against *Shigella* and Enterotoxigenic *Escherichia coli*: A summary of the 2018 VASE Conference

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ABSTRACT

PATH hosted the second Vaccines Against *Shigella* and Enterotoxigenic *Escherichia coli* (VASE) Conference in Mexico City in June 2018, again providing experts from around the world an opportunity to participate in a highly collaborative forum to discuss progress in the development of new enteric vaccines. Through a combination of plenary sessions and posters, keynote presentations, and workshops, the 2018 VASE Conference aimed to accelerate communication and progress among those working to achieve the goal of licensed vaccines against these two bacterial pathogens. Many presentations recognized the importance of diarrheal disease and long-term sequelae caused by infections with *Shigella* and enterotoxigenic *E. coli* (ETEC). Other presentations explored new strategies for vaccine development, including the search for novel, possibly conserved, antigens for more effective vaccines. Much progress is being made as some vaccine candidates are now moving through clinical trials. Research presented in oral and poster presentations at the VASE Conference covered a range of topics, including: the global burden of disease, epidemiology, and health economics; host parameters and genomics that predict responses to infection and disease; preclinical evaluations of vaccine antigens and models of enteric diseases; and vaccine candidates in clinical trials and human challenge studies. This article reviews key points and highlighted research presented in each of the plenary conference sessions and poster presentations at the 2018 VASE Conference.

1. Introduction

On June 12–14, 2018 PATH hosted the second biennial Vaccines Against *Shigella* and ETEC (VASE) Conference in Mexico City. This conference brought together almost 200 scientists representing 27 countries. New findings related to vaccines against *Shigella* and ETEC were reported in 48 posters and 22 oral presentations. In addition to the oral and poster presentations from the submitted abstracts, the conference included two keynote addresses. Dr. Jaime Sepulveda of the University of California, San Francisco presented his views on shrinking the diarrhea mortality global gap by 2030, including a cost-effectiveness analysis of future *Shigella* and ETEC vaccines. Dr. Timothy Hand from the University of Pittsburgh presented his work on modeling the effects of malnutrition and dysbiosis of the gut microbiome on the efficacy of oral vaccination, a highly relevant topic for *Shigella* and ETEC vaccine development. In addition to the formal meeting content, there were ten facilitated workshop discussions, which allowed smaller groups of conference participants to engage in deeper discussions on topics related to enteric vaccines that were of interest to them.

This special issue of *Vaccine* provides an overview of the major topics presented at the 2018 VASE Conference in an effort to share the content of the meeting more broadly with the enteric vaccine field. This article reviews key points and highlighted research presented in each of the plenary conference sessions and the poster presentations. The final conference agenda and abstracts booklet are available on the VASE Conference website (www.vaseconference.org), and each presentation mentioned in this article is referenced using its assigned identifier code. The additional articles that follow this overview capture the presentations and discussions that took place in each of the workshop sessions.

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2. Global burden of disease, epidemiology, and health economics

2.1. Burden, epidemiology, and etiology

Results from several multi-country studies on disease burden reflect the global scope of diarrheal disease. In the Malnutrition and Enteric Disease Study, a prospective birth cohort study in eight sites in South America, Africa, and Asia, investigators found that 64% of diarrhea episodes could be attributed to an infectious agent (GB069). The top five pathogens were *Shigella*/enteroinvasive *E. coli* (EIEC), sapovirus, rotavirus, adenovirus 40/41, and ETEC. The researchers estimate that a *Shigella* and ETEC vaccine would reduce 43.4 episodes per 100 child years. A 36-month prospective case-control study (Vaccine Impact on Diarrhea in Africa) of moderate-to-severe diarrhea (MSD) in The Gambia, Mali, and Kenya assessed the incidence, etiology, and consequences of MSD among children 0–59 months old following introduction of rotavirus vaccine (GB081). In children 0–11 months old, the attributable fraction of *Cryptosporidium* was equal to or higher than rotavirus, and in Kenya norovirus strain GII is comparable to rotavirus. *Shigella* is now predominate in The Gambia and Kenya, and is the most important pathogen among older children in these two countries as well as Mali.

National and sub-national disease burden estimates are also important to consider. In a cross-sectional study of the Malayali tribe of South India, the prevalence of underweight children less than five years old was 47%, with 24% reporting diarrhea in the last month (GB028). Two studies provided results on *Shigella*/EIEC and *Cryptosporidium*-associated diarrhea in Ghanaian children less than five years old. Of children with bloody diarrhea seeking care, 34% of 400 stool samples were gene positive for EIEC and *Shigella* spp. with most infections detected from birth to 18 months old and peak infection at 13–18 months of age (GB017). In a study of *Cryptosporidium* spp. conducted in rural Gambian children, 12% of 1929 diarrhea cases and 4.8% of 2962 controls were pathogen positive (GB089).

A study in Lusaka, Zambia, explored diarrhea etiology and the presence of coinfections among children less than five years old by using a Luminex x-Tag gastrointestinal multiplex PCR panel to simultaneously detect enteric viruses, bacteria and protozoa from the stool samples (GB092). This system facilitates co-detection of pathogens, thereby yielding percentages which total >100%. The leading causes of diarrhea were rotavirus (68%), adenovirus (42%), ETEC (42%), salmonella (38%), and giardia (38%). Coinfections were common with *Shigella* (39% of 933 rotavirus infections and 44% of 582 adenovirus). Children infected with two or more enteropathogens were 2.6 times more likely to have severe disease.

Another study examined ETEC prevalence in hospital and community settings in different climates of Mexico (GB102). Results showed that ETEC remains an important etiological agent in Mexico with LT-ETEC strains causing diarrhea, and ST-ETEC strains only identified in hospitalized cases.

2.2. Phenotypes and genotypes

Insights into genomic diversity may aid investigations of virulence and the development of diagnostics and vaccines. The genetic diversity of 269 CFA/I and CS6 ST-only strains from diarrhea cases was studied through functional characterizations and comparative genomics; results showed 85% of the genomes were in six lineages (GB080). Cuba's National Program of Integrated Surveillance includes enteropathogens associated with diarrhea (PRE100). ETEC ranks second among isolates in children under five years of age. Of 300 ETEC isolates, ST toxin-producing strains predominate followed by LT, and then LTST strains. Of 200 *Shigella* isolates, the most common strains are *S. sonnei*, followed by *S. flexneri, S. boydii*, and *S. dysenteriae*. Another study examined the prevalence of CFA/I, TibA, and EtpA adhesion genes among ETEC isolates from Mexican children (GB082). Results suggest that children hospitalized with diarrhea compared to community cases with symptomatic and asymptomatic ETEC diarrhea were eight times more likely to have ETEC adhesin genes. In Honduras, investigators explored virulence genes and microbiological features of *E. coli* strains identified in handmade cheese, water sources, and from children less than three years of age (GB030). The main pathotype was enteropathogenic *E. coli* with 100 strains found in humans and cheese. Second was ETEC, which was present from all sources, and 29% of the strains amplified one or more toxin genes.

One multi-country study measured the prevalence of virulence genes among ETEC clinical isolates from Colombia, Peru, and Mexico (GB032). LT expression was most common in Colombia (62%) and Peru (58%), and LTST expression was seen in Mexico (41%). Other virulence genes seen commonly in some countries included CS21, Irp2, and EatA. ST2332, ST10, and ST443 were the most common Sequence Type in Colombia, Peru, and Mexico.

2.3. Infection risk

Lewis blood group antigens were studied as a marker for increased ETEC risk, and zinc deficiency was evaluated as a risk for all-cause diarrhea. In Nicaragua, investigators tested if children with Lewis blood group "a" antigen have more symptomatic than asymptomatic ETEC infections as previously shown in a Bangladesh study (GB073). ETEC episodes were detected in 14% of the children, with 14.3% of the ETEC positive cases in the symptomatic and 14.0% of the asymptomatic children. No association was detected between Lewis phenotype and symptomatic infections, suggesting that a non-secretor status is a host feature affecting ETEC susceptibility.

In Bangladeshi children, investigators tested whether zinc deficiency was a risk for diarrhea (GB103). Bacteria-associated diarrhea among infants with and without zinc deficiency was 66% vs. 48% ($p = 0.008$). Virus-associated diarrhea was four times higher and median time to illness was 27 vs. 33 weeks in non-deficient vs. zinc-deficient infants.

2.4. Diagnostics

A novel, simple, rapid (~60 min), and inexpensive assay has been developed to detect ETEC (LT, STh, STp genes) and *Shigella* (IpaH gene) (GB054). The assay involves a six-minute sample preparation directly from stool with unique lyophilized reaction strips and uses an established looped mediated isothermal amplification platform. High correlations in sensitivity and specificity were seen in frozen samples from Bangladesh, Mexico, and Guatemala, compared to qPCR, for LT, STh, and STp. The prevalence of *Shigella* in these samples was too low to test the assay.

In another diagnostic method, dried filter paper was evaluated for use for specimen preservation and detection of diarrheal pathogens and shipped long distances for testing (GB105). Researchers tested 396 cholera negative specimens on filter paper from patients in Cameroon for ETEC genes (LT, STh, and STp) and *Shigella* (IpaH) using PCR methods. These samples facilitated the identification of pathogens in children living in remote, underserved locations in resource-poor countries.

2.5. Costs and sequelae

Several studies examined the long-term health impacts of diarrhea and related cost estimates. A cross-sectional study in Malawi

assessed the direct and indirect household costs of illness and direct treatment costs in health facilities for cholera episodes. Average costs to patients' household and treatment/health facilities was US$66 and US$60, respectively, in 2016 (GB044). Also, the cost-effectiveness of a reactive oral cholera vaccination campaign that considered direct and indirect effects was conducted in Malawi (GB043). Relative to the Malawi gross domestic product per capita, a reactive oral cholera vaccine campaign was a cost-effective intervention especially when considering indirect vaccine effects.

The cost-effectiveness of ETEC and *Shigella* vaccines was assessed in 79 low- and lower middle-income countries (GB059, GB104). In children less than five years of age, investigators modeled *Shigella* and ETEC mortality, induced stunting from symptomatic infections, and mortality due to infections from *Shigella*/ETEC-associated stunting. According to the model, from years 2025–2034, ETEC and *Shigella* episodes would induce 289,600 and 397,400 deaths respectively, with most deaths occurring in Africa. The ETEC episodes would result in 29 million stunted children, and *Shigella* episodes would result in another 45 million. Vaccination (60% efficacy) would prevent 92,000 ETEC and 126,600 *Shigella* direct deaths and 48,800 ETEC-and 60,800 *Shigella*-associated stunting deaths from other infectious diseases over the next 10 years.

Another study explored whether symptomatic and asymptomatic infections would affect linear growth in children residing in seven low-resource settings (GB065). Diarrhea episodes caused by bacteria and protozoa, but not viruses, caused a temporary reduction in length for age (LAZ) but was not maintained. However, high asymptomatic burden from *Shigella*/EIEC, EAEC, *Campylobacter*, and Giardia were associated with significant decrements in LAZ at two years of age. Interventions that prevent exposure to these pathogens may increase mean length by two years of age.

3. Host parameters and genomics that predict responses to infection and disease

3.1. Controlled human infection models (CHIMs)

In one CHIM study, an ST-only strain of ETEC, TW11681, was utilized as challenge strain, with TW11681 DNA quantitated from stools, and CFA/I and YhgJ serum titers measured (GEN068). All subjects had detectible TW11681 DNA, though six demonstrated a TW11681 DNA peak at days 2–5 post challenge, of which two developed diarrhea and five had abdominal pains or cramps. The subjects with symptoms also demonstrated an increase in CFA/I-specific antibodies. In another study, subjects were challenged independently with ETEC ST strain TW11681 and strain TW10722 (GEN101). Isolated peripheral blood mononuclear cells were examined by mass cytometry with markers that identify cTfh cells and plasmablasts (gut-homing and IgA+ subtypes).

Sera was generated from human volunteers with three different interventions: two vaccines (whole-cell *S. flexneri* 2a vaccine [Sf2aWC] and live attenuated *S. flexneri* 2a vaccine strain [CVD 1204]), and the challenge strain, wild-type *S. flexneri* 2a 2457T (GEN078). These sera were tested against a *Shigella* proteome microarray consisting of 2177 proteins, developed to represent the conserved core of four of the most prevelant *Shigella* species. Reactivity to classical vaccine targets was confirmed and potential vaccine targets identified.

Another study used CHIM to test the potential of rifaximin as chemoprophylaxis for the prevention of campylobacteriosis (GEN056). Rifaximin appeared to have minimal impact on the resident gut microbiota, with most subjects returning to their original (day 0) microbiota after infection and antibiotic treatment, demonstrating that the impact of the pathogen on the microbiota can be measured.

3.2. Animal models

One study focused on the generation of a transgenic mouse model that can not only serve as a model for human familial diarrhea syndrome, but also for testing the efficacy of toxoid vaccines focused on ST (GEN087). A single mutation, S840I, is a hyperactive mutation in receptor guanylyl cyclase C (GC-C). Activation of GC-C results in production of cGMP, which regulates fluid and ion secretion, cell proliferation, and gut immune responses.

BALB/c and CD1 mice were orally immunized with the double-mutant of heat-labile toxin (dmLT), with and without U-OMP19 (ADJ055). U-OMP19 increased the immunogenicity of dmLT and of toxin neutralizing antibodies, and induced significant protection against oral challenge with LT in BALB/c and CD1 patent mouse gut assays.

A rhesus macaque model for *Shigella* was utilized to test a commercially available hyperimmune bovine colostrum product (HBC) for its ability to prevent and treat shigellosis (GEN047). HBC was generated in dairy cows hyperimmunized with selected homogenized ETEC cells and is available as Travelan™. Polyclonal antibodies in HBC also target other diarrheal pathogens in a less specific manner. Studies were initiated to test whether a seven-day course of orally administered Travelan™ would be tolerable and effective in juvenile, naïve rhesus macaques for both prevention and treatment of shigellosis.

C57BL/6 mice with mouse microbiome (MMB) or stably colonized with a human gut microbiome (HMB) were gavaged with EHEC strain 86-24 or ETEC strain H10407 (GEN046). Groups of MMB and HMB were streptomycin (SM) treated prior to challenge. The presence of the HMB rendered the "humanized" mice susceptible to EHEC without the need for SM treatment. However, human microbiota was not sufficient to induce susceptibility to infection by ETEC, which did require SM treatment. Thus, a human microbiome renders the mice susceptible to infection by 86-24, but not H10407.

3.3. Cell models and assays

Chinese Hamster Ovary cells were modified by adding specific glycosyltransferases to express Lewis Lea or Leb determinants on N- and O-glycans (GEN019). CfaB, as well as four related fimbriae, CS1, CS2, CS4, and CS14 bind significantly more to engineered Lea expressing cells, and as well showed by docking analysis that there were likely three amino acids, Glu25, Asn27, and Thr29 in the combining site of CfaB and related proteins important receptor binding. An *in vitro* LT neutralization assay that can rapidly test human and animal sera was designed and optimized (ADJ077) and subsequently standardized across laboratories. LT toxin reduces NF-kB signaling after Toll-like receptor agonist treatment in HCT116 cells. Serum from immunized human and rabbit samples inhibited the LT-reduction activity and can be used to improve evaluation of clinical responses to ETEC vaccines.

A serum bactericidal assay (SBA) has been developed and tested against three *Shigella* serotypes (GEN031), accomplished by directly measuring the killing of target bacteria at multiple dilutions of samples and was harmonized between several laboratories. The SBA was characterized with human sera against *S. flexneri* 2a, 3a, and *S. sonnei*. Inhibition of bacteriocidal activity was demonstrated with homologous, but not heterologous LPS.

Three candidate stool biomarkers: myeloperoxidase (MPO), calprotectin (CALP), and alpha-1 anti-trypsin (AAT) were assayed to measure gut inflammation (MPO and CALP) and gut leakiness/permeability (AAT) (GEN085). The primary outcome was stunting,

defined in Zambian children under age five with height-for-age z-score below −2. Stool samples from a total of 219 children were studied, and while there was no evidence of independent effects of MPO, CALP, and AAT on risk of stunting, there was strong evidence of synergistic effects of the fecal markers MPO and CALP on risk of stunting.

3.4. Host parameters

More powerful immunologic tools are now available to more deeply study the specific immune responses of lymphocyte subsets. To characterize the systemic humoral and cellular immune responses in natural ETEC infection, analysis of CD4+ T cells, memory T-cells (CD4+CD45RO+) and cytokine responses to different ETEC antigens was conducted (GEN038). Significant increases in memory T cells to LTB and dmLT was seen at early convalescence (day 7) as contrasted to what is seen at day 2 (acute stage) and in healthy participants. There was no significant increase in T cell proliferation seen for ST or EatA. Increased IFN-g and Il-13 were observed at day 7 in comparison to day 2, suggesting that a mixed Th1 and Th2 response was generated. Plasma levels of IgG, IgA, and IgM directed at LTB were seen at early convalescent stage. This demonstrates that antigen-specific memory T cell responses develop at early convalescent stages after infection that correlate with development of antigen-specific systemic B-cell responses, suggesting that T-cell responses to ETEC antigens are important for the generation of stable B-cell responses.

The interaction between *Shigella* and human lymphocytes necessary for the generation of adaptive immunity has been studied in depth (GEN016). *Shigella* target B-cell subsets, which leads to their apoptosis via a mechanism dependent on the type 3 secretion system (T3SS) component IpaD and TLR2. In addition, *Shigella* target T lymphocytes, arresting their migration *in vitro* and *in vivo*. T3SS effector injection that does not result in cell invasion now appears to be the main targeting mechanism towards lymphocytes, and T-cell targeting is dependent on their glycosylation, with such targeting impacting the formation of the immunological synapse. These new insights thus suggest a revision of the role of T3SS in *Shigella* pathogenesis is in order.

3.5. Genomics and proteomics

ETEC vaccine ACE527 was administered to subjects with and without dmLT, and the subjects were challenged six months later. Significantly higher protection was afforded when ACE527 was administered with dmLT. Fecal IgA from these subjects was evaluated using ETEC proteome microarrays comprised of proteins expressed using the *In Vitro* Transcription Translation (IVTT) system, with additional ETEC-purified antigens (GEN072). Strong responses were observed to purified colonization factors, novel antigens, and toxins as well as to many IVTT proteins. Following H10407 challenge, FliC responses were stronger in protected over unprotected subjects. Additionally, responses were seen to IVTT versions of CS3, EatA, YghJ, and the flagellar hook-associated protein 1. An *E. coli* pan-genomic protein array was also developed containing 7366 full-length or fragmented diarrheagenic *E. coli* proteins (GEN062). This expansive microarray was used to assess antibody responses in subjects with culture-confirmed ETEC-associated watery diarrhea participating in a travelers' diarrhea treatment trial. Serum IgG and IgA from acute and convalescent subjects were tested. The microarray allows the identification of novel antigens associated with travelers' diarrhea.

A *Shigella* proteome microarray consisting of 2177 proteins representing the conserved core of the four major *Shigella* species was generated and tested, and interrogated with serum samples from human vaccine trials of Sf2aWC and CVD 1204, as well as from CHIM challenge strain *S. flexneri* 2a 2457T. Samples from CVD 1204, a live attenuated vaccine, and the CHIM study demonstrated strong and broad responses to multiple *Shigella* species (GEN078).

4. Preclinical evaluations of vaccine antigens and models of enteric disease

4.1. Shigella

4.1.1. Cell-based antigens

A live attenuated *Shigella* vaccine candidate, ShigETEC, which lacks O-antigen expression and is attenuated by deletion of IpaB and IpaC genes is under development (PRE061). The cells express LT-B and detoxified ST as a fusion protein to contribute to coverage against ETEC, which may be augmented by the natural presence of cell surface antigens for both ETEC and *Shigella*. Functional antibodies against LT and ST were detected following immunization. ShigETEC is protective in the murine lung shigellosis model in a species- and serotype-independent manner. Analysis of >400 sera from Bangladeshi adults found high levels of antibody to the O-antigen negative ShigETEC vaccine strain.

A combined *Shigella*-ETEC vaccine was constructed from the attenuated *Shigella* CVD1208S by engineering the ETEC CFA/I-encoding operon as well as the heat-labile toxin (LT) A2 and B subunits into the chromosome to form CVD1208S-122 (PRE070). This construct protected guinea pigs against challenge with wild-type *Shigella*. Researchers also investigated the feasibility of using an inactivated form of the vaccine. Comparison of cells inactivated with formalin, heat, or B-propriolactone found similar serum and mucosal anti-*S. flexneri* LPS serum IgG and mucosal IgA responses in guinea pigs. Vaccines following all three inactivation methods induced strong serum and mucosal antibody responses against the ETEC antigens expressed.

Two novel methods were tested to inactivate another *Shigella*-ETEC hybrid vaccine: creation of cell ghosts and exposure to gamma irradiation (PRE029). Following both methods of inactivation, the cells were immunogenic for *Shigella* and ETEC antigens and protected mice from subsequent challenge with *Shigella*.

An inactivated whole-cell *Shigella* vaccine approach using a heat-killed multi-serotype *Shigella* (HKMS) vaccine to provide protection in different animal models was studied (PRE091). The findings revealed that immunization with HKMS protects against bacteria-induced lethality and systemic inflammatory response largely through Th1 and Th17 responses.

Studies of cytokine expression following challenge with different *Shigella* species (*S. flexneri* 2a, *S. sonnei*, and *S. dysenteriae* 1) found differences in cytokine responses (PRE018), indicating the serotype difference in innate and adaptive immune responses. A physiologically relevant system was established using a human colonoid model containing multiple cell types including goblet cells, entero-endocrine cells, Paneth cells, and colonocytes (PRE039). This model system makes possible establishment of host cytokine and gene expression responses that can be used as correlates of immunogenicity and reactogenicity to enable more rapid advancement of the most promising vaccine candidates into clinical trials.

4.1.2. Subcellular antigens

Active investigations are ongoing to develop a second-generation Invaplex vaccine consisting of a complex of LPS extracted from *Shigella* in combination with recombinant forms of IpaB and IpaC (InvaplexAR) (PRE071). The complex was found to be heat stable up to 80 °C. Thus, the structure of InvaplexAR remains constant over time, temperature, and total concentration.

Work is also underway to annotate the proteins which are otherwise known as "hypothetical" and to characterize the same computationally with the help of various bioinformatics tools and databases for the genome of *S. dysenteriae* 197 (PRE022). These studies enabled identification of proteins for further work on a computational pipeline for a better understanding of the drug resistance and pathogenesis mechanisms in *S. dysenteriae* which could help to develop novel mechanisms for disease treatment.

4.2. ETEC

4.2.1. Colonization factor-based vaccine antigens

Immunization of *Aotus nancymae* NHP with CfaEB, a fimbrial tip adhesin of class 5a fimbriae, resulted in 75% protective efficacy against heterologous challenge with ETEC expressing CS14 (PRE051). Immunization of NHP with a second antigen, CfaEp3, composed of CfaEB fused to the pilins of CS14 (CsuA2) and CS4 (CsfA), provided 84% protection against CS14-expressing ETEC and 27% protection against CFA/I-expressing ETEC.

Investigators used mice transgenic for human immunoglobulin genes to produce humanized monoclonals targeting the CfaE tip adhesion of CFA/I (PRE098). Oral administration of these antibodies inhibited colonization of ETEC H10407 in mice and have the potential to block ETEC colonization and serve as immunoprophylaxis to prevent ETEC infection.

The repeating major subunit from CS21, LngA, was characterized as a monomer or as fusions of alleles from different strains, for immunogenicity, compared to monomers of the tip adhesin, LngB (PRE096). The recently characterized CS30, which is related to 987P found on pig ETEC isolates, was shown to adhere to human intestinal cells as well as glycosphingolipids found on human and pig intestine. This suggests that CS30 ETEC strains could bind to different hosts and may suggest a need for an ETEC vaccine that protects against infections from both human and animal-associated ETEC strains.

In order to identify the most promising ETEC antigen candidates, an integrated mathematical approach was used to evaluate a number of vaccine antigen-adjuvant-formulation combinations in terms of the quality and magnitude of immune responses (PRE084). Investigators found that antigen and adjuvant dose and formulation choice were important for optimizing vaccine immunogenicity. Mathematical modeling represents new technology that may contribute to the identification of the promising antigen candidates.

4.2.2. ST toxoid-based antigens

A genetic fusion composed of a mutant ST toxoid fused to mutant LT toxin (3xSTaN12S-mLTR192G/L211A) was compared to a chemical conjugate composed of ST toxoid conjugated to BSA (BSA-ST-A14T) (PRE048). Both antigens were immunogenic in mice inducing anti-ST antibodies that were able to neutralize ST toxin activity in T84 cells.

An advance in purification processes for ST or ST mutant derivatives was described wherein a genetic fusion was engineered to encode native ST or ST mutant derivatives fused to the disulfide bond isomerase DsbC, a 6X His tag for purification, and a tobacco Etch virus protease cleavage site to release free ST with no additional residues (PRE053). This process was shown to allow efficient purification of ST or mutant derivatives with expected characteristics, biological activities, and antigenic properties.

An innovative method for developing ST conjugate antigens, named ST-Secreted Conjugate, was non-toxic when applied to T84 cells and immunogenic in mice producing anti-ST IgG that neutralized ST (PRE012). This process could speed the development of an antigen that neutralizes ST.

CHIMs were developed for ST-only ETEC strains (PRE064; PRE066). Researchers tested two different ETEC strains in CHIM studies wherein volunteers ingested escalating doses of ETEC strain TW10722(O115:H5, STh-CS5 CS6) or ETEC strain TW11681 (O19:H45, STh-CFA/I CS21). It was concluded that using relatively large doses of TW10722 would be safe and efficient for testing an ST-based vaccine.

4.2.3. Combined colonization factor and ST vaccine antigens

One subunit approach that has advanced is MEFA (multi-epitope fusion antigen). The antigen named MEFA CFA/I/II/IV-3xSTaN125-mnLTG192G/L211 induced antibodies in mice that were able to inhibit adhesion of each fimbrial type-expressing ETEC in cell culture and neutralize ST and LT toxin (CMB020). In further studies, researchers combined this MEFA antigen with the *Shigella* DBF antigen to generate MACE (multi-antigen combination enteric vaccine) for protection against *Shigella* and ETEC (PRE097). Immunization of mice with MACE induced 90% protection against lethal *Shigella* challenge in the lung model.

4.2.4. Other new ETEC antigens

An *in silico* strategy was used to search for high potential peptide epitopes to bind to different MHC Class I molecules from different human populations (PRE013). The analysis identified nine highly promiscuous antigens derived from 4915 proteins conserved among ETEC strain E24377A. The results suggest that the epitopes designed based on these analyses could manifest enduring immunity against diarrhea.

A conserved antigen for broad protection against ETEC, the ETEC Skp protein was tested as a vaccine candidate (ADJ023). Following immunization of mice, researchers observed that the display of Skp on OMVs in the absence of exogenous adjuvant was equally as effective as the Skp-GST fusion administered with cholera toxin in inducing responses protective against lethal pulmonary challenge with ETEC.

Investigators developed and utilized a novel mass spectrometry-based technique, named BEMAP, to map previously unexplored O-linked glycoproteins from ETEC (PRE088). They identified YghJ as a novel glycosylated vaccine candidate. Glycosylated and non-glycosylated variants of YghJ were purified and used to immunize rats which produced high affinity antibodies to the glycan-peptide moiety of glycosylated YghJ. Further studies demonstrated that both forms of YghJ are recognized by serum from pre- and post-H10407-challenged volunteers. O-linked bacterial glycoproteins may constitute an important reservoir of novel vaccine candidates with superior immunogenicity.

5. Vaccine candidates in clinical trials and human challenge

5.1. Shigella

5.1.1. Polysaccharide-conjugate candidates

A bioconjugate vaccine that conjugates *Shigella* O-antigen to recombinant exotoxin A of *Pseudomonas aeruginosa* recently advanced through a Phase 2 CHIM study and found preliminary evidence of efficacy (CL036). An in-depth analysis was conducted aimed at teasing out associations between a broad array of host-immunological responses through use of a recently published disease severity score compared to traditional endpoints. In addition to demonstrating the advantage of the *Shigella* severity score over traditional dichotomous outcomes, the variety of analysis models used showed that multiple immune parameters all predicted protection. Another Phase 2 CHIM study analyzed B-cell memory responses among the volunteers (CL042). At 10–16 months post-

vaccination/challenge, memory B-cells against *S. flexneri* 2a LPS were detected (IgG ALS) at multiple time-points, demonstrating that the Flexyn2a bioconjugate vaccine induces memory B-cells against LPS.

In a Phase 1 first-in-human study of a synthetic carbohydrate-based conjugate vaccine, the prototype vaccine was given in a three-dose intramuscular regimen at two dose levels with and without alum in 64 volunteers (CL067). Results demonstrated it is a safe vaccine, and a variety of robust immune responses were seen with the highest dose group (10 μg) with a clear alum adjuvant effect. Furthermore, memory B cells were elicited and functional antibodies were generated.

5.1.2. Whole-cell candidates

The functional antibody and cytokine responses from a recent WRSS1 (live oral *Shigella sonnei*) vaccine in Bangladeshi adults and children were promising (CL033). Adults exhibited an enhanced Th1 response, which is similar in phenotype to experimental challenge of naïve US volunteers, however children demonstrated an induction of a Th17A response in the presence of inflammatory cytokines, reflecting a different immune response pathway in absence of a Th1 response. SBA responses in children were enhanced with booster doses of vaccine.

A comparison of SBA and opsonophagocytic killing antibodies (OPKA) was made with material from recent Phase 1 trials of inactivated (killed) *S. flexneri* 2a and *S. flexneri* 2a CVD1208S (live attenuated) (CL079). SBA and OPKA antibody responses peaked on day 7 for both constructs and the magnitude and persistence of these responses was dependent upon strength of vaccine priming. Discrimination between killed whole-cell and live attenuated responses revealed that, for the given vaccine constructs and regimens tested, the killed whole-cell vaccine achieved lesser SBA and OPKA response rates (~40%) compared to the live attenuated construct (~60%). Observations from the previously mentioned WRSS1 study, showed that the level of pre-existing titers was an important determinant of post-vaccine SBA and OPKA responses.

5.1.3. Subcellular complex approach

An *S. flexneri* 2a Invaplex intranasal vaccine against *S. sonnei* and *S. flexneri* 3a elicited cross-protective immune responses (SBA) (CL040). Vaccination with the Sf2a LPS/IpaB/IpaC construct induced functional antibodies against Sf2a and Sf3a, but not *S. sonnei*, supporting the notion of similarity between LPS for Sf2a and Sf3a serotypes, and that LPS is the major antigenic target associated with bactericidal responses.

5.1.4. CHIM

A recent trial focused on establishment and optimal dose finding of a *S. sonnei* 53G lyophilized CHIM studied the immune responses elicited (CL035). LPS-specific serum IgG and IgA responses peaked on day 14 after infection. IpaB and IpaC responses were more variable and generally lower for IpaC compared to IpaB. SBA titers also peaked at day 14. In addition, challenge induced LPS-specific antibody secreting cells with gut homing markers and levels of intestinal inflammation were elevated after challenge. Markers of disease severity included fecal calprotectin, myeloperoxidase, and LPS-specific IgA α4β7-positive ASC responses. More severe disease and dysentery were associated with higher IgA α4β7-positive ASC responses. Finally, baseline parameters of higher LPS-specific IgA titers and SBA titers were associated with disease resistance, supporting other observations and potential immune correlates of protection.

5.2. ETEC

5.2.1. Killed whole-cell approaches

A Phase 1/2 trial of the ETVAX candidate in descending-age groups in Bangladesh had promising results (CL037). In this trial, adjuvanted and non-adjuvanted two-dose regimens (0 and 14 days) were sequentially age-deescalated from adults to children to infants with associated decreases in dose-levels (full, ½, ¼, 1/8) to evaluate safety and immunogencity. Half of an adult dose was safe in older children and young children, and a ¼ dose was safe in the infant cohort. Adjuvanted and non-adjuvanted doses were equivalently tolerated. A deeper analysis of mucosal immune responses in the aforementioned study was also conducted (CL041). ETVAX induced mucosal responses to all primary vaccine antigens in all age groups, though levels varied. In general, mucosal responses decreased with age and were not dose-dependent (within age). There were high frequencies of mucosal immune responses to vaccine antigens in the 6–11 month old placebo recipients, suggesting a high background rate of ETEC infection and possible interference with vaccine-induced responses.

In addition to clinical advancement of ETVAX in the pediatric population, it has advanced into a Phase 2 field trial among travelers from Finland visiting Grand Popo, Benin (CL106). While trial results are not anticipated until the second quarter of 2019, the design and progress of an 800-person randomized controlled trial was described. Interestingly, 50–70% of vaccine participants have reported travelers' diarrhea, suggesting a high burden of illness.

5.2.2. ETEC CHIM development

A comprehensive analysis of anti-CS6 and LT serum, mucosal, and cellular immune responses to an ETEC B7A (CS6, LT, ST) CHIM found that a minority of subjects generated primary immune responses to CS6 and LT as measured by serum, ALS, and various mucosal (fecal, saliva) responses (CL099). Memory B cell responses (2-fold rise) were more common for IgA to CS6 (~70%) and IgG to LTB (~70%). In general, IgA responses were higher to CS6, and IgG responses were higher to LT for the various humoral and mucosal assays. CD4+ T cell proliferation against CS6 and LT peptides were a more sensitive parameter to detect infection by B7A compared to antibody levels.

5.2.3. Other clinical advancements for enteric vaccines

A Dukoral (cholera) booster study among previously primed adult volunteers with the full two-dose Dukoral vaccine had promising results (CL057). Among 52 adult Swedish volunteers who received a booster vaccine dose four months after the primary series, it was found that a 1/25 dose was sufficient to stimulate a mucosal ALS IgA antibody response with similar antibody avidity to CTB, but not towards serum IgA or IgG titers against CTB.

A new Dot-Blot technique that establishes a less expensive, easier, and quicker assay to identify polysaccharide vaccines may offer a more optimized method to monitor the production and stability of antigen in vaccine manufacturing (CL093). Similarly, the use of high-resolution liquid chromatography to evaluate the molecular integrity of the Vi polysaccharide from *S. typhi* conjugate vaccine is being developed by the Finlay Institute (CL094). They found that the HPLC method for assessing vaccine integrity is accurate and specific and could be used for routine analysis in process manufacturing of this vaccine.

6. Conclusion

The 2018 VASE Conference summarized here continued to accelerate communication and progress among those working in the enteric vaccine field to make *Shigella* and ETEC vaccines a

reality. The meeting provided a highly collaborative environment for this work, as demonstrated by the numerous side meetings and discussions conducted among the researchers working on these vaccines. PATH is optimistic that support for future VASE Conferences will continue in 2020 and beyond.

Conflict of interest

The authors declared that there is no conflict of interest.