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The intriguing interaction of *Escherichia coli* with the host environment and innovative strategies to interfere with colonization: A summary of the 2019 *E. coli* and the Mucosal Immune System meeting.

Eric Cox[a], Meryem Aloulou[b], James M Fleckenstein[c], Christina Schäffer[d], Åsa Sjöling[e], Stephanie Schüller[f], Kurt Hanevik[g], Bert Devriendt[a], Weiping Zhang[h], Ann-Mari Svennerholm[i], Edward G. Dudley[j]

[a]*Laboratory of Immunology, Faculty of Veterinary Medicine, Ghent University, Belgium*

[b] *Toulouse Purpan Physiopathology Center, INSERM U1043, CNRS UMR5282, Toulouse III University, Toulouse, France*

[c] *Division of Infectious Diseases, Department of Medicine, Washington University School of Medicine, Saint Louis, Missouri, USA*

[d] *Department of NanoBiotechnology,* NanoGlycobiology *unit, Universität für Bodenkultur Wien, Vienna Austria*

[e] *Department of Microbiology, Tumor and Cell Biology (MTC), Karolinska Institutet, Stockholm, Sweden*

[f] *Norwich Medical School, University of East Anglia, Norwich, UK*

[g] *Department of Clinical Science, Faculty of Medicine, University of Bergen and Norwegian National Advisory Unit on Tropical Infectious Diseases, Department of Medicine, Haukeland University Hospital, Bergen, Norway*

[h] *Department of Pathobiology, University of Illinois at Urbana-Champaign, Illinois, USA*

[i] *Department of Microbiology and Immunology, Institute of Biomedicine University of Gothenburg, Sweden*

[j]*The Pennsylvania State University, and Director of E. coli Reference Center University Park, PA 16802. USA*

Abstract

The 3rd *E. coli* and the Mucosal Immune System (ECMIS) meeting was held at Ghent University in Belgium from June 2-5, 2019. It brought together an international group of scientists interested in mechanisms of colonization, host response, and vaccine development. ECMIS distinguishes itself from related meetings on these enteropathogens by providing a greater emphasis on animal health and disease, and covering a broad range of pathotypes including enterohemorrhagic, enteropathogenic, enterotoxigenic, enteroaggregative, and extraintestinal pathogenic *E. coli*. As it is well-established that the genus *Shigella* represents a subspecies of *E. coli*, these organisms along with related enteroinvasive *E. coli* are also included. In addition, *Tannerella forsythia,* a periodontal pathogen, was presented as an example of a pathogen which uses its surface glycans for mucosal interaction. This review summarizes several highlights from the 2019 meeting and major advances to our understanding of the biology of these pathogens and their impact on the host.

Introduction

The gut microbiome is a diverse community of more than 100 trillion microorganisms which influence mucosal and systemic immune functions via production of metabolites, virulence factors and through interactions with other members of the microbiota. Most bacteria in the gut belong to one of eight phyla, with the phylum *Proteobacteria* accounting for ca. 2.1% of the population. Among these, the majority classify as *Enterobacteriaceae*, with *Escherichia coli* by far the most abundant species (1). A recent phylogenetic study of human-derived *E. coli* suggested a highly dynamic nature with turnover in the order of months to years (2). The authors suggest, based on data of Faith *et al* (3), that this might also be the case for the rest of the microbiome. Thus, the potential for clonal turnover to change gut function is great. Understanding how this might influence the host or how host factors affect the microbiome is challenging.

The conference on *E. coli* and the Mucosal Immune System in 2019 (ECMIS-2019) was the 3rd conference in a series of conferences of which the first one was held in 2011, exactly 100 years after the death of Theodor Escherich. The meetings are organized to bring together basic scientists and clinicians working on *E. coli* and the mucosal immune system in particular focusing on the interaction of these intriguing pathogens with the mucosal epithelium, and to exchange knowledge on the pathogenicity of different types of *E. coli* over species. Whereas the first meeting in 2011 focused on differences between infections in different species, the second meeting in 2015 rather addressed mechanisms of different *E. coli* pathogens independent of species. This 3rd conference addressed some new insights in the interactions between host, pathogen and its environment and how these interactions steer host and/or pathogen. Furthermore, several examples were presented of how this interaction can be exploited to control *E. coli* infections. More information on this last conference can also be found at www.ecmis.ugent.be

The mucosal immune system and modulation of the host by *E. coli*

The main function of the immune system is to protect the host from pathogens. The mammalian gut harbors large numbers of diverse microbes, which establish a strong relationship with the immune system, ensuring host homeostasis and consequently supporting health. The microbes have strong potential to generate immunoglobulin A (IgA), the most abundantly produced antibody isotype, which promotes maintenance of non-invasive commensal bacteria, immune tolerance, and neutralization of invasive pathogens through multiple mechanisms. Supporting evidence for physiologic relevance comes from studies in patients with selective IgA deficiency, who exhibit an increased susceptibility to autoimmune diseases (4). IgA synthesis occurs at different gut-associated lymphoid tissues (GALT), either in organized tissues such as Peyer's patches and mesenteric lymph nodes, or by dispersed B cells in the lamina propria in isolated lymphoid follicles. Diversification of the IgA repertoire, primarily via T cell-dependent pathways, is required to maintain gut homeostasis and ensure mucosal defense. Dr. Meryem Aloulou (Center for Pathophysiology of Toulouse Purpan) began the session "Modulation of the Host", by describing the crucial role of follicular T cells to support B cell maturation in germinal centers (GC), where positive and negative regulatory roles are classically assigned to T follicular helper (Tfh) and regulatory (Tfr) cells, respectively (5). GCs represent critical sites in which B cell responses are amplified and refined in specificity and isotype, leading to the generation of high-affinity memory B cells and long-lived plasma cells. Tfh cells regulate GC B cells and lead to their maturation through somatic hypermutation (SHM) and class switch recombination (CSR), brought about by the expression of activation-induced cytidine deaminase (AID). Interestingly, *AIDG23S* mice carrying a knocked-in mutation of the *AID* gene, which causes specific defects in SHM, developed hyperplasia of GCs in GALTs, dysbiosis of the microbiota and more susceptibility to infection, indicating that SHM is essential in maintaining intestinal homeostasis and mucosal defense (6). GC Tfh cells are thought to be the positive regulators of this process, while Tfr cells, a subset of $Foxp3^{+}$ regulatory T (Treg) cells, are negative regulators. Gut Treg cells, however, in addition to suppressing inflammation and preserving immune tolerance, are also known to promote GC and IgA responses by generating GC T cells, ultimately resulting in the diversification of gut microbiota (7, 8). Gut Treg depletion, in fact, causes a rapid loss of specific IgA responses in the intestine. Overall, Tfh and Treg cells function not so much in opposition but in a mutualistic relationship to regulate the GC reaction in the gut, maintain a diverse and healthy gut microbiota, and foster immune homeostasis. The exact mechanisms by which Treg and Tfh cells cooperation achieve these homeostatic and symbiotic functions are still poorly understood. Therefore, understanding the mechanism of these processes and their regulation will facilitate the development of new strategies for prevention or treatment of gut disorders.

Another mechanism to modulate the host immune response is used by *Shigella*. It is well known that several rounds of infection with *Shigella* are needed to prime antibody responses, which are of short duration. Dr. Katja Brunner (Institut Pasteur) of the group led by Dr. Armelle Phalipon presented research providing insights into antibody suppression. *Shigella* can induce B cell death by invading the lymphocytes, and, as demonstrated using different mutants, by interaction of the type three secretion system (T3SS) needle tip adhesin IpaD with TLR2 on B cells. For apoptosis to occur bacterial co-signals are required which sensitize the B cells to apoptosis and upregulate TLR2 (9). Another mechanism was demonstrated for *in vitro* activated human blood B- and $CD4^{+}$ T-, and $CD8^{+}$ T-lymphocytes, but also B- and T-lymphocytes residing in the colonic mucosa. *Shigella* can inject these cells via the type III secretion system without invading them (10). T cell activation enhances expression of GM1 gangliosides, which interact with the O-antigen-moiety of *Shigella* lipopolysaccharide, making these activated T cells more susceptible for T3SS-mediated injection (11). So far, the only outcomes of this direct targeting of activated T cells are the impairment of CD4+ T cell dynamics and migration, mediated by the T3SS effector IpgD.

In the third presentation of this session, Dr. James Fleckenstein (Washington University School of Medicine), described new virulence factors from human ETEC strains, namely EtpA and EatA (reviewed in 13). EtpA is an extracellular adhesin, while EatA is a member of the serine protease autotransporter of the *Enterobacteriaceae* family and acts as a mucinase to degrade host MUC2. Degradation enhances epithelial access and ETEC adhesion, including that mediated the EtpA-mediated bridging of flagella with *N*-acetylgalactosamine (GalNAc) exposed on the surface of epithelial cells. Affinity is highest for terminal GalNAc of blood group A, which might explain the more severe disease in humans with this blood group (14).

Type 1 fimbriae (F1) also can play a role in ETEC interaction with the mucosa (15). Lastly, an excellent example of the host-pathogen interaction mediated by by ETEC heat-labile toxin (LT) was presented. In this model, initial delivery of LT triggers upregulation expression of CEACAM6 molecules on intestinal epithelial cells, which then serve as critical receptors for FimH, the tip adhesin of F1. While it has been suggested that ETEC use their toxins to propel organisms back into the environment, these studies suggest a more sophisticated scenario where LT is exploited to enhance a transient epithelial niche on small intestinal enterocytes.

Modulation of *E. coli* by the host

It has become increasingly evident that host factors present in the gastrointestinal tract impact virulence and growth of pathogenic bacteria. In the intestines intrinsic factors of different origin are sensed by invading pathogens and used to modulate gene and protein expression. In the session "Modulation of *E. coli* by the Host" Drs. Åsa Sjöling (Karolinska Institute), Stephanie Schüller (University of East Anglia), and Guoqiang Zhu (Yangzhou University) presented recent data on how pathogenic *E. coli* respond to different host factors.

The first talk by Dr. Sjöling described the ETEC response to bile stress encountered in the gastrointestinal tract. The bile components secreted by the gallbladder are reabsorbed by epithelial cells through the jejunum and ileum. Remaining bile acids may be converted to secondary bile acids by resident microbiota, mainly in the large intestine. Regulation of virulence and biofilm formation in response to specific concentrations of bile has been reported in a number of enteropathogenic bacteria (16, 17).

Human ETEC isolates expressing the colonization factors CS5 and CS6 belong to a globally spread and highly virulent lineage (18). Isolates of this lineage respond specifically to the bile salt sodium glycocholate (NaGCH), which not only induces specific expression of colonization factor CS5 (16, 19), but also an entire regulon of virulence factors located on a virulence plasmid as well as on the chromosome. Dr. Sjöling explained how this induction is governed by the transcription factor CsvR (Coli surface virulence factor regulator) located upstream of the plasmid-encoded CS5-operon. CsvR also regulates motility by down-regulation of flagellar operons located on the chromosome. Altogether the results indicate that bile salt sensing induces a large virulence regulon, controlling the initial states of attachment to the host. Oxygen regulation is an important factor in the gut since pathogenic species in the gastrointestinal (GI) tract are often facultative anaerobes that might thrive in presence of higher levels of oxygen. Oxygen levels decrease through the GI tract and a radial gradient is also present with oxygen levels diffusing from the intestinal mucosa towards the anaerobic gut lumen (20). Dr. Schüller introduced a microaerobic diffusion Chamber system to determine the influence of oxygen and human colonic epithelium on virulence gene expression in enteroaggregative *E. coli* (EAEC). While oxygen induced expression of the transcription factor AggR and its dependent adhesion factors AAF and dispersin, physical contact with host cells triggered subsequent expression of the mucinase Pic and the cytotoxins HlyE and Pet. Interestingly, host cell-mediated virulence gene induction occurred independently of the master regulator AggR (21, 22).

Bacteria use quorum sensing to signal a coordinated gene expression within a bacterial population. The acyl-homoserine-lactones (AHL) are produced and sensed by Gram-negative species to communicate and recent findings indicate that homologues are secreted by eukaryotic cells thereby mediating interkingdom signaling. Dr. Zhu reported findings that exogenous and endogenously produced AHL activate acid resistance regulons and stress responses in enterohemorrhagic *E. coli* (EHEC) thereby facilitating survival in low pH environments.

An interesting connection was revealed in this session, contrasting intestinal colonization strategies used by different *E. coli* pathotypes. AggR and CsvR are both members of the AraC-family of transcriptional regulators and activate adherence by distinct pathogens in response to different environmental cues. Interestingly, AggR activates dispersin in EAEC, and CsvR (23) the dispersin-like protein CexE in ETEC, as well as the putative secretion systems encoded by the *aatPABCD* operon. Hence, *E. coli* as well as other enteropathogens share conserved transcription factors and responses to host stimuli. Interestingly, both AHL and bile sensing in EHEC have an opposite effect on colonization by downregulating the locus of enterocyte effacement (LEE) (24, 25). EHEC as well as EAEC primarily colonize colonic epithelium where bile salt concentrations are lower than in the proximal small intestine, where ETEC is preferentially found. Differences in regulatory circuits may explain the spatial preferences. In summary, increased knowledge of the most important factors sensed at the site of infection might reveal novel targets to limit enteropathogenic disease.

Modulation of *E. coli* by the environment.

The bacterial pathogenesis field appreciates that the study of virulence mechanisms and gene expression needs to consider impacts of other microorganisms and metabolites in the environment. Enterohaemorrhagic *E. coli* (EHEC) O157:H7 is a serious foodborne pathogen most commonly transmitted to humans through contaminated beef and fresh produce. Strains of O157:H7 differ in their carriage of virulence genes, however human disease requires the T3SS-associated gene for intimin (*eae*), and one or more genes encoding for Stx1 and/or Stx2, the two isoforms of Shiga toxin (Stx). A number of publications describe mechanisms by which gut commensals regulate *eae*. This session explored how the gut microbiome influences the expression and toxicity of Stx.

As Dr. Frederic Auvray (Institut de Recherche en Santé Digestive) detailed in his talk, "Overview of Stx phages diversity and their role in virulence and evolution of *Escherichia coli*", genes for Stx are encoded within lambdoid bacteriophages. These phages are genetically diverse, and capable of jumping to other *E. coli* including other pathogenic variants resulting in newly appreciated "hybrid" types. Excision may also lead to loss of prophage from O157:H7 and other Shiga toxin-producing *E. coli* (STEC), which can complicate interpretation of diagnostic assays. Induction of the phage is known to increase Stx production, and often this is achieved in the laboratory through addition of DNA damaging agents such as mitomycin C, fluoroquinolones or hydrogen peroxide.

Dr. Edward Dudley (The Pennsylvania State University) described in the talk "Commensal *E. coli* that enhance toxin production by *E. coli* O157:H7" known mechanisms by which non-O157:H7 *E. coli* can enhance virulence potential. This talk presented a newly discovered mechanism (26), involving a previously unknown microcin produced by a strain designated 0.1229. Co-culture of O157:H7 with 0.1229 leads to a *recA*-dependent enhancement of Stx production *in vitro*. Co-inoculated germ-free mice also exhibited more serious signs of disease than mice inoculated with either *E. coli* alone. These data demonstrate that non-Stx producing *E. coli* that naturally colonize the intestines may accelerate the course of disease.

To the contrary, Dr. Mononmani Soundararajan (Institute for Molecular Infection Biology) demonstrated that some *E. coli* dampen toxin production in the talk "Inactivation of *stx*-phages by probiotic *E. coli* strain Nissle 1917". Nissle 1917 (EcN) is a well-established probiotic strain and is the active component of the commercial product sold under the name Mutaflor. This study demonstrated that EcN, when incubated with an *stx*-converting bacteriophage, leads to a 2-log inactivation as measured by phage plaque assays. While the exact mechanism is unclear, heat-killed EcN exhibited similar activity, while treatment with Proteinase K abolished it, suggesting heat-stable protein(s) are responsible. The laboratory strain *E. coli* K-12, when co-cultured with O157:H7, increased Stx production, and previous work of others has shown that this mechanism involves *stx*-converting phage infection of the non-pathogenic strain. This talk demonstrated that in a triculture, where O157:H7, EcN, and K-12 are grown together, both Stx- and phage levels are reduced compared to the co-culture lacking EcN. These data demonstrate that probiotics including EcN may decrease the severity of O157:H7 disease.

Lastly, Dr. Anne Kijewski (Norwegian Institute of Life Sciences) provided evidence that microbial metabolites, specifically vitamin K, may play a role in modulating virulence of O157:H7. While vitamin K naturally occurs within the intestinal tract of humans, individual differences in concentration occur due to diet, host factors, and microbial communities present. Through investigating different chemical forms of vitamin K, it was discovered that menadione and menadione bisulfite both inhibited the growth of *E. coli* O157:H7 strain EDL933 in laboratory broth. Addition of these compounds also decreased Stx toxin production and gene transcription, and decreased *stx*-converting phage levels, when bacteria were grown in the presence of hydrogen peroxide or ciprofloxacin. This treatment also increased O157:H7 survival, collectively suggesting these vitamin K derivatives dampen phage induction normally resulting from DNA damaging agents. Several DNA damaging agents including ciprofloxacin and mitomycin C induce cellular filamentation of O157:H7, and this phenotype was also inhibited by menadione and menadione sodium bisulfate.

Collectively, the talks in this session provided a new appreciation of how the intestinal environment, especially other *E. coli* strains, may direct the severity of disease outcome during an O157:H7 infection. Future work is needed to understand whether results also apply to non-O157 STEC, which are collectively a more common cause of human illness than O157:H7. Additionally, previous studies demonstrated that extracts from fecal bacteria can reduce Stx production, and the work presented on vitamin K may provide us with insights into the possible mechanism(s) behind such observations.

A role of bacterial cell surface glycoproteins in colonization of host cells

Cell surface-associated glycosylation systems translate into a molecular barcode that is pivotal to the pathogenicity of several bacteria, mediating distinct bacteria-host interactions and increasing bacterial fitness in their niche (27). Thus, for understanding of the pathogenesis of bacterial infections, insight into glyco-compound biosynthesis is instrumental. However, due their secondary gene product-nature this is a challenging endeavour (28, 29).

Dr. Christina Schäffer (BOKU University of Natural Resources and Life Sciences) began the "Host-pathogen interaction at the receptor level" session, presenting as example her work on glycobiology-based strategies of the Gram-negative anaerobe *Tannerella forsythia* which support its status as a periodontal pathogen (30). This pathogen is gaining attention not only as a cause of periodontitis – globally, the most common inflammatory disease of bacterial origin – but also due to its link to systemic diseases. It is covered by a 2D crystalline cell surface (S-) layer that displays a unique protein glycosylation encoded by a general protein *O*-glycosylation system (31, 32). The BOKU research group found that the localization of *T. forsythia* within dental plaque varied depending on changes in the S-layer glycan, which also affected aggregation with and the prevalence of other bacteria present in a multispecies biofilm model (33). Immune response profiling of primary monocytes and human oral keratinocytes (HOK) revealed that truncation of the *T. forsythia* glycan leads to significant reduction of IL-1β and regulates macrophage inflammatory protein-1. HOK infected with *T. forsythia* produce IL-1Ra, chemokines and VEGF (34). Overall, the *T. forsythia* S-layer and attached sugars contribute to dampening the immune response to initial infection, mediate persistence of the bacterium in the host and, hence, play a pivotal role in orchestrating the bacterial virulence. As future aims it will be important to deepen our understanding of the vast mechanisms bacteria possess for protein glycosylation to devise novel strategies for designing vaccine formulations and protein therapeutics, based on synthetic glycobiology approaches.

The knowledge on interaction of adhesion factors of the bacteria with host cell glycans can also be used to develop strategies to prevent colonization. The research group of Dr. Eric Cox (Ghent University) has demonstrated that porcine F18$^+$ ETEC and/or Stx2e-producing F18$^+$ *E. coli* (STEC) interact via their fimbrial tip adhesin with glycosphingolipids having blood group ABH determinants on a type-1 core. The relative binding affinity to different blood group determinants decreases in the order B5 type 1 and A6 type1, A7 type I and B7 type 1, H5 type 1, A7 type 4, A8 type 1 and A9 type 1, with the latter having the weakest interaction (35). Ten mg per mL PBS of the A6 type 1 oligosaccharide was able to decrease binding to intestinal villi by 73% suggesting that the sugar could be used as a decoy receptor to decrease intestinal colonization. By conjugating the oligosaccharide on a carrier, the concentration needed for 70% inhibition was significantly decreased. Experiments using a small intestinal segment perfusion model demonstrated that this was sufficient for the host to reabsorb intestinal fluid secretion due to infections with $F18^+$ ETEC. Supplementing feed or water of piglets with the decoy receptor significantly reduced duration and height of fecal excretion of and $F18^+$ STEC strain, showing the potential of this strategy to control infection in piglets.

Piglets which suckle their dam are protected against ETEC infection by milk antibodies that interfere with binding of the fimbrial adhesins of ETEC to the mucosa, but at weaning this protection disappears and severe ETEC-induced diarrhea can occur. The VIB research group (Ghent University-VIB) of Dr. Vikram Virdi demonstrated that the antigen-binding variable domain of the llama heavy chain-only antibody (VHH), specific for the adhesin of F4+ fimbriae, grafted onto porcine IgA Fc and expressed in *Arabidopsis* seed was able to neutralize the infection of piglets with an $F4^+$ ETEC strain (36). VHHs can survive harsh chemical and temperature conditions yet remain functional. In that first study co-transformation of VHH-IgA with the porcine joining chain and secretory component led to the production of light-chain devoid, assembled multivalent dimeric, and secretory IgA-like antibodies. The produced antibodies, a mixture of monomeric, dimeric and secretory IgA significantly reduced infection.

Unexpectedly, this group demonstrated in a second study that the monomeric IgA (mVHH-IgA) format against ETEC delivered orally in feed is sufficient to prevent ETEC bacterial attachment, and to lower the shedding of the challenge ETEC bacteria, thus protecting piglets similarly as the SIgA format (37). Furthermore, they showed that mVHH-IgAs can be produced efficiently in soybean seeds and a *Pichia pastoris* yeast cell production platform. Crushed soybean seeds expressing mVHH-IgA, or the dried medium from *Pichia* secreting mVHH-IgA, when orally delivered in a feed formulation, protected the piglets from the ETEC challenge. The convenient scalability and frugal downstream processing make these anti-ETEC mVHH-IgAs most suitable for translation as a safe alternative prophylaxis to antibiotics. Moreover, given the anatomical organ size similarity, the in-piglet model results are highly relevant for translation of oral mVHH-IgA applications for human GI infections.

New vaccine strategies against enterotoxigenic *Escherichia coli* (ETEC)

Vaccination is considered an effective prevention option for ETEC induced diarrhoea. Indeed, vaccinating pregnant livestock animals to provide protective maternal antibodies to suckling newborns largely prevents neonatal diarrhea in young animals particularly pigs (38). However, though a few vaccine candidates have been under clinical studies (39-41), there are still no vaccines licensed against ETEC associated diarrhea for humans (42, 43).

Using controlled human challenge models (CHIMs) is a cost- and time efficient way to test new prevention strategies among which new vaccine candidates (44). Such models already exist for ETEC disease, but there is a need for models that use relevant ETEC strains circulating in low-and-middle-income countries. Some vaccine candidates require specific toxin or colonization factor (CF) profiles in the challenge strain, for example testing a heat-stable toxin (ST)-based candidate would require absence of heat-labile toxin (LT) to avoid the contribution of LT to diarrheal stool output, the main outcome measure in a challenge model.

Efforts to develop a model based on a STh only epidemiologically relevant strain was presented by Dr. K Hanevik (University of Bergen). An inoculum of 10^{10} CFU of the STh only ETEC strain TW10722 was observed to cause an overall diarrhea attack risk of 78% in healthy human volunteers (45). However, a good immunological correlate of protection for ETEC disease is still missing (46). While ETEC specific small intestinal IgA antibodies are thought to be an important contributor to protection against symptomatic ETEC infection, measuring it is both impractical and inaccurate due to the location of infection and the dilution/contaminant effects of intestinal content.

The use of CHIMs has a large potential to increase understanding of ETEC pathophysiology and the search for potential correlates of protection (44). An adequate antibody response is dependent on $CD4^+$ T cell helper cell involvement (47). Dr. Hanevik showed that ETEC infection elicited a rapid and long-lasting human CD4 T cell response against CFs CS5, CS6, and the ETEC mucinase YghJ. These responses correlated with serum anti-CS5 and anti-CS6 IgA levels. Further experiments should examine which particular T cell subtypes are involved, and how this correlates with ETEC specific IgA intestinal lavage and with protection against ETEC.

Key challenges in developing effective vaccines against ETEC diarrhea in humans include heterogeneity among ETEC strains and difficulty in inducing robust local mucosal immunity (42, 43). Over 25 immunologically different colonization factors (CFs) and two very distinctive enterotoxins (Sta (with two variants STh and STp) and LT) have been identified from ETEC strains isolated from human diarrhea patients. ETEC bacteria producing any one or two CFs and either or both enterotoxins can cause diarrhea in children and international travelers. To overcome these challenges, new strategies have been implemented for developing effective ETEC vaccines. This includes high expression of multiple ETEC CFs in a vaccine product, identification of conservative antigens among ETEC strains, and application of an epitope- and structure-based vaccinology platform to induce antibodies protecting against heterogeneous ETEC strains. To enhance vaccine candidates in stimulating local mucosal immunity, mucosal adjuvants double mutant LT (dmLT; $LT_{R192G/L211A}$), LTB, CTB, LT and CT B subunit hybrid (LCTB), as well as aminopeptidase N (APN)-specific antibody formats were applied to increase antigen uptake by small intestinal epithelial cells and thus local mucosal immune responses. Several of these strategies were explored in the ECMIS 2019 symposium.

Dr. Ann-Mari Svennerholm (University of Gothenburg) presented results from several clinical trials of an oral inactivated ETEC vaccine comprising four recombinant ETEC strains overexpressing the most prevalent human ETEC CFs (*i.e.*, CFA/I, CS3, CS5 and CS6) in combination with an LCTB toxoid (ETVAX) (39, 40) and given alone or together with dmLT adjuvant in Swedish adults and in decreasing age groups (45 years to 6 months of age) in Bangladesh. These studies showed that the vaccine is safe and induced strong mucosal immune responses against all the primary vaccine antigens determined by IgA antibody in lymphocyte secretions (ALS) and/or fecal SIgA antibody responses in a majority of the vaccinees (39, 40). Furthermore, the vaccine was shown to induce a mucosal immunological memory for at least 1-2 years after primary vaccination (49). Additionally, dmLT adjuvant was demonstrated an effective adjuvant to enhance ETVAX in inducing mucosal immunity in Bangladesh children. Thus, addition of dmLT adjuvant to the vaccine significantly enhanced mucosal immune responses against CFs and the O antigen (O78 LPS) of ETVAX in infants 6-11 months of age.

Different from the cocktail vaccine strategy, Dr. Weiping Zhang (University of Illinois) presented the epitope- and structure-based multiepitope fusion antigen (MEFA) vaccinology platform to develop broadly protective ETEC subunit vaccines. A combination of two MEFA proteins, CFA/I/II/IV MEFA which applied CFA/I subunit CfaB backbone to present neutralizing epitopes of CFA/II (CS1 - CS3) and CFA/IV (CS4 - CS6) and toxoid fusion MEFA $3xSTa_{N12S}$-$mnLT_{R192G/L211A}$ of which three copies of STa toxoid STa_{N12S} were presented by the monomeric LT mutant (a single peptide with one LTB subunit peptide genetically fused to one LTA subunit peptide with mutations at residues 192 and 211), was shown to induce antibodies that broadly inhibited adherence of ETEC bacteria producing any of the seven most important ETEC adhesins (CFA/I, CS1 - CS6) and neutralized enterotoxicity of both toxins (LT, STa) (50). Moreover, antibodies derived from CFA/I/II/IV MEFA and toxoid fusion protected against ETEC diarrhea in a pig challenge model, suggesting the potential application of these two proteins for a broadly protective multivalent ETEC subunit vaccine. Additionally, Dr. Duan from Yanzhou University presented that antibodies induced by toxoid fusion $3xSTa_{N12S}$-$mnLT_{R192G/L211A}$ protein had little cross reactivity to guanylin and uroguanylin (51). Researchers from the Henry Jackson Foundation and the Naval Medical Research Center examined the application of recombinant ETEC adhesin proteins CfaEB of CFA/I and CssBA of CS6 as carrier proteins for antigens of *Campylobacter jejuni* and *Shigella flexneri*, and protection against ETEC adherence. From a non-human primate immunization study, they reported that *Aotus nancymaae* monkeys immunized with HS23/36-CfaEB were protected when challenged with ETEC and *C. jejuni*. Recombinant CssBA alone was also evaluated as vaccine against CS6 ETEC strains. In contrast to the multivalent vaccine strategy, conservative antigen vaccine approach was also discussed.

Researchers also presented recent advances in inducing small intestinal mucosal immunity. Researchers from Ghent University presented data on antibody-mediated targeting of vaccine antigens to aminopeptidase N (also known as CD13), an apical membrane protein in enterocytes involved in transcytosis of F4 fimbriae (52). A key hurdle in oral subunit vaccines is poor transport of vaccine antigens across the epithelial barrier (53). This might be surmounted by their targeted delivery to APN. Upon oral administration to piglets, the selective delivery of vaccine antigens, as fused antigens or encapsulated in microparticles, to

APN by antibodies resulted in their transport across the small intestinal barrier and the induction of antigen-specific systemic and mucosal IgA^+ antibody secreting cells (54-56).

Progress on vaccines against pig post-weaning diarrhea (PWD) was presented as well. Coliprotec® F4, an oral vaccine licensed in some European countries by Elanco Animal Health, was shown to improve pig growth performance (based on daily weight gain) during the first three weeks of the post-weaning period. Pigs immunized with the oral live bivalent *E. coli* F4/F18 (Coliprotec® F4/F18) showed similar technical performance parameters and a significant reduction in medication use, compared to pigs treated with colistin. Additionally, researchers in the US examined the MEFA platform to include neutralizing epitopes of F4 and F18 fimbriae and toxins LT, STa, STb and Stx2e to develop a broadly protective vaccine against PWD (57, 58).

While developing effective vaccines against ETEC-associated diarrhea remains to be challenging, progress has been made from recent research. Novel vaccine technologies include those presented at ECMIS-2019 and continuous efforts from research groups can accelerate ETEC vaccine development and potentially lead to the licensing of effective vaccines for children's, travelers', and pig post-weaning diarrhea.

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