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A Tale of Two Bacterial Enteropathogens and One Multivalent Vaccine

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Introduction

Two bacterial enteric pathogens, *Shigella* and enterotoxigenic *Escherichia coli* (ETEC), are considered prototypical disparate ends of the spectrum from the perspective of pathogenesis. *Shigella* is the paradigm of a locally invasive pathogen that invades and destroys gut mucosa, often resulting clinically in gross blood and mucus in diarrheal stools (dysentery). In contrast, ETEC represents one of the best examples of a non-invasive bacterial pathogen that does not anatomically damage intestinal mucosa but rather elicits intestinal secretion and diminished absorption consequent to the effects of the heat-stable and heat-labile enterotoxins that it produces. Enterotoxin-induced intestinal secretion can result in copious purging, overt dehydration, shock and death in frail young infant hosts.

Professor Philippe Sansonetti is widely recognized as the premier authority on the pathogenesis of disease caused by *Shigella*. His decades of research describing the interaction between this human host-restricted pathogen and the intestinal mucosa collectively comprise one of the most extraordinary contributions on bacterial pathogenesis. His discoveries and use of novel technologies have contributed widely to the field of microbial pathogenesis in general and have served as inspiration to generations of investigators. The Sansonetti laboratory at the Institut Pasteur served as a site to which the best and brightest came to study under his tutelage and upon their departure to establish their own independent research units elsewhere, Professor Sansonetti's scientific progeny came to constitute a network that includes many of the world's leading research microbiologists and cell biologists. This paper is meant to serve as a tribute to Philippe Sansonetti and to honor him as a colleague, collaborator and friend.

Shigella and Enterotoxigenic Escherichia coli

Whilst *Shigella* and ETEC exhibit highly divergent pathogenesis, they nevertheless share some important commonalities that are relevant to the development of practical, well-tolerated and efficacious vaccines. *Shigella* and ETEC are each recognized as one of the top four enteric pathogens that cause diarrheal illness in young children in developing countries and as leading causes of young child deaths (Kotloff et al., 2013;Liu et al., 2016). These two

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bacterial pathogens are also major etiologic agents of travelers' diarrhea among persons from industrialized countries who visit developing regions of the world (Bourgeois et al., 2016;Hyams et al., 1991;Porter et al., 2017). Thus, a single vaccine that could target both these pathogens is highly desirable and would greatly enhance the vaccine's cost-effectiveness, an important attribute in the modern era. These pathogens also share a major impediment for vaccine developers in that each exhibits broad serotype or antigenic heterogeneity. Thus, rational, evidence-based strategies must be articulated to achieve broad-spectrum protection against the antigenic heterogeneity that each pathogen displays.

Shigella

Shigella and ETEC are classified as members of the enterobacteriaceae and are phylogenetically indistinguishable. It has been argued by some, based on genomics, that *Shigella* species should be taxonomically included within *E. coli* (Pettengill et al., 2015)

However, counter arguments to keep *Shigella* as a distinct genus include the clinical manifestations of mucosally invasive disease and epidemiologic features including the small inoculum size capable of causing shigellosis (DuPont et al., 1989;Levine et al., 1973) and direct fecal oral contact spread as the main mode of transmission (Sahl et al., 2015;Strockbine and Maurelli, 2005). Despite high levels of genomic homology, a set of unique virulence factors encoded by each pathogen confers very different pathogenic mechanisms that result in distinct clinical outcomes (Table 1).

The key virulence factors of *Shigella* are encoded on a large virulence plasmid that was characterized extensively by Sansonetti and colleagues (Sansonetti and Phalipon, 1996). Indeed, it was the seminal research of Sansonetti, working with the late Samuel B. Formal and Dennis Kopecko in the Walter Reed Army Institute of Research in Washington, D.C., that identified the virulence plasmid as responsible for host cell invasion capacity, the distinguishing pathogenic property of *Shigella* (Sansonetti et al., 1981;Sansonetti et al., 1982a;Sansonetti et al., 1982b;Sansonetti et al., 1983). Sansonetti and colleagues proceeded to characterize key regions of this plasmid that harbored genes encoding invasion capability, secreted effectors, and a unique cell-to-cell spread phenotype that utilized polymerization of host cell actin (Baudry et al., 1987;Bernardini et al., 1989;Hale et al., 1983;Maurelli et al., 1985).

ETEC

In contrast to the invasive phenotype of *Shigella*, ETEC cause disease by attaching to the small intestine via colonization factors (CFs) and elaborating heat labile (LT) and/or heat stable (ST) toxin(s) that cause dysfunction of ion transport and result in watery diarrhea (Levine, 1987). The genes encoding CFs and toxins are often located on plasmids in ETEC and isolates often harbor genes encoding more than one CF.

Protective antigens of *Shigella*, antigenic heterogeneity and vaccine strategies

Epidemiologic field studies and volunteer re-challenge studies document that an initial clinical episode of shigellosis confers circa 75% protection against clinical illness upon re-exposure to the homologous *Shigella* serotype. Other observations indicate that the

protective immune responses are directed against the *Shigella* O antigens that define serotype (Levine et al., 2007). However, that fact that multiple serotypes of *Shigella* contribute to the overall global burden of disease make antigenic diversity an obstacle that hinders a simple vaccine development strategy. Epidemiologically important *Shigella* serotypes include *S. sonnei* and the 15 *S. flexneri* serotypes and subtypes. *S. sonnei*, consisting of a single serotype, is the most important strain in industrialized countries and accounts for ~23% isolates in less developed regions of the world. *S. flexneri* is the most important species globally and comprises 15 serotypes. In contrast, whereas *S. dysenteriae* has 15 serotypes and *S. boydii* includes 19 serotypes, they account collectively for only 10.4% of cases worldwide (Livio et al., 2014). Thus, a broadly protective *Shigella* vaccine must provide protection against the 16 epidemiologically important serotypes (Livio et al., 2014).

While many *Shigella* vaccine strategies have been pursued (reviewed in (Ashkenazi and Cohen, 2013;Barry et al., 2013;Levine et al., 2007;Mani et al., 2016), the current leading approaches include: 1) parenteral vaccines that deliver chemically purified or synthetic *Shigella* OPS antigens as conjugates to carrier proteins, genetic bioconjugates (Riddle et al., 2016) or as general outer membrane vesicles derived from *Shigella* serotypes (Launay et al., 2017) (Launay et al., 2017), or; 2) live attenuated vaccine strains administered as live oral vaccines.

Recently, Mullard, Phalipon, Sansonetti and colleagues have pursued a synthetic carbohydrate-based conjugate approach to *Shigella* vaccine development. A series of elegant technical advances facilitated the biochemical synthesis of *Shigella* O-antigen specific carbohydrate antigens that were conjugated to a protein carrier (Phalipon et al., 2006;Phalipon et al., 2009;van der Put et al., 2016). These vaccine candidates have been demonstrated to be safe and immunogenic in volunteers (Cohen et al., 2017) and are advancing to evaluation using the controlled human infection model.

Early generations of live oral vaccines were shown to protect against natural exposure to wild type pathogens in field trials (Levine et al., 1976;Mel et al., 1965) and to protect vaccinated U.S. volunteers against experimental exposure to wild type organisms in the course of volunteer challenge studies (Coster et al., 1999;DuPont et al., 1972). The historical difficulty of this approach was in achieving the optimal balance of safety by attenuation, while retaining adequate immunogenicity to ensure protection.

Sansonetti and colleagues developed live attenuated *Shigella* vaccine strains based on their discovery of the role of VirG/IcsA in catalyzing cell-to-cell spread by polymerization of host cell actin (Bernardini et al., 1989). The first candidates were based on the fundamental mutation in *icsA* and included additional mutations in either *iuc:iut*, encoding the iron scavenging siderophore aerobactin production and transport (Fontaine et al., 1990;Sansonetti and Arondel, 1989) or *ompB*, encoding an important osmoregulatory protein (Sansonetti et al., 1991). One candidate, *S. flexneri* 2a vaccine strain SC602 was shown to induce protective efficacy against severe diarrhea and dysentery challenge in a controlled human infection model in North American volunteers (Coster et al., 1999;Katz et al., 2004). The narrow window of safety exhibited by this vaccine complicated its further clinical

development. In clinical trials in U.S. volunteers, SC602 was highly reactogenic when administered in doses above 10^4 CFU. When tested in adults and children in Bangladesh, SC602 did not cause adverse reactions. However, it also failed to elicit immune responses (Rahman et al., 2011).

Investigators at the CVD pursued an alternative strategy based on inactivation of genes encoding critical enzymes in metabolic pathways, namely *guaBA* (Noriega et al., 1996) and genes encoding two newly discovered enterotoxins of *Shigella*. The observations that *Shigella* infection includes early clinical manifestations of watery diarrhea (Kinsey et al., 1976;Rout et al., 1975) and that live attenuated vaccine strains still caused watery diarrhea in volunteers prompted CVD investigators to identify enterotoxins that elicit secretory diarrhea. *Shigella* enterotoxin 1 (ShET1) is encoded by the *set*1A and B genes that are located on the chromosome of *S. flexneri* 2a and 2b strains (Fasano et al., 1995;Fasano et al., 1997;Livio et al., 2014;Noriega et al., 1995). *Shigella* enterotoxin 2 (ShET2) is encoded by *sen*, a gene found on the virulence plasmid of all *Shigella* strains (Nataro et al., 1995). The attenuation resulting from deletion of *guaBA* in *S. flexneri* 2a vaccine strain CVD 1204 and the importance of deletions in enterotoxin-encoding genes *set* and *sen* to achieve a higher level of safety in vaccine strain CVD 1208 were established in volunteer studies (Kotloff et al., 2004;Kotloff et al., 2007). CVD 1208S has been manufactured as a cGMP lot and is advancing to challenge studies.

Our strategy for constructing a vaccine that confers broad protection against *Shigella* infection is informed by epidemiologic studies that identified *S. sonnei* (single serotype) and *S. flexneri* serotypes 2a, 3a and 6 as the most prevalent serotypes isolated from young children with moderate-to-severe diarrhea (Livio et al., 2014) (Table 2). Inclusion of these 4 serotypes would provide direct protection against ~64% *Shigella* strains (Livio 2014). Recently we have included an additional attenuated *S. flexneri* strain of serotype 1b to the multivalent vaccine to broaden direct coverage up to ~72% of epidemiologically relevant, disease-associated, *Shigella* serotypes. However it is anticipated that based on cross reactivity among serotypes within the vaccine with other important serotypes that are not in the vaccine but that share O group or type antigens with the vaccine strains, it may be possible to protect against up to 89% of the epidemiologically relevant *Shigella* serotypes. (Levine et al., 2007;Noriega et al., 1999a). Live attenuated versions of each of these serotypes have been engineered with *guaBA* and *sen* deletions and demonstrated to be safe, immunogenic, and protective against wild type challenge in animal models (DeLaine et al., 2016). While no *S. dysenteriae* 1 strains were isolated in GEMS, the exceptional virulence of this serotype that expresses Shiga toxin, causes severe disease with complications, and has been responsible for epidemics and pandemics, argues for its inclusion either in a broadly protective vaccine or as a monovalent vaccine to be kept in a potential emerging pathogen stockpile. In the event of re-appearance and resurgence of *S. dysenteriae* 1, such a stockpile could help limit the spread of Shiga dysentery. A Shiga toxin-negative, attenuated derivative of *S. dysenteriae* 1 has been developed (Wu et al., 2011). Furthermore, we have shown that a mixed inoculum composed of two or three live attenuated strains of *Shigella* can induce immune responses to all components and provide protection against each component serotype (DeLaine et al., 2016;Noriega et al., 1999b). Immunological readouts include serum and mucosal antibody titers as well as antibody secreting cells (ASC) or

antibodies in lymphocyte secretions (ALS) to serotype-specific LPS O-antigen. Recent studies have supported the use of functional assays including serum bactericidal (SBA) and opsonophagocytosis inhibition (OPA) assays as potential correlates of protection (Shimanovich et al., 2017).

Protective antigens of ETEC, antigenic heterogeneity and vaccine strategies

In contrast to the invasive phenotype of *Shigella*, ETEC cause disease by attaching to the small intestine via colonization factors (CFs) and elaborating heat labile (LT) and/or heat stable (ST) toxins which cause watery diarrhea. Epidemiological and volunteer studies support the protective capacity of antibodies that block colonization factors to prevent disease (Levine et al., 2019). Clinical isolates of ETEC express a multitude of different CFs. There are 7 major CFs that have been firmly associated with isolates that cause disease in young children including CFA/I, and CS1 through CS6 (Vidal et al., 2019). While a variety of minor CFs have been identified, with just a few exceptions (CS7 and CS17), convincing evidence of their association with disease has not been established through epidemiological studies or volunteer challenges (Vidal et al., 2019). Recent analysis identified one minor CF, CS14, as being both common and significantly associated with diarrheal disease (Vidal et al., 2019). Our strategy to provide broad coverage against ETEC includes antigens to induce colonization blocking immune responses against all the major CFs (i.e., CFA/I, CS1-CS6) plus CS14 (Table 3) (Levine et al., 2019). In addition, an antigen to induce LT-toxin neutralizing antibodies is included to cover a subset of LT-only ETEC strains that are associated with diarrhea in specific populations (Mansour et al., 2014;Steinsland et al., 2002).

Specifically, we have engineered four live attenuated *Shgella* strains to constitutively express critical ETEC antigens (Table 3). This multivalent *Shigella*-ETEC approach was supported by a clinical trial wherein an earlier prototype *Shigella*-ETEC candidate consisting of *S. flexneri* 2a strain CVD 1208S harboring a stabilized plasmid encoding CFA/I and LThA2B, CVD 1208S(pCFA/I-LTB), was used to immunize volunteers. This vaccine was well tolerated but despite the use of a highly engineered plasmid system to ensure stable maintenance, the plasmid was lost from *Shigella* within the host GI tract. Plasmid loss was verified by characterization of bacteria shed in volunteer stool samples. Notwithstanding these disappointing results, a subset of volunteers shed the vaccine strain in which the plasmid was stably maintained in the *Shigella* live vector. Notably, those vaccinees mounted robust immune responses to both the *Shigella* live vector as well as ETEC antigens. This observation served as proof of principle that the combined *Shigella*-ETEC strategy could be effective if a more genetically-stabilized vaccine strain could be developed. To this end, we have now engineered live *Shigella* vaccine strains to express key ETEC antigens from chromosomal loci. The resultant *Shigella*-ETEC hybrid vaccine strains are remarkably stable, express morphologically correct fimbriae on the surface, elicit strong immune responses and confer protection against wild type challenge in animal models (Figure 1) (Barry et al., 2016).

There is no good animal model that recapitulates ETEC disease; therefore in vitro assays are utilized to measure functional immune responses. Hemagglutination inhibition (HAI) is

considered a proxy for inhibition of binding to human intestine (Baker et al., 2009;Cravioto et al., 1982) and HAI antibodies titers are one measure of functional responses to the ETEC component of these vaccine candidates.

Our most advanced *Shigella*-ETEC candidate, *S. flexneri* 2a vaccine strain CVD 1208S-122, which expresses CFA/I and LThA2B from the chromosome, is safe and immunogenic in animal models and confers protective efficacy against wild type *Shigella* challenge (manuscript in preparation, VED abstract). This vaccine is currently being manufactured as a cGMP lot and a clinical development plan has been prepared. Anticipated positive outcomes from initial clinical studies will pave the way for testing combinations of the *Shigella*-ETEC hybrid strains that constitute a broadly protective vaccine against two important pathogens.

Epilogue

The pioneering work of Sansonetti and subsequent work by his multitude of collaborators and trainees have advanced the field of *Shigella* research and facilitated the development of novel interventions against this globally important human pathogen. Confirmation of the pathogenic impact of *Shigella* and ETEC on the most vulnerable populations has converged with advances in technology to result in an exciting era of vaccine development and the advancement of multiple novel vaccine strategies. Our combined *Shigella*-ETEC approach has the potential to expand protection to individuals at risk of disease by two important pathogens. All investigators who work on *Shigella* in the modern era owe a debt to Philippe Sansonetti for his many ground breaking discoveries and insights about this pathogen. These insights have paved the way for a new generation of *Shigella* vaccines.

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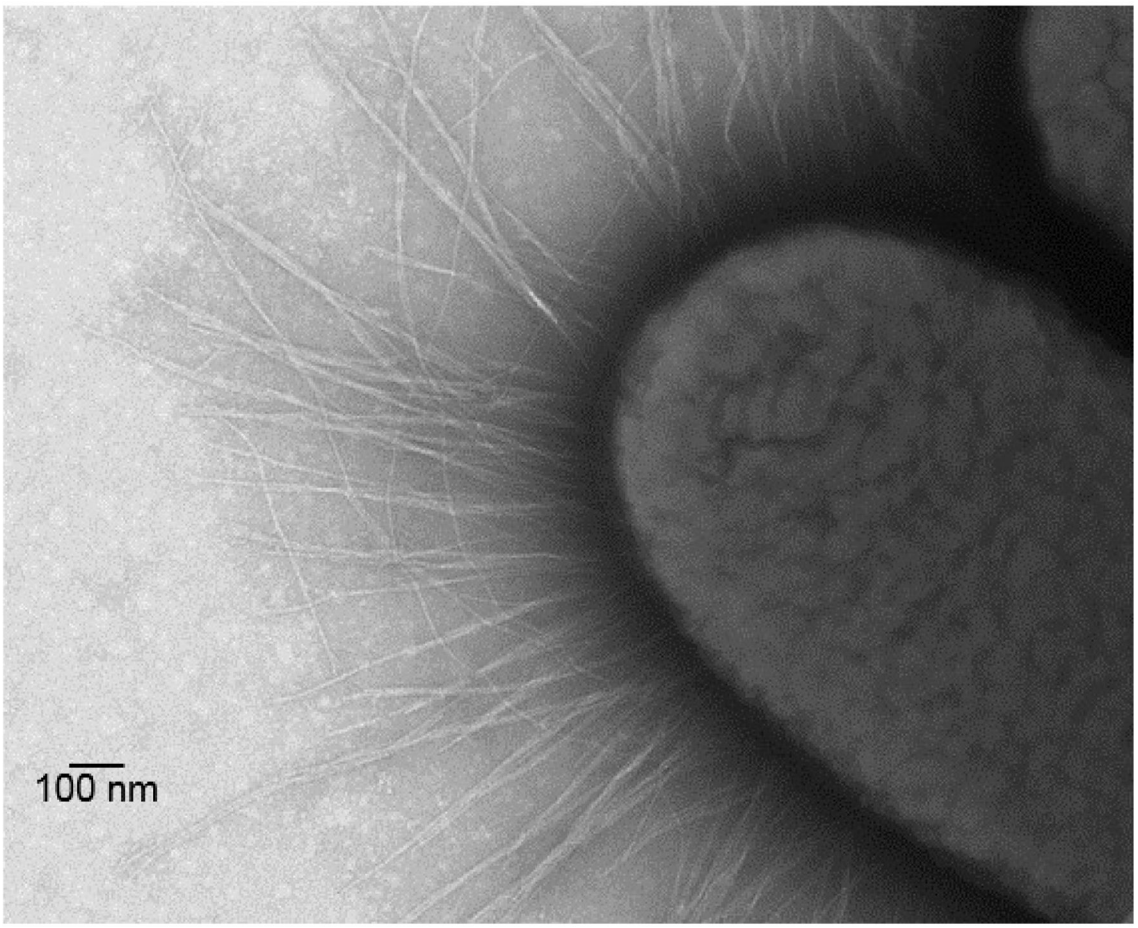

Figure 1.

Transmission electron micrograph of *S. flexneri* 2a vaccine strain CVD 1208S expressing CFA/I fimbriae. CVD 1208S-CFA/I was suspended on a 300 mesh Formvar coated grid and stained with 2% ammonium molybdate. The image was examined using a JEOL electron microscope JEM-1200EX and is shown at 1500X; the scale bar represents 100 nm.

Table 1.

Differences and Similarities between *Shigella* and ETEC

	Shigella	**ETEC**
Taxonomic Classification; Genomic Content	enterobacteriaceae; ~4Mb genome with ~220kb virulence plasmid	enterobacteriaceae; ~4Mb genome with plasmid(s) encoding CFs and toxins
Strain Diversity in Relation to Key Antigens	Diversity based on LPS O-antigen serotypes	Diversity based colonization factors and toxins
Clinical Manifestations	Initial watery diarrhea followed by dysentery (blood + mucus in stool)	Profuse Watery diarrhea
Key Virulence Factors	Invasion mechanisms, secreted effectors and enterotoxins	Attachment factors and LT and/or ST toxins
Key Pathogenic Mechanism	Invasion, cell-to-cell spread, and induction of inflammation	Attachment and delivery of toxins (LT and/or ST) that promote GI ion transport dysregulation
Vaccine Development Strategies	Oral: Live attenuated Killed whole cell Parenteral: Conserved antigens O-Antigen conjugates (synthetic, non-synthetic) Combinations (GEMMA, Invaplex)	Oral: Killed Whole Cell Multivalent Live vectored Live attenuated Parenteral: Conserved antigen
Vaccine Target Populations	Young children in developing countries Specific populations in developed countries (daycare centers and custodial institutions) Travelers including military personnel	Young children in developing countries Travelers including military personnel

Table 2.

Strategy for *Shigella* serotype inclusion in a broadly protective vaccine based on *Shigella* Serotype Distribution in GEMS (Livio et al., 2014)

Strain/ serotype	**% Total (GEMS)**		
S. sonnei	**23.7%**		
***S. flexneri* serotype**		**Type- and/or Group-antigen Expressed**	
		Type- antigen	**Group-antigen**
2a	20.2%	**II**	**3,4**
6	11.0%	**VI**	
2b	10.9%	**II**	**7,8**
3a	9.4%		**7,8**
1b	7.5%	**I**	**6**
4a	2.9%	IV	**3,4**
7a	2.0%	VII	
X	1.0%		**7,8**
Y	0.4%		**3,4**
5b	0.3%	V	**7,8**
1a	0.3%	**I**	**3,4**
3b	0.1%		**3,4; 6**
4b	0	IV	**6**

Bold: serotypes and antigens included in CVD vaccine;

Table 3.

CVD multivalent *Shigella*-ETEC Strategy

***Shigella* Strain**	**Vaccine Name**	**ETEC Antigen(s)**
S. sonnei	CVD 1233S	CS2, CS3
S. flexneri 2a	CVD 1208S	CFA/I, LThA2B
S. flexneri 3a	CVD 1213	CS1, CS5
S. flexneri 6	CVD 1215	CS4, CS6
S. flexneri 1b	CVD 1224	CS14
S. dysenteriae 1	CVD1254	StxB