Combination vaccines against diarrheal diseases

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Introduction

Several recent large scale studies of global diarrheal disease burden and epidemiology, renewed recognition of multiple diarrheal diseases remain a leading cause of global childhood mortality and morbidity. Several recent epidemiological studies highlight the rate of diarrheal diseases in different parts of the world and draw attention to the impact on childhood growth and survival. Despite the well-documented global burden of diarrheal diseases, currently there are no combination diarrheal vaccines, only licensed vaccines for rotavirus and cholera, and Salmonella typhi-based vaccines for typhoid fever. The recognition of the impact of diarrheal episodes on infant growth, as seen in resource-poor countries, has spurred action from governmental and non-governmental agencies to accelerate research toward affordable and effective vaccines against diarrheal diseases. Both travelers and children in endemic countries will benefit from a combination diarrheal vaccine, but it can be argued that the greater proportion of any positive impact will be on the public health status of the latter. The history of combination pediatric vaccines indicate that monovalent or single disease vaccines are typically licensed first prior to formulation in a combination vaccine, and that the combinations themselves undergo periodic revision in response to need for improvement in safety or potential for wider coverage of important pediatric pathogens. Nevertheless combination pediatric vaccines have proven to be an effective tool in limiting or eradicating communicable childhood diseases worldwide. The landscape of diarrheal vaccine candidates indicates that there now several in active development that offer options for potential testing of combinations to combat those bacterial and viral pathogens responsible for the heaviest disease burden—rotavirus, ETEC, Shigella, Campylobacter, V. cholera and Salmonella.

Global burden of diarrheal diseases

The Global Burden of Disease (GBD) study, 2010, estimated that although annual rates of childhood mortality due to diarrheal diseases have decreased from 2.5 million in 1990 to 1.4 million in 2010, the number of deaths in children <5 years of age due to diarrheal diseases remain significant.1, 2 The burden of childhood diarrhea in 2010 amounted to almost a billion episodes with 2% being severe episodes with an estimated >500,000 deaths in children 1–4 years of age.3-7 Globally, diarrheal disease also remains one of the leading causes of disability-adjusted life years or DALYs.8,9 Nearly 3-quarters of childhood diarrhea and pneumonia are concentrated in 15 countries, with the highest burden in Africa, South and South East (SE) Asia.10 In the GBD 2010 study, rotavirus was identified to be the most important pathogen contributing to the global rates of disease followed by Cryptosporidia, the 2 together causing one-third of the diarrheal diseases in children <4 years of age.1 The remaining share of the disease burden was attributed to several gram negative bacteria that include enterotoxigenic E. coli (ETEC), enteropathogenic E. coli (EPEC), Shigella,
Campylobacter, V. cholera, Salmonella, with a high percentage of cases having no identified pathogens.\(^1,8,11\) (Table 1).

The recently published Global Enteric Multicenter Study (GEMS) is a large-scale survey of the incidence and causative agents of moderate-to-severe diarrheal disease in young children 0–59 months of age residing in low income parts of 7 countries in Africa and South Asia.\(^12-15\) The GEMS study pointed out that just 4 pathogens contributed to the majority of moderate to severe diarrhea and these included rotavirus, ETEC, Shigella and Cryptosporidia. Other pathogens such as Aeromonas, V. cholera and Campylobacter were more region-specific. Among the youngest children 0–11 months of age, rotavirus and ETEC encoding heat stable toxin (ST) was often the leading agents of moderate-to-severe-diarrhea while in the older children ages 12–59 months, Shigella was either the lead agent or among the top 2 lead agents overall (Table 1). The GEMS study also followed a birth cohort till 2 years of age and provided additional evidence that children who have repeated episodes of moderate to severe diarrhea are comparatively underweight, stunted in physical growth, and have decreased cognitive functions as compared to age-controlled children with no disease.\(^16-19\)

**Traveler’s diarrhea (TD)**

In the US and other developed countries the risk of diarrheal diseases is low, and when it occurs, is usually self-limiting and underreported and often treated with over-the-counter medications and antibiotics. The FoodNet program in the US that conducts population-based surveillance for laboratory-confirmed cases of diarrhea indicated that in 2011 there were 18,964 laboratory confirmed cases of diarrhea with 4398 hospitalizations and 82 deaths. Of these cases 41% and 36% were due to Salmonella and Campylobacter species, followed by Shigella (8.1%) and Cryptosporidia (8%).\(^20\) Campylobacter infections are commonly associated with eating contaminated chicken, and 80% of these cases can be eliminated by proper poultry farming and cooking. Although ETEC has been recognized as an etiological agent of diarrhea since the 1960’s, poor detection methods resulted in infrequent identification of ETEC as a cause of food-related gastroenteritis. Traditional methods of detection included culture, animal bioassays and later ELISA assays with antibodies to toxins.\(^21\) More recent molecular based techniques have shown higher sensitivities for detection.\(^22\)

Diarrheal diseases are important causes of morbidity and mortality in Sub-Saharan Africa, North Africa and the Middle East, South and SE Asia, Central Asia, and several parts of Latin America.\(^8,23\) Travelers including military populations who visit these endemic regions risk incurring diarrhea from multiple causes due to lack of pre-existing immunity.\(^24-28\) The Centers for Disease Control, Atlanta, GA (CDC) notes that “Traveler’s Diarrheal Disease (TD) is the most predictable travel-related illness,” and that attack rates can be as high as 70%.\(^29\) As seen in Table 1, diarrhea is caused by infection with a number of bacterial pathogens as well as several viruses and parasites.\(^30,31\) In an age of globalization, millions of travelers move from one part of the world to another for business, pleasure or outreach. Some of these regions are recognized to be endemic for diarrheal disease. A case can therefore be made for a commercially viable TD vaccine that protect travelers against the discomfort, not to mention, wastage of time and expenses incurred due to treatment of episodes of diarrhea.

There are region-specific distribution patterns of diarrheal pathogens that need to be taken into account for vaccine development.\(^8\) In one study ETEC was found to be the most common pathogen identified overall and shared the primary burden of TD disease (30–35%) in Africa, South Asia (India), and Latin America while enterogaegregative E. coli (EAEC) was the second most common pathogen identified in traveler’s who had visited Latin America (24.7%) and South Asia (16%).\(^32\) In SE Asia Campylobacter was more prevalent (32%) with Salmonella and V. cholera causing an equal share of disease episodes (9%). EPEC was also identified in 14.3%, 18% and 7.7% in traveler’s returning from Latin America, SE Asia and Africa. Shigella species were most commonly found in TD in Africa and South Asia (8–9%).\(^33\) Among the viruses,
norovirus, rotavirus and adenovirus were the main etiologic agents causing diarrheal disease while the most prevalent parasites causing diarrhea were *Cyclospora, Cryptosporidium, Entamoeba* and *Giardia*. In 40–50% of the cases no pathogen was identified indicating that better detection methods may be needed to obtain a more complete list of pathogens causing diarrhea. It is believed that 80% of all TD are caused by bacterial species that are transmitted by contaminated food and water. Currently the most common agents of TD are ETEC, followed by EAEC, *C. jejuni*, *Shigella* spp and *Salmonella* spp.

Prophylactic antibiotics may be prescribed, but increased resistance to commonly used drugs such as fluoroquinolones has been observed in *Campylobacter, Shigella* and *Salmonella*. Although TD is generally an acute, short-term infection, in some cases it can trigger chronic conditions such as idiopathic inflammatory bowel disease. Thus there is significant potential benefit to vaccination of travelers to high-risk parts of the world.

Based on prevalence, severity of disease caused by the pathogen and stage of current clinical development, Table 2 provides an initial list of pathogens for which vaccines could be developed within the next 10 years. Development of a multi-cause childhood diarrheal disease vaccine, as well as a TD vaccine, that will require antigens from multiple pathogens can be developed pursuing a pathway that has been successfully employed for the formulation of several modern-day multivalent pediatric combination vaccines. A brief history of combination vaccine is also provided.

### History of combination vaccines

One of the earliest examples of a combination vaccine was a typhoid-paratyphoid A and B vaccine (TAB) that was administered by the intradermal route (ID) and was composed of *S. typhi, S. paratyphi* A and *S. paratyphi* B. The TAB vaccine contained 1–2 × 10⁷ CFU/ml of the enteric organisms suspended in 0.5% phenol-saline. Later, TAB was combined with a tetanus vaccine (TABT) and introduced into the British Army, Royal Navy and Air Force. The ID route reportedly caused minimal reactivity and elicited higher agglutinating antibody titers. TAB was also combined with heat-killed and phenol-preserved *V. cholera* strain Ogawa (TAB/Ch) and evaluated singly or combined and administered by the ID route. The combined TAB/Ch vaccine contained 5 × 10⁶ CFU of enteric organisms and 8 × 10⁶ CFU of cholera organisms per ml suspended in 0.5% phenol saline. The combined vaccine in this case was obtained by adding equal quantities of double-strength TAB and cholera vaccines. Human volunteers were vaccinated ID with 2 0.1 ml volumes of TAB/Ch spaced 21 days apart. The data indicated 2–8 fold higher agglutinin responses to the O-antigens of *S. typhi* and *V. cholera* and was validated by animal studies in mice. The conclusions drawn from this study was that a combined enteric and cholera vaccine could be given ID and was preferable over the administration of the 2 components separately. Thus ID vaccination with a combined vaccine became a routine procedure in the British Services. This vaccine however, did not meet with the same success in the US because the immunity patterns to the 3 different pathogens were not duplicated.

### Modern era of combination vaccines

The Food and Drug Administration (FDA) defines "combination vaccine" as 2 or more vaccines that have been combined by the manufacturer or supplied as vaccine components that are formulated to be combined immediately before administration. However, in practice, it is most likely that a combination vaccine is supplied as a pre-blended, single entity. The combination is intended to either prevent multiple diseases or a single disease caused by different strains or serotypes. Since multiple species of bacteria, viruses and parasites can cause

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**Table 2. Diarrheal disease-causing pathogens for combination vaccine development**

| Bacteria                                                                 | References |
|--------------------------------------------------------------------------|------------|
| ETEC† enterotoxigenic *E. coli*, most common diarrheal pathogen in TD, strains produce heat stable (ST) and/or heat labile (LT) toxins, ST-ETEC most associated with disease | 6,8,10,14,21,108,113,120,122,123,131,135,141,147 |
| EAEC enterogroupative *E. coli*, important emerging cause of persistent pediatric diarrhea, important cause of TD in S. Asia, Latin America, Africa | 8,37,40,156 |
| EPEC enteropathogenic *E. coli*, important cause of diarrhea related illness in children <5 years of age, identified by Hec 2 cell cultures, presence of adherence plasmid and intimin gene | 1,6,8,12,14 |
| *Shigella* cause of bacillary dysentery, *S. sonnet* prevalent in developed countries, *S. flexneri* 2a, 3a, 6 in developing countries, 8-12% of all cause diarrhea | 12,15,24,25,39,45,111,118,121,125,140,142 |
| *Campylobacter* associated in the US mostly with contaminated chicken, important cause of diarrheal illness in TD in SE Asia | 8,25,32,38,114-116 |
| *Vibrio cholera* causes high mortality rates during epidemics due to voluminous watery diarrhea leading to dehydration, endemic in some countries, seen in SE Asia, in TD | 4,5,19,48,107,110,136 |
| *Salmonella typhi* causes typhoid, Important cause of enteric fever | 8,5,95-97,100 |
| *Salmonella* important cause of diarrheal illness in children and in adults in developed and in developing non-typoidal countries, in TD | 1,41,42,98,99,155 |

| Viruses                                                                 | References |
|--------------------------------------------------------------------------|------------|
| Rotavirus** most important cause of hospitalization for childhood diarrhea in the US and high mortality rates due to diarrhea in the developing world | 6-8,101-106 |
| Norovirus higher risk for severe disease in the elderly, transmission in day-care centers, cruise ships, nursing homes, military, in TD, in children <5 years in developing countries | 32,36,143,157 |

| Parasites                                                               | References |
|--------------------------------------------------------------------------|------------|
| Cryptosporidium high global mortality rates from diarrhea in the developing world | 14,32,34,143,154 |
| Entamoeba acute and persistent diarrhea in children in developing countries, bloody stools | 6,10,19,23,32,34 |

*Vaccine candidates in clinical trial.

**Licensed vaccines available TD, Traveler’s diarrhea.
diarrheal disease, vaccine development must consider the population to be vaccinated (Table 1). In the case of diarrheal diseases in endemic parts of the world there would be special attention to the age range of the recipients as most of them would be very young children. Other factors to consider would be the etiological burden and attack rates in these populations, consideration of severity of disease, health care-seeking attitudes within the population, requirement for hospital care, complex treatment regimen, post-infectious sequelae, and prevalent species of each type of organism that is responsible for disease burden.\textsuperscript{14,46,55-57} Combination vaccines against diarrheal diseases could be a mixture of 1) live or whole-cell-killed vaccines that are reconstituted and mixed before oral ingestion 2) subunit vaccines against individual pathogens that are reconstituted and administered as a single injection or 3) whole cell and subunit vaccines, including DNA vaccines, given concurrently.\textsuperscript{57} Another category would be live vaccine organisms expressing heterologous antigens (vectored vaccines). Whole cell or subunit vaccines may each contain single or multiple antigens from a single pathogenic species or multiple antigens from several species that cause diarrheal disease. Some of these changes could also be engineered at the manufacturing step. Adjuvants may be added during reconstitution of the vaccine or manufactured along with the antigen. The stepwise development of several combination vaccines for pediatric use provides a case study as strategies for a combination vaccine against diarrheal diseases are contemplated.

**Current pediatric combination vaccines**

The inactivated polio vaccine (IPV) is composed of inactivated poliovirus types 1, 2, 3 vaccine strains and is an example of a combination vaccine that protects against multiple variants of a single disease.\textsuperscript{58-60} The diphtheria, tetanus and pertussis (whooping cough) vaccine (DTP & DTaP) and the measles-mumps-rubella (MMR) vaccines are examples of combination vaccines that protect against multiple diseases and are comprised of previously licensed monovalent vaccines.\textsuperscript{61-66} The MMR vaccine is a trivalent mixture of live attenuated viruses of 3 diseases administered via injection. Although individual licensed vaccines against all 3 diseases were available since the 1960s, the 3 vaccines were combined in 1971 to become the MMR vaccine.\textsuperscript{66} DTP & DTaP are subunit-based vaccines composed of purified antigens from *Corynebacterium diptheriae*, *Clostridium tetani* and *Bordetella pertussis*.\textsuperscript{67-69} Prior to the development of DTP, monovalent toxoid-based vaccines were available to protect against diphtheria, tetanus and pertussis. However, in 1948 DTP was licensed by the FDA. The DTP vaccine became the first version of a combined diphtheria, tetanus and pertussis vaccine that was routinely administered to children from the 1940’s to the mid 1990s.\textsuperscript{69,70} Since then, individual components in the combination vaccine have been replaced by other antigens to improve the safety profile. For example, due to reported long term neurological effects with the whole-cell pertussis vaccine component of the DTP vaccine (also referred to as DTwP), an acellular version, DTaP, using purified pertussis antigens was incorporated into a new formulation and approved in 1991 for use in the US.\textsuperscript{68,69} Two versions of DTaP exist, one with 3 and the other with 5 pertussis antigens (DTaP\textsubscript{3} and DTaP\textsubscript{5}).\textsuperscript{71} Because DTaP uses fewer purified antigens than the whole-cell vaccines, it is less reactogenic and therefore considered safer, but it is also more expensive. Recent research suggests that DTwP is more effective than DTaP in conferring immunity due to DTaP’s narrower antigen base.\textsuperscript{72-73} DTaP vaccines contain alum as the adjuvant and the vaccines from different manufacturers differ mainly in the number, amount and detoxification of the pertussis components. Additionally, other vaccines have been added to DTaP, such as IPV, vaccines for hepatitis B (HepB) and *Haemophilus influenzae* type B (Hib), to obtain licensed vaccines for DTaP-IPV, DTaP-IPV-Hib, DTaP-HepB-IPV and the latest combination that is DTaP-HepB-Hib/IPV hexavalent vaccine.\textsuperscript{74-83} In each case aluminum hydroxide or aluminum phosphate is the adjuvant. At the same time various combinations of HepB, pneumococcal conjugate vaccine (PCV) and pneumococcal polysaccharide-based vaccines, *Neisseria meningitidis* serogroup C and Y tetanus toxoid conjugate vaccines, that are either separately coadministered or combined in a single injection with routine pediatric vaccines are also being evaluated in clinical trials for safety and for demonstration of lack of immunological interference with other coadministered vaccine antigens.\textsuperscript{84-91} The potential advantages of such combination vaccines accrue from giving fewer injections that protect against several diseases, accommodating the administration of the combination vaccines within the WHO-recommended Expanded Program of Immunization (EPI) schedule, lowering pain and anxiety to the infants and caregivers, reducing overhead costs for administration and vaccine storage and overall increasing vaccine coverage and compliance.

An important factor in the formulation of licensed combination vaccines is the availability of vaccine-induced immune measures that correlate with protection against a specific disease.\textsuperscript{92} For diarrheal vaccines against individual pathogens, correlates or surrogates of protection will be required before vaccines can be combined successfully. When two or more vaccines are combined, evaluation of immunological interference is judged by determining the levels of antibodies that are known to confer protection against a specific pathogen.\textsuperscript{92} Most currently used vaccines act through functional antibodies such as bactericidal or opsonophagocytic antibodies against encapsulated bacteria such as Hib, pneumococci and meningococci or anti-toxin antibodies to diphtheria, tetanus and pertussis toxins. For example, a bactericidal serum antibody level of 0.15 ug/ml and 0.18–0.35 ug/ml has been determined to be sufficient for protection against bacteremia caused by Hib and pneumococci respectively.\textsuperscript{92} These correlates may vary with the population, exposure rates, serotype and clinical end-point.\textsuperscript{93} Anti-toxin antibody levels of 0.1 ug/ml for tetanus and diphtheria and 5–10 units of anti-pertussis toxin antibody levels for pertussis correlate with protection. The most commonly reported example of immune interference during the formulation of a combination vaccine is the lower antibody titer to the Hib component of a DTaP-based combination vaccine.\textsuperscript{93,94} The exact mechanism for this reduced response is not understood although interference of the Hib-conjugate with free unconjugated tetanus toxoid, interaction with the alum adjuvant and other factors have been advanced.\textsuperscript{94} The polio vaccines (IPV,
OPV) elicit antibodies that prevent viremia and neutralization titers of 1:8 or 1:4 are considered protective.92

Assuming that there are licensed vaccines already in use and correlates of protection are known, a major question during development of a combination vaccine is when and how to combine 2 different vaccines. Further, as the safety and efficacy of initial combination vaccines become established, individual combinations may become combined with others to create a single entity with expanded coverage. Obviously vaccines cannot be mixed at will except when specifically approved by the FDA and packaged for that purpose. The Hib conjugate vaccine (ActHIB, Sanofi and Hiberix, GSK) was introduced in the US in 1987 for use in children 2 months–18 months and is composed of purified polyribosylribitol phosphate (PRP) capsular polysaccharide of Hib conjugated with tetanus toxoid (PRP-T). It is manufactured as a lyophilized powder and is reconstituted at the time of delivery with either saline to obtain the monovalent vaccine or combined with DTP or DTaP vaccine to obtain a combination DTP-Hib or DTaP-Hib vaccine.78,82 The more recent development of a licensed liquid pentavalent vaccine DTaP₅-IPV-Hib (Pediael, Sanofi Pasteur, licensed 2008) for infants and toddlers is made up of 3 different combination vaccines protecting against 5 diseases, diphtheria, tetanus, pertussis, polio and Hib.83 The licensing of this pentavalent vaccine was based on the observation that no clinically important differences in the safety or immunologic profiles were noted between infants receiving the pentavalent vaccine and those that received separately administered DTaP, IPV and Hib vaccines.79 A fully liquid combination vaccine has the advantage of delivering an accurate dose with fewer injections for the infant. Such combination multivalent vaccines results in increased vaccine compliance and enables more infants to be safely immunized and to complete their immunization regimens against multiple childhood diseases more successfully.

Current licensed diarrheal vaccines

Currently there are licensed vaccines against only 2 strictly diarrheal pathogens, rotavirus and V. cholera. Both vaccines are orally administered. Two licensed vaccines against S. typhi, an oral live attenuated vaccine, Ty21a, and a subunit vaccine based on the Vi polysaccharide antigen are administered to protect against typhoid fever.11,41,95-97 A number of live and subunit Salmonella vaccine candidates have been evaluated for non-typhoidal Salmonella but this area of effort will not be discussed further in this review.98-100

Rotavirus is the most common cause of severe gastroenteritis in infants and young children worldwide and causes approximately half a million deaths each year among children aged <5 years, with >80% of deaths occurring in developing countries. Two live attenuated oral vaccines, RotaTeq® (RV5, Merck) and Rotarix® (RV1, GSK) have been licensed in over 100 countries and are recommended for administration concurrently with DTaP-IPV vaccines at 2, 4, 6 months or at 6, 10, and 14 weeks in developing countries.101,102 Clinical trials have indicated that 2 doses of live attenuated rotavirus vaccines given orally to infants did not interfere with the immune responses to concurrently administered intramuscular injections of DTaP, Hib, HepB and PCV.103 Combining 2 different routes of immunization for different vaccines is another example of maximizing the efficiency of infant immunization. The licensed rotavirus vaccines have reduced the rates of hospitalization in the US by ~80%.104,105 However, in endemic countries like India and Pakistan, the rotavirus vaccines have shown reduced efficacy indicating that higher doses and more doses may be required to immunize such an endemic population.106 Similar observations have been previously noted with live polio and cholera vaccines. RotaTeq® (RV5) is a human-bovine reassortant which was licensed by the FDA in February 2006 and Rotarix® (RV1) is a human rotavirus strain attenuated by multiple passages through cultured cells that was licensed by the FDA in April 2008. The Biologics Licensure Application (BLA) for RV1 contained 6 phase II trials and 5 phase III trials and the BLA for RV5 contained 3 phase III trials (see WHO website on rotavirus vaccines).

Two licensed oral cholera vaccines exist to reduce the burden of disease in endemic regions and during outbreaks.107 Dukoral, composed of whole cell killed V. cholera combined with a recombinant cholera toxin B subunit (WC-rBS) has shown very high short-term protection in different age-groups in Bangladesh and Peru and significant long-term efficacy in endemic populations.108 Dukoral has also been used as a traveler’s diarrheal vaccine against ETEC infections. The cholera toxin B subunit shares sequence homology with the B subunit of the heat labile toxin (LT) of ETEC and is believed to confer this cross protection. Shanchol, that was developed at the International Vaccine Institute in Seoul, Korea, and licensed in India in 2011, is a bivalent vaccine containing whole cell-killed bivalent cholera vaccine against O1 and O139 serotypes.109 A live attenuated cholera vaccine CVD 103-HgR that has demonstrated efficacy in multiple populations has been licensed in several countries and is currently in the US licensure process.110

Current landscape of vaccine development for enteric pathogens

It was generally believed that mucosal pathogens would require intestinal immunity and therefore the oral route would be the most immunogenic route of vaccine administration. Oral delivery would mimic the course of natural infection that is known to confer immunity against many diarrheal diseases. It would also be the easiest and cheapest for vaccine delivery. However, with the evaluation of non-oral routes of immunization such as intranasal, intradermal, and sublingual, in addition to the conventional intramuscular route, killed whole-cell and subunit vaccines are becoming more attractive for reasons of safety as well as the capability to deliver both a systemic and a mucosal immune response.

A key consideration for a combination diarrheal vaccine will be the list of antigens to be included for obtaining broad protection against multiple pathogens. For example, in Shigella, current data indicates that protection is serotype-specific and is based on the O-antigen type of the LPS molecule. Shigella has more than 50 serotypes suggesting that vaccines against all 50 serotypes may be needed for 100% protection. However, epidemiology studies show that 4–6 serotypes predominate and vaccine candidates
against S. sonnei and S. flexneri serotypes 2a, 3a and 6 may suffice to protect 80% of shigellosis worldwide. 111 Therefore an effective O antigen-based vaccine against Shigella must contain the respective component from these 4 prevalent serotypes. There are efforts to develop a Shigella vaccine based on the highly conserved Type III secretion system antigens IpaB and IpaD 112 but proof of concept in humans remains to be addressed. ETEC strains are made up of almost 100 different O-antigenic types and express a heat-labile (LT) and a heat-stable enterotoxin (ST) along with more than 25 different colonization factors (CFs) or coli surface antigens (CS) that enable the bacteria to colonize the small intestine and induce diarrhea. While several CFs predominate, such as CFA/I, CS1-CS7, CS14, 17 and 21, 30–50% of ETEC strains do not express an identifiable CF on their surface. Approximately 30% of ETEC strains express either LT or ST while the remaining express both enterotoxin types. ETEC vaccine development is directed toward immune responses to LT, ST, the predominant CFs and their tip adhesins. 108,113 Campylobacter species express a capsule and lipooligosaccharide and using a traditional Penner serotyping scheme, 47 different Campylobacter serotypes have been described. 114,115 A capsule conjugate vaccine against Campylobacter that has shown promise in non-human primates will have to take into consideration the predominant capsule types circulating in the world. A combination diarrheal vaccine against any 2 or 3 of these enteric pathogens will therefore be composed of multiple antigens from each species.

There are 3 main issues in the field of diarrheal disease vaccine development: 1) to determine whether live, whole-cell killed or subunit vaccines provides the best strategy for obtaining durable protection 2) to determine how many live attenuated or whole cell-killed strains or how many antigens will comprise an efficacious vaccine against an individual pathogen listed in Table 1 and 3) what are the correlates of protection for each vaccine so that when monospecific vaccines are combined, immune responses can be quantitated and immunological interferences due to the combination can be evaluated. Currently there are no licensed vaccines against Campylobacter, non-typhoidal Salmonella, ETEC, EAEC, Cryptosporidia and Shigella. A limited number of live attenuated, killed whole-cell and subunit vaccine candidates have been previously evaluated in Phase 1, 2b and even Phase 3 clinical trials for some of these pathogens and results obtained have provided valuable information on safety and immunogenicity, although correlates of protection for any of the diarrheal diseases-causing pathogens are lacking. 108,116-124 A Phase 3 evaluation of S. sonnei and S. flexneri 2a O-specific polysaccharide conjugate was completed in children 1–4 years of age in Israel. 121 The S. sonnei conjugate showed 71% efficacy in 3–4 year old, an efficacy rate previously demonstrated with similar conjugates in Israeli adults. Efficacy was minimal in children less than 3 years of age. 121 A phase 3 study was also conducted with a skin-patch vaccine containing heat-labile toxin (LT) from ETEC. This was carried out in a population of travelers to Mexico and Guatemala. 124 The LT patch vaccine did not protect travelers against diarrhea caused by ETEC or other organisms. With increasing knowledge of bacterial pathogenesis and identification of novel and better characterized antigens, a number of promising candidates are currently in the development phase with some entering clinical trials. Based on current developmental stage, monovalent vaccines against ETEC, Shigella and Campylobacter may become a reality in the next decade, constituting the first step toward the development of a combination diarrhea vaccine. 113,114,118,125-127 Although correlates of protection for all 3 pathogens are unclear, mucosal responses along with systemic responses will be important for determining protection. Since ETEC and Shigella vaccine development have had a relatively earlier start, Tables 3 and 4 lists some of the promising strategies that are being implemented for these 2 pathogens. 122,125,128-142 For Campylobacter, a capsule conjugate vaccine is currently in clinical trials. 114,115

### Table 3. Current ETEC vaccine landscape

| Type | Preclinical | Phase 1, route, dose | Ref. |
|------|-------------|----------------------|------|
| Live, ACE527<sup>a</sup> | + | oral 10<sup>10</sup>–10<sup>11</sup> | 120,121,131 |
| Formalin killed cells overexpressing CFs with LTCB<sup>b</sup> | + | oral 10<sup>10</sup>–10<sup>11</sup> 2 doses, d0, d14 | 131,141,147 |
| Fimbrial tip protein | + | ongoing study, TCI & ID multiple doses | 113,123 |
| dmLT<sup>c</sup> | + | adjuvant | 24,131 |
| EtpA glycoprotein | + | | 128 |
| STa toxoid fusions<sup>d</sup> | + | | 127 |
| Plant-based<sup>e</sup> | + | | 129 |
| e.g. MucoRice-CTB<sup>f</sup> | + | | 136 |

<sup>a</sup> composed of 3 strains expressing CFA/I & LTB, CS5, CS6 & LTB, CS1, CS2, CS3 & LTB, tac promoter-driven genes integrated on chromosome.

<sup>b</sup> hybrid LTb/CTB toxoid, provides better LTb neutralizing responses than CTB in animals, CFs, colonization factors.

<sup>c</sup> double-mutated LT, LTR192G/L211A, to be used mainly as an adjuvant with vaccine candidates.

<sup>d</sup> STb toxoid with amino acid substitutions that is fused to carrier protein such as LTB, CTB or a CFA subunit.

<sup>e</sup> CTB and LTB also expressed in potatoes, carrots, corn, tobacco, CTB can provide some protection against LT-ETEC.

<sup>f</sup> rice-expressed CTB with a KDEL signal at the C-terminal of CTB, has been fed orally to mice and non-human primates.

Combination vaccines for diarrheal diseases

Traveler’s, including members of the military and young children living in endemic parts of the world are the most in need of vaccines against diarrheal diseases. 4,26,28,143,144,145 A combination vaccine would greatly simplify immunization of these target populations.
groups, but the nature of the combination vaccine is likely very different for each group.

Based on recent data on infection rates, ETEC and Campylobacter are reasonable targets for an initial combination vaccine against TD. There are currently no vaccines licensed in the US for either pathogen. Dukoral, an oral cholera vaccine, is being used in some countries as a TD vaccine against ETEC on the basis of cross-reactivity of the B subunits of the respective toxins. In most cases, no prescription is needed for Dukoral and the vaccine can be self-administered. Vaccination requires 2 doses and the traveler is advised to take the last dose at least 1 week prior to travel. Ideally, similar to Dukoral, a combination TD vaccine could be self-administered by the oral route, which is the only practical route for this approach. A combination of subunit vaccines administered parenterally is also feasible, but this would require more close coordination between the traveler and the travel physician. Since travelers to endemic countries are sensitive to the high risk of diarrheal disease, there is likely a fairly strong market for TD vaccines in the more developed part of the world. Considerations for a combination diarrhea vaccine for young children living in resource-poor endemic parts of the world are much more complex compared to the scenario for TD vaccines. Some key factors include geographical disease burden and coverage of the most relevant pathogens, route of vaccination, a defined schedule for immunization of the very young, who may already be on a vaccination regimen based on an EPI schedule, and options for manufacture of the vaccine. Based on the recent findings from the GBD 2010 study and the GEMS study, vaccines against ETEC and Shigella, along with the licensed rotavirus vaccine, would have the greatest impact on improving the health status of the most vulnerable – the children under 5 living in endemic parts of the world. However, both the GEMS and the GBD 2010 studies have indicated that a vaccine against Cryptosporidium has to be taken into consideration although clinical data for a vaccine against Cryptosporidium is lacking. In SE. Asia where Campylobacter is responsible for 30% of diarrheal disease, a combination vaccine against ETEC, Shigella, and Campylobacter will be protective against 70–80% of the diarrhea seen in that region. Overall, the path of development of a combination diarrheal vaccine would be similar to the steps used to license current pediatric vaccines.

There are no vaccines currently available for either ETEC, Shigella or Campylobacter but there are multiple development efforts ongoing that may yield suitable candidates for either a stand-alone or a combination vaccine (Tables 3 and 4). If the vaccine were to be delivered orally, consideration must be given to potential interference by the tropical enteropathy that is often observed in malnourished young children, and the general need for multiple oral doses to obtain protective immunity in these children. Another challenge is the size or volume of the inoculum, which likely cannot exceed a few milliliters for the youngest children, and the palatability of the vaccine vehicle. Nonetheless, live or inactivated whole cells offer one possible option for a combination ETEC-Shigella vaccine. Parenteral vaccines are also being developed for ETEC, Shigella and Campylobacter and the challenge here may be how to optimize mucosal immunity by immunization through a non-mucosal route. A hypothetical scenario for an initial combination vaccine, either a live or whole-cell killed combination vaccine or a subunit vaccine against these 2 pathogens is schematized in Figures 1 and 2. Such a licensed initial combination vaccine could be further expanded to include either existing vaccine candidates such as for cholera, rotavirus and typhoidal Salmonella or combined with newer vaccines as they are developed against other diarrheal pathogens (Figs. 1 and 2). Novel adjuvants such as dmLT may be able to stimulate mucosal as well as systemic immune responses even when the vaccine is given by injection rather than orally. Some of the issues affecting each type of combination is also outlined (Figs. 1 and 2).

Ideally, combination diarrheal vaccines could be given in accordance with the in-country EPI schedule, which typically includes the first 6, 10 and 14 weeks of life (2, 4, and 6 months of age in the US). Vaccines against childhood illnesses such tuberculosis, diphtheria, pertussis, tetanus, polio and measles are

### Table 4. Current Shigella vaccine landscape

| Type                             | Preclinical | Phase 1, route, dose, | Ref     |
|----------------------------------|-------------|-----------------------|---------|
| Live CVD seriesa                  | +           | oral, $10^9$–$10^{10}$| 118,140,142 |
| Live WRAIR seriesb                | +           | ongoing               | 118,134,138,140,142 |
| Live S. typhi Ty21a with Shigella LPS genes | +           | oral, $10^9$–$10^{10}$| 117,125 |
| Formalin killed cells, trivalent | +           | Intranasal            | 14,119,125-126 |
| Invaplexd                        | +           | parenteral            | 121,125,142 |
| LPS conjugates                    | +           | parenteral            | 133,142 |
| Synthetic oligo-saccharides      | +           |                      | 132     |
| Bioglycoconjugatesf               | +           |                      | 139     |
| Purified Ipa proteins             | +           |                      | 137     |
| OMf vesicles                      | +           |                      |         |

*a* based on *guaBA* mutations, multiple serotypes, limited intracellular replication.

*b* based on *virG(csA)* mutations, multiple serotypes, inability to spread intercellularly.

*c* trivalent product with *S. sonnei, S. flexneri* 2a and 3a.

**d** combination of serotype-specific LPS and conserved antigens IpaB and IpaC proteins, LPS from different serotypes can be substituted.

**e** LPS conjugates with CRM, a non-toxic recombinant variant of diphtheria toxin with a single amino acid substitution.

**f** Shigella O-antigen expressed in an *E. coli* with *Campylobacter* glycosylation secretion machinery.

**g** OM, outer membrane.

**h** Campylobacter will be protective against 70–80% of the diarrhea seen in that region. Overall, the path of development of a combination diarrheal vaccine would be similar to the steps used to license current pediatric vaccines.

**i** There are no vaccines currently available for either ETEC, Shigella or Campylobacter but there are multiple development efforts ongoing that may yield suitable candidates for either a stand-alone or a combination vaccine (Tables 3 and 4). If the vaccine were to be delivered orally, consideration must be given to potential interference by the tropical enteropathy that is often observed in malnourished young children, and the general need for multiple oral doses to obtain protective immunity in these children. Another challenge is the size or volume of the inoculum, which likely cannot exceed a few milliliters for the youngest children, and the palatability of the vaccine vehicle. Nonetheless, live or inactivated whole cells offer one possible option for a combination ETEC-Shigella vaccine. Parenteral vaccines are also being developed for ETEC, Shigella and Campylobacter and the challenge here may be how to optimize mucosal immunity by immunization through a non-mucosal route. A hypothetical scenario for an initial combination vaccine, either a live or whole-cell killed combination vaccine or a subunit vaccine against these 2 pathogens is schematized in Figures 1 and 2. Such a licensed initial combination vaccine could be further expanded to include either existing vaccine candidates such as for cholera, rotavirus and typhoidal Salmonella or combined with newer vaccines as they are developed against other diarrheal pathogens (Figs. 1 and 2). Novel adjuvants such as dmLT may be able to stimulate mucosal as well as systemic immune responses even when the vaccine is given by injection rather than orally. Some of the issues affecting each type of combination is also outlined (Figs. 1 and 2).

**j** Ideally, combination diarrheal vaccines could be given in accordance with the in-country EPI schedule, which typically includes the first 6, 10 and 14 weeks of life (2, 4, and 6 months of age in the US). Vaccines against childhood illnesses such tuberculosis, diphtheria, pertussis, tetanus, polio and measles are
usually given on the EPI schedule. Most of these are given by the parenteral route, although oral polio vaccine is still being used in many parts of the world. However, the licensed rotavirus vaccines are given orally and are shown to be efficacious given concurrently with the EPI vaccines. If a combination diarrhea vaccine were to be incorporated, one critical step would be to demonstrate that the new vaccine does not interfere with immunogenicity or efficacy of the EPI vaccines already in place. An ETEC and Shigella combination is an obvious option, but others may be considered. Since rotavirus and ETEC are of greatest threat to younger children, a combination vaccine against these may be an attractive approach (Figs. 1 and 2). Existing oral rotavirus vaccines are already being taken up in parts of the developing world for early childhood immunization. Shigella becomes important in children after the first year of life, so vaccination at a later age may optimize efficacy. A Shigella vaccine could be given at 9 and 12 months, concurrent with the schedule for measles vaccine. For this scenario, a combination Shigella-typhoid vaccine could be considered, since both are invasive pathogens that tend to affect older children. Recent studies have indicated that as few as 4 serotypes of Shigella can protect against >80% of shigellosis worldwide. There are oral and parenteral vaccines for typhoid already, but these are not currently used in children under 2 years of age. The injectable version and the oral version is only for those aged 6 years and above. Nonetheless, recent studies indicate that the oral typhoid vaccine may be safe and immunogenic in younger children ages 2–5. For this potential Shigella/typhoid combination, there is much developmental work to be done.

A combination diarrheal vaccine against multiple pathogens will require development of efficacious vaccines against individual bacterial pathogens. The combination itself will depend upon whether it serves as a pediatric vaccine or a TD vaccine. A combination pediatric vaccine could be given in conjunction with other childhood immunizations. Interestingly, studies on the infant’s immune system has indicated that neonates are capable of mounting a protective immune response to vaccines within hours of birth and that they are capable of generating both a humoral and cellular immune responses to pathogens. The neonates immune system has the capacity to respond to extremely large numbers of antigens and one study estimated that each infant has the theoretical capacity to respond to at least 10,000 vaccines, with each vaccine composed of 100 antigens. Response of B cells in infants to T-independent antigens such as polysaccharides are considerably less than adults till they reach 2 years of age. Thus vaccines against the most common diarrheal disease-causing pathogens can be accommodated within the schedule of childhood immunizations, whether given as live oral vaccines such as the rotavirus vaccine, or whole-cell killed and subunit vaccines. Whether administered concurrently or sequentially, the vaccination should not negatively affect the safety and immunogenicity of the routine pediatric vaccines. For example, the

Figure 1. Hypothetical scenario for combination live or whole cell-killed diarrheal vaccine.
The MMRV vaccine, a combined measles, mumps, rubella and varicella vaccine, has been proposed as a replacement for the MMR vaccine to simplify administration of the vaccines. However, preliminary data indicating a rate of fever-induced seizure of 9 per 10,000 vaccinations with MMRV, as opposed to 4 per 10,000 for separate MMR and varicella injections has convinced US health officials not to recommend use of MMRV vaccine over separate injections.

As greater understanding of pathogenesis and other vaccines come into effect for *Salmonella*, EAEC and *Cryptosporidia*, these can be added stepwise to the mixture analogous to the manner in which current pediatric vaccines are formulated. For the infant group the combination vaccine, be it live or subunit, could be given as part of the normal pediatric vaccination schedule while a traveler’s diarrheal vaccine, given as 2 or 3 doses, could be scheduled to be taken ahead of the planned travel dates. In this context it may be worth mentioning that a norovirus vaccine, based on virus-like particles, has shown promising safety and efficacy in limited Phase 1 and 2 trials. Such a vaccine could be used for high-risk populations or situations such as the elderly, in day care, on cruise ships, nursing homes or in the military.

**Manufacturing issues for development of a combination diarrheal vaccine**

Under manufacturing issues, there are several key considerations relating to formulation (Table 5). Of primary importance is the compatibility of the individual components:

- The potential for immunological interference should be evaluated in an animal model to detect effects of the combination on potency and immunogenicity. For diarrhea vaccines, mucosal immunity would be a key parameter for evaluation when determining compatibility. Here again, some knowledge can be gained from evaluating the formulation of pediatric combination vaccines. As described above, the most commonly reported example of immune interference in DTaP-based vaccines is the reduction in antibody titers to the Hib component of the vaccine PRP antigens. Consistent with the clinical data this interference has also been seen in animal models. Several explanations have been provided including interference of tetanus toxoid and the FHA pertussis antigen with Hib and incompatibility with the alum adjuvant in DTaP vaccines. As a result, the DTaP-Hib vaccines have been licensed in Europe but not in the US.
- Depending on the nature of the components, formulation of the final combination must be assessed by a battery of physicochemical, biochemical and biological assays. For example, in the case of live attenuated bacterial strains combined in a single formulation, assays would be needed to determine the viability and potency of each strain. If the combination is one of subunit proteins, there may be challenges in terms of identifying preservatives, excipients and delivery vehicles that effectively maintain the stability of each component and yet do not interfere with required assays. If the combination vaccine includes an adjuvant, additional areas of assessment would be required.
Table 5. Manufacturing issues for combination vaccines

- Use of human-derived or animal-derived materials and use of preservatives in manufactured products can become an issue.
- Developing a formulation process for the combination vaccine that ensures lot to lot consistency, minimizes interference between antigens and maintains safety and efficacy profile similar to the individual components.
- Manufacturing process should be amenable to scale-up production; the profile of the final product should mimic the safety, efficacy, potency and preclinical testing profile of the pilot scale vaccine.
- Combination vaccine must show acceptable toxicity levels, animal studies may be necessary and animal data must reflect clinical results.
- Multiple clinical trials will be necessary for determining safety, efficacy, and impact of different vaccination schedules in infants and adults.
- Manufacturing product should pass all regulatory rules of the FDA for licensure.

*Abridged from Vose, J. 2001, CID, 33: S334–339, Van Hoof, J. CID, 2001, 33: S346–350.

In addition to lack of any negative effect on safety or immunogenicity of any component, the selected adjuvant should not interfere with assays for each component in the final product. Besides alum, 2 other adjuvants AS03 and AS04 have been licensed in the US. AS03 is a oil-in-water emulsion of D,L-alphatocopherol (vitamin E) and squalene and an emulsifier polysorbate 80 and AS04 is aluminum hydroxide and monophosphoryl lipid A. New mucosal adjuvants such as dmLT or liposomes that may be particularly useful for diarrhea vaccines are also being investigated.159,160 If a subunit enteric combination vaccine is formulated, non-alum based adjuvants such as the oil-in-water emulsion MF59 and polylactide coglycolide (PLG) microparticles, could also be evaluated.161 New approaches are needed to evaluate formulation of non-alum-based adjuvants as part of a combination diarrhea vaccine.

- Demonstration of potency, which may be a reflection of vaccine stability, is likely the most important and challenging part of product testing. Potency is often demonstrated by immunogenicity in animal models but development of improved in vitro assays to measure batch-to-batch consistency of vaccine production would be useful to reduce reliance on animal testing. For example, antibody-based ELISA assays for quantifying diphtheria toxoid antigen in DTP-based combination vaccines have several advantages over other biochemical and biophysical methods due to their sensitivity and to the fact that they can be used on the final combined product even in the presence of antigen adsorbed to the adjuvant alum. Whether supplied as a pre-blended formulation or separate components for co-administration at the time of vaccination, the FDA guideline states that in general the potency requirement of each component in the combination should comply with the potency requirement for each component as a stand-alone product. For vaccines already licensed, potency standards would have been previously established. However, since there are so few vaccines licensed for diarrheal diseases, the combination vaccine may be one of new candidates for which potency will have to be established for each as part of the vaccine development plan.

The FDA guidance on clinical evaluation of safety and immunogenicity requires that the immunogenicity of combination vaccines should not be decreased when compared to those of individual components delivered simultaneously. In the case of licensed components such as those for childhood diseases, in some cases they are given simultaneously (oral rotavirus vaccine given along with intramuscularly administered DTP-based vaccines), and in other cases, such as DTP-based combination vaccines, they are formulated together for a single delivery. For example, Kinrix is a combination pediatric vaccine of DTaP-IPV (GSK, licensed 2008) that is injected into the muscle in a 4-dose EPI schedule. The diphtheria and tetanus toxins are extracted from the respective cultures, detoxified with formaldehyde, and individually adsorbed to aluminum phosphate.162 Pertussis toxin (PT), filamentous haemagglutinin antigen (FHA) and pertactin (PRN) are isolated separately from the supernatant of B. pertussis cultures.162 Fimbrial antigens (FIM) are copurified from bacterial cells. The pertussis antigens are purified by sequential filtration, salt precipitation, ultrafiltrations and chromatography. Glutaraldehyde and formaldehyde are used to detoxify PT and FHA respectively. The individual antigens are adsorbed separately onto aluminum phosphate (AlPO4). Polioviruses types 1, 2, 3 are each grown in separate cultures of human fetal lung cells (MRC-5). The viral suspensions are inactivated by formaldehyde after concentration by ultrafiltration and liquid chromatography. The monovalent IPV’s are combined to produce the trivalent poliovirus concentrate.162 The adsorbed DTP antigens are combined with aluminum phosphate as an adjuvant and water for injection. The trivalent polio concentrate is then added and the DTaP-IPV component is diluted to its final concentration.162 Again, because of the very limited number of licensed diarrhea vaccines, there may be very little pre-existing data to support a combination vaccine. On the other hand, most diarrheal vaccines will be administered either orally or through an alternate mucosal route, so the simultaneous administration of the individual components as a mixture may be nearly the same as the administration of a pre-blended combination vaccine formulation. Additional information on manufacture, testing and clinical trials of combination vaccines is available in the FDA website.162

Conclusion

Despite the well-documented global burden of diarrheal diseases, currently there are no combination diarrheal vaccines to protect against the diversity of enteric pathogens which include bacteria, viruses and parasites. The recent success with rotavirus and cholera vaccines has stimulated developmental efforts to advance vaccine candidates against other diarrheal pathogens such as ETEC, Shigella, Salmonella, Campylobacter and Norovirus. In the future, research will also be directed toward combining monovalent vaccines against
individual pathogens to provide broader coverage against multiple causes of diarrheal disease. The American College of Immunization practices (ACIP) recommends that combination vaccines be used whenever possible. As new vaccines that target previously unaddressed diseases are added to the vaccination calendar, the use and improvement of currently available combination vaccines will be paramount if high vaccine coverage is to be maintained. Areas for future research would be to increase compatibility of antigens, testing more potent adjuvants and developing better methods to monitor vaccine production and potency.

In an article summarizing the issues concerning combination vaccines for less developed countries, the question was raised as to “how can vaccines of special epidemiological importance for the developing world be developed”. Successful development of an affordable combination vaccine against diarrheal diseases for pediatric public health will rely on subsidy by international organizations, potential private sector markets among developed world travelers and the middle to upper income class of developing countries, and collaboration between the commercial manufacturer (who likely hold the patents and IP rights) and a local producer. Ultimately governments and health-policy decision makers in individual countries will determine if such combination diarrheal vaccines benefit their population and add value to the well-being of their children.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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1488 Volume 11 Issue 6

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