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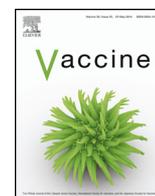
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Nontyphoidal salmonella disease: Current status of vaccine research and development



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ABSTRACT

Among more than 2500 nontyphoidal *Salmonella enterica* (NTS) serovars, *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis account for approximately fifty percent of all human isolates of NTS reported globally. The global incidence of NTS gastroenteritis in 2010 was estimated to be 93 million cases, approximately 80 million of which were contracted via food-borne transmission. It is estimated that 155,000 deaths resulted from NTS in 2010. NTS also causes severe, extra-intestinal, invasive bacteremia, referred to as invasive nontyphoidal *Salmonella* (iNTS) disease. iNTS disease usually presents as a febrile illness, frequently without gastrointestinal symptoms, in both adults and children. Symptoms of iNTS are similar to malaria, often including fever (>90%) and splenomegaly (>40%). The underlying reasons for the high rates of iNTS disease in Africa are still being elucidated. Evidence from animal and human studies supports the feasibility of developing a safe and effective vaccine against iNTS. Both antibodies and complement can kill *Salmonella* species *in vitro*. Proof-of-principle studies in animal models have demonstrated efficacy for live attenuated and subunit vaccines that target the O-antigens, flagellin proteins, and other outer membrane proteins of serovars Typhimurium and Enteritidis. More recently, a novel delivery strategy for NTS vaccines has been developed: the Generalized Modules for Membrane Antigens (GMMA) technology which presents surface polysaccharides and outer membrane proteins in their native conformation. GMMA technology is self-adjuvanting, as it delivers multiple pathogen-associated molecular pattern molecules. GMMA may be particularly relevant for low- and middle-income countries as it has the potential for high immunologic potency at a low cost and involves a relatively simple production process without the need for complex conjugation. Several vaccines for the predominant NTS serovars Typhimurium and Enteritidis, are currently under development.

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The genus *Salmonella* belongs to the Enterobacteriaceae family and comprises Gram-negative, non-spore-forming, facultative anaerobic bacilli [1]. *Salmonella enterica* subspecies *enterica* serovar Typhi and *Salmonella* Paratyphi A and B cause enteric fever, a systemic febrile illness that only occurs in humans and is distinguished from the more common self-limited acute gastroenteritis caused by other *Salmonella* serotypes. Non-typhoidal *Salmonella* (NTS) infect a variety of hosts and are frequently zoonotic in origin [2]. Of

the more than 2,500 NTS serovars, *Salmonella* Typhimurium and *Salmonella* Enteritidis account for approximately 50% of all human isolates of NTS reported globally. NTS has been recognized as a major cause of invasive bacterial infections in young children and HIV-infected individuals in sub-Saharan Africa as well as elderly and immunocompromised individuals worldwide [3,4].

The global incidence of NTS gastroenteritis was estimated to be 93 million cases in 2010; approximately 80 million contracted the infection via food-borne transmission [3]. It is estimated that 155,000 deaths resulted from NTS that year. NTS also causes severe, extra-intestinal, invasive bacteremia, referred to as invasive nontyphoidal *Salmonella* (iNTS) disease [2]. A recent estimate suggests that, globally, there are 49 cases (range of 30–94) of iNTS per

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100,000 population which means that 3.4 (range 2.1–6.5) million cases occur globally each year [5]. In Africa, the iNTS incidence is much higher (227 [range of 142–341] cases per 100,000). iNTS disease usually presents as a febrile illness, frequently without gastrointestinal symptoms, in both adults and children. Symptoms of iNTS are similar to malaria, often including fever (>90%) and splenomegaly (>40%). The underlying reason for the high rates of iNTS disease in Africa are still being elucidated; however, there are several established contributing factors that include increased invasiveness of distinct clades specific to Africa (e.g. *Salmonella* Typhimurium ST313), compromised host immunity in those with HIV infection, malaria, malnutrition, and increased opportunities for transmission (e.g., through contaminated water supplies). NTS bacteremia is particularly virulent in HIV-infected African adults who have a mortality rate of 47 per cent and recurrence rate of 43 per cent [6]. As a whole, the iNTS case fatality rate is estimated to be ~20% translating into 681,316 (range of 415,164 to 1,301,520) deaths annually [5]. Although mortality is lower in high income countries, the economic burden of NTS in those countries is still significant. In the United States, NTS costs US\$3.3 billion per year, with a loss of 17,000 quality-adjusted life years, the most of any food-borne pathogen [3]. iNTS disease has been overshadowed in the past by other diseases for which better data available, such as malaria, and HIV. The gaps in knowledge about the epidemiology of iNTS, however, are starting to close.

Because the typical clinical presentation of iNTS disease is non-specific, diagnosis is often difficult in resource-limited settings. Blood or bone-marrow culture may be used to diagnose cases of bacteremia. Polymerase chain reaction (PCR) on stool samples can potentially aid in the rapid diagnosis of *Salmonella* and multiplex PCR may serve as a means to identify specific invasive *Salmonella* serovars [7]. Serum ELISA is helpful in detecting past *Salmonella* infections, but is less useful for iNTS diagnosis for acute infections [8]. iNTS disease is primarily treated with antibiotics, whose class and duration are chosen on the basis of cost, availability, local patterns of resistance and treatment response. Treatment failure is of increasing concern in HIV-infected individuals and those infected with antibiotic-resistant strains (e.g. ST313). One approach toward overcoming these obstacles is to treat people with antibiotics that have optimal intracellular penetration, such as fluoroquinolones [9], although resistance to this class of antibiotics is increasing as well. The global burden of iNTS disease is likely to continue rising in absolute numbers and in the relative proportion of bacteremia cases, particularly as antimicrobial resistance becomes more prevalent and licensed vaccines reduce the incidence of other major causes of bacteremia, such as *Streptococcus pneumoniae* and *Haemophilus influenzae* b. As available tools for treatment become less effective, the development of effective vaccines will rise in priority for disease control efforts [2].

1. Biological feasibility for vaccine development

Effective *Salmonella* Typhi vaccines have been successfully licensed and administered to millions of people. Evidence from animal and human studies supports the feasibility of vaccine development against NTS as well. Both antibodies and complement can kill *Salmonella* species *in vitro*. Epidemiologic studies in sub-Saharan Africa have shown that the development of antibodies against NTS corresponds with a decrease in age-related incidence of iNTS disease, and that serum antibodies have corresponding *in vitro* bactericidal activity partly by mediating intracellular oxidation [9,10]. However, one African study found that high antibody titers against *Salmonella* lipopolysaccharide (LPS) O-antigen were associated with impaired *in vitro* serum killing of *Salmonella* Typhimurium in a proportion of HIV-infected Malawian adults [10].

The *in vivo* significance of this observation is not clear, as anti-LPS antibodies have bactericidal activity, protecting against NTS challenge in mouse models [9,10].

2. General approaches to vaccine development for low- and middle-income markets

Proof-of-principle studies have demonstrated efficacy, in animal models, of live-attenuated and subunit vaccines that target the O-antigens, flagellin proteins, and other outer membrane proteins of *Salmonella* Typhimurium and *Salmonella* Enteritidis. The relatively poor immunogenicity of purified O-antigens can be significantly enhanced through chemical linkage to carrier proteins. The subunit glycoconjugation approach specifically links LPS-derived O polysaccharide to carrier proteins and has been successful as, unlike *Salmonella* Typhi, the NTS are not encapsulated. More recently, a novel delivery strategy for NTS vaccines has been developed: the Generalized Modules for Membrane Antigens (GMMA) technology presents surface polysaccharides and outer membrane proteins in their native conformation. GMMA technology is self-adjuncting, as it delivers multiple pathogen-associated molecular pattern molecules. GMMA may be particularly relevant for low- and middle-income countries as it has the potential for high immunologic potency at a low cost and involves a relatively simple production process without the need for complex conjugation.

The development of recombinant or purified protein vaccines based on surface or outer membrane protein antigens, such as flagellin and porins OmpC, F and D offer the potential for broad-spectrum coverage due to targeting of conserved antigens. A reverse vaccinology approach using bioinformatics analysis of whole genome sequences from clinically important serovars may facilitate identification of additional conserved antigens. However, manufacturing complexities in purifying outer membrane proteins with the appropriate conformation may obviate the utility of porins as immunogens. Other promising vaccine approaches against iNTS disease include live attenuated candidates, which can be delivered orally and induce robust mucosal and T cell immunity.

Vaccines for iNTS will also need to target 2–4 month old infants, before the peak incidence at age 12 months. Programmatic field implementation in children could integrate directly with existing Expanded Program on Immunization schedules, potentially at 6, 10, and 14 weeks, or at 9 months concomitant with measles vaccination. This schedule will allow for programmatic introduction, as well as will enable children to be protected at an earlier age when they are at higher risk of disease. Vaccine implementation would likely also include populations infected with HIV. In higher income countries, NTS vaccines could also target the elderly who experience a high case-fatality rate of up to 50%.

3. Technical and regulatory assessment

In 2013, the World Health Organization provided guidance on the regulation and prequalification of typhoid conjugate vaccines [11] [12]. Although no such pathway is available for NTS vaccines, the experience with typhoid vaccines may serve as a good model to adapt. There are relatively robust animal models that can be used to evaluate preclinical data. Mice, for example, are permissive to *Salmonella* Typhimurium and *Salmonella* Enteritidis systemic infection that begins via entry through the gut mucosa and spreads through the lymphatic system. In untreated mice, infection manifests as invasive disease without gastroenteritis. To produce an NTS enterocolitis infection, mice must be pre-treated with streptomycin or other antibiotics prior to bacterial challenge. There are important differences to consider between mouse and human infections,

Table 1
Development status of current nontyphoidal *Salmonella* vaccine candidates (POC = proof-of-concept trial).

Candidate name/identifier	Preclinical	Phase I	Phase II	POC	Phase III
CVD 1931 (<i>Salmonella</i> Typhimurium Δ guaBA Δ clpX) and CVD 1944 (<i>Salmonella</i> Enteritidis R11 Δ guaBA Δ clpX), live oral [UMB]	X				
Bivalent COPS: FliC O:1,4[5],12:H:i+O:1,9,12:H:g,m conjugates using CVD 1925 and CVD 1943 reagent strains [UMB; Bharat Biotech; Wellcome Trust]	X				
Bivalent iNTS-GMMA [NVGH]	X				
Bivalent conjugate (O:1,4[5],12-CRM197+O:1,9,12-CRM197) [NVGH]	X				
OmpD [University of Birmingham]	X				
O:4,12-TT [NIH]	X				
WT05 [Microscience Limited]		X			

such as the inability of mouse complement to kill NTS *in vitro* [12]. Although immunologic correlates of protection have not yet been identified, data from Malawi have shown that acquisition of circulating antibodies to *Salmonella* Typhimurium, including the surface lipopolysaccharide, is associated with a lower risk of NTS bacteremia, particularly in the first few months of life when maternal antibodies are still present [10]. *In vitro* assays are available to quantify the serum bactericidal activity (SBA) of antibodies induced by *Salmonella* Typhimurium and *Salmonella* Enteritidis infection [15] and to assess the opsonophagocytic killing activity of anti-*Salmonella* antibodies [8].

4. Status of vaccine R&D activities

Several vaccines for *Salmonella* Typhimurium and *Salmonella* Enteritidis are under development, some of which are bivalent for both serovars (Table 1). It is unclear if these vaccine candidates will be protective against both gastroenteritis and invasive disease, but it has been proposed that a multivalent vaccine that targets 5–6 serovars could protect against the most relevant forms of gastroenteritis and invasive *Salmonella* worldwide [13,14]. The Center for Vaccine Development at the University of Maryland, Baltimore (UMB) is developing live-attenuated, oral vaccines for both *Salmonella* Typhimurium (CVD 1931, derived from a wild-type *Salmonella* Typhimurium strain from the ST313 genotype circulating in sub-Saharan Africa) and *Salmonella* Enteritidis (CVD 1944, derived from wild-type invasive *Salmonella* Enteritidis). These attenuated strains induce seroconversion (four-fold or greater antibody titer rise) of functional anti-LPS and anti-flagellin antibodies and are also expected to elicit robust cell-mediated immunity, a key component required for the resolution of *Salmonella* infections. Furthermore, CVD 1921, a prototype attenuated *Salmonella* Typhimurium vaccine strain, has been shown to be safe and well-tolerated in immunocompromised non-human primates [15].

Microscience Limited has published results from the only live attenuated NTS vaccine candidate to be tested in humans. The vaccine, WT05, is derived from a gastroenteritis-associated strain of *Salmonella* Typhimurium and is attenuated by deletion of the *aroC* and the *ssaV* genes. A Phase 1 clinical trial of this product found there to be prolonged stool shedding of the vaccine strain in healthy volunteers for up to 23 days. In 1992, the US National Institutes of Health (NIH) published pre-clinical research on an NTS conjugate vaccine for *Salmonella* Typhimurium that linked O:4 to tetanus toxoid (O:4-TT) [16]. This O:4-TT conjugate vaccine was able to protect mice against lethal challenge with wild-type *Salmonella* Typhimurium.

UMB has developed a bivalent, NTS conjugate vaccine candidate that covalently links the core and O-polysaccharides (COPS; both core and O polysaccharide are components of LPS) of *Salmonella* Typhimurium and *Salmonella* Enteritidis to the homologous Phase 1 flagellin subunits, respectively. It is hypothesized that by using

a *Salmonella* protective protein antigen instead of a heterologous pathogen protein (e.g., tetanus toxoid, CRM197), enhanced efficacy may be achieved as antibodies would be directed towards two independent protective targets on the bacterial surface. It has been shown that flagellin elicits a robust antibody response and that flagellin alone is protective in a mouse model of invasive NTS infection. UMB has created reagent strains of *Salmonella* Typhimurium and *Salmonella* Enteritidis that hyper-express flagellin that are valuable for the economical and safe purification of components for the NTS COPS-FliC conjugate vaccines. UMB has also developed high-yield, low-cost methods to biochemically purify COPS and flagellin from liquid growth cultures of these strains. *Salmonella* Enteritidis COPS-FliC conjugates were able to elicit protective antibodies in preclinical studies with both components of the conjugate eliciting *Salmonella*-specific immunity and protection maintained at very low vaccine doses [14]. This vaccine is being advanced in partnership with Bharat Biotech of India and the Wellcome Trust.

The Novartis Vaccines Institute for Global Health (NVGH, a GSK company) has also developed a bivalent O-antigen polysaccharide-CRM₁₉₇ conjugate vaccine targeting both *Salmonella* Typhimurium and *Salmonella* Enteritidis [17]. The institute's current efforts are focused on the advancement of the GMMA platform for Gram-negative bacteria, including NTS vaccine production. Bacterial genetic modifications are introduced into *Salmonella* Typhimurium and *Salmonella* Enteritidis parent strains to increase membrane blebbing of small (50–90 nm) immunogenic particles and to detoxify lipid A. In mice, NTS GMMA vaccines are at least as immunogenic at comparable doses of O-antigen glycoconjugate vaccines (NVGH, unpublished data). *Salmonella* GMMA reactogenicity remains to be evaluated in humans; however, *Shigella sonnei* GMMA was safe and immunogenic in a Phase 1 trial in adults [18]. The ease of manufacturing GMMA and its dose-sparing potential has influenced NVGH's vaccine development prioritization. The NVGH bivalent conjugate vaccine is available for further development if the GMMA platform does not perform as expected.

The University of Birmingham has proposed a different protein-based vaccine candidate consisting of outer membrane protein D (OmpD), purified from whole bacteria [19]. Studies of porin-deficient bacteria showed that OmpD (absent in *Salmonella* Typhi) is a viable target for antibody protection against iNTS. It should be noted that several other vaccines against *Salmonella* Typhimurium and *Salmonella* Enteritidis are available and/or under development for use in veterinary medicine and commercial food production, particularly in the raising of livestock and other key animal carriers, most notably chickens.

5. Likelihood for financing

iNTS disease has yet to be recognized as a major priority for vaccine development either by global health policy institutions or funding agencies, even though there is a significant burden of

childhood mortality associated with this disease [20]. It has been proposed that until bacterial bloodstream infections are recognized as a distinct cause of mortality, iNTS disease will remain a significant, but neglected, disease of developing countries. Although typhoid conjugate vaccines were designated a priority by the GAVI Alliance in 2008, no such steps have been taken for vaccines against iNTS. Funding for research to date has come from the Wellcome Trust, the Bill & Melinda Gates Foundation and NIH, but progress through clinical development, especially large Phase 3 efficacy trials, will require funding from a variety of sources, including vaccine-manufacturing partners in potential target markets.

Conflicts of interest

Drs. Tennant and Simon are inventors on the following US patents: “Broad spectrum vaccine against non-typhoidal *Salmonella*” (patent number 9,050,283) and “Broad spectrum vaccine against typhoidal and non-typhoidal *Salmonella* disease” (patent number 9,011,871); Dr. MacLennan is a former employee of the Novartis Vaccines Institute for Global Health and recipient of a Clinical Research Fellowship from GlaxoSmithKline; Dr. Martin is an employee of GSK; Dr. Khan declares no conflict of interest.

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