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Vaccines against invasive *Salmonella* disease
Current status and future directions

Calman A MacLennan[1,2,*], Laura B Martin[1], and Francesca Micoli[1]

[1]Novartis Vaccines Institute for Global Health; Siena, Italy; [2]Medical Research Council Centre for Immune Regulation and Clinical Immunology Service; Institute of Biomedical Research, School of Immunity and Infection; College of Medicine and Dental Sciences; University of Birmingham; Birmingham, UK

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Abbreviations: iNTS, invasive nontyphoidal *Salmonella*; NTS, nontyphoidal *Salmonella*; CPS, capsular polysaccharide; Ti2, T-independent type 2; TLR5, toll-like receptor 5; *r*EPA, recombinant *Pseudomonas aeruginosa* exoprotein A; IVI, International Vaccine Institute; NVGH, Novartis Vaccines Institute for Global Health; CVD, Center for Vaccine Development; EPI, Expanded Programme on Immunization; TT, tetanus toxoid; DT, diphtheria toxoid; CRM_{197}, nontoxic recombinant form of diphtheria toxin; PCMV, protein capsular matrix vaccine; GMMA, Generalized Modules for Membrane Antigens; PAMPs, pathogen-associated molecular patterns; LAV, live attenuated vaccine

Though primarily enteric pathogens, *Salmonellae* are responsible for a considerable yet under-appreciated global burden of invasive disease. In South and South-East Asia, this manifests as enteric fever caused by serovars Typhi and Paratyphi A. In sub-Saharan Africa, a similar disease burden results from invasive nontyphoidal *Salmonellae,* principally serovars Typhimurium and Enteritidis. The existing Ty21a live-attenuated and Vi capsular polysaccharide vaccines target *S.* Typhi and are not effective in young children where the burden of invasive *Salmonella* disease is highest. After years of lack of investment in new *Salmonella* vaccines, recent times have seen increased interest in the area led by emerging-market manufacturers, global health vaccine institutes and academic partners. New glycoconjugate vaccines against *S.* Typhi are becoming available with similar vaccines against other invasive serovars in development. With other new vaccines under investigation, including live-attenuated, protein-based and GMMA vaccines, now is an exciting time for the *Salmonella* vaccine field.

Protection Against more than Diarrhea

Serovars of the Gram-negative bacterium *Salmonella enterica* are usually associated with food-borne diarrheal illness in high-income countries. Such gastrointestinal disease is normally self-limiting and rarely life-threatening. Perhaps surprisingly, *Salmonella* has not been identified as a principal etiological agent of diarrhea in developing countries.[1] Nevertheless, *Salmonellae* are responsible for a huge global disease burden through two forms of invasive illness: enteric fever and invasive nontyphoidal *Salmonella* (iNTS) disease. Enteric fever is principally caused by *Salmonella enterica* serovar Typhi (*S.* Typhi), for which the disease is also called typhoid fever, and *S.* Paratyphi A. Disease due to both serovars is a major problem in South and South-East Asia (**Fig. 1A**). *S.* Typhi is the leading pathogen isolated from blood cultures in South Asia,[2] though in some areas enteric fever caused by *S.* Paratyphi A is more common.[5] The annual global burden of disease due to typhoid fever was estimated at 21.7 million cases in 2000 with a case-fatality rate of 1% resulting in 217000 deaths.[3] Pre-school and school-aged children are the most affected age groups.[6-8] The global burden of disease attributable to *S.* Paratyphi A in 2000 was 5.4 million cases.[3]

In contrast, iNTS disease is a neglected disease and is mainly a problem in sub-Saharan Africa (**Fig. 1B**). Published global burden of disease estimates are not currently available, though case fatality rates, at 20–25%,[9] are much higher than for enteric fever, with an overall annual mortality likely to be well in excess of 100,000 and not dissimilar from that of enteric fever. In sub-Saharan African countries, nontyphoidal *Salmonellae* (NTS) are either the leading or next most common pathogenic blood culture isolate after pneumococcus,[4] for which vaccines are available and are being implemented in the region. The two main serovars responsible for iNTS disease are Typhimurium and Enteritidis and the two groups most affected by iNTS disease are children under two years and HIV-infected individuals. Fever surveillance across 12 sites in sub-Saharan Africa during the RTS,S/AS01 malaria vaccine phase 3 trials gave an incidence of *Salmonella* bacteremia in children under two years of around 500/100000 children/year.[10] It is not clear why invasive *Salmonella* disease is a problem in the developing world and not in high-income countries, particularly with respect to iNTS disease. This could be due to differences in transmission, host immunity or the bacteria themselves.[11]

Multi-locus sequencing typing (MLST) has been used to trace the evolutionary history of *S.* Typhi. This revealed the expansion of haplotype H58 in Asia and Africa associated with the acquisition of resistance to fluoroquinolones over the past 20 y.[12] Whole genome studies of *Salmonella* isolates from Africa have identified new clades associated with iNTS disease, in particular, the *S.* Typhimurium ST313 pathovar.[13,14]

*Correspondence to: Calman A MacLennan; Email: calman.maclennan@novartis.com
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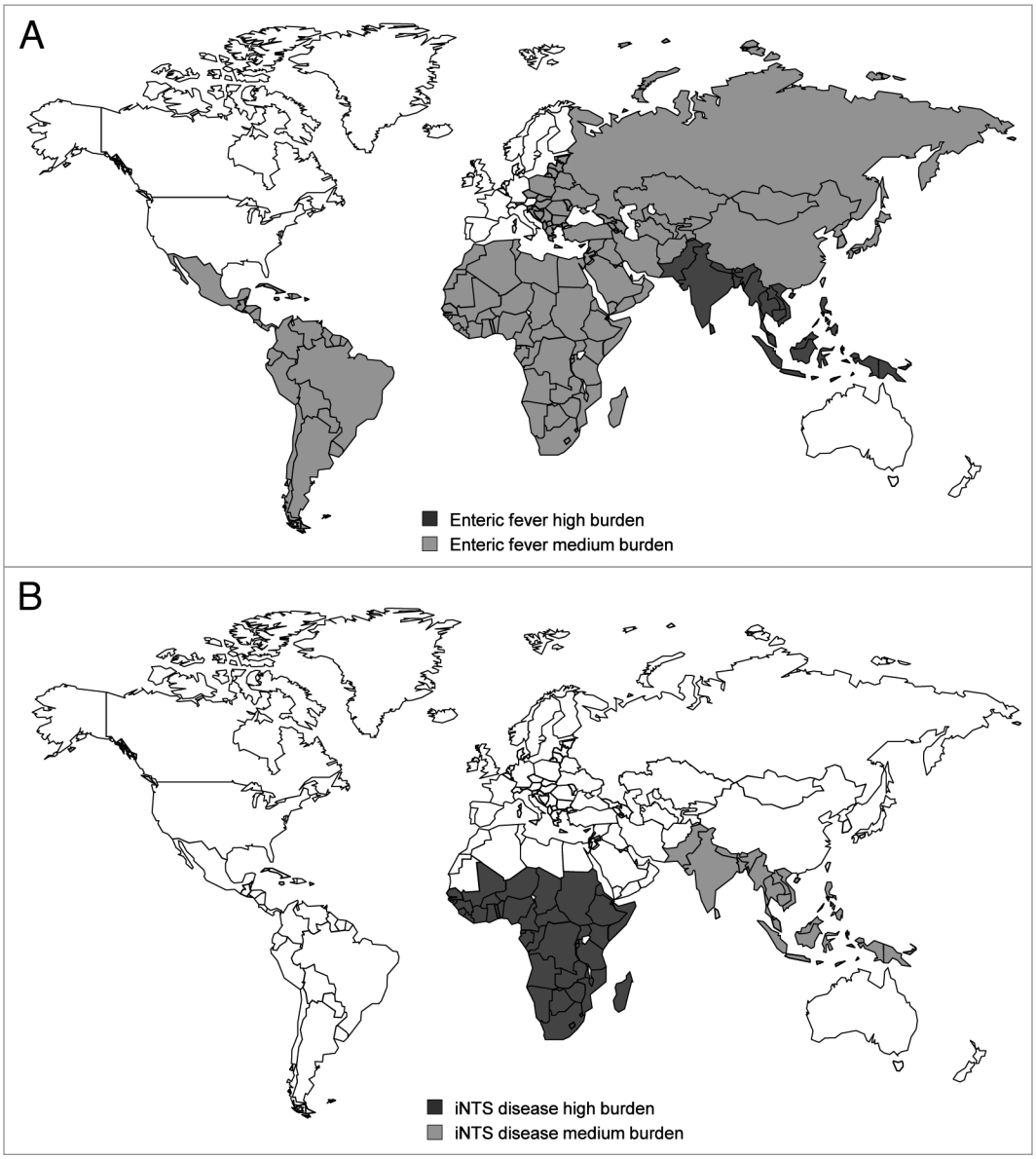

Figure 1. Geographical distribution of A. enteric fever and B. invasive nontyphoidal *Salmonella* (iNTS) disease indicating countries with high (> 100 cases/100,000 population/year) and medium (10–100 cases/100,000 population/year) disease burden. Based on data from references 2-4.

S. Typhimurium ST313 is characterized by genome degradation and pseudogene formation, similar to that found in *S.* Typhi, suggesting that it may be passing through an evolutionary bottleneck.[13] The genomic differences present in the ST313 clade, compared with NTS strains in developed countries, could underlie alternative pathways of transmission which for ST313 appear to be primarily human-to-human, rather than zoonotic or through contaminated food products, as is common in industrialised nations.[15,16] The emergence of the two known clades of ST313 has been traced back to independent origins in Malawi and DRC around 50 y ago and their transmission through sub-Saharan Africa has been linked to the occurrence of the HIV/AIDS pandemic in Africa.[14]

Why New Vaccines against *Salmonella* are Needed

There are two widely-available forms of vaccine licensed for use against *Salmonella*,[17,18] yet neither have been implemented at country level. These are the live attenuated vaccine, Ty21a,[17,18] and Vi capsular polysaccharide (Vi CPS).[19] Part of the reason for their lack of use in at-risk populations is their poor immunogenicity in young children. Neither are licensed for use in under two year olds.[17,18] There are other associated problems with these vaccines (see section "Past and Current Vaccines") and both are targeted against *S.* Typhi, with no vaccine currently available against the other three key invasive serovars of *Salmonella enterica*, Paratyphi A, Typhimurium and Enteritidis. Although

invasive forms of *Salmonella* disease are amenable to antibiotics, increasing frequencies of multi-drug resistance among invasive isolates threaten the effectiveness of such treatment.[12,20] In Malawi, around 90% of iNTS isolates are multi-drug resistant.[20]

A key problem with the effective management of invasive *Salmonella* disease, particularly in Africa, is the lack of appropriate diagnostic facilities. Currently, these infections can only be detected by microbiological culture, and facilities for this are rare in developing countries, particularly in Africa. The Widal test, based on the detection of antibodies to the O- and H-antigens of *S.* Typhi by agglutination with patient serum, has been used in the past for diagnosis of typhoid fever. However, the sensitivity and specificity of this test is low, particularly in endemic areas where prior exposure to *S.* Typhi is common.[21] Clinical diagnosis is difficult and for iNTS disease is simply not possible since there are no signs and symptoms that distinguish it from a number of other common infections. Fever is often the only presenting feature for iNTS disease resulting in confusion with malaria with which iNTS is well known to be associated.[22] iNTS disease can also result in the same clinical presentation as severe pneumonia,[23] the currently recommended antibiotic treatment for which is often ineffective against iNTS.

Even where blood culture facilities are available and a definitive diagnosis is possible, the clinical demise of individuals with iNTS disease is often rapid, with around half of children who die during their admission succumbing before the blood culture results are available. This often fatal condition is emphasized by the fact that the 20–25% case fatality rate comes from studies at sites where blood culture facilities are available. Another downside to these diagnostic problems is their contribution to the continuing poor awareness of invasive *Salmonella* infections, particularly iNTS disease, as a significant global disease burden. In short, new vaccines against *Salmonella* have the potential to make an enormous impact on global health.

Table 1. Potential vaccine coverage of main invasive *Salmonella enterica* serovars by candidate antigens

Clinical presentation	Enteric fever		iNTS[a] disease	
Serovar of *S. enterica*	Typhi	Paratyphi A	Typhimurium	Enteritidis
Antigen				
A. Polysaccharide				
1. Vi	+	-	-	-
2. O:2	-	+	-	-
3. O:4,5	-	-	+	-
4. O:9	+	-	-	+
B. Protein				
1. Omp F/Omp C	+	+	+	+
2. Omp D	-	+	+	+
3. Other	+/-[d]	+/-[d]	+/-[d]	+/-[d]
C. Mixed				
1. LAV[b]	+/-[e]	+/-[e]	+/-[e]	+/-[e]
2. GMMA[c]	+/-[e]	+/-[e]	+/-[e]	+/-[e]

[a]iNTS, invasive nontyphoidal *Salmonella* ; [b]LAV, Live Attenuated Vaccine; [c]GMMA, Generalized Modules for Membrane Antigens; [d]+/– for 'Other' protein antigens indicates dependency on identity of antigen; [e]+/– for 'LAV' and 'GMMA' indicates dependency on choice of production strain and presence/expression levels of key antigens in production strain and target serovar.[47]

Understanding the Modalities of Protective Immunity

Since *Salmonellae* are facultative intracellular pathogens, they are able to survive both extracellularly and within the intracellular niche in monocytes and macrophages. This presents a challenge to vaccine developers, since humoral immunity is key for dealing with extracellular bacteria, while cellular immunity, mediated by both $CD4^+$ and $CD8^+$ T cells,[24,25] is required to eliminate bacteria within the monocyte/macrophage. Animal studies show that both modalities of immunity are required for the efficient control and elimination of *Salmonella*. *Salmonellae* have a conserved set of genes that allow them to survive within macrophages and mutations in these genes lead to a loss of virulence.[26] Mice with defects in the oxidative burst mechanism[27,28] and mice lacking T cells[29] are unable to properly control *Salmonella* infections. Passive transfer studies indicate an important role for antibodies in protection against *Salmonella*.[24,30] Antibodies have a key role in facilitating uptake and killing of *Salmonella* by phagocytes and preventing spread of disease which occurs principally via the blood,[31] while T cells are required for the elimination of persistent infection within phagocytes.[32,33]

Studies in individuals with primary immunodeficiencies support the importance of cell-mediated immunity for protection against *Salmonella* disease. Patients with chronic granulomatous disease caused by defects in the oxidative burst mechanism,[34,35] and those with deficiencies of T helper 1 cell activation, caused by deletions in the IL-12/23-IFNγ cytokine axis,[36-38] are particularly susceptible to *Salmonella* disease. The best evidence for the role of antibodies in protection against *Salmonella* disease in man comes from efficacy studies of the Vi CPS vaccine, since this vaccine induces antibodies and not T cells. The importance of antibodies against iNTS disease in man is indicated by recent field studies from sub-Saharan Africa which have shown that the age-related prevalence of iNTS disease declines as specific antibody is acquired.[39] Accompanying mechanistic laboratory studies have demonstrated that such antibodies have functional activity against the invasive disease-causing isolates, both through activation and deposition of complement on the bacterial membrane resulting in cell-free killing[39] and through opsonisation for efficient uptake and killing by phagocytic cells.[40] However, the time

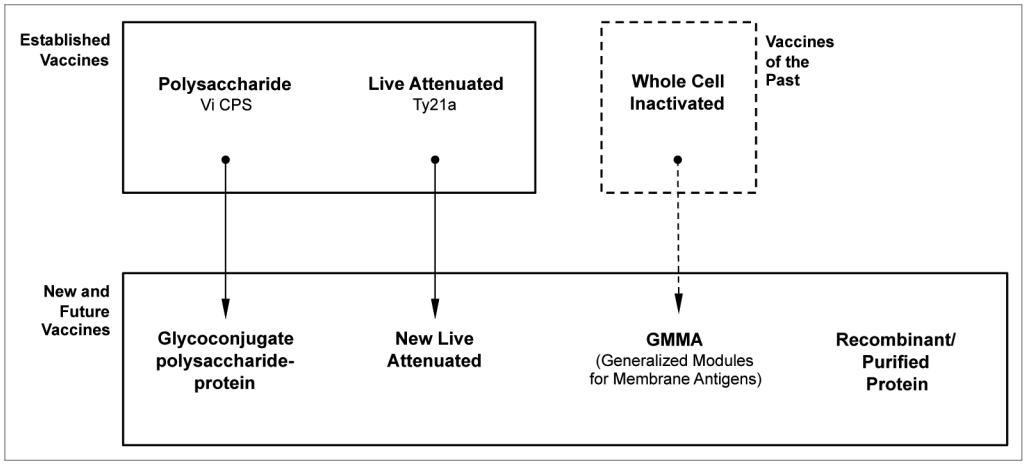

Figure 2. Established and new *Salmonella* vaccines, and how they relate to each other. (Adapted with permission from reference 47).

required for killing of extracellular *Salmonella* is sufficient for the bacteria to escape into the intracellular niche where they can no longer be targeted by antibody.[41]

Hence, the evidence from human studies also supports a role for both antibodies and cell-mediated immunity in protection against invasive *Salmonella* disease. The well-recognized clinical association between HIV infection and iNTS disease, particularly among individuals with CD4 counts below 200 cells/μl,[42] also appears to provide clear support for the importance of cell mediated immunity in defense against *Salmonella*. However, the effects of HIV infection on host immunity are widespread, and there is recent evidence to indicate that HIV also increases susceptibility to NTS through dysregulation of humoral immunity[43] and cytokine responses,[44] and disruption of the integrity of the gastrointestinal mucosa.[45] Vaccine strategies that induce protective mucosal immunity to prevent NTS gastroenteritis and invasion from the gastrointestinal tract could be particularly beneficial. No such association has been found between HIV infection and typhoid fever. Intriguingly, a study from Tanzania found that HIV-infected individuals were less susceptible to typhoid fever than those without HIV infection.[46] These findings suggest differences in the immune mechanisms responsible for protection against iNTS disease and typhoid fever.

Targets of Protective Immunity

Antibodies mediating protection against *Salmonella* necessarily need to target moieties on the outer surface of the bacterium (**Table 1**). The Vi antigen, which forms a polysaccharide capsule around *S*. Typhi (hence Vi CPS) and also *S*. Paratyphi C and *S*. Dublin, O-antigen of lipopolysaccharide (somatic antigen) and flagellin (H-antigen), which forms the flagella of *Salmonella*, are all highly immunogenic. Antisera against Vi, O- and H-antigens have long been used for the typing of *Salmonella* serovars according to the Kauffmann-White scheme.[48,49] All have been proposed as vaccine candidates with Vi CPS currently in use as a vaccine against typhoid fever.

Because the key iNTS serovars, Typhimurium and Enteritidis, are not host restricted to man, much work has been possible on NTS disease in the mouse model of salmonellosis. Experimental O-antigen-based conjugate vaccines can induce protection against otherwise lethal *Salmonella* challenge in mice.[50,51] Passive transfer of monoclonal antibodies targeting O-antigen also protect against challenge,[52,53] and most bactericidal antibody following immunisation of animals with heat-killed invasive African ST313 *S*. Typhimurium is directed against O-antigen.[54] In man, antibodies against O-antigen are present in adults and children from an early age in both African[55] and US[56] populations and have bactericidal activity. However, high levels of antibodies against O-antigen in some HIV-infected African adults are associated with a lack of in vitro killing of *Salmonella*.[43] While the in vivo significance of this finding is not clear, the implication is that antibody to O-antigen does not protect HIV-infected individuals from iNTS disease.

Host restriction of *S*. Typhi to man has resulted in less animal work in relation to the Vi antigen, though genetic manipulation of NTS strains to express Vi[57] can help overcome this obstacle. Nevertheless, the bactericidal capacity of antibodies raised to Vi in rabbits and mice has been known for many years,[58] and there is an inverse relationship between incidence of typhoid fever and serum bactericidal titer against *S*. Typhi.[59] The strongest evidence for the importance of antibodies targeting Vi is the protective efficacy data for the Vi CPS vaccine[17-19] and from Phase 3 studies with candidate Vi conjugate vaccine.[60-64]

While surface polysaccharides, by nature, are T-independent type 2 antigens (Ti2 antigens), and require conjugation to carrier proteins to induce T-dependent antibody responses,[64,65] *Salmonella* proteins are able to recruit T cell help without further manipulation. Flagellin is the only *Salmonella* surface typing antigen that is a protein and therefore has been investigated for its ability to generate protective immune responses. Both immunisation with flagellin alone[50,67,68] or as the carrier protein for an O-antigen glycoconjugate vaccine[50,69] has been shown to induce protection in mice. Flagellin is also the key ligand for toll-like receptor 5 (TLR5) and through this interaction is involved in innate signaling to the immune system

Table 2. Advantages and disadvantages of past, present and future vaccines against *Salmonella enterica*

Vaccine	Advantages	Disadvantages
A Vaccines of the Past		
Whole Cell Inactivated	73% 3-y efficacy	Reactogenicity
B Established Vaccines		
1. Vi CPS	Single dose	Not licensed for infants
	Low reactogenicity	Lack of memory response
	WHO prequalification	Lack of affinity maturation
		Only protects against *S.* Typhi
2. Ty21a	Oral administration	Not licensed for infants
	Some cross-protection against *S.* Paratyphi B	Requires multiple doses
C New and Future Vaccines		
1. Vi glycoconjugate	Higher efficacy than current vaccines	Only protects against *S.* Typhi
	T-dependent antibody response	
	Memory induction	
	Affinity maturation	
	Low reactogenicity	
2. O-antigen glycoconjugate	As for Vi glycoconjugates	Only protects against serovars with same O-antigen specificity
3. New Live Attenuated	*Salmonella*-specific B and T cell immunity	Attenuating for optimal balance of immunity and reactogenicity
	Clearance of residual infection	Breadth of coverage may be limited by insufficient expression of key antigens
		Possibility of disease in immunocompromised subjects
4. Recombinant Proteins	*Salmonella*-specific B and T cell immunity	Issues with antigen conformation may limit ability to induce effective B cell response
	Potential for pan-specific immunity	
	Low reactogenicity	
5. Proteins purified from whole *Salmonellae*	*Salmonella*-specific B and T cell immunity	Difficulties with purification of integral membrane proteins
	Potential for pan-specific immunity	
	Low reactogenicity	
6. GMMA	*Salmonella*-specific B and T cell immunity	Balance of reactogenicity and immunogenicity in man not currently known
	Potential for pan-specific immunity	
	Enrichment of membrane antigens	
	Ease of manufacture Low cost-of-goods	

Adapted with permission from reference 47.

and can have immunomodulatory effects in mice.[69-71] Potential problems with flagellin as a vaccine candidate are that in some serovars, notably *S.* Typhimurium, but not the other three main invasive serovars, there can be phase variable expression, and, in addition, it is not constitutively expressed by *Salmonella* during infection.

While *Salmonella* O-antigens and flagellin are antigenically diverse and vary between serovars, some surface proteins are highly-conserved and thus have potential for use in broadly protective vaccines. Monoclonal antibodies raised against *Salmonella* outer membrane proteins protect against challenge following passive transfer,[53] indicating their potential utility in vaccines. Much attention has been given to the porins which constitute particularly abundant outer membrane proteins. Immunisation with OmpC and OmpF,[72] and OmpD[73] has been shown to protect mice in challenge studies. These are widely-conserved proteins, although OmpD is not expressed by *S.* Typhi. As they have multiple membrane-spanning domains, their production for use in

Table 3. Vaccines currently available and in development against *S.*Typhi

Name	Description	Developer	Stage of development	References
Ty21a	Live attenuated	Vivotif (Crucell)	Licensed for adults and children > 5 y	17,18,104-111
Vi CPS	Vi Polysaccharide	Typherix (GSK), Typhim Vi (Sanofi), Typbar Vi (Bharat Biotech), Typho Vi (BioMed); Vax-tyVi (Finlay Institute); > 6 other endemic countries manufacturers	Licensed for adults and children ≥ 2 y	19,101,102, 112-115
Vi-TT	Vi Conjugate	Peda-Typh (BioMed)	Licensed in India	63,117
		Typbar-TCV (Bharat Biotech)	Licensed in India	63,118
Vi-rEPA	Vi Conjugate	National Institutes for Health	Phase 3	60-62
		Lanzhou Institute (China)	Licensed in China	63
Vi-CRM[197]	Vi Conjugate	NVGH (technology transfer to Biological E underway)	Phase 2	119,120
Vi-DT	Vi Conjugate	International Vaccine Institute (IVI)/Shanta Biotech	Phase 1	63,121-123
Vi conjugated to fusion protein PsaA-PdT	Vi Conjugate	Harvard Medical School	Preclinical	124
O:9-DT	O:9 Conjugate	International Vaccine Institute (IVI)	Preclinical	125
M01ZH09	Live attenuated	Emergent Biosolutions	Phase 2 in adults and children; evaluation in *S.* Typhi human challenge	126-131
CVD 909	Live attenuated	University of Maryland	Phase 2	132-136
Ty800	Live attenuated	Avant Immunotherapeutics	Phase 2	137,138
OmpC and OmpF	Outer membrane protein	Instituto Mexicano del Seguro Social	Phase 1 in Mexico	139,140

vaccines is not straightforward. Protein arrays have enabled the screening of sera for antibodies targeting thousands of *Salmonella* proteins. This approach has recently been used to demonstrate a common immune signature shared by mice immunised with live attenuated *Salmonella* and African children convalescing following iNTS disease, and to identify other potential candidate antigens, including SseB.[75] These protein arrays have enabled the discrimination of immune responses of Vietnamese patients with acute typhoid from healthy controls[76] and Bangladeshi patients with typhoid from those who are febrile due to other causes.[77] Although the importance of T cell-mediated immunity for protection against *Salmonella* is well-known, far less work has been performed in order to identify the relevant T cell antigens, compared with B cells antigens. Interestingly, a recent study has found that the important T cell antigens are likely to be surface-associated in *Salmonella*,[78] indicating that surface proteins may act as both B cell and T cell antigens.

The Challenge Of Changing Epidemiology and Breadth Of Coverage

As well as understanding the modalities and targets of protective immunity for *Salmonella* vaccines, it is important to understand which serovars of *Salmonella enterica* need to be targeted by vaccines. A drawback of the currently-available vaccines is that they are all directed against *S.* Typhi. As mentioned earlier, *S.* Paratyphi A causes enteric fever with the same geographic distribution as *S.* Typhi, and the diseases are clinically indistinguishable.[78] Hence, for South and South-East Asia, a vaccine that can protect against both serovars would be more valuable than a vaccine that is restricted to one. In sub-Saharan Africa, the same is true for *S.* Typhimurium and *S.* Enteritidis, indicating the importance of a vaccine that can protect against both iNTS serovars for this region.

With subunit vaccines based on antigens that are specific to single serovars or groups of serovars (e.g., Vi, the O-antigens and flagellin), broad coverage can be achieved by combining multiple subunits, as has been done for the multivalent pneumococcal and meningococcal conjugate vaccines. However, this has the disadvantage of increasing costs, especially when glycoconjugate technology is used. As the invasive forms of *Salmonella* disease are a major problem in some of the poorest countries, vaccine affordability is a key consideration. A further drawback to the use of serovar-specific antigens for vaccine development is the evolving epidemiology of invasive *Salmonella* disease. In the time it takes to develop a new vaccine (minimum ten years), the epidemiology may change considerably. There is evidence for this occurring already in Africa, where *S.* Typhi has become an increasing problem in some areas. This appears to be partly driven by the spread of *S.* Typhi H58 into Africa from Asia[79] and has been a particular problem in urban slums with poor sanitation.[80,81] Surprisingly, in Blantyre, Malawi, where typhoid fever has previously been uncommon compared with iNTS disease, in the last year *S.* Typhi has been isolated more frequently from the blood of patients admitted to hospital than nontyphoidal serovars.[83]

Other nontyphoidal serovars are a problem in specific parts of sub-Saharan Africa, such as Dublin and Stanleyville in Mali,[84] and could take over from Typhimurium and Enteritidis as the major cause of iNTS disease. Nevertheless, O-antigen-based conjugate

Table 4. Vaccines in development against *S.* Paratyphi A

Name	Description	Developer	Stage of development	References
O:2-TT	O:2 Conjugate	NIH	Phase 2	141,142
		Technology transfer from NIH to Lanzhou Institute (China)	Phase 2	143
		Technology transfer from NIH to Chengdu Institute (China)	Preclinical	143
		Changchun Institute of Biological Products	Preclinical	143
O:2-DT*	O:2 Conjugate	IVI	Preclinical	143
O:2-CRM_{197}†	O:2 Conjugate	NVGH (technology transfer to Biological E underway)	Preclinical	144,145
CVD 1902‡	Live attenuated	University of Maryland	Phase 1	

Notes: *development in combination with corresponding Vi-DT conjugate against *S.* Typhi; †development in combination with corresponding Vi-CRM_{197} conjugate against *S.* Typhi; ‡development in combination with CVD 909

vaccines should offer cross-protection against other non-encapsulated serovars within the same serogroup. Hence, O:1,4,[5],12 (to give its full O-antigen designation) *S.* Typhimurium conjugates should protect against other O:4 group (formerly B group) serovars,[49] including Stanleyville (O:1,4,[5],12,27) and Paratyphi B (O:1,4,[5],12), since all express the dominant O:4 antigen. Likewise, O:1,9,12 *S.* Enteritidis conjugates should protect against O:9 group (formerly D group) serovars, such as Dublin (also O:1,9,12) and potentially Typhi (O:9,12[Vi]), provided the O-specific antibodies gain access through the Vi capsule to their target antigen. In view of the potential for cross-protection within serogroups, it has been proposed that a multivalent vaccine composed of 5–6 conjugates could cover almost all invasive *Salmonella* disease.[85] An alternative strategy for developing vaccines with broad coverage is to use protein antigens that are highly conserved among different serovars of *Salmonella*. The increasing number of available whole genome sequences from different invasive *Salmonella* field isolates,[13,14,86] combined with the reverse vaccinology approach,[87] can facilitate the identification of such antigens.

Past and Current Vaccines

The first type of vaccine against *Salmonella*, an inactivated whole cell vaccine, was in use for over 100 y (**Fig. 2**, **Table 2**). Like Ty21a and Vi CPS, the inactivated whole cell vaccine targeted *S.* Typhi and was never implemented at a country-wide level. It was introduced in 1896,[88] and used extensively by the British[89] and US[90] military resulting in a dramatic reduction in cases of typhoid fever and associated deaths. Of the three *Salmonella* vaccines, the inactivated whole cell vaccine has been the most effective with a three year cumulative efficacy of 73%.[17] The major drawback, and reason why this vaccine is no longer used,[17] is its high level of reactogenicity[91,92] which, while previously tolerated by military personnel, is unacceptable for general use.

Ty21a and Vi CPS are fascinating for the immunologist and vaccinologist as they act through completely different, though still-to-be-properly-defined, mechanisms. Ty21a is a live attenuated vaccine that was developed through non-specific chemical mutagenesis.[93] Though derived from the Vi-expressing *S.* Typhi Ty2 strain, surprisingly Ty21a does not express the Vi antigen and so none of its effects can be attributed to an immune response to this antigen. In contrast, the Vi CPS vaccine consists of purified Vi polysaccharide, although a recent study has suggested that other *Salmonella* components may be present in the vaccine.[94] There are reduced rates of seroconversion following immunization with Ty21a in young children compared with adults.[95] As an enteric-coated capsule, it is licensed for use in adults and children over five years. Multiple doses (routinely three) are needed and there are issues with thermal stability emphasizing the importance of a robust cold-chain. Despite these drawbacks, Ty21a has a cumulative three-dose efficacy of 51%[17,18] and there is evidence that it can induce herd protection.[96] The vaccine may also be amenable to increased thermal stability using a modified freeze-drying process.[97]

As a live attenuated vaccine, Ty21a has good potential to induce T cell immunity and cross-protection against non-Typhi serovars. Indeed, there is clinical evidence for some cross-protection against *S.* Paratyphi B[98] and in vitro evidence for the induction of antibody-secreting cells with cross-reactivity against *S.* Paratyphi A and B.[99,100] Such studies suggest that Ty21a may be acting primarily through the humoral immune response that it elicits, rather than through cell-mediated immunity. Recent evidence suggests that much of the B cell response is directed against O:9 which would indicate potential utility for the vaccine against the principal iNTS serovar, *S.* Enteritidis.[101] *S.* Enteritidis, in common with *S.* Typhi, expresses O:9.

Being a Ti2 antigen, the Vi CPS is unlikely to be immunogenic in infants and is only licensed for children over two years of age. Its effectiveness in children between two and five years is uncertain, since two cluster randomized-controlled trials in this age group, in Kolkata[102] and Karachi,[103] gave differing results, with protective efficacy only demonstrated in the Kolkata study. The lack of T cell help in the immune response to pure polysaccharide vaccines classically results in a lack of immunoglobulin class-switching, affinity maturation through somatic hypermutation in germinal centers, in addition to a lack of immunogenicity in infants and young children.[65,66] Further common findings are a lack of induction of immunological memory as well as limited duration of antibody response and hyporesponsiveness to subsequent vaccination.[104] In this respect, it is perhaps surprising that Vi CPS has a similar three year efficacy against typhoid fever to that of Ty21a, at 55%,[17,18] despite their different mechanisms of action. In contrast to Ty21a, the efficacy of Vi CPS is for a single vaccination dose, though Vi CPS has similar cold chain requirements. It is interesting to speculate what efficacy would result if both vaccines

Table 5. Vaccines in development against iNTS disease*

Name	Description	Developer	Stage of development	References
O:4,5/O:9-flagellin	O:4,5/O:9 Conjugate	University of Maryland	Preclinical	50,69
O:4,12-TT	O:4-TT Conjugate	NIH	Preclinical	51
Os-po	O:4-porin Conjugate	National Bacteriology Laboratory, Stockholm	Preclinical	146
O:4,5/O:9-CRM_{197}	O:4,5/O:9 Conjugate	NVGH	Preclinical	145
WT05	Live attenuated	Microscience, Wokingham Berkshire	Phase 1	147
CVD 1921 and CVD 1941	Live attenuated	University of Maryland	Preclinical	148
S. Typhimuirum ruvB mutant	Live attenuated	Seoul National University	Preclinical	149
Salmonella hfq deletion mutant	Live attenuated	Indian Institute of Science Bangalore	Preclinical	150
SA186	Live attenuated	Istituto Superiore di Sanità Roma	Preclinical	151
MT13	Live attenuated	KIIT University Odisha	Preclinical	152
Various	Live attenuated, DNA adenine methylase mutants	University of California, Santa Barbara	Preclinical	153,154
Various	Live attenuated, regulated delayed attenuation	Arizona State University	Preclinical	155-157
Porins	*S.* Typhimurium porins	National Bacteriology Laboratory, Stockholm	Preclinical	146
OmpD	Outer membrane protein	University of Birmingham, UK	Preclinical	73
S. Typhimurium and *S.* Enteritidis GMMA	Generalized Modules for Membrane Antigens	NVGH	Preclinical	65,158,159

*an exhaustive list, particularly of all candidate vaccines in preclinical studies, is beyond the scope of this review

were given together, as their mechanisms of protection may well act in a complementary synergistic manner. As far as we are aware, no clinical trial has been conducted to investigate this. A further difference in the immune response elicited by Vi CPS and Ty21a is found in the profile of homing receptor expression in the circulating plasmablasts induced. Those resulting from immunization with Vi CPS have a systemic homing profile with the large majority of cells expressing L-selectin, while plasmablasts after vaccination with Ty21a have very high expression of the mucosal homing receptor $\alpha_4\beta_7$,[94] similar to what occurs following natural infection.

New Vaccines

With the limitations of the two existing *Salmonella* vaccines, particularly their lack of effectiveness in young children, along with their lack of widespread uptake in endemic countries, the *Salmonella* community and global health policy makers are keenly awaiting the arrival of new vaccines against *Salmonella* (**Table 3, 4 and 5**). This has been a long wait given that the first Phase 3 study of a Vi glycoconjugate vaccine was reported over 12 y ago.[60] This study found 91% efficacy with a vaccine consisting of Vi conjugated to recombinant *Pseudomonas aeruginosa* exoprotein A (Vi-*r*EPA) in Vietnamese children aged two to five years after 27 mo follow up, with 89% efficacy after 46 mo.[61] The delay can most likely be attributed to the lack of a clear commercial incentive for developing vaccines against *Salmonella*.[66] Invasive *Salmonella* infections are principally diseases of low-income countries. Nevertheless, in recent years there has been enhanced activity in the field of vaccine development against *Salmonella*, particularly in the development of conjugate vaccines against *S.* Typhi, with several different companies and institutions involved. These initiatives have partly been driven by the expanding network of vaccine manufacturers in the emerging economies, particularly India (BioMed, Shantha Biotechnics and Bharat Biotech International) and China (Lanzhou Institute of Biological Products). Several other manufacturers in the Developing Countries Vaccine Manufacturers Network,[159] such as the Finlay Institute, Biological E, Biofarma, Chengdu, SK Chemical and EuBiologics are also developing vaccines against *Salmonella*. Impetus for the development of *Salmonella* vaccines has also come from global health vaccine institutes, particularly the International Vaccine Institute (IVI)[161] in Seoul, South Korea, and Novartis Vaccines Institute for Global Health (NVGH)[162] in Siena, Italy, as well as key academic institutions, notably the National Institutes of Health, USA, and Center for Vaccine Development (CVD) at the University of Maryland, Baltimore, USA.[163]

Such vaccines offer the prospect of inducing improved levels of protection over current vaccines and, importantly, providing protective immunity in children under two years of age where invasive *Salmonella* disease, particularly iNTS disease, is a particular problem. Therefore, these vaccines could be administered as part of national Expanded Programmes on Immunization (EPI), thus reducing delivery costs. Vaccines against typhoid and enteric fevers would likely be given at nine months,[164] prior to the peak

in age-related incidence in the second year of life,[7] and vaccines against iNTS to young infants between two and four months of age, since peak incidence occurs around one year.[39,165] While the first new vaccines against typhoid fever have already been licensed for in-country use in India and China, vaccines against iNTS disease lag a long way behind, despite the comparable burden of disease they cause. Since both enteric fever and iNTS disease are health problems primarily in low-income countries, this delay is likely to be attributable to a general lack of appreciation and awareness of the problem of iNTS disease in the global health community.[11]

Glycoconjugate Vaccines

Glycoconjugates are the most advanced of the new generation of vaccines against *Salmonella* and offer the advantages described above over pure polysaccharide vaccines such as Vi CPS. *Salmonella* glycoconjugate vaccines have the potential to recruit T cell help to the production of antibodies against Vi and O-antigen surface polysaccharides through covalent linkage to protein carrier molecules, thereby effectively converting these polysaccharides from T-independent to T-dependent antigens. This approach had been successfully applied to other encapsulated bacteria, particularly *Hemophilus influenzae* b, meningococcus and pneumococcus. Although *S.* Typhi is the only encapsulated serovar among the four *Salmonella* serovars responsible for the majority of invasive *Salmonella* disease, evidence from animal studies supports the development of conjugate vaccines against the three other principal invasive serovars through conjugation of their lipopolysaccharide O-antigens (O:1,2,12, O:1,4,[5],12 and O:1,9,12, for *S.* Paratyphi A, Typhimurium and Enteritidis, respectively)[49] to suitable carrier proteins.[85]

The glycoconjugate strategy for new *Salmonella* vaccines is principally an antibody approach. The majority of *Salmonella* conjugate vaccines employ the familiar carrier proteins, tetanus toxoid (TT), diphtheria toxoid (DT) and the nontoxic recombinant form of diphtheria toxin (CRM_{197}), as well as the less-commonly used protein, *r*EPA. The former three carrier proteins have been used extensively in existing glycoconjugate vaccines, are known to be safe and effective at inducing T-dependent responses to the carbohydrate moiety, but do not result in an immune response against any other *Salmonella* antigens. Vi-TT and Vi-*r*EPA vaccines are already licensed for in-country used in India and China. None of these carriers are *Salmonella* proteins. It has been argued that glycoconjugates employing *Salmonella* proteins could be more effective than those with exogenous carriers, as they would target the immune response to two different *Salmonella* antigens instead of one.[85,146] Conjugation to *Salmonella* proteins also offers the possibility of inducing *Salmonella*-specific T cells. 35 y ago, Svenson and colleagues showed that O:4 conjugated to Typhimurium porins gave better protection in mice that vaccination with porin vaccines alone or an O:4-DT conjugate.[146] More recently, investigators at the CVD have found enhanced immunogenicity and protective effect using iNTS vaccines with O:4,5 and O:9 conjugated to *Salmonella* flagellin compared with flagellin alone.[50,68]

Although only licensed in India, BioMed has been the first company to license a *Salmonella* conjugate vaccine: a Vi-TT vaccine, Peda Typh.[117] Another Indian company, Bharat Biotech has registered their Vi-TT vaccine (Typbar-TT) in India, and the Lanzhou Institute of Biological Products, in partnership with the US National Institutes of Health, licensed its Vi-*r*EPA vaccine in China.[62] The Vi-CRM_{197} vaccine developed by NVGH[166-168] was tested in Phase 1 and 2 trials in adults in Europe,[119] followed by Phase 2 trials evaluation in adults, children and infants in India, Pakistan and the Philippines.[120] While being far more immunogenic than Vi CPS vaccine, anti-Vi response following revaccination was lower than after primary vaccination. Vi-CRM_{197} technology is currently being transferred to Biological E. Meanwhile, the International Vaccine Institute and Shanta Biotech have developed a Vi-DT vaccine.[121-123] A related approach to that of the Vi glycoconjugate vaccines is protein capsular matrix vaccine (PCMV) technology by the Matrivax Research and Development Corporation that entraps the Vi polysaccharide in a matrix of CRM_{197}.[169]

A key issue with these Vi-conjugate vaccines is that they offer no protection themselves against enteric fever caused by *S.* Paratyphi A or strains of Typhi that might not express Vi. Phase 1 and 2 clinical studies with an O:2-TT vaccine targeted against *S.* Paratyphi A[141] were conducted 14 y ago in Vietnamese adults and children by the NIH group and found to be safe and immunogenic.[142] The technology for this vaccine has subsequently been transferred to the Chengdu and Lanzhou Institutes of Biological Products, with the Lanzhou Institute currently conducting a Phase 2 trial with the vaccine. Other O:2 glycoconjugates using DT and CRM_{197} have been developed and tested in preclinical studies by IVI[143,170] and NVGH[144,145] respectively. Both have been developed alongside Vi conjugate vaccines in order to be used in bivalent combinations and protect against both main forms of enteric fever. The NVGH O:2-CRM_{197} technology is also being transferred to Biological E as part of a bivalent vaccine with Vi-CRM_{197}. As mentioned previously, the development of conjugate vaccines against iNTS has lagged behind those for enteric fever, despite early preclinical proof of concept studies in mice at the National Bacteriology Laboratory in Sweden, using, among other candidate vaccines, O:4 conjugated to porin,[146] and at the NIH with O:4-TT conjugates.[51] In addition to the O:4,5- and O:9-flagellin vaccines of CVD, the technology for which is being transferred to Bharat Biotech, NVGH has developed and tested similar vaccines conjugated to CRM_{197} in preclinical studies.[145] Given the emergence and spread of typhoid fever in sub-Saharan Africa, it will be important to know whether O:9 conjugates can protect against *S.* Typhi as well as *S.* Enteritidis.

Live-Attenuated Vaccines

Although new live-attenuated vaccines have received less attention recently compared with the glycoconjugate vaccines,

this vaccine strategy has a number of potential advantages. Live-attenuated vaccines have excellent ability to elicit *Salmonella*-specific T cell responses required for clearance of residual infection, can be given orally and have good capacity to induce mucosal immunity through lymphocyte expression of mucosal homing receptors.[101] The delivery of multiple *Salmonella* antigens to the immune system raises the possibility of inducing broad protective coverage across *Salmonella* serovars. Molecular biology has advanced greatly since the time when Ty21a was developed using random mutagenesis. The ability to introduce targeted mutations and genetic modifications combined with the full availability of the bacterial genomes from whole genome sequencing has considerably improved the capacity to rationally design new live-attenuated vaccines. The major challenge in the development of live-attenuated vaccines is in attaining an optimal level of attenuation without compromising immunogenicity. Attenuation is required both to prevent persistent infection and disease from the vaccine itself, a particularly important consideration in populations such as those in sub-Saharan Africa with high prevalence of HIV infection, and to minimise reactogenicity. Unfortunately, immunogenicity often decreases alongside reactogenicity when *Salmonella* are attenuated.[171] A further consideration is to develop live vaccines that require fewer doses than Ty21a and are more heat-stable.

Three live attenuated vaccines against typhoid fever have been developed and tested in Phase 2 trials. CVD 909 is the latest in a series of live-attenuated vaccines developed by CVD. It has attenuating mutations in the *aroC*, *aroD* and *htrA* genes, but is distinguished from its predecessors by the replacement of the P_{tviA} promoter, which controls Vi expression, with the strong constitutive P_{tac} promoter. This ensures constitutive Vi expression,[132-135] which has been lacking from many live attenuated *S*. Typhi vaccines, either through complete lack of expression (as for Ty21a) or due to switching off of Vi expression during in vivo infection. The Ty800 vaccine developed by Avant Immunotherapeutics has a disrupted *aroC* gene and mutated *ssaV* gene.[137,138] M01ZH09 from Emergent Biosolutions has mutations in the *Pho/PhoQ* regulator genes.[126-129] All are based on the Ty2 parent strain and have good safety, tolerability and immunogenicity profiles inducing mucosal as well as systemic antibodies. As for the glycoconjugates, live attenuated oral vaccines against *S*. Paratyphi A and iNTS are further behind on the development pathway. CVD has tested its candidate live *S*. Paratyphi A vaccine CVD 1902 in a Phase 1 trial with a plan to use this in combination with CVD 909 to protect against both forms of enteric fever.

CVD have also developed and conducted preclinical studies with live iNTS vaccines. These consist of *S*. Typhimurium and *S*. Enteritidis with deleted *guaBA* and *clpP* genes and could protect against lethal challenge with the homologous serovar in mice.[148] The only live-attenuated iNTS vaccine to be tested to date in man is WT05, a *S*. Typhimurium with the same *aroC* and *ssaV* attenuations as *S*. Typhi Ty800. This Phase 1 study found prolonged stool shedding in volunteers for up to 23 d,[147] and the vaccine has not been tested further. Many other candidate live attenuated *S*. Typhimurium vaccines have been tested in mice,[149-152] but none have so far been into clinical trials. These include *Salmonella* stains attenuated through mutation of DNA adenine methylase which acts as a global regulator of gene expression.[153,154] Another promising strategy is the introduction of mutations in *Salmonella* that lead to regulated delayed attenuation in vivo through mechanisms including dependency on key nutrients that are absent in host tissues, and programmed lysis.[155-157]

Protein-Based Subunit Vaccines and GMMA

The main alternative subunit approach to *Salmonella* vaccines from the glycoconjugates is the development of vaccines from recombinant or purified proteins.[74,139,140] These potentially have the advantage of cross-protection if carefully selected through bioinformatic analysis of whole genome sequences as part of a reverse vaccinology approach to vaccine antigen discovery.[87] As proteins, such vaccines can induce both antibody and T cell responses. There are issues with preserving the conformation of proteins with multiple membrane-spanning domains that can result in the induction of antibodies with poor function on immunization. Such proteins may be better prepared by purification from whole *Salmonella* rather than recombinant technology.[140]

A better knowledge of the B cell and T cell epitopes of *Salmonella* would be of great help for advancing the protein-based vaccine approach. The proteins that have received most attention to date have been flagellin and porins OmpC, F and D. As described earlier, preclinical studies in the mouse model have demonstrated promise for all of these antigens with immunization resulting in protection against *Salmonella* challenge.[50,69,73,74] OmpC and F induce long-lasting antibody responses in mice[139] and have been found to be safe and immunogenic when tested in a Phase 1 study in man.[140] However, such vaccines are not necessarily amenable to simple production methods. The importance of preserving the correct conformation of such antigens has been indicated by the failure of recombinant *Salmonella* porins to protect mice.[172]

An innovative strategy to maintain the conformational integrity of *Salmonella* antigens, while avoiding laborious purification steps, is GMMA technology. GMMA (Generalized Modules for Membrane Antigens)[159,174] are small particles of 50 to 90 nm diameter consisting of blebs of outer membrane. Their shedding from the surface of Gram-negative bacteria, such as *Salmonella*, is enhanced following the genetic deletion of proteins that span the periplasm and serve to maintain the integrity of the inner and outer membranes. Deletion of the *tolR* gene of *Salmonella* and *Shigella*[66,158,159] results in the upregulation of this shedding process enabling the production of very high vaccine substance yields. Further deletions of genes, such as those encoding the late acyltransferases HtrB[174] and MsbB,[175] resulting in the removal of acyl groups from the lipid A moiety of LPS, are incorporated to reduce reactogenicity. Unlike the case with live-attenuated vaccines, there is no possibility of infection. Purification is straightforward and economical, consisting of two tangential flow filtration steps.[159]

Preclinical studies indicate that GMMA vaccines can deliver both surface polysaccharides and outer membrane proteins to the

immune system and that they are more immunogenic than glycoconjugate vaccines. This is likely to be partly because antigens are delivered in their correct conformation and orientation, but also through the self-adjuvanting properties of GMMA which deliver innate signals through TLR ligands and other pathogen-associated molecular patterns (PAMPs). GMMA also have good potential to induce *Salmonella*-specific T cell immunity. The combination of high yield and a simple production process makes this a highly-affordable technology, particularly suited for the development of vaccines for low- and middle-income countries, where cost-of-goods is a key consideration.[66] As the reactogenicity relative to the immunogenicity of GMMA vaccines in man is currently unknown, clinical trials (currently underway for a *Shigella sonnei* GMMA vaccine) are required to assess the safety and tolerability of this vaccine platform.

Conclusion

The licensure and large-scale implementation of *S*. Typhi glycoconjugate vaccines, foreseeable in the next few years, represents a major step forward for global health. The introduction of glycoconjugate vaccines has been a successful public health intervention against other encapsulated bacteria, particularly among infants and young children. It will be important to drive forward first vaccines against the other principal invasive *Salmonella* serovars: Paratyphi A, Typhimurium and Enteritidis. Improved surveillance of invasive *Salmonella* disease across the developing world, which is currently inadequate, will be key for assessing the impact of such new vaccines and detecting changes in the epidemiology of *Salmonella* disease which may be accelerated by their implementation. A key unresolved scientific question is whether the glycoconjugates, which rely on the induction of antibodies against surface polysaccharides as their principal mechanism of action, will be sufficient to effectively deal with the global problem of invasive *Salmonella* disease, particularly iNTS disease. While glycoconjugates are set to make a major impact, the possibility of serovar replacement and the challenge of iNTS disease among HIV-infected Africans, where lack of antibodies to *Salmonella* does not appear to underlie susceptibility to NTS, support the development of second and third generation vaccines. These include new live-attenuated, protein-based and GMMA-based vaccines, which could potentially induce broader protection together with *Salmonella*-specific T cell responses.

Potential conflict of interest

C.A.M., L.B.M. and F.M. are employees of the Novartis Vaccines Institute for Global Health. C.A.M. is the recipient of a clinical research fellowship from GlaxoSmithKline.

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