

**The background, role and approach for development of a Controlled Human Infection Model
for nontyphoidal *Salmonella***

Calman A. MacLennan^{1,2}

¹Jenner Institute, Nuffield Department of Medicine, University of Oxford, OX3 7DQ, United
Kingdom

²Bill & Melinda Gates Foundation, 62 Buckingham Gate, London, SW1E 6AJ, United Kingdom

Email

calman.maclennan@ndm.ox.ac.uk

Abstract

Nontyphoidal *Salmonella* (NTS) is responsible for a major global burden of disease and economic loss, particularly in low- and middle-income countries. It is designated a priority pathogen by the WHO for vaccine development and, with new impetus from vaccine developers, the establishment of an NTS controlled human infection model (CHIM) is timely and valuable. The broadly dichotomous clinical presentations of diarrhoea or invasive disease, commonly bacteraemia, present significant challenges to the development of an NTS CHIM. Nevertheless, if successful, such a CHIM will be invaluable for understanding the pathogenesis of NTS disease, identifying correlates of protection and advancing candidate vaccines towards licensure. This article describes the background case for a CHIM for NTS, the role of such a CHIM and outlines a potential approach to its development.

Keywords

Nontyphoidal/Salmonella/CHIM/human challenge/vaccine/correlates of protection/pathogenesis

Abbreviations

AMR antimicrobial resistance

cfu/ml colony-forming units/millilitre

CCR9 C-C motif chemokine receptor 9

CD3 cluster of differentiation 3

CHIM controlled human infection model

CLA cutaneous lymphocyte-associated antigen

CMO contract manufacturing organisation

COP correlate of protection

CRP C-reactive protein

CXCR5 C-X-C chemokine receptor 5

CyTOF cytometry by time of flight

DALYs disability-adjusted life years

DNA deoxyribonucleic acid

ELISA enzyme-linked immunosorbent assay

ELISpot enzyme-linked immune absorbent spot

EMA European Medicines Agency

ESR erythrocyte sedimentation rate

MHRA Medicines and Healthcare Products Regulatory Agency

FDA Food & Drug Administration

GBD Global Burden of Disease

GI gastrointestinal

GMMA generalised modules for membrane antigens

GMP good manufacturing practice

GVGH GSK Vaccine Institute for Global Health

HIC high-income country

HIV human immunodeficiency virus

HLA-DR human leukocyte antigen – DR isotype

ICOS inducible T cell co-stimulator

IFN γ interferon- γ

IgG immunoglobulin G

IL12/23 interleukin 12/23

I-FABP intestinal fatty acid-binding protein

iNTS invasive nontyphoidal *Salmonella*

LAL *Limulus* amoebocyte lysate

LMIC low- and middle-income country

LPS lipopolysaccharide

MHRA Medicines and Healthcare Products Regulatory Agency

MIP-1 β macrophage inflammatory protein-1 β

MLST multi-locus sequence typing

mOMV mutant-derived outer membrane vesicles

NTS nontyphoidal *Salmonella*

OGD oesophago-gastro-duodenoscopy

PBMC peripheral blood mononuclear cells

qPCR quantitative polymerase chain reaction

RNA ribonucleic acid

ST19 sequence type 19

S. Typhimurium Salmonella enterica serovar Typhimurium

TCR $\gamma\delta$ $\gamma\delta$ T cell receptor

TCV typhoid conjugate vaccine

TFH cell T follicular helper cell

TNF α tumour necrosis factor α

TT tetanus toxod

US United States

WHO World Health Organisation

1. Background

1.1 Disease burden of nontyphoidal *Salmonella*

Disease in humans due to nontyphoidal serovars of *Salmonella* (NTS) presents in two main forms, as diarrhoea or invasive disease, most commonly bacteraemia. The Global Burden of Disease 2019 report (GBD 2019) estimates NTS diarrhoea to be responsible for 61,647 deaths and 4.27 million disability-adjusted life years (DALYs - years lost due to ill-health, disability or early death) annually. Most of this disease burden, 39,493 deaths and 3.50 million DALYs, is in children under 5 years of age (GBD 2019 Diseases and Injuries Collaborators 2020). Diarrhoea is the commonest cause of death worldwide in children under five years of age after pneumonia. Low- and middle-income countries (LMICs), particularly in Africa and Asia, bear the main burden of this mortality. Invasive NTS (iNTS) disease is a considerable problem in sub-Saharan Africa where NTS is often the commonest cause of bacterial blood stream infections known as bacteraemia (Reddy et al. 2010). Although much less frequent than NTS diarrhoea, the high iNTS disease case fatality rate of around 20% makes this a major under-recognised cause of global mortality. iNTS disease is responsible for an estimated 79,046 deaths and 6.11 million DALYs annually, with 49,869 and 4.32 million of these respectively among children under 5 years (GBD 2019 Diseases and Injuries Collaborators 2020).

The relationship between NTS diarrhoea and invasive disease is not properly understood. NTS diarrhoea commonly occurs in all countries and immune-competent individuals of all ages are potentially susceptible. In contrast, iNTS disease mostly affects immune-naïve infants and young children, children with well-recognised comorbidities including malaria and malnutrition, and HIV-infected individuals of all ages (Gilchrist and MacLennan 2019). Intriguingly, iNTS disease often occurs in the absence of diarrhoea. Nevertheless, the GI tract appears to be the portal of entry for NTS with transmission through the faeco-oral route.

There are growing levels of antimicrobial resistance among *Salmonella* strains (Kariuki et al. 2015). A lack of pathognomonic presentation for both NTS diarrhoea, which is indistinguishable from other forms of diarrhoea, and iNTS disease, which most commonly presents as fever alone, precludes clinical diagnosis (Gilchrist and MacLennan 2019). While qPCR cards have been developed for detection of NTS in stool in the research setting, there is no rapid affordable diagnostic test, and stool and/or blood culture, if available, are required to make a diagnosis.

1.2 Disease strains

There are over 2,000 serovars of NTS. *Salmonella enterica* serovars Typhimurium and Enteritidis (*S. Typhimurium* and *S. Enteritidis*) are the commonest serovars responsible for both diarrhoeal and invasive disease in humans. *S. Typhimurium* isolated from diarrhoeal cases most commonly belong to the ST19 MLST (multi-locus sequence typing) type. Invasive African *S. Typhimurium* mainly belong to a new pathovar, ST313. This is characterised by resistance to multiple antibiotics and genome degradation with pseudogene formation, similar to that seen with *S. Typhi* and *S. Paratyphi A*, the agents of enteric fever. These genetic changes may have led to an adaptation to an invasive lifestyle (Kingsley et al. 2009, Okoro et al. 2012). Similarly, distinct lineages of *S. Enteritidis* from Africa associated with invasive disease show evidence of genome degradation in contrast with lineages responsible for enterocolitis (Feasey et al. 2016).

1.3 Pathogenesis

Unlike *S. Typhi* and *S. Paratyphi A* which are human-restricted pathogens, many NTS serovars including Typhimurium and Enteritidis cause disease in a range of animals and NTS infections have been studied extensively in mice. While animal models of NTS infection can be of considerable benefit to our understanding of disease pathogenesis, they are not fully representative of disease in man, particularly in the context of correlates of protection and vaccine development. Our

comparative lack of knowledge of NTS infection in man has hampered progress of vaccine development against NTS.

In immunocompetent subjects globally, NTS commonly presents as a self-limiting enterocolitis with nausea, vomiting, watery diarrhoea and abdominal pain (Hohmann 2001). Shedding of NTS from the gastrointestinal tract can persist following diarrhoea, and may be increased by antimicrobial therapy. Secondary bacteraemia can develop in up to 5% of individuals. In immunosuppressed and immunodeficient subjects in LMICs, particularly in Africa, including immune naïve young children, NTS bacteraemia can occur without diarrhoea, and fever is often the only clinical finding. Diarrhoea is due to the induction of an inflammatory enterocolitis which potentially favours NTS over the normal gastrointestinal microbiota (Stecher et al. 2007).

Primary NTS bacteraemia, akin to enteric fever, appears to avoid induction of an inflammatory response at the gastrointestinal mucosa. Whether NTS then invade via M cells of Peyer's Patches and disseminate by infecting underlying macrophages, as with typhoid fever, is not known. The relationship between diarrhoeagenic NTS and invasive NTS disease is unclear. Animal data support the concept of translocation of NTS from the gastrointestinal lumen into the bloodstream to cause invasive disease (Raffatellu et al. 2008)

1.4 Immunity

A full review of immunity to NTS is beyond the scope of this article but will be touched on further in Point 3 below, 'Approach to NTS CHIM development'. Briefly, evidence from studies in humans and animals, including subjects with immune deficiencies, implicates the importance of antibody and T cells, as well as modalities of innate immunity for protection against NTS disease (Gilchrist et al. 2015). *Salmonella* is a facultative intracellular pathogen adapted to survive in macrophages and

genetic defects in the IL12/23-IFN γ -axis are associated with high susceptibility (MacLennan et al. 2004, Gilchrist et al. 2015). Animal models indicate that T cell immunity is required for full elimination of infection from the intracellular reservoir of NTS within macrophages (Mastroeni et al. 1993, Salerno-Goncalves et al. 2002).

However, *Salmonella* is also present in the extracellular space and a growing number of studies indicate that antibodies are important for protection, particularly in Africa where the burden of iNTS disease is highest (MacLennan et al. 2008, Gondwe et al. 2010, Nyirenda et al. 2014, MacLennan 2014). Typhoid conjugate vaccines that target an antibody response to the Vi capsule are effective in preventing clinical disease (MacLennan et al. 2014, Shakya et al. 2019), providing further support of a role for antibody in immunity to *Salmonella*. However, of some concern, HIV-infected Africans with high levels of antibodies against O-antigen inhibit in vitro killing of *S. Typhimurium* (MacLennan et al. 2010). To date, there is no proven immunological correlate of protection for NTS disease, either diarrhoeagenic or invasive.

1.5 Vaccine Development

In view of the high global burden of both diarrhoea and invasive disease attributable to NTS, increasing levels of antimicrobial resistance (AMR) and lack of affordable diagnostics, there is a clear need for a vaccine. No licensed vaccine currently exists for use in man. This situation contrasts with the state of vaccines against typhoid fever, where typhoid conjugate vaccines consisting of Vi polysaccharide covalently linked to a carrier protein are set to supersede unconjugated Vi polysaccharide vaccines and the live attenuated vaccine, Ty21a (Shakya et al. 2019). Until relatively recently, only one NTS vaccine had entered a clinical trial. This was the WT05 live attenuated *S. Typhimurium* vaccine which was tested in a phase 1 study almost 20 years ago (Hindle et al. 2002).

Due to variable immunogenicity and prolonged shedding of the vaccine strain, further development was halted.

However, several new candidate NTS vaccines are currently in clinical development targeted primarily at preventing iNTS disease. A bivalent glycoconjugate vaccine consisting of the O-antigens of *S. Typhimurium* (O:4) and *S. Enteritidis* (O:9) conjugated to flagellin derived from both respective *Salmonella* serovars has been developed through a partnership between the University of Maryland and Bharat Biotech International Limited (Baliban et al. 2018). This is currently being evaluated in a Phase 1 clinical trial as part of a trivalent vaccine in combination with Bharat Biotech's Vi-TT typhoid conjugate vaccine, Typbar TCV. The other lead NTS candidate vaccine is a bivalent mutant-derived outer membrane vesicle (mOMV) vaccine, referred to as a 'GMMA' (Generalized Modules for Membrane Antigens) vaccine, developed by the GSK Vaccines Institute for Global Health (Micoli et al. 2018).

Both vaccines have been designed to induce a strong antibody response against the O-antigen of *S. Typhimurium* and *S. Enteritidis* lipopolysaccharide. There is evidence, indicated above, to indicate that such an approach will be protective in otherwise immunocompetent infants who lack these antibodies (MacLennan et al. 2008, Gondwe et al. 2010, Nyirenda et al. 2014), but it is not certain whether the immune response elicited will be sufficient for protection. Along with the absence of a correlate of protection, it is not known whether an NTS vaccine must induce *Salmonella*-specific T cells. The vaccines should induce sufficient mucosal immunity to prevent diarrhoea, as well as systemic immunity to protect against invasive disease. In addition, the finding of high levels of antibodies to *Salmonella* O-antigen in HIV-infected individuals (MacLennan et al. 2010) suggests that these vaccines may not protect this high-risk group.

Nevertheless, NTS is recognised by the WHO Product Development Vaccine Advisory Committee as a priority pathogen for vaccine development (Giersing 2019). This in part is due to the threat posed to global health by AMR among NTS isolates (Boston Consulting Group 2018), in addition to the direct potential impact on global health described above. As with the University of Maryland/Bharat Biotech approach, other vaccine developers are exploring the possibility of combination vaccines against multiple serotypes of *S. enterica*, including *S. Typhimurium*, Enteritidis, Typhi and Paratyphi A. Similar to multivalent vaccines against pneumococcus and meningococcus, such vaccines promise to provide broader coverage against *Salmonella* by protecting against both enteric fever (typhoid and paratyphoid A) and nontyphoidal *Salmonella* disease.

2 Role of a controlled human infection model (CHIM) for nontyphoidal *Salmonella*

Currently there is no (CHIM) for NTS. As for vaccine development, this contrasts with the situation for typhoid. A well-established typhoid CHIM at the University of Oxford has been used to investigate the immunopathogenesis of typhoid fever (Darton et al. 2016) and assess efficacy of typhoid vaccines (Jin et al. 2017). Recently, a CHIM for paratyphoid A has also been set up at Oxford (Dobinson et al. 2017). Given the complexity and questions concerning NTS disease outlined above, and the need to accelerate vaccine development against NTS, there are multiple reasons to develop a CHIM for NTS.

A CHIM could have enormous value for understanding the pathogenesis of NTS disease, both diarrhoeagenic and invasive disease and the relationship between these disease types. By studying immune responses before and after challenge, valuable insights will be gained into the nature of protective immunity against NTS, together with the possibility of identifying correlates of protection. An NTS CHIM would be invaluable in directly supporting the development of vaccines against NTS, both in the context of providing an early indication of vaccine efficacy and for supporting licensure in late-stage development. A CHIM could also help identify new target antigens for second generation NTS vaccine development and biomarkers for NTS diagnostics.

There are several important issues to address and unknowns to consider in order to successfully establish a first NTS CHIM. Foremost, with NTS able to present as both diarrhoea and invasive disease, there is uncertainty as to what clinical presentation will be seen in volunteers post-challenge. CHIMs are normally conducted in healthy adults in high-income countries (HICs), and so the relevance to infection and disease among LMIC children, who have the greatest burden of NTS disease, needs to be considered. Clinical presentation is likely to vary depending on the serovar, MLST type/pathovar and strain of *Salmonella* used in the challenge, so selection of the challenge

strain is critical. Safety is paramount and needs particular attention given high case fatality rates seen with invasive disease. Disease endpoints, both clinical and microbiological, need to be defined and will depend on the clinical presentation observed. Finally, which clinical samples to take and when, as well as which immunological investigations to conduct, will be key for understanding protective immunity and correlates of protection. The following are a selection of research questions that could be addressed by a CHIM for NTS:

Pathogenesis

1. What is the pathogenesis of NTS disease in man?
2. What is the time course of NTS disease?
3. What is the duration of carriage of NTS?
4. What is the infecting dose of NTS required to cause disease in man?
5. Can ingestion of NTS results in invasive disease in immunocompetent adults?
6. What is the relationship between diarrhoeagenic and invasive NTS disease?
7. Does NTS disease caused by *S. Typhimurium* differ from that caused by *S. Enteritidis*?
8. To what extent is the clinical presentation of NTS disease determined by MLST type/pathovar and strain?

Immunology

9. Is induction of antibody to O-antigen sufficient to protect against NTS disease in man?
10. Do high levels of antibody to O-antigen abrogate protection in immunocompetent adults?
11. Is induction of a *Salmonella*-specific T cell response necessary for protection in man?
12. What are the correlates of protection (COP) against invasive and/or diarrhoeagenic NTS disease?
13. What is the threshold level necessary for protection of any proven COP (threshold of protection)?

14. What are the correlates of susceptibility to NTS disease in man?

Vaccinology

15. Does a specific candidate vaccine have efficacy against NTS disease?

16. What candidate antigens are most suitable for incorporation into second generation NTS vaccines?

Diagnostics

17. What biomarkers are most suitable for use as diagnostics for NTS disease?

3 Approach to NTS CHIM development

3.1 Safety

Safety, as with any clinical intervention, is of utmost importance in the development of a new infectious challenge model. For an NTS CHIM, this is even more so because of the potential severity of disease and high case fatality rate often associated with field cases of iNTS disease, even though NTS bacteraemia with systemic disease is very uncommon in immunocompetent adults. Therefore, the CHIM must be conducted with a high level of vigilance for any indication of possible compromise to subject safety.

Many safety considerations are discussed below in the context of strain selection, but safety considerations are also a key for clinical and microbiological endpoints and the selection of volunteers, and the following steps should be considered:

1. Establishment of an independent Data Safety Monitoring Board of experts from the field to review the preparations and conduct of the CHIM.
2. A low threshold for commencing antibiotic therapy. Ciprofloxacin has excellent intracellular penetration and so should be highly effective at clearing residual infection.
4. A study doctor and study nurse on standby 24 hours a day to provide clinical care during the study.
4. Inclusion of robust Quality Assurance steps in the preparation of the NTS challenge strain prior to administration to participants.

Whatever approach is adopted will need approval by the relevant regulatory body depending on location of the model, for example the FDA (Food & Drug Administration), EMA (European Medicines Agency) or MHRA (Medicines and Healthcare Products Regulatory Agency). Early discussion with regulators is strongly recommended. In addition, a thorough risk assessment and

careful ethical review will be necessary. Initial dosing of volunteers will need to be performed in a cautious step-wise manner beginning at a low dose of inoculum.

3.2 Strain selection

Several considerations are required in relation to NTS strain selection for a CHIM, in particular, which serovar and pathovar to use and whether to use a genetically-attenuated strain. In relation serovar, the key choice is between *S. Typhimurium* and *S. Enteritidis*, as these are the two serovars responsible for the greatest burden of NTS disease globally, constituting up to 90% of clinical disease in many settings. Given the global consensus that an NTS vaccine is most needed for Africa, and that *S. Typhimurium* is the dominant serovar in this continent, an *S. Typhimurium* strain is the logical choice for a first challenge strain. There are also safety considerations that favour selection of *S. Typhimurium* over *S. Enteritidis*. With iNTS disease, higher case fatality rates have been observed with *S. Enteritidis* compared with *S. Typhimurium* (Still et al. 2020) and *S. Enteritidis* generally has higher levels of complement resistance compared with *S. Typhimurium* (Boyd et al. 2014). Also, from a safety perspective, being able to halt rapidly infection in a volunteer is key, so the selected strain should be fully sensitive to antibiotics.

If *S. Typhimurium* is the chosen serovar, the next decision concerns which pathovar. In part, this relates to what form of clinical presentation is being looked for in terms of diarrhoea and/or bacteraemia, and, linked to this, safety considerations. As mentioned, in the context of global NTS disease, there are two principle pathovars of *S. Typhimurium* distinguished by MLST-typing as ST19 and ST313. ST19 is more commonly associated with diarrhoeagenic disease and ST313 with invasive disease. However the situation is not straightforward. ST19 isolates can cause invasive disease (Okoro et al. 2012) and similarly, ST313 isolates can cause diarrhoea (Kariuki and Onsare

2015). Also, it is unclear what disease presentation will be observed in healthy adults who volunteer for an NTS CHIM, mostly likely in a HIC setting.

From a safety perspective, invasive disease is associated with a high case fatality rate. Nevertheless this is usually in the context of HIV-infected adults (Gordon et al. 2002) and young children who are immune-naïve and/or have significant medical comorbidities such as malaria (MacLennan et al. 2017, Gilchrist and MacLennan 2019). iNTS disease is rarely seen in healthy immunocompetent adults and poor outcome in this group is rarer still. Therefore, in the carefully-monitored environment of a CHIM, the risks of severe disease from an ST313 *S. Typhimurium* are likely to be very low. In relation to safety, there are considerations for ST19 strains and diarrheagenic presentations. Prolonged shedding of NTS in the stool is a possible consequence of challenge with NTS and this may be more likely when clinical presentation is with enterocolitis rather than invasive disease.

A further consideration is whether targeted genetic mutations should be introduced into the challenge strain. The primary reason for this approach would be to reduce the risk of severe disease and poor outcome through attenuation of the challenge strain, similar to the approach adopted with live attenuated vaccines. A disadvantage of this approach is uncertainty of the relevance of findings from a CHIM using an attenuated compared to a wild-type challenge strain. Additionally, attenuations may introduce problems of their own. For example, genetic attenuation of a candidate *S. Typhimurium* vaccine strain resulted in prolonged *Salmonella* excretion for a period of three months among volunteers (Hindle et al. 2002). Therefore, the case to use a genetically-modified challenge strain is not clear.

The selected strain needs to be fully characterised, both phenotypically (Lanzilao et al. 2015) and genomically (Kingsley et al. 2009) and have confirmed lack of antibiotic resistance. A full set of analytical procedures will need to be developed and used to establish release criteria for manufactured lots. The selected strain should be passaged to ensure stability and clonality, whole genome sequence determined by PacBio sequencing, and full proteome by LC-(QTOF)-MS mass spectroscopy. A suitably qualified contract manufacturing organisation (CMO) is required for the preparation of master and working cell banks and production of bacterial lots under good manufacturing practice (GMP). It is recommended that GMP lots of bacteria be manufactured at 10^3 , 10^4 and 10^5 cfu/ml and ongoing stability studies conducted.

3.3 Endpoints

Consideration needs to be given to the clinical, microbiological and immunological endpoints of an NTS CHIM. From a safety perspective, careful monitoring will be necessary to understand when to commence antibiotic therapy and any necessary supportive medical intervention that may be required in the case of severe disease. For these reasons, the CHIM may need to be established with volunteers admitted to an inpatient clinical trial facility with full medical back-up including resuscitation facilities. However, if the safety profile of the NTS CHIM proves similar to that of the typhoid CHIM, outpatient monitoring with a minimum of daily visits may be possible.

With safety concerns associated with invasive disease, daily monitoring of peripheral blood will be needed to assess whether NTS has translocated from the gastrointestinal tract to the bloodstream. This would normally be performed by blood culturing for the presence of *Salmonella*. However, in view of the 24 to 48 hours required to obtain a positive culture, together with concerns about potential progression of iNTS disease, a rapid molecular detection method such as qPCR would be preferable. This may need to be developed specifically for the model. Since iNTS disease often

presents with fever alone, careful temperature monitoring will be required, along with other vital signs. Isolated fever is often observed in the typhoid CHIM. If this proves to be the main presentation in the NTS CHIM, it might be possible to adopt similar clinical endpoints to the typhoid CHIM based on oral temperature and/or presence of NTS in the blood (Darton et al. 2016).

Ingestion of NTS may well result in diarrhoeagenic disease. Here, lessons about study design and endpoints can be derived from CHIM for other diarrhoeagenic pathogens, particularly *Shigella*, where CHIMs have been established at three sites in the US (MacLennan et al. 2019a). Consensus has been reached among the *Shigella* community concerning CHIM study design (Talaat et al. 2019), clinical endpoints (MacLennan et al. 2019b) and immunological sampling and endpoints (Kaminski et al. 2019). An important distinction needs to be drawn between infection alone, as assessed by the presence of NTS challenge strain in the stool, and disease characterised by clinical manifestations. For diarrhoeagenic pathogens, while detection of pathogen in the stool is important in relation to duration of shedding, clinical endpoint criteria are critical. Such criteria may be set at a particular level of severity as determined by frequency and volume of stool, and presence of other clinical features (MacLennan et al. 2019b).

Considering the uncertainty about clinical presentation, the following is proposed as criteria for an endpoint for NTS salmonellosis:

- 3 or more liquid or watery stools (Bristol stool chart 6 or 7) in 24 hours, or
- Fever $\geq 38.0^{\circ}\text{C}$ for 12 hours, or
- Gram-negative bacteria detected in the blood by blood culture or molecular method

Immunological sampling, measurements and endpoints are key for understanding the immune response to NTS infection and will be critical for establishing the presence of a correlate of protection against NTS. A priori planning of sample timing, assessment and storage needs to take place long before a clinical study starts.

3.4 Volunteer Recruitment

As with all aspect of CHIM, volunteer recruitment needs to be considered primarily from a safety perspective. For this reason, in line with other CHIM, it is recommended that healthy non-pregnant adult volunteers aged 18 to 65 are recruited who can give full informed consent. Each participant should be screened to ensure no concurrent medical condition or immunocompromise, no prior episodes of *Salmonella* disease, no history of psychiatric disease, and no previous immunisation with a *Salmonella* vaccine. It will also be important to check that volunteers do not have any comorbidity associated with iNTS disease that might result in a poor outcome. Specifically, volunteers should be assessed to ensure that they do not have HIV infection or other impairment of immunity, either primary or secondary, including use of immunosuppressive or immunomodulatory medication. Malaria and recent history of malaria need to be specifically excluded, together with anaemia, malnutrition and sickle cell disease. Rare cases of iNTS disease in HICs have been associated with neoplasia and this must be carefully excluded too.

A further consideration in volunteer recruitment is background immunity to NTS which is likely to impact the likelihood of disease in volunteers. There is currently no standardised ELISA (enzyme-linked immunosorbent assay) or other assay for assessing background immunity to NTS. However, published data suggest that acquisition of antibody to NTS is acquired rapidly in the first two years of life both in the African context, where iNTS disease is most prevalent (MacLennan et al. 2008, Nyirenda et al. 2014, Stockdale et al. 2019), in Asia (de Alwis et al. 2019) and in HIC settings

(Trebicka et al. 2013). With the exception of HIV-infected adults, iNTS disease is largely restricted to children under two years of age with a relative sparing of children under six months, corresponding to the age at which maternal antibody protects against disease (MacLennan et al. 2008). This immunoepidemiological observation supports the concept that low levels of specific antibody protects against iNTS disease in the context of an otherwise intact but naïve immune system. If correct, the possibility of invasive disease in healthy adult volunteers is very low.

The situation is less clear in relation to immunity and susceptibility to NTS enterocolitis, since this disease presentation occurs at all ages including adult travellers (Majowicz et al. 2010) and military personnel (Williams et al. 2017). Together, these epidemiological observations suggest that a diarrhoeagenic disease presentation is more likely than invasive disease following exposure of healthy adult volunteers to NTS in the context of a CHIM. Due to the uncertainty concerning the type and level of baseline immunity needed to impact disease presentation, it is currently unclear whether volunteers with high levels of pre-existing NTS antibodies should be excluded. Measurement of immunity to NTS pre-challenge will be essential to gain insight into potential correlates of protection, and must include a broad range of immunological indices, as suggested below.

3.5 Study Design

In relation to other aspects of study design, valuable lessons can be learned from established CHIM to other enteric and diarrheal pathogens such as *Shigella* (Talaat et al. 2019) and typhoid (Darton et al. 2016) and paratyphoid A (Dobinson et al. 2017). After ensuring safety considerations are met, the priority is to determine the dose required to cause clinical disease in 60-75% of participants. The following is a suggestion of a reasonable approach for establishing a *Shigella* CHIM with timeline for immunisation, challenge and follow up in **Figure 1**:

Following enrolment and informed consent, baseline bloods and stool samples, together with GI mucosal samples from the duodenum and sigmoid colon are taken. The infecting dose required to induce clinical disease will need to be determined in a step-wise dose-escalation manner (**Figure 2**). As this dose is currently unknown, experience from establishing CHIM for other *Salmonellae*, typhoid and paratyphoid A, suggests that 10^3 bacteria is a reasonable starting dose. Five participants will be given 10^3 *Salmonellae* by the oral route, after drinking sodium bicarbonate solution to neutralise gastric acid. As this is a new CHIM, there will be a time interval of one week between the first subject and second subject ingesting the challenge strain, and a further week before the next four subjects receive the challenge strain both at the initiation of the study and with any increase in bacterial challenge dose. To ensure the safety of the model, participants will be monitored in an inpatient facility from the time of ingesting the *Salmonella* preparation until their symptoms have resolved following antibiotic treatment (or fourteen days if they remain asymptomatic), and then daily as outpatients.

Blood, stool, urine and saliva samples will be taken daily permitting prompt assessment of fulfilment of endpoint criteria discussed above (see below). On reaching the endpoint, participants will be started on oral ciprofloxacin for seven days. Onset of clinical salmonellosis may be rapid since symptoms have been reported to develop within 5 to 72 hours of exposure. Intravenous fluids for rehydration and intravenous antibiotics need to be available together with a full suite of resuscitation equipment. Should volunteers not develop clinical disease, they will be treated with ciprofloxacin fourteen days after the challenge (**Figure 1**).

There should be a strong emphasis on infection control including:

- Handwashing after toileting
- No preparation or handling of food that will be consumed by others
- No exposure to young children or immunocompromised individuals

Depending on the clinical attack rate, the next round of 5 volunteers will be given the same dose of *Salmonella* or the next higher dose, until it is possible to demonstrate a dose that gives an attack rate of 60-75% in 20 volunteers (Sandler and Douek 2012).

3.6 Clinical and routine blood indices

It is important to take the full opportunity to study clinical, immunological, and microbiological aspects of the infection during the inpatient phase of the study and at subsequent follow up visits. As a limited amount is known about the natural history of NTS disease in man, the clinical course of infection should be carefully monitored alongside routine haematological and biochemical blood analysis. At each time point the recording of symptom history and conducting of clinical examinations will include:

- Temperature, pulse and blood pressure
- Bristol stool chart with details of frequency, form and volume of stool
- Symptom chart including recording of abdominal pain, cramps and vomiting

Blood will be taken for a full blood count including white cell differential, blood culture (and molecular diagnosis of *Salmonella*, if available), creatinine, urea and electrolytes, liver function tests, and the inflammatory markers, CRP and ESR.

3.7 Tissue sampling and immune monitoring

Careful tissue sampling and immune monitoring is key for identification of:

- The form and magnitude of immune response required for protective immunity
- Correlates of protection
- Candidate antigens for second-generation vaccines

Given existing evidence of roles for antibodies, T cells and innate immunity for protection against NTS discussed above, it will be important to adopt a comprehensive approach to the immune profiling of peripheral blood and gastrointestinal (GI) mucosal tissue from study volunteers. A plan is required for both inpatient sampling and follow-up visits post discharge and it is suggested that these be conducted at day 21, 28, and month 2, 3, 6, 9 and 12 post-challenge.

To better understand the association between diarrhoea and invasive disease, assessment of translocation of *Salmonella* and *Salmonella* products, particularly lipopolysaccharide (LPS), to the blood stream will be of value. Translocation of viable *Salmonella* will result in bacterial challenge and antigenic stimulation in the bloodstream, and drive systemic inflammation. Suggested measurements in the blood include LPS by *Limulus* amoebocyte lysate (LAL) assay; immunological markers of translocation, including soluble CD14 and LPS binding protein; and endotoxin core IgM which falls with increasing LPS translocation (Sandler and Douek 2012). In addition to CRP and ESR, systemic inflammation can be gauged by plasma cytokine and chemokine levels, frequencies of activated CD4⁺ and CD8⁺ T cells co-expressing CD38 and HLA-DR, and D-dimer. I-FABP can be tested as a marker of intestinal damage. These markers are not specific for *Salmonella* and so need to be interpreted in the context of confirmed NTS disease.

3.7.1 Antibodies

IgG, IgA and IgM antibodies to O-antigen and flagellin should be determined by ELISA as a minimum, and ideally to a broad range of *Salmonella* proteins using high throughput protein array technology (Lee et al. 2012). This approach will profile the antibody repertoires in participant sera with a high *Salmonella* proteome coverage. Functional antibody assays need to be conducted, such as serum bactericidal assays and opsonophagocytic assays, alongside avidity and affinity studies and specific IgG subclass analysis.

3.7.2 Cells

A broad panel of lymphocyte markers will allow full characterisation of total and specific T and B cell subsets in peripheral blood mononuclear cells (PBMC) and mucosal tissue preparations with and without prior in vitro stimulation with NTS. Sample analysis can be conducted by ELISpot (enzyme-linked immune absorbent spot), multicolour flow cytometry or CyTOF (cytometry by time of flight) platforms and the following are suggested marker panels:

- To investigate simultaneously T and B lymphocytes, natural killer cells, monocytes and dendritic cell subsets:
CD3, CD4, CD8, CD14, CD33, CD19, CD20, CD16, CD56, CD11c, IgD, IgA, TCR $\gamma\delta$, CD45RA, CD26, CD27, CD123, CD161, CD127.
- To characterize cell activation and identify effector cells differentiating upon infection:
CD25, CD38, HLA-DR, CD86, ICOS, CD57, Ki67 and Bcl2.
- To characterize homing profiles to the gut of effector cells:
CCR9, CCR10 and $\alpha 4\beta 7$ for trafficking to the gut, CLA (to distinguish skin/oral cavity homing subsets), CCR7 and CD62L/L-Selectin (for central memory and naïve subsets/lymph node homing), CXCR5 (for germinal centre TFH cells – T follicular helper cell), CCR6 and CXCR3.
- To define functional profile of effector cells:

IFN- γ , IL-17, MIP-1 β , IL-22, TNF α , IL-2.

3.7.3 Complement

Complement is key for in bactericidal (MacLennan et al. 2008) and opsonophagocytic antibody-mediated killing of NTS (Gondwe et al. 2010) and so measurement of C3, C4, and functional levels of classical and alternative pathways of complement activation is recommended.

3.7.4 Neutrophils

Susceptibility to NTS infections of patients with chronic granulomatous disease indicates an important role for phagocytic cells in immunity to *Salmonella* (Gilchrist et al. 2015). Neutrophil and monocyte phagocytosis and oxidative burst can be determined by flow cytometry.

3.7.5 Microbiology and microbiota

In order to colonize the intestinal tract, *Salmonella* must first contend with the indigenous microbiota for nutrients and ecological niche. Thus, an individual's colonisation resistance mediated by the microbiota can vary based on the composition of the microbiota. Study of the intestinal microbiota of challenged volunteers will help understand the role of human microbiota in NTS colonisation resistance.

Key to our understanding of NTS infections will be detection or lack of detection of bacteraemia following challenge with NTS delivered by the oral route. Duration of faecal shedding will also give insight into the potential for transmission. Blood, stool, oropharyngeal swabs and urine will be collected at each visit for NTS culture and molecular diagnosis. Any retrieved isolates should be sequenced in order to provide information about the micro-evolution of NTS in man. Changes in the

microbiome following challenge will provide insight into the role of microbiota disturbance in understanding pathogenesis.

3.7.6 Gastrointestinal Mucosal Tissue

The immunological responses that occurs following ingestion of the *Salmonella* challenge dose will first occur at the intestinal mucosa. Although peripheral blood is easier to access and assess than intestinal mucosa, changes in blood do not necessarily reflect what is occurring at the site of infection. Research endoscopy, including oesophago-gastro-duodenoscopy (OGD) and flexible sigmoidoscopy can be performed pre- and post-challenge to obtain mucosal tissue from the upper and lower gastrointestinal (GI) tract to examine innate and adaptive responses to NTS.

3.7.7 RNA and DNA

Samples prepared under conditions to preserve RNA should be stored to allow transcriptomic studies that can complement immunological work and provide confirmation at the molecular level for new cellular insights. DNA can be prepared from participants in order to conduct HLA typing to correlate with antibody and T cell responses.

3.7.8 Biobanking

Biobanking of serum, PBMC, NTS isolates and tissue samples will be critical for subsequent experimental analysis guided by the results of the initial panel of assays.

4. Conclusion

With candidate NTS vaccine entering the clinic for the first time in many years, now is the opportune time for developing a CHIM to facilitate NTS vaccine development. Despite our knowledge of NTS disease from studies in mice, vaccine development requires a better understanding of the pathogenesis of NTS disease in man. Key aetiological questions need to be answered, such as the relationship between diarrhoeagenic and invasive forms of NTS disease. Such understanding can only be gleaned from clinical studies and a CHIM provides an ideal format for such work.

In addition to understanding pathogenesis, an NTS CHIM will provide important insights into the modalities of protective immunity and help establish correlates and thresholds of protection. To date, observational studies of disease in the field have highlighted immune parameters associated with susceptibility and protection from disease. A CHIM will provide key additional evidence to help establish mechanisms of protection.

Finally and perhaps most importantly, a CHIM will enable the early establishment of vaccine efficacy and help up-select candidates for further development and derisk lengthy expensive Phase 3 field efficacy trials. Though safety issues have been highlighted throughout this article, a cautious step-wise approach to developing a CHIM for NTS is possible and will help advance our understanding of this key global health threat and support the rapid development of affordable vaccines.

Figure Legends**1. Nontyphoidal *Salmonella* (NTS) Controlled Human Infection Model timeline**

Note that antibiotics are given at day 14 unless indicated earlier due to development of clinical salmonellosis.

2. Flow diagram for establishing dose of the oral nontyphoidal *Salmonella* (NTS) inoculum

resulting in salmonellosis in 60 to 75% of volunteers starting with 10^3 bacteria and increasing to 10^4 and 10^5 as required. At each new dose, for safety reasons, the inoculum will be given to one volunteer first, a second volunteer after one week and the remaining three volunteers a week later. No more than five volunteers will be given the inoculum at one time.

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