Understanding typhoid disease; a controlled human infection model of typhoid fever

A thesis submitted in partial fulfilment of the requirement

for the degree of Doctor of Philosophy

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Hilary 2013

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Abstract

Typhoid disease, caused by infection with *S.* Typhi, is a significant cause of mortality and morbidity in resource–poor countries. Efforts have been made to generate a new generation of vaccines that are efficacious and can be given to infants, but have been hindered by a poor understanding of the protective immune response to *S.* Typhi infection, and in particular by the absence of a correlate of protection.

Controlled human infection studies ('challenge studies') provide a model for investigating infectious diseases and appraising novel vaccines, including in typhoid disease. This DPhil described the development of a human challenge model of typhoid fever using *S.* Typhi Quailes strain administered to healthy adults in a sodium bicarbonate buffer. The careful characterisation and manufactured of the strain is described. Following ingestion of 10^3 CFU of *S.* Typhi 55% of participants developed typhoid disease, whilst ingestion of 10^4 CFU gave a higher attack rate of 65%. At this attack rate vaccine efficacy against human challenge should be demonstrable with a modest sample size. Validity of the model in the appraisal of vaccines was demonstrated using Ty21a, a live, oral, attenuated vaccine. Protective efficacy of Ty21a compared to placebo against challenge was 35%, comparable to that observed in some endemic settings, and the estimated protection in the first year after vaccination in Cochrane meta-analysis.

Clinical, microbiological and humoral immune responses were investigated in participants challenged during model development. Typhoid disease was associated with a high fever in most, but not all participants, and a range of symptoms. Severity of disease was variable, and included asymptomatic bacteraemia, as well as fever and symptoms in participants in whom bacteraemia could not be demonstrated. Typhoid disease was associated with a strong humoral immune response to the flagellin and lipopolysaccharide antigens of *S.* Typhi but not the Vi polysaccharide capsule. Humoral immune responses were not demonstrated in participants without typhoid fever. There was a dose-response relationship to the clinical, microbiological and humoral responses with participants challenged with 10^4 CFU having more marked responses than those challenged with 10^3 CFU.

Future success of challenge studies relies on the willing participation of healthy adult volunteers. The motivations for participation, and experiences of participants, were appraised by questionnaire. Whilst financial compensation was an important motivator, it was not the sole motivator. Participants were positive about their experiences, and most would participate again.

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Lead doctor	Claire Waddington
Lead nurse	Kathryn Haworth
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Perceptions of participants in the challenge study

Development of questionnaire	Claire Waddington
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Abbreviations

Alk Phos	Alkaline phosphatase
ALT	Alanine transaminase
API	Analytical profile index
BSA	Bovine serum albumin
CDC	Center for Disease Control (USA)
CFU	Colony forming units
CRP	C-reactive protein
DNA	Deoxyribonucleic acid
dH_2O	Distilled water
ELISA	Enzyme linked immunosorbent assay
ELISpot	Enzyme linked immunosorbent spot
ESR	Erythrocyte sedimentation rate
FCS	Foetal calf serum
GCP	Good Clinical Practice
GMC	Geometric mean concentration
GMP	Good manufacturing practice
GP	General practitioner
H (antigen)	Flagellin antigen of S. Typhi
Hb	Haemoglobin
HPA	Health Protection Agency
Ig (A, M, G)	Immunoglobulin
IL	Interleukin
IMP	Investigational medicinal product
LFT	Liver function test
LPS	Lipopolysacharide

MCB	Master cell bank
MHRA	Medicines for Healthcare Regulatory Authority
MLST	Multilocus sequence typing
NBBS	Newborn bovine serum
NHS	National Health Service
O (antigen)	Lipopolysaccharide antigen of S. Typhi
OUH	Oxford University Hospitals
OVG	Oxford Vaccine Group
OVG 2009/10	Clinical study entitled 'Understanding typhoid disease; Developing a *Salmonella* Typhi challenge model in health adults.
OVG 2011/02	Clinical study entitled 'Understanding typhoid disease after vaccination: a single centre, randomised, double-blind, placebo-controlled study to evaluate M01ZH09 in a healthy adult challenge model, using Ty21a vaccine as a positive control.
OR	Odds ratio
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with tween
PBSTM	Milk in phosphate buffered saline with tween
Pl	Platelets
SAE	Serious adverse events
SNP	Single nucleotide polymorphism
SOP	Standard operating procedure
TD	Typhoid disease
TLR	Toll-like receptor

TMB	Ultra-sensitive tetramethylbenzidine
TSA	Tryptone soy agar
TSB	Tryptone soy broth
Vi	Virulence factor polysaccharide capsule of S. Typhi
WCB	Working cell bank
WCC	Total white cell count
WHO	World Health Organisation
XLD	Xylose lysine deoxycholate agar

1. Introduction

Typhoid disease results from systemic infection with *Salmonella enterica* subspecies *enterica* serovar Typhi (*S*. Typhi). Paratyphoid fever is a similar illness caused by serovars Paratyphi A, B or C. Typhoid disease is common in resource-poor regions of the world, where inadequate sanitation facilitates disease spread via faecal-oral transmission. Typhoid disease produces a wide range of non-specific symptoms and is often clinically indistinguishable from many other diseases, both infectious and non-infectious. Prompt treatment minimises illness severity, but late presentation to health care facilities often delays initiation of appropriate antibiotic therapy. Treatment delay is compounded by a lack of reliable diagnostic tests.

For over 100 years, prevention of typhoid disease has been attempted through vaccination[1]. Three vaccines for typhoid disease exist currently, but are only moderately efficacious and unsuitable for infant immunisation. The implementation of existing vaccines and development of new efficacious vaccines has been hindered by a lack of understanding of typhoid immunobiology. *S*. Typhi is a human restricted pathogen and requires a relatively high dose to cause infection, and therefore the development of a suitable vaccine could lead to the eradication of typhoid disease. To reach this goal, an improved understanding of the immunobiology of typhoid disease is needed.

1.1. Epidemiology

1.1.1. Disease incidence and geographical distribution

In 2010 typhoid disease was estimated to have affected 26.9 million people, and to have a 1% mortality rate,[2] a higher incidence than has previously been estimated.[3] Significant limitations in the data used to generate morbidity and mortality estimates exist[2,4] and the true burden of enteric disease may be considerably higher than worldwide incidence

estimates suggest.[3,4] Resource-poor countries frequently lack the health care and public health infrastructure to provide reliable data.[3,5-7] Consequently, calculations of worldwide disease burden relied on extrapolation of limited data to a geographically wide area, and included data from vaccine studies which may have deliberately been undertaken in high incidence areas.[2] This is likely to be inaccurate, as data from both Asia[8] and Africa[9] have demonstrated considerable temporal and geographical variation in disease rates over relatively small areas, with the highest incidence in densely populated urban areas. For example, in Kenya, overall incidence in an urban slum was calculated as 247 cases per 100,000 person-years, compared to 29 cases per 100,000 person years in a rural setting.[9]

Data are also weakened by difficulties in precise case ascertainment. Historically, cases of clinical enteric fever have been attributed to *S.* Typhi but an increasing burden of paratyphoid disease in many regions, makes this unreliable.[10] Data from population-based blood culture studies, although specific, are few in number,[8] and consistently demonstrate a higher disease prevalence than is suggested by public health figures.[7] Similarly, sero-epidemiological studies based on detection of antibodies to typhoid antigens suggest higher rates of infection than those studies using clinical or microbiological case detection, with up to 80% of residents in endemic regions showing evidence of past infection.[11,12] Microbiological isolation of *S.* Typhi provides a definitive diagnosis, but blood culture sensitivity is limited to 50% to 60% and therefore underestimates the true number of cases.[13] Blood culture facilities are also of limited availability in many endemic regions.[14]

Although Asia was previously thought to experience the majority of the disease burden,[3] a high burden of disease is increasingly recognised in Africa.[2,15] Acute febrile illness is a common cause of admission to hospital in Africa,[16,17] and has traditionally been attributed principally to malaria and HIV-associated disease.[15,18] In contrast to non-typhoidal Salmonella, *S.* Typhi is not associated with HIV infection.[19] This attribution may be inaccurate however. Malaria has been shown to be is over diagnosed in febrile patients[18] and the increased use of bed-nets and anti-retroviral therapy in HIV infected patients has

led to a decline in malaria prevalence and HIV associated disease in some regions of Africa over the last decade.[20] Bacterial infection, including that due to *S.* Typhi and other, non-typhoidal, *Salmonella* serovars may now therefore account for a significantly higher proportion of cases of acute febrile illness numbers of infections than was previously recognised. A meta-analysis of community-acquired bloodstream infections across Africa estimated that *S.* Typhi accounted for 9.9% of all isolates, although the vast majority of these isolates were from North Africa where HIV prevalence was lowest.[15] Overall, non-typhoidal *Salmonella* predominated as a cause of bloodstream infection, especially in children, and was a leading cause of illness in those with HIV.[15] Although this meta-analysis showed considerable regional variation in the incidence of typhoid disease, and was limited by the extrapolation of limited data across a diverse continent and people, the relatively high burden of *S.* Typhi infection in parts of Africa has been supported by other data. For example in a study of children under 15 years of age in Ghana, 12.4% of blood culture isolates were due to *S.* Typhi, equivalent to an incidence of approximately 190/100,000 per year.[21] In Nigeria also, *S.* Typhi accounted for 20.9% of isolates in febrile children less than 5 years of age, despite high pre-culture antimicrobial use.[22] Disease rates in Zanzibar match those seen in many high incidence regions of Asia, with 58% of blood culture isolates identified as *S.* Typhi.[20] Comparison of blood culture isolates with clinical diagnoses in Africa showed that 55% of children with *S.* Typhi bacteraemia were diagnosed clinically with malaria.[22] Although these data show the importance of typhoid in Africa at the clinical front-line, the false attribution of febrile illness to malaria has hindered the recognition of typhoid fever as a major cause of disease in Africa,[15,22] supporting the idea that typhoid disease is an under recognised problem in Africa and highlighting the need for better diagnostics and preventative measures in resource-poor countries. Conversely, the disease burden in previously highly endemic areas in parts of Asia may be falling, particularly in areas where significant investments in infrastructure have been made. For example, a 15 year retrospective review of blood culture results in Vietnam showed that, while *S.* Typhi

had accounted for 74% of positive blood cultures in 1994, the proportion by 2008 was 6.2%.[23] This is explained by an absolute reduction in numbers as well as a proportionate reduction, with increasing incidence of non-typhoidal Salmonella associated with the rising HIV prevalence in Vietnam.

1.1.2. Age related burden of disease

The majority of the global typhoid burden is borne by children and adolescents, with an estimated 57.7% of all cases in low and middle income countries occurring in those under 5 years of age.[2] In typhoid endemic countries children in the 2-4 year age group have the highest disease incidence.[24-27] This burden of disease in young children was previously under recognised[28-30] primarily due to the non-specific nature of typhoid disease symptoms,[31] lower admission rates,[32-34] frequent comorbidity leading to disease misclassification,[35] and difficulty in drawing sufficient quantities of blood for diagnostic cultures to be performed.[36] This was highlighted by active community surveillance of typhoid disease, conducted as part of the "Diseases Of the Most Impoverished" (DOMI) programme which calculated a disease incidence of 27.3 per 1000 person years in those aged less than 5 years, compared to 11.7 in those aged 11-19 years and 1.1 in those aged 19-40 years of age.[25] Similarly, studies in Bangladesh and India have shown that the highest *S.* Typhi isolation rate occurred in the second year of life,[26] and in those aged 2-3 years[37] respectively. These data highlight the need to develop vaccines for typhoid disease that are suitable for infants.[26,35,38]

1.2. Nomenclature

The Salmonella genus, a member of *Enterobacteriaceae* family, is large and diverse and has a much-debated nomenclature.[39] Increased understanding of the genomic evolution of organisms has required revision of the classification system. The currently accepted classification, outlined in Opinion 80 of the Judicial Commission of the International

Committee of the Systematics of Prokaryotes, is an amalgamation of the scientific rigour of the Bacteriological Code applied to the current understanding of the relationship between isolates coupled with the historical terminology that has become familiar to clinicians.[40]

Two species exist within the Salmonella genus, *Salmonella bongori* and *Salmonella enterica.*[41] The *Salmonella enterica* species, to which *S.* Typhi belongs, is in turn divided into six subspecies. These are named *enterica, salamae, arizonae, diarizonae, houtenae* and *idica,* and are differentiated by antigenic and biochemical characteristics, as well as genetic phylogeny. Subspecies *enterica* is the largest of the subspecies, accounting for around three fifths (1531 of 2557) of the recognised isolates within the species enterica.

Salmonella bongori and the six subspecies of *enterica* are further divided into serovars based on surface antigen expression. There is a high degree of variation in surface antigens within the genus, possibly driven by immune selection,[42] and hence a large number of serovars. The species *Salmonella enterica* is much larger than *Salmonella bongori*, with *Salmonella enterica* accounting for 2557 of the 2579 currently recognised *Salmonella* serovars. Classification is determined by the White- Kauffmann-Le Minor scheme, which identifies serovars on the basis of three surface antigens; the O (lipopolysaccharide), H (flagella) and K (capsular) antigens.[43]

The naming of Salmonella serovars has changed over time.[41,44] Serovars were initially considered species, and hence italicised, and were based on the resulting clinical syndrome or host (for example *Salmonella abortus-equi*). The increasing recognition that many serovars caused disease beyond the syndrome or host with which they were initially associated, led to naming based on the geographic origin of the first isolate (e.g. *Salmonella london*). By the time it was recognised that these were serovars (traditionally given antigenic formulas, not names) rather than species, many of the names were well established. In an attempt to avoid confusion, the serovar names of *Salmonella enterica* subsp. *enterica* have been kept, but are not italicised and the first letter capitalised. The less well known serovars of other

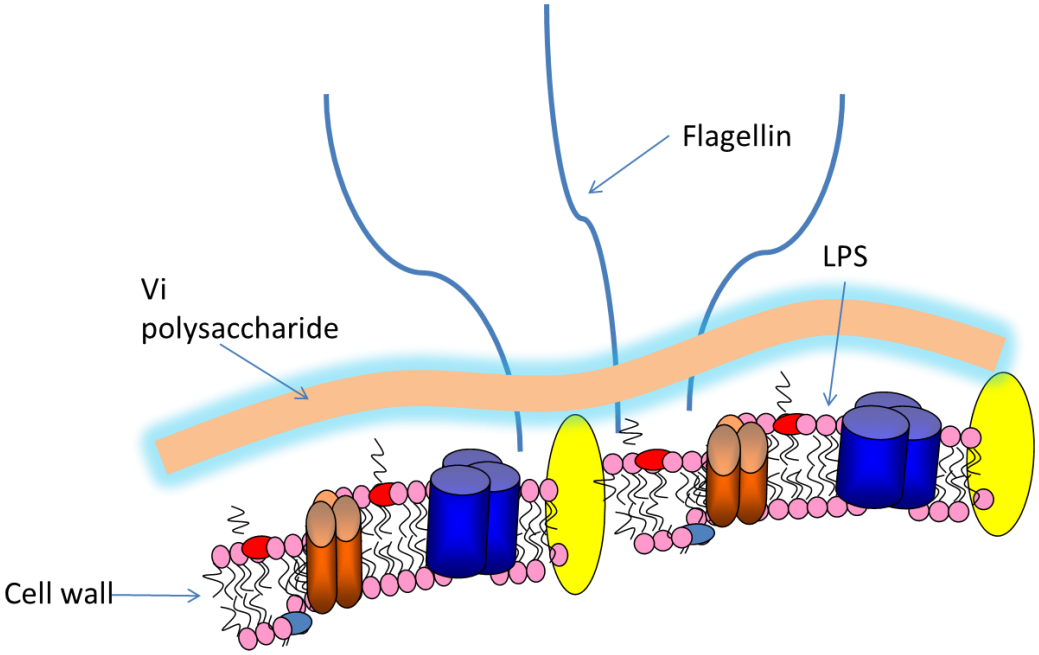
subspecies are designated by the antigenic formula. Hence, the taxonomy of *Salmonella* Typhi is designated as *Salmonella enterica* subspecies *enterica* serovar Typhi.

1.3. Bacteriology

S. Typhi, the causative agent of typhoid disease was first identified by Eberth in 1880.[45] The first direct demonstration of Koch's disease causation postulates in humans came in 1904, when 13 volunteers ingested a "heat killed" vaccine preparation of the Dorset strain, which turned out to have been incompletely inactivated. Ten of the volunteers developed typhoid disease between 6 and 28 days later.[46] This was the first known challenge of humans with viable virulent *S*. Typhi, albeit non-intentional. Members of the Salmonella genus are Gram-negative motile bacilli and are non-lactose fermenting, oxidase and urease negative and facultatively anaerobic. When grown on blood agar, colonies appear as moist, smooth, white colonies two to three millimetres in diameter.[47,48] *S*. Typhi is weak producer of hydrogen sulphide from thiosulfate metabolism, which can give rise to colonies with a black centre when plated on selective media.[47] Due to the risk of laboratory acquired typhoid disease[49,50] samples which potentially contain *S*. Typhi should be processed in a containment level 3 laboratory.[51]

The surface expressed antigens of *S*. Typhi are important for antigenic classification, as well as understanding *S*. Typhi virulence and the immunological response to infection. The principal antigens are shown in Figure 1.

Figure 1: Diagrammatic representation of the principal surface expressed antigens of *S.* Typhi, showing the flagellin 'H' antigen, the lipopolysaccharide (LPS) 'O' antigen and the virulence factor (Vi) polysaccharide capsule in relation to the bacterial cell wall



Antigenic classification by the White- Kauffmann- Le Minor scheme places *S.* Typhi in *Salmonella* serogroup D, the members of which all possess the somatic O9 lipopolysaccharide antigen, antigenic factor 012.[40] The antigenic virulence factor polysaccharide capsule (Vi) is characteristic of *S.* Typhi, only occasionally occurring in other serovars including Dublin and Hirschfeldii (paratyphoid C) as well as in *Citrobacter freundii.*[52] It can be detected by agglutination with Vi antiserum, and can prevent agglutination with O antiserum. Hence, for correct antigenic classification the Vi capsule may need to be removed by heating.

S. Typhi typically has a single, monophasic flagella with the H1-d antigen encoded on the *fliC* locus,[53] which agglutinates polyvalent H and single factor H antisera. Flagella are important for bacterial motility and may play a role in cell entry and exit.[54] Variations in flagella antigens may influence infectivity or pathogenicity through changes in gut mucosal or Toll-Like Receptor interactions.[55] Rare variants of the flagella antigen have been described, including isolates that exhibit phase variation in which two flagellin genes are

alternatively expressed.[56] This occurs in up to 16% of isolates from Indonesia, which alternatively express the H1-j antigen.[54] A high degree of genetic homology between H1-d and H1-j exists,[53] but the H1-j serotype appears to be less motile on agar, less invasive of cell-lines *in vitro* and causes less severe clinical illness compared to the H1-d serotype.[54] H1-j isolates tend to be isolated from older individuals compared to the H1-d flagella type, possibly because exposure to the more common H1-d antigen in early life has led to protective immunity against this flagella type but not against H1-j isolates.[54] Two further flagella antigen variations have also been detected in Indonesia and are named Ind[55] and z66.[53] The z66 variant is rare and is found as a phase two flagellum to either the d or j flagella.[57]

There is limited genomic variation between *S.* Typhi isolates, and considerable genomic overlap between *S.* Typhi and other members of the *Salmonella enterica* species, as well as with other *Enterobacteriaceae*.[58] *S.* Typhi isolates compared by multi-locus sequence typing (MLST) in which the nucleotide sequences of pre-defined internal genomic fragments are compared, have demonstrated that *S.* Typhi isolates are clonal in origin.[59] *S.* Typhi is relatively young in bacterial times, with an estimated age of between 15,000 and 150,000 years, based on the rarity of mutations.[59] Despite variation in antigenicity and pathogenicity between members of *Salmonella enterica* species[60] there is only 1% variation in the core genetic elements.[61] Most, if not all, of the *Enterobacteriaceae* also share a set of 'core genes' which are responsible for the 'household functions' required for survival in the gut microenvironment.[62] These core genes are usually found arranged in the same order along the chromosome, a feature referred to as synteny.[61] The *Salmonella enterica* species diverged from *Escherichia coli* approximately 100 million years ago,[63] but considerable genetic homology remains, with 45% to 55% of the genome shared.[60] When core, non-variable genes of the *Salmonella enterica* sp. are compared to conserved homologues within *Escherichia coli* there is 83% overlap.[62] This genetic homology reflects the ability of both to survive in the gut microenvironment, leading to co-linear evolution since their genetic divergence.[7]

S. Typhi is restricted to the human host,[58] and causes systemic rather than the luminal pathology seen with most *Salmonella enterica* serovars.[64,65] This may account for the presence of genetically redundant pseudogenes, which constitute 5% of the *S*. Typhi genome.[58] These genes, when of known function, are involved in host interaction, virulence and housekeeping functions.[7] Genes related to intraluminal survival in the gut are pseudogenes in both *S*. Typhi and *S*. Paratyphi[58] reflecting the extra-luminal lifecycle and pathology of these bacteria.

Alongside the highly conserved, core genetic elements are distinct, virulence-associated areas termed Salmonella Pathogenicity Islands (SPIs), thought to have been acquired by relatively recent horizontal transfer of genetic material.[66] SPI-1 and SPI-2 encode type III secretion systems, consisting of needle-like complexes that allow bacteria to inject the host cell with effector proteins, hijacking cell function and moderating the immune response.[42,67] *S*. Typhi SPI-1 is almost identical to the SPI-1 of *Salmonella* Typhimurium, except for the absence of one gene, *avrA*, important in modulating the intestinal mucosal response to infection.[68] The absence of this gene in *S*. Typhi may partially account for the difference in gut pathogenesis between these two serovars.

S. Typhi SPI-7 is the largest genomic island to be identified so far in *Salmonella*, and encodes the Vi polysaccharide capsule[69] on the *viaB* locus.[52,70] The majority of *S*. Typhi genomes sequenced so far include SPI-7,[71] suggesting that it conveys a strong selective advantage. SPI-7 acquisition is thought to have occurred relatively recently, probably via a phage or conjugative transposon.[52] SPI-7 insertion causes physical imbalance in the *S*. Typhi genome, probably accounting for the relatively unusual feature of plasticity, in which DNA segments are arranged in different confirmations, to increase physical balance, and optimising genetic fitness.[72] SPI-7 instability results in its frequent loss during strain storage and passage.[69] The widespread use of vaccines based on the Vi antigen may exert a selection pressure on this unstable genomic region, whose out-selection from *S*. Typhi isolates would theoretically result in vaccine failure.[52] Vi-positive strains have almost twice

the attack rate of Vi-negative strains when used in human challenge experiments, suggesting that Vi expression considerably enhances strain virulence.[73] Vi production has an anti-inflammatory effect in *in vitro* models by masking the presence of Pathogen Associated Molecular Patterns (PAMPs) including those recognised by Toll-like receptors.[74,75] Vi expression is determined by environmental osmolarity, with down regulation in high osmolarity environments such as the gut, allowing the secretion of SPI-1 effector proteins and enhancement of the bacterial adhesion and invasion.[76]

1.4. Acute typhoid disease

1.4.1. Pathogenesis

Detailed studies of pathogenesis have been limited by *S*. Typhi being a human-restricted pathogen, with many findings extrapolated from the mouse model of typhoid disease based on *S*. Typhimurium infection, or from studies of human cell-line infection.[77]

Following transmission via the faecal-oral route, ingested organisms must survive passage through the acidic conditions of the stomach before adhering to and invading the gut mucosa.[7] Low stomach pH is an important defence mechanism, with those taking antacids or with previous gastrectomy at increased risk of typhoid disease.[7] Infection rates depend on the size of the infecting inoculum, with previous human challenge studies showing a linear relationship between the challenge dose and the attack rate.[73] Disease could not be induced by challenge with 10^3 CFU, whereas 98% of those challenged with 10^9 CFU developed illness.

Bacterial fimbriae, and perhaps other bacterial adhesins that have not been identified, are important in adherence to the gut epithelium.[58] The combination of the 12 fimbrial operons encoded on the *S*. Typhi genome is unique to *S*. Typhi, and may partly dictate disease pathogenesis.[64] The cystic fibrosis transmembrane conductance regulator (CFTR) may be an important site of attachment.[78] Cells expressing the most common CFTR

mutation translocated 86% fewer *S*. Typhi into the sub-mucosa than wild-type CFTR,[78] suggesting a protective advantage in those with CFTR mutations.

One mechanism of invasion relies on exploitation of specialised epithelial cells, termed Microfold (M) cells, overlying lymphoid-associated tissue, including Peyer's patches.[79,80] M cells perform antigenic sampling, transferring material across the epithelium to the underlying lymphoid tissue, and *S*. Typhi uses this to invade.[80] A further, separate method of invasion is by direct invasion of enterocytes, forming a vacuole, which is then released from the basal pole of the cell.[81] A further mechanism of invasion may use selective epithelial cell death by *S*. Typhi to form pores in the gut mucosa thorough which it can invade.[82]

Following gut invasion, *S*. Typhi can invade mononuclear phagocytic cells, including macrophages,[83] some of which stay in the lymphoid tissue of the small intestine and some of which drain to mesenteric lymph nodes. A brief primary bacteraemia is thought to occur that disseminates *S*. Typhi to the reticulo-endothelial system of the liver, spleen and bone marrow.[84] This primary bacteraemia was investigated in previous typhoid challenge studies in which four men were challenged with an ID^{95} dose of *S*. Typhi and then commenced 24 hours later on chloramphenicol for 7 or 28 days.[85] In one of two men given a 7 day course, typhoid disease occurred 9 days after finishing antibiotics, suggesting that *S*. Typhi must have reached a protected (intracellular) niche within 24 hours,[85] where it was shielded from the effect of antibiotic action. Further, all four men developed antibody responses identical to those of participants who had not received early antibiotics. This indirectly supported the idea of a primary, asymptomatic bacteraemia occurring, shortly after ingestion and several days prior to the onset of clinical symptoms.

S. Typhi incubation occurs within the intracellular niche of the phagocytic cells of the reticulo-endothelial system.[7] Direct culture of the buffy coat of blood has demonstrated S. Typhi within leukocytes,[86] and, correspondingly, quantitative assays have shown that the number of CFU isolated from a given volume of blood are higher when a cell lysing agent

is added to the culture.[13] The duration of the incubation phase of infection is probably determined by the virulence of the organism, the host response to infection and the number of bacteria, and can be prolonged with incubation periods of 56 days documented.[73,87] The relatively long incubation period for *S.* Typhi and the limited clonal diversity (and hence antigenic variation)[88] suggests that it occupies an immune privileged site *in vivo*.

After incubation, *S.* Typhi is released from the intracellular niche, causing a second bacteraemia accompanied by the classic symptoms of typhoid disease.[84] Blood concentrations of organisms are higher in children,[26] possibly reflecting a lack of pre-existing immunity, and in those infected with multi-drug resistant strains, suggesting increased virulence of these strains.[86] Organisms are removed by the kuppfer cells of the liver from where they are excreted in bile leading to reinvasion of the small bowel, as well as excretion of bacteria in the stool. At this stage the small bowel can become inflamed, leading to ulceration, necrosis and haemorrhage.[89,90]

Haemorrhagic necrosis of Peyer's patches in the terminal ileum is the most common serious complication of typhoid disease.[84] Necrosis and inflammation leads in turn to perforation, peritonitis and sepsis.[91-93] Acute inflammation of the gut may result from immunological priming during initial ingestion and invasion, followed by immune-mediated inflammatory damage on re-exposure to *S.* Typhi when it is excreted in the bile following the secondary bacteraemia.[91] Histological examination shows initial diffuse enterocolitis, with mucosal hypertrophy due to a macrophage-rich hyperplasia and then necrosis, usually causing a single perforation.[90,94] After illness has abated, complete recovery with no scarring occurs.[90] Histological evidence of enterocolitis can be found in the absence of any gastrointestinal symptoms, although there is a positive correlation between the severity of systemic symptoms and histological changes.[90] Whilst some studies have found an association between gut perforation and increasing duration of symptoms,[92] others have found the opposite[95] possibly due to differences in available diagnostic resources.

Perforation is more common in males than females, possibly due to higher environmental exposure and consequent immunological priming of Peyer's patches, or due to gender related differences in the inflammatory response.[95]

1.4.2. Clinical illness

Fever is the most frequent and universal symptom of typhoid disease with other frequent symptoms being malaise, chills, anorexia, diarrhoea, headache, weight loss, abdominal pain and rash.[96,97] Nausea, constipation, myalgia, arthralgia and cough are also reported.[29,98,99] Severity of symptoms is highly variable, with some patients able to continue normal activity and some requiring in-patient care.[84 99]

Specific physical signs are frequently absent, but diffuse abdominal tenderness is common, and hepatosplenomegaly and/or lymphadenopathy may also be present.[7,97] Rose spots, a transient, fine, blanching maculopapular rash, usually starting on the trunk and spreading to the arms and legs, are said to be pathognomonic for typhoid disease, but are reported with a range of frequency from as few as 3% of cases, up to 40%.[7,97] A relative bradycardia (Faget's sign) is also described.[7] *S.* Typhi disease can lead to a variety of other rarer clinical entities including meningitis, septic arthritis and osteomyelitis.[42]

Complications of typhoid disease increase in frequency with longer duration of untreated disease, with the majority of complications occurring in patients who have been unwell for three weeks or more.[7] Meta-analysis has shown an overall small bowel perforation rate of 3%, and with an associated mortality of 40%,[100] accounting for the majority of the mortality from typhoid disease.[92,93] Cerebral dysfunction, including delirium and coma, is another feared complication of severe typhoid disease, which in the absence of prompt antibiotic therapy and steroids is associated with 20% mortality.[101]

The peripheral white cell count is often low in typhoid disease[84] with frank leukopenia developing in up to one third of patients.[96,97,99] Platelet counts are also decreased, with moderate thrombocytopenia occurring in approximately one third of patients.[96] Clotting

studies may demonstrate evidence of disseminated intravascular coagulation, but this rarely manifests clinically.[102] Liver function tests are often deranged, occurring in the vast majority of patients in some series.[96,99] The most frequent abnormality is a rise in liver enzymes, with decreased albumin levels.[96] Clinically significant hepatic dysfunction is rare.[10]

The range and frequency of symptoms in children is similar to that observed in adults, with fever again being the commonest presenting feature.[103] Gastrointestinal disturbance is more likely to a manifest as diarrhoea than constipation compared to adults.[103] Although children can exhibit severe disease[104], even bacteraemic disease can be mild.[25,38] Neonatal typhoid disease is described, but is rare.[7] Manifestations include severe sepsis neonatorum and chronic faecal carriage.[105-107]

Typhoid disease in pregnancy led to abortion or premature labour in 60% to 80% of cases and maternal mortality in 15% in the pre-antibiotic era. With appropriate antibiotic treatment, however, typhoid disease is no more severe than in non-pregnant patients.[108] Indeed, antibiotic treated typhoid disease in pregnancy may be less severe than in non-pregnant patients; a review of 80 pregnant women documented a lower complication rate in pregnancy compared to non-pregnant controls (8% vs. 23% respectively).[109] This difference may be due to a different immunological response to typhoid disease in pregnancy, in which skewing of the immune system towards T-helper 2 pattern is seen to prevent foetal rejection.[109]

The mortality rate from typhoid disease varies widely depending on the population studied[7], and has decreased substantially since the discovery of effective antibiotics.[110] Infants may have a higher mortality than older children, with mortality rates in hospitalised infants up to 4 times that of older children.[104,111] Infection with multi-drug resistant strains may also increase mortality.[104]

Relapse of typhoid disease can occur after a significant asymptomatic period, occurring an average of 22 days (range 8 to 40 days) after the resolution of the first episode.[112] Relapse rates in untreated typhoid disease are around 10%[73] and may be strain variable.[113] Some

apparent relapses may be reinfection, although molecular typing has confirmed that 90% of relapses are due to the same strain as the initial infection.[112] High rates of 15% to 20% are seen after chloramphenicol treatment, suggesting that antibiotics interfere with the development of protective immunity.[114] Newer antibiotics are associated with lower relapse rates, with relapse reported in 5% and 1.5% of those treated with ceftriaxone and fluoroquinolones respectively.[112] Relapses are usually of milder severity and respond more rapidly to antibiotics compared to the index illness.[112,114]

1.5. Chronic carriage of *S.* Typhi

In 1902, Koch postulated that healthy people could carry disease causing organisms, serving as reservoirs of infection to bridge the gap between disease outbreaks.[115] Ongoing excretion of *S*. Typhi in the stool of asymptomatic individuals following disease resolution was shown shortly afterwards.[115] It was later apparent that prolonged excretion of *S*. Typhi in stool could occur in people without a history of illness, with chronic carriers emotively described as "*living storehouses and factories of disease*".[116]

The role of chronic carriers in disease transmission was famously illustrated by Mary Mallon (Typhoid Mary) in the early part of the 20th century.[115] Working as a cook, Mary Mallon infected between 26 and 54 people before being requested by public health officials to find alternative employment.[117] She refused, and was eventually incarcerated until her death, in order to protect public health. In the absence of treatment, incarceration of chronic carriers continued thorough out the 20th century, including in the UK, where it recently emerged that 43 female typhoid carriers had been placed in an asylum from 1907 to the asylum's closure in 1992.[118] Outbreaks resulting from a single carrier can be significantly larger than was documented for Mary Mallon. In the UK for example, 207 cases over 13 years were eventually traced to a cowman and milker referred to as Mr N.[115] Chronic carriers of *S*. Typhi remain a source of domestically acquired typhoid disease in resource-rich settings.[119]

Up to 10% of untreated patients with acute typhoid disease excrete *S*. Typhi in their stools for three months after infection;[7] 1 to 4% will continue to excrete *S*. Typhi beyond this and are considered to be 'chronic carriers'.[10] Faecal bacterial loads in chronic carriers are high, with between 10^6 and 10^{10} organisms per gram of faeces.[120] Chronic carriers who work as food handlers are at increased risk of transmitting the disease, as illustrated in the historical cases of Mary Mallon and Mr N. Indeed, during Mr N's employment as a sailor there was no evidence of secondary spread.[115]

Gallstones are the principal risk factor for developing chronic carriage,[121] with gallstones detected in approximately 90% of chronic carriers, compared to 10% of those who do not become chronic carriers after acute infection.[122] Gallstones allow attachment of the bacteria and biofilm formation,[123] which in turn protects the bacteria from the antimicrobial and emulsifying action of bile[124]. Exposure of *S*. Typhi to bile increased production of the 'O' lipopolysaccharide capsule, which in turn forms the extracellular matrix essential to biofilm formation.[123] Biofilm formation has been demonstrated on gallstones obtained following cholecystectomy[125], and on gallstones inoculated ex-vivo with *S*. Typhi.[123] This chronic persistent infection is not a benign process, with biliary cancer occurring as a sequela to chronic carriage in some people.[126,127]

Detecting typhoid carriers is difficult. Approximately a quarter of them have no history of typhoid infection[7], and carriage can persist asymptomatically for decades.[128,129] Carriers can be identified by screening of stool specimens, but this can be labour intensive.[130] Carriers usually have high levels of anti-Vi antibodies, and this has been used as a method of detection in an outbreak setting[131] but is of limited use in endemic regions where chronic exposure leads to a high levels of anti-Vi antibodies in the general population.[132] The wide spread use of Vi-vaccines in the future will further limit the use of Vi antibody detection of chronic carriers.

Treatment strategies for chronic *S*. Typhi carriage are limited. Intensive anti-microbial therapy can cure some individuals, with high-dose ampicillin for 28 days having been

shown to cure five out of six chronic carriers in whom it was trialled.[133] Increasing the duration of treatment to 90 days did not improve the cure rates.[134] Successful eradication of chronic infection with ciprofloxacin has also been reported.[135] However, antibiotic therapy alone does not cure in all patients, likely due to biofilm formation by *S*. Typhi on gallstones preventing antibiotic penetration.[125] Cholecystectomy is more effective for patients with gallstones, achieving cure in approximately 75%.[125] Combining surgery and antibiotics improves the outcome further,[136] but carriage still persists post-operatively in some patients[128,137] and is prohibitively costly in many typhoid endemic regions. Ideally those infected with *S*. Typhi should be excluded from food handling and care of vulnerable groups in order to break transmission.[4,138]

1.6. Diagnosis of typhoid disease

Prompt diagnosis and treatment of typhoid infection decreases disease complications and limits the opportunity for disease spread.[7] However, achieving a prompt and reliable diagnosis is difficult. Typhoid disease cannot be reliably distinguished on clinical grounds from other diseases common in endemic regions, including malaria, tuberculosis, dengue fever and brucellosis.[7] Diagnostic tests that are both sensitive and specific are lacking, especially in endemic regions, where laboratory facilities are frequently limited.[139] New, reliable diagnostic tests are needed to prevent delays in treatment, minimise morbidity and mortality, prevent inappropriate use of antimicrobials and provide reliable epidemiological data.

The discovery by Widal in the late 19th century that sera from patients with typhoid disease agglutinated typhoid bacilli formed the basis of the Widal test, which has been used for the diagnosis of typhoid disease ever since.[140] The current Widal test detects the titre of agglutinating antibodies to the O (lipopolysaccharide) and H (flagella) antigens by serial dilution.[141,142] The demonstration of a four-fold rise in antibody titre between acute and convalescent samples is considered diagnostic.[142] However, the Widal test and other

serological tests are limited by the high residual background levels from previous exposure in endemic regions, and cross reactivity with other antigens,[142-148] paired acute and convalescent samples are frequently not available. Furthermore, serological tests do not distinguish *S.* Typhi and *S.* Paratyphi infection. Newer generation serological tests, based on detection of antibody response to typhoid antigens in urine,[149,150] serum[143,151-157] have been developed, but have failed to overcome the limitations of the Widal assay in field testing.[158-160]

Culture of *S.* Typhi or *S.* Paratyphi from blood or bone marrow is diagnostic, and allows antibiotic sensitivity testing of the strain, but is not achieved in all patients.[13] Sensitivity is variably reported between 30% and 90%[161-163] and several days of incubation may be needed before a positive result is obtained. The bacterial load in blood during acute typhoid disease is relatively low, with a median count of one colony-forming unit per ml of blood, limiting sensitivity.[86] Increasing the volume of blood cultured may increase sensitivity.[86] Bacterial load decreases with increasing duration of illness[86,162] reducing sensitivity further when presentation is delayed.[162] Sensitivity of blood culture may be improved by the use of bile containing media,[164] which inhibits the growth of skin contaminants and results in blood cell lysis, releasing intracellular bacteria and providing haem as a growth medium. However, bile media also inhibits many pathological bacteria limiting its use as a general isolation media.[13] More recently the use of another blood-lysing agent, digitonin, has been shown to be as effective as ox-bile media, whilst allowing the growth of other pathological bacteria,[13] and may play a role in the future. Sensitivity of blood culture is limited further by prior use of antimicrobial agents, a frequent occurrence in many resource poor countries.[22]

Culture of bone marrow is more sensitive than that of blood, and is the gold standard diagnostic test for typhoid disease.[48] Sensitivity rates of 90% are reported, even with prior antimicrobial administration.[161,163] Simultaneous culture of both blood and bone marrow has shown that blood culture detects only two-thirds of *S.* Typhi infections diagnosed by bone marrow culture.[162] The higher sensitivity of bone marrow compared to blood is

probably a proxy of the significantly higher concentrations of bacteria in the bone marrow during acute disease.[13] Furthermore, the bacterial counts in bone marrow do not decrease with increasing duration of illness, and counts are only minimally affected by prior use of antibiotics.[165] The invasive nature of bone marrow aspiration limits bone marrow culture in clinical practice[4,10] but can be useful if repeat blood cultures are negative or if previous antibiotic therapy has been used.

Duodenal string culture devices serve as a method for sampling bile-stained duodenal fluid and can be useful in achieving a bacteriological diagnosis.[166] Although application is limited by the poor tolerability of this method in ill patients[167] and young children,[163] the addition of duodenal string cultures to blood culture and stool culture can improved overall diagnostic sensitivity.[167]

Detection of *S*. Typhi in stool demonstrates exposure, and as such is a useful adjuvant to the diagnosis of acute typhoid disease. Excretion of *S*. Typhi in stool can be demonstrated in 45% to 65% of those with acute typhoid disease with the use of 2 or more samples of suitable volume.[13,163] Yields may be higher in children compared to adults.[163] Patients with higher levels of bacteria in their blood are also more likely to excrete *S*. Typhi in their stools.[86] Selenite enrichment media aids the isolation of *Salmonella* species by inhibiting the growth of other bowel organisms.[168] Pick-up rates of up to 65% have been shown in children during acute disease,[163] possibly due to the use of rectal swab enhancing isolation, or due to high excretion rates in children.

Isolation of *S*. Typhi from clinical samples must also be reported to those responsible for public health as outlined in the Public Health (Control of Disease) Act 1984.[169] It has been a requirement to report *S*. Typhi disease in London from 1891, and in the rest of the England and Wales from 1899.[169] Originally, responsibility for reporting lay with the head of the household or the landlord, but it is now a medical practitioner's responsibility. Microbiologists must consider these wider implications when *S*. Typhi is isolated.

An alternative approach to the diagnosis of typhoid disease is the use of PCR to detect microbial DNA. Theoretically, methods detecting DNA should be specific, applicable to many different samples, and not be affected by viability of the organism. However, the low concentrations of microbial DNA in clinical samples has proved limiting. The amount of mammalian DNA considerably outweighs the amount of bacterial DNA, potentially leading to false-positive results from non-specific binding, as has been shown with assays based on the detection of 16s rDNA from microbes.[170] The volume of blood available from patients is often small, especially among children. To overcome these problems, a novel approach was used by Zhou *et al.*[171,172] A short period of culture in ox-bile, previously shown to be the optimal culture media for *S*. Typhi,[173] was coupled with a PCR, targeting the flagellin gene,[171] previously shown to highly specific for *S*. Typhi.[174] At concentrations up to 2.4%, the bile-containing media facilitates sustained growth of *S*. Typhi whilst lysing blood cells, thereby theoretically allowing liberation of DNA from these intracellular organisms and inhibiting any bactericidal action of blood. After a minimum of 3 hours incubation this approach detected DNA from 4 ml of blood spiked with 3 CFU of *S*. Typhi. This showed that this combined approach was rapid but sensitive, and offer hope that this method can be used for reliable diagnosis in patient samples. The use of this method in controlled human typhoid disease model should provide further evidence supporting the use of this novel diagnostic method.

1.7. Treatment of typhoid disease

Effective antibiotic treatment of for typhoid disease with Chloramphenicol was first described in 1948.[175] Chloramphenicol led to a rapid resolution of clinical typhoid disease,[176] and was shown to decrease mortality from 26% in the pre-antibiotic era to 5% in a retrospective analysis of patients in Indonesia.[110] Chloramphenicol also decreased complication rates and time to fever clearance.[110]

S. Typhi resistance to chloramphenicol was described just two years after its role in the treatment of typhoid disease was reported.[177] By 1975, 80% of isolates from Vietnam were resistant;[178] resistance was mediated by high molecular weight, self-transferable plasmids.[7] Although other antibiotics, including co-trimoxazole,[179] ampicillin and amoxicillin were also found to be effective against both susceptible and chloramphenicol resistant *S*. Typhi,[180] multidrug resistant *S*. Typhi isolates, defined as strains resistant to all these first line antibiotics[181] were reported in South Asia in 1980, and became widespread by the mid-1990s.[104,182] The emergence of multidrug resistant *S*. Typhi has been a major setback in the control of typhoid disease, and presents the real threat that untreatable typhoid disease will re-emerge.[7] Reassuringly, data from Nepal,[183] India[184] and Vietnam[23] suggest that the prevalence of multi-drug resistant strains may be decreasing.

The prevalence of multidrug resistant strains is geographically variable.[8,185] For example, the prevalence of multidrug resistant strains in community isolates was 7% in India, 22% in Vietnam and 65% from Pakistan, whilst strains from Indonesia and China showed no multidrug resistance.[8] Although resistance rarely emerges during treatment of the individual patient,[186] clonal dissemination of individual multidrug resistant strains[112,187] as well as transfer of plasmids encoding the resistance genes[177] facilitates their geographical spread. In the absence of global surveillance for drug resistant *S*. Typhi, the true worldwide burden and distribution of antimicrobial resistance is unknown,[188] and therefore selection of antibiotic treatment in the returning traveller is difficult.[185,188]

1.7.1. Quinolone treatment

Fluoroquinolone antibiotics (principally ciprofloxacin and ofloxacin) are the first choice in the treatment of multi-drug resistant *S*. Typhi disease.[48] Fluoroquinolones are concentrated in bile, urine, macrophages and the bowel, act intra-cellularly and have oral bioavailability equivalent to that achieved from intravenous administration, making them extremely useful in the treatment of typhoid disease.[189] Their efficacy in the treatment of multi-drug resistant

strains has been established in randomised trials.[190,191] Pooled data shows an overall clinical cure rate of 97.9%, microbiological cure rate of 99.6%, a mean fever clearance time of 3.9 days.[7]

Although fluoroquinolones are not licenced for use in children because of concerns regarding toxicity to developing joints,[192] off-licence use in cases of clinical need has provided data supporting their safety in children.[193,194] Direct evidence for the safe use of fluoroquinolones to treat typhoid disease in children was provided by a study of 326 Vietnamese children aged 1 and 14 years, followed up for growth for 2 years after treatment, which showed comparable growth to age matched controls and no evidence of joint toxicity.[195] Short course fluoroquinolone treatment which limits potential toxicity, have been shown to be effective.[196-198]

The first case of illness caused by a *S*. Typhi with reduced sensitivity to fluoroquinolones was reported in 1992.[199] These strains have since been associated with slower resolution of clinical symptoms, longer time to defeverescence, the need for higher dose therapy and more prolonged therapy and treatment failure.[200,201] Since intermediate resistant strains were first reported, their worldwide prevalence has rapidly increased. For example, isolates from Vietnam showed an increase in intermediate susceptibility prevalence from 4% in 1993 to 76% in 1998,[200] and a large survey of isolates in India showed that between 1998 and 2001 the proportion of intermediate resistant isolates rose from 2% to 40%.[202] In a twelve month period in the United States, 9% of *S*. Typhi strains acquired abroad had decreased ciprofloxacin susceptibility, and in keeping with data from endemic countries, these patients had a longer time to fever clearance and experienced more treatment failures.[203] These data highlight the importance in strain isolation and antimicrobial sensitivity testing where possible. The prevalence of such strains, especially in Asia, suggests that ciprofloxacin is no longer a reliable empirical therapy for typhoid disease.[185] Reduced sensitivity strains are of a single clone, haplotype H58,[204] which appears to have spread worldwide from areas of high anti-microbial usage via clonal expansion.[182]

Detecting reduced fluoroquinolone susceptibility by disc-diffusion methods is unreliable. Decreased susceptibility to nalidixic acid may be a proxy for resistance,[205] but The British Society of Antimicrobial Chemotherapy recommends formal testing of the minimum inhibitory concentration for ciprofloxacin[206] as strains showing full susceptibility to nalidixic acid can have decreased ciprofloxacin sensitivity.[205] Isolates with a minimum inhibitory concentration for ciprofloxacin between 0.125 and 1 milligram per litre are deemed to have decreased ciprofloxacin susceptibility.[206] Strains of *S*. Typhi that are fully resistant to ciprofloxacin (minimum inhibitory concentration > 2.0 milligram per litre) have also now been reported.[207,208]

Fluoroquinolone resistant strains remain susceptible to ceftriaxone and azithromycin.[185,200] Ceftriaxone achieves high cure rates,[209] and is as effective as chloramphenicol in randomised trials when given for 3[210,211] or 7 days.[212] Resistance to ceftriaxone has been reported, but is sporadic.[213] However, third generation cephalosporins are inferior to fluoroquinolone treatment and require parenteral administration.[196,214] Azithromycin exhibits good intracellular penetration and a long elimination half-life of 72 hours, allowing once daily dosing and relatively short courses of treatment.[215] Azithromycin is more potent than the first line agents against *Salmonella* species when tested *in vitro*.[216,217] Clinical response to azithromycin is good, and may be preferential to that seen with fluoroquinolones and ceftriaxone.[215] However, azithromycin is expensive, limiting its usefulness in endemic countries,[218] and community trials have not been conducted.

1.8. Prevention of typhoid disease

1.8.1. Sanitation and clean water

Humans are the only host and reservoir for *S*. Typhi.[84] A lack of basic hygiene and sanitation infrastructure facilitates the spread of *S*. Typhi by the faecal–oral route.[219] Transmission is almost always via contaminated water and food, with direct human-to-human transmission only rarely reported.[84,220] Chronic problems with water and sanitation infrastructure lead to endemic disease,[221] whilst acute disruption to water supply[181] and faecal contamination of food[222,223] can cause disease outbreaks and epidemics. The importance of clean water in the prevention of endemic typhoid disease has been recognised since the end of 19th Century.[224] During the early part of the 20th century, resource-rich countries saw a marked decline in the incidence of typhoid disease following on from the routine provision of sanitation and clean water.[181,225]

The dramatic effect that the loss of clean water supplies can have on disease rates was illustrated in the typhoid disease epidemic in Tajikistan between 1996-7, when a period of civil unrest led to a marked decline in standards of water chlorination.[226] In a six month period 1% of the capital city's population were affected by typhoid disease.[226]

Direct evidence for the effect of poor sanitation infrastructure in countries with endemic typhoid disease has been provided by case-control studies of risk factors. A lack of a household toilet has been identified as a risk factor in Indonesia (odds ratio (OR) of 2.2)[227] and the use of a latrine identified as protective in Bangladesh (OR 0.1).[228] Drinking untreated water has been shown to be a risk factor for disease in Vietnam (OR 3.9),[229] 3.0 in Uzbekistan (OR 3.0),[230] Bangladesh (OR 12.1).[228] However, several studies have failed to identify contamination of the household water supply as a risk factor for disease acquisition, despite high levels of contamination in both case and control households.[79,102,242] This may be due to the relatively low numbers of *S*. Typhi ingested when contaminated water is the vehicle of transmission compared to that ingested with contaminated food,[223] as well as the ability to boil water in one's own home.[35,227,231]

Seasonal variation in the supply of clean water is thought to account for observed fluctuations in disease rates.[4,27] In Pakistan, Indonesia and Vietnam, for example, the disease rates peak during the dry season when the quality of water deteriorates due to a lack of fresh supplies.[27,38,232] In other regions, typhoid incidence is reported to increase in the rainy season,[26,233] due to high flow of water overwhelming water sanitation systems that separate sewage from clean water.[233]

Transmission of *S*. Typhi via contaminated food is also an important source of disease. Particular food groups such as ice cream[231,234] and raw vegetables,[235] are associated with increased risk of typhoid disease, and food prepared outside the home is also more likely to be contaminated than food prepared in the home.[231,234,235] These findings reflect the difficulty in preparing clean food in the absence of running water.

1.8.2. Typhoid disease in resource-rich countries

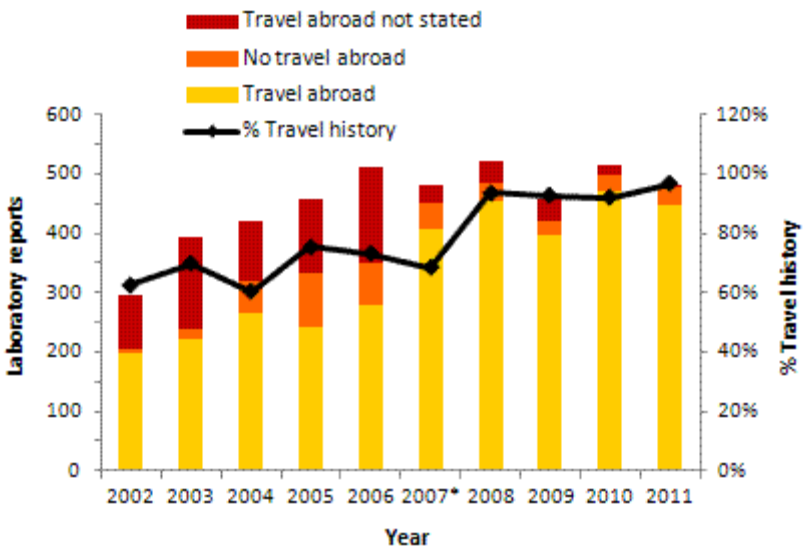
Within resource-rich countries, the percentage of cases of typhoid disease occurring in individuals with a preceding travel history is rising decade on decade,[188,236] although the actual rates of cases per number of travellers has decreased.[237] In laboratory-based surveillance in the United States, 81% of isolates were recovered from patients with a travel history, especially those who had visited Asia.[188] A separate review of cases reported in the period 1994 to 1999 in the United States attributed 74% to travel, of which 53% were acquired in the Indian subcontinent.[238]

In the UK, the number of reported cases of enteric disease rose since the mid-1990s[239] but appears to have stabilised (Figure 2).[119] In 2011 there was a total of 493 cases reported, of which 261 were due to *S.* Typhi.[119]

Figure 2: Travel history of laboratory confirmed cases of enteric fever due to *S.* Typhi and *S.* Paratyphi in the UK between 2002 and 2011.

Source: Health Protection Agency;

http://www.hpa.org.uk/web/HPAweb&HPAwebStandard/HPAweb_C/1259152344471



The vast majority of cases are travel related cases (92% in 2011), with individuals returning from visiting friends and relatives (VFRs) in endemic countries accounting for at least 80% of cases;[119] this demographic is also the least likely to have taken pre-travel advice.[239]

Vaccination against typhoid disease is recommended for most travellers over 18 months of age who are travelling from the UK to countries in Africa, South Asia, the Middle East, Central and South America and the Caribbean.[240,241] Efficacy studies for typhoid vaccines in the prevention of travel associated disease have not been conducted, but only a very small number of patients with travel-related typhoid disease have received a vaccine prior to travel[236,238] suggesting a failure to vaccinate rather than a failure of the vaccines. For example, of the 294 cases of enteric fever occurring in the UK in 2006-2007, only 54 had received a typhoid vaccine in the last 3 years, in whom a total of 12 cases of *S*. Typhi disease occurred (42 had *S*. Paratyphi disease).[239] The majority of travel associated typhoid disease occurs in relatively young travellers, with students being the most frequently reported occupational group[236,238] and a median age of 27 years for UK reported cases in 2011.[119] Young people may have limited contact with health care providers, limiting the opportunities for public health advice. There is a relatively high incidence of cases in travellers under two years of age, accounting for 2.6% of the 2011 reported cases in the UK,[119] and 7% of cases in one series.[238] This emphasises the need to develop a vaccine suitable for this vulnerable population. The impact of recent concerns over the quality of some Vi polysaccharide vaccines used in the UK between early 2011 and their withdrawal in October 2012[242] is not known, although reassuringly there was no increase in cases in 2011 compared to 2010.[119] The effect of the current limitations of typhoid vaccine supply[243] is not known.

The majority of domestically acquired typhoid disease result from contact with an infected traveller, with small numbers of cases due to contact with chronic carriers.[119] Small outbreaks of typhoid disease can follow transmission from contaminated food.[236,238] Diagnostic delay may increase mortality from domestically acquired disease compared to travel-related disease,[237] although this is not consistently reported.[236] Clinical microbiologists may also be at increased risk due to laboratory exposure,[49,238] as may general medical personnel caring for those with typhoid disease.[236] For some domestically acquired disease, a potential source cannot be identified.[119]

1.9. The immune response to *S.* Typhi

The immune response to typhoid disease is complicated and incompletely understood.[42] How protection is mediated is not known, and no correlates of protection exist for typhoid vaccines.

To establish systemic infection, *S.* Typhi must survive passage through to the small bowel, invade across the gut wall and disseminate throughout the body. In doing so, the bacterium must survive numerous encounters with the immune system, and indeed, appears to be particularly adept at doing so. Natural immunity after disease is minimal; rechallenge with 10^5 CFU of *S.* Typhi in 22 participants with previous typhoid disease gave an attack rate of 23%, only marginally less than the 30% observed in typhoid naïve participants challenged concurrently with the same dose of *S.* Typhi.[244] This concurs with an epidemiological study of two separate typhoid outbreaks, in which the attack rate was 20.4% in men with a previous history of typhoid disease and 31.2% in men only exposed during the second outbreak.[245] The poor immunity that results from previous *S.* Typhi infection suggests that the organism is able to subvert the normal immune response. Correspondingly, genomic sequencing has shown very limited variation even in the actively secreted proteins of *S.* Typhi,[88] implying a lack of immune driven selection. The description of asymptomatic *S.* Typhi bacteraemia also suggests that an immune driven inflammatory response to infection

is not universal.[246] However, a detailed understanding of the interaction between *S*. Typhi and the immune system has been held back by the absence of a suitable animal model of typhoid disease. Evidence has been mainly extrapolated from *S*. Typhimurium infection in mice, which results in a typhoid-like disease.[42] However *S*. Typhimurium differs from *S*. Typhi not only in its virulence factor and antigen expression,[42] but also in the disease that it causes in humans,[247] intrinsically limiting the applicability of the model. The recent development of a humanised mouse model of typhoid disease is likely to allow a more accurate profile of the immune response[248,249] in the future.

1.9.1. B cell immunity in salmonellosis

A characteristic feature of *Salmonella* bacteria is their ability to establish intracellular infection, including within cells of the immune system[250] where the bacteria can avoid destruction by antibody and complement. Significant areas of the *Salmonella* genome are dedicated to the invasion and immune-modulation of target cells[251], and *Salmonella* that are unable to invade and survive intra-cellularly have been shown to be avirulent in mice.[252] Clearance of this intra-cellular infection is likely to occur via T cell mediated immunity[253], but evidence suggests that B cells and antibody also play an important role in protecting against and controlling *Salmonella* infection. Immunised B cell deficient mice are more susceptible to *S*. Typhimurium infection than immunised wild type mice.[254,255] Passive transfer of antibody to B cell deficient mice has been shown to be protective by some[256] but not all[254] studies. Mice deficient in B cell development cannot control *Salmonella* replication, but this susceptibility has been shown to be at least partially reversed by the passive transfer of antibody in some studies.[257] However, the role of B cells in *Salmonella* infection must extend beyond that of antibody production as B cell deficient mice who are passively immunised with immune serum still succumb to *Salmonella* infection at a greater rate than B cell competent mice, and mice with B cells, but who lacking secreted or class switched antibody, were not significantly more prone to infection compared to wild type

mice.[258] Instead, B cells are thought to be a critical adjuvant to the T-cell response.[258-260] B cell receptor activation by *Salmonella* may result in bacteria phagocytosis, which rather than causing apoptosis, results in antibody production, as well as MHC class II presentation and CD4+ T cell activation.[261] B cells that have internalised *Salmonella* have also been shown to cross present antigen via MHC class I, activating cytotoxic T cells.[262] Clearly, protection against and control of *Salmonella* infection requires a complicated interplay between both B and T cells.

Direct evidence for the role of antibody in providing protection against *S*. Typhi infection is afforded by the efficacy of the Vi polysaccharide vaccine. This vaccine induces anti-Vi antibodies alone, and is at least moderately effective at preventing typhoid disease.[263-265] The Vi capsule shields the bacteria from the immune system, preventing opsonisation of LPS via complement-3 binding.[266] The Vi capsule also facilitates mucosal invasion by inhibiting Toll-like receptor (TLR) signalling, preventing induction of innate immune cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL) IL-8 and IL-6.[74] Anti-Vi antibody induced by vaccination is thought to work either by opsonisation and induction of Fc receptor mediated phagocytosis and/or serum killing during an extracellular phase.[42] However, protective immunity must also be mediated by other factors, as the Ty21a vaccine, which lacks the Vi antigen, also provides moderate protection.[267]

The interaction of *S*. Typhi with B cells and antibody is likely to occur at different points in the infection cycle. The first encounter with antibody occurs following ingestion, when *S*. Typhi must evade not only killing by gastric acid, digestive enzymes, and bile salts, but also secretory IgA.[268] Invasion across the gut wall brings *S*. Typhi into contact with underlying immune cells, including B cells.[80] Invasion is thought to occur principally via the microfold (M) cells overlying Peyer's patches.[79,269] M cells contribute to immune surveillance by sampling and transporting antigens from the gut lumen across the epithelium to the underlying immune cells, a mechanism that *S*. Typhi exploits to allow invasion.[80] *S*. Typhi may also invade via phagocytosis by dendritic cells, which reach by a paracellular route into

the gut epithelium and pull the bacteria into the tissues.[270] Mouse models using oral challenge with *Salmonella* have suggested that B cells play a significant role in protection at this point in the infection cycle.[255] The oral challenge dose required to induce disease in mice is significantly higher than the intravenous dose, implying that significant bacterial clearance occurs after oral challenge.[255] However knockout mice with genetic deletion ($Ig\mu^{-/-}$) that causes failure of B cell maturation and hence antibody production, are considerably more susceptible to oral infection than wild type mice[255], suggesting that this clearance of bacteria is B cell dependent. Similarly, mice that have the polymeric immunoglobulin receptor knocked out and therefore unable to bind and transport IgA and IgM to the mucosa have increased susceptibility to infection.[271] Despite this immune encounter immediately upon invasion, *S.* Typhi infection does not result in local inflammation, polymorphonuclear cell influx and diarrhoea typical of other *Salmonella* infections, but silently invades and disseminates.[42]

Following invasion, *S.* Typhi is thought to disseminate through the reticulo-endothelial system. This is likely to occur principally in endosomal compartments within macrophages,[250,272,273] but may also involve extracellular bacteria that spread through efferent lymphatics and in blood.[42] Extracellular bacteria at this point will be vulnerable to antibody mediated killing.[42,274]

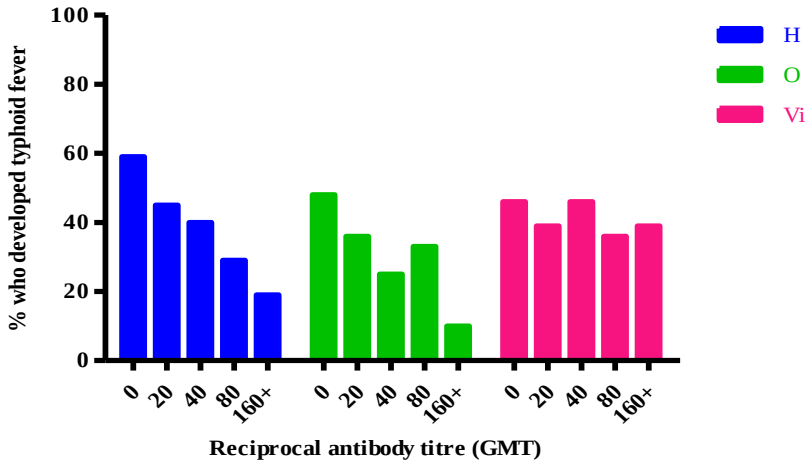
Having disseminated systemically, a period of replication occurs, principally in the intracellular haven of infected tissues.[42] Continuous spread from infected foci to establish new sites of infection is one of the hallmarks of *Salmonella* infection, occurring as a 'hit and run' process to allow the bacteria to stay ahead of the immune system.[275] One mechanism of spread is thought to be macrophage cell lysis,[276] which releases bacteria into the extracellular space, where it is once again exposed to B cells and antibody,[275] possibly preventing cell to cell transmission of *S.* Typhi.[277]

1.9.2. The antibody response to *S.* Typhi surface expressed antigens in humans

Antibody responses to surface expressed O and H antigens of S. Typhi during acute typhoid disease occurs in the majority of patients, and forms the basis of the Widal test,[141,142] with a four-fold rise in titre between acute and convalescent samples considered diagnostic.[142] It is recognised however that an antibody rise does not always occur in typhoid fever, even in blood culture confirmed cases.[278] This may be due to obtaining an acute phase sample late on in the course of illness, antibiotics attenuation of the rise or high background titres masking a rise.[278] Human challenge studies provide a unique opportunity to provide a detailed kinetic profile of the immune profile in an immunologically naïve population, where these confounding variables can be controlled.

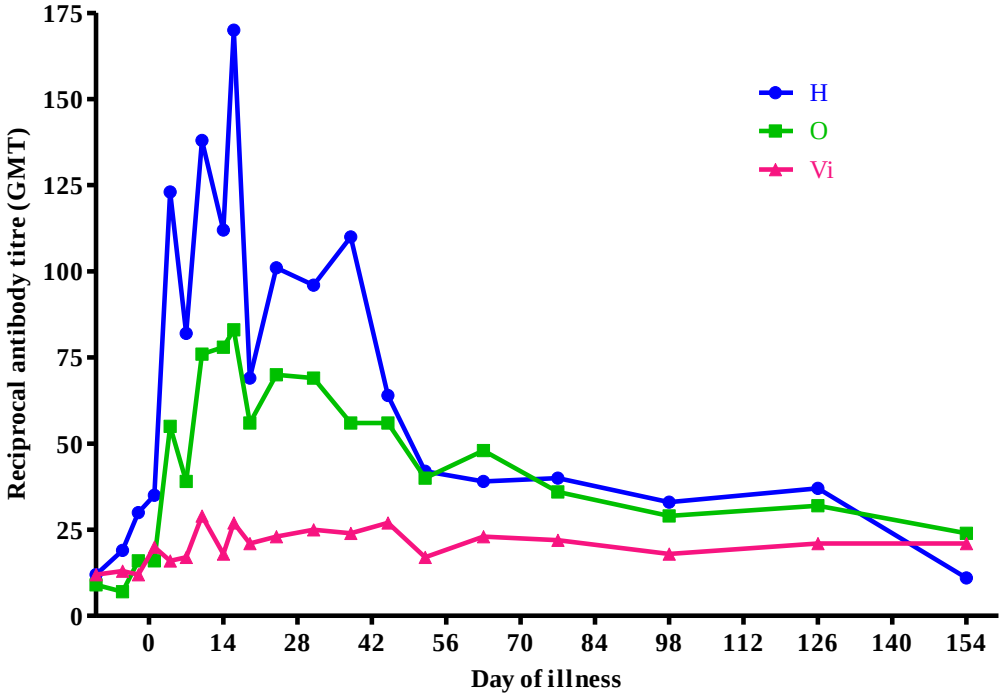
Total antibody responses to the O, H and Vi antigens were studied in the previous human challenge models of typhoid disease, conducted at the University of Maryland.[87] The percentage of participants who developed typhoid fever following challenge, shown by their baseline antibody titre is shown in Figure 3. Participants frequently had pre-existing antibody, with baseline anti-H antibody levels, and to a lesser extent, anti-O antibody correlating with subsequent protection against typhoid disease, shown by the lower percentage of participants developing typhoid disease with progressive rise in titres.[85,87,279] The role of H antibody in protection was supported by the correlation between H antibody responses and efficacy in field trials of killed whole-cell vaccines.[84] Correspondingly, the trial of a vaccine prepared from a strain that lacked the H antigen failed to convey protection.[280] There was no correlation between Vi antibodies and protection.[87]

Figure 3: The percentage of participants who developed typhoid fever following challenge with *S.* Typhi, according to their baseline reciprocal total antibody titres to the H, O and Vi antigens, in previous human challenge studies conducted at the University of Maryland. Reproduced from 'Induced typhoid fever and experimental typhoid vaccines – a study of 1886 volunteers' [87]



The kinetics of the antibody response in those who developed typhoid fever following challenge is shown in Figure 4.

Figure 4: The kinetics of the total antibody response to the H, O and Vi antigens by day of illness in participants who developed typhoid fever following challenge with *S.* Typhi in previous human challenge studies. Reproduced from 'Induced typhoid fever and experimental typhoid vaccines – a study of 1886 volunteers[87]



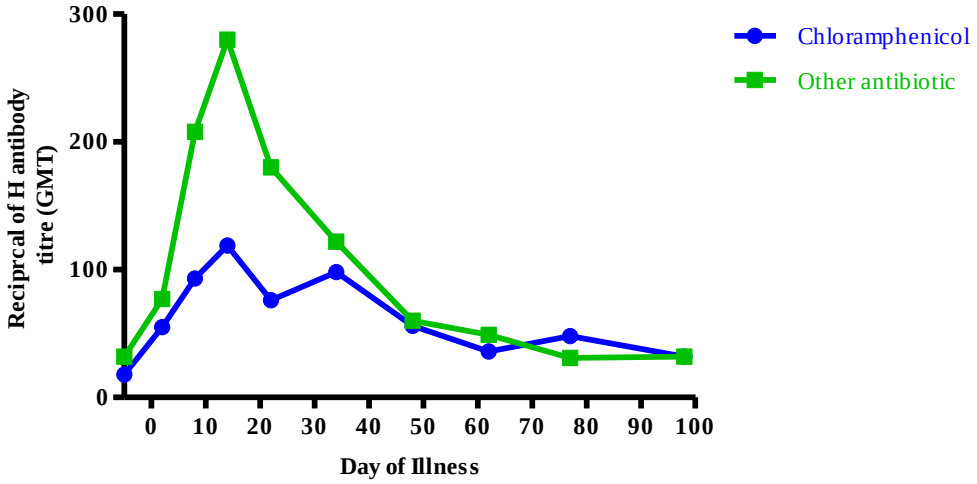
Anti-O and anti-H antibodies rose early in the incubation period, while the Vi antibodies rose later.[281] The response to the Vi antigen was least marked. Antibody levels declined after the third week of disease returning to the baseline after 14 to 22 weeks. This response was not universal however, with 9% of those diagnosed with typhoid fever exhibiting no rise in any of the 3 antibodies.[87] Table 1 shows the percentage of participants in whom a fourfold rise over baseline antibody concentration was seen following challenge. It can be seen that antibody response were not restricted to those with typhoid fever, with significant rises in antibody, seen in both those with illness and without, most frequently with anti-H antibody.[87]

Table 1: The percentage of participants with a fourfold rise in total antibody concentrations to the H, O and Vi antigens following challenge with 10^5 CFU of *S.* Typhi, grouped by those who developed typhoid fever of sufficient severity to warrant antibiotic treatment, and those that did not develop typhoid fever. Reproduced from 'Induced typhoid fever and experimental typhoid vaccines – a study of 1886 volunteers'[87]

	Per cent with ≥ 4-fold rise in antibody titre over baseline		
Antibody	**Typhoid fever requiring treatment**	**No typhoid fever**	**Total***
H	73	24	43
O	74	11	38
Vi	40	22	30
*excludes those developing illness that were not treated (data not available)			

The effect of chloramphenicol treatment on the antibody response is shown in Figure 5. Chloramphenicol therapy attenuated the antibody response, mostly notably to the H antigen, and to a lesser extent to the LPS antigen, but does not affect anti-Vi responses. This is notable because chloramphenicol treatment was also associated with increased rates of illness relapse, compared to that observed in untreated typhoid disease, suggesting that chloramphenicol treatment led to a suppression of the immune response that predisposed to subsequent relapse.

Figure 5: The kinetics of the total antibody response to the H antigen in participants with typhoid fever treated with chloramphenicol or other antibiotics (principally amoxicillin) following challenge with *S.* Typhi. Reproduced from 'Induced typhoid fever and experimental typhoid vaccines – a study of 1886 volunteers[87]



These data support the idea that there is a significant B cell response to *S.* Typhi. To progress the understanding of immunity in typhoid disease more detailed data from human studies is needed.

1.10. Current vaccination strategies

Despite a poor understanding of typhoid immunity, three typhoid vaccines have been licensed to-date: a killed whole cell vaccine, a live attenuated oral vaccine (Ty21a) and a Vi polysaccharide parenteral vaccine. All have their limitations, and new vaccines are needed to overcome these limitations.

1.10.1. Killed whole cell vaccines

Vaccines designed to confer protection against typhoid disease were developed as early as the end of the 19th century.[1,282] Early vaccines were killed whole-cell vaccines, inactivated by heat and phenol or acetone. Heat inactivated typhoid vaccines were studied in several army units, but a statistical review by Pearson in 1904 based on data from soldiers in India and from those immunised in the South African war concluded that studies of these vaccines

were inadequate and evidence of efficacy was lacking.[283] Despite this, heat inactivated typhoid vaccines were routinely used by the British army for many years.[282] An outbreak of typhoid disease in 1946 provided the first data supporting the efficacy of the killed whole cell vaccine, with an attack rate of 12.1% in unimmunised individuals compared to 0.5% in those who had been immunised as part of the military.[284]

The true efficacy of the killed-whole cell vaccines was first prospectively investigated in a series of WHO sponsored randomised controlled trials in endemic regions in the 1950's and 1960's.[285-294] The first of these trials was conducted in 1953 by the Yugoslav Typhoid Commission, and compared the heat-inactivated, phenol-preserved vaccine to the alcohol-inactivated and preserved vaccine.[289] A further control group received a vaccine against *Shigella flexneri*. The heat-phenol inactivated vaccine was demonstrated to be the most efficacious, with 70% protection against culture-confirmed typhoid disease.[289] Efficacy was shown to persist for at least 5 years.[290,291]

Although efficacious, the use of these vaccines was limited by their reactogenicity. Killed whole cell vaccines contain components of Gram negative bacteria, including lipopolysaccharide, that elicit profound systemic and local reactions due to the innate responses of the immune and inflammatory systems.[264] Fever rates after vaccination were approximately 20-25% and local reactions occurred in 40-50% of vaccinees.[286,290,292] Reactions were of sufficient severity to lead to an absentee rate of approximately 15%.[286,290,292] Despite this, these vaccines were successfully used to control typhoid disease in Thailand[295] but have now become obsolete.[264,282,296]

With the description in the 1940s of chloramphenicol as an effective treatment for typhoid disease, the pressure to develop suitable vaccination strategies was much reduced. The outbreak of chloramphenicol resistant strains in the 1970 once more focused attention on developing vaccines that could be introduced as part of public health programmes.[297]

1.10.2. Oral vaccination against typhoid disease

Oral vaccines were developed in the hope that they would be less reactogenic and easier to administer compared to killed whole-cell vaccines, as well as allowing stimulation of mucosal immunity.[244] Initial attempts focused on oral ingestion of the available whole-cell vaccines. Oral ingestion of the heat-killed, alcohol preserved vaccine was well tolerated and gave a good serological response,[298] but failed to demonstrate efficacy in vaccine-challenge studies.[244] Similarly, efficacy could not be demonstrated in a large field trial in India.[299] This led to the abandonment of using the existing whole cell vaccines as oral vaccines.

In attempts to make a more efficacious vaccine, live attenuated vaccines were developed. The first prototype was a streptomycin-dependent mutant *S*. Typhi oral typhoid vaccine but the vaccine lost efficacy after lyophilisation.[267] A live, attenuated strain of *S*. Typhi that was successfully used as an oral vaccine was first described in 1975.[300] Referred to as Ty21a, the strain had been derived by nonspecific chemical mutagenesis of the parent wild-type *S*. Typhi strain Ty2, resulting in over 20 different mutations.[300] Mutation of the *galE* gene, which rendered the strain unable to produce Vi polysaccharide, was initially thought to be the most significant of these mutations, but this was contradicted by the later finding that a mutation of *galE* alone failed to attenuate Ty2.[301] Initial trials of the Ty21a vaccine were conducted in a human challenge model at the University of Maryland.[267] After vaccination, volunteers were challenged with *S*. Typhi Quailes strain from which a protective efficacy of 87% was demonstrated.[267]

The first randomised, placebo- controlled, double-blind field trials of Ty21a were conducted in Egypt, between 1978 and 1981.[302] These demonstrated that the Ty21a vaccine was safe and had a protective efficacy of 95% in the field.[302] Later trials in Chile showed a lower efficacy of 67%[303] but did demonstrate an additional herd-protection benefit.[304]

Through extensive trial experience with Ty21a and the post-licensure data, the safety and tolerability of Ty21a is now well established.[267,305] However, several significant limitations exist. The overall efficacy of the vaccine is moderate, with meta-analysis showing 51%

protection over 3 years.[181] The current commercially-available Ty21a vaccine is a lyophilised formulation in enteric-coated capsules, that requires several doses to be effective.[306] This is difficult to administer to young children[307] and is less immunogenic in infants when compared with older children.[303] This vaccine has therefore not been licensed for use in children below 6 years of age. Although a "liquid" formulation of the vaccine (reconstitution of lyophilised vaccine buffer and water) has been shown to be practical, well tolerated and immunogenic, [307] it is not currently being manufactured.

1.10.3. Vi polysaccharide vaccine

The virulence (Vi) polysaccharide capsule of *S*. Typhi consists of a repeating homopolymer of alpha-1,4,2-deoxy-2-N-acetylgalacturonic acid, and was first recognised as a virulence factor by Felix in 1934.[308] The importance of the Vi polysaccharide capsule in *S*. Typhi pathogenicity was highlighted in early studies which demonstrated Vi expression in 84 of 86 pathological isolates, with the remaining two specimens possibly having been rendered Vi negative by multiple passage.[308] This was later corroborated by data from blood culture isolates.[264]

Initial experiments using Vi as a vaccine antigen in the early 1950s[309] showed that, although injected purified Vi was immunogenic, it failed to protect against experimental human challenge.[73,310] It was latter recognised that failure was due to Vi denaturation during laboratory isolation.[311]

The finding that killed whole cell vaccines containing the Vi antigen were more efficacious than those that did not[294] renewed interest in Vi as a vaccine antigen. The successful isolation of non-denatured purified Vi polysaccharide[296,312] (using the same detergent that had been used in the isolation of the polysaccharide for the meningococcal vaccine[313]), paved the way for Vi to be developed as a vaccine candidate.[264] Safety studies showed that when contamination by LPS was minimal, the Vi vaccine was well tolerated.[265] The

vaccines generated anti-Vi antibodies in the majority of recipients and these antibodies persisted for at least three years.[314]

These initial safety studies were followed up with field trials to look at the safety and efficacy of a 25μg dose of non-denatured, purified Vi polysaccharide given as a single subcutaneous dose. Having established safety in 274 people in Nepal as part of a pilot study, Acharya *et al.* conducted a large trial involving 6438 participants between the ages of 5 and 44 years of age randomised to receive either the Vi polysaccharide vaccine or pneumococcal polysaccharide vaccine as control.[315] The vaccine was well tolerated, and around 75% of participants had a rise in anti-Vi antibodies of four fold or more.[315] Vaccine efficacy against culture-positive typhoid disease was 72% over 17 months of follow up.[315] A similar study in South Africa study in a randomised- double blind trial of 11,384 school-aged children demonstrated 64% efficacy over 21 months' passive surveillance.[263] The findings from these two field trials demonstrated that the Vi polysaccharide vaccine was safe and effective in endemic regions; the Vi polysaccharide vaccine was licensed in the United States in 1994.[181] This study also provided evidence for the potential of the Vi vaccine to induce herd immunity; with a vaccine uptake of 60% in the Vi clusters, protective efficacy in unvaccinated members of the Vi-clusters was 57%.[316]

However, significant limitations of the Vi polysaccharide vaccine remain. The administration of the vaccine via the parenteral rather than the oral route requires the use of trained health personnel and probably decreases the acceptability of the vaccine among target populations.[317] As with other T-cell independent polysaccharide vaccines, the Vi polysaccharide does not generate immunological memory and is not boosted by repeated vaccination.[85,309,318] The efficacy of the Vi vaccine in infants is unknown,[11] however, polysaccharide vaccine is unlikely to be efficacious in early childhood due to immaturity of the splenic marginal zone which appears to be required for T-cell independent responses.[319] The Vi polysaccharide vaccine is further limited by the short duration of efficacy of 2-3

years,[320,321] and introduction into the EPI childhood schedule would therefore be of limited value if co-administered with other routine vaccines during infancy.

1.10.4. Use of currently available typhoid vaccines for control of endemic disease

The discovery of effective and relatively cheap antibiotic treatment for typhoid disease in 1948[175] decreased the drive to find effective typhoid vaccination strategies for disease control.[322] The emergence of drug resistant strains of *S.* Typhi has renewed focus on vaccination for disease control. Currently available typhoid vaccines were recommended for use in school aged children in endemic regions by the WHO and reiterated in 2008.[181] Despite this, routine typhoid immunisation use is limited.[323-325]

Where immunisation programmes have been used, they have successfully decreased the burden of disease.[326] The killed whole-cell vaccine was used in children in Thailand, and although no formal appraisal was done, the incidence of typhoid disease sharply declined.[326] Similarly, disease in Cuba was controlled using the whole cell vaccine, and has been maintained by the use of Vi polysaccharide vaccine in 2010.[326] The Vi polysaccharide vaccine alone in school children has successfully controlled disease in China.[325] Public support for vaccination, including a willingness to pay for vaccines, has been demonstrated in Vietnam,[327] China,[328] and Indonesia,[329] further supporting the idea that introduction of vaccination in endemic settings would be successful.

Economic analysis of the burden of typhoid disease has been undertaken to determine if routine vaccination is economically worthwhile. The cost per case of typhoid disease has been found to be hugely variable depending on the study setting and the proportion of health care funded by the state.[330] For example, in Delhi the cost was calculated at US$18.94 per case,[24] whereas in slum areas of Kolkata, where inpatient care was more often required, costs were US$101 per case, a considerable financial burden in an area where average household income was US$50 per month.[331] When considered in the wider context

of net costs per Disease Adjusted Life Year (DALY) compared to per-capita gross domestic product, prevention of disease by introduction of typhoid vaccination would be very cost effective.[329] Despite this favourable economic assessment, the cost of typhoid vaccination would account for a significant proportion of the state spending on health care in many countries, and as such is unlikely to be provided free of charge.[332] Various approaches to funding the implementation of a successful typhoid vaccination programme without significantly increasing public spending have been studied.[333] Charging adults but not children for immunisation, or charging an equal user-fee for all ages, would both enable the introduction of economically successful vaccination programmes.[333]

Although direct and herd immunity have been demonstrated for both Ty21a[303] and Vi polysaccharide vaccines,[316] the latter is likely to be preferentially used.[324] Firstly, the Vi polysaccharide vaccine is theoretically more heat stable than the Ty21a vaccine,[324] which is advantageous in resource poor countries in which maintenance of a cold chain is logistically difficult. Secondly, the Vi polysaccharide vaccine only requires a single dose in comparison to the three or four dose schedule needed for the Ty21a vaccine. Thirdly, Vi vaccine can be locally and economically produced, with successful technology transfer to China demonstrated in 1990.[334] Locally produced vaccine had 69% efficacy over 18 months, comparable to that observed in previous efficacy studies.[335]

Despite the demonstration that the use of existing typhoid vaccines for the control of endemic typhoid disease would be efficacious, supported by local populations, and economically viable, most endemic countries have not implemented routing vaccination.[24] Reluctance to implement vaccination programmes has been attributed to the absence of country specific disease incidence data, and preference for perceived longer-term solutions such as improved water and sanitation provision.[336] It is hoped with increasing realisation of the importance of typhoid disease, successful vaccination programmes may yet be implemented.[323]

1.10.5. Novel vaccine strategies

The development of novel vaccines has been facilitated by scientific advances allowing precise manipulation of bacteria to introduce attenuating mutations.[337] Approaches to strain attenuation, including mutations in biochemical pathways, heat shock proteins, regulatory genes and putative virulence genes have all been tried.

1.10.5.1. Novel oral vaccines

Oral vaccination offers the opportunity to induce gut mucosal immunity as well being a logistically easier way to deliver large scale vaccination programmes. Experience with the Ty21a vaccine has demonstrated that it is exceptionally well tolerated and easy to administer,[303] but its application is limited by its immunogenicity and formulation in capsules.[84] Efforts to increase the immunogenicity of oral typhoid vaccines without compromising safety and tolerability have resulted in several novel oral, single-dose vaccines which are currently being evaluated in clinical trials. These include *S.* Typhi-derived strains M01ZH09, CVD 909 and Ty800. Along the way, several theoretically useful vaccine strains have failed to deliver due to insufficient attenuation or lack of immunogenicity[85] and have highlighted the difficulties in predicting the results of genetic manipulation of parent strains.

The difficulties in understanding the human immune response to genetically manipulated strains was illustrated by a Vi-positive variant of vaccine strain Ty21a.[338] Given the protection afforded by Vi antibody induced by immunisation with Vi polysaccharide[315,339] it was thought that the immunogenicity of Ty21a may be enhanced by reinserting the *viaB* gene, allowing Vi capsule expression.[338] However, immunogenicity studies failed to show any response to Vi or improved immunogenicity over Ty21a.[340]

The live, attenuated vaccine M01ZH09, based on the parent Ty2 strain, contains two independently attenuating gene deletions.[341] One, a mutation of the *aroC* gene, prevents synthesis of aromatic amino acids required for bacterial growth. The second mutation is in

the *ssaV* gene, causing structural abnormality in the specialised type III secretion system encoded by SPI-2.[342] Evidence from the mouse model of typhoid disease using *S.* Typhimurium suggests that the absence of this secretion system leads to an inability of *S.* Typhi to survive within macrophages.[343] Intracellular survival is critical for the systemic spread of *S.* Typhi, and hence the *ssaV* mutation also inhibits systemic spread of the bacteria.[344] In trials of this vaccine to date, systemic infection with the vaccine strain has not been observed.[342,345]

The safety and immunogenicity of M01ZH09 has been studied in trials in the US, UK and Vietnam. The vaccine is given as a single oral dose and has been well tolerated at all tested doses with mild, self-limiting, gastrointestinal side effects being the most commonly reported adverse event.[345] Immunogenicity studies have shown both IgA and IgG responses to the lipopolysaccharide (LPS) of the vaccine.[342,346] Immunogenicity and acceptability has been shown in children aged 5 to 14 years during field trials in Vietnam.[346] Phase III trials of M01ZH09 are required to move this vaccine forwards to licensure. In the absence of definite correlates of protection, however, phase III trials would need to be large and of sufficient duration to demonstrate a significant reduction in the local incidence of typhoid disease. This makes these trials prohibitively costly.

The vaccine strain CVD 909 is the latest in a developmental process that commenced with prototype vaccine CVD 908, and progressed via the CVD 908-*htrA* strain. Strain CVD 908 is derived from Ty2 is attenuated by mutations in *aroC* and *aroD*, that rendered it nutritionally auxotrophic for aromatic compounds that are in limited concentrations in human tissue.[347] However, although immunogenic and well tolerated in high doses in humans[347], silent, self-resolving vaccinaemia occurred between 4 and 8 days after ingesting the vaccine strain.[347] Interestingly, the same mutation in a different parent strain (ISP1820) was even less attenuated than CVD 908, causing symptomatic bacteraemia[348], illustrating the variable result of attenuation depending on the parent strain. Vaccinaemia in itself is not necessarily a contraindication to licensure, with established vaccines including yellow

fever, rubella and live polio vaccines inducing vaccinaemia[85] but the requirement for enhanced pre-licensure safety data was felt to be prohibitive and efforts moved towards further attenuation to prevent vaccinaemia. The potential attenuating effect of deletion of the *htrA* gene which codes for a heat shock protein that facilitates survival and replication[349] was initially demonstrated in the *S.* Typhimurium mouse model of typhoid disease.[350] Deletion of *htrA* in CVD 908 did not decrease immunogenicity compared to that observed with CVD 908, and importantly did not result in vaccinaemia.[351,352]

CVD 908-*htrA* was further modified to express the Vi polysaccharide. In contrast to immunogenicity findings when Vi expression was restored in Ty21a, insertion of Vi into CVD 908-*htrA* was partially successful, with the majority of volunteers given the highest dose of the vaccine developing an antibody secreting cell response to the Vi antigen.[353] More recently, the demonstration that CVD 909 induced anti Vi responses that could be boosted by later administration of the Vi polysaccharide vaccine provided evidence of a memory B cell response to the oral vaccine.[354]

A further approach to attenuation was used in the development of Ty800 vaccine. The *phoP*/*phoQ* regulator region of the Ty2 parent strain was deleted, altering the transcription of multiple virulence properties regulated by this region, including those allowing survival within macrophages and phagosomes.[355] In dose finding trials, a single dose of this vaccine was shown to be well tolerated and immunogenic.[356] Unfortunately, the development of this promising vaccine did not progress beyond phase 1 human clinical studies.

1.10.5.2. Novel parenteral vaccines

Conjugation of the Vi polysaccharide to carrier proteins to form a protein-polysaccharide conjugate vaccine offers many potential advantages over pure Vi polysaccharide vaccine. Similar to other vaccines where polysaccharide is conjugated to carrier proteins, a Vi-conjugate vaccine has potential to induce a T-cell dependent response, with antibody affinity maturation, subclass switching and immunological memory that can be boosted[85,319]

and protection in infants. [319] Examination of responses to pure Vi polysaccharide and Vi conjugate vaccines in mice has also suggested that conjugate vaccines may avoid the hypo-responsiveness observed with plain polysaccharide vaccines in which the immune response to subsequent exposure to the vaccine is attenuated with a second dose of the (plain polysaccharide) vaccine.[357] Another recently described approach to vaccine development is the use of a lipopolysaccharide –conjugate vaccine[358] which is currently in pre-clinical studies.

The successful conjugation of Vi to a protein carrier was initially described in 1987.[359] Conjugation to a nontoxic, recombinant protein (*Pseudomonas aeruginosa* exotoxin A) elicited strong antibody response that persisted for at least 26 weeks in human volunteers.[360] Subsequent field trials of this vaccine in endemic Vietnam showed immunogenicity in children as young as two years of age, and also demonstrated a booster response on repeat administration.[361] In an efficacy trial two doses of the Vi polysaccharide–protein conjugate vaccine administered 6 weeks apart was safe, immunogenic and had a protective efficacy of 91.5% in children aged 2–5 years over 27 months' follow-up.[362]

Following on from this ground work, a number of manufactures are pursuing development of Vi-conjugate vaccines using a variety of protein carriers.[363,364] Although one conjugate vaccine is already licensed in India (but has not been prequalified by the WHO), the majority of these new generation vaccines will complete licensure trials in the next few years.[365] The identification of Vi-negative strains from patients with typhoid disease [71,366] has raised concerns that the immuno-protection from vaccines derived from the Vi antigen may be limited.[367] The widespread use of vaccines based on the Vi antigen may serve to exert selection pressure for Vi negative strains, hence potentially limiting the lifetime of these vaccines. To-date there are no data to support this hypothesis, although there has not been any widespread introduction of Vi vaccines in order to definitively refute this idea.[319]

1.11. Challenge models of typhoid disease

The vaccination and subsequent exposure of James Phipps to smallpox infected material by Edward Jenner in May 1796 could be considered the origin of both vaccination and of human challenge studies. The eventual eradication of smallpox by vaccination illustrates the significant benefit that can be derived from the application of human challenge with infectious agents.[368] Since that time, controlled human infection studies (or 'challenge studies'), in which participants are deliberately infected with pathogens, have provided an understanding of the aetiology, transmission, treatment and prophylaxis of many infectious diseases. For many pathogens, especially those that are human-restricted, such as *S.* Typhi, challenge studies provide the only biologically relevant model of infection.[369-371]

The significant worldwide burden of typhoid disease has been established[2,117,228,372], but costly changes to infrastructure and living standards, needed to prevent the faecal-oral transmission of *S.* Typhi, are occurring only at a discouraging pace[23]. Effective typhoid vaccines may provide medium term disease control[365] but currently available vaccines have significant limitations.[85,373] The development of novel vaccines that overcome these limitations is hindered by poor understanding of both infection and vaccine-derived protective immunity.[85,373,374] Vaccine development is a long, costly and challenging process, and many potential vaccines fail on the path to licensure.[375] The poor understanding of typhoid immunobiology, and, in particular, the absence of a correlate of immunity that could be used in efficacy trials, was highlighted as a rate-limiting factor in the slow introduction of Ty21a[375]. A human challenge model of typhoid disease may provide a way to overcome some of these limitations in understanding, and allow the appraisal of novel typhoid vaccines.

A human challenge model of typhoid disease, in which participants ingested virulent *S.* Typhi in milk, was previously established at the University of Maryland,[85] in order to directly measure the efficacy of typhoid vaccines in preventing clinical illness.[46] These

investigations provided valuable insight into the disease and its pathogenesis, and into the nature of typhoid immunity.[90,244] Human challenge experiments have not been conducted with *S.* Typhi since the termination of the Maryland typhoid programme in 1974, due to concerns regarding the use of prisoners as participants.[376] In the intervening period, advances have been made in the understanding of the immunobiology of infection and vaccination, host-pathogen interactions and the technology to investigate immune responses, host-susceptibilities and diagnostic biomarkers has been radically transformed.

Re-establishing a challenge model of typhoid disease could have the potential to lead to many new insights into this disease and its prevention. Demonstrating the efficacy of novel vaccines using a human challenge model could save time and cost in vaccine development, providing confidence for investment in phase III trials. Progress from demonstration of immunogenicity in phase II to phase III trials is especially difficult in situations where no correlate of protection is available.

As well as providing evidence for the value of challenge studies, the work conducted in Maryland, along with data from other enteric challenge studies, provided an understanding of the attack rate at various challenge doses, the influence of pre-existing immunity in participants on reported attack rates, the importance of strain selection, the safety of *S.* Typhi challenge and the potential influence of diagnostic definitions on reported outcome, all of which are of direct relevance in re-establishing a challenge model.

1.11.1. Historical perspective

Vaccination against typhoid disease and its subsequent appraisal by human challenge with *S.* Typhi has existed for over 100 years. The first typhoid vaccine, developed by Wright in 1896[377], was administered to two medical officers, one of whom underwent subsequent challenge by injection into the flank with 'virulent typhoid bacilli'.[377,378] Illness did not occur, and this was taken as evidence of successful vaccination.[378] On the basis of this finding, vaccination of military personal became commonplace; in the absence of properly

controlled field trials however, controversy regarding true vaccine efficacy remained.[378] Field trials of killed whole-cell vaccines were later conducted in hyper-endemic regions, where despite the demonstration of protective efficacy, the contribution of chronic, low grade exposure in subsequent vaccine efficacy remained unclear.[46] Human challenge studies provided a unique opportunity to study killed-whole cell vaccine efficacy in a previously unexposed population, providing the impetus to develop challenge models of typhoid disease.[46]

In 1952, two men were challenged with wild type *S*. Typhi Ty2 strain at the University of Maryland, marking the start of a long programme of *S*. Typhi challenge experiments, principally aimed at vaccine appraisal.[378] Detailed dose-finding studies using *S*. Typhi Quailes strain were later performed, and demonstrated that for 50% of participants to developed illness (the attack rate), 10^7 colony forming units (CFU) of *S*. Typhi needed to be ingested (the challenge dose). The attack rate with a challenge dose of 10^5 CFU was 25%.[73] Further insights into the disease, its pathogenesis, and into the nature of typhoid immunity afforded by the model are summarised in Table 2.[90,244] The model was used to quantitatively assess the human host response to challenge and directly measure the efficacy of typhoid fever vaccines in preventing clinical illness[46] (Table 3). Over the subsequent 16 years (1959-1974) 1886 participants received both established and novel experimental vaccines against typhoid disease, of whom 762 were challenged with a single strain of *S*. Typhi, the 'Quailes' strain.[73,87]

Table 2: A summary of findings related to typhoid pathogenesis from previous human challenge models of typhoid fever

Study purpose	Participants	Findings	First author	Year	Reference
Dose finding studies	213 males	$ID_{25} = 10^5$ CFU Increasing does increases attack rate and decrease incubation period. Clinical description of typhoid fever.	Hornick	1970	[73]
Asymptomatic primary bacteraemia description	Case report of 2 participants from vaccine studies	Description of two bacteraemic participants that failed to manifest clinical typhoid fever	Snyder	1963	[246]
Determine changes in serum complement and properdin levels during acute typhoid fever and subsequent treatment in six participants	6 participants participating in vaccine studies	Complement levels rise and properdin levels fall during acute typhoid fever.	Schubart	1964	[379]
Determination of histopathological changes in the gut during acute typhoid.	6 participants in vaccine studies.	Gut biopsies performed on 6 participants with typhoid, showing enteritis in acute disease that healed without scarring	Sprinz	1966	[90]
To examine changes in whole blood amino acids during acute typhoid fever	17 males participating in vaccine studies.	9/10 men who developed typhoid fever had a significant rise in amino acid levels during the incubation period. Illness onset decreased concentration to below baseline.	Feigin	1968	[380]
Examine effect of parenteral chloramphenicol treatment for typhoid	24 unimmunised control participants from vaccine studies.	Parenteral route increased duration of clinical illness. Inferiority to oral route demonstrated.	DuPont	1970	[281]
Effects of streptomycin pre-treatment	4 male participants	Allowed disease to be induced in one of four participants following challenge with 10^3 CFU, a dose too low to induce disease normally.	Hornick	1970	[73]
Comparison of response to challenge with different strains of *S*. Typhi	86 participants given 1 of 5 different strains.	Vi positive strains of *S*. Typhi associated with twice the attack rate of Vi negative strains	Hornick	1970	[73]
Role of endotoxin in pathogenesis	Not stated.	Examination and comparison of response to endotoxin challenge and *S*. Typhi challenge. Participants rendered tolerant to endotoxin did not have a different clinical course after *S*. Typhi challenge	Greisman Hornick	1961 1970	[114,381]
Examination of effect of early chloramphenicol therapy in prophylaxis against typhoid fever	4 participants treated 24 hours after challenge, 2 for 7 days and 2 for 28 days.	One participant developed typhoid fever after 7 days of chloramphenicol therapy. All had an immunological response consistent with clinical disease.	Hornick	1970	[73]
Rechallenge with homologous strain of *S*. Typhi to determine protection from prior infection	22 participants rechallenged within 12 months, compared with 34 controls from vaccine trials	23% protection from previous infection	DuPont	1971	[244]

Table 3: A summary of findings from previous vaccine-challenge studies of typhoid fever

Vaccine	Finding	Challenge dose	First named author	Year	Reference
Killed parenteral whole cell vaccine	Following immunisation, one subject was injected with viable *S*. Typhi. Typhoid fever did not develop.	Unknown	Wright	1896	[377]
Heat treated oral vaccine	10/13 immunised volunteers developed *S*. Typhi between 6 and 28 days later, demonstrating Koch's postulates for *S*. Typhi.	Unknown	Tigertt, reported in 1959	1904	[382]
Parenteral acetone-killed whole cell vaccines	No protection.	10^7 CFU (ID_{50})	Hornick	1967	[378]
Parenteral acetone-killed whole cell vaccines	67% efficacy.	10^5 CFU (ID_{25})	Hornick	1967	[378]
Parenteral phenol-heat-inactivated vaccine	No protection	10^7 CFU (ID_{50})	Hornick	1967	[378]
Parenteral phenol-heat-inactivated vaccine	75% efficacy	10^5 CFU (ID_{25})	Hornick	1967	[378]
Parenteral, denatured Vi polysaccharide	No protection	10^5 CFU	Hornick	1970	[73]
Killed oral vaccine, containing Ty2 organisms inactivated by acetone and freeze drying, in enteric capsules ($100x10^9$ CFU per dose)	15% efficacy at twice the manufacturer's recommended dose (12 tablets)	10^5 CFU	DuPont	1971	[244]
Freshly harvested attenuated streptomycin-dependent oral vaccine	66% efficacy	10^5 CFU	DuPont	1970	[383]
Lyophilised attenuated streptomycin-dependent oral vaccine	19% (non-significant)	10^5 CFU	Levine	1976	[7]
Ty21a grown with exogenous galactose	87% efficacy	10^5 CFU	Gilman	1977	[267]
Ty21a grown without exogenous galactose	50% efficacy	10^5 CFU	Gilman	1977	[267]

1.11.2. Strain and challenge dose selection

The importance of strain selection in human challenge studies has been highlighted by the Academy of Medical Scientists.[370] Strain selection must prioritise participant safety, whilst considering model consistency and scientific validity.

The initial challenge of two participants in 1952 used a challenge dose of $6x10^6$ colony forming units (CFU) of wild-type strain Ty2.[378] Neither participant developed clinical infection, nor were serological responses demonstrated,[378] and the challenge experiment was deemed to have failed. Subsequent studies in chimpanzees demonstrated that a very large inoculum of Ty2 was required to induce disease in that animal model that most closely approximates human typhoid infection.[384]

In 1959 a new strain, given at a much higher dose, was successfully used to induce typhoid fever. This strain was a wild-type, Vi-expressing, phage type D-1 strain isolated in 1958 from the gallbladder of a chronic carrier, Mrs Quailes, after whom it was named.[73] This particular strain was selected as it had recently demonstrated virulence by causing disease in Mrs Quailes' family.[378]

Although the use of the Quailes strain had been successful, the contribution of strain-variable virulence and antigenic factors to pathogenicity was unclear. The Quailes strain was known to express the Vi polysaccharide capsule, named because of association with virulence. To investigate the effects of Vi and other antigen expression on clinical disease, challenges were conducted with three Vi-expressing wild-type strains (Quailes, Ty2 and Zermatt), a Vi-negative stain (Ty2W) derived from Ty2 and a naturally occurring Vi and H (flagellin antigen) negative strain (O-901) at a dose of 10^7 CFU.[73] The wild-type, Vi positive strains demonstrated an attack rate twice that of Vi negative strains (51% vs. 26%), illustrating the importance of Vi capsule in virulence.[73] However, the clinical severity of resulting disease did not appear to be strain dependent, and was described as 'typical enteric fever'.[73] As the vast majority of naturally occurring *S.* Typhi strains are both Vi and H

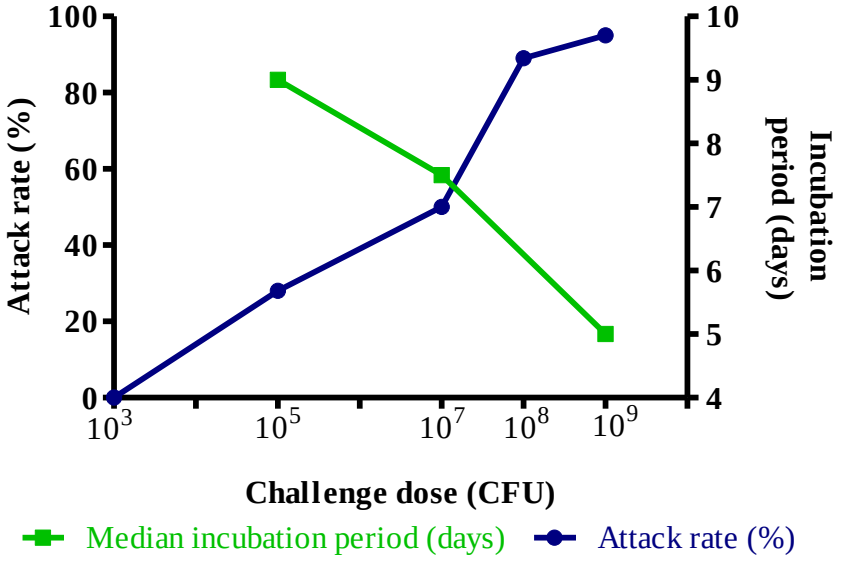
antigen positive,[385] the Quailes strain would seem to be ideal for use in a model of 'natural' typhoid exposure, and this strain was used for the subsequent challenge studies.

To allow appraisal of vaccine candidates in the typhoid challenge model the number of CFU of *S.* Typhi Quailes strain that need to be ingested to produce disease at a given rate (the attack rate) had to be determined. During initial dose-finding studies with Quailes strain, 119 participants gargled, and then swallowed between 10^3 and 10^9 CFU of Quailes strain suspended in 30 ml to 45 ml of milk.[73,85] The attack rate correlated directly with challenge dose; the incubation period was inversely correlated.[73] Interestingly, once illness occurred the clinical course was comparable irrespective of the challenge dose.[73]

Table 4: The attack rate and incubation period with different challenge doses of *S.* Typhi Quailes strain given in milk, showing increasing attack rate and decreasing incubation period with larger challenge doses. Reproduced from 'Typhoid fever: pathogenesis and immunologic control', R.B Hornick *et al,* 1970[73]

Challenge dose of *S.* Typhi (CFU)	Number of volunteers challenged	Number diagnosed with typhoid fever (%)	Median incubation period in days (range)
10^9	42	40 (95)	5 (3-32)
10^8	9	8 (89)	
10^7	32	16 (50)	7.5 (4-56)
10^5	116	32 (28)	9 (6-33)
10^3	14	0	

Figure 6: The attack rate and incubation period with different challenge doses of *S.* Typhi Quailes strain given in milk in previous human challenge studies, showing increasing attack rate and decreasing incubation period with larger challenge doses. Reproduced from Typhoid fever: pathogenesis and immunologic control, R.B Hornick *et al,* 1970[73]



The ID_{50} dose was 10^7 CFU, with a median incubation period of 7.5 days, whilst 10^9 CFU had a 95% attack rate after 5 day incubation. There was no illness noted after a dose of 10^3 CFU.[73] The incubation period was highly variable, ranging from 4 to 56 days after 10^7 CFU. Although increased disease severity with a higher challenge dose is seen with some enteric infections, this is not universally true, and notably did not appear to occur with *S.* Typhi challenge. In early experiments with *E. coli*, for example, there was a clear relationship between dose and severity of illness, with doses of $6.5x10^9$ CFU and higher, causing a 100% attack rate, with 50% of participants developing severe disease, compared to a $5.3x10^8$ CFU dose, causing a 67% attack rate and no severe illness.[386] Previous *S.* Typhi models and analyses of outbreaks have shown a fairly uniform severity of illness, irrespective of dose,[73,223,387] providing reassurance that the challenge dose can be safely increased in needed to produce a desired attack rate.

Although increased challenge dose was shown to increase attack rates safely, large bacterial loads risked overwhelming vaccine protective efficacy.[388] For example, appraisal of a

transcutaneous immunisation against enterotoxigenic *E. coli* in a challenge model did not demonstrate vaccine efficacy, and it was hypothesised that this may be in part due to a higher challenge dose than would be encountered in the natural environment[389]. Similarly, previous studies of *S.* Typhi vaccines, known to be effective in the field, were overwhelmed by a challenge dose that gave a 50% attack rate in controls.[73]

1.11.3. Diagnostic definitions and attack rates

Prospectively defining a diagnostic endpoint for a symptomatically diverse disease such as typhoid fever, for which there is no reliable laboratory diagnostic test, is difficult.[390] It is however essential to accurately classify disease outcomes so that attack rates can be accurately determined.

In early Maryland typhoid challenge studies, a stringent case definition of typhoid fever was used. Typhoid fever was defined as an oral temperature of 103°F (equivalent to 39.4°C) persisting for 24 to 36 hours.[73] Temperatures were usually only measured in the morning during the incubation period, possibly missing initial, early rises in temperature likely to occur in the evening due to diurnal fluctuation.[390] Symptoms of typhoid were routinely recorded, but were not part of the diagnostic definition. This contrasts with *Shigella* challenge models in which diarrhoea in addition to temperature, were used as a marker of infection.[391] Similarly, blood and stool samples were also routinely cultured following *S.* Typhi challenge, but again microbiological findings did not form part of the diagnostic definition.[244] Microbiological endpoints have been successfully used in human challenge models. For example studies of cholera, illness was classified on the basis of culture of Vibrios from stool, as well as by the presence of diarrhoea.[392,393]

The stringent definition may have excluded milder cases from the case definition.[390] Typhoid fever severity in the Maryland model was relatively uniform, and did not vary with challenge dose, possibly a reflection of the exclusion of those with less severe disease.[73] Retrospective analysis of challenge outcome has examined the influence that different

diagnostic definitions would have had on the reported attack rate and disease severity (Table 5).[390]

Table 5: Variations in the reported attack rate with different endpoint definitions of illness for participants challenged with different doses of *S.* Typhi Quailes strain given in milk in previous challenge studies[390]

Challenge dose of *S.* Typhi (CFU)	Definition of illness*			
	Temperature ≥37.8°C for 12 hrs, peak ≥38.3°C (%)	Antibiotics given for clinical illness	Temperature ≥38.3°C for 12 hrs	Temperature ≥39.4°C for 36 hrs^
10^{8-9}	24/25 (96.0)	30/34 (88.2)	24/25 (96.0)	18/25 (72.0)
10^{7}	13/27 (48.1)	13/27 (48.1)	13/27 (48.1)	8/27 (29.6)
10^{5}	83/200 (41.5)	72/204 (35.3)	77/200 (38.5)	52/200 (26.0)
10^{3}	0/13 (0)	0/13 (0)	0/13 (0)	0/13 (0.0)
*37.8°C = 100°F, 38.3°C = 101°F, 39.4°C = 103°F, hrs = hours ^original definition used				

The effects of reassessing the disease definition on the reported attack rate were marked, not least due to the (relatively) small numbers of participants involved. A less strict definition using a temperature greater than 100°F (37.8°C) for 12 hours with a peak of greater than 101°F (38.3°C), would have increased reported attack rates from, for example, 26% to 41.5% at the 10^5 dose.[390] Furthermore, with less strict definition, peak temperature correlated with the log transformed dose given and with the number of symptoms and signs, albeit weakly.[390] Having a clearly defined endpoint in any challenge study is vital. Nevertheless these findings demonstrate the complexities of defining clinical typhoid disease, and the effect that slight variations in definition may have on study findings. Whilst ascertainment of cases may be more accurate if disease is allowed to progress to fulfil a stringent disease endpoint, conversely there is the need to prevent unnecessary participant harm that may come from delay in diagnosis.[87]

1.11.4. Pre-existing immunity

Early challenge studies using *S.* Typhi[394] and other enteric pathogens[386,395] were undertaken in male volunteers, who were inmates at the Maryland House of Correction, Jessup, Maryland. Although these inmates were apparently healthy, willing[396,397] and readily contained, their participation raises ethical[376] as well as scientific concerns. Prisoners represented a very narrow demographic profile, influencing the applicability of the model to the wider population. Many had previously served in the U.S. military, where vaccination against *S.* Typhi with whole-cell killed vaccines had originally been introduced in 1911[398] and was routine for troops deployed overseas during World War II, the Korean War and the Vietnam War. In a retrospective analysis of 305 participants challenged with 10^5 CFU, the attack rate in 105 military veterans and 200 men without military service was 20% and 48% respectively,[46] representing an apparent 58% protective effect from previous military service. This difference in attack rate likely reflects substantial pre-existing immunity in US military veterans. Protective efficacy of the Ty21a vaccine in the Maryland typhoid model was 87%, higher than that observed in many subsequent field-trials,[303,317,373,399-401] possibly due in part to pre-existing immunity from military service. These findings demonstrate the importance of challenging an immunologically naïve cohort in order to have an accurate, reproducible attack rate that can determine the protective efficacy of a vaccine in an antigenically naïve cohort.

1.11.5. Safety

Over the course of the Maryland studies, 762 participants ingested the Quailes strain, providing a wealth of data on safety. Although strains other than the Quailes strain have been well characterised, they have not been extensively used in challenge models and lack the associated safety data.

Presumably consequent to the prompt administration of appropriate antibiotic therapy, remarkably few participants developed complications during the course of the typhoid

challenge studies in Maryland. One participant developed mild haemolytic anaemia and another had 'several' episodes of temporary confusion,[73] on the background of previous (undisclosed) psychiatric history.[87] Chronic carriage of *S.* Typhi, in which prolonged stool excretion of *S.* Typhi occurs mainly due to biofilm formation of pre-existing gallstones,[402] occurred in one participant with previously undiagnosed gallstones, and resolved following cholecystectomy.[175] Gastrointestinal bleeding, the most feared complication of typhoid disease, the risk of which is now recognised to increase with prolongation of the interval prior to treatment,[7] occurred in one participant during a relapse of typhoid fever, following an initial, untreated episode.[87] One participant required intravenous fluid replacement for diarrhoea.[87] Two further reported complications – one, a pleural effusion, and one, the onset of diabetes, occurred outside the immediate study period and were not thought to be related.[87]

1.11.6. Vehicles for ingestion

Gastric acid is one of the first lines of defence against enteric infection, and the ability of bacteria to survive this will influence the subsequent attack rate.[403,404] Organisms vary in their sensitivity to acid.[405] Although culture of *S.* Typhi from gastric aspirates up to 30 minutes after challenge has been possible[73] it is highly variable between individuals and may contribute to variable attack rates. An indication of the deleterious effect of gastric acid on attack rates following *S.* Typhi challenge was shown in experiments using streptomycin pre-treatment, known to increase the pH of the gastrointestinal tract in mice. One of four men pre-treated with streptomycin before challenge with $1x10^3$ CFU of *S.* Typhi developed disease[73], previously unreported at this challenge dose. More direct evidence was provided by measurement of gastric pH with cholera challenge studies, where low gastric pH in the 24 hours prior to challenge correlated with the subsequent total diarrhoeal volume.[406]

The use of sodium bicarbonate as a gastric acid buffer was first described with *Shigella* challenge experiments in 1946.[407] However, no measure of gastric acid was attempted, and

there were no data to allow comparison of attack rate and symptoms, and consequently little attention was given to this aspect of the model. Sodium bicarbonate ingestion prior to swallowing attenuated *Shigella* strains was later shown to increase faecal excretion compared to that observed without pre-feeding (33% vs. 100%),[408] suggesting increased gastric survival of bacteria with sodium bicarbonate. In *V. cholerae* challenge studies, sodium bicarbonate allowed induction of diarrhoeal disease in the majority of those challenged (9 of 13 participants) with 10^4 bacteria, much lower than the 10^8 CFU needed when milk was used.[403] Findings were similar with *E. coli*[409], virulent *Shigella*[410] and *C. jejuni*[411] challenge models. Quantitative data on the effect of gastric pH were obtained from gastric aspirates of 37 men in cholera challenge studies, and showed that, after overnight fast, all but 2 had a gastric pH less than 2.[403] Thirty minutes after ingestion of sodium bicarbonate, gastric pH remained elevated (pH >5) in over 50% of volunteers.[403] Participants with elevated pH at 30 minutes subsequently had higher attack rates at both the 10^4 dose (4/4 vs. 1/5 participants) and the 10^6 dose (9/10 vs. 5/7 participants).[403]

The Quailes strain used in the original *S.* Typhi challenge studies was stored at -70°C in milk, and prior to challenge, was reconstituted, subcultured for 6 hours at 37°C and harvested prior to re-suspension in milk ready for ingestion.[73] Sodium bicarbonate pre-treatment has never been used in *S.* Typhi challenge, although the acid sensitivity of the organism is recognised.[405]

1.12. Aims and objectives of this thesis

This thesis aims to detail the development of a 21st century human challenge model of typhoid disease through the following objectives:

- To develop and carefully characterise a frozen cell bank of *S.* Typhi suitable for use in human challenge studies of typhoid disease
- To develop a safe and reproducible model of typhoid disease in healthy adults

- To characterise the clinical response of participants challenged with *S*. Typhi
- To characterise the microbiological response to *S*. Typhi challenge in healthy adults
- To characterise the humoral immune response to *S*. Typhi challenge in healthy adults
- To validate the challenge model as a mechanism to appraise typhoid vaccines
- To investigate the acceptability of the human challenge model to participants.

2. Laboratory material and methods

2.1. Buffers and reagents

2.1.1. Phosphate buffered saline (PBS)

Five PBS tablets (Sigma-Aldrich, Dorset, UK; P4417) were added to 1 litre of sterile, pyrogen free water (s.d. H_2O; Baxter, Berkshire, U.K; UKF7114). The pH was adjusted to between 7.2 and 7.4 by the addition of 10M sodium hydroxide (NaOH; Sigma-Aldrich; S5881) or 50% hydrochloric acid (HCl; Sigma-Aldrich; S814-8) as appropriate. PBS was stored at room temperature for up to six months.

2.1.2. One times PBS with 0.05% tween (1x PBST)

Five hundred microliters of the detergent Tween- 20 (Sigma-Aldrich; P1754) was added to 1 litre of PBS, prepared as above. PBST was stored at room temperature for up to six months.

2.1.3. Ten times PBST (0.05% tween) (10x PBST)/ ELISA wash buffer

Sodium chloride (NaCl, 80 g/l; Sigma-Aldrich; S7653), potassium chloride (KCl, 3.14 g/l; Sigma-Aldrich; P3911), di-sodium hydrogen phosphate heptahydrate ($Na_2HPO_4.7H_2O$, 20.6 g/l; Sigma-Aldrich; S9390) and potassium dihydrogen orthophosphate (KH_2PO_4, 1.6 g/l; VWR International, Poole, U.K; 26936) were dissolved in 800 ml of s.d.H_2O, and then 5 ml of Tween-20 was added. The final volume was adjusted to 1litre with s.d.H_2O. The pH was corrected using to 7.2 to 7.4 as before. Prior to use, 900 ml of s.d. H_2O was added to 100 ml of 10x PBST and the pH corrected to 7.2 to 7.4. This was kept at room temperature for up to six months.

2.1.4. Carbonate–bicarbonate buffer

The content of one capsule of carbonate-bicarbonate (Sigma-Aldrich; C3041) was dissolved in 100 ml of s.d. H_2O, and pH corrected to 9.6. This was stored at room temperature for up to one month.

2.1.5. 10% sodium deoxycholate

One gram of sodium deoxycholate (Sigma-Aldrich; D6750) was dissolved in 10 ml of s.d. H_20, from which 1 ml aliquots were prepared and frozen at 20°C for up to 1 month.

2.1.6. 5% milk in PBST (PBSTM)

Fifty grams of skimmed milk powder (Sigma-Aldrich; 70166) was dissolved in 1L of PBST. This was stored at 4°C for up to one week.

2.1.7. 1% bovine serum albumin (BSA)

Ten grams of lyophilised BSA (Sigma-Aldrich; A2153) was dissolved in 1L of PBST. This was stored at 4°C for up to 1 month.

2.1.8. 1% foetal calf serum (FCS)

Ten millilitres of FCS (Sigma-Aldrich; N4637) was added to 990 ml of PBST and mixed by stirring. This was stored at 4°C for up to 1 month.

2.1.9. RPMI with L-glutamine, penicillin, and streptomycin/ R_0 medium

L-glutamine at 2mM (Sigma-Aldrich; G7513) and 5 ml of penicillin-streptomycin solution (50 U/ml and 0.05 g/ml respectively; Sigma-Aldrich; P4458) were added to 500 ml of RPMI-1640 with phenol red indicator and 25 mM hepes modification (Sigma-Aldrich; R5886). R_0 solution was stored at 4°C for up to one week.

2.1.10. R_0 with 5% new-born bovine serum (NBBS) / R_{10} medium

Fifty millilitres of new born bovine serum (NBBS; Sigma-Aldrich; N4637) was added to 450 ml of R_0 and mixed. R_{10} was stored at 4°C for up to one week.

2.1.11. Vi polysaccharide antigen

S. Typhi Vi polysaccharide at 5mg/ml concentration (donated by Sanofi–Pasteur).

2.1.12. H antigen

The H antigen was prepared in house. *S*. Typhi Quailes strain was inoculated into 1 ml of tryptic soy broth (TSB; Oxoid, Basingstoke, UK; CM1016) and incubated overnight at 37°C. The following day, the 99 ml of TSB was added to give a 1:100 dilution, and incubated at 37°C until the optical density (O.D) at 600nm was 0.6 (approximately 3 hours). To collect the bacterial cells, the suspension was centrifuged at 4000 g for 20 minutes at 4°C. The supernatant was removed and the cell pellet resuspended in 5 ml of TSB. Cells were then homogenised using 3, 45 second cycles with Ultra-Turrax T10 (IKA-work, Staufen, Germany). The suspension was then centrifuged as before, and the supernatant removed and filtered using a 0.2 μL filter. Absence of viable bacterial cells was confirmed by overnight culture of 100 μL of the suspension on triptone soy agar (TSA; Oxoid; CM0131). The filtrate was then ultra-centrifuged at 28500 g for 1 hour at 4°C. The pellet was resuspended in 1/1000 of the volume in water, and the protein concentration verified using a bicinchonic acid assay (BCA kit; Sigma-Aldrich; BCA-1). The H assay preparation used in this study was at a final concentration of 50μg/ml.

2.1.13. Lipopolysaccharide (LPS) antigen

Ten milligrams of lyophilised LPS (Sigma-Aldrich; L2387) was dissolved in s.d. H_20 and stored at -20°C for up to 6 months in 1 ml aliquots.

2.1.14. Other reagents

Detection antibodies for the enzyme linked immunoassays (ELISA): Goat anti-human immunoglobulin (Ig) G, IgM and IgA specific-horse radish peroxidase (HRP) conjugates (all AbD Serotec, Oxford, UK; STAR97P, STAR145P, and STAR141P respectively). HRP-conjugate substrate: Ultra-sensitive tetramethylbenzidine solution (TMB; Sigma-Aldrich T0440). Stopping solution: 2M sulphuric acid (H_2SO_4).

2.2. Equipment

2.2.1. ELISA equipment

MaxiSorp 96 well ELISA plates (Thermo Scientific Nunc; 442404), multichannel electronic pipettes (Biohit), automated plate washer (Bio-tek), and a plate reader with 450nm filter (Multiskan EX, Thermoscientific) with Ascent^{TM} analysis software were used.

2.2.2. Enzyme linked immunospot (ELISpot)

The AID ELISpot Reader System, consisting of an optical reader (AID ELR03m), and image analysis software, AID ELISpot version 4.0, Autoimmun Diagnosticka), were used to count spots on the ELISpot plates.

2.3. General Laboratory methods

2.3.1. Sample handling

Samples were labelled with participant's study code and the time point at which the sample was obtained. Following processing, sera for storage were separated into 2 equal aliquots that were labelled with the same participant details. Prior to ELISA assay, sera were thawed and assigned a random number from a password-controlled list prepared by an independent statistician to ensure blinding. Sample labelling was checked by two members of the laboratory team. The plasma cell ELISpot assay was performed in real time, and therefore was performed un-blinded.

2.3.2. Serum separation

Whole blood was collected in serum separating tubes and left to clot for a minimum of 30 minutes (at room temp) and maximum of 24 hours at 4°C. Clotted samples were centrifuged at 3000g, for 10 minutes and the sera removed. Equal aliquots of sera were place into two cryovials and stored at -80°C until required.

2.3.3. Peripheral Blood Mononuclear Cell (PBMC) separation for ELISpot

Blood was collected in 10 ml tubes coated with ethylenediaminetetraacetic acid (EDTA; Becton Dickinson, Oxford, UK). On receipt, blood was diluted 1:2 with R_0 medium. Separation of PBMCs was by density graded centrifugation over Lymphoprep (Axis-Shield, Dundee, UK). PBMCs were then washed twice in R_0. Cell numbers per ml of cell suspension were estimated by mixing 50 µl of cell suspension with 50 µl each of PBS and 0.4% trypan blue (Sigma- Aldrich), giving a dilution factor of 3. From this mixture, 10 µl was added to a haemocytometer for cell counting. Cells were counted in duplicate and averaged. The total number of cells was calculated using the formula:

Total cell number = average number of cells counted x dilution factor x volume of cell suspension.

Following washing, cells were diluted to a final concentration of 2.5 x 10^6 cells per ml in R_{10}. If there were sufficient cells, a 5.0x10^6 cells per ml concentration was also prepared.

2.3.4. *Ex vivo* ELISpot assay for measurement of IgG, IgM and IgG antigen specific plasma cells against the Vi, H and LPS antigens of *S.* Typhi

2.3.4.1. Plate coating

ELISpot were performed using MultiScreen 96-well ELISpot plates with a 0.45 μm surfactant free mixed cellulose ester membrane (Milipore, Darmstadt, Germany; MAHAS4510). Antigens for plate coating were diluted to the correct concentration in carbonate-bicarbonate buffer, as per Table 6, and 100 μl /well added to the plate as per the plate layout (Figure 7). Pan-antibody, which binds any antibody, was included as a positive control. Coated plates were sealed and incubated overnight at 4°C.

Table 6: Antigen concentrations required for coating a 96-well plate for *ex vivo* ELISpot measurement of plasma cell counts in PBMCs from participants in the human challenge model of typhoid fever

Antigen	Stock concentration	Concentration required for ELISpot	Dilution factor
LPS	10 mg/ml	10 μg/ml	1:1000
H	50 mg/ml	10 μg/ml	1:5000
Vi	5 mg/ml	2.0 μg/ml	1:2500
Pan-antibody	5 mg/ml	20 μg/ml	1:250

Figure 7: Plate layout for a 96-well plate for *ex vivo* ELISpot measurement of plasma cell counts in PBMCs from participants in the human challenge model of typhoid fever

	1	2	3	4	5	6	7	8	9	10	11	12
A	LPS αIgG	LPS αIgM	LPS αIgA	Vi αIgG	Vi αIgM	Vi αIgA	LPS αIgG	LPS αIgM	LPS αIgA	Vi αIgG	Vi αIgM	Vi αIgA
B												
C												
D												
E	H αIgG	H αIgM	H αIgA	Pan-Ab αIgG	Pan-Ab αIgM	Pan-Ab αIgA	H αIgG	H αIgM	H αIgA	Pan-Ab αIgG	Pan-Ab αIgM	Pan-Ab αIgA
F												
G												
H												

$2.5x10^5$ cells/well

$5.0x10^5$ cells/well

2.3.4.2. Plate blocking

After washing 6 times with 200 µl/ well of PBS, plates were blocked with 200 µl/ well of R_{10} medium and incubated for 1 hour at 37°C in 5% CO_2.

2.3.4.3. Samples

Cells prepared to required concentrations were added at 100 µl/well as per the plate plan (Figure 7Figure 7). The plate was then incubated at 37°C in 5% CO_2 for 24 hours.

2.3.4.4. Plate development

Following incubation, cells were discarded and the plate wells washed 5 times with PBST. Anti-human IgG, IgM and IgA chain-specific alkaline phosphatase conjugate was diluted 1:5000 in R_{10} medium, and added to the plate at 100 µl per well as per Figure 7. Plates were then incubated for 4 hours at room temperature, before washing 5 times with PBST (200µl /well) followed by 2 washes with 200 µl /well of s.d. H_2O. The substrate, 5-bromo-4-chloro-3-indolyl phosphate in nitroblue tetrazolium dissolved in aqueous

dimethylformamide (Bio-Rad Laboratories, Hercules, USA; 170-6432), was added at 100 µl/ well. Spots were allowed to develop until the background started to darken. To stop the reaction 200 µl/well of water was added. Plates were dried in a drying oven, before counting using the AID ELISpot reader.

2.3.5. LPS ELISA

2.3.5.1. Plate coating

Plates were coated with 100 µl of 14.8 µg/ml of LPS in PBS with 0.1% sodium deoxycholate. This was prepared by mixing 18 µg of LPS (concentration, 10 mg/ml) in 12 ml of PBS with 120 µl of 10% sodium deoxycholate added. Plates were sealed and incubated at 4°C overnight.

2.3.5.2. Preparation of standard and quality control sera

A standard serum (nominal concentration of 30,000 units/ml) for the LPS ELISA was supplied by Emergent Biosolutions (UK) and was formed from pooled sera from participants vaccinated with M01ZH09, a live attenuated *S*. Typhi vaccine.

An intermediate positive control serum, with a nominal concentration of 300 units/ml was prepared by mixing 300 µl of immune sera with29700 ml of 1%BSA. Standard sera and quality control samples were prepared by diluting the intermediate positive control in 1% BSA, as per Table 7.

Table 7: Preparation of quality control sera from intermediate positive control sera for the use in an LPS ELISA to detect antibody in sera from participants challenged with S. Typhi in a human challenge model

	Nominal concentration (units/ml)	Volume of 300 units/ml intermediate positive control (µl)	Volume of 1% BSA (µl)	Total volume (µl)
High quality control	48	3200	16800	20000
Medium quality control	26	1716	18084	19800
Low quality control	14	924	18876	19800
Standard solution	1 ELISA unit	136	344	480

2.3.5.3. Plate blocking

After washing 5 times with PBST, 300µl per well of 1% BSA was added, and incubated at room temperature for 1 hour.

2.3.5.4. Sera

After washing the plate five times with PBST, 200 µl of test sera, diluted to a starting concentration of 1:50 in 1% BSA, were added to the first row of the plate and then doubly diluted in 1% BSA seven times to a final concentration of 1:6400 in 100 µl per well as per the plate plan (Figure 8). Standard sera (200 µl) was added and double diluted seven times in 1% BSA added at 100 µl per well. The high, medium and low quality control sera were added 100 µl per well. Blank control wells contained 100µl of 1% BSA alone. Sera were incubated for 90 minutes at room temperature.

Figure 8: Layout of a 96-well plate for ELISA assays to detect IgG, IgM and IgA to the Vi, H and LPS antigens of *S.* Typhi in serum samples from adult participants challenged with *S.* Typhi in the development of a challenge model of typhoid disease

	1, 2	3, 4	5,6	7, 8	9, 10	11, 12
a	Standard sera	Hi QC	Sample 1	Sample 2	Sample 3	Sample 4
b		Medium QC				
c		Low QC				
d		Blank				
e		Blank				
f		Blank				
g		Blank				
h		Blank				
QC = quality control, → = double dilute down plate, Blank = PBSTM alone						

2.3.5.5. Conjugate antibody

Plates were washed five times with PBST before adding 100 µl per well of conjugate antibody, diluted in 1% BSA to 1:20000 for anti-human IgG, and 1:5000 for anti-human IgA and IgM. Plates were incubated at room temperature for one hour.

2.3.5.6. Substrate

Plates were washed as before, 100 µl per well of TMB added to each well and incubated for 30 minutes at room temperature out of direct light. The reaction was stopped by adding 50 µl of 2M H_2SO_4 to each well. The optical density of each well was read at 450 nm using the plate reader.

2.3.6. Quantification of *S.* Typhi in blood

At the time of typhoid diagnosis, 10ml of whole blood was collected into an Isolator 10 tube (BC0507, Oxoid) containing purified saponin (28g/litre), a cell lysing agent, allowing release of intracellular *S.* Typhi, sodium polyanetholsulphonate (15.3g/litre) to

anticoagulate the sample and inhibit phagocytosis, and polypropylene glycol (8mg/litre) to inhibit foaming.

The sample was transported at room temperature and processed immediately upon receipt. The tube was centrifuged at 3000g for 30 minutes. Using an ISOSTAT supernatant pipette (BC0509; Oxoid), the supernatant was removed and discarded. The remaining sample was vortexed for 10 seconds and then plated in equal quantities on to two XLD agar plates (Oxoid; PO0164A) and spread using a sterile spreader. Plates were incubated at 37°C for 24 hours. Following incubation, the total number of colonies per plate was counted. Colonies morphologically in keeping with *S.* Typhi were confirmed as *S.* Typhi by slide agglutination.

2.4. Data management

Statistical analysis was performed using Prism version 5 (Graphpad Software, La Jolla, USA) and Excel 2010 (Microsoft, Washington, USA).

3. Assay development and validation

3.1. Development and validation of a Vi polysaccharide ELISA

3.1.1. Introduction

The importance of the anti-Vi antibody in protection against *S.* Typhi infection is demonstrated by the protective efficacy of the Vi-polysaccharide vaccine.[263,314,315] However, there is no standardised, validated ELISA for detection anti-Vi antibody. ELISA techniques have been used to detect the anti Vi response to the Vi vaccine[412-414] and to the Vi-conjugate vaccines[362], as well as for the detection of chronic carriers.[415] In this section the development of a Vi polysaccharide ELISA for measuring the changes in Vi antibodies that occurs during acute infection is described.

3.1.2. Protocol for the Vi polysaccharide ELISA

3.1.2.1. Plate coating

Nunc MaxiSorp flat-bottomed 96 well plates were coated with 100 µl per well of Vi antigen at a concentration of 1 µg/ml, prepared by adding 2.4 µl of Vi antigen to 12 ml of carbonate-bicarbonate buffer, per plate. Plates were incubated at 37°C for 3 hours before washing with 300 µl per well of PBST five times.

3.1.2.2. Plate blocking

To block non-specific antibody binding, plates were blocked with 300 µl per well of PBSTM, and incubated overnight at 4°C.

3.1.2.3. Sera

After washing five times with 300 µl per well of PBST, 200 µl of test sera, diluted to a starting concentration of 1:50 in PBSTM, were added to the first row of the plate and then serially diluted, in seven two fold dilutions, in PBSTM to a final concentration of 1:6400 in 100 µl per well (Figure 8). For IgG ELISAs, the initial standard sera dilution was 1:100 in PBSTM. For IgM and IgA ELISAs the starting dilution of the standard sera was 1:50. Standard sera (prepared as described in 3.1.3) was double diluted seven times in PBSTM and added at 100µl per well. Positive quality control sera (obtained as described in 3.1.3) were added at three dilutions to each plate at 100 µl per well. For the IgG ELISA, the dilutions were 1:100, 1:200 and 1:400, for the IgM ELISA, 1:25, 1:50 and 1:100 and for the IgA ELISA 1:50, 1:100 and 1:200. Blank control wells contained 100µl of PBSTM alone. Sera were incubated for 90 minutes at 37°C.

3.1.2.4. Detection antibody (IgG, IgM and IgA-HRP-conjugates)

Plates were washed five times with PBST before adding 100 µl per well of detection antibody, diluted in PBSTM to 1:20000 for anti-human IgG, and 1:5000 for anti-human IgA and IgM. Plates were incubated at 37°C for one hour.

3.1.2.5. Substrate

Plates were washed as before, and 100 µl of TMB added to each well and incubated for 30 minutes at room temperature out of direct light. The reaction was stopped by adding 50 µl of 2M H_2SO_4 per well and the optical density at 450 nm (O.D 450-nm) measured.

3.1.3. Vi ELISA development

The protocol described above was developed by determining in turn the optimal antigen coating concentration, blocking agent, detection antibody dilution and serum dilutions. Specificity was determined by pre-adsorbing sera with the Vi antigen. The inter-plate and day to day intra-assay variation was also established.

3.1.3.1. Standards and controls

A standard serum for the Vi ELISA was created by combining post-vaccination sera from 6 individuals, vaccinated for occupational health and travel reasons between 4 and 6 weeks prior to obtaining sera. Pooled sera were mixed overnight at 4°C. The first dilution of the standard curve was assigned a value of 1 ELISA unit. A positive quality control (QC) serum was obtained from an individual vaccinated 4 weeks prior to sampling with the Vi polysaccharide vaccine. For both standard and QC sera, anonymised, 100μl aliquots were prepared and frozen at -20°C until required. Defrosted aliquots were kept at 4°C for a maximum of 7 days.

3.1.3.2. Determining optimal antigen coating concentration

Vi antigen was diluted in carbonate-bicarbonate buffer to give three different concentrations of 1 μg/ml, 2 μg/ml and 4 μg/ml for comparison and 100 μg/well added. The ELISA was performed as described in section 3.1.2. Serum from a recently vaccinated individual was compared at a dilution of 1:25 and 1:50 in duplicate wells. Blank control wells that contained no serum were also included. The mean O.D 450-nm value obtained for each serum at each antigen coating concentration was compared.

3.1.3.3. Determining optimal blocking agent

Blocking with 1% and 5% milk was compared with 1% and 5% BSA. Each blocking agent was added at 300 μL per well to three columns of the plate. The ELISA was performed as in section 3.1.2. Serum from both a non-vaccinated individual and the positive control were diluted 1:200 and tested in duplicate for each blocking agent. The mean O.D 450-nm values obtained under each blocking condition were compared.

3.1.3.4. Determining the optimal detection antibody dilutions

The detection antibodies, goat anti-human-IgG, -IgM and –IgA-HRP, were diluted at 1:5000, 1:10000, 1:15000, 1:20000, 1:30000 and 1:40000 in PBSTM. Serum from a non-

vaccinated and a positive control was diluted at 1:200. The mean O.D 450-nm values from the 8 repeats for each HRP-conjugated detection antibody dilution were then compared.

3.1.3.5. Development the optimal starting concentration of standard serum

The starting dilution of the standard serum was determined by comparing the O.D-450nm resulting from dilutions of 1:10, 1:25, 1:50, 1:100, 1:200, 1:400, 1:800 and 1:1600 of the standard serum in PBSTM. Serum from a non-vaccinated individual was tested at the same dilutions as a negative control. The ELISAs were performed in duplicate as described in section 3.1.2. The mean O.D 450-nm values were used to generate standard curves, which were then compared.

3.1.3.6. Determining the optimum starting dilution for test sera

The optimal starting dilution for all test sera was determined by comparing sera from two recently vaccinated individuals, plus an individual from an endemic country, to give a range of results that may be expected in participants challenged with *S*. Typhi in the model. Serial double dilutions of the sera, starting at a 1:50, were tested in duplicate for each of the detection antibodies and the mean O.D 450-nm values compared. The ELISA was performed as in section 3.1.2.

3.1.3.7. Determining specificity of the ELISA

To determine specificity, a serum pre-adsorbtion assay was used in which the positive control serum was pre-incubated with increasing amounts of Vi antigen, allowing the anti-Vi antibody to be adsorbed by antigen, resulting in decreasing O.D 450-nm when used in the ELISA. A negative serum was used as a control. The Vi antigen concentrations 5 μg/ml, 10 μg/ml, 20 μg/ml, 40 μg/ml, 80 μg/ml and 160 μg/ml were prepared in s.d. H_2O and mixed in equal volume with sera diluted at 1:50 and incubated for 2 hours at room temperature. The ELISA was then performed as in section 3.1.2. The assay was performed in duplicate wells and the mean O.D 450-nm values calculated.

3.1.3.8. Intra-plate variation

To determine the cross-plate variation in O.D 450-nm, serum from a recently vaccinated individual was diluted 1:200 in PBSTM and added to each well of the plate. The rest of the assay was described in 3.1.2. The mean O.D 450-nm across the plate was calculated, along with the standard deviation and co-efficient of variation.

3.1.3.9. Inter-plate validation

To determine the day to day variability of the ELISA, serum from a recently immunised individual was tested on 5 different days at dilutions of 1:50, 1:100 and 1:200. The rest of the assay was as described in section 3.1.2. The mean O.D 450-nm of the duplicate wells was calculated. The mean O.D 450-nm over 5 days was then calculated along with the standard deviation and co-efficient of variation.

3.1.4. Results

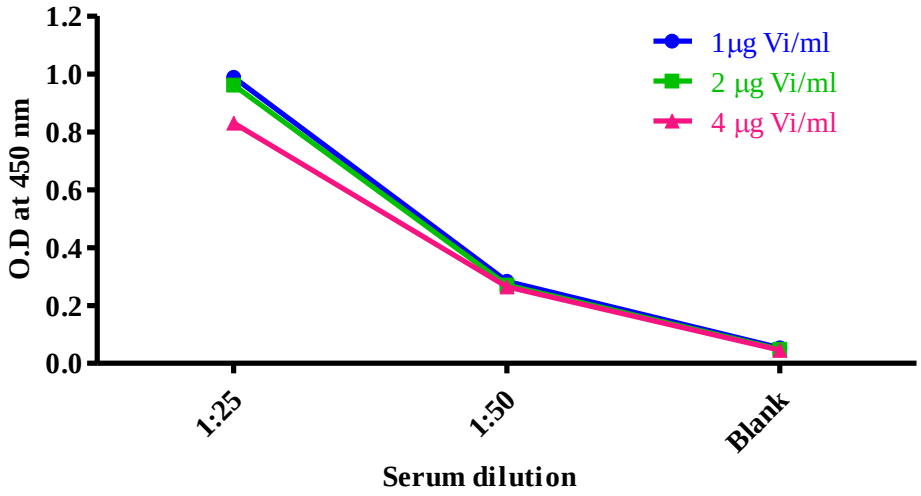
3.1.4.1. Optimal antigen coating concentration

The O.D 450-nm of a serum tested on 3 different coating concentrations of Vi antigen can be seen in Table 8 and Figure 9.

Table 8: Comparison of the O.D 450 nm obtained using 3 different Vi antigen coating concentrations with serum at a 1:25 and 1:50 dilution, and blank control in the development of a Vi ELISA

	Mean O.D 450 nm of duplicate wells		
	Vi antigen coating concentration		
	1 μg/ml	2 μg/ml	4 μg/ml
1:25 serum dilution	0.989	0.962	0.831
1:50 serum dilution	0.284	0.270	0.265
Blank control (no sera)	0.054	0.049	0.046

Figure 9: Comparison of the O.D 450 nm obtained using 3 different Vi antigen coating concentrations with serum at a 1:25 and 1:50 dilution, and blank control in the development of a Vi ELISA



There was little difference in OD obtained for serum tested on antigen coating concentrations of 1 µg/ml and 2 µg/ml. With a coating concentration of 4 µg/ml there was a slight decrease in O.D 450 nm, possibly due to unbound antigen binding to and adsorbing antibody out of solution before it could bind to the plate. The lowest antigen concentration of 1 µg/ml was therefore used.

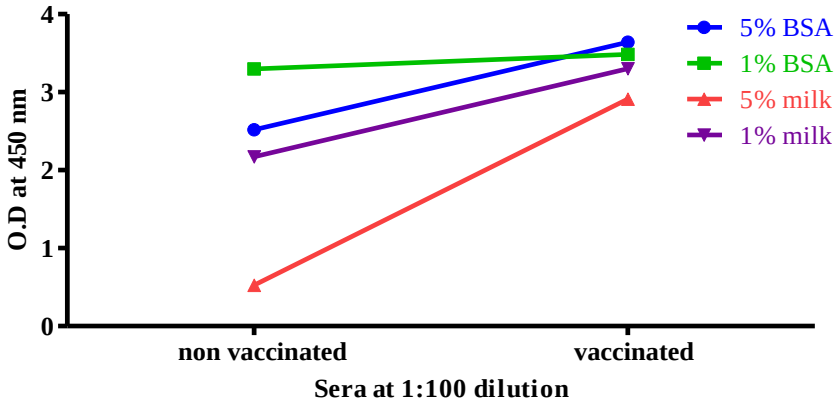
3.1.4.2. Optimal blocking agent

The O.D 450 nm values obtained for both a positive control and a non-vaccinated control serum, diluted at 1:100 tested on plates blocked with different blocking buffers can be seen in Table 9 and Figure 10.

Table 9: Comparison of the O.D 450 nm obtained using 4 blocking preparations with serum at a 1:100 dilution from a non-vaccinated and vaccinated individual in the development of a Vi ELISA

Blocking preparation	Non –vaccinated	Positive control
	Mean O.D 450nm of duplicate wells	
5% BSA	2.52	3.64
1% BSA	3.30	3.48
5% milk	0.53	2.91
1% milk	2.17	3.30

Figure 10: Comparison of the O.D 450 nm obtained using 4 blocking preparations with serum at a 1:100 dilution from a non-vaccinated and vaccinated individual in the development of a Vi ELISA



The optimum blocking agent was 5% milk as this gave the best differentiation between sera with high antibody levels (post-vaccination sera) and low antibody levels (non-vaccinated sera).

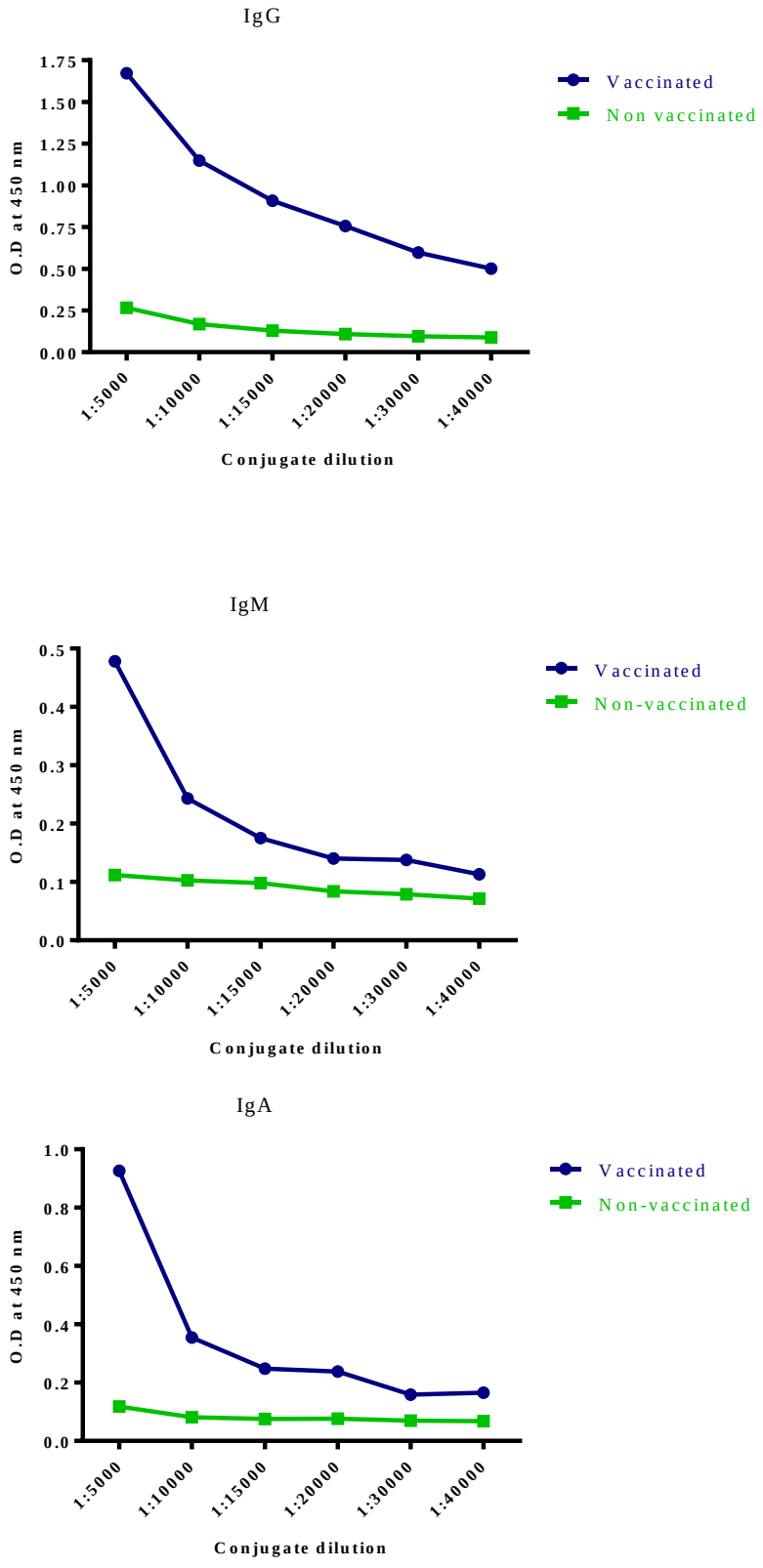
3.1.4.3. Optimum detection antibody concentration

The variation in OD-450 nm with the different detection antibody dilutions can be seen in Table 10 and Figure 11.

Table 10: The effect of detection antibody dilution on the mean O.D 450 nm of duplicate wells, obtained for sera (1:200) from a vaccinated and a non-vaccinated individual in the development of a Vi ELISA

Conjugate antibody	Serum source	Detection antibody dilution					
		1:5000	1:10000	1:15000	1:20000	1:30000	1:40000
		Mean O.D 450nm of duplicate wells					
IgG	Vaccinated	1.67	1.15	0.91	0.76	0.60	0.50
	Non-vaccinated	0.27	0.17	0.13	0.11	0.10	0.09
IgM	Vaccinated	0.48	0.24	0.18	0.14	0.14	0.11
	Non-vaccinated	0.12	0.10	0.10	0.08	0.08	0.07
IgA	Vaccinated	0.93	0.35	0.25	0.24	0.16	0.17
	Non-vaccinated	0.11	0.10	0.08	0.08	0.07	0.07

Figure 11: The effect of detection antibody dilution on the mean O.D 450 nm of duplicate wells, obtained for sera (1:200) from a vaccinated and a non-vaccinated individual in the development of a Vi ELISA



A clear difference in the OD-450nm of post-vaccination serum and non-vaccinated serum was achieved with all of the detection antibodies. A dilution of 1:20000 (anti-IgG); 1:5000 (anti-IgM and IgA) were chosen for use in the ELISAs. At these dilutions, blank wells had an O.D-450 nm of less than 0.1.

3.1.4.4. Development of the standard curves

The O.D- 450 nm values for each standard serum dilution following detection with either anti-IgG, IgM or IgA are shown in Table 11 and Figure 12.

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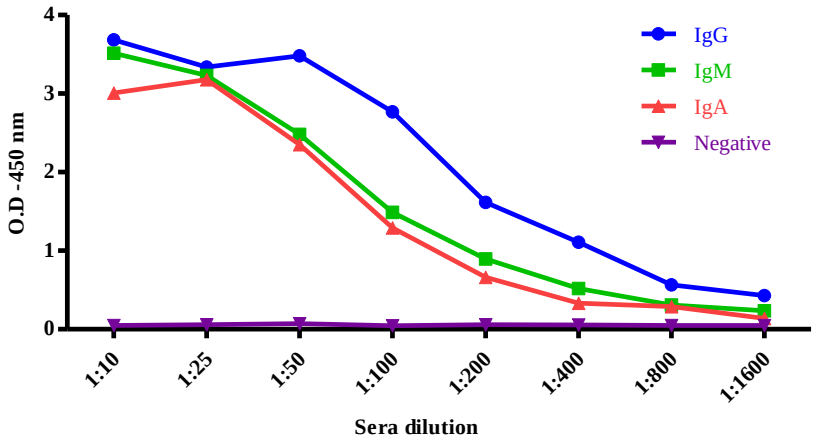
Table 11: Change in mean O.D- 450 nm of duplicate wells with progressive dilutions of standard serum from 1:10 to 1:1600 for each of the detection antibodies in the development of a Vi ELISA

Dilution of standard sera	Detection antibody		
	IgG	IgM	IgA
	Mean O.D at 450nm of duplicate wells		
1:10	3.69	3.51	3.01
1:25	3.34	3.23	3.18
1:50	3.48	2.48	2.35
1:100	2.77	1.49	1.29
1:200	1.61	0.90	0.66
1:400	1.11	0.52	0.33
1:800	0.57	0.31	0.29
1:1600	0.43	0.24	0.14

Figure 12: Change in mean O.D- 450 nm of duplicate wells with progressive dilutions of standard serum from 1:10 to 1:1600 for each of the detection antibodies in the development of a Vi ELISA



There is evidence of pro-zoning (falsely low results resulting from excess serum antibody being unable to bind proportionately to the coating antigen). Dilution of the serum overcomes this, resulting in increasing OD values until dilutions of 1:50 for IgG and 1:25 for IgM and IgA. Hence the optimal starting concentration for the standard serum for IgG was 1:100, for IgM 1:25 and IgA 1:50.

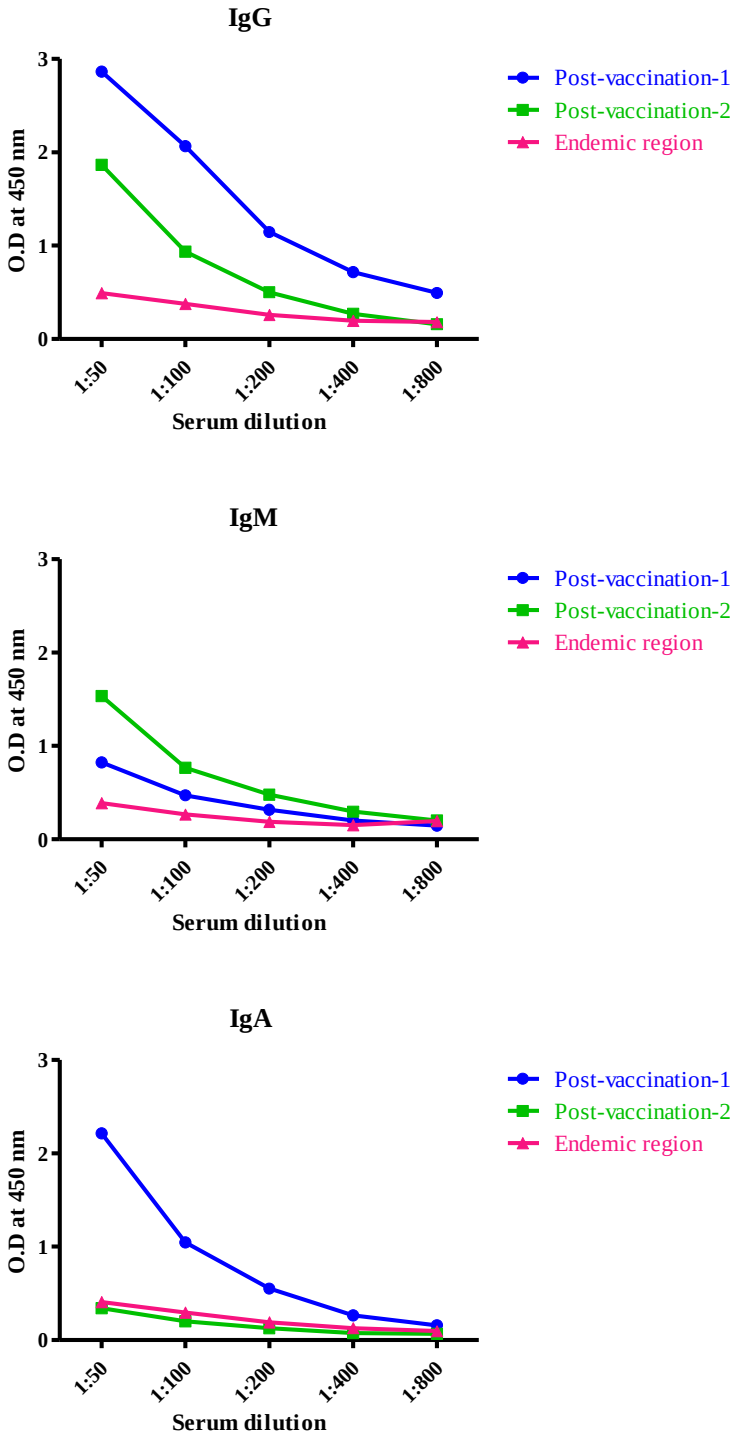
3.1.4.5. Optimal dilution of test sera

The change in O.D-450 nm with progressive dilutions of 3 different sera with each detection antibody is shown in Table 12 and Figure 13.

Table 12: Comparison of the mean OD-450 nm of duplicate wells for 3 sera, (2 post vaccination, and 1 from a previous resident of a typhoid endemic country), at progressive dilutions from 1:50 to 1:800 for each of the detection antibodies used in the development of a Vi ELISA

Antibody	Sera	Serum dilution				
		1:50	1:100	1:200	1:400	1:800
		Mean O.D-450 nm of duplicate wells				
IgG	Post-vaccination -1	2.86	2.07	1.15	0.72	0.49
	Post vaccination -2	1.87	0.94	0.5	0.49	0.16
	Endemic region	0.49	0.38	0.26	0.20	0.18
IgM	Post-vaccination -1	0.824	0.471	0.317	0.201	0.147
	Post vaccination -2	1.534	0.767	0.48	0.297	0.2
	Endemic region	0.388	0.266	0.188	0.152	0.196
IgA	Post-vaccination -1	2.215	1.046	0.55	0.263	0.159
	Post vaccination -2	0.342	0.2	0.125	0.076	0.066
	Endemic region	0.407	0.294	0.192	0.128	0.096

Figure 13: Comparison of the mean OD-450 nm of duplicate wells for 3 sera, (2 post vaccination, and 1 from a previous resident of a typhoid endemic country), at progressive dilutions from 1:50 to 1:800 for each of the detection antibodies used in the development of a Vi ELISA



For all Ig isotypes, serial dilution of serum from a starting dilution of 1:50 generated OD values that fell within the range of the standard curve, therefore this was chosen as the

starting dilution. IgM antibody concentrations were lower than that seen for IgG and IgA, which may be explained by a long term immune response expected from repeated vaccination or long term residence in an endemic country. The IgM response in immunologically naïve challenged participants is likely to be higher.

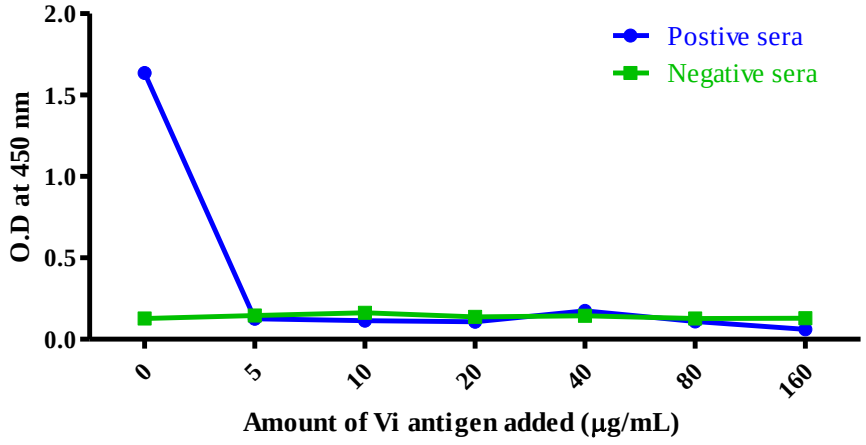
3.1.4.6. Specificity

Specificity, as determined by pre-adsorption of test sera with Vi antigen, is shown in Table 13 and Figure 14.

Table 13: ELISA specificity shown by the decrease in mean OD-450 nm when Vi antigen is added to the serum prior to the ELISA, pre-adsorbing the antibody present in the positive serum, in the validation of a Vi ELISA

	Amount of Vi antigen used for pre-adsorption (µg/ mL)						
	0	**5**	**10**	**20**	40	80	160
	O.D-450 nm						
Positive sera	1.636	0.126	0.114	0.107	0.175	0.11	0.061
Negative sera	0.128	0.146	0.162	0.137	0.144	0.127	0.13

Figure 14: ELISA specificity shown by the decrease in mean OD-450 nm when Vi antigen is added to the serum prior to the ELISA, pre-adsorbing the antibody present in the positive serum, in the validation of a Vi ELISA



The ELISA had high specificity, shown by the marked decline in O.D 450 nm when small amounts of Vi antigen are added to the positive serum. The negative serum did not pre-adsorb, due to the absence of Vi antibody.

3.1.4.7. Intra-plate variation

Results of the intra-plate variability are shown in Table 14.

Table 14: Validation of intra-plate variability performed on post vaccination serum diluted 1:50 across one plate for each of IgG, IgM and IgA. The standard deviation and co-efficient for each plate are shown

	Conjugate antibody		
	IgG	**IgM**	**IgA**
Average O.D-450 nm	0.876	0.401	0.363
Standard deviation	0.069	0.38	0.27
Co-efficient of variability (%)	7.98	9.55	7.62

There was very little intra-plate variation for all 3 assays, confirming assay precision.

3.1.4.8. Inter-plate validation

Variability in the O.D- 450 nm readings for sera at a 1:50, 1:100 and 1:200 dilution over 5 days with each of the conjugate antibodies is shown in Table 15.

Table 15: Inter-plate variation over 5 days for sera at 3 dilutions with IgG, IgM and IgA detection antibodies. The mean OD-450 nm over 5 days, along with the standard deviation and co-efficient of variation are shown

Day	IgG			IgM			IgA		
	Serum dilution								
	1:50	1:100	1:200	1:50	1:100	1:200	1:50	1:100	1:200
Day 1	0.53	0.26	0.14	0.48	0.27	0.15	0.53	0.26	0.14
Day 2	0.59	0.26	0.13	0.49	0.26	0.13	0.59	0.26	0.13
Day 3	0.52	0.27	0.13	0.53	0.27	0.14	0.52	0.27	0.13
Day 4	0.54	0.26	0.13	0.50	0.26	0.15	0.54	0.26	0.13
Day 5	0.54	0.28	0.13	0.51	0.26	0.14	0.54	0.28	0.13
Mean	0.54	0.26	0.13	0.50	0.26	0.14	0.54	0.26	0.13
Standard deviation	0.03	0.01	0.01	0.02	0.00	0.01	0.03	0.01	0.01
Coefficient of variability (%)	5.09	2.80	4.41	3.88	0.82	4.90	5.09	2.80	4.41

Variability in the assay over 5 days was low, confirming the validity of the assay.

3.1.5. Discussion

To develop a Vi polysaccharide ELISA to measure the IgG, IgM and IgA response to challenge, the optimal coating concentration, blocking agent and conjugate antibody dilutions were determined. A standard serum and positive QC serum was formed and starting dilutions for each sera for each conjugate antibody determined. Specificity was confirmed by a pre-adsorbance assay. The accuracy and precision of the assay was validated by determining intra-plate and inter-plate variability.

3.2. Development and validation of a H (flagellin) ELISA

3.2.1. Introduction

Antibodies to the H (flagellin) antigen of *S*. Typhi rise following acute infection. This, along with changes in antibodies to the O (lipopolysaccharide) antigen forms the basis of the diagnostic Widal test.[142] Anti- H antibodies were also suggested as a correlate of protection in early typhoid challenge studies[73] and field trials of killed whole-cell vaccines.[84] There is no standardised ELISA for measuring antibody responses to the H antigen. This section describes the development of such an ELISA for the assessment of the kinetic response following challenge with *S*. Typhi in the development of a human challenge model.

3.2.2. Protocol for the H ELISA

3.2.2.1. Plate coating

Nunc MaxiSorb 96 well flat bottomed plates were coated with 100µl per well of H antigen at a concentration of 10µg/ml, prepared by adding 2.4µl of H antigen to 12 ml of carbonate-bicarbonate buffer per plate. Plates were incubated at 4°C overnight.

3.2.2.2. Plate blocking

After washing with 300µl per well of PBST five times, 300µl per well of 1% FCS was added, and incubated for 1 hour at room temperature.

3.2.2.3. Test serum, QC and standard serum dilutions

After washing five times with PBST, 200 µl of test sera, diluted to a starting concentration of 1:50 in 1% FCS, were added to the first row of the plate and then doubly diluted in 1% FCS seven times to a final concentration of 1:6400 in 100 µl per well as per the plate plan

(Figure 8). For IgG ELISAs, the standard serum starting dilution was 1:100 in 1% FCS. For IgM ELISAs the starting dilution was 1:50, and for IgA ELISAs 1:25. The standard serum was double diluted seven times in 1% FCS and added at 100µl per well. The Positive QC was added at 1:50, 1:100 and 1:200 to each plate at 100 µl per well. Blank control wells contained 100µl of 1% FCS alone. The sera were incubated for 90 minutes at room temperature.

3.2.2.4. Detection antibodies (anti-IgG, IgM and IgA)

Plates were washed five times with PBST before adding 100 µl per well of detection antibody diluted in 1% FCS to 1:20000 for anti-human IgG, and 1:5000 for anti-human IgA and IgM. Plates were incubated at room temperature for one hour.

3.2.2.5. Substrate

Plates were washed as before, and 100 µl per well of TMB added to each well and incubated for 30 minutes at room temperature out of direct light. The reaction was stopped by adding 50 µl of 2M H_2SO_4 to each well. The optical density of each well was read at 450 nm using the plate reader.

3.2.3. H ELISA development

The protocol described above was developed following the same steps as described for the Vi ELISA.

3.2.3.1. Standards and controls

A standard serum was prepared by combining sera obtained post-challenge from 10 participants in the clinical study by overnight mixing at 4°C. Aliquots of 100µl were prepared and frozen at -20°C until required. Defrosted aliquots were kept at 4°C for a maximum of 7 days. The first dilution of the standard curve was assigned a value of 1 ELISA unit. A positive QC serum was obtained from an individual with acute typhoid disease.

3.2.3.2. Optimal antigen coating concentration

H antigen was diluted in carbonate-bicarbonate buffer to give a starting concentration of 20 μg/ml. From this a further 5 concentrations were prepared by serial double dilution to a final concentration of 0.75 μg/ml. Each of the 6 concentrations was added to 2 columns of the plate at 100 μg/well. Three separate assay buffers (1% BSA, 1% FCS, 5% milk) were used for blocking, sera dilution and conjugate antibody dilution. Positive QC was used at a 1:50 dilution. Anti-human IgG was used as a conjugate at a 1:10000 dilution. Other steps were as described in 3.2.2. Assays were performed in duplicate. The mean O.D-450nm obtained with each concentration were compared.

3.2.3.3. Optimal blocking agent

To determine the optimal blocking agent, 5% PBSTM, 1% BSA and 1% FCS were compared. Each blocking agent was added at 300 μl per well to three columns of the plate. Anti-human IgG was used as a conjugate at a 1:10000 dilution. The rest of the assay was performed as described in 3.2.2. Sera from a non-vaccinated individual and the positive QC serum were diluted to 1:200 and assayed in duplicate for each blocking agent. The mean ODs were compared.

3.2.3.4. Development of a standard curve and optimal detection antibody dilution

Sera obtained from 4 participants diagnosed with typhoid disease were pooled and mixed overnight at 4^{o}C. Starting dilution for the standard sera was determined by comparing serial double dilutions of 1:25 to 1:3200. Anti-human IgG, IgA and IgM γ-chain specific detection antibodies were diluted at 1:5000, 1:10000, 1:20000, 1:40000, 1:80000 and 1:100000 and the O.D-450 nm resulting from each sera and detection antibody dilution were compared. Assays were performed in duplicate and the mean O.D-450 nm compared.

3.2.3.5. Optimum starting dilutions for test sera

Optimal starting dilutions for test sera were determined by serial double dilutions of the sera from two recently vaccinated individuals, and an individual from an endemic country, starting at a 1:50 dilution, in duplicate. The rest of the protocol was as described in section 3.2.2.

3.2.3.6. Determining specificity

H-ELISA specificity was determined by a pre-adsorbance assay, as described for Vi-ELISA. H antigen dilutions of 5 μg/ml, 10 μg/ml, 20 μg/ml, 40 μg/ml, 80 μg/ml and 160 μg/ml concentrations were prepared in s.d. H_2O. A serum dilution of 1:25 was prepared and mixed with equal volumes of each of the H antigen dilutions. These were incubated for 2 hours at room temperature. The ELISA was then preformed as described in section 3.2.2. The assay was performed in duplicate and the mean O.D 450 nm values calculated.

3.2.3.7. Intra-plate variation

To determine the intra-plate variation in O.D-450 nm, serum from a recently vaccinated individual was diluted at 1:200 in 1% FCS and added to each well of the plate. The rest of the assay was as described in section 3.2.2.

3.2.3.8. Inter-plate validation

To determine the day to day variability in the ELISA assay the standard curve and positive control sera were assayed over 5 days to determine the variation in O.D-450 nm.

3.2.4. Results

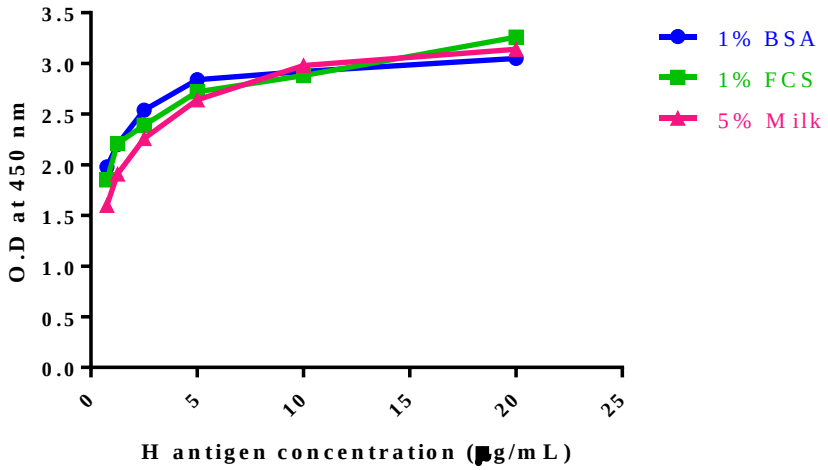
3.2.4.1. Optimal H antigen coating concentration

Comparison of the O.D450 nm generated at the 6 test concentrations of H antigen can be seen in Table 16 and Figure 15.

Table 16: Comparison of the O.D 450 nm obtained from test serum diluted 1:50 using different H antigen coating concentrations and assay buffers, in the development of an H ELISA

H antigen concentration	Assay buffer		
	1% BSA	1% FCS	5% Milk
	Average O.D at 450 nm of duplicate wells		
20 µg/ ml	3.05	3.26	3.14
10 µg/ ml	2.92	2.88	2.98
5 µg/ ml	2.84	2.72	2.64
2.5 µg/ ml	2.54	2.39	2.26
1.25 µg/ ml	2.20	2.21	1.91
0.75 µg/ ml	1.98	1.85	1.60

Figure 15: Comparison of the O.D 450 nm obtained from test serum diluted 1:50 using different H antigen coating concentrations and assay buffers, in the development of an H ELISA



At concentrations less than 10 µg/ml, there is insufficient antigen so that the antigen becomes the limiting factor in the ELISA, shown by the decline in O.D-450 nm with each decrease in antigen concentration below 10 µg/ml. There is little difference between the 10 µg/ml and 20 µg/ml concentrations. Therefore 10 µg/ml was used to coat the ELISA plates. The blocking buffer appeared to have no effect on the optimal antigen coating concentration.

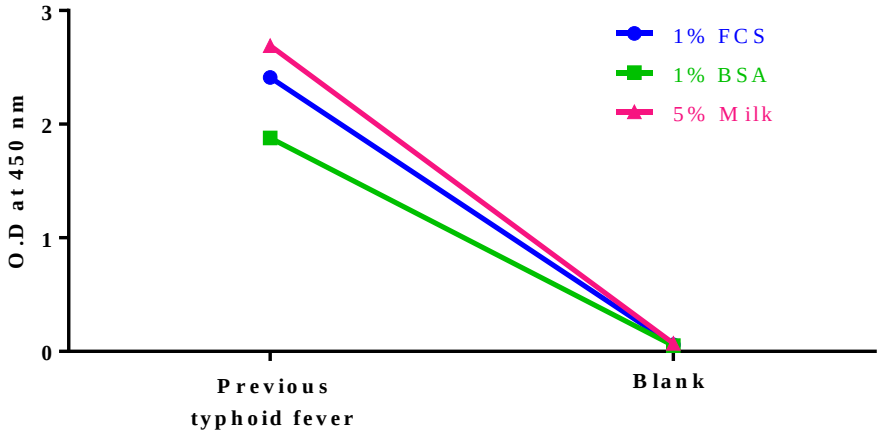
3.2.4.2. Optimal blocking agent

Comparison of the mean O.D-450 nm of duplicate wells obtained using serum from an individual with previous typhoid disease and blank control wells with 3 different blocking agents is shown in Table 17 and Figure 16.

Table 17: Comparison of the effect of 3 different blocking preparations on the O.D-450nm obtained from serum from an individual with previous typhoid disease and blank control wells in the development of an H antigen ELISA

Blocking preparation	Previous typhoid disease	Blank (no sera)
	Average O.D at 450 nm of duplicate wells	
1% FCS	2.41	0.06
1% BSA	1.88	0.05
5% milk	2.69	0.07

Figure 16: Comparison of the effect of 3 different blocking preparations on the O.D-450nm obtained from serum from an individual with previous typhoid disease and blank control wells in the development of an H antigen ELISA



Blank wells had low O.D-450 nm with all 3 blocking agents. FCS and milk blocking agents gave the highest O.D-450 nm for the positive sera. FCS was chosen as the blocking agent due to compatibility with the plate washing equipment.

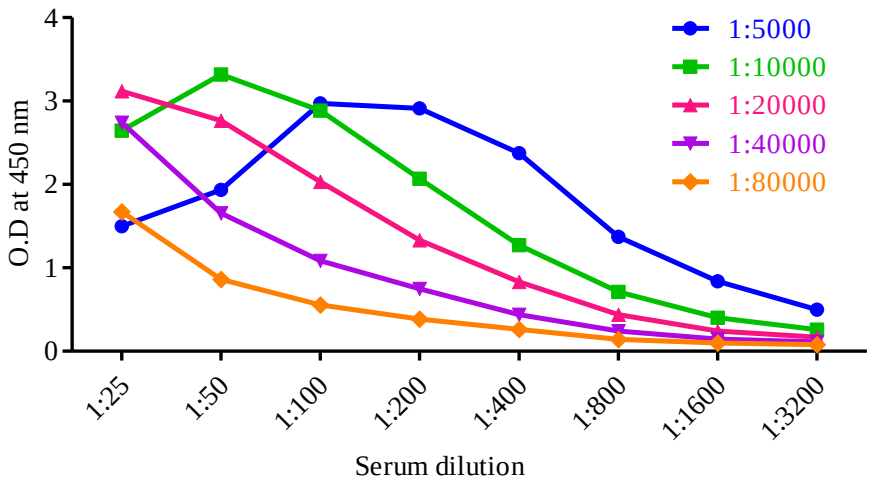
3.2.4.3. Development of a standard curve and optimal detection antibody dilution

Variation in O.D-450 nm with different dilutions of standard sera and anti-IgG detection antibody dilutions can be seen in Table 18 and Figure 17.

Table 18: Effect of anti-IgG detection antibody dilution on the O.D-450nm obtained for standard serum dilution curves in the development of an H ELISA

Serum dilution	Detection antibody dilution				
	1:5000	1:10000	1:20000	1:40000	1:80000
	O.D-450 nm				
1:25	1.497	2.645	3.118	2.743	1.673
1:50	1.934	3.320	2.766	1.654	0.862
1:100	2.970	2.884	2.032	1.086	0.555
1:200	2.910	2.068	1.330	0.749	0.384
1:400	2.376	1.272	0.831	0.440	0.262
1:800	1.373	0.712	0.440	0.242	0.143
1:1600	0.838	0.403	0.243	0.144	0.099
1:3200	0.498	0.258	0.169	0.111	0.079

Figure 17: Effect of anti-IgG detection antibody dilution on the O.D-450nm obtained for standard serum dilution curves in the development of an H ELISA



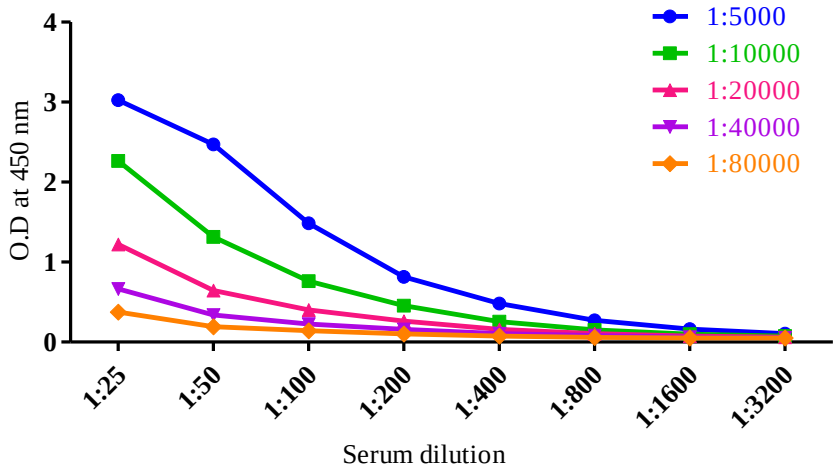
There was evidence of pro-zoning with detection antibody dilutions of ≤1:20000. A 1:100 dilution of standard serum in conjunction with 1:20000 dilution of detection antibody generated a high OD-450 nm without pro-zoning. Therefore, a 1:20000 anti-IgG conjugate dilution and a starting dilution of 1:100 of the standard sera was chosen.

Variation in O.D with different dilutions of standard sera and anti-IgM detection antibody dilutions can be seen in Table 19 and Figure 18.

Table 19: Effect of anti-IgM detection antibody dilution on the O.D-450nm obtained for standard serum dilution curves in the development of an H ELISA

Serum dilution	Detection antibody dilution				
	1:5000	1:10000	1:20000	1:40000	1:80000
	O.D-450 nm				
1:25	3.0265	2.2665	1.222	0.665	0.374
1:50	2.473	1.3145	0.6465	0.339	0.191
1:100	1.486	0.7625	0.4015	0.2235	0.1405
1:200	0.8165	0.4545	0.261	0.158	0.103
1:400	0.481	0.2565	0.157	0.104	0.075
1:800	0.2715	0.1505	0.104	0.081	0.06
1:1600	0.1625	0.0995	0.0765	0.061	0.053
1:3200	0.1055	0.0745	0.0605	0.0545	0.05

Figure 18: Effect of anti-IgM detection antibody dilution on the O.D-450nm obtained for standard serum dilution curves in the development of an H ELISA



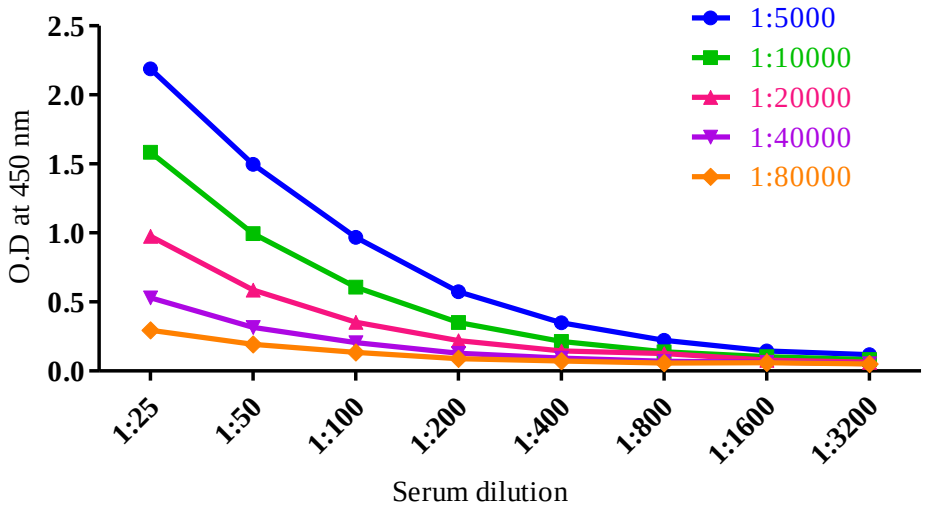
Highest OD-450 nm values were achieved with an anti-IgM detection antibody dilution of 1:5000. A starting dilution of 1:50 for the standard serum ensured good linearity of the standard curve, and therefore these dilutions were used in further experiments.

Variation in O.D with different dilutions of standard sera and anti-IgA detection antibody dilutions can be seen in Table 20 and Figure 19.

Table 20: Effect of anti-IgA detection antibody dilution on the O.D-450nm obtained for standard serum dilution curves in the development of an H ELISA

Serum dilution	Detection antibody dilutions				
	1:5000	1:10000	1:20000	1:40000	1:80000
	O.D-450 nm				
1:25	2.189	1.585	0.975	0.5305	0.295
1:50	1.497	0.9945	0.587	0.3165	0.1935
1:100	0.968	0.6075	0.353	0.2045	0.1335
1:200	0.574	0.352	0.219	0.1285	0.089
1:400	0.35	0.214	0.144	0.093	0.0725
1:800	0.2215	0.139	0.126	0.0705	0.0565
1:1600	0.145	0.103	0.0775	0.0625	0.0595
1:3200	0.118	0.082	0.064	0.053	0.0505

Figure 19: Effect of anti-IgA detection antibody dilution on the O.D-450nm obtained for standard serum dilution curves in the development of an H ELISA



A 1:5000 dilution of anti-IgA conjugate antibody and a 1:25 starting dilution for the standard curve gave the highest O.D-450 nm, with good linearity of the standard curve, and therefore these dilutions were used in the assay.

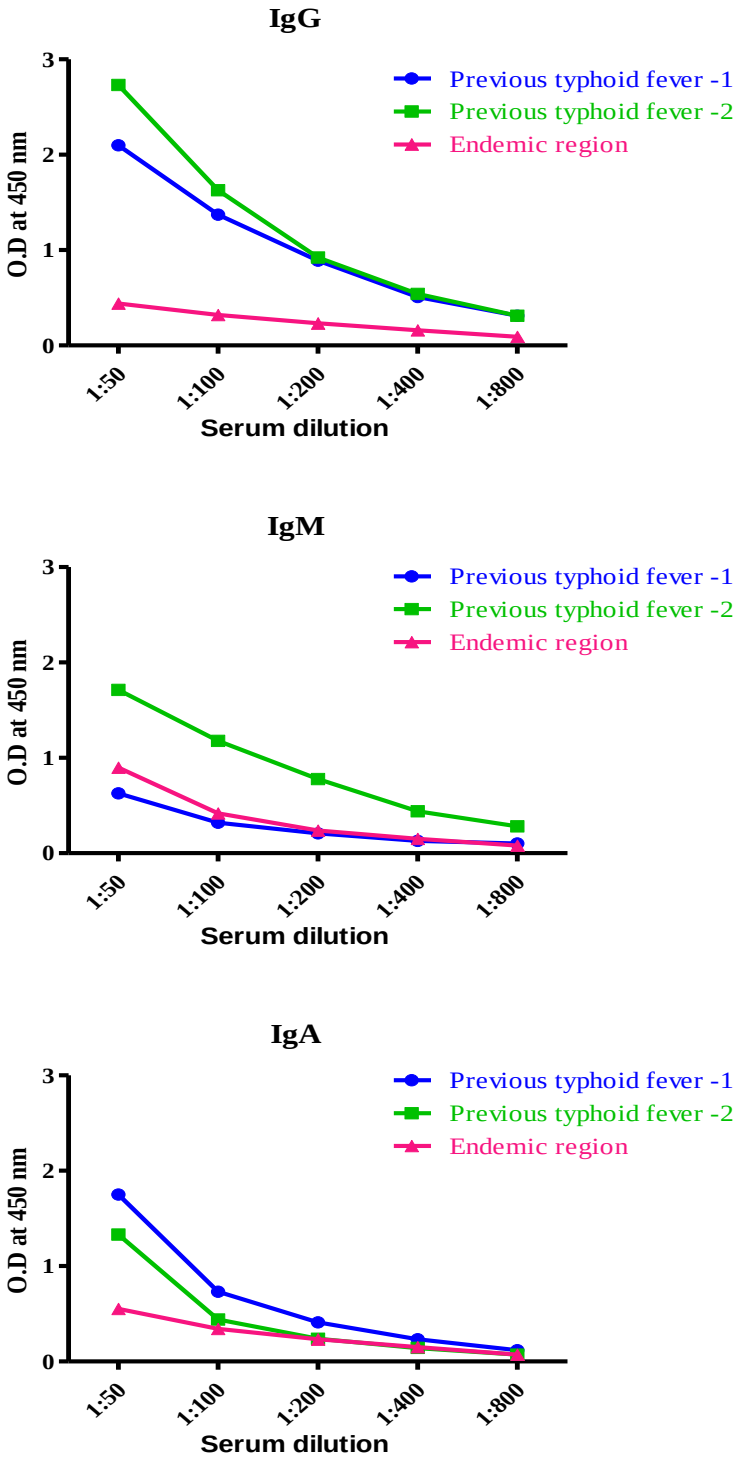
3.2.4.4. Optimal dilution of test sera

The change in O.D-450 nm with progressive sera dilutions for each conjugate antibody is shown in Table 21 and Figure 20.

Table 21: Comparison of the OD-450 nm from 3 sera (2 from people with a history of confirmed typhoid disease in the previous month and 1 from someone previously resident in a typhoid endemic country) at progressive dilutions from 1:50 to 1:800, with IgG, IgM and IgA detection antibodies in the development of an H ELISA

Antibody	Sera	Sera dilution				
		1:50	1:100	1:200	1:400	1:800
		O.D-450 nm				
IgG	Previous typhoid disease -1	2.10	1.37	0.89	0.51	0.31
	Previous typhoid disease -2	2.73	1.63	0.92	0.54	0.31
	Endemic region	0.44	0.32	0.23	0.16	0.09
IgM	Previous typhoid disease -1	0.628	0.32	0.21	0.13	0.10
	Previous typhoid disease -2	1.71	1.18	0.78	0.44	0.28
	Endemic region	0.90	0.42	0.24	0.15	0.08
IgA	Previous typhoid disease -1	1.75	0.73	0.41	0.23	0.12
	Previous typhoid disease -2	1.33	0.44	0.24	0.14	0.07
	Endemic region	0.55	0.34	0.23	0.15	0.07

Figure 20: Comparison of the OD-450 nm from 3 sera (2 from people with a history of confirmed typhoid disease in the previous month and 1 from someone previously resident in a typhoid endemic country) at progressive dilutions from 1:50 to 1:800, with IgG, IgM and IgA detection antibodies in the development of an H ELISA



For all iso-types, serial dilution from an initial dilution of 1:50 gave a reading within the range of the standard curve, so this was chosen as the starting concentration.

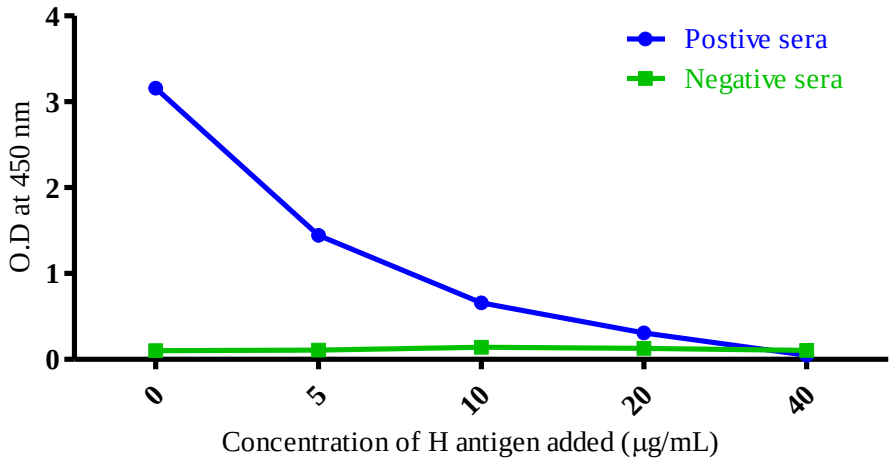
3.2.4.5. Specificity

Results for the pre-adsorbtion specificity experiment are shown in Table 22 and Figure 21.

Table 22: Demonstration of ELISA specificity using a pre-adsorption assay, showing decreasing OD- 450 nm when H antigen is added to the serum prior to the ELISA, pre-adsorbing the antibody in the positive serum, but not affecting the negative serum

Serum used	Concentration of H antigen used for preadsorbtion (µg/ ml)				
	0	5	10	20	40
	O.D-450 nm				
Positive sera	3.157	1.446	0.658	0.307	0.047
Negative sera	0.1025	0.10875	0.14	0.1285	0.106

Figure 21: Demonstration of ELISA specificity using a pre-adsorption assay, showing decreasing OD- 450 nm when H antigen is added to the serum prior to the ELISA, pre-adsorbing the antibody in the positive serum, but not affecting the negative serum



The ELISA had high specificity, shown by the marked decline in O.D 450 nm when a small amount of H antigen was added to the positive serum. The negative serum did not pre-adsorb, demonstrating that there was no non-specific binding.

3.2.4.6. Intra-plate variation

Results of the intra-plate variability are shown in Table 23.

Table 23: Validation of intra-plate variability, performed on post vaccination serum at a 1:50 dilution across one plate for each of IgG, IgM and IgA detection antibodies. The standard deviation and co-efficient for each plate are shown

	Detection antibody		
	IgG	**IgM**	**IgA**
Average O.D-450 nm	2.63	1.033	0.43
Standard deviation	0.25	0.07	0.04
Co-efficient of variation (%)	9.58	7.08	10.09

There was very little intra-plate variation, confirming assay precision.

3.2.4.7. Inter-plate validation

Variability in the O.D-450 nm readings for sera at a 1:50, 1:100 and 1:200 dilution over 5 days with each of the conjugate antibodies is shown in Table 24.

Table 24: Inter-plate variation over 5 days for sera at 3 dilutions with IgG, IgM and IgA detection antibodies. The mean OD-450 nm value over 5 days, along with the standard deviation and co-efficient of variation are shown

Day	Detection antibody								
	IgG			IgM			IgA		
	Serum dilution								
	1:50	1:100	1:200	1:50	1:100	1:200	1:50	1:100	1:200
Day 1	0.70	0.40	0.16	1.00	0.40	0.20	0.75	0.41	0.21
Day 2	0.60	0.35	0.17	0.90	0.40	0.20	0.60	0.27	0.19
Day 3	0.65	0.30	0.13	0.75	0.38	0.20	0.70	0.32	0.17
Day 4	0.75	0.32	0.20	0.75	0.30	0.20	0.60	0.35	0.18
Day 5	0.65	0.32	0.16	1.00	0.40	0.19	0.85	0.34	0.22
Mean	0.67	0.34	0.16	0.88	0.38	0.20	0.70	0.34	0.19
Standard deviation	0.06	0.04	0.03	0.13	0.04	0.00	0.11	0.05	0.02
Coefficient variability (%)	8.51	11.53	15.30	14.26	11.53	2.26	15.15	15.00	10.69

Variability in the assay over 5 days was low, confirming the validity of the assay.

3.2.5. Discussion

An H antigen ELISA to measure the IgG, IgM and IgA response to challenge, was developed by determining the optimal coating concentration, blocking agent and conjugate antibody dilutions. A standard serum and positive QC serum was formed and starting dilutions for each sera for each conjugate antibody determined. Specificity was confirmed by a pre-adsorbance assay. The accuracy and precision of the assay was validated.

4. Development and preparation of a challenge strain

4.1. Introduction

In developing a human challenge model for use in the appraisal of vaccine candidates, due consideration to strain selection, the number of organisms to be ingested ('challenge dose'), the preparation of the strain and the vehicle in which the strain is administered must be given. This chapter outlines the processes used to develop the strain for the challenge model of typhoid disease.

4.1.1. Rationale for dose selection

For the future use of the model vaccine-challenge efficacy studies, the attack rate needed to be sufficiently high so that efficacy could be demonstrated with a manageable number of participants. A high attack rate can be achieved by increasing the number of bacteria ingested, with both *S.* Typhi and *C. jejuni*, exhibiting a simple linear relationship between dose and attack rate, and an inverse relationship between dose and incubation period.[73,411] However, although an attack rate of 100% in placebo immunised participants would allow the fewest numbers of participants to be used in vaccine-challenge efficacy studies, the dose of bacteria needed to induce such an attack rate is likely to be significantly higher than typically would be encountered in the field, risking increased disease severity, and/or overwhelming vaccine protective efficacy.

Power calculations for the number of participants needed to demonstrate vaccine efficacy at different attack rates (Table 25) were used to inform the decision on target attack rate in the model.

Table 25: The number of participants that would be required to demonstrate different levels of vaccine efficacy with 90% and 80% power at the 1% and 5% significance level depending on the attack rates in placebo immunised participants, in a vaccine-challenge model of typhoid disease

Attack rate in novel vaccine group	Attack rate placebo immunised group	Vaccine efficacy	Number of participants required in each group			
			90% power, 5 % significance level	90% power, 1% significance level	80% power, 5% significance level	80% power, 1% significance level
10%	30%	66.7%	92	126	71	102
10%	40%	75.0%	48	66	38	54
10%	50%	80.0%	30	41	24	34
10%	60%	83.3%	21	28	17	24
10%	70%	85.7%	15	20	12	17
10%	80%	87.5%	11	15	9	13
10%	90%	88.9%	8	11	7	10
10%	100%	90.0%	6	8	5	7
20%	40%	50.0%	118	164	91	131
20%	50%	60.0%	57	79	45	64
20%	60%	66.7%	34	47	27	38
20%	70%	71.4%	22	31	18	25
20%	80%	75.0%	16	21	13	18
20%	90%	77.8%	11	15	9	13
20%	100%	80.0%	8	11	7	9
30%	60%	50.0%	62	86	48	69
30%	70%	57.1%	36	49	28	40
30%	80%	62.5%	22	31	18	25
30%	90%	66.7%	15	20	12	17
30%	100%	70.0%	10	14	9	12

With an attack rate of 60% in placebo vaccinated controls, a vaccine of protective efficacy of 83% would reduce the attack rate to 10%, and could be demonstrated with 90% power and 5% significance by comparing two groups of 21 participants (see Table 25). If the attack rate in the immunologically naive group fell to 50, 30 participants would be needed per group to demonstrate a protective effect of vaccination of 80% with 90% power (1-β) at the 5% significance level (α). A target attack rate of 60-75% was selected in an attempt to

provide a balance between having an attack rate that was high enough to allow vaccine efficacy to be demonstrated in a reasonable number of participants and the risk of increasing disease severity and overwhelming vaccine protection with a high challenge inoculum.

Sodium bicarbonate pre-treatment has never been used in *S.* Typhi challenge, although the acid sensitivity of the organism is recognised.[405] Sodium bicarbonate use in previous enteric challenge studies has suggested that its use can be reasonably expected to decrease the number of bacteria needed to achieve a given attack rate by 2-3 logs.[403,410,416] Participants in the previous *S.* Typhi challenge studies frequently had elevated titres of antibodies to *S.* Typhi antigens prior to challenge, probably due to vaccination during previous military service and natural exposure.[73] Assuming the exclusion of those with a history of typhoid vaccination and/or living in a disease endemic country in the current study leads to a more immunologically naïve population compared to previous models, and assuming that sodium bicarbonate use has the same effect on the attack rate following *S.* Typhi challenge as has been shown with other enteric organisms, an initial challenge dose of 10^3 CFU is likely to be the lowest dose that would result in the desired attack rate. Hence, this dose was chosen for manufacture, along with doses of 10^4 CFU and 10^5 CFU so that the challenge dose could be increased if the attack rate was less than required. A 10^7 CFU dose was prepared to serve as a reserve stock of the strain.

4.1.2. Preparation of challenge strains

Freshly cultured organisms have been used in the vast majority of enteric challenge studies, including the previous typhoid challenge studies, requiring large cohorts of participants to be challenged simultaneously to minimise variability. Use of frozen preparations of challenge strains preparations may aid uniformity, allowing fewer participants to be challenged at any given point. A comparison between freshly harvested and frozen (with no post-freezing incubation) challenge strains was undertaken in the cholera challenge

model.[417,418] No difference in the incubation period or the immune response was seen. This has not been examined with other bacteria, but these findings provide reassurance that using frozen stocks from a single batch does not compromise model integrity.

4.1.3. Regulatory requirements

Investigational Medicinal Products (IMPs) are defined as 'a pharmaceutical form of an active substance or placebo being tested or used as a reference in a clinical trial'.[419] Good Manufacturing Practice (GMP) guidelines outline the processes needed to ensure the quality of medicinal products.[420] Within the UK, IMP and GMP regulations are enforced by the UK Medicines and Healthcare Products Regulatory Authority (MHRA). The status of bacteria for use as a challenge agent has been confirmed as not being an IMP by the MHRA. However, the MHRA has advised that strain should be prepared 'in the spirit of Good Manufacturing Practice (GMP)'.

The generation of a Master Cell Bank (MCB) facilitates subsequent GMP manufacture by allowing the detailed characterisation of the master strain from which all subsequent strains are derived. Based on a single clone, a MCB provides a cell reserve that is frozen in time, thereby preserving the characteristics of the cell and preventing contamination and deterioration. A microbiological profile of the MCB is developed to confirm identity and purity and to understand the long term stability of the MCB. The development of such a profile provides essential information to facilitate the manufacturing process. A Working Cell Bank (WCB) is formed directly from the MCB and provides cells directly for the manufacturing process. This two tiered system provides a practical way for the manufacturing that allows return to the original cell line if required.

For GMP standards to be satisfied, manufacturing itself must take place in a facility licensed by the MHRA as having the premises, processes, equipment and quality control processes in place to ensure the required standard in order to manufacture to GMP

standards.[420] There is a further requirement for category three laboratory standards to be used when manufacturing *S*. Typhi.

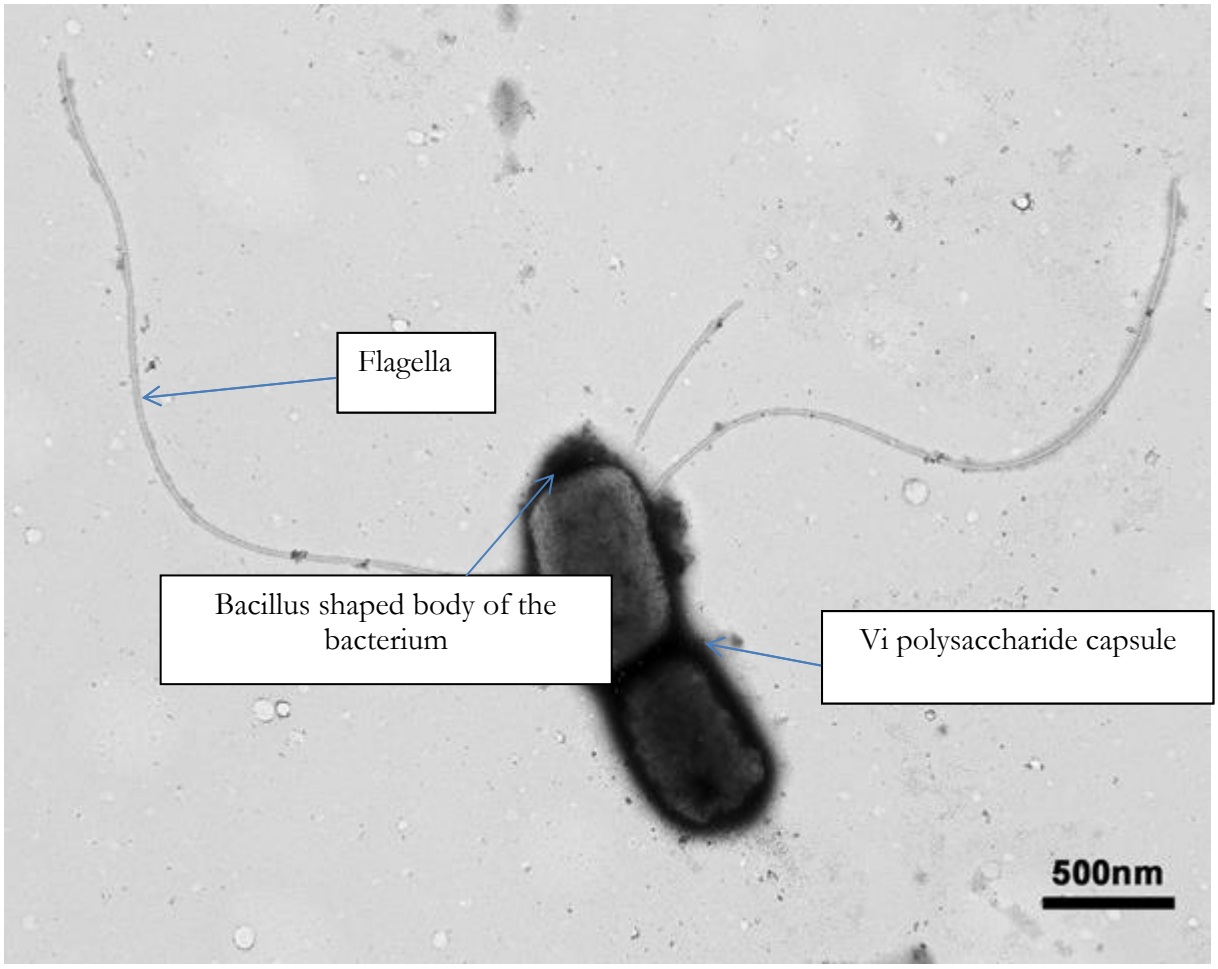
4.2. Methods

4.2.1. Strain selection

S. Typhi Quailes strain (Figure 22) was selected as the challenge organism given the wealth of safety and response data available for it. The Quailes strain had previously been selected for challenge studies as it was a fresh, clinical isolate that had not been subject to repeated passage, in contrast to other well established strains such as Ty2. An isolate of the *S*. Typhi Quailes strain was suspended in milk and frozen at -80°C during the 1970's at the University of Maryland, who added the strain to the collection at The Centers for Disease Control and Prevention (CDC), U.S.A in 2008. A Material Transfer Agreement (MTA) was established with the University of Maryland, allowing a sub-culture of this strain to be obtained by The University of Oxford for use in challenge studies. A fresh sub-culture of the Quailes strain was prepared by CDC on a microbial slope and frozen to -80°C prior to shipping to the University of Oxford.

Information on the growth media that had been used to propagate the original Quailes strain cell line was not available, and in particular was not certified as being free of prions that could cause transmissible spongiform encephalopathy (TSE-free media), and as such was not suitable for human consumption. This limitation is addressed in section 4.2.3.

Figure 22: Electron micrograph photo of *S.* Typhi Quailes strain showing the bacillus bacteria surrounded by the Vi polysaccharide capsule and the presence of polar flagella. Photo courtesy of the University of Oxford



4.2.2. Expansion cell bank

The *S.* Typhi isolate was streaked on to trypticase soy agar (TSA, Oxoid) and incubated overnight at 37°C. A single colony was inoculated into 100 ml of tryptone soy broth (TSB) and incubated in a shaking incubator at 37°C, 200 RPM, until the optical density at 600 nm (O.D 600 nm) was approximately 1. An 80% glycerol solution was made by dissolving 40 g of glycerol (Sigma) in 50 ml of sterile, filtered water (Sigma). This was added to the cultured *S.* Typhi at a 1:5 concentration to form the expansion cell bank. From this, 1ml aliquots were prepared and frozen at -80°C.

4.2.3. Master cell bank preparation

4.2.3.1. Formation

To remove any possible contamination of the original strain with transmissible spongiform encephalopathy (TSE) causing agents from prior animal-derived media, 3 rounds of sub-culture in TSE-free media were used to 'flush out' the original media. Liquid culture was used to reduce the risk of clonal selection. An aliquot of the expansion cell bank was defrosted for 10 minutes at room temperature and vortexed. From this, 200µl was removed and added to 200 ml of TSB and mixed in a shaking incubator at 37°C at 200 revolutions per minute (RPM) for 5 hours. The optical density of the culture at 600 nm (O.D 600) was measured to ensure adequate growth (target O.D 600 of 1.0). 200µl of the culture was then removed and added to a further 200ml of TSB and incubated overnight at room temperature. The O.D 600 was measured the following day and a volume of the culture equivalent to 200 µL of a 1.0 O.D 600 solution was added to 400 ml of TSB and was incubated at 37°C at 200 RPM until an O.D 600 of 1.0 was reached. 100 ml of 80% glycerol (prepared as above) was added and mixed. The MCB was divided into 1ml aliquots and frozen at 70°C.

4.2.3.2. Identity testing

Approximately 10µl of the master cell bank was inoculated on to TSA agar and incubated overnight. Two colonies were selected at random and inoculated into 5 ml of saline, before being identified using API 20E (Biomerieux, Basingstoke, UK) according to the manufacturer's instructions.

Antigenic classification was confirmed by slide agglutination. Two large colonies were suspended in saline and emulsified, from which a 2µl loop-full was dabbed on a slide, and a drop of test antisera added and lightly mixed by rocking the slide. A positive agglutination was recorded if a grainy solution with a clear background was observed on mixing within 20 seconds. To remove the Vi antigen and unmask the O antigen for testing, a dense

suspension of the bacteria in saline was made in a test tube and boiled for 30 minutes. For testing of the 'H' agglutination, a 10μL loop of the MCB was inoculated into tryptone soya broth (TSB, Oxoid Ltd, UK) and incubated overnight before being tested with anti-H agglutinating sera as above.

Antimicrobial sensitivity was determined using disc diffusion sensitivity testing[421] against ampicillin (10 μg), ceftazadime (30 μg), imipenem (10 μg), tazobactam (10 μg), amoxicillin with clavulanic acid (20/10 μg), cephelothin (30 μg), ceftriaxone (30 μg), gentamicin (10 μg), meropenem (10 μg), trimethoprim (5 μg), naladixic acid (30 μg), cefuroxime (30 μg), azithromycin (15 μg) (all from BBL Sensi-Disc Susceptibility Test Discs, B.D). The minimum inhibitory concentration (MIC) for ciprofloxacin was also measured by Etest strips (Bio-Stat). A suspension of the *S.* Typhi Quailes strain from the MCB was prepared to a density of 0.5 McFarland in 5 ml of sterile distilled water and then plated on to Iso-Sensitest agar (ISA; Oxoid, Basingstoke, UK) with a cotton wool swab, to obtain semi-confluent growth. The plates were allowed to dry before antibiotic discs were applied (6 per plate). The plates were incubated in air, overnight at 37°C. The diameters of the zones of inhibition were measured the next day to the nearest millimetre.

4.2.3.3. Purity testing

Purity testing was conducted to measure the bacterial count of the MCB and to ensure that it was free from unwanted pathogenic micro-organisms, specifically *Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus, Staphlococcus aureus, Salmonella* Typhimurium, *Aspergillus niger,* and *Candida albicans.*

The total aerobic count was measured by diluting 100 μl of a vial from the MCB in 900 μl of sterile water and mixing. This was serially repeated to achieve a starting dilution of 10^{-5} of the original mixture. Of this, 100 μl was plated on to each of 5 TSA plates (Oxoid) which were incubated for 48 hours at 30°C. Six colonies were selected at random and identified by gram stain and serum agglutination as above.

To detect other micro-organisms (microbial limit testing), the MCB was cultured in enrichment broth before being plated on to selective agar as outlined in Table 26. Nine millilitres of fluid lactose medium enrichment broth and 9 ml of fluid Soybean-casine digest medium were added separately to a sterile universal. To each enrichment broth, 1 ml of the MCB was added and mixed. Each broth was incubated for 48 hours at 35°C. Where required, 1 ml of the enrichment was then added to 9 ml of the secondary enrichment broth, mixed and incubated at 35°C for a further 24 hours. Following enrichment culture, a 10 μL loop was then used to inoculate selective agar plates in duplicate. Plates were inspected daily for 3 days and any colonies growing were identified by gram staining and sera agglutination as above.

Table 26 Enrichment broth and selective agar used for the detection of micro-organism contamination in the Master Cell Bank of *S.* Typhi Quailes strain

Target organism	Enrichment	Selective agar
Escherichia coli	Fluid lactose medium	MacConkey agar incubated at 35°C for 3 days
Pseudomonas aeruginosa	Fluid lactose medium Fluid Soybean-casine digest medium	Cetrimide agar incubated at 35°C for 3 days
Bacillus cereus	Fluid Soybean-casine digest medium	Bacillus cereus agar incubated at 35°C for 3 days
Staphlococcus aureus	Fluid Soybean-casine digest medium	Baird-parker agar incubated at 32°C for 3 days
Salmonella Typhimurium	Fluid lactose medium (primary enrichment) Fluid tetrathionate medium (secondary enrichment broth) Fluid selenite-cystine medium (secondary enrichment broth)	Xylose-lysine-deoxycholate (XLD) agar incubated at 35°C for 3 days.
Aspergillus niger	Sabouraud broth	Acidified potato dextrose agar, incubated for 72 hours at 30°C followed by 5 days at room temperature.
Candida albicans	Sabouraud broth	Sabouraud dextrose agar, incubated for 72 hours at 30°C followed by 5 days at room temperature.

4.2.3.4. Genetic sequencing

An original isolate as obtained from the CDC and an isolate from the MCB were sequenced by the Wellcome Trust Sanger Institute, Hinxton, to determine if any genetic changes had occurred in strain development. The isolates were also compared to Ty2, a wild-type *S.* Typhi strain. Frozen isolates of the original strain and the MCB were inoculated directly on to Lysogeny broth (LB; Sigma-Aldrich) and cultured overnight. DNA was isolated from each of the cultures and sequencing using Illumina sequencing technologies. Single nucleotide polymorphisms (SNPs) comparative to the Ty2 strain were

identified using sequences mapping maq software. SNPs were filtered for quality score and read depth cut off.

4.2.4. GMP manufacture

4.2.4.1. Reagents

M9S media

Five hundred millilitres of sterile distilled H_2O (s.d. H_2O) was heated to 60°C. Ten grams of soya peptone (Solabia, Cedex, France; F-A1603), 17.1 g of disodium hydrogen phosphate, 3.0 g of potassium dihydrogen phosphate, 0.5 g of sodium chloride and 1.0 g of ammonium chloride were added to the water in order, and mixed by stirring. Four hundred and ninety three grams of magnesium sulphate was dissolved in 175 ml of s.d. H_20 and added to the mixture, followed by 14.7 mg of calcium chloride dissolved in 85 ml of s.d. H_2O. The mixture was made up to 945 ml with s.d. H_2O, and cooled to room temperature. The pH was verified to be in the range of 7.0 +/- 0.2. The M9S media was then autoclaved at 125°C for 20 minutes.

10% glucose solution

Twenty grams of glucose (BDH; 284504S) was dissolved in 200 ml of s.d. H_20, and autoclaved to 121°C for 20 minutes.

M9S-20% sucrose

Five hundred millilitres of s.d. H_2O was heated to 60°C. Ten grams of soya peptone (Solabia, Cedex, France; A160700), 17.1 g of disodium hydrogen phosphate (VWR; 301565Y), 3.0 g of potassium dihydrogen phosphate (VWR; 102035C), 0.5 g of sodium chloride (Sigma; 10241AP) and 1.0 g of ammonium chloride (VWR; 100173D) were added to the water in order, and stirred until dissolved. Four hundred and ninety three grams of magnesium sulphate (VWR; 25167.298) and 14.7 mg of calcium chloride (Sigma; C8106)

was dissolved in 100 ml of s.d. H_2O and added to the mixture and autoclaved as before. Separately, 200 g of sucrose (Sigma; S3929) was dissolved in 500 ml of s.d. H_2O and filtered twice with sterile 0.22 μm polyethersulfone filter units. In a laminar flow hood, the 500 ml of M9S solution and 500 ml sucrose solution were combined in a 1 L bottle and stored at 4°C for up to one month.

4.2.4.2. Preparation of *S.* Typhi (Quailes strain) M9S+10% sucrose

One litre of M9S media and 200 ml of 10% glucose were placed in an incubator at 37°C to pre-warm. One vial of *S*. Typhi Quailes strain was removed from the MCB and thawed for 15 minutes at room temperature. One hundred millilitres of M9S media was added to each of two 500 ml Corning baffled flasks and warmed to 37°C, and 100 μL added to each of the flasks, and 5.5 ml of 10% glucose added. The MCB vial was mixed by inversion 5 times, and 100 μL added to each flask. The remaining 800 μL of the MCB vial was sent for quality control analysis to verify a pure culture. The Corning flasks were incubated at 37°C at 200 RPM for 5 to 6 hours, until the O.D 600 of the solution reached 1 to 1.5. To make the Active Substance Mastermix (ASM), the contents of both Corning flasks were added to a 2 L bottle, and 1 L of pre-chilled M9S-20% sucrose along with 800 ml pre-chilled M9S media added, and the contents stirred for 15 minutes using a magnetic stirrer. One millilitre of the solution was used to measure the viable cell count (VCC- T_0), and 0.5 ml tested for culture purity. The ASM was stored at 4°C for a maximum of 19 hours. Following storage, a further 1 ml of the ASM was removed for VCC measurement to ensure that microbial growth had not occurred.

Final doses were formulated in 1300 ml of M9S- 10% sucrose, made by mixing 650 ml each of M9S +10% sucrose and M9S in a 2 L bottle, pre-chilled to 4°C. It was estimated that there would be a 20% loss in viable cells on freezing, hence the target doses pre-freezing were 1.3 $x10^7$, $1.2x10^5$, $1.2x10^4$, and $1.2x10^3$ CFU/ml.

To calculate the amount of ASM in 1300 ml, the following formula was used:

$$\frac{\text{Desired dose CFU/mL x1300mL}}{\text{VCC } T_0 \text{ CFU/mL}}$$

The calculated ASM volume was removed from M9S- 10% sucrose solution, and the ASM added. The solution was mixed by stirring for 15 minutes at a temperature of 4°C. The doses were then dispensed in 1 ml volumes into cryovials using a dispensing pump and frozen at -80°C.

Figure 23: Class II microbiological safety cabinet used for the manufacture of *S.* Typhi Quailes strain at the Health Protection Agency's Porton Down GMP facility

4.2.4.3. Viable cell count (VCC) estimation and stability

Frozen vials were defrosted at room temperature for 20 minutes to 30 minutes. Samples were serially diluted so that the number of colonies on a plate was in the range of 30 to 300 CFU. The initial dilution was made by adding 500 ml of the sample to 4.5 ml of maximum recovery diluent (MRD; Sigma-Aldrich; 07233) to form a 10^1 dilution. One millilitre of this was then added to 9 ml of MRD serially until the concentration was suitable for counting.

From the final dilution, 100 µL was plated on to Brain-Heart Infusion agar plates (BHI-agar; Oxoid; CM1136) in triplicate and spread using a sterile hockey stick style spreader. Plates were incubated at 37°C for 17 to 26 hours before counting. To calculate the VCC, the mean bacterial count from the 3 plates was calculated, and multiplied by the dilution factor to give a mean count per sample.

From each dose level, 48 vials were selected for stability testing at 3, 6, 9, 12, 18, 24, 30 and 36 months post manufacture. Vials were batched into groups of 200, and 1 vial from each group taken for each time point so that changes in VCC across the batch could be estimated.

4.2.4.4. Quality control

Vials were selected at random from each manufactured dose level for quality control testing. Identity testing was by Gram stain, API 20E and agglutination as above. Antibiotic sensitivity testing was by disc diffusion as before.

Microbial limits were pre-defined as the sample must contain no pathogenic bacteria, namely *Escherichia coli*, *Pseudomonas spp.*, *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella spp.* (other than *S.* Typhi), or yeasts and moulds as no more than 100 non-pathogenic bacteria and 20 fungi/ ml of the strain material. Microbial limits testing and species identification was performed by Beckman Coulter Genomics (North Carolina, U.S.A). For the detection of yeasts and moulds, Acidified Potato Dextrose Agar medium was heated to 50°C and poured into 2 plates per dose. Separately, Sabouraud Dextrose Agar Medium with Kanamycin was heated to 50°C and poured into two plates for each dose level. To all plates, 1 ml of warm lactic acid (10%) was added, followed by 1 ml of each dose. Plates were mixed by swirling. Agar was allowed to solidify, and then incubated at 22°C for 3 days. Growth was checked on days 4 and 8. Detection of other microbial contaminants was as above. Any growth was confirmed as *S.* Typhi by gram strain, slide agglutination and API 20E as above.

Total aerobic counts were measured by plating of 100 μL of *S.* Typhi Quailes strain direct from the vial on to each of 6 M9 minimal agar plates. Plates were incubated at 32°C for 48 hours.

4.3. Preparation of *S.* Typhi for ingestion by participants

4.3.1. Stability in bicarbonate

To determine the stability of the challenge strain at different temperatures once it had been prepared for ingestion, 0.87 g of sodium bicarbonate (Waitrose Limited, Bracknell, UK) was dissolved in 50 ml of water (Volvic Mineral water 500 ml, Danone, France). Four hundred microliters of a vial containing $1x10^5$ CFU/ml and 600 μL of the $1x10^4$ CFU/ml vial were mixed to make a concentration of approximately $2.8x10^4$ CFU/ml, and 330 μL were added to each of 2 aliquots of 10 ml of the bicarbonate solution. One aliquot was incubated at room temperature and 1 aliquot was kept on ice. At hourly intervals 100 μL were plated on TSA agar in triplicate.

4.3.2. Preparation of *S.* Typhi for ingestion

The expected recovery rate from each vial was approximately 60%. To generate a dose that was in the range of $1\text{-}5x10^3$ CFU from the manufactured vials containing $1x10^4$ CFU/ml, a 200μl sample per participant was used.

The solution for ingestion was prepared in a category two biological safety cabinet within a category three containment laboratory that had been industrially cleaned and was used solely for the purposes of preparing the solution immediately prior to ingestion. The challenge inoculant was prepared by a study investigator and double checked by a second investigator.

Water (Volvic) and sodium bicarbonate (Waitrose Limited) used for the preparation were commercially available food products and did not require regulatory approval.

4.3.2.1. Sodium bicarbonate pre-treatment

For each participant, 120 ml of water was added to 2.1g of sodium bicarbonate and shaken to dissolve.

4.3.2.2. *S.* Typhi challenge solution

Depending on the number of volunteers to be challenged, a variable amount of sodium bicarbonate solution was prepared, such that 30 ml per participant and 30 ml of excess solution for dose testing was available. Sodium bicarbonate was added to a container and dissolved to give the correct concentration (0.5g sodium bicarbonate/30 ml water). Two vials containing one log higher CFU of *S.* Typhi Quailes strain than required for challenge were selected at random and defrosted at room temperature and vortexed to ensure mixing. From each vial, 900 µl was taken and mixed together by vortexing. One hundred microliters of the mixed strain per 30 ml of sodium bicarbonate solution was added to the sodium bicarbonate solution and vortexed. 30 ml of the sodium bicarbonate – *S.* Typhi Quailes strain mixture was aliquoted into separate screw top containers (one per participant) and placed on ice. The challenge mixture was used within two hours. The remaining 30 ml was plated out to measure the exact challenge dose (see dose testing). Containers for the samples were single use and disposed of after autoclaving following ingestion by the volunteer.

4.3.3. Dose testing

From the original challenge inoculum, 3 x 2 ml aliquots were taken and centrifuged at 7200 *g* for 5 minutes. From the supernatant, 1.9 ml was discarded and then the pellet containing the bacteria was resuspended in the remaining solution by vortexing. The entire contents of each vial was then plated out on to Trypticase soy agar (TSA) and incubated overnight at 37°C in 5% carbon dioxide. The number of colonies on each plate was counted the next day and the count averaged across the three plates to give the average count in 2 ml of challenge solution. This number was then multiplied by 15 to give the overall number of CFU that the volunteer had ingested.

4.4. Results

4.4.1. Generation of a Master Cell Bank

A master cell bank of 200 vials of *S*. Typhi Quailes strain was successfully generated. The identity of the contents was verified as *S*. Typhi with 99.6% certainty by API 20E testing on two colonies selected at random (identification number 60(4*)04540). On agglutination testing, the strain agglutinated with 09, H-d and Vi antigens and failed to agglutinate 04 (negative control), confirming the identity as *S*. Typhi.

Results of antimicrobial sensitivity testing are shown in Table 27. The MIC of ciprofloxacin by E test was 0.016 μg/ml confirming sensitivity. The strain was fully susceptible to all antimicrobials tested.

Table 27: Results of antimicrobial sensitivity testing for the *S.* Typhi Quailes strain Master Cell Bank, showing full sensitivity of the strain to all antimicrobials tested

Antibiotic	Zone size observed (mm)	Zone size required to demonstrate sensitivity	Interpretation
Ampicillin	35	15	Sensitive
Ceftazadime	39	30	Sensitive
Imipenem	34	21	Sensitive
Tazobactam	32	22	Sensitive
Amoxicillin/ clavulanic acid	35	15	Sensitive
Cephelothin	38	27	Sensitive
Ceftriaxone	44	28	Sensitive
Gentamicin	27	20	Sensitive
Meropenem	42	27	Sensitive
Trimethoprim	32	20	Sensitive
Naladixic Acid	29	N/A	Sensitive
Cefuroxime	34	20	Sensitive
Azithromycin	22	19	Sensitive

The total aerobic counts were calculated from a sample of the MCB diluted to 10^{-6}. The results from the five plates are shown in Table 28.

Table 28: Number of cells counted from 5 TSA plates inoculated with 100 µg of the *S.* Typhi Quailes strain Master Cell Bank, diluted to 10^{-6} for determination of the total aerobic count

Isolate	Count 1	Count 2	Count 3	Count 4	Count 5	Mean	Count/ml
MCB	288	290	342	312	335	313	3.1×10^8

Colonies observed were of a single colonial morphology, and 6 randomly selected colonies were confirmed by slide agglutination as presumptive *Salmonella* spp. (O9+, O2-). No other colony types were observed.

Results of the microbial limits testing are shown in Table 29. Colonies growing on Sabouraud, Potato Dextrose Agar, XLD and Maconkey Agar identified were confirmed as *S*. Typhi by Gram stain and agglutination testing.

Table 29: Results of microbial limit testing undertaken during the development of a Master Cell Bank of *S.* Typhi Quailes strain, showing a pure culture

Media	Growth at 24h	Growth at 48h	Growth at 96h
Cetrimide Agar	NG	NG	NG
Maconkey Agar	NLF*, No *E. coli*	NLF*, No *E. coli*	NLF*, No *E. coli*
Bacillus cereus Agar	NG	NG	NG
Baird Parker Agar	NG	NG	NG
Sabouraud Agar	Faint growth of translucent colonies. No Candida or fungi*	Faint growth of translucent colonies. No Candida or fungi*	Faint growth of translucent colonies. No Candida or fungi*
Potato Dextrose Agar	As Sabouraud	As Sabouraud	As Sabouraud
XLD	NLF	NLF	NLF
NLF = non lactose fermenter. NG = no growth			

Both the original strain and the MCB strain were genomically characterised with a read sequence depth of 308, and 288 respectively. Both isolates were identical. Comparison to Ty2 showed 14 single nucleotide polymorphisms.

4.4.2. GMP manufacture of *S.* Typhi Quailes strain

The viable cell counts for the manufactured strains at each of the 4 target doses are shown in Table 30. All dose levels were above the target doses. For the 1-5x10^3 and 1-5x10^4 dose levels, the counts were approximately one log higher than specified. These two doses were therefore relabelled so that the 10^3 dose level became the 10^4 level and the 10^4 became the 10^5 dose, and are from here on in referred to as the relabelled value. A 10^3 dose level was

not released. The original 10^5 dose level was not further developed and not released. The 10^7 dose was accepted at the manufactured level as it was to serve as cell stock.

Table 30: Actual cell counts obtained immediately pre-freezing from each of the 4 dose levels of *S.* Typhi Quailes strain manufactured to GMP standards

Repeat	Target dose (CFU)			
	1-5x10^7	**1-5x10^5**	**1-5x10^4**	**1-5 x10^3**
	Counts obtained (CFU)			
1	6.375 x10^7	7.43x10^5	1.760x10^5	1.24x10^4
2	5.600 x10^7	4.62x10^5	1.720 x10^5	1.32x10^4
3	5.275 x10^7	7.89x10^5	1.170 x10^5	1.31x10^4
4	5.175 x10^7	8.10x10^5	1.165 x10^5	1.48x10^4
5	4.075 x10^7	8.43x10^5	1.135 x10^5	1.43x10^4
6	6.650 x10^7	5.88x10^5	1.385 x10^5	1.11x10^4
7	5.475 x10^7	7.49x10^5	1.495 x10^5	1.40x10^4
8	5.600 x10^7	7.76x10^5	1.590 x10^5	1.33x10^4
9	5.150 x10^7	7.64x10^5	1.250 x10^5	1.34x10^4
10	4.575 x10^7	7.53x10^5	1.095 x10^5	1.26x10^4
Average (mean) count	5.39500 x10^7	7.28x10^5	1.38x10^5	1.32x10^4

Microbial limits testing confirmed that the vial contents were a pure culture at all doses, without contamination. The culture was confirmed as *S*. Typhi Quailes strain.

Stability data over 24 months for the GMP released doses are shown in Table 31, Figure 24, Table 32, Figure 25, Table 33 and Figure 26.

Table 31: Stability data for the 10^7 CFU *S.* Typhi dose manufactured to GMP standards, showing the viable cell count in different vial number groups at 3, 6, 9, 12, 18 and 24 months after manufacture

Vial group	Time point (months)					
	3	6	9	12	18	24
	Viable cell count ($CFUx10^7$)					
1-200	6.821	7.874	7.819	6.672	7.049	7.205
201-400	5.896	6.880	6.221	6.289	6.738	5.991
401-600	4.908	4.664	5.422	3.934	5.021	4.614
601-800	3.642	4.282	3.547	4.568	3.997	3.694
801-1000	2.967	3.256	3.748	3.290	2.769	3.235
1001-1200	2.775	3.923	2.768	2.740	2.748	3.039

Figure 24: Stability data for the 10^7 CFU *S.* Typhi dose manufactured to GMP standards, showing the viable cell count in different vial number groups at 3, 6, 9, 12, 18 and 24 months after manufacture

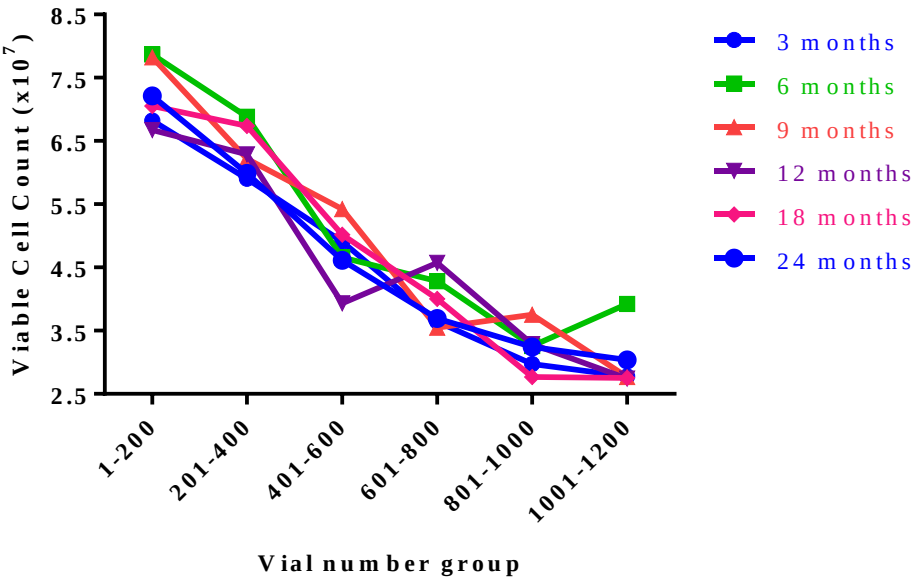


Table 32: Stability data for the 10^5 CFU *S.* Typhi dose manufactured to GMP standards, showing the viable cell count in different vial number groups at 3, 6, 9, 12, 18 and 24 months after manufacture

Vial group	Time point (months)					
	3	6	9	12	18	24
	Viable cell count ($CFUx10^5$)					
1-200	1.940	1.263	0.782	1.799	1.170	0.657
201-400	1.780	0.728	1.299	1.817	1.336	1.568
401-600	1.281	1.471	1.571	1.397	1.501	1.191
601-800	1.614	1.487	1.487	1.545	1.382	0.927
801-1000	1.108	0.9093	0.909	1.276x	1.323	1.197
1001-1200	0.976	1.028	1.028	1.070	1.061	0.9553

Figure 25: Stability data for the 10^5 CFU *S.* Typhi dose manufactured to GMP standards, showing the viable cell count in different vial number groups at 3, 6, 9, 12, 18 and 24 months after manufacture

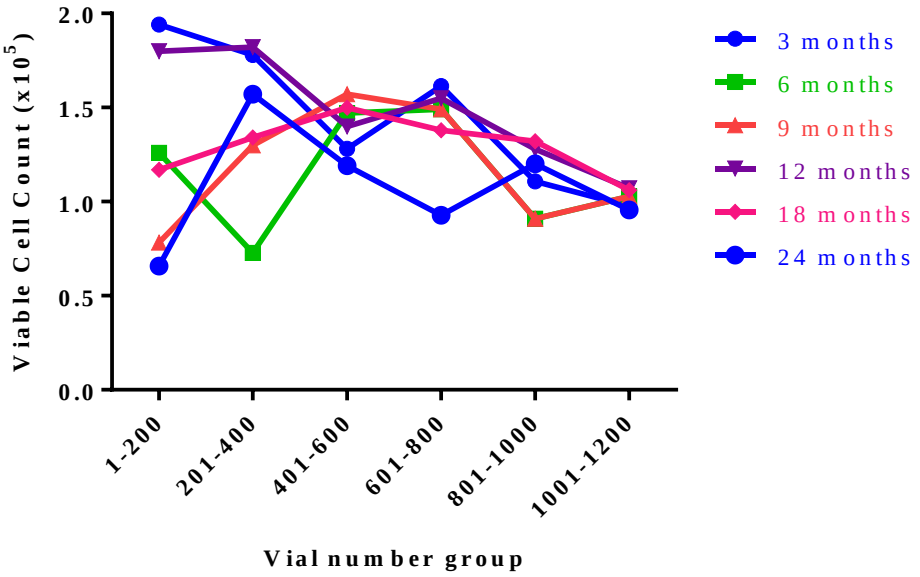
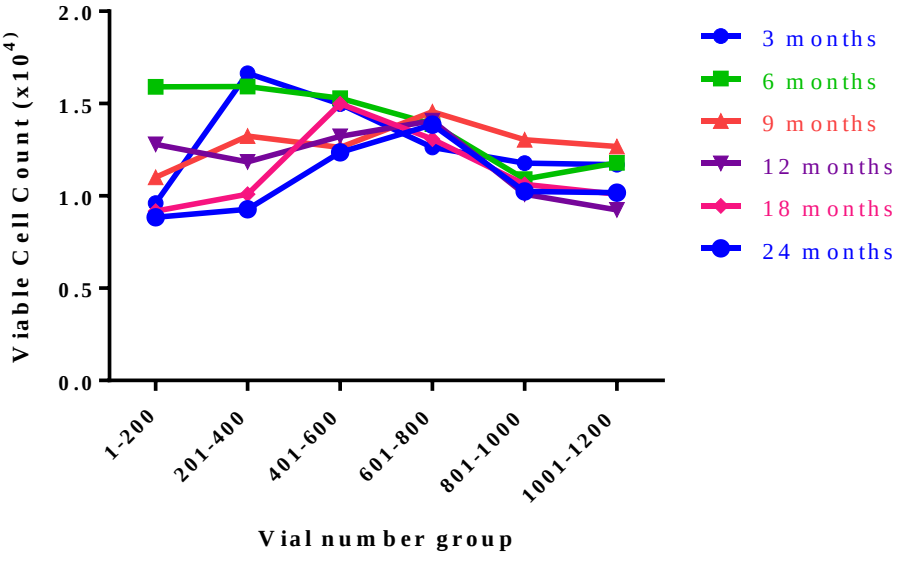


Table 33: Stability data for the 10^4 CFU *S.* Typhi dose manufactured to GMP standards, showing the viable cell count in different vial number groups at 3, 6, 9, 12, 18 and 24 months after manufacture

Vial group	Time point (months)					
	3	6	9	12	18	24
	Viable cell count (CFUx10^4)					
1-200	0.961	1.587	1.095	1.283	0.917	0.884
201-400	1.664	1.593	1.324	1.184	1.009	0.928
401-600	1.497	1.529	1.263	1.323	1.500	1.235
601-800	1.262	1.391	1.455	1.408	1.307	1.386
801-1000	1.178	1.086	1.304	1.010	1.063	1.024
1001-1200	1.169	1.183	1.268	0.924	1.009	1.017

Figure 26: Stability data for the 10^4 CFU *S.* Typhi dose manufactured to GMP standards, showing the viable cell count in different vial number groups at 3, 6, 9, 12, 18 and 24 months after manufacture

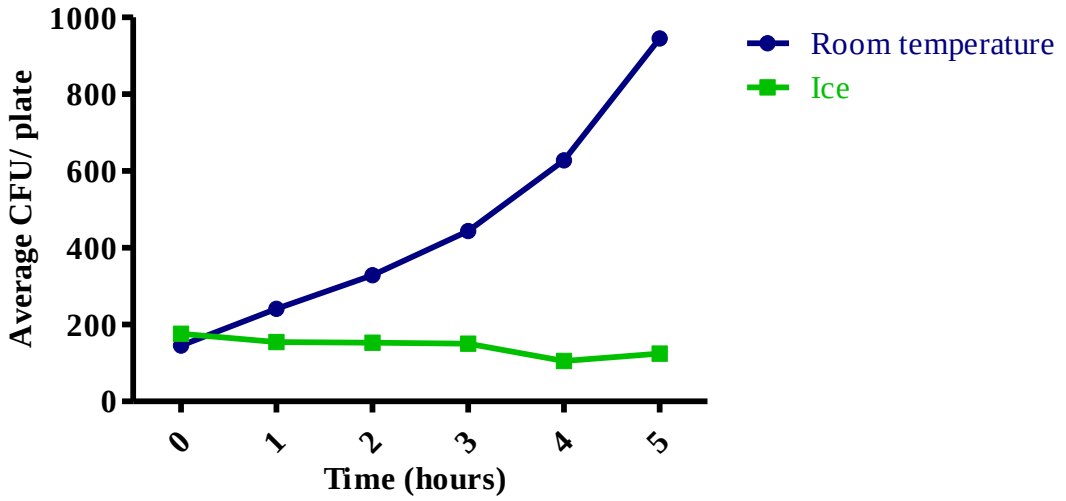


At the 10^7 CFU dose, the VCC decreases with increasing vial number, suggesting there was a loss of cell viability during the vial filling process during manufacture. This did not occur with the 10^5 CFU and 10^4 CFU doses. Stability of the manufactured product over time is high, with no obvious loss in VCC with time at any dose level.

4.4.3. Challenge dose

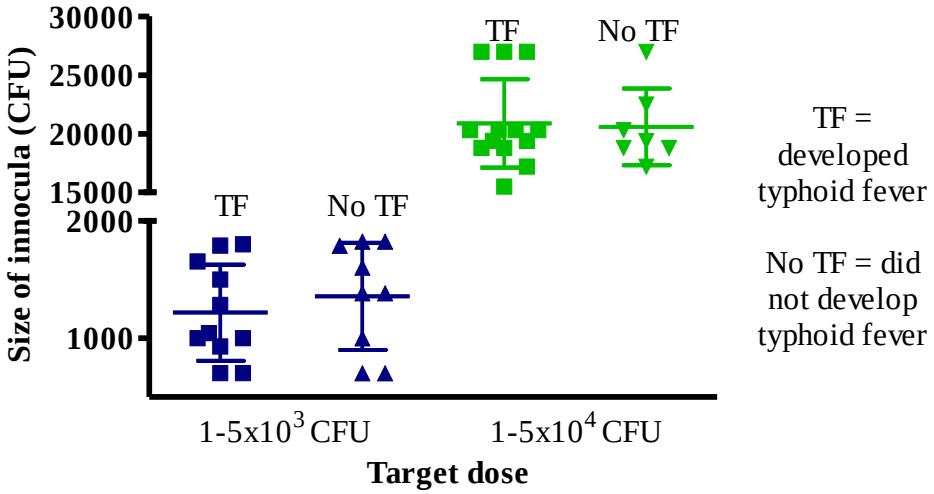
The stability of the GMP Quailes strain in bicarbonate is shown in Figure 27. A constant cell count was seen when the sample was incubated on ice for 3 hours, after which time there was a slight loss of viability. When incubated at room temperature, microbial replication occurred, reflected by an increase in cell number.

Figure 27: Stability of the viable cell count of *S.* Typhi Quailes strain in bicarbonate over time when incubated on ice and at room temperature



The actual challenge doses prepared for participant ingestion are shown in Figure 28. With a target challenge dose of 1-5x10^3, the mean average actual dose administered was 1.28x10^3 CFU (range 0.705x10^3 to 1.880x10^3) of *S.* Typhi Quailes strain. With a target challenge dose of 1-5x10^4 CFU, the mean average actual dose was 2.07x10^4 CFU (range 1.55x10^4-2.70x10^4). There was no significant difference in dose received between those who did and did not develop typhoid disease (10^3 CFU challenge dose, p=0.47, 10^4 CFU challenge dose, p=0.81).

Figure 28: Actual challenge dose of *S.* Typhi Quailes strain received by participants challenged with target doses of 1-5x10³ CFU or 1-5 x10⁴ CFU during the development of a human challenge model of typhoid disease, shown as the average dose and 95% confidence interval, for those who did and did not develop typhoid disease



4.5. Discussion

The strain selected for the challenge studies was *S.* Typhi Quailes strain as it had been safely used in previous challenge studies. This strain was isolated in 1958, and it is possible that current strains of *S.* Typhi that are circulating are significantly different. However, this is unlikely as *S.* Typhi isolates show a remarkable degree of genetic homology[59]. This was confirmed by the genetic sequencing of the strain that showed a very small amount of genetic variation from the wild-type Ty2 strain, and no alteration in the Quailes strain genome on passage. A further limitation of using a historic strain was that the documentation of strain storage and manipulation since its original isolation was very limited. Establishing a MCB allows future use of the strain to be based on the careful characterisation outlined in this chapter.

The doses selected for manufacture were based on the likely numbers of organisms that would need to be ingested in a subsequent challenge study to induce an attack rate of 60% to 75% in immunologically naïve participants. This attack rate was selected because, for the

model to be practical method for novel vaccine appraisal, only a moderate number of participants could be enrolled so as to limit the cost of using the model and make the model logistically feasible. Furthermore, there is an ethical argument for keeping participant numbers in such a study to a minimum. For a novel typhoid vaccine to offer an improvement over currently available vaccines, efficacy would need to be approximately 80%, which, with an attack rate of 60% to 75% in placebo immunised participants, could be delivered with the necessary limited numbers of participants. Although a higher attack rate would have allowed a smaller number of participants to be used whilst maintaining statistical power, this would have increased the risk that vaccine efficacy could be overwhelmed by the high challenge dose. Indeed, even at the chosen attack rate, vaccine protective efficacy may be overwhelmed, as was demonstrated in previous challenge studies using killed whole cell typhoid vaccines. These studies, also using *S.* Typhi Quailes strain, showed that vaccine protective efficacy could not be demonstrated using a challenge dose that gave a 50% attack rate in placebo immunised participants, but when the attack rate in the placebo group was lowered to 25%, vaccine protective efficacy could be shown at a similar rate to field trials.[73] Despite the risk of overwhelming protective efficacy, it can be argued that novel vaccines need to be able to induce more robust protection compared to previous typhoid vaccines to be of practical use in the field, and therefore a model with an attack rate of 60% to 75% therefore provides a reasonable and practical model for vaccine appraisal.

Previous challenge studies have not manufactured strains to the standards of GMP. GMP manufacture provided reassurance that the challenge strain was a pure culture, free from TSE contamination and fit for ingestion by participants. However, the biological variability of bacterial strains and the requirement for category 3 containment of *S.* Typhi led to manufacturing challenges and expense. The four manufactured doses were higher than the specification, probably due to ongoing bacterial growth during the hold step of manufacture and dose dispensing. For the 10^7 CFU preparation there was a loss of cell

viability with increasing vial number. This was thought to be due to cell death during the time taken to fill the vials, due to limited nutrient supply that was critical with the higher numbers of bacteria, although this was not formally investigated. As this dose was to serve as a reserve stock rather than stock for challenge it was not felt to be of critical importance. Meeting specification was most difficult for the lower number of challenge organisms where even low rates of ongoing bacterial replication have a large impact on the total number of organisms. This variability is consistent with that seen in live attenuated vaccines that are also manufactured by GMP. For example, Ty21a is manufactured to be in the range of 2-6.8x10^9 CFU[306]. These data have shown that while GMP manufacture can be used to ensure that challenge strain is manufactured to the highest standards, biological variation limits the specificity of the process.

Previous challenge studies have used freshly harvested organisms, but this required large numbers of participants to be challenged at once to maintain challenge dose consistency. Manufacturing each dose in bulk allowed relatively consistent doses to be given between participant cohorts. The viability of the frozen strain over time is stable, allowing the manufactured stock to be used for future challenge studies. The stability of the prepared challenge dose on ice allowed the strain to be prepared and transported to the clinic room for challenge without the cell number increasing. The data shown here have demonstrated that bulk manufacture of stock that is then frozen and reconstituted is an effective means of administration providing consistent challenge to small cohorts of participants.

4.6. Conclusion

The *S.* Typhi Quailes strain was selected, obtained, characterised, manufactured and prepared for challenge to GMP standards. The Quailes strain was used to establish the challenge model, which will be described in the next chapter.

5. Development of human challenge model of *S.* Typhi for use in vaccine appraisal

5.1. Introduction

This chapter will outline the processes undertaken in developing a safe, reproducible typhoid challenge model with sufficient attack rate for use in future vaccine appraisal.

5.1.1. Diagnostic definitions

The importance of diagnostic definitions in challenge models has been demonstrated by the significant variability in the reported attack rate with different diagnostic definitions in a retrospective analysis of data from previous challenge studies.[390] The Maryland studies used a stringent clinical definition based on a sustained fever for 24 hours or more,[73] and did not include a microbiological endpoint. This may have led to the exclusion of milder cases of disease.[390] For the current challenge study, a clinical endpoint based on the presence of sustained fever as well as a microbiological endpoint of *S.* Typhi bacteraemia was used to try and accurately diagnose all cases of typhoid disease, whilst maximising participant safety.

Although culturing of *S.* Typhi from blood is diagnostic for typhoid disease, sensitivity is variable between 30 per cent and 90 per cent[161,162] and several days of incubation may be needed before a positive result is obtained. Hence, a microbiological definition alone would be insufficient to detect all cases of typhoid disease. Following invasion of *S.* Typhi, it is thought to disseminate by a primary bacteraemia. Any *S.* Typhi bacteraemia occurring within 5 days of challenge was felt likely to reflect this primary bacteraemia and not true typhoid disease; hence this period was excluded from the diagnostic definition.

Fever is the most universal symptom of typhoid disease[73,188] and forms the most reliable basis for a symptom-based definition of disease. Fever occurring before 72 hours after

challenge was unlikely to be due to typhoid disease but rather pre-existing or inter-current illness, and therefore this period after challenge was excluded from the definition.

5.1.2. Participant selection and risk minimisation

Careful consideration of participant selection, monitoring and treatment is needed to ensure that a challenge model holds scientific validity, is safe and ethical. Previous typhoid challenge studies provided evidence for some aspects of the study protocol; for others, such as the safe limits of blood volume that can be taken for sampling and the risks of secondary transmission of infection, evidence from the wider literature is needed.

Previous typhoid challenge studies included participants thought to have pre-existing immunity from vaccination during military service and natural exposure, and this significantly influenced the subsequent attack rates.[87] This illustrates the importance of challenging an immunologically naïve cohort in order to have an accurate, reproducible attack rate that can determine the protective efficacy of a vaccine in an antigenically naïve cohort. However, in the absence of any marker of typhoid immunity, screening for this is impossible. In establishing a modern day model of typhoid disease, potential sources of pre-existing immunity are travel vaccination[241], residence in an endemic region[422], and previous enteric infection[244]. Excluding individuals with a history of vaccination for travel, occupational exposure, or residence in an endemic setting, is likely to be the most reliable way of ensuring uniform, reproducible susceptibility to challenge, and a naive population in which to study disease immunobiology.

5.1.3. High-risk groups

Typhoid disease is a systemic illness with a wide variety of manifestations.[7] When considering human challenge models of typhoid disease and its risk, it is pragmatic to enrol only participants who are in excellent physical health. There are however no data to suggest that particular co-morbidities confer increased susceptibility to typhoid disease. However,

particular risks may pertain to those with psychiatric morbidity, where a formal diagnosis may be lacking, and those at risk of being chronic carriers.

Neuro-psychiatric manifestations of typhoid disease include confusion, delirium, and psychosis[423]. Symptoms induced by challenge experiments may cause psychological distress over and above that experienced in day-to-day life.[424] 'Several' participants in the early Maryland typhoid challenge studies experienced episodes of confusion and psychosis.[73] Those with a preceding history of psychiatric illness may be more vulnerable to such symptoms/complications.[73] The Hospital Depression and Anxiety Scale (HADS),[425,426] originally developed to detect anxiety and depression in non-psychiatric outpatients, has been shown to be sensitive and specific in detecting psychological ill health, and correlates with longer self-administered questionnaires on psychological wellbeing.[427] Although not validated in the setting of a challenge studies, the HADS may be useful as a method of screening potential participants for risk of psychiatric manifestations following typhoid challenge, and monitoring psychological well-being during the study. In addition, exclusion of those with significant psychiatric history and alcohol or drug dependence should help exclude high-risk individuals.

Chronic carriage of *S*. Typhi occurs primarily in people with gallstones.[402] A single case of chronic carriage occurred in the previous Maryland typhoid challenge studies in a participant who was subsequently found to have undiagnosed gallstones.[175] Ultrasound is a reliable way of detecting gallstones,[428] allowing the exclusion of participants at high-risk of chronic carriage. In addition, the treatment of all participants with quinolone antibiotics is the optimum therapy to allow clearance of *S*. Typhi from the biliary tract and gut, preventing chronic carriage.[429-431] These two approaches should prevent chronic carriage in the challenge population.

5.1.4. Treatment of typhoid disease

Serious complications of typhoid disease, particularly that of gastrointestinal bleeding and perforation, increase with treatment delay,[7] typically occurring in people with untreated typhoid disease of two or more weeks duration.[432] This was illustrated in the Maryland challenge studies, where the sole case of gut perforation occurred in a participant experiencing a relapse of typhoid disease following an initial, untreated episode.[87] This highlights the need for prompt antibiotic treatment of all participants, irrespective of initial clinical illness severity. Quinolone antibiotics are considered the optimal treatment as they safe, well tolerated and lead to rapid resolution of symptoms and clearance of the *S.* Typhi.[195,433] Clinical relapse after quinolone treatment is rare, occurring less frequently than following course of 'older' antibiotics such as chloramphenicol.[197,429,434-436]

5.1.5. Blood sampling

There are no guidelines on the safe volume of blood donation in clinical studies involving adults. Studies of iatrogenic blood loss have focused on high-risk groups such as those in intensive care[437] in whom co-morbidity, trauma, surgery, prolonged admission and poor nutritional status often lead to a low admission haemoglobin, and to further decline during illness. Blood donors provide a more appropriate reference group as they are generally healthy and are pre-screened for factors that could increase the risks of donation. However limits on who should donate blood, and what constitutes a safe volume and frequency of donation vary considerably and are rarely evidenced based.[438,439] Frequency of blood donation varies from between 8 weeks in countries including Canada and 16 weeks in the UK.[438]

Although the volume of blood donation is more uniform across countries, the principal reason for limiting the volume is the risk of syncope rather than changes in haemoglobin.[440] Acute volume change is a major contributing factor in syncope, with the standard 525ml blood donation volume constituting up to 15% of circulating volume of the physically

smallest donors.[441] The risk of iron depletion is less well characterised. Long-term, frequent blood donors have been shown to be at risk of depleted iron stores with frequent donation over two years or more.[442] In the setting of a challenge study, monitoring haemoglobin concentrations, pre-exclusion of anaemic patients, limiting the duration of frequent blood sampling and the total blood volume taken in line with that taken by the National Blood Transfusion service (total taken in one year, 1410 ml), represents a safe approach to blood sampling whilst maximising the scientific merit of the study.

5.1.6. Public health considerations

Faecal-oral transmission of enteric infection occurs in countries with poor sanitation infrastructure, where interrupting the transmission cycle is difficult.[3] Chronic carriers, who excrete large numbers of organisms, are thought to be the principal source of infection.[402] Transmission from acute cases is thought to be relatively rare, as the number of bacteria excreted are fewer and rarely sufficient to overcome natural host defences such as stomach acid.[405] Effective antibiotic treatment in acute cases clears *S*. Typhi from stool, further limiting infectivity.[436,443] The only (rarely) reported exception is direct contact transmission by ano-lingual sexual contact.[220,444]

High levels of sanitation in the U.K. have led to a marked decrease in the incidence of domestically acquired typhoid disease since the turn of the 20th century. Typhoid disease in travellers is usually diagnosed several weeks after return, allowing a prolonged period of excretion and exposure to contacts before enteric precautions can be implemented and antibiotic therapy instigated.[188,445] Despite this delay, secondary transmission of *S*. Typhi within households in the U.K. is exceptionally rare and reflects the impact that good levels of sanitation have on disease transmission.[446] In a study of 44 cases of typhoid disease in London, only one case of secondary transmission occurred.[447] Similarly, in a case series collected over 15 years in Scotland, only 6 of 267 cases were attributable to secondary transmission.[446] The number of indigenously acquired cases within the UK has further

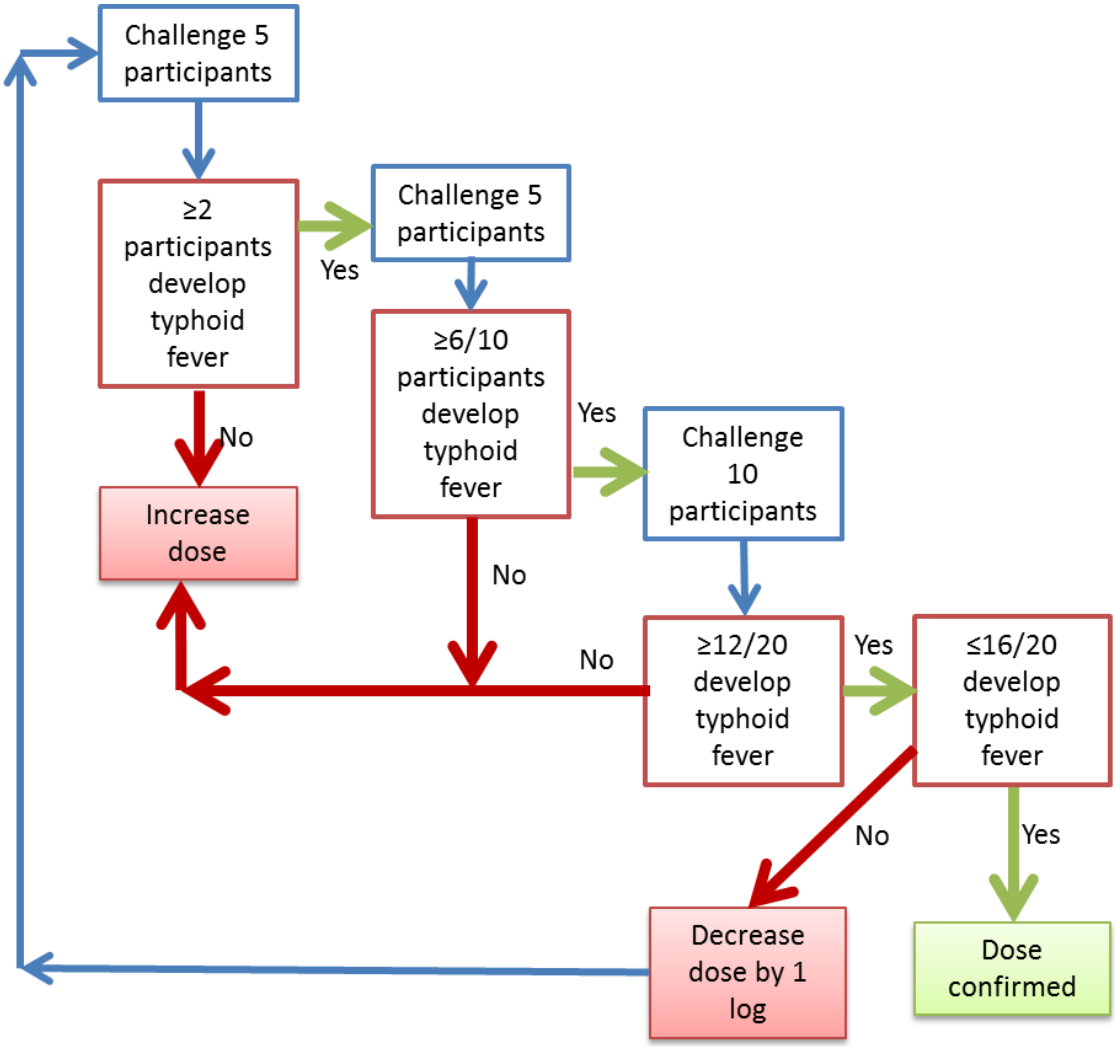
fallen since 1967,[448] possibly reflecting ongoing improvements in clean water and sanitation. Employment involving food handing provides a potential route for disease transmission [115,372] with epidemic outbreaks of typhoid disease in resource-rich countries almost always confined to unknowingly infected food handlers, often those who are chronic carriers.[188,231] Those working with young children, and immuno-suppressed individuals are pragmatically excluded from work during the period of infectivity in the UK.[449]

Previous typhoid challenge studies were conducted in closed units, and therefore there is no direct evidence of the risk of secondary transmission. Prompt treatment with antibiotics after the onset of typhoid disease will decrease the period of infectivity, and education of participants on the importance of hand hygiene will allow interruption of the transmission cycle. The exclusion of food handlers and social and care workers in contact with vulnerable populations avoids exposing those at increased risk.

5.2. Rationale for study design

As outlined in Chapter 4, an attack rate of 60%-75% was decided as the desired attack rate for the model to allow future testing of vaccine efficacy. To determine the dose needed to give the desired attack rate whilst limiting the number of participants exposed to *S*. Typhi, participants were allocated a dose of *S*. Typhi Quailes strain sequentially by date of challenge, based on the attack rate in the preceding participants (Figure 29). The probabilities of using different numbers of participants to satisfy the design algorithm given the true attack rate for a dose are shown in Table 34. The algorithm decision points for deciding to either continue at the same dose, to escalate or de-escalate were made to minimise the chances of pursuing an incorrect dose whilst maximising the chances of demonstrating the correct dose in 20 participants.

Figure 29: Study design algorhythm in a study to determine the dose of *S.* Typhi required to give an attack rate of 60-75% in a cohort of 20 participants.



Initially 5 participants are challenged and followed up for 14 days. If the attack rate is sufficient (≥ 2/5) a further 5 participants are challenged and followed for 14 days. If the combined attack rate of these first two cohorts is ≥6 /10, a further cohort of 10 participants is challenged. If the attack rate is insufficient at any decision point, the dose is increased and the algorhythm restarted. If the final attack rate is ≥16/20, the algorhythm is restarted with a log lower CFU.

Table 34: The probability of each outcome in the study design algorithm occurring given the true attack rate at any given dose, giving a statistical indication of the number of participants potentially used in establishing a model with a 60-75% attack rate (highlighted in pink)

True attack rate at selected dose:	Probability that all criteria are satisfied	Probability that dose is escalated after challenge of first 5 participants	Probability that dose is escalated after challenge of 10 participants	Probability that 20 participants are challenged before dose escalated or accepted	Probability that dose is de-escalated after 20 participants challenged
5%	0	0.977	0.023	0	0
10%	0	0.919	0.081	0	0
20%	0	0.737	0.256	0.006	0
30%	0.002	0.528	0.425	0.046	0.000
40%	0.027	0.337	0.499	0.164	0.000
50%	0.134	0.188	0.44	0.372	0.006
60%	0.346	0.087	0.286	0.627	0.051
70%	0.481	0.031	0.124	0.845	0.237
75%	0.432	0.016	0.066	0.918	0.415
80%	0.304	0.007	0.028	0.965	0.629
90%	0.04	0	0.001	0.998	0.957
95%	0.002	0	0	1	0.997

5.3. Methods

5.3.1. Study design

An observational, dose-escalation, outpatient trial of oral challenge with a starting dose of 1-5x10^3 CFU of *S.* Typhi Quailes strain administered in sodium bicarbonate to sequential cohorts of 5, 5 and 10 healthy adult participants was conducted.

Participants were allocated a dose of *S.* Typhi Quailes strain sequentially depending on the attack rate in the preceding participants (see statistics section). The first participant at each challenge dose was challenged alone and, in the absence of any safety concerns, subsequent

participants were challenged after 14 days. Safety data were reviewed by the Data Safety Monitoring Committee prior to an increase in challenge dose. Dose allocation was single–blinded such that participants were not aware of the challenge dose but study staff were.

5.3.2. Study organisation and approvals

The study (OVG 2009/10) was compliant with Good Clinical Practice (GCP) regulations and with the principles set out in the Declaration of Helsinki. The University of Oxford was the study Sponsor. Ethical approval was granted by the National Research Ethics Service through the Oxfordshire Research Ethics Committee. Local National Health Service (NHS) Research and Development approval was granted by the Oxford Radcliffe Hospitals NHS Trust. Study monitoring was provided by the University of Oxford Clinical Trials Research Governance department.

5.3.3. Inclusion and exclusion criteria

Healthy, adult participants aged 18 to 60 years who were likely to be immunologically naïve to *S.* Typhi (i.e. no history of typhoid disease, vaccination against typhoid disease or residence in a typhoid endemic region for more than six months) were recruited. Exclusion criteria included: significant medical history; gall bladder disease (as determined by ultrasound); history of antibiotic allergy; food-handlers; those in contact with susceptible third parties (including health care workers and carers of young children, the elderly and the immunocompromised); previous psychiatric history requiring inpatient admission; moderate or severe anxiety or depression as detected by the Hospital Anxiety and Depression score.

5.3.4. Primary objective

The primary objective was to determine the dose of *S.* Typhi Quailes strain in colony forming units (CFU) needed to produce a clinical or laboratory attack rate of between 60% and 75% in a cohort of 20 adults, when ingested with a sodium bicarbonate buffer.

5.3.5. Secondary objectives:

1) To describe the clinical and laboratory features of the host responses following challenge with *S*. Typhi Quailes strain

2) To describe the development of humoral immunity following infection with *S*. Typhi Quailes strain

3) To appraise the participant experience of taking part in a challenge study.

5.3.6. Recruitment

Information about the study was disseminated at student events and by sending emails to previous participants in similar trials and to University of Oxford colleges. Those who were interested in participating were provided with a detailed study information booklet at least 24 hours before being seen for a screening visit. A brief telephone screening procedure was undertaken, outlining the principal study requirements and the main inclusion and exclusion criteria.

Potential participants attended a full screening visit, where, following informed consent, inclusion or exclusion criteria were assessed by a full medical including:

- vaccination and medication history
- physical examination (including electrocardiogram to demonstrate potentially undiagnosed cardiac abnormality)
- blood, urine (for pregnancy testing and dipstick testing to screen for renal disease)
- stool testing for enteric pathogens
- gallbladder ultrasound of the gallbladder
- psychological screening
- Laboratory testing, including full blood count, liver and renal function tests, inflammatory markers, total IgA and anti-endomysial antibodies, all performed by the Oxford Radcliffe NHS Trust laboratories using standard approved methods.

Results were assessed by a study doctor as being either normal, abnormal but not significant or abnormal and significant. Medical history was confirmed with a participant's general practitioner with the participant's express consent. Simultaneous enrolment in other clinical trials was queried on the TOPS (The Over-volunteering Prevention System) database.[450]

5.3.7. Participant challenge

On the day of challenge (Day 0), the participant's consent was confirmed and an interim medical history obtained prior to challenge. Female participants underwent urinary pregnancy testing. The HADS questionnaire was repeated. Baseline blood, urine, saliva and faecal samples were obtained. Participants fasted for 90 minutes before ingesting the sodium bicarbonate solution to ensure rapid and uniform gastric emptying (Figure 30).

Figure 30: Sodium bicarbonate solution given to participants prior to challenge to neutralise stomach acid in a human challenge model of typhoid disease

After one minute the challenge solution, containing the required dose of *S.* Typhi, was drunk (Figure 31).

Figure 31: A participant swallowing S. Typhi Quailes strain in sodium bicarbonate buffer as part of a human challenge study of typhoid disease

Participants then fasted for a further 90 minutes to prevent any influence food may have on gastric clearance or pH. Following challenge the Health Protection Unit and the participant's GP were informed. Prevention of secondary transmission guidance, together with liquid soap and paper towels were provided. Participants were taught and observed practicing good hand-hygiene techniques.

5.3.8. Definition of Illness

Typhoid disease was defined as:

- positive blood culture for *S*. Typhi after day 7 post challenge

Or

- oral temperature ≥38°C persisting for 12 hours without anti-pyretic medication or other means to lower fever occurring after 72 hours post challenge

Or

- positive blood culture for *S*. Typhi after day 5 with the presence of an objective sign of typhoid disease such as fever ≥38°C, as determined by a study doctor

The earliest indication that a participant had a *S*. Typhi bacteraemia was the identification of Gram negative bacilli on Gram staining of blood culture broths. Formal identification of

organisms as *S.* Typhi took a minimum of a further 24 hours. Participants in whom a Gram negative bacillus was identified were therefore defined as having typhoid disease for the purposes of clinical management (including antibiotic treatment) and blood, urine, faecal and saliva sample collection and processing.

5.3.9. Follow-up of participants

Participants were reviewed at least daily and were telephoned every evening for 2 weeks. Participants detailed symptoms of fever, headache, malaise, anorexia, abdominal pain, nausea/vomiting, myalgias, arthralgias, cough, rash, diarrhoea and constipation in a diary card that was reviewed at clinic visits. Symptom severity was graded by impact on daily activity (mild – no interference with daily activity, moderate – some interference, severe – unable to perform activities of daily living and life threatening – requiring hospital admission or death). Oral temperature was recorded a minimum of twice daily. Participants with fever >38°C or possible bacteraemia were reviewed by a study doctor. Participants were discussed with a consultant infectious disease physician if they had evidence of severe typhoid disease, could not tolerate oral antibiotics, or as required.

Table 35: Diagnostic criteria for severe typhoid disease used in a human challenge model of typhoid disease.

Severe typhoid disease
oral temperature ≥40°C
systolic blood pressure ≤85 mmHg
lethargy or confusion
gastrointestinal bleeding
gastrointestinal perforation
severe laboratory abnormality

5.3.10. Treatment

Indications for antibiotic therapy were the diagnosis of typhoid disease, symptoms severe enough to prevent all normal activity, clinical necessity as judged by a study doctor, or reaching day 14 after challenge. Participants were treated with ciprofloxacin 500mg twice daily for 14 days. Participants were directly observed or telephoned to remind them to take the antibiotic dose. Second line treatment for participants intolerant to ciprofloxacin was azithromycin 500mg once daily for 14 days (or until 14 days of total antibiotics taken if prior doses with ciprofloxacin had already been received). Following completion of antibiotics, participants were screened for excretion of *S*. Typhi in 2 stool samples obtained a week apart, in accordance with public health guidance[130].

5.3.11. Sampling

Daily samples of blood, saliva (using sponge collection device; Oracol; Malvern Medial Supplies, Worcester, UK), mid-stream urine and stool specimens were collected. Blood was drawn according to the schedule shown in Table 36. Participants diagnosed with typhoid disease had bloods drawn as per Table 37 from diagnosis for 4 days. Blood culture, quantitative blood culture, C- reactive protein (CRP), full blood count (FBC), urea, creatinine and electrolytes (U&E) and liver function tests (LFTs) were assayed immediately by ORH clinical laboratories, according to NHS SOPs. Other samples were processed for freezing at -80°C.

Table 36: Schedule of blood tests for participants in a human challenge model of typhoid disease

Sampling time point	Investigation performed (amount of whole blood taken from participant)										
	Blood culture (10ml aerobic)	Polymerase Chain Reaction (5ml)	Antibody Secreting Cells (25ml)	Cell Mediated immunity (35- 45 ml)	Full Blood Count (1ml)	CRP, U&E, LFT (2ml)	Serum Bactericidal Assay (3ml)	Antibody measurement (5ml)	Cytokine (2ml)	Functional genomics (3ml)	Total volume of whole blood taken (ml)
Challenge			*	*	*	*	*	*	*	*	116
Hour 6	*	*								*	18
Hour 12	*	*							*	*	20
Hour 24	*	*		*	*	*	*		*	*	61
Hour 36	*	*								*	18
Day 2	*	*		*				*	*	*	70
Day 3	*	*							*	*	20
Day 4	*	*		*	*	*	*	*	*	*	76
Day 5	*	*							*	*	20
Day 6	*	*			*	*			*	*	23
Day 7	*	*	*	*				*	*	*	95
Day 8	*	*			*	*				*	21
Day 9	*	*	*	*						*	88
Day 10	*	*			*	*	*	*	*	*	31
Day 11	*	*								*	18
Day 12	*	*			*	*				*	21
Day 13	*	*								*	18
Day 14	*	*		*	*	*	*	*	*	*	73

*= sample taken. Sampling point is relative to point of challenge. Blood culture, PCR and functional genomics were obtained at every sampling point. Other assays were timed to maximise information whilst not taking excessive volumes of blood from volunteers.

Table 37: Schedule of blood tests for participants diagnosed with typhoid disease in a human challenge model of typhoid disease

Sampling time point	Investigation performed (amount of whole blood taken from participant)											
	Blood culture (10ml aerobic)	PCR (5ml)	Blood culture quantification (10ml)	Antibody Secreting Cells (25ml)	Cell Mediated immunity (35- 45 ml)	Full Blood Count (1ml)	CRP, U&E, LFT (2ml)	Serum Bactericidal Assay (3ml)	Antibody measurement (5ml)	Cytokine (2ml)	Functional genomics (3ml)	Total volume of whole blood (ml)
TD Hour 0	*	*	*			*	*	*			*	34
TD +Hour 6	*	*									*	18
TD +Hour 12	*	*						*			*	21
TD +Hour 24	*	*				*	*	*			*	24
TD +Hour 36	*	*									*	18
TD +Hour 48	*	*		*	*	*	*	*	*	*	*	101
TD +Hour 72	*	*						*			*	21
TD +Hour 96	*	*			*	*	*	*	*	*	*	76

Participants satisfying the definition of typhoid fever had blood samples taken as per rows labelled 'TD'. This replaced samples that would have otherwise been due.

*= sample taken. TD= typhoid diagnosis point. Sampling point is relative to point of challenge. Blood culture, PCR and functional genomics were obtained at every sampling point. Other assays were timed to maximise information whilst not taking excessive volumes of blood from volunteers.

5.3.12. Public health management

The Health Protection Agency (HPA) provided independent public health oversight. They were informed of all those challenged, those developing typhoid disease and those with *S*. Typhi isolated on stool culture. The HPA reference laboratory was provided with an isolate of the Quailes strain to allow comparison in the event of a community acquired case being detected. In addition any breaches in precautions that may have resulted in another individual coming into contact with the excreta of a participant would have been reported to the proper officer/health protection unit.

5.3.13. Statistics

The confidence interval around any observed attack rate is shown in Table 38.

Table 38: Confidence intervals around the observed attack rate in twenty participants in a human challenge model of typhoid disease

Observed attack rate in 20 participants	95% Confidence interval for attack rate
50%	27% to 73%
60%	36% to 81%
70%	46% to 88%
80%	56% to 94%
90%	68% to 98.8%

The attack rate was calculated in the per protocol population by dividing the number of participants diagnosed with typhoid disease by the total number of participants challenged with S. Typhi at each dose. Participants who did not reach the primary endpoint and were treated or withdrawn before day 14 were not included in the per protocol analysis.

Comparisons of secondary endpoints between infected and uninfected participants were descriptive and exploratory.

5.3.14. Data recording

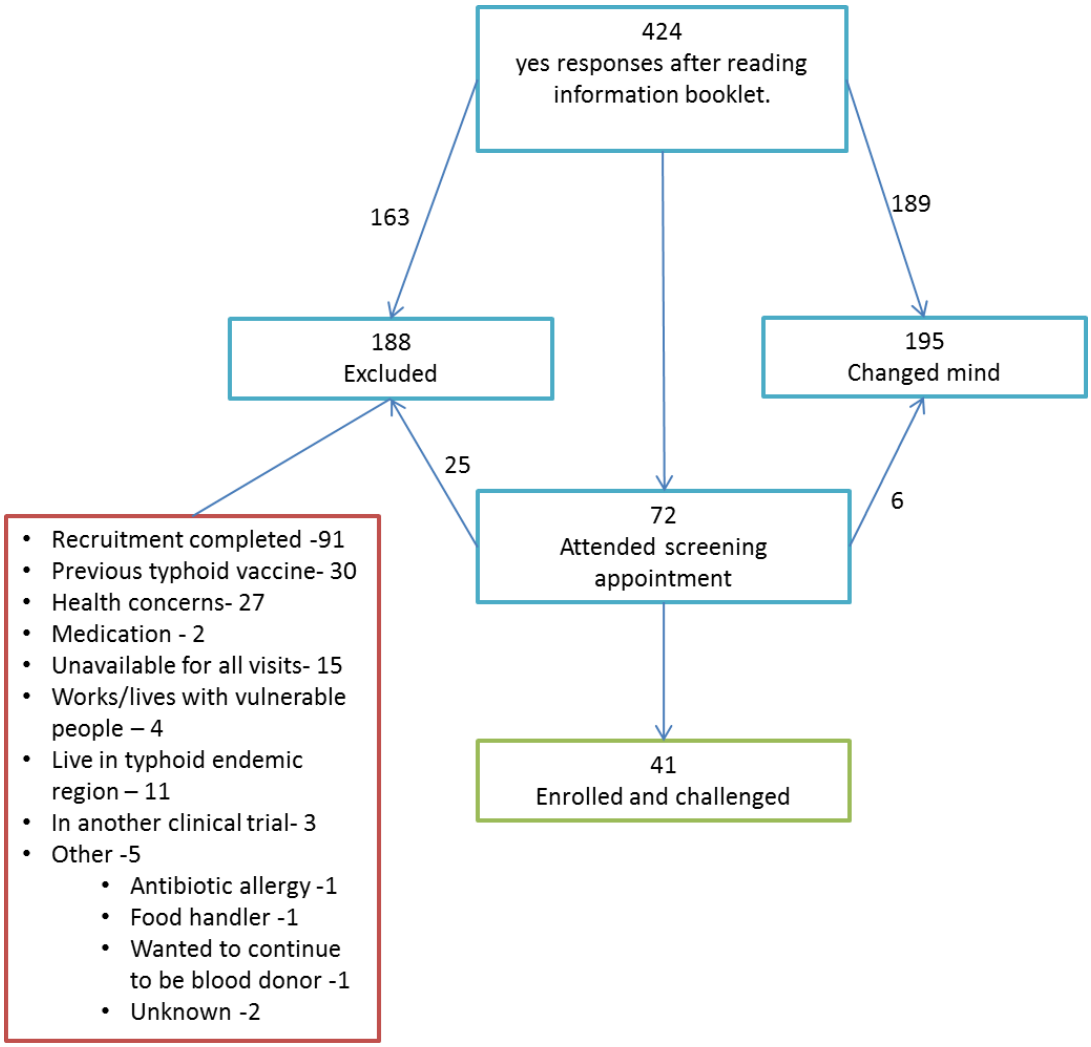
Diary card data was transcribed using OpenClinica clinical trial software (OpenClinica Community edition version 2.1, Massachusetts, USA). Data was extracted and analysed using Microsoft Excel (2010 edition, Microsoft, Washington, USA) and GraphPad Prism (version 5, GraphPad, Inc., La Jolla, USA).

5.4. Results

5.4.1. Recruitment

After reading the information booklet, 424 individuals expressed an interest in being screened for enrolment. Following telephone screening, 189 changed their minds, and 163 were excluded. 72 participants attended screening visits, of whom 25 were excluded, and 6 changed their minds. A total of 41 participants were challenged. This is shown in Figure 32.

Figure 32: Consort diagram showing participant recruitment for the study 'Understanding typhoid disease: Developing a *S.* Typhi challenge model in healthy adults'



5.4.2. Study participants

Forty one participants were challenged in two cohorts; cohort one included 21 participants, challenged with a target dose of 1-5x10^3 CFU *S.* Typhi, cohort two included 20 participants receiving a target dose of 1-5 x10^4 CFU. One participant in cohort one was treated before the primary end point was reached and was therefore excluded from the per-protocol analysis. Baseline characteristics of the two cohorts, shown as those who developed typhoid disease and those that did not are shown in Table 39.

Table 39: Baseline characteristics of 40 participants in the study 'Understanding typhoid disease: Developing a *S.* Typhi challenge model in healthy adults', shown by as those who did and did not develop typhoid disease or not, following challenged with either 1-5x10^3 CFU or 1-5 x10^4 CFU of *S.* Typhi Quailes strain

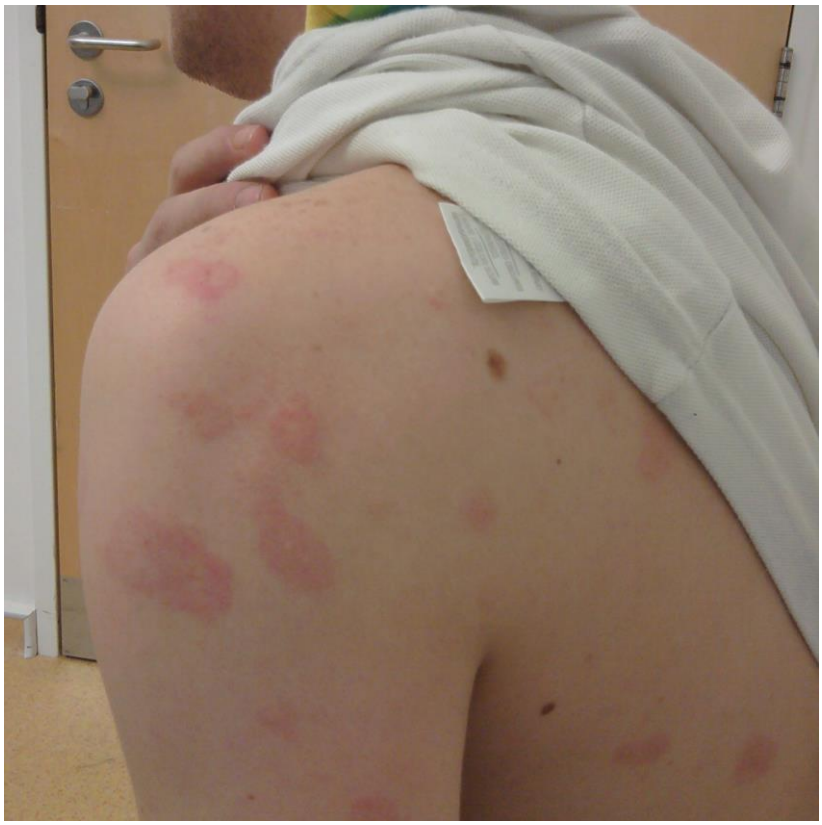
Cohort 1 (Challenge dose, 1-5 x10^3 CFU)			
		Typhoid disease	**No typhoid disease**
Gender	No. of males	11	5
	No. of females	0	4
Age	Median (range)	26.7 (19.4-46.5)	27.5 (20.7-46.3)
Ethnicity	Caucasian	10	9
	Other	1	0
Cigarettes smoked per day	Median per day (range)	0 (0-20)	0 (0-0)
Alcohol intake	Median per week (range)	6 (0-24)	4 (0-12)
Cohort 2 (Challenge dose 1-5 x10^4 CFU)			
		Typhoid disease	**No typhoid disease**
Gender	No. of males	8	4
	No. of females	5	3
Age	Median(range)	27.4 (19.6-44.4)	25.5 (19.8-45.1)
Ethnicity	Caucasian	12	7
	Afro-Caribbean	1	0
Cigarettes smoked	Median per day (range)	0 (0-8)	1 (0-15)
Alcohol intake	Median per week (range)	4 (0-30)	10 (0-20)

5.4.3. Safety

None of the 41 challenge participants required in-patient care or had life-threatening sequelae. One participant was treated prior to meeting the primary end points due to symptoms preventing all activities of daily living. There were no participants with severe typhoid disease in cohort one. In cohort two, 2 out of 20 participants had severe typhoid disease on the basis of fever greater than 40°C. Two participants had possible transient orchitis associated with typhoid disease. Two participants stopped antibiotic courses early,

one due to rapid rise in alkaline phosphatase and one due to diarrhoea (antibiotics were switched to azithromycin). One participant developed erythema multiforme, likely from ciprofloxacin, at the end of the antibiotic course (Figure 33).

Figure 33: Erythema multiforme rash in a participant treated with ciprofloxacin following challenge with *S.* Typhi



The number of participants with symptoms of anxiety and depression as classified by the HAD score is shown in Table 40. Changes in HADS are shown in Table 41. No participants experienced severe psychological symptoms, and only one participant experienced moderate symptoms at any time point. One participant experienced a large increase in anxiety symptoms, with a rise of 11 points over baseline).

Table 40: Levels of anxiety and depression as classified by the HAD score in 40 participants at day 0, day 7 and day 14 after challenge in the study 'Understanding typhoid disease: Developing a *S.* Typhi challenge model in healthy adults'

	HADS –Anxiety (number of participants)			HADS- Depression (number of participants)		
	Day 0	**Day 7**	**Day 14**	**Day 0**	**Day 7**	**Day 14**
Normal	35/40	36/40	37/40	38/40	40/40	39/40
Mild	4/40	3/40	2/40	2/40	0	1/40
Moderate	1/40	1/40	1/40	0	0	0
Severe	0	0	0	0	0	0

Table 41: Changes in HAD score at days 7 and 14 after challenge with *S.* Typhi relative to baseline value (day 0) in 40 participants in the study 'Understanding typhoid disease: Developing a *S.* Typhi challenge model in healthy adults'

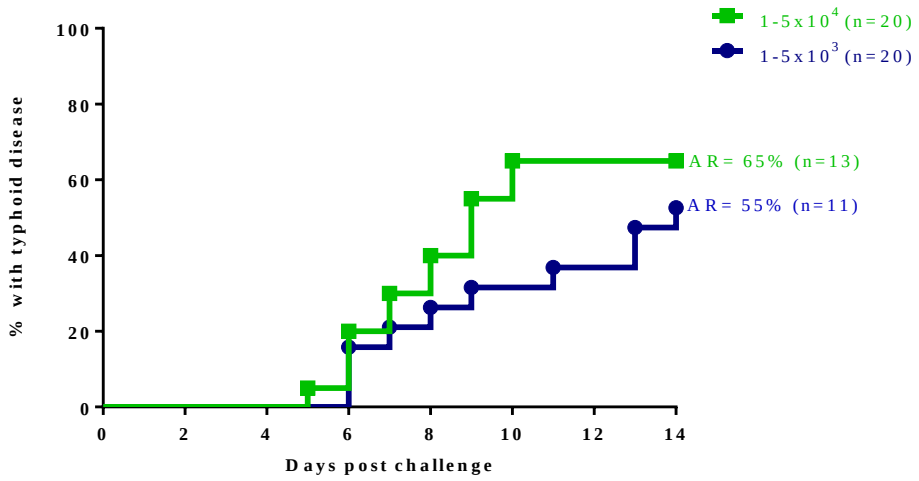
Change in HAD score relative to baseline (day 0)	Number of participants (range of change in HADS score)			
	Day 7 - Anxiety	**Day 7 - Depression**	**Day 14- Anxiety**	**Day 14- Depression**
Increase	8 (1-6)	14 (1-5)	4 (1-11)	10 (1-8)
Decrease	20 (1-5)	7 (1-3)	29 (1-6)	9 (1-3)
No change	12	19	7	21

Secondary transmission to household and sexual contacts was not reported. No participants have continued to excrete *S.* Typhi in their stools after antibiotics.

5.4.4. Primary endpoint

In cohort 1, 11 of 20 participants developed typhoid disease (attack rate of 55%). In cohort 2, 13 of 20 participants developed typhoid disease (attack rate 65%) according to the *a priori* endpoint definition (Figure 34). This difference did not reach statistical significance (Fisher's exact test, p=0.75).

Figure 34: Time to infection curve for participants who developed typhoid disease following challenge with either 1-5x10^3 CFU or 1-5x10^4 CFU of *S.* Typhi Quailes strain on day 0 in the study 'Understanding typhoid disease: Developing a *S.* Typhi challenge model in healthy adults'



There was a trend towards decreased incubation period with the higher challenge dose. Onset of illness appeared to occur earlier for those given a higher dose compared to those given the lower dose (cohort 2, 5-10 days, cohort 1 5-13 days) but this did not achieve significance (log-rank test p= 0.32). Criteria by which people were diagnosed are shown in Table 42.

Table 42: The number of participants that were diagnosed with typhoid disease by each of the diagnostic criteria following challenge with either 10^3 CFU or 10^4 CFU of *S.* Typhi in the study 'Understanding typhoid disease: Developing a *S.* Typhi challenge model in healthy adults'

Challenge dose	Diagnostic criteria for typhoid disease				Total number of participants diagnosed with typhoid (%)
	Number of participants with bacteraemia 5 days post challenge with objective sign of typhoid fever (%)	Number of participants with bacteraemia ONLY, 7 days post challenge (%)	Number of participants with temperature >38°C for 12 hours 3 days after challenge ONLY (%)	Number of participants with bacteraemia and temperature >38°C for 12 7 days after challenge hours (%)	
1-5 x10^3 CFU (n=20)	3 (15)	4 (20)	1 (5)	3 (15)	11 (55)
1-5 x10^4 CFU (n=20)	4 (20)	4 (20)	2 (10)	3 (15)	13 (65)
Total (n=40)	7 (17.5)	8 (20)	3 (7.5)	6 (15)	24 (60)

Of the 8 participants diagnosed with typhoid disease due to bacteraemia only 7 days after challenge, 4 were subsequently febrile, with oral temperatures of 38°C or more (1 participant challenged with 10^3 CFU, and 3 challenged with 10^4 CFU).

5.5. Discussion

5.5.1. Attack rate

When ingested with a sodium bicarbonate buffer, 1-5x10^4 CFU of *S.* Typhi Quailes strain led 65% of participants to develop typhoid disease. With this attack rate, the model could be employed in the future to appraise novel vaccines, as the model would have sufficient power to demonstrate improved vaccine efficacy of potential novel vaccines over currently licenced vaccines in a moderate number of participants.[451]

The attack rate observed with ingestion of a lower number of organisms ($1x10^3$ CFU of *S.* Typhi Quailes strain) was 55%. This is the first time that infection has been successfully induced at this low a challenge dose in a challenge model. In previous challenge studies that used milk rather than sodium bicarbonate as a buffer, infection could not be induced with $1x10^3$ CFU and in participants receiving $1x\ 10^7$ CFU, the attack rate was just 50%.[85] The uniformly higher attack rates seen when using a sodium bicarbonate buffer rather than milk is consistent with data from related enteric disease human challenge models where a sodium bicarbonate buffer has been used.[452,453] For example, in *Shigella flexneri* challenge studies an attack rate of 92% (11 of 12 volunteers) was seen in the sodium bicarbonate group compared to 50-60% when a milk buffer was used.[410] These increased attack rates following sodium bicarbonate pre-treatment compared to milk reflect the sensitivity of *S.* Typhi to stomach acid, which has been examined in-vitro. *S.* Typhi incubated at pH 2.5 for 2 hours, mimicking the gut environment, had less than 0.001% bacterial survival.[405] Notably, *Salmonella* species were significantly more sensitive to the effects of acid than *Shigella* species and most species of *E. coli* tested.[405]

Given the trend towards a dose- response observed at the two doses examined, it may have been possible to induce a higher attack rate with a further increase in challenge dose. However, this would have risked overwhelming any protection afforded by a vaccine. The model described in this report will be more reflective of wild-type infection, where attack rates are modest[223] and hence more relevant for vaccine appraisal.

5.5.2. Diagnostic definitions

Typhoid disease was diagnosed in those with *S.* Typhi bacteraemia and/or persistent fever. Although scientific integrity demands clear endpoints, the clinical nature of this study highlighted several grey areas around this definition of disease. One participant developed symptoms typical of typhoid disease as well as a temperature of 37.8°C on the evening of day 10, progressing to 38.4°C by the next morning. At this point, treatment was

commenced in view of the extent of the participant's symptoms. The participant did not satisfy the study's primary endpoint definition as the fever abated before 12 hours had elapsed and *S*. Typhi was not isolated from the blood. In all probability this participant genuinely exhibited typhoid disease, but was excluded from the *per protocol* analysis of the primary endpoint. Another participant also experienced symptoms consistent with typhoid disease and recorded an oral temperature over 38°C; the fever did not endure long enough to meet the primary endpoint. Although the endpoint of 12 hours of fever was considerably more conservative than the 2 consecutive days that was used in the Maryland challenge studies[87] it may none the less have been too stringent, and indeed may exclude milder cases of disease.[390] Given the rarity of a high fever in adults, it is highly plausible that any documented fever over 38°C in the 14 days after challenge is attributable to typhoid disease, especially when associated with other compatible infection symptoms. The vast majority of participants with fever had blood culture confirmation of typhoid disease later on. Furthermore, for participants with typhoid, this period of untreated disease was unpleasant, and it is ethically important to consider reducing this period in future studies.

Four participants had bacteraemia with *S*. Typhi with no associated febrile response. In a field trial setting, these participants would not have been diagnosed with typhoid fever as blood cultures would only be obtained in febrile patients. These participants may have developed fever had antibiotic therapy not been initiated. However, it is not known whether it is safe to leave asymptomatic *S*. Typhi bacteraemia untreated. One approach may have been to admit these participants for close observation and to only commence antibiotics if clinical illness ensued.

All un-diagnosed participants were treated with antibiotics at 14 days after challenge. This cut off point was chosen to ensure safety of the model, and to make sure the model could be practically delivered. Complications of typhoid fever are known to increase in incidence with a longer duration of infection,[7] and therefore it was felt that it was safest to treat all participants by day 14 to ensure sub-clinical infection was not untreated for a prolonged

period. It was also felt that increasing the period of intense follow up in the trial may affect recruitment and increase the cost of the trial. In limiting the follow up period to 14 days there is however a risk that the true attack rate for each challenge dose was underestimated, as further cases may have been diagnosed had participants not been treated. This is likely to be particularly true at the lower 10^3 CFU challenge dose, where the incubation period was longer than was observed at the 10^4 CFU challenge dose. Indeed at the 10^3 CFU challenge dose, cases were diagnosed right up until the day 14 cut off. This trend is also supported by data from the Maryland studies, where incubation period was inversely related to challenge dose, and was extremely prolonged in some participants.[73] For example, in those ingesting $1x10^5$ CFU, the incubation period range was 3 to 52 days, and 18% of cases occurred after 3 weeks.[73] This raises the question as to whether the period of follow–up for the primary endpoint could be increased, for example to 21 days. Although complication rates increase with delayed treatment of typhoid disease, there is no clear evidence that prolonged incubation periods affect clinical outcome or the complication rate, and indeed in this study those diagnosed later in the follow up period were no more unwell than those diagnosed early on. To limit the cost implications and burden on participants, daily visits could be extended beyond 14 days only in participants that had not been diagnosed with disease by day 14 rather than in all participants.

It can be seen that determining strict case definitions in a human challenge models is challenging. The numbers of participants in challenge studies is usually small, and therefore determining whether each participant meets the case definition or not has a significant impact on the reported attack rates. For example, had either of the participants who developed fever but did not meet the case definition been considered cases in the analysis, the attack rate following ingestion of 10^3 CFU would have been 60%, satisfying the primary objective of the study. Having established in this study that challenge is safe and well tolerated, a more flexible approach to case follow up may be appropriate in future

challenge studies, for example by allowing a longer incubation period and not treating asymptomatic bacteraemia. This may make the model more representative of field studies.

5.5.3. Safety

Clinical illness was well tolerated by all participants, with no participants requiring admission for in-patient care. Previous challenge trials have also demonstrated that challenge with *S.* Typhi Quailes strain is a safe and effective mechanism to examine the immunobiology, pathogenesis and vaccination strategies for typhoid disease.[73,378] One participant in the previous trials became a chronic carrier and was later found to have gallstones. Cholecystectomy led to a resolution of his carrier status. No participants in this study experienced persistent excretion of *S.* Typhi in their stools. No cases of secondary transmission have been detected, providing reassurance that the measures taken were sufficient to protect public health.

Variations in participant reported anxiety and depression as measured by the HAD score were observed. The normal variability of the score is not known. Only one participant experienced a significant increase in anxiety. The participant reported that this was due to external circumstance, and was happy to continue in the study. The HAD score, although not developed for this purpose, may provide a useful measure of anxiety and depression for participants in the study where both the nature of the illness and the study may have adverse impacts on psychological health. Further validation of the use of the HAD score for this purpose is needed.

5.6. Conclusions

A challenge model of typhoid disease using *S.* Typhi Quailes strain at a dose of 1-5 x10^4 CFU gives an attack rate that should allow future vaccine efficacy studies to be conducted with a moderate number of participants. The safety of challenging participants with a category 3 pathogen in an outpatient setting has been demonstrated. Establishing the

model has provided a unique opportunity to gather detailed data on the clinical response to *S*. Typhi challenge, and this data will be presented in subsequent chapters.

6. Clinical response to *S.* Typhi Challenge

6.1. Introduction

Typhoid disease has a variable, non-specific, clinical presentation with reported symptoms and severity depending on the population studied.[7,454] Data from field studies regarding the clinical course of infection is limited by case selection bias, and difficulties in accurately identifying cases.[7] Fever is reported to be the most universal symptom of typhoid disease,[87] with other symptoms including malaise, myalgia, arthralgia, anorexia, gastrointestinal disturbance, cough and headache.[96,97] Similar symptoms are reported in endemic settings, suggesting that chronic exposure and any pre-existing immunity does little to influence the course of the disease once established.[29,98,99,104] Illness severity is partially predicted by the interval between disease onset and treatment, but even allowing for this, severity is highly variable, with some patients able to continue normal activity and some requiring in-patient care.[99]

The systemic inflammatory and immune response to typhoid disease, together with direct bacterial invasion of the bone marrow, is reflected in abnormalities of laboratory blood values/parameters. Haematopoietic cell concentrations are frequently reduced, and thrombocytopenia in particular is a common finding.[96,102] Leukocyte counts are often low, especially given the degree of fever and toxicity[104], with frank leukopenia may occur.[96,97,99] Liver enzymes are usually elevated, and infection may be associated with decreased albumin and raised bilirubin concentrations.[7,96,99]

Findings in previous *S.* Typhi challenge studies conducted in Maryland were consistent with the symptoms reported in naturally occurring typhoid disease. Fever, rising in a step-wise fashion over three days, was the first sign of illness and was followed by headache and abdominal pain, associated with tenderness on palpation, and then anorexia, myalgia and

fatigue.[73] Although the incubation period varied the resulting illness was comparable irrespective of challenge dose.[73] Treatment with chloramphenicol led to defervescence after an average of 3.5 days of treatment.[73,378]

The human challenge model presented in Chapter 5 provided a unique opportunity to prospectively study the clinical features of typhoid infection. This chapter describes the clinical response of participants challenged with 10^3 or 10^4 CFU of *S.* Typhi (Quailes strain) during the development of a human challenge model of typhoid disease. Comparison between those who did and did not develop typhoid disease at each challenge dose will be presented.

6.2. Methods

A full description of participant follow-up is presented in Chapter 5. Solicited symptoms were self-recorded by diary card. Diary cards were reviewed daily by study investigators to verify symptoms reported and their severity. Temperature was measured by oral thermometry in the morning and evening and at any other time that participants felt feverish or unwell. Blood pressure and pulse rate measurements were performed at morning visits. Laboratory tests were performed according to the schedule outlined in Chapter 5 by Oxford University Hospitals pathology laboratories according to their standard procedures. Additional clinical reviews and blood sampling were arranged if indicated at the discretion of study doctors.

6.2.1. Statistical analysis

Diary card data was transcribed using OpenClinica clinical trial software (OpenClinica Community edition version 2.1, Massachusetts, USA). Data was extracted and analysed using Microsoft Excel (2010 edition, Microsoft, Washington, USA) and GraphPad Prism (version 5, GraphPad, Inc., La Jolla, USA).

Participants were grouped according to if they met the diagnostic definition of typhoid disease or not at each challenge dose (10^3 and 10^4 CFU). Mean averages and 95% confidences intervals are presented for comparative descriptive analyses. Formal statistical comparisons were not performed as the study was not powered for clinical endpoints.

6.3. Results

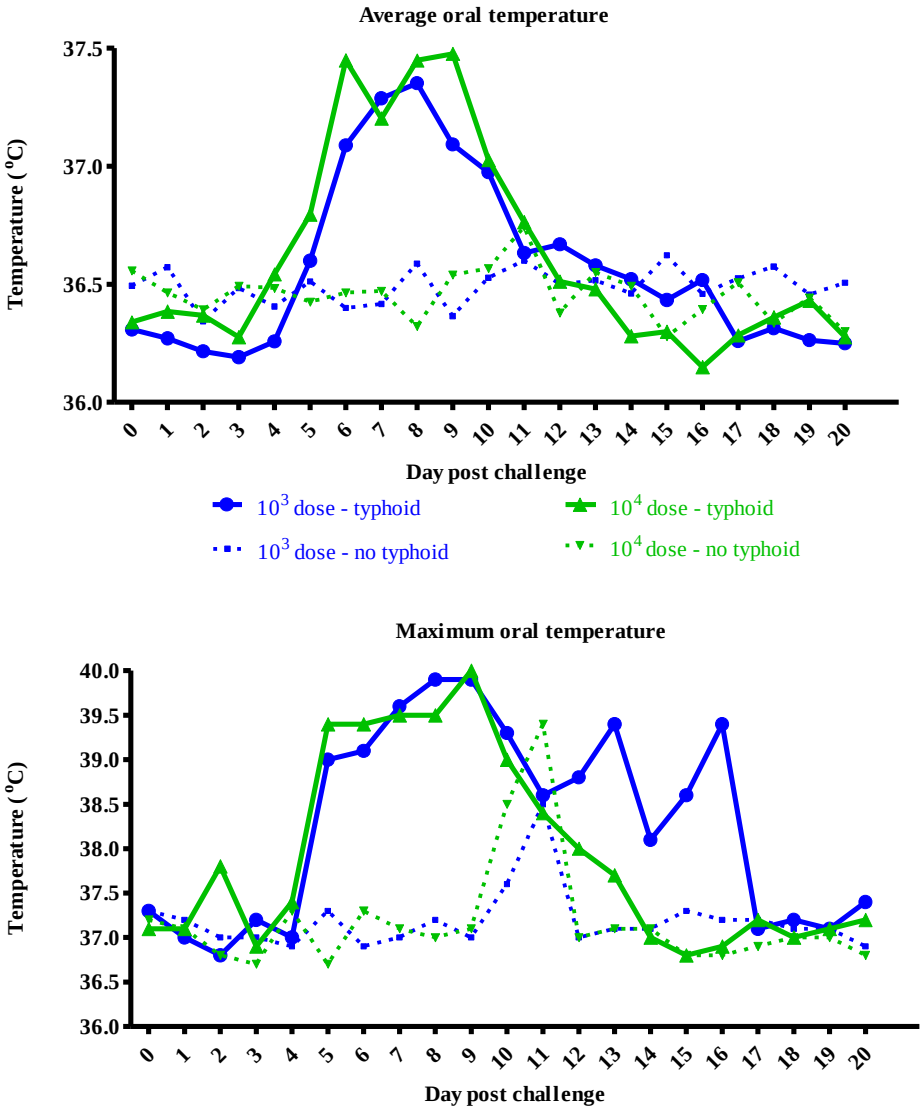
6.3.1. Oral temperature kinetics in all participants

The mean and maximum oral temperatures in the 20 days following challenge are shown in Table 43 and Figure 35. Oral temperatures rose from day 4 onwards in those who developed typhoid disease. The average temperature rose slightly earlier in those challenged with 10^4 CFU *S*. Typhi, and reached a slightly higher maximum compared to those challenged with 10^3 CFU. Four participants (3 challenged with 10^3 CFU and 1 challenged with 10^4 CFU) who were bacteraemic with *S*. Typhi did not have temperatures greater than the diagnostic threshold of 38°C. Maximum temperatures in these 4 participants were 37.3, 37.2, 37.5 and 37.5°C respectively. Notably, two participants, one from each challenge dose, developed temperatures above 38°C; neither was sustained for the 12 hours required to meet the primary endpoint and so remained 'undiagnosed'. One of these participants was symptomatic, reporting muscle pain, loss of appetite, abdominal pain and nausea, but this, along with the temperature self-resolved after a few hours. The other participant remained asymptomatic throughout the study period, and neither was bacteraemic.

Table 43: Maximum (max.) and mean oral temperatures over the 20 days following challenge (Day 0) with 10^3 or 10^4 CFU of *S.* Typhi Quailes strain in a human challenge model of typhoid disease, with temperatures above the limit of normal (37.6°C) highlighted in pale pink and temperatures above the diagnostic threshold for typhoid disease (38°C) highlighted in dark pink

Challenge outcome	Challenge dose (CFU)		Oral temperature (°C)																			
			Day 0	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 8**	**Day 9**	**Day 10**	**Day 11**	**Day 12**	**Day 13**	**Day 14**	**Day 15**	**Day 16**	**Day 17**	**Day 18**	**Day 19**	**Day 20**
Developed typhoid disease	10^3 (n=11)	Max.	37.3	37.0	36.8	37.2	37.0	39.0	39.1	39.6	39.9	39.9	39.3	38.6	38.8	39.4	38.1	38.6	39.4	37.1	37.2	37.1	37.4
		Mean	36.3	36.3	36.2	36.2	36.3	36.6	37.1	37.3	37.4	37.1	37.0	36.6	36.7	36.6	36.5	36.4	36.5	36.3	36.3	36.3	36.3
	10^4 (n=13)	Max.	37.1	37.1	37.8	36.9	37.4	39.4	39.4	39.5	39.5	40.0	39.0	38.4	38.0	37.7	37.0	36.8	36.9	37.2	37.0	37.1	37.2
		Mean	36.3	36.4	36.4	36.3	36.5	36.8	37.5	37.2	37.4	37.5	37.0	36.8	36.5	36.5	36.3	36.3	36.2	36.3	36.4	36.4	36.3
Did not develop typhoid disease	10^3 (n=9)	Max.	37.3	37.2	37.0	37.0	36.9	37.3	36.9	37.0	37.2	37.0	37.6	38.5	37.0	37.1	37.1	37.3	37.2	37.2	37.1	37.1	36.9
		Mean	36.5	36.6	36.3	36.5	36.4	36.5	36.4	36.4	36.6	36.4	36.5	36.6	36.5	36.5	36.5	36.6	36.5	36.5	36.6	36.5	36.5
	10^4 (n=7)	Max.	37.2	37.1	36.8	36.7	37.3	36.7	37.3	37.1	37.0	37.1	38.5	39.4	37.0	37.1	37.1	36.8	36.8	36.9	37.0	37.0	36.8
		Mean	36.6	36.5	36.4	36.5	36.5	36.4	36.5	36.5	36.3	36.5	36.6	36.7	36.4	36.6	36.5	36.3	36.4	36.5	36.3	36.4	36.3

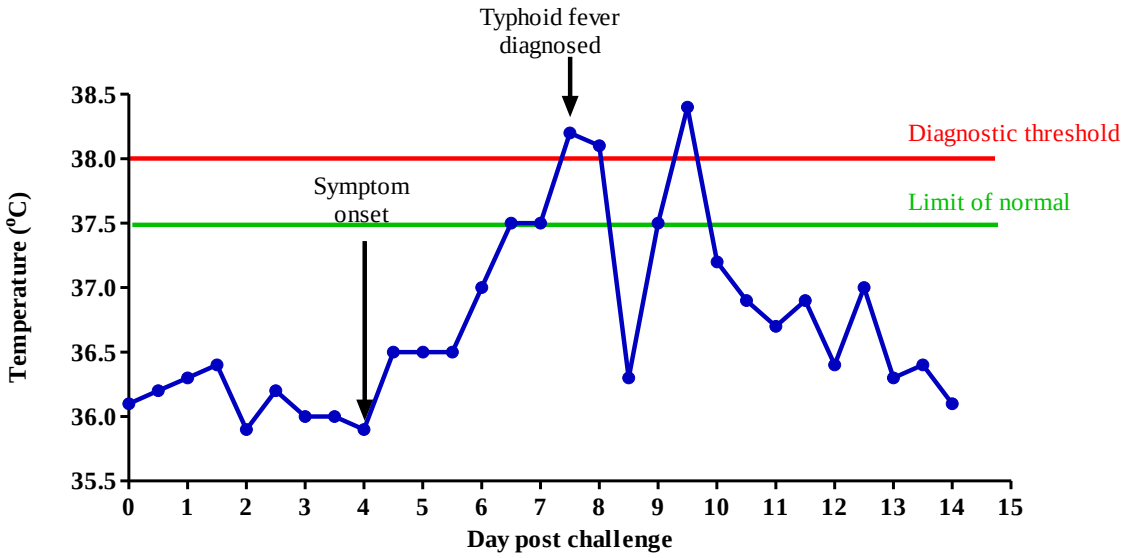
Figure 35: Maximum (max.) and mean oral temperatures over the 20 days following challenge (Day 0) with 10^3 or 10^4 CFU of *S.* Typhi Quailes strain in a human challenge model of typhoid disease



6.3.2. Oral temperature changes relative to the point of diagnosis of typhoid disease

A stepwise pattern in temperature elevation was noted in the many participants with typhoid disease. A typical example is shown in Figure 36. The diagnosis of typhoid disease was made at the peak of temperature.

Figure 36: Morning and evening oral temperature readings over the 14 days following challenge with 10^3 CFU of *S.* Typhi on day 0 in a participant diagnosed with typhoid disease, showing temperature instability and step-wise rise in temperature between days 4 and 8 after challenge, at which point the diagnostic definition for typhoid disease was satisfied

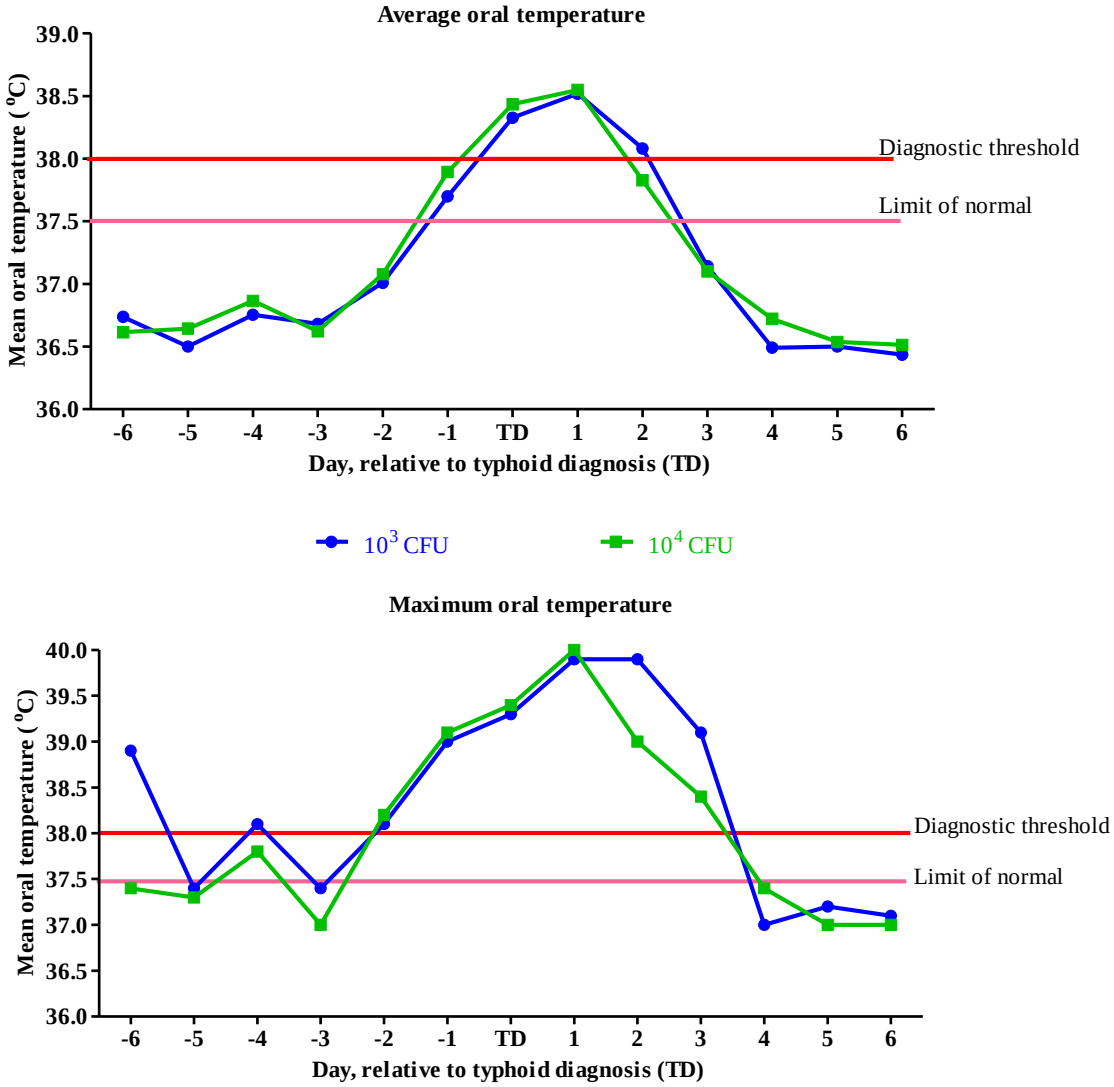


Daily maximum and average oral temperature relative to the diagnosis of typhoid disease in participants who developed typhoid disease following challenge with 10^3 or 10^4 CFU are shown in Table 44 and Figure 37.

Table 44: Maximum and mean oral temperatures in participants diagnosed with typhoid disease following challenge with 10^3 or 10^4 CFU of *S.* Typhi, relative to the point of diagnosis of typhoid disease (TD) in a human challenge model of typhoid disease

Challenge dose (CFU)		Oral temperature (°C)												
		-6	-5	-4	-3	-2	-1	TD	+1	+2	+3	+4	+5	+6
10^3 (n=11)	Maximum	38.9	37.4	38.1	38.2	38.1	39.0	39.3	39.9	39.9	39.1	37.0	37.2	37.1
	Mean	36.7	36.5	36.8	36.7	37.0	37.7	38.3	38.5	38.1	37.1	36.5	36.5	36.4
10^4 (n=13)	Maximum	37.4	37.3	37.8	37.0	38.2	39.1	39.4	40.0	39.0	38.4	37.4	37.0	37.0
	Mean	36.6	36.6	36.9	36.6	37.1	37.9	38.4	38.6	37.8	37.1	36.7	36.5	36.5

Figure 37: Maximum and mean oral temperatures in participants diagnosed with typhoid disease following challenge with 10^3 or 10^4 CFU of *S.* Typhi, relative to the point of diagnosis of typhoid disease (TD) in a human challenge model of typhoid disease



Elevated oral temperature occurred on average 2 days prior to diagnosis of typhoid disease, peaked the day after diagnosis and then declined gradually, returning to normal by 4 days after diagnosis. Of those with typhoid disease, participants challenged with 10^3 CFU had an average of 2.63 days with a temperature ≥38°C, and with 10^4 CFU had an average of 2.61 days. The maximum oral temperature observed in those developing typhoid was similar at both dose levels (10^3 CFU, 39.9°C, 10^4 CFU, 40°C).

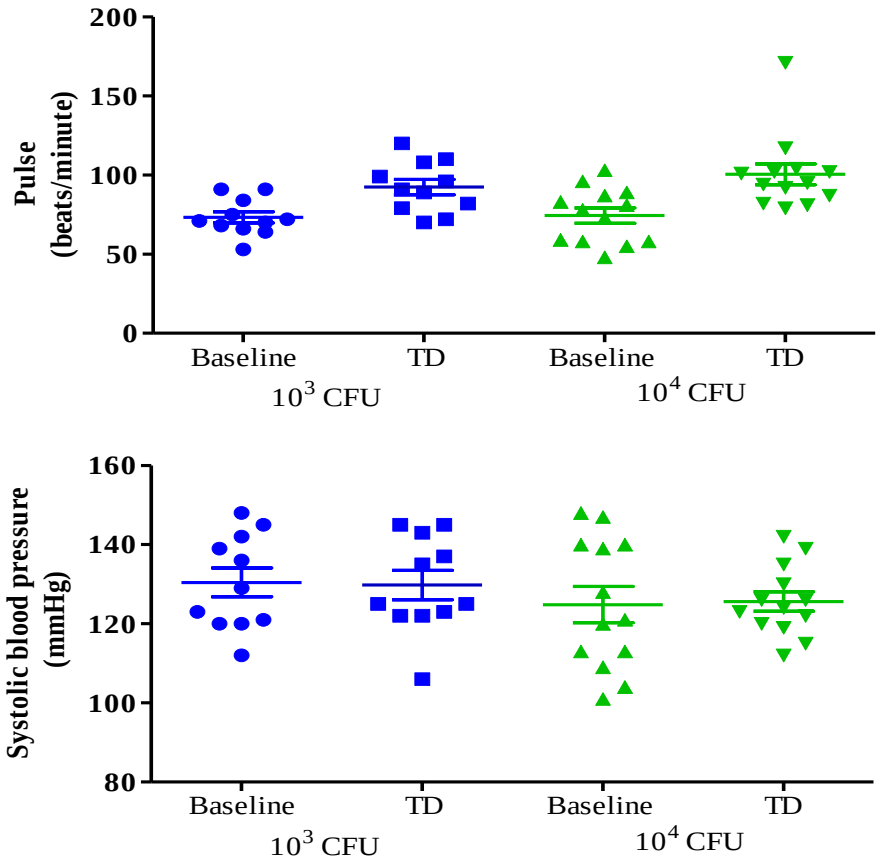
6.3.3. Changes in pulse and systolic blood pressure with typhoid disease

The changes in average pulse and systolic blood pressure for participants diagnosed with typhoid disease are shown in Table 45 and Figure 38.

Table 45: Mean pulse and systolic blood pressure at baseline (Day 0) and at typhoid diagnosis for participants diagnosed with typhoid disease following challenge with 10^3 or 10^4 CFU of *S.* Typhi

	Time point	Challenge dose	
		10^3 CFU (n=11)	**10^4 CFU (n=13)**
Mean pulse (95% CI)	Baseline (Day 0)	73.2 (65.4-81.0)	74.5 (63.9-85.0)
	Typhoid diagnosis	92.4 (81.6-103.2)	100.5 (86.2-115.0)
Mean systolic blood pressure (95% CI)	Baseline (Day 0)	130.5 (122.3-138.6)	124.8 (114.9-134.8)
	Typhoid diagnosis	129.8 (121.6-138.0)	125.6 (120.2-131.0)

Figure 38: Mean pulse and systolic blood pressure at baseline (Day 0) and at typhoid diagnosis for participants diagnosed with typhoid disease following challenge with 10^3 or 10^4 CFU of *S.* Typhi



The diagnosis of typhoid disease was associated with tachycardia relative to baseline, but, given the degree of fever in the majority of participants, the tachycardia was relatively modest. The average increase in pulse was higher following challenge with 10^4 CFU than with 10^3 CFU (19.2 vs. 26.0 beats per minute). There was no change is systolic blood pressure.

6.3.4. Solicited symptoms

Diary card data for solicited symptoms were available for all participants, and are shown in Table 46, Table 47, Table 48, Table 49, Figure 39, Figure 40 and Figure 41.

Table 46: The number of participants diagnosed WITH typhoid disease who reported each solicited symptom on each day following challenge (day 0) with 10^3 CFU of *S.* Typhi in a human challenge model of typhoid disease

Challenge dose (CFU)	Symptom	Number of participants affected (%)															
		Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15
10^3 (n=11)	Headache	0 (0.0)	0 (0.0)	2 (18.2)	3 (27.3)	1 (9.1)	3 (27.3)	5 (45.5)	4 (36.4)	6 (54.5)	6 (54.5)	4 (36.4)	4 (36.4)	3 (27.3)	2 (18.2)	4 (36.4)	5 (45.5)
	Generally unwell	0 (0.0)	1 (9.1)	2 (18.2)	3 (27.3)	2 (18.2)	3 (27.3)	6 (54.5)	7 (63.6)	6 (54.5)	6 (54.5)	5 (45.5)	4 (36.4)	1 (9.1)	2 (18.2)	3 (27.3)	3 (27.3)
	Loss of appetite	0 (0.0)	1 (9.1)	0 (0.0)	2 (18.2)	1 (9.1)	3 (27.3)	4 (36.4)	5 (45.5)	5 (45.5)	5 (45.5)	4 (36.4)	3 (27.3)	1 (9.1)	1 (9.1)	0 (0.0)	1 (9.1)
	Abdominal pain	0 (0.0)	1 (9.1)	1 (9.1)	1 (9.1)	2 (18.2)	3 (27.3)	3 (27.3)	3 (27.3)	3 (27.3)	4 (36.4)	5 (45.5)	2 (18.2)	1 (9.1)	2 (18.2)	1 (9.1)	0 (0.0)
	Nausea and vomiting	0 (0.0)	0 (0.0)	1 (9.1)	2 (18.2)	1 (9.1)	2 (18.2)	2 (18.2)	5 (45.5)	2 (18.2)	3 (27.3)	3 (27.3)	1 (9.1)	1 (9.1)	1 (9.1)	1 (9.1)	1 (9.1)
	Myalgia	0 (0.0)	0 (0.0)	0 (0.0)	1 (9.1)	1 (9.1)	4 (36.4)	4 (36.4)	4 (36.4)	4 (36.4)	6 (54.5)	4 (36.4)	1 (9.1)	1 (9.1)	2 (18.2)	3 (27.3)	2 (18.2)
	Arthralgia	0 (0.0)	0 (0.0)	0 (0.0)	1 (9.1)	2 (18.2)	2 (18.2)	4 (36.4)	3 (27.3)	2 (18.2)	4 (36.4)	2 (18.2)	1 (9.1)	0 (0.0)	1 (9.1)	1 (9.1)	3 (27.3)
	Cough	0 (0.0)	1 (9.1)	0 (0.0)	1 (9.1)	1 (9.1)	1 (9.1)	2 (18.2)	4 (36.4)	3 (27.3)	6 (54.5)	5 (45.5)	1 (9.1)	1 (9.1)	2 (18.2)	2 (18.2)	1 (9.1)
	Diarrhoea	0 (0.0)	0 (0.0)	0 (0.0)	1 (9.1)	1 (9.1)	1 (9.1)	1 (9.1)	0 (0.0)	0 (0.0)	0 (0.0)	1 (9.1)	1 (9.1)	0 (0.0)	1 (9.1)	0 (0.0)	0 (0.0)
	Constipation	0 (0.0)	1 (9.1)	1 (9.1)	2 (18.2)	1 (9.1)	0 (0.0)	1 (9.1)	3 (27.3)	3 (27.3)	4 (36.4)	6 (54.5)	2 (18.2)	1 (9.1)	1 (9.1)	2 (18.2)	1 (9.1)
	Total number of symptoms per participant	0	0.5	0.6	1.5	1.2	2.0	2.9	3.5	3.1	4.0	3.5	1.8	0.9	1.4	1.5	1.5

Table 47: The number of participants diagnosed WITH typhoid disease who reported each solicited symptom on each day following challenge (day 0) with 10^4 CFU of *S.* Typhi in a human challenge model of typhoid disease

Challenge dose (CFU)	Symptom	Number of participants affected (%)															
		Day 0	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 8**	**Day 9**	**Day 10**	**Day 11**	**Day 12**	**Day 13**	**Day 14**	**Day 15**
10^4 (n=13)	Headache	2 (15.4)	0 (0.0)	0 (0.0)	2 (15.4)	3 (23.1)	8 (61.5)	6 (46.2)	9 (69.2)	10 (76.9)	9 (69.2)	9 (69.2)	5 (38.5)	4 (30.8)	4 (30.8)	2 (15.4)	1 (7.7)
	Generally unwell	1 (7.7)	1 (7.7)	1 (7.7)	2 (15.4)	4 (30.8)	8 (61.5)	9 (69.2)	10 (76.9)	12 (92.3)	9 (69.2)	9 (69.2)	7 (53.8)	4 (30.8)	3 (23.1)	2 (15.4)	2 (15.4)
	Loss of appetite	0 (0.0)	0 (0.0)	0 (0.0)	1 (7.7)	1 (7.7)	4 (30.8)	8 (61.5)	9 (69.2)	11 (84.6)	8 (61.5)	6 (46.2)	4 (30.8)	3 (23.1)	1 (7.7)	0 (0.0)	0 (0.0)
	Abdominal pain	1 (7.7)	1 (7.7)	1 (7.7)	1 (7.7)	3 (23.1)	5 (38.5)	5 (38.5)	7 (53.8)	6 (46.2)	5 (38.5)	4 (30.8)	4 (30.8)	2 (15.4)	2 (15.4)	2 (15.4)	2 (15.4)
	Nausea and vomiting	0 (0.0)	0 (0.0)	0 (0.0)	1 (7.7)	2 (15.4)	3 (23.1)	4 (30.8)	6 (46.2)	9 (69.2)	4 (30.8)	4 (30.8)	4 (30.8)	1 (7.7)	1 (7.7)	3 (23.1)	1 (7.7)
	Myalgia	1 (7.7)	0 (0.0)	1 (7.7)	0 (0.0)	2 (15.4)	5 (38.5)	6 (46.2)	8 (61.5)	10 (76.9)	8 (61.5)	6 (46.2)	5 (38.5)	3 (23.1)	1 (7.7)	1 (7.7)	0 (0.0)
	Arthralgia	0 (0.0)	0 (0.0)	1 (7.7)	2 (15.4)	0 (0.0)	4 (30.8)	6 (46.2)	9 (69.2)	8 (61.5)	7 (53.8)	5 (38.5)	3 (23.1)	2 (15.4)	1 (7.7)	1 (7.7)	0 (0.0)
	Cough	1 (7.7)	0 (0.0)	0 (0.0)	1 (7.7)	1 (7.7)	1 (7.7)	0 (0.0)	3 (23.1)	5 (38.5)	5 (38.5)	4 (30.8)	2 (15.4)	1 (7.7)	1 (7.7)	1 (7.7)	1 (7.7)
	Diarrhoea	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (15.4)	1 (7.7)	1 (7.7)	2 (15.4)	4 (30.8)	1 (7.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Constipation	0 (0.0)	1 (7.7)	0 (0.0)	1 (7.7)	0 (0.0)	2 (15.4)	2 (15.4)	2 (15.4)	1 (7.7)	0 (0.0)	3 (23.1)	3 (23.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Total number of symptoms per participant	0.5	0.2	0.3	0.8	1.4	3.2	3.6	5.0	5.8	4.3	3.8	2.8	1.5	1.1	0.9	0.5

Table 48: The number of participants WITHOUT typhoid disease reporting each solicited symptom for each day following challenge (day 0) with 10^3 CFU of S. Typhi in a human challenge model of typhoid disease

Challenge dose (CFU)	Symptom	Number of participants affected (%)															
		Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15
10^3 (n=9)	Headache	3 (33.3)	2 (22.2)	1 (11.1)	2 (22.2)	0 (0.0)	0 (0.0)	0 (0.0)	1 (11.1)	1 (11.1)	1 (11.1)	1 (11.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (11.1)
	Generally unwell	0 (0.0)	0 (0.0)	0 (0.0)	1 (11.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (11.1)	0 (0.0)	2 (22.2)	1 (11.1)	1 (11.1)	0 (0.0)	0 (0.0)	0 (0.0)
	Loss of appetite	1 (11.1)	0 (0.0)	1 (11.1)	0 (0.0)	0 (0.0)	0 (0.0)	1 (11.1)	1 (11.1)	0 (0.0)	0 (0.0)	1 (11.1)	1 (11.1)	0 (0.0)	0 (0.0)	0 (0.0)	1 (11.1)
	Abdominal pain	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (11.1)	0 (0.0)	1 (11.1)	1 (11.1)	1 (11.1)	0 (0.0)	0 (0.0)	1 (11.1)
	Nausea and vomiting	0 (0.0)	0 (0.0)	1 (11.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (11.1)	1 (11.1)	0 (0.0)	0 (0.0)	0 (0.0)	1 (11.1)
	Myalgia	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (22.2)	2 (22.2)	1 (11.1)	0 (0.0)	0 (0.0)	0 (0.0)
	Arthralgia	0 (0.0)	0 (0.0)	0 (0.0)	1 (11.1)	1 (11.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Cough	1 (11.1)	0 (0.0)	2 (22.2)	2 (22.2)	2 (22.2)	2 (22.2)	2 (22.2)	1 (11.1)	2 (22.2)	2 (22.2)	1 (11.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Diarrhoea	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (11.1)	0 (0.0)	1 (11.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Constipation	0 (0.0)	1 (11.1)	1 (11.1)	2 (22.2)	2 (22.2)	1 (11.1)	1 (11.1)	1 (11.1)	1 (11.1)	2 (22.2)	1 (11.1)	1 (11.1)	1 (11.1)	0 (0.0)	0 (0.0)	0 (0.0)
	Total number of symptoms per participant	0.6	0.3	0.7	0.9	0.6	0.3	0.4	0.4	0.8	0.6	1.2	0.8	0.4	0.0	0.0	0.4

Table 49: The number of participants WITHOUT typhoid disease reporting each solicited symptom for each day following challenge (day 0) with 10^4 CFU of S. Typhi in a human challenge model of typhoid disease

Challenge dose (CFU)	Symptom	Number of participants affected (%)															
		Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15
10^4 (n=7)	Headache	1 (14.3)	1 (14.3)	0 (0.0)	1 (14.3)	2 (28.6)	0 (0.0)	1 (14.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (14.3)	0 (0.0)	0 (0.0)
	Generally unwell	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (14.3)	1 (14.3)	1 (14.3)	1 (14.3)	1 (14.3)	0 (0.0)	0 (0.0)
	Loss of appetite	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (14.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (14.3)
	Abdominal pain	1 (14.3)	0 (0.0)	0 (0.0)	0 (0.0)	1 (14.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Nausea and vomiting	1 (14.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Myalgia	0 (0.0)	1 (14.3)	0 (0.0)	1 (14.3)	1 (14.3)	1 (14.3)	1 (14.3)	0 (0.0)	2 (28.6)	1 (14.3)	1 (14.3)	2 (28.6)	1 (14.3)	1 (14.3)	0 (0.0)	0 (0.0)
	Arthralgia	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Cough	1 (14.3)	1 (14.3)	1 (14.3)	1 (14.3)	1 (14.3)	1 (14.3)	1 (14.3)	1 (14.3)	1 (14.3)	1 (14.3)	1 (14.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Diarrhoea	0 (0.0)	0 (0.0)	0 (0.0)	1 (14.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Constipation	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (14.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (14.3)	0 (0.0)	0 (0.0)	0 (0.0)	1 (14.3)	0 (0.0)
	Total number of symptoms per participant	0.6	0.4	0.1	0.6	0.7	0.6	0.4	0.1	0.4	0.4	0.6	0.4	0.3	0.4	0.1	0.1

Figure 39: The total number of solicited symptoms reported per participant on each day following challenge (day 0) with either 10^3 CFU or 10^4 CFU, in those who were (typhoid) or were not (no typhoid) diagnosed with typhoid fever, in a human challenge model of typhoid disease

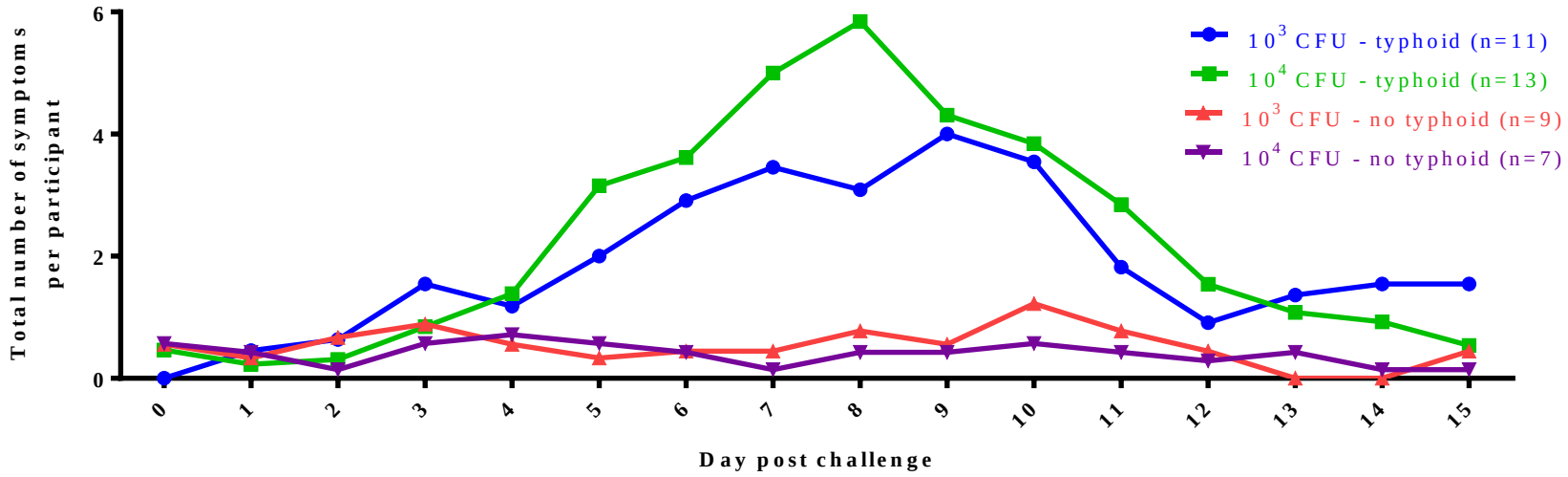


Figure 40: The proportion of participants reporting each systemic solicited symptom on each day following challenge with either 10^3 CFU or 10^4 CFU of *S.* Typhi, in a human challenge model of typhoid disease

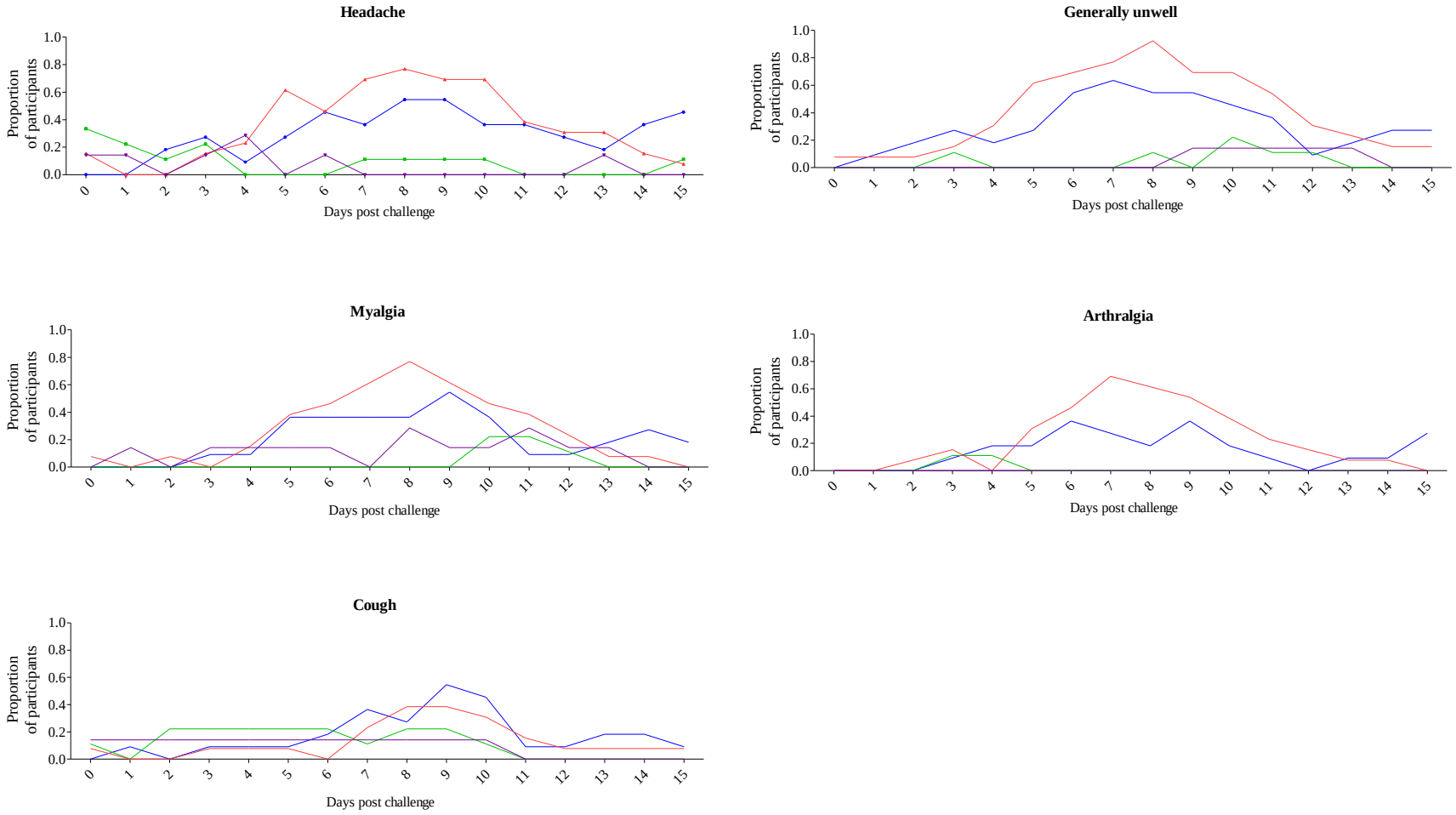
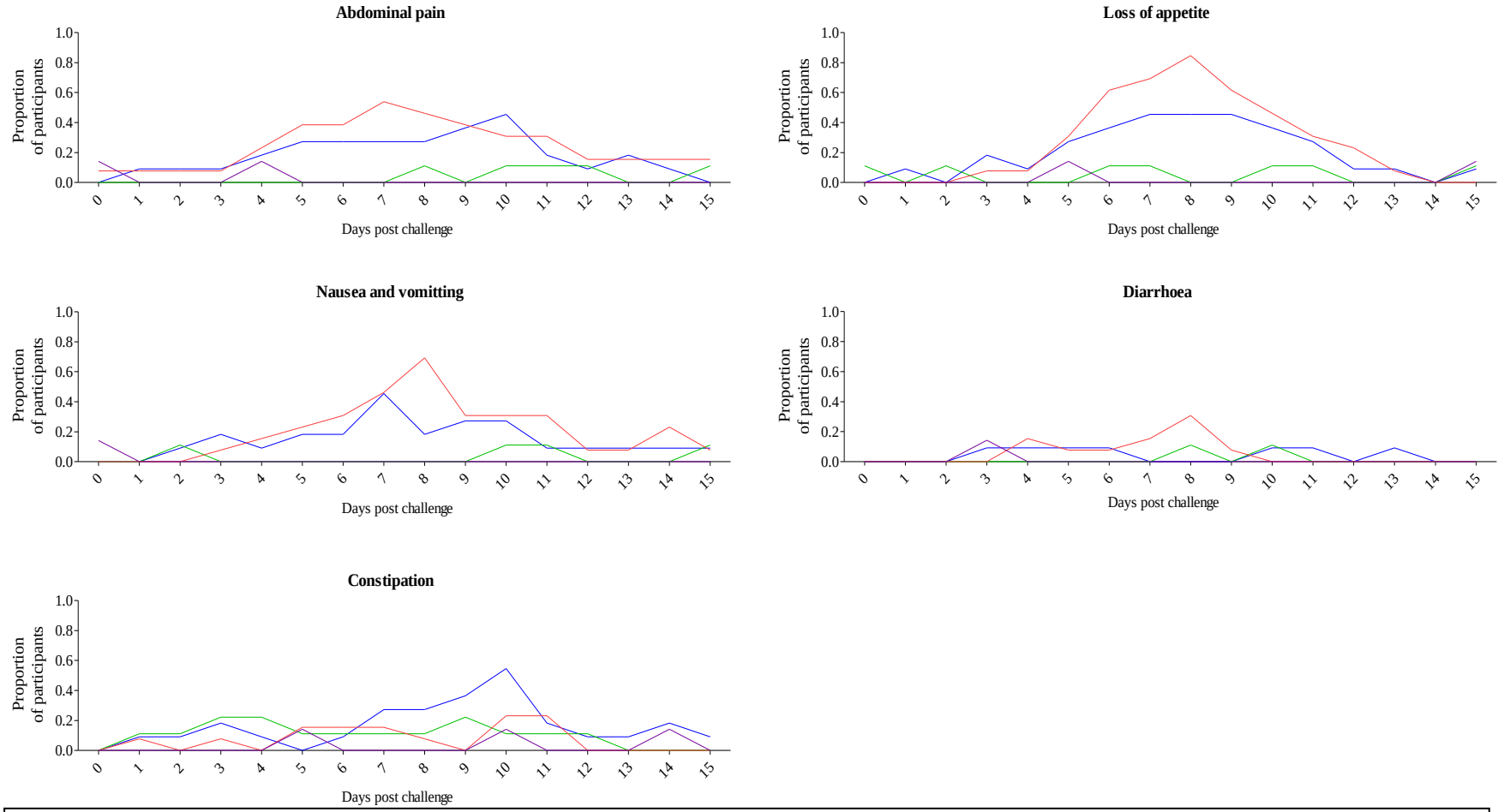


Figure 41: The proportion of participants reporting each gastro-intestinal solicited symptom on each day following challenge with either 10^3 CFU or 10^4 CFU of *S.* Typhi, in a human challenge model of typhoid disease



Participants developing typhoid disease became symptomatic from 4 days after challenge, and remained more symptomatic than those not diagnosed with typhoid disease until day 13. For those diagnosed with typhoid disease, the number of symptoms was related to disease onset (Figure 42, Figure 43 and Figure 44). Symptom reporting increased from day 1 prior to diagnosis of typhoid disease, and peaked on the day of diagnosis and day after diagnosis. Symptom onset occurred at the same time as oral temperature started to rise.

Figure 42: The average number of solicited symptoms reported per participant, relative to the day of typhoid diagnosis (TD) in participants challenged with 10^3 and 10^4 CFU of *S.* Typhi in a human challenge model of typhoid disease

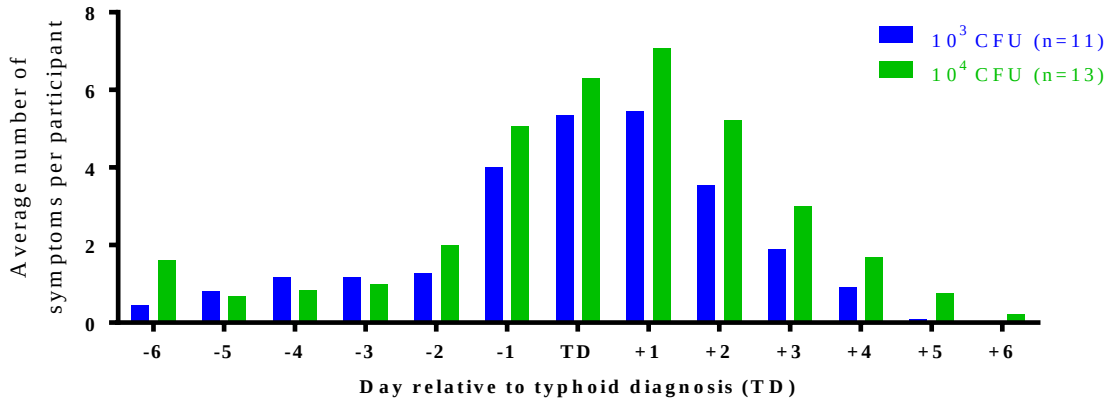


Figure 43: The proportion of participants reporting each solicited systemic symptom relative to the day of typhoid disease diagnosis in participants challenged with 10^3 CFU (n=11) and 10^4 CFU (n=13) of *S.* Typhi in a human challenge model of typhoid disease

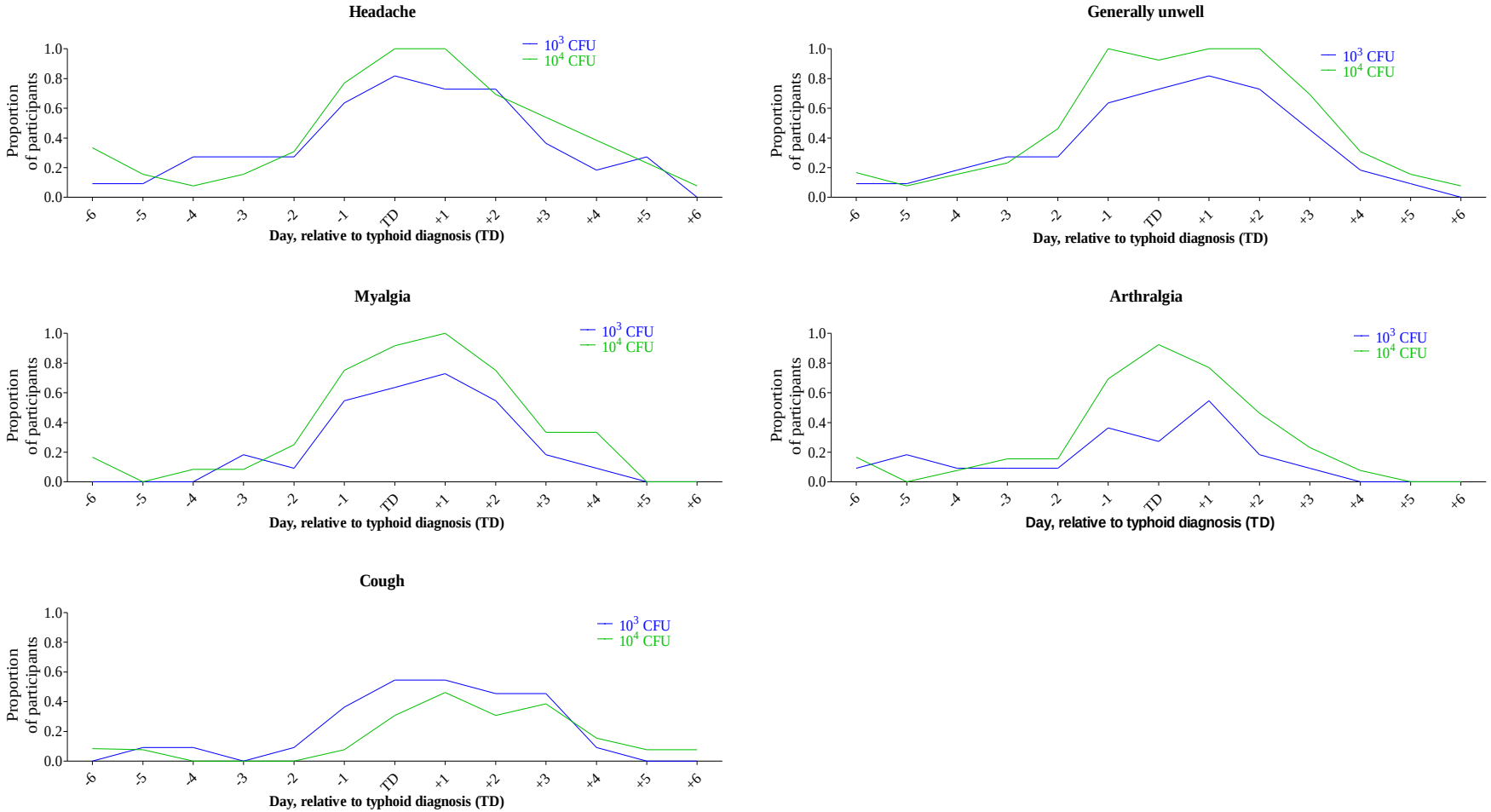
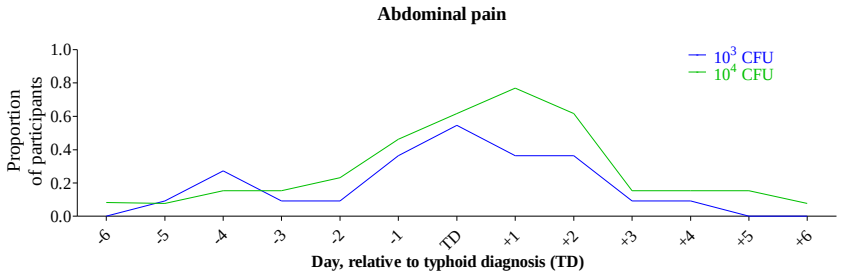
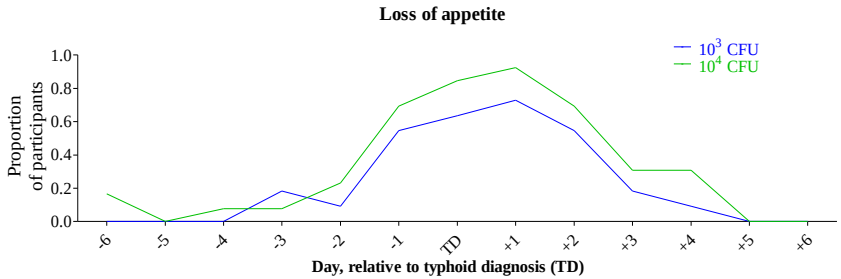
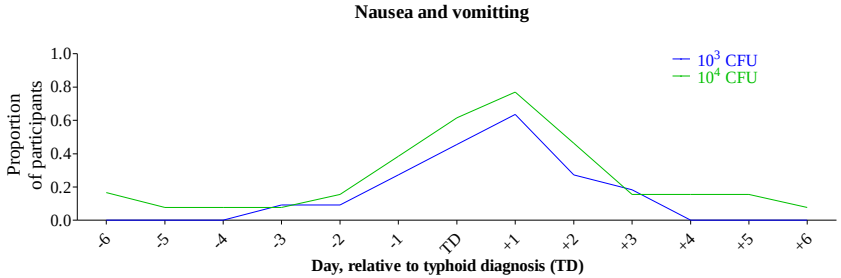
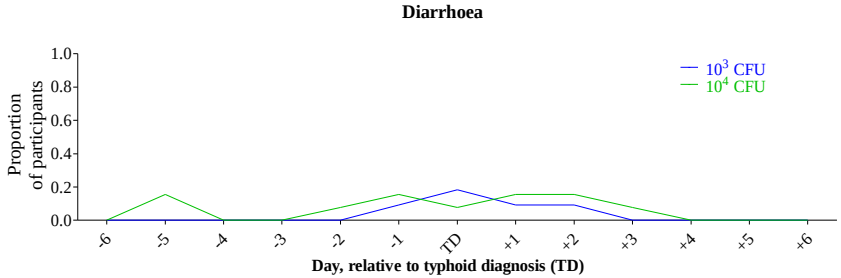


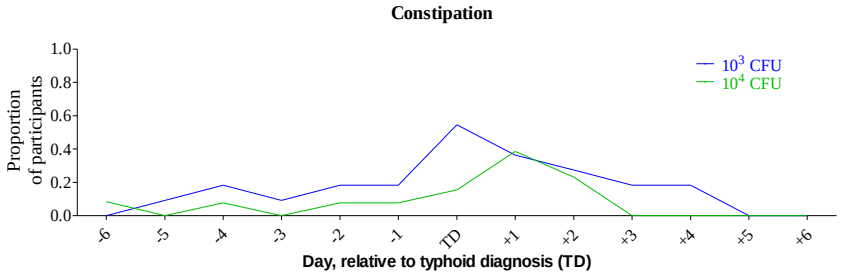
Figure 44: The proportion of participants reporting each solicited gastrointestinal symptom relative to the day of typhoid disease diagnosis in participants challenged with 10^3 CFU (n=11) and 10^4 CFU (n=13) of *S.* Typhi in a human challenge model of typhoid disease











There was a dose-response relationship to extent of symptoms observed, with participants who developed typhoid disease following the 10^3 CFU challenge dose reporting fewer symptoms than those developing typhoid disease following the 10^4 CFU challenge dose (Figure 42, Figure 43 and Figure 44). This dose-response relationship was true for all symptoms, apart from constipation and cough that affected a higher proportion of the challenged with 10^3 CFU than those challenged with 10^4 CFU.

The overall incidence, duration and severity of each solicited symptom are shown in Table 50.

Table 50: The proportion of participants affected, duration and severity for each solicited symptoms in participants who did and did not develop typhoid disease following challenge either 10^3 or 10^4 CFU of *S.* Typhi in the development of a human challenge model of typhoid disease

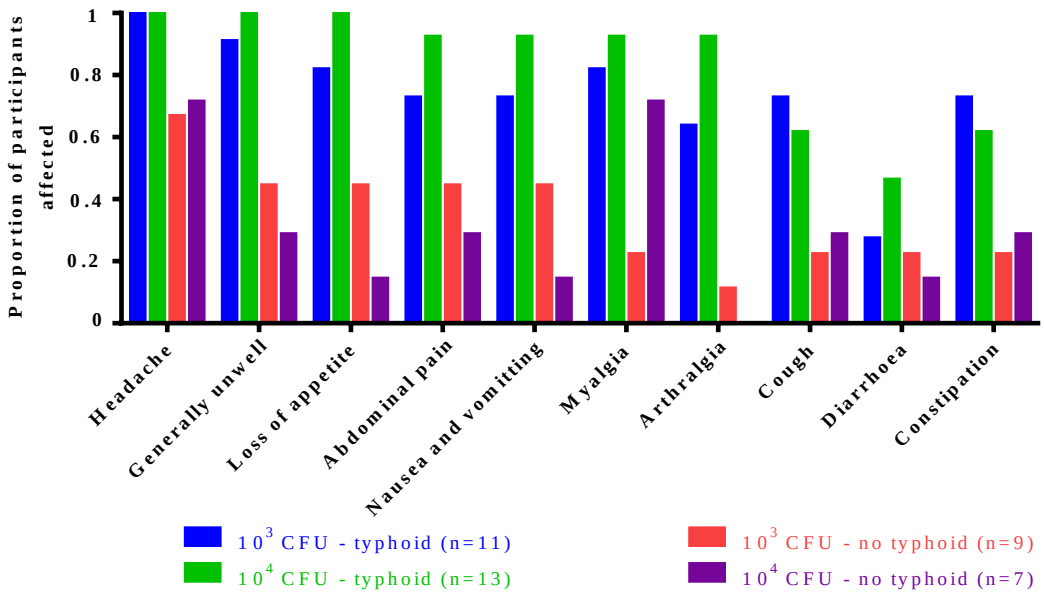
Symptom		Challenge dose (CFU)	Proportion affected (%)	Average duration of symptom (days)	No. days reported mild (% of total)	No. days reported Moderate (% of total)	No. days reported severe (% of total)
Headache	Developed typhoid disease	10^3	11/11 (100)	4.7	21 (40.4)	16 (30.8)	15 (28.8)
		10^4	13/13 (100)	5.7	43 (58.1)	23 (31.1)	8 (10.8)
	Did not develop typhoid disease	10^3	6/9 (66.7)	2.2	13 (100.0)	0 (0.0)	0 (0.0)
		10^4	5/7 (71.4)	1.4	6 (85.7)	1 (14.3)	0 (0.0)
Generally unwell	Developed typhoid disease	10^3	10/11 (90.9)	5.4	18 (33.3)	23 (42.6)	13 (24.1)
		10^4	13/13 (100)	6.5	43 (51.2)	26 (31.0)	15 (17.9)
	Did not develop typhoid disease	10^3	4/9 (44.4)	1.5	4 (66.7)	1 (16.7)	1 (16.7)
		10^4	2/7 (28.6)	2.5	3 (60.0)	1 (20.0)	1 (20.0)
Loss of appetite	Developed typhoid disease	10^3	9/11 (81.8)	4.0	21 (58.3)	8 (22.2)	7 (19.4)
		10^4	13/13 (100)	4.3	26 (46.4)	25 (44.6)	5 (8.9)
	Did not develop typhoid disease	10^3	4/9 (44.4)	1.8	6 (85.7)	0 (0)	1 (14.3)
		10^4	1/7 (14.3)	2.0	2 (100)	0 (0)	0 (0)
Abdominal pain	Developed typhoid disease	10^3	8/11 (72.7)	4.0	21 (65.6)	10 (31.3)	1 (3.1)
		10^4	12/13 92.3	4.3	35 (68.6)	14 (27.5)	2 (3.9)
	Did not develop typhoid disease	10^3	4/9 (44.4)	1.3	4 (80.0)	1 (20.0)	0 (0)
		10^4	2/7 (28.6)	1.0	2 (100)	0 (0)	0 (0)

Symptom		Challenge dose (CFU)	Proportion affected (%)	Average duration of symptom (days)	No. days reported mild (% of total)	No. days reported Moderate (% of total)	No. days reported severe (% of total)
Nausea &vomiting	Developed typhoid disease	10^3	8/11 (72.7)	3.3	18 (69.2)	6 (23.1)	2 (7.7)
		10^4	12/13 (92.3)	3.6	24 (55.8)	14 (32.6)	5 (11.6)
	Did not develop typhoid disease	10^3	4/9 (44.4)	1.0	3 (75.0)	1 (25.0)	0 (0)
		10^4	1/7 (14.3)	1.0	0 (0)	0 (0)	1 (100)
Myalgia	Developed typhoid disease	10^3	9/11 (81.8)	4.1	14 (37.8)	21 (56.8)	2 (5.4)
		10^4	12/13 (92.3)	4.8	32 (56.1)	18 (31.6)	7 (12.3)
	Did not develop typhoid disease	10^3	2/9 (22.2)	2.5	4 (80.0)	1 (20.0)	0 (0)
		10^4	5/7 (71.4)	2.6	8 (61.5)	4 (30.8)	1 (7.7)
Arthralgia	Developed typhoid disease	10^3	7/13 (63.6)	3.7	16 (61.5)	7 (26.9)	3 (11.5)
		10^4	12/13 (92.3)	4.1	30 (61.2)	13 (26.5)	6 (12.2)
	Did not develop typhoid disease	10^3	1/9 (11.1)	2.0	1 (50.0)	1 (50.0)	0 (0)
		10^4	0/7 (0)	N/A	N/A	N/A	N/A
Cough	Developed typhoid disease	10^3	8/11 (72.7)	3.9	29 (93.5)	2 (6.5)	0 (0)
		10^4	8/13 (61.5)	3.4	25 (92.6)	2 (7.4)	0 (0)
	Did not develop typhoid disease	10^3	2/9 (22.2)	8.5	17 (100))	0 (0)	0 (0)
		10^4	2/7 (28.6)	5.5	11 (100)	0 (0)	0 (0)

Symptom		Challenge dose (CFU)	Proportion affected (%)	Average duration of symptom (days)	No. days reported mild (% of total)	No. days reported Moderate (% of total)	No. days reported severe (% of total)
Constipation	Developed typhoid disease	10^3	8/11 (72.7)	3.6	17 (58.6)	7 (24.1)	5 (17.2)
		10^4	8/13 (61.5)	1.9	7 (46.7)	6 (40.0)	2 (13.3)
	Did not develop typhoid disease	10^3	2/9 (22.2)	7.5	14 (93.3)	1 (6.7)	0 (0)
		10^4	2/7 (28.6)	1.5	2 (66.7)	1 (33.3)	0 (0)
Diarrhoea	Developed typhoid disease	10^3	3/11 (27.3)	2.3	7 (100)	0 (0)	0 (0)
		10^4	6/13 (46.2)	1.8	7 (63.0)	3 (27.0)	1 (9.0)
	Did not develop typhoid disease	10^3	2/9 (22.2)	1.0	2 (100)	0 (0)	0 (0)
		10^4	1/7 (14.3)	1.0	1 (100)	0 (0)	0 (0)

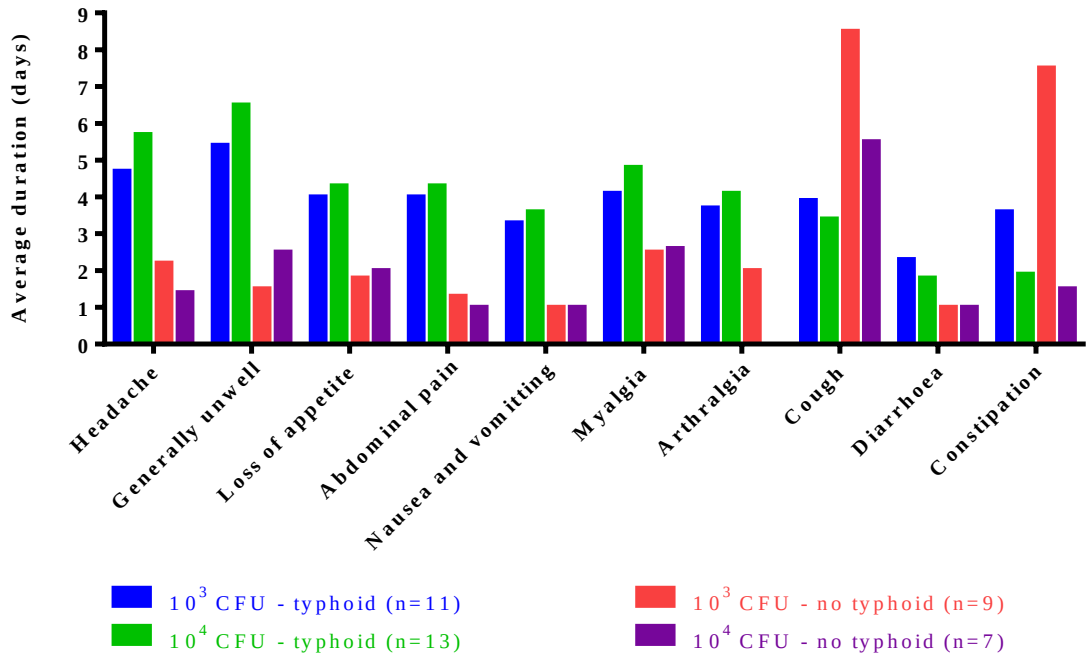
All solicited symptoms affected more participants with typhoid disease than without typhoid disease (Figure 45). Headache was universally experienced by participants with typhoid disease, but was also frequently reported in those that did not have typhoid disease. Of those with typhoid disease, a higher proportion of participants challenged with 10^4 CFU experienced being generally unwell, loss of appetite, abdominal pain, nausea and vomiting, arthralgia and diarrhoea compared to those receiving the 10^3 challenge dose. Constipation was more frequent following challenge with 10^3 CFU than with the 10^4 CFU, whereas the opposite was true for diarrhoea, which was more frequent following 10^4 CFU compared to 10^3 CFU. All of the solicited symptoms were also reported in those who did not develop typhoid disease, possibly reflecting reporting bias and anxiety at the possibility of developing typhoid disease. Arthralgia was the most discriminatory symptom, occurring in 19 of the 24 participants with typhoid disease, but only 1 of the 16 who did not have typhoid disease.

Figure 45: The proportion of participants reporting each solicited symptom at any point during the 14 days following challenge with 10^3 or 10^4 CFU of S. Typhi, for participants developing typhoid disease (typhoid) or not (no typhoid)



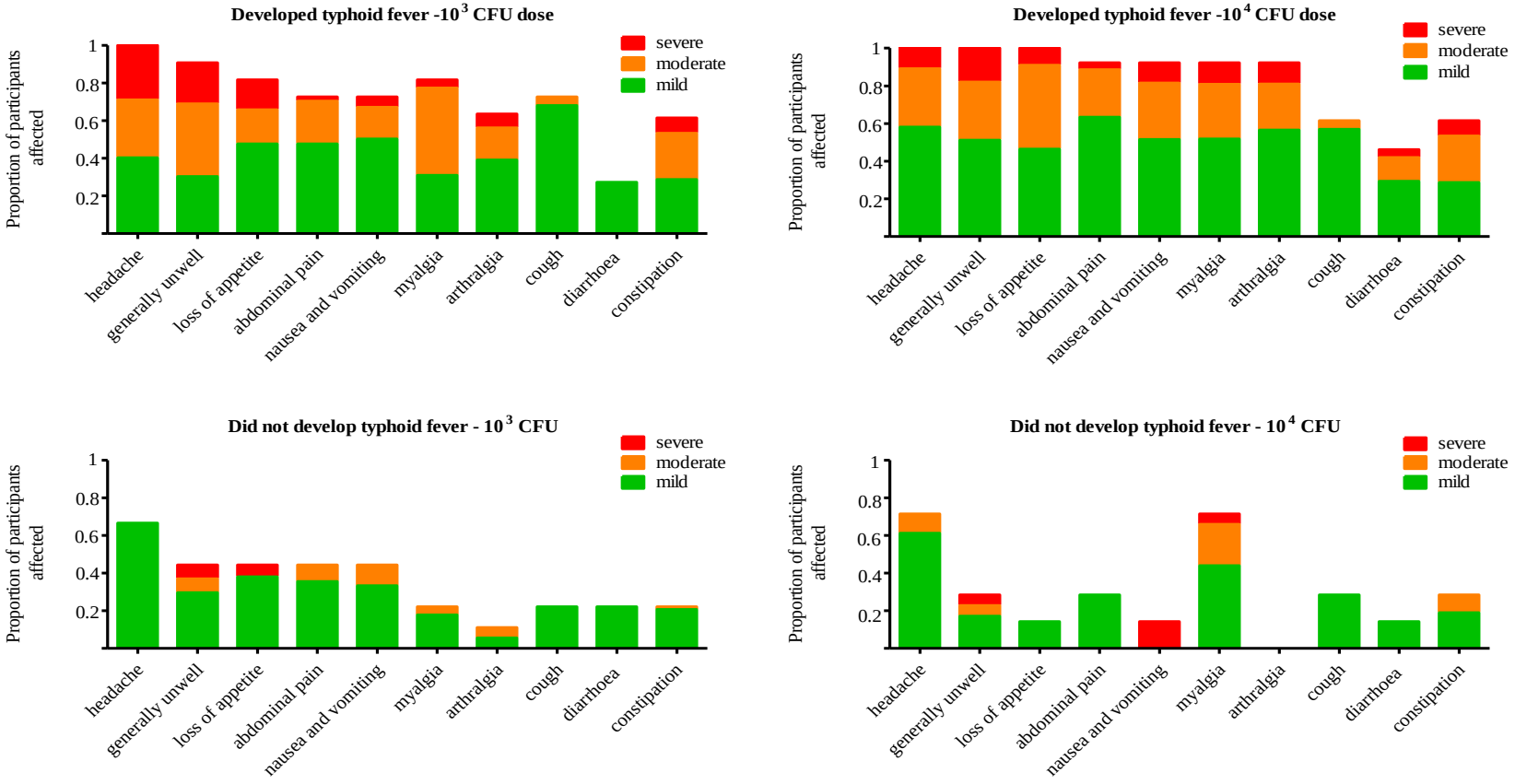
In participants developing typhoid disease, symptoms tended to endure for slightly longer in those challenged with 10^4 CFU *S.* Typhi compared to those challenged with 10^3 CFU, except for cough, diarrhoea and constipation (Figure 46). Headache and feeling generally unwell were the most enduring features of typhoid disease. Cough and constipation lasted longest in those without typhoid disease, possibly reflecting the high background prevalence of these symptoms.

Figure 46: The average duration of each solicited symptom in the 14 days following challenge with 10^3 or 10^4 CFU of *S.* Typhi, for participants developing typhoid disease (typhoid) or not (no typhoid)



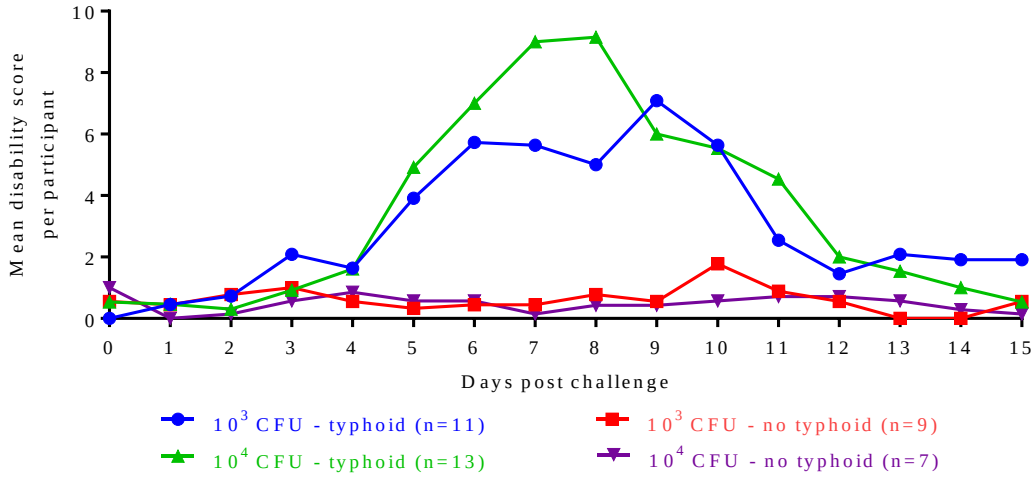
The proportion of each symptom reported as mild (did not limit normal daily activity), moderate (limited some daily activity) or severe (prevents daily activity) is shown in Figure 47.

Figure 47: Participant rated symptom severity for each solicited symptom for participants developing typhoid disease and or not, following challenge with either 10^3 or 10^4 CFU of *S.* Typhi in a human challenge model of typhoid disease. Severity grading is based on interference with activities of daily living (ADL) as mild (did not interfere with ADLs), moderate (interfered with some but not all ADLs) or severe (prevented all ADLs)



Reported symptoms were mainly of mild or moderate severity. No participant experienced severe cough, but some participants experienced all other symptoms of a severity that prevented all normal daily activity. As would be expected, severe symptoms were more common in those developing typhoid disease compared to those who did not. Participants with typhoid disease reported a range of disease severity. One participant with *S.* Typhi bacteraemia was essentially asymptomatic, reporting only a mild headache on the 2 days after diagnosis, and had a maximum temperature of 37.2°C. A further 3 participants with typhoid disease reported mild symptoms only, and 2 participants reported moderate symptoms only. Hence, although the majority reported at least one symptom preventing all normal daily activity when diagnosed with typhoid disease, 4 out of 11 participants challenged with 10^3 CFU and 2 out of 13 participants challenged with 10^4 CFU did not report any severe symptoms. There was no overall difference in the average number of severe symptoms reported per participant at each dose (4.4 10^3 CFU, 4.5 10^4 CFU), nor was any difference in severity of individual symptoms in those with typhoid fever following challenge with 10^3 CFU compared to those challenge with 10^4 CFU. However, the overall disability per participant (calculated as the sum of mild symptoms, weighted as 1, moderate symptoms, weighted as 2 and severe symptoms, weighted as 3) was greater in those challenge with the higher challenge dose (Figure 48).

Figure 48: Mean disability score for participants who did (typhoid) or did not (no typhoid) develop typhoid fever following challenge with either 10^3 or 10^4 CFU of *S.* Typhi in a human challenge model of typhoid disease



6.3.5. Laboratory findings

Results of biochemistry and haematology assays in participants are shown in Table 51and Table 52.

Table 51: Mean values and 95% confidence intervals of biochemistry assays for participants who developed typhoid disease or not following challenge with 10^3 or 10^4 CFU of *S.* Typhi

Assay	Outcome of challenge	Challenge dose	Mean value (95% CI)						
			Day 1	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
C- reactive protein (mg/L)	Developed typhoid disease	10^3 CFU	1.9 (0.0-3.7)	1.2 (-0.2-2.6)	15.6 (-3.0-34.2)	47.9 (5.1-90.8)	29.8 (9.4-50.2)	20.5 (9.1-31.9)	14.0 (5.1-22.9)
		10^4 CFU	1.8 (-0.4-4.0)	1.5 (-0.4-3.5)	20.4 (2.2-38.5)	58.1 (28.8-87.3)	53.8 (26.8-80.8)	33.9 (13.5-54.2)	18.5 (5.8-31.2)
	Did not develop typhoid disease	10^3 CFU	1.4 (-1.5-4.2)	1.0 (-1.1-3.2)	0.6 (-0.5-1.6)	0.7 (-0.5-1.8)	2.3 (-2.8-7.4)	5.7 (-3.3-14.8)	3.2 (-2.7-9.0)
		10^4 CFU	1.7 (-1.4-4.7)	0.4 (-0.1-0.8)	0.8 (-0.5-2.2)	0.4 (-0.4-1.2)	4.2 (-4.8-13.2)	1.1 (-0.5-2.7)	0.5 (-0.1-1.1)
ESR (mm/hr.)	Developed typhoid disease	10^3 CFU	1.9 (0.2-3.6)	2.3 (0.0-4.6)	1.7 (-0.7-4.1)	3.7 (-1.3-8.7)	7.4 (1.2-13.6)	2.5 (-1.0-5.9)	6.5 (2.5-10.6)
		10^4 CFU	3.4 (0.9-5.9)	1.7 (-0.1-3.5)	6.3 (2.0-10.6)	11.1 (4.6-17.6)	9.2 (0.8-17.5)	9.2 (0.1-18.2)	8.6 (-2.2-19.4)
	Did not develop typhoid disease	10^3 CFU	2.0 (-0.3-4.3)	2.1 (-0.1-4.4)	2.2 (0.8-3.6)	3.7 (2.0-5.3)	2.5 (-1.0-6.0)	1.6 (-0.4-3.6)	4.6 (0.3-8.8)
		10^4 CFU	2.3 (-0.8-5.4)	2.0 (-0.1-4.1)	4.0 (0.8-7.2)	6.9 (-2.8-16.5)	1.6 (-0.1-3.2)	2.3 (-0.1-4.8)	20.6 (-13.0-54.1)
Albumin (g/L)	Developed typhoid disease	10^3 CFU	44.64 (43.01-46.26)	44.44 (42.81-46.08)	45.55 (44.03-47.06)	44.36 (42.68-46.04)	44.82 (42.9-46.74)	44.18 (42.24-46.12)	45 4(3.1-46.9)
		10^4 CFU	46.23 (45.07-47.39)	45.77 (45.16-46.38)	45.38 (43.96-46.81)	45.58 (44.55-46.61)	44.18 (42.51-45.85)	43.92 (42.55-45.28)	43.54 (42.17-44.9)
	Did not develop typhoid disease	10^3 CFU	44.33 (43.25-45.42)	45.11 (44.4-45.82)	45.0 (43.82-46.18)	45.44 (43.95-46.94)	46 (44.46-47.54)	44.75 (43.59-45.91)	44.13 (42.15-46.1)
		10^4 CFU	46.29 (44.54-48.03)	45.43 (43.44-47.42)	45.57 (43.19-47.95)	45.33 (42.79-47.88)	42.43 (40.11-44.75)	43.8 3(9.46-48.14)	45.14 (42.85-47.44)
Amylase (IU/L)	Developed typhoid disease	10^3 CFU	55.6 (44.4-66.9)	52.6 (36.6-68.6)	56.0 (45.4-66.6)	53.2 (44.1-62.3)	55.0 (38.9-71.1)	53.6 (41.1-66.0)	59.9 (46.9-72.9)
		10^4 CFU	63.9 (54.4-73.3)	63.9 (53.4-74.3)	60.0 (48.7-71.3)	52.8 (41.0-64.6)	51.4 (43.9-59.0)	54.5 (46.0-62.9)	60.2 (52.1-68.2)
	Did not develop typhoid disease	10^3 CFU	60.3 (46.8-73.8)	59.9 (45.2-74.5)	56.3 (49.2-63.3)	59.3 (50.3-68.2)	56.9 (49.7-64.1)	55.1 (45.2-65.1)	60.6 (48.5-72.6)
		10^4 CFU	49.33 (40.03-58.64)	49.67 (38.67-60.67)	51.67 (40.16-63.18)	47.6 (37.08-58.12)	51.5 (39.58-63.42)	57.25 (49.41-65.09)	51.17 (42.43-59.91)

Assay	Outcome of challenge	Challenge dose	Mean value (95% CI)						
			Day 1	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
Bilirubin (µmol/L	Developed typhoid disease	10^3 CFU	13.5 (8.0-18.9)	13.3 (10.1-16.6)	11.7 (9.7-13.8)	12.5 (9.1-15.8)	10.5 (8.0-13.0)	9.4 (6.9-11.9)	8.4 (6.6-10.1)
		10^4 CFU	12.7 (8.5-16.9)	12.2 (9.6-14.7)	10.9 (8.2-13.5)	9.8 (7.5-12.0)	7.9 (5.7-10.2)	6.5 (4.5-8.5)	6.4 (5.1-7.7)
	Did not develop typhoid	10^3 CFU	10.0 (6.3-13.7)	10.6 (8.5-12.7)	9.4 (6.7-12.1)	11.0 (7.2-14.8)	9.6 (6.2-12.9)	8.4 (6.4-10.3)	8.0 (5.7-10.3)
		10^4 CFU	11.1 (4.4-17.8)	11.3 (8.0-14.6)	10.9 (7.2-14.5)	10.8 (8.2-13.4)	8.3 (5.6-11.0)	11.4 (9.1-13.7)	9.3 (7.2-11.3)
Alanine transaminase (IU/L)	Developed typhoid disease	10^3 CFU	22.3 (13.6-31.0)	20.9 (10.7-31.1)	24.5 (15.1-33.8)	32.4 (17.8-46.9)	49.6 (17.0-82.3)	91.7 (-6.5-190.0)	107.6 (-21.6-236.9)
		10^4 CFU	16.9 (14.2-19.6)	16.1 (14.1-18.0)	18.5 (14.7-22.2)	24.6 (17.7-31.5)	23.8 (17.5-30.2)	34.3 (21.3-47.3)	66.2 (30.1-102.2)
	Did not develop typhoid disease	10^3 CFU	23.4 (6.1-40.8)	29.6 (11.2-47.9)	34.8 (8.1-61.4)	33.6 (8.5-58.7)	33.0 (9.1-56.9)	21.5 (11.0-32.0)	26.3 (11.7-41.0)
		10^4 CFU	18.9 (10.2-27.5)	15.7 (11.7-19.7)	16.7 (14.2-19.2)	17.7 (12.9-22.5)	18.3 (11.5-25.1)	16.2 (10.7-21.7)	15.4 (12.4-18.5)
Alkaline Phosphatase (IU/L)	Developed typhoid disease	10^3 CFU	138.2 (120.6-155.8)	131.7 (114.3-149.1)	144.9 (124.6-165.2)	145.9 (120.0-171.8)	158.6 (111.9-205.4)	154.5 (115.4-193.6)	157.2 (120.2-194.1)
		10^4 CFU	139.6 (127.9-151.3)	133.6 (124.5-142.7)	134.8 (123.9-145.8)	134.9 (125.3-144.5)	131.7 (122.7-140.8)	137.8 (121.1-154.6)	144.5 (125.4-163.7)
	Did not develop typhoid disease	10^3 CFU	138.0 (112.7-163.3)	135.2 (108.6-161.9)	132.4 (109.0-155.9)	135.1 (110.9-159.3)	133.6 (108.2-158.9)	136.4 (108.1-164.6)	135.3 (108.6-162.1)
		10^4 CFU	140.6 (116.7-164.4)	133.9 (114.5-153.2)	135.1 (115.0-155.3)	130.2 (107.2-153.1)	134.0 (109.8-158.2)	125.0 (85.2-164.8)	131.0 (105.4-156.6)

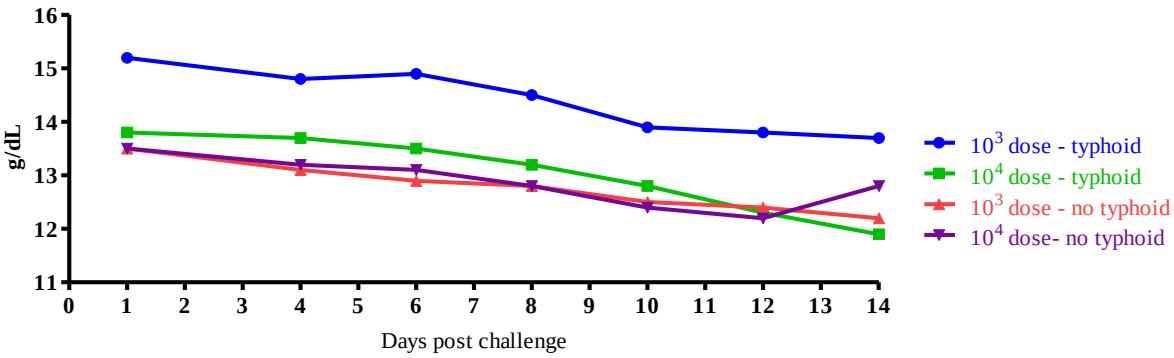
Table 52: Mean values and 95% confidence intervals of biochemistry assays for participants who developed typhoid disease or not following challenge with 10^3 or 10^4 CFU of *S.* Typhi

Assay		Challenge Dose	Mean value (95% CI)						
			Day 1	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
Haemoglobin (g/dl)	Developed typhoid disease	10^3 CFU	15.2 (14.8-15.6)	14.8 (14.3-15.3)	14.9 (14.4-15.4)	14.5 (13.9-15.1)	13.9 (13.2-14.6)	13.8 (13.2-14.4)	13.7 (13.1-14.3)
		10^4 CFU	13.8 (12.8-14.7)	13.7 (13.1-14.2)	13.5 (12.7-14.3)	13.2 (12.4-14.0)	12.8 (12.1-13.6)	12.3 (11.7-12.8)	11.9 (11.0-12.7)
	Did not develop typhoid disease	10^3 CFU	13.5 (12.5-14.5)	13.1 (12.0-14.2)	12.9 (12.1-13.8)	12.8 (11.9-13.6)	12.5 (11.6-13.5)	12.4 (11.1-13.7)	12.2 (11.1-13.3)
		10^4 CFU	13.5 (12.8-14.3)	13.2 (12.2-14.2)	13.1 (12.1-14.1)	12.8 (12.0-13.7)	12.4 (11.2-13.5)	12.2 (11.1-13.3)	12.8 (11.4-14.3)
Platelets (10^9/L)	Developed typhoid disease	10^3 CFU	200.5 (173.0-228.1)	205.1 (177.3-232.9)	196.8 (163.6-230.0)	201.8 (149.1-254.5)	167.8 (136.7-198.9)	204.5 (178.1-230.8)	242.0 (194.6-289.4)
		10^4 CFU	225.8 (172.7-279.0)	240.4 (214.5-266.2)	215.2 (187.8-242.5)	190.2 (160.8-219.7)	193.7 (169.2-218.3)	232.3 (206.3-258.3)	300.3 (251.8-348.8)
	Did not develop typhoid disease	10^3 CFU	211.1 (175.0-247.2)	201.4 (158.1-244.8)	221.3 (189.6-253.0)	227.8 (190.9-264.6)	231.1 (191.3-270.9)	225.6 (188.5-262.7)	232.4 (204.8-260.1)
		10^4 CFU	225.0 (199.7-250.3)	229.6 (198.8-260.4)	237.1 (202.9-271.4)	244.4 (206.7-282.1)	228.3 (179.1-277.4)	240.7 (186.9-294.4)	222.3 (166.4-278.3)
Total white cell count (10^9/L)	Developed typhoid disease	10^3 CFU	7.0 (5.6-8.3)	6.8 (5.1-8.4)	7.6 (6.0-9.2)	6.6 (4.4-8.7)	5.7 (4.2-7.1)	5.8 (4.9-6.6)	6.0 (5.1-7.0)
		10^4 CFU	6.7 (5.0-8.4)	6.4 (5.6-7.2)	6.6 (6.0-7.2)	5.5 (4.4-6.7)	5.6 (4.0-7.2)	4.7 (3.9-5.5)	5.5 (4.8-6.3)
	Did not develop typhoid disease	10^3 CFU	5.4 (4.7-6.0)	6.0 (5.0-6.9)	5.8 (5.2-6.4)	5.4 (4.7-6.2)	5.4 (3.9-6.8)	4.9 (4.0-5.8)	5.1 (4.3-6.0)
		10^4 CFU	5.9 (5.0-6.7)	5.4 (4.3-6.5)	5.5 (4.5-6.4)	5.4 (4.5-6.2)	4.8 (3.9-5.6)	5.1 (4.2-5.9)	5.2 (3.9-6.5)
Neutrophils (10^9/L)	Developed typhoid disease	10^3 CFU	3.8 (3.0-4.6)	3.6 (2.5-4.6)	4.9 (3.5-6.3)	4.3 (2.5-6.1)	3.5 (2.3-4.8)	3.1 (2.4-3.8)	3.3 (2.6-4.0)
		10^4 CFU	3.6 (2.3-4.8)	3.9 (3.1-4.6)	4.6 (4.0-5.3)	3.8 (2.8-4.7)	3.7 (2.1-5.3)	2.2 (1.4-3.0)	2.7 (2.1-3.3)
	Did not develop typhoid disease	10^3 CFU	2.9 (2.1-3.7)	3.0 (1.9-4.0)	3.1 (2.5-3.6)	2.8 (2.3-3.3)	3.0 (1.5-4.6)	2.5 (2.1-2.9)	2.8 (2.1-3.5)
		10^4 CFU	2.8 (2.3-3.3)	2.7 (2.0-3.3)	2.9 (2.2-3.6)	2.8 (2.4-3.3)	2.3 (1.8-2.9)	2.6 (2.1-3.0)	2.9 (2.1-3.7)

Assay		Challenge Dose	Mean value (95% CI)						
			Day 1	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
Lymphocytes (10^9/L)	Developed typhoid disease	10^3 CFU	2.5 (1.8-3.1)	2.4 (1.8-3.0)	1.8 (1.2-2.3)	1.6 (1.1-2.0)	1.4 (1.0-1.7)	1.8 (1.3-2.3)	1.9 (1.3-2.6)
		10^4 CFU	2.4 (1.8-2.9)	1.8 (1.5-2.0)	1.3 (1.0-1.6)	1.1 (0.9-1.2)	1.2 (0.8-1.5)	1.8 (1.5-2.2)	2.2 (1.9-2.4)
	Did not develop typhoid disease	10^3 CFU	1.9 (1.5-2.2)	2.3 (1.7-3.0)	2.1 (1.5-2.6)	2.0 (1.5-2.5)	1.8 (1.2-2.3)	1.7 (1.1-2.3)	1.7 (1.2-2.2)
		10^4 CFU	2.1 (1.2-3.0)	1.9 (1.6-2.2)	1.8 (1.5-2.0)	1.8 (1.4-2.1)	1.9 (1.5-2.2)	1.8 (1.3-2.3)	1.6 (1.2-2.1)
Monocytes (10^9/L)	Developed typhoid disease	10^3 CFU	0.6 (0.5-0.7)	0.6 (0.4-0.7)	0.8 (0.6-0.9)	0.7 (0.5-0.9)	0.7 (0.5-0.8)	0.6 (0.5-0.8)	0.6 (0.5-0.7)
		10^4 CFU	0.5 (0.4-0.7)	0.6 (0.5-0.7)	0.6 (0.5-0.7)	0.6 (0.5-0.7)	0.7 (0.5-0.8)	0.6 (0.4-0.7)	0.5 (0.4-0.6)
	Did not develop typhoid disease	10^3 CFU	0.4 (0.4-0.5)	0.5 (0.4-0.5)	0.5 (0.4-0.5)	0.5 (0.4-0.6)	0.4 (0.3-0.5)	0.5 (0.4-0.6)	0.5 (0.4-0.5)
		10^4 CFU	0.5 (0.3-0.7)	0.4 (0.3-0.5)	0.4 (0.3-0.5)	0.4 (0.3-0.4)	0.4 (0.3-0.4)	0.4 (0.3-0.4)	0.3 (0.2-0.5)
Eosinophils (10^9/L)	Developed typhoid disease	10^3 CFU	0.2 (0.1-0.3)	0.2 (0.1-0.3)	0.1 (0.0-0.2)	0.1 (0.0-0.1)	0.1 (0.0-0.1)	0.1 (0.1-0.2)	0.1 (0.1-0.2)
		10^4 CFU	0.2 (0.1-0.3)	0.2 (0.1-0.3)	0.1 (0.0-0.1)	0.0 (0.0-0.1)	0.0 (0.0-0.1)	0.1 (0.0-0.1)	0.2 (0.1-0.2)
	Did not develop typhoid disease	10^3 CFU	0.2 (0.1-0.2)	0.2 (0.1-0.3)	0.2 (0.1-0.2)	0.1 (0.1-0.2)	0.1 (0.1-0.2)	0.2 (0.1-0.2)	0.1 (0.1-0.2)
		10^4 CFU	0.5 (0.1-1.1)	0.4 (0.0-0.8)	0.4 (0.1-0.6)	0.4 (0.1-0.7)	0.3 (0.1-0.5)	0.3 (0.1-0.5)	0.3 (0.0-0.6)

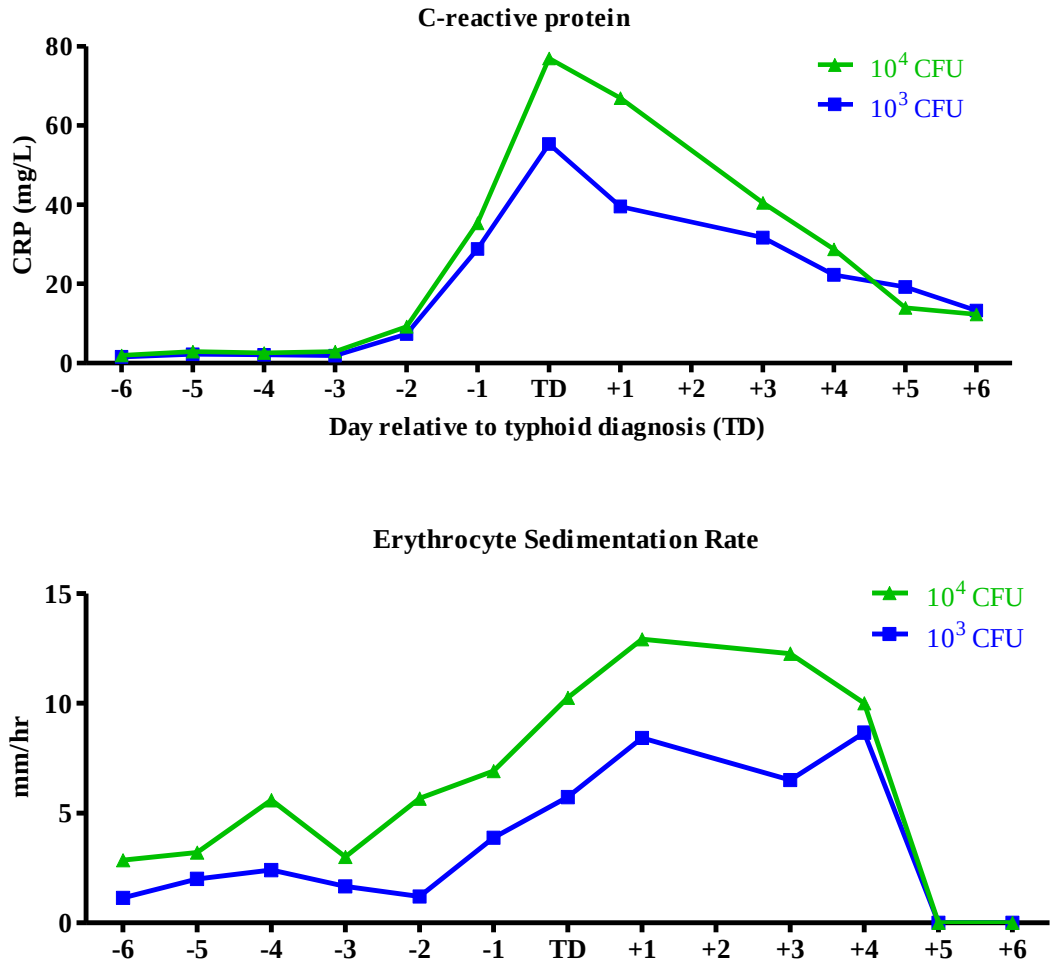
Haemoglobin measurements decreased in all participants (Figure 49). Other laboratory parameters did not alter in participants not diagnosed with typhoid disease.

Figure 49: Change in mean haemoglobin concentration for participants who did and did not developed typhoid disease following challenge with 10^3 or 10^4 CFU of *S.* Typhi



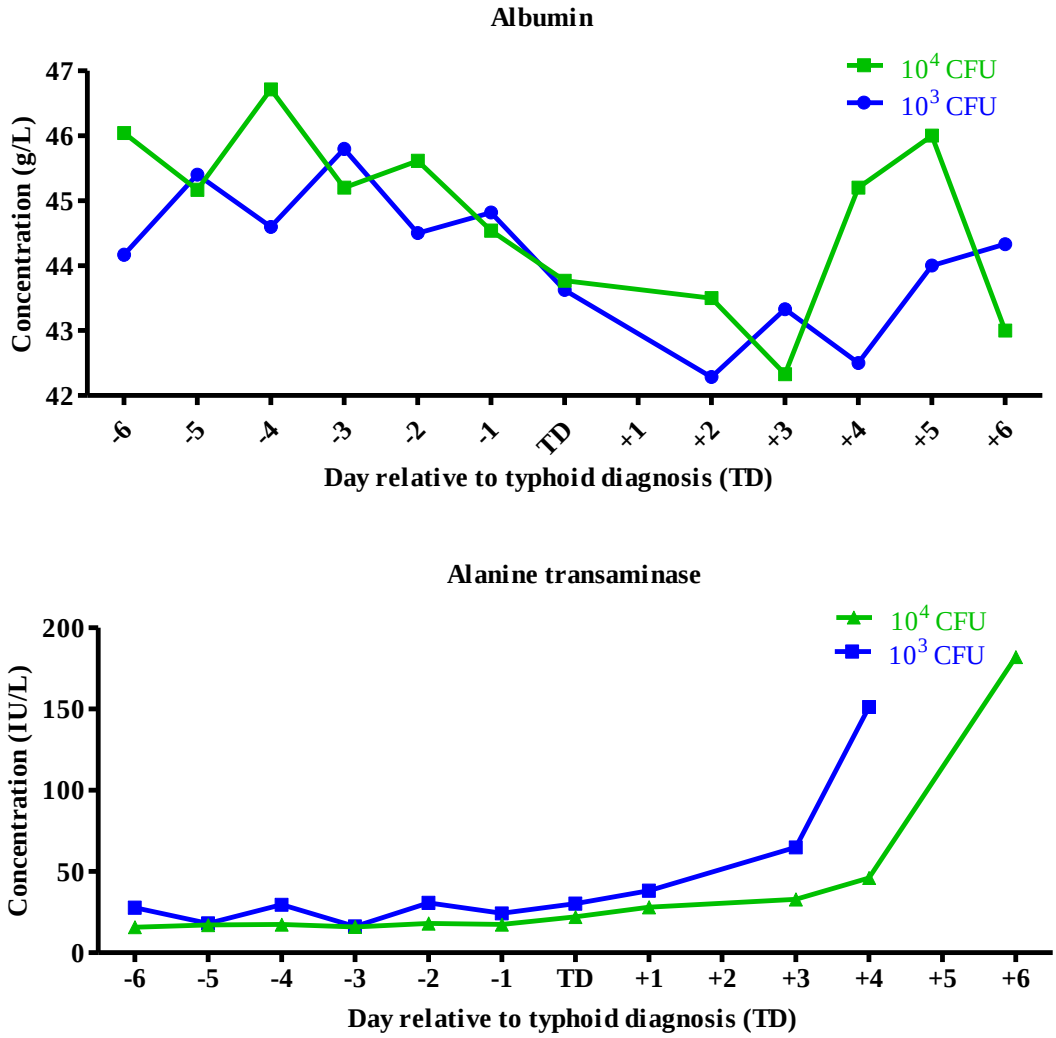
For participants who developed typhoid disease, changes were related to the onset of typhoid disease. A systemic inflammatory response was apparent from 3 days prior to diagnosis (Figure 50), and peaked either at typhoid diagnosis (C-reactive protein; CRP) or the day after (erythrocyte sedimentation rate; ESR). Interestingly, only one participant, previously noted as having an essentially asymptomatic bacteraemia, did not have a notable rise in CRP (maximum value 5.8 μg/L). All other participants with typhoid disease had a raised CRP, with a greater response in those challenged with 10^3 CFU compared to those challenged with 10^4 CFU. The ESR response was more variable, with only 3 participants challenged with 10^3 CFU and 8 participants challenged with 10^4 CFU having an ESR greater than 10 mm/hr.

Figure 50: Changes in the mean C-reactive protein concentration and erythrocyte sedimentation rate relative to the point where typhoid was diagnosed (TD) in participants challenged with 10^3 or 10^4 CFU of *S.* Typhi



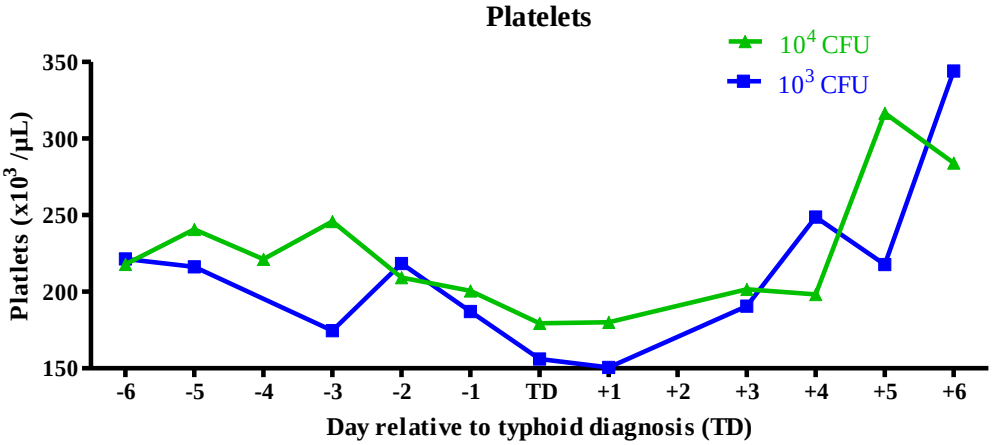
In keeping with the inflammatory response, there was a decrease in the albumin concentration and a rise in the alanine transaminase (ALT) concentration (Figure 51). The ALT showed a marked increase in some participants at the very end of the challenge period, later than other laboratory abnormalities. This may have been due in part to the effects of ciprofloxacin therapy, although participants did not report any other side effects of therapy. Renal function remained stable throughout in all participants (data not shown).

Figure 51: Changes in mean albumin and alanine transaminase (ALT) concentration relative to the point where typhoid was diagnosed (TD) in participants challenged with 10^3 or 10^4 CFU of *S.* Typhi.



Changes in the platelet count of participants diagnosed with typhoid disease are shown in Figure 52.

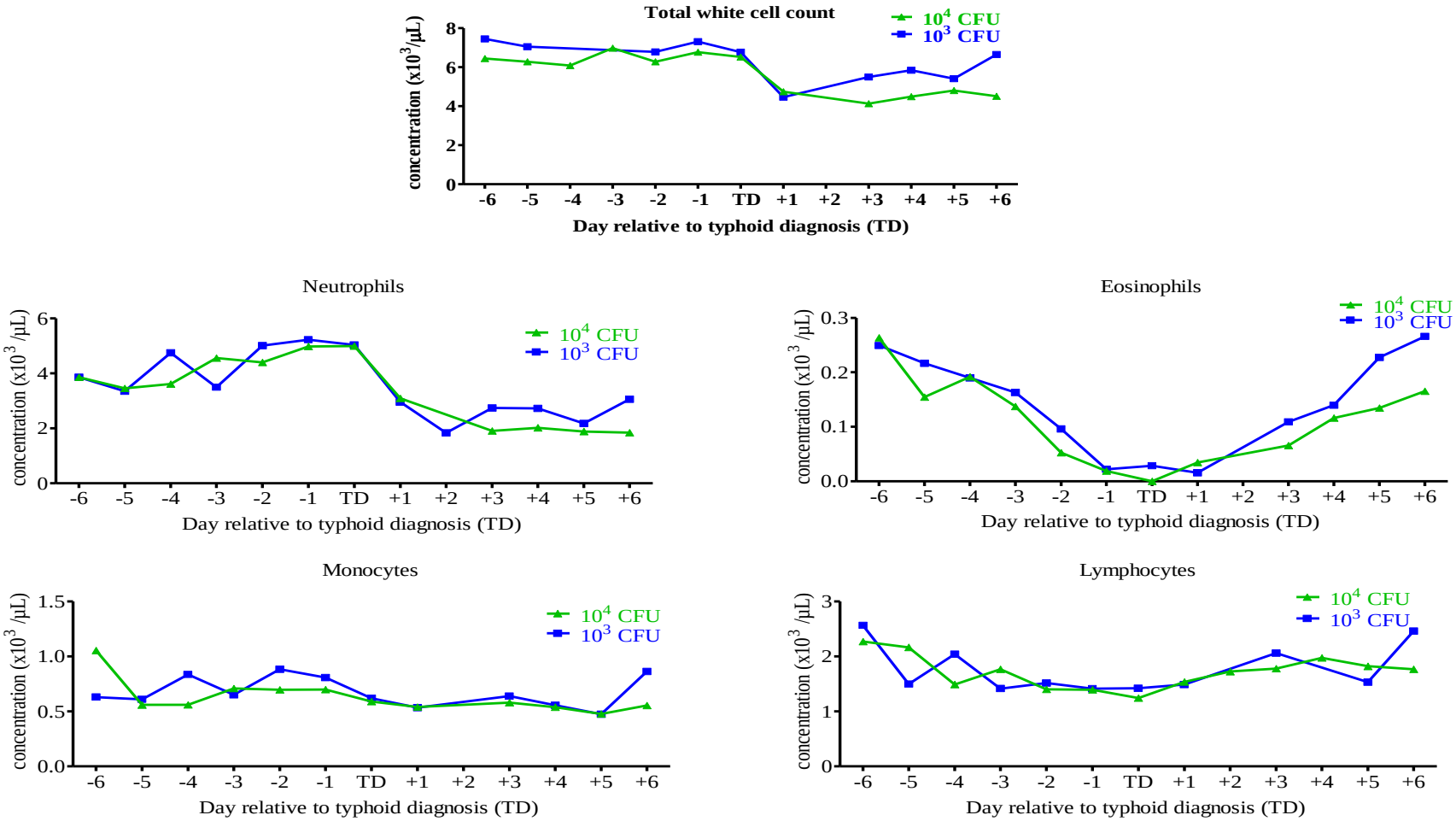
Figure 52: Changes in mean platelet count relative to the point where typhoid was diagnosed (TD) in participants challenged with 10^3 or 10^4 CFU of *S.* Typhi.



Significant decreases in the platelet count were seen from 3 days prior to typhoid diagnosis, with the lowest value being $54x10^9$/ml. All participants with typhoid disease had a decrease in platelet count at typhoid diagnosis relative to baseline. The average drop was slightly greater in those challenged with 10^4 CFU compared to those challenged with 10^3 CFU (80.6 $x10^3$/µL vs. 76.3 $x10^3$/µL respectively). Eleven of the twenty-four participants with typhoid disease (6 challenged with 10^3 CFU and 5 challenged with 10^4 CFU) had platelet counts below the limit of normal ($150x10^3$ µL). No complications associated with this decrease were seen. In the latter period of the study there was a rebound thrombocytosis.

Changes to the total and differential white cell count are shown in

Figure 53: Changes in total white cell count and differential white cell count relative to the point where typhoid was diagnosed (TD) in participants challenged with 10^3 or 10^4 CFU of *S.* Typhi.



The total white cell count decreased after typhoid diagnosis, apparent as a drop in the neutrophil and eosinophil count. The eosinophil count declined from 6 days prior to diagnosis of typhoid disease and was the earliest of all the blood parameters to change. All but one participant had a decrease in eosinophil count relative to baseline.

6.4. Discussion

Patients with typhoid disease report a wide range of symptoms of varying severity.[7] Participants in this study similarly reported a range of symptoms and severity. There was a trend for the larger challenge doses to be associated with increased duration, frequency and severity of many of the parameters examined, suggesting that at least some of the variation in clinical typhoid disease is dependent on size of the infecting dose.

6.4.1. Symptoms and signs of typhoid disease

Fever is the most universal sign of typhoid disease.[188] Although the vast majority of participants in this study diagnosed with typhoid disease were febrile, this was not universally true, with 4 bacteraemic participants failing to reach the diagnostic threshold for temperature of 38°C. Headache was universally experienced by all participants, with feeling generally unwell, loss of appetite, abdominal pain, nausea and vomiting, myalgia and arthralgia also affecting the vast majority of participants. Detailed symptom data are not available from previous *S*. Typhi challenge studies, but the range of symptoms observed are comparable between the two studies,[87] as well as being consistent with reports from endemic[7,38] and non-endemic settings[445,455] where pre-existing immunity from chronic exposure is unlikely to influence symptomology. Headache and anorexia were the most frequently occurring symptoms in previous typhoid challenge studies, occurring in approximately 90% of participants. Malaise, abdominal pain, arthralgia and myalgia were described as occurring in 50% of participants on at least one day of illness.[87] Constipation and cough occurred in 40% and 30% respectively. Although the relative order of

frequency of these symptoms is similar, symptoms were reported with higher frequency in this study compared to previous challenge studies. The proportion of participants reporting each symptom in this study is also higher than has been reported in other adult cohorts. In a UK series reported by Clark *et al*, for example, headache was reported by 62% of the cohort, diarrhoea by 59%, malaise by 46%, abdominal pain by 44%, vomiting by 38% and cough by 31%.[445] Similarly, in 22 adult patients admitted to a Parisian hospital, fever was present in all patients, headache in 82%, diarrhoea in 50%, fatigue in 45%, abdominal pain in 36%, nausea in 23% and vomiting in 23%.[455]. Differences may be partly attributed to the different methods of data collection, with previous challenge studies and patient cohorts using physician interview, whereas participant completed diary cards were used in this study. The use of diary cards may lead to solicitation bias and in the absence of a control group who were not challenged with *S*. Typhi, the rate of symptom reporting in non-exposed individuals is not known. Differences may also be partly due to the challenge dose used, with those challenged with 10^4 CFU in this study more symptomatic that those challenged with 10^3 CFU, suggesting that perhaps typhoid disease in previous typhoid challenge studies and in the field results from a comparatively low challenge dose.

Wide variation in the severity of typhoid disease is well described. For example, in the series reported by Clark *et al*, 2 of 61 patients were only admitted when *S*. Typhi bacteraemia was detected on samples taken in the assessment of disease.[445] In typhoid–endemic, resource–poor countries between 60% and 90% of patients diagnosed with typhoid disease are treated in the community, suggesting that the majority of patients have a relatively mild disease course.[25,38] Similarly, the severity of symptoms varied considerably between participants in this study. Whilst the majority of those diagnosed with typhoid disease had temperatures greater than 38°C and had symptoms that prevented all normal daily activity, this was not universally observed.

Participants in whom typhoid disease was not diagnosed were also noted to be symptomatic during the study period. There was no clear peak in symptoms as was seen in

those with typhoid disease, and the reporting of these symptoms may therefore reflect solicitation bias from the use of diary cards or the background prevalence of the solicited symptoms. Arthralgia was the most discriminatory symptom, perhaps reflecting the low background prevalence of this symptom. Two participants who did not meet the diagnostic definition for typhoid disease had unsustained fevers of 38°C or more, one of whom also reported symptoms of typhoid disease. It is possible that these participants had typhoid disease but not of sufficient severity to meet the diagnostic definition.

6.4.2. Laboratory features of typhoid disease

Laboratory tests on participants with typhoid disease showed a systemic inflammatory response, with a marked rise in CRP, ESR, and ALT and a fall in albumin. The ALT rise occurred later than other changes. Abnormalities of liver function with typhoid disease have been frequently reported,[7,99,456,457] and the data from our participants corresponds with this. The ALT rise was relatively late in the follow up period, occurring several days after the initiation of ciprofloxacin therapy. *S*. Typhi is known to reside in the reticulo-endothelial system, including the liver.[91] Hepatocytes may be the last haven of infection for *S*. Typhi, with the late ALT rise reflecting eventual destruction of infected hepatocytes.

Abnormalities in coagulation profiles of patients with typhoid disease, including disseminated intravascular coagulation and thrombocytopenia have been well described.[102] A decrease in platelet count was observed in all participants with typhoid disease in this study, with thrombocytopenia apparent in 11 of 24 participants with typhoid. These findings are in keeping with a study of 28 Vietnamese patients (24 of whom were blood culture positive), in which 17 patients were thrombocytopenic.[102] A more recent study of patients diagnosed over a 10 year period in the UK found a lower rate of thrombocytopenia, affecting 16% of the cohort.[445] Thrombocytopenia, although common, was asymptomatic in the Vietnamese study, and similarly, none of the participants in this study experienced symptoms of thrombocytopenia including bleeding. In the latter period

of this study there appeared to be a rebound in platelets to above the baseline value. Both the initial decrease and the rebound in count is in keeping with the data from the Maryland challenge studies, where a similar pattern was noted.[73] Rebound thrombocytosis has been noted in resolution of other conditions that are associated with bone marrow suppression, including after trauma, acute infection and alcohol withdrawal in alcoholic patients.[458-461] The pathology of this phenomenon is not completely understood, but it has been speculated that bone marrow recovery in the setting of cytokine stimulation may be the cause[459,461] and this may similarly be the case in the setting of typhoid disease.

The median haemoglobin concentration dropped in both the group of participants that developed typhoid disease and the group that did not. The volume of blood sampled during the first 28 days of the study was 1107ml. This significant blood volume may account for the decrease. Decrease in haemoglobin concentration is widely described in *S.* Typhi infection,[445,462] and this may also be part of the aetiology in some participants although there was no obvious difference in the decrease between those who did and did not develop typhoid disease. As well as a decrease in haemoglobin concentration, a decrease in white cell count, neutrophil count and lymphocyte count is commonly observed in typhoid disease,[7,445] and is thought to be due to bone marrow involvement and the effects of acute infection. The findings in this study were again in keeping with these previous observations. The eosinophil concentration almost universally decreased in those with typhoid disease and did so early in disease. This is in keeping with two case series of 28 and 54 children with typhoid fever in India, which demonstrated eosinopenia in 71.4% and 86.6% respectively.[457,463] Similarly, 10 of 17 returning travellers with typhoid fever were recently reported as having an absolute eosinophil count of 0 by automated differential.[457] Eosinopenia also occurs in other infectious conditions, and is a marker for sepsis in patients on intensive care units.[464,465] The mechanism of eosinopenia in infection, and more specifically in typhoid disease, is unclear. In health eosinophils principally reside at mucosal surfaces, including the gut mucosa,[466] and it is possible that increased marginalisation of

eosinophils during infection at the gut mucosa may account for the early and marked decrease.[467] Eosinophils have been shown to have anti-bacterial activity, particularly against Gram negative bacteria,[468] and it is possible that the early eosinophil decrease reflects recruitment to the gut mucosa is an early attempt to control infection.

6.4.3. Dose related severity of illness

Challenge studies provide a unique opportunity to directly control the infecting dose while measuring the outcome of infection. Participants developing typhoid disease following challenge with the higher dose of 10^4 CFU of *S.* Typhi had higher average temperatures, pulse rates at diagnosis, number of symptoms, duration of symptoms, and a higher rise in blood inflammatory markers compared to those challenged with 10^3 CFU. Together these findings suggest a direct relationship between challenge dose and severity of illness. Typhoid disease severity in previous challenge studies was reported as being relatively uniform, and did not vary with challenge dose.[73] However, this may have been due in part to the exclusion of milder cases under the stringent disease definition.[390] When a more permissive definition of illness was applied in a retrospective analysis of the data, peak temperature positively correlated with the log-transformed dose given and with the number of symptoms and signs, albeit weakly,[390] a finding that would support the data presented in this chapter. The only other available data on dose-severity relationships in typhoid disease is based on retrospective analysis of outbreaks of disease.[223] These analyses of outbreaks rely on crude measurements for both challenge dose and severity, with case fatality being the only marker of severity used.

A dose-effect relationship is important in implementing and evaluating public health interventions. Interventions such as vaccination or preliminary improvements in water and sanitation may serve to reduce the dose of bacteria that an individual is exposed to, rather than to prevent exposure entirely. In the setting of a dose response relationship, interventions acting to decrease exposure may have a greater impact on severe rather than

total disease.[469] The data presented in this chapter provide the first data that suggests there may be a dose-effect relationship between the dose of *S.* Typhi and illness severity and highlight the unique opportunity to appraise these relationships afforded by the carefully controlled setting of a challenge study. This possible dose-response relationship will be further investigated by examining the genomic, cytokine and microbiological response using samples obtained in this study.

6.5. Conclusion

Typhoid disease induced by human challenge with *S.* Typhi Quailes strain induces symptoms in keeping with classical typhoid disease. The extent of symptoms and severity is variable between individuals, ranging from asymptomatic bacteraemia to illness that prevents all normal daily activities. A systemic inflammatory response is apparent on laboratory blood testing, together with suppression of haematopoietic cells. Some of the variation in response is accounted for by the size of the challenge dose, a finding that is important for planning and monitoring public health interventions.

7. Microbiological response to *S.* Typhi challenge

7.1. Introduction

Prompt diagnosis and treatment of typhoid disease decreases disease complications and limits opportunity for disease spread.[7] As has been shown in Chapter 6, typhoid disease causes a diverse range of clinical symptoms, many of which also occur in other diseases common in endemic countries including malaria, tuberculosis, dengue fever and brucellosis,[7] making diagnosis on based clinical symptoms alone difficult. Isolation of *S.* Typhi from blood is diagnostic but not always achieved,[13] partly due to the low concentrations of organisms in blood.[86] Isolation of *S.* Typhi from stool samples provides evidence of exposure but is not in itself diagnostic. This chapter presents the microbiological findings in participants challenged as part of establishing a typhoid disease model.

7.1.1. Blood culture

Blood culture has been employed as a diagnostic method in typhoid disease for over 100 years.[470] Isolation of *S.* Typhi by blood culture allows definitive diagnosis and antibiotic sensitivity testing, but is limited by variable sensitivity between 30% and 90%.[161-163]

Compared to other bacteraemic illnesses, the bacterial load in blood during acute typhoid disease is low, with a median count of one colony-forming unit per ml of blood and hence the volume of blood cultured is an important predictor of sensitivity.[86] Bone marrow culture is considerably more sensitive than blood culture, and this has been shown to be a product of 15 fold greater bacterial concentration in this compartment.[13]

Blood bacterial load in typhoid disease decreases with increasing duration of illness,[86,162] further reducing the sensitivity of blood culture when presentation is delayed. For example, blood culture positivity rates decreased from 80% in untreated patients presenting in the

first week to 30% in those presenting in the third week of illness in a study in Pakistan.[162] Similarly in children diagnosed with typhoid disease in Peru, blood culture was positive in 70% of those presenting in the first week of illness, but only positive in 22% of those presenting after 7 days.[163]

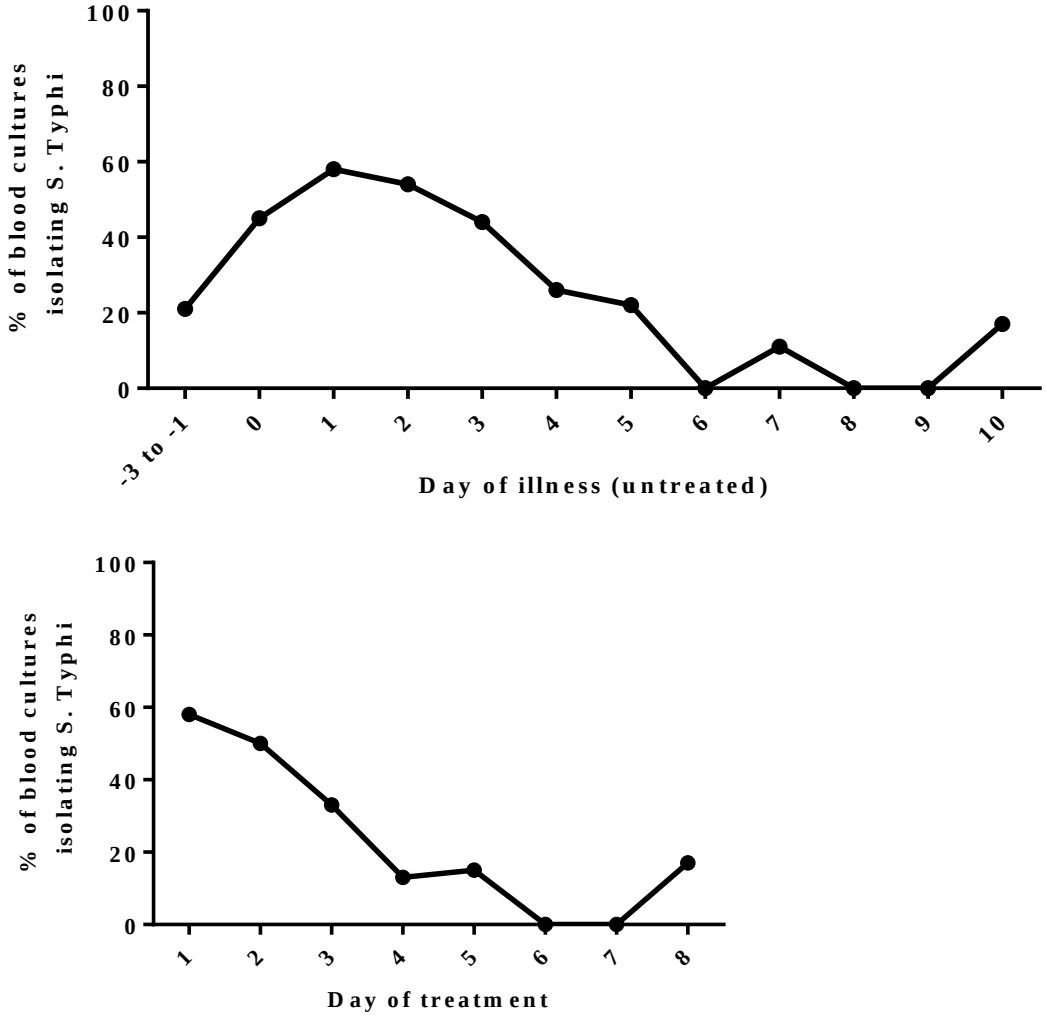
7.1.2. Stool culture

Stool culture can be a useful aid to diagnosis in acute typhoid disease and is important in the detection of carrier status. Single stool cultures isolate *S.* Typhi in 13% to 14% of patients with acute typhoid disease.[86] Multiple specimens have been shown to increase yields, with three stool cultures detecting 31.6% of patients, compared to 13.4% from single culture.[13] The volume of stool cultured is also an important determinant of isolation rates, with a 10% increase when 2 grams are cultured compared to 1 gram.[13] The rate of stool culture positivity is also positively correlated with the bacterial concentration in blood.[86] Detection of *S.* Typhi is enhanced by selenite enrichment media that inhibits the growth of other bowel organisms.[168]

7.1.3. Microbiological findings from previous typhoid challenge studies

Limited microbiological investigation was performed during previous typhoid challenge studies.[87] Blood and stool culture was routinely performed, but did not form part of the diagnostic definition of typhoid disease. Participants challenged with 10^5 CFU of *S.* Typhi, which accounted for the majority of those challenged, had at least one blood culture taken during illness (average number of blood cultures per participant, 5.8), mostly during the first 3 days of illness.[87] Overall, bacteraemia was detected in 75% of those with clinical typhoid disease. Bacteraemia rates fell with increasing duration of infection, and with antimicrobial therapy (Figure 54).

Figure 54: The percentage of the total blood cultures from which *S.* Typhi was isolated for each day of clinical illness (top graph) or day of antibiotic treatment (bottom graph) in participants challenge with *S.* Typhi in previous challenge studies conducted at the University of Maryland, showing decreasing isolation rates with increasing duration of illness and antibiotic therapy. Reproduced from data presented in 'Induced typhoid disease and experimental typhoid vaccines' – a study of 1886 volunteers, T.E. Woodward[87]



Detailed examination of bacteraemia took place in earlier challenge studies, and demonstrated a range of clinical response to bacteraemia. At one extreme, two of the first 64 men challenged were bacteraemic and asymptomatic:[246] one had ingested 10^8 CFU, was bacteraemic for seven days, with only a mild rise in CRP and a slight headache; the other, who received 10^9 CFU, was bacteraemic for one day, and again only experienced a slight

rise in CRP. A later report included several further challenge studies, and described an additional 3 men who remained asymptomatic and afebrile whilst bacteraemic, 5 men with mild symptoms but no temperature response, and 6 participants where bacteraemia preceded symptoms by up to 17 days.[87] The presence of a 'silent' Gram-negative bacteraemia may reflect the organism's ability to hide from the immune system, possibly within the intracellular space, thus limiting activation of the inflammatory response.[73]

Detailed results of stool culture from participants in the Maryland studies were not published, but detailed in an end of study report by T.E. Woodward.[87] Stool culture demonstrated transit of *S.* Typhi in the immediate post oral challenge period, with cultures positive in the two days after challenge.[73] Although early stool culture positivity was associated with subsequent development of typhoid disease, this was not absolute; participants with negative stool cultures went on to develop typhoid disease, and vice versa.[87] Indeed, some participants who remained asymptomatic from typhoid disease were noted to excrete *S.* Typhi in their stools for several weeks. The temporal pattern of stool shedding was similar in both those who did and those who did not develop typhoid disease, with the frequency of excretion peaking two weeks after challenge and remaining for 3 weeks before declining rapidly, such that all participants were negative by 6 weeks after challenge.[87] Since only those who developed typhoid disease were treated with antibiotics, it was concluded that this was the natural pattern of shedding.

7.2. Material and methods

7.2.1. Qualitative blood culture

Prior to venepuncture, skin was cleaned with 1.5 ml of 2% chlorhexidine gluconate in 70% isopropyl (ChloraPrep FREPP applicator system, CareFusion, UK). The required volume of blood was obtained using a sterile non-touch technique from the anterior cubital fossa using a sterile 22G butterfly needle, and was added to BACTEC Plus Aerobic/ F culture

vials (Becton, Dickinson and Company, New Jersey, USA). Ten millilitres of blood was cultured, except when participants were diagnosed with typhoid disease when 5 ml was cultured by standard methods.

Blood cultures were incubated using BACTEC FX SYSTEM (Becton, Dickinson and Company (BD)) by the Microbiology laboratory, Oxford University Hospitals (OUH), according to the current operating procedure (M-SOP-017 Blood Culture, OUH, NHS Trust). This culture system is based on the detection of carbon dioxide released by micro-organisms by a sensor disc at the bottom of the culture. Blood culture bottles that were detected as having an increase in carbon dioxide were removed from the BACTEC machine for microbial identification by microscopy and culture. Direct microscopy was performed using Gram staining. For microbial culture of Gram-negative organisms (including *S*. Typhi), one drop of blood/broth mixture was plated directly on to blood agar (incubated overnight, anaerobically at 37°C; Oxoid; Basingstoke, UK; CM00331) and chocolate agar (incubated overnight, aerobically, at 37°C; Becton, Dickinson and Company). A further drop of blood was diluted in 1 mL of peptone water (Oxoid; CM0509), before being plated on to MacConkey agar (incubated overnight, at 37°C in air; Oxoid; CM0007), chromogenic (CHROMagar Orientation) agar (incubated at 37°C in air; CHROMagar Microbiology, New Jersey, USA). For simultaneous antimicrobial testing, iso-senitest agar (incubated at 37°C in air; Oxoid; CM0471) and Wilkins-charlgren anaerobe agar (incubated at 37°C anaerobically; Oxoid; CM0619) were plated with the peptone water inoculum to give semi-confluent growth, before adding antimicrobial sensitivity and metronidazole discs respectively.

7.2.2. Stool culture

Stool samples were self-collected by participants and stored at room temperature. The time of sampling was noted. Routine stool cultures and screening for enteric pathogens was performed by the Microbiology Laboratory (OUH) according to the standard procedures

(M-SOP-111 Stool Culture, OUH, NHS Trust). A pea-sized amount of stool (approximately 1 g) was inoculated into selenite broth (Oxoid; CM0395) and mixed by vortexing. After overnight incubation at 37°C, 5µl of the selenite, obtained from the top of the broth culture, was inoculated onto chromogenic agar (Salmonella Plus agar, E&O laboratories; Bonnybridge, Scotland; PP1071) and incubated aerobically at 37°C for 18 to 24 hours.

7.2.3. Microbial identification

Colonies phenotypically compatible as *S*. Typhi colonies cultured from blood or stool had morphology confirmed by Gram staining, and further identified by Analytical Profile Index (API)-10S (bioMerieux, France) and agglutination with *S*. Typhi antisera. Isolates were retained for phage typing.

For API testing, one colony was inoculated into 5ml of saline and added to the API strip according to manufacturer's instructions. The strip was incubated at 37°C overnight before being read according to the manufacturer's instructions.

Speciation was by slide agglutination, according to the Kauffman and White scheme. One to two large colonies were suspended in saline and emulsified. Two micro-litres were dabbed on to a slide, and 2µl of test antisera added and lightly mixed by rocking the slide. Positive agglutination occurred within 20 seconds and could be seen by a grainy solution on mixing. To remove the Vi antigen and unmask the O antigen for testing, a dense suspension of the bacteria in saline was made in a test tube and boiled for 30 minutes.

7.2.4. Antibiotic sensitivity testing

Antimicrobial susceptibility was determined by disc sensitivity testing which included: ampicillin (10 µg), trimethoprim (125 µg), chloramphenicol (10 µg), ertapenam (10 µg) ceftriaxone (30 µg), ciprofloxacin (5 µg) and nalidixic acid (30 µg; all supplied by Becton, Dickinson and Company). The minimum inhibitory concentration (MIC) for ciprofloxacin was measured by Etest (AB Biodisk). Iso-Sensitest agar (ISA; Oxoid, Basingstoke, UK)

were streaked with the inoculated peptone water, to obtain semi-confluent growth. The plates were allowed to dry before antibiotic discs were applied (6 per plate). The plates were incubated in air, overnight at 37°C. The diameters of the zones of inhibition were measured the next day to the nearest millimetre.

7.2.5. Quantitative blood culture

Methods for quantitative blood culture are outlined in Chapter 2. In brief, 10 mL of whole blood was inoculated into Isolator 10 tubes when typhoid disease was diagnosed. The sample was processed immediately by centrifugation and plating on to XLD agar. Colonies were counted after 24 hours.

7.3. Results

7.3.1. Blood cultures

Over the course of the study 692 blood cultures were sent, an average of 17.3 per participant (Table 53). From this, *S*. Typhi was isolated from 87, 17 were contaminated with organisms other than *S*. Typhi and 588 were negative. Hence, the overall *S*. Typhi positivity rate was 12.6%, and contamination rate was 2.5%.

Table 53: Results of daily blood cultures obtained from participants challenged with either 10^3 CFU or 10^4 CFU of *S.* Typhi over the 14 days following challenge, in the development of a human challenge model of typhoid disease

	Challenge dose of *S.* Typhi (CFU)		Total
	10^3	10^4	
No. of blood cultures isolating *S.* Typhi /total number of blood cultures (%)	19/305 (12.0%)	34/353 (13.1%)	53/658 (12.6)
No. of participants bacteraemic/ total number with typhoid disease (%)	10/11 (90.9%)	11/13 (84.6%)	21/24 (87.5%)
No. positive blood cultures/ participants with typhoid disease	1.7	2.6	2.2
No. of positive blood cultures / participant with *S.* Typhi bacteraemia	1.9	3.1	2.5

The number of positive blood cultures and proportion of participants that were bacteraemic per day of follow-up are shown in Table 54 and Figure 55.

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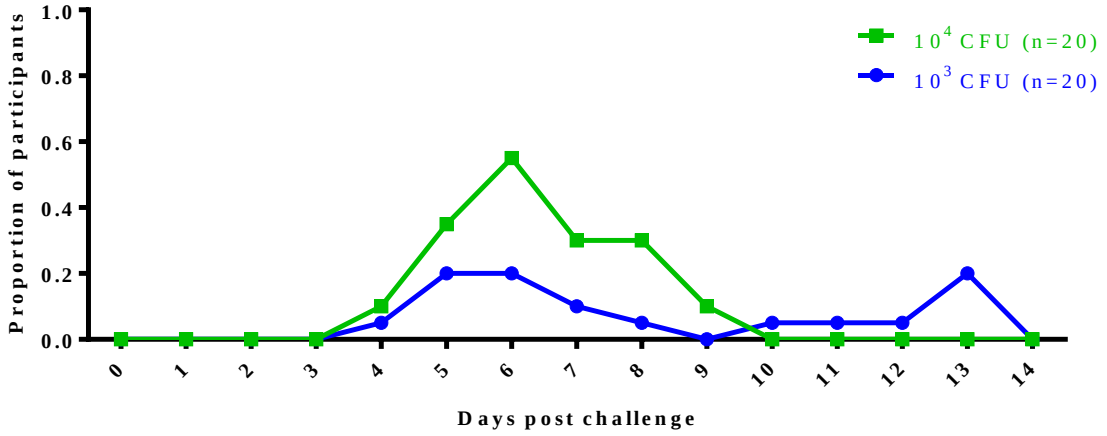
Table 54: The results of daily blood cultures obtained from participants challenged on day 0 with either 10^3 CFU or 10^4 CFU of *S.* Typhi in a human challenge model of typhoid disease, showing the total number of blood cultures from which *S.* Typhi was isolated, the proportion of all participants that were bacteraemic with *S.* Typhi and the proportion of participants diagnosed with typhoid disease in the 14 days after challenge in whom bacteraemia was detected

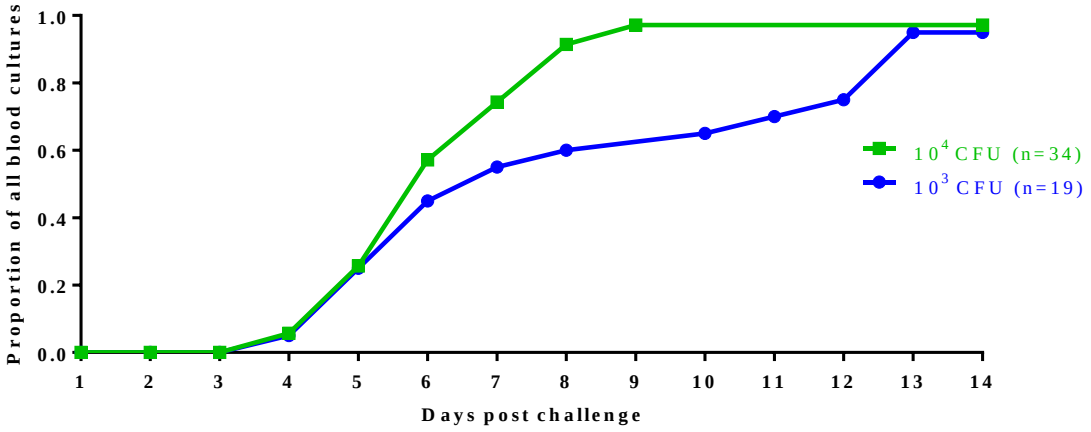
Challenge dose of *S.* Typhi (CFU)		Days following challenge (Day 0) with *S.* Typhi														
		Day 0	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 8**	**Day 9**	**Day 10**	**Day 11**	**Day 12**	**Day 13**	**Day 14**
10^3 (n=20)	Total number of blood cultures isolating *S.* Typhi	0	0	0	0	1	4	4	2	1	0	1	1	1	4	0
	Proportion of all participants with *S.* Typhi bacteraemia	0	0	0	0	0.05	0.2	0.2	0.1	0.05	0	0.05	0.05	0.05	0.2	0
	Proportion of participants who developed typhoid disease with *S.* Typhi bacteraemia	0	0	0	0	0.09	0.36	0.36	0.18	0.09	0	0.09	0.09	0.09	0.36	0
10^4 (n=20)	Total number of blood cultures isolating *S.* Typhi	0	0	0	0	2	7	11	6	6	2	0	0	0	0	0
	Proportion of all participants with *S.* Typhi bacteraemia	0	0	0	0	0.1	0.35	0.55	0.3	0.3	0.1	0	0	0	0	0
	Proportion of participants who developed typhoid disease with *S.* Typhi bacteraemia	0	0	0	0	0.15	0.54	0.85	0.46	0.46	0.15	0	0	0	0	0

Figure 55: The proportion of participants in whom *S.* Typhi bacteraemia was detected following challenge on day 0 with either 10^3 CFU or 10^4 CFU of *S.* Typhi in a human challenge model of typhoid disease



In keeping with the increased attack rate observed following challenge with 10^4 CFU *S.* Typhi, the rate of bacteraemia was also higher, compared to that seen in those challenged with 10^3 CFU. The kinetics of isolation of *S.* Typhi from blood culture following challenge is seen in Figure 56.

Figure 56: The kinetics of *S.* Typhi isolation in blood cultures obtained each day from participants challenged with either 10^3 or 10^4 CFU of *S.* Typhi on day 0 in the development of a human challenge model of typhoid disease.



The earliest bacteraemia occurred on day 4 following challenge for each dose. Blood cultures from participants challenged with 10^4 CFU became positive earlier and over a

narrower time frame compared to those receiving 10^3 CFU. The latest point of bacteraemia for participants challenged with 10^4 CFU was at day 9 whilst those challenged with the 10^3 challenge dose became bacteraemic up to day 13 following challenge. Whereas blood cultures collected after 12 hours of antibiotics were sterile in all those challenged with 10^3 CFU, bacteraemia persisted for 24 hours in 3 patients challenged with 10^4 CFU. No participants were bacteraemic 24 hours after antibiotic therapy had commenced. All *S.* Typhi isolates were fully sensitive to all antimicrobials.

7.3.2. Stool culture

A total of 541 stool cultures was sent, (average 13.5 per participant) of which 54 were positive (positivity rate of 10%), as shown in Table 55.

Table 55: Results of stool cultures obtained over the 14 days after challenge (day 0) from participants challenged with either 10^3 CFU or 10^4 CFU of *S.* Typhi in a human challenge model of typhoid disease

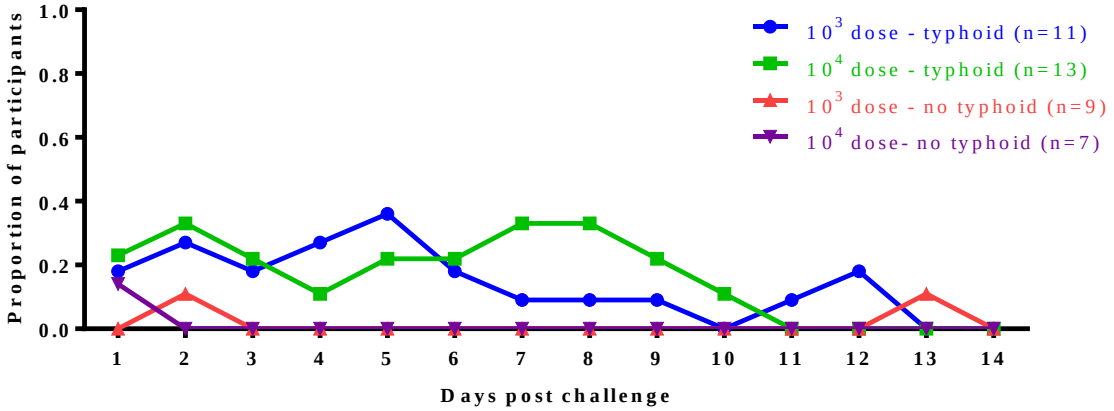
	Develop typhoid disease			**Did not develop typhoid disease**		
	10^3 CFU challenge dose (n=11)	**10^4 CFU challenge dose (n=13)**	**Total (n=24)**	**10^3 CFU challenge dose (n=9)**	**10^4 CFU challenge dose (n=7)**	**Total (n=16)**
Total number of stool cultures sent	151	178	329	116	96	212
Average number stool cultures sent per participant	13.7	13.7	13.7	12.8	13.7	13.25
Number (%) positive for *S.* Typhi	22 (14.6)	29 (16.3)	51 (15.5)	2 (1.7)	1 (1.0)	3 (1.4)
Number positive to *S.* Typhi/ participant	2.00	2.23	2.13	0.22	0.14	0.18

Participants who were diagnosed with typhoid disease had more positive stool samples than those without typhoid disease (average of 2.13 vs. 0.18 respectively). Of those with typhoid disease, 8 of 11 participants challenged with 10^3 CFU and 11 of 13 challenged with 10^4 CFU were stool culture positive during the 14 days following challenge. For those not developing typhoid disease, 2 of 9 and 1 of 7 participants challenged with 10^3 CFU and 10^4 CFU respectively excreted *S.* Typhi.

Table 56: The results of stool cultures obtained over 14 days from participants challenged on day 0 with either 10^3 CFU or 10^4 CFU of *S.* Typhi in a human challenge model of typhoid disease, showing the total number of stool cultures from which *S.* Typhi was isolated, and the proportion of participants that were stool culture positive for *S.* Typhi, for participants who did and did not develop typhoid disease

	Challenge dose (CFU)		Day post challenge (day 0) with *S.* Typhi													
			Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
Developed typhoid disease	10^3 (n=11)	Total number of stool culturing *S.* Typhi / total sent	2/11	3/11	2/11	3/9	4/11	2/11	1/11	1/11	1/11	0/11	1/11	2/10	0/11	0/11
		Proportion of participants stool culture positive for *S.* Typhi	0.18	0.27	0.18	0.27	0.36	0.18	0.09	0.09	0.09	0.00	0.09	0.18	0.00	0.00
	10^4 (n=13)	Total number of stool culturing *S.* Typhi / total sent	3/12	3/9	2/8	1/9	2/9	2/9	3/9	3/8	2/9	1/9	0/9	0/9	0/9	0/9
		Proportion of participants stool culture positive for *S.* Typhi	0.23	0.33	0.22	0.11	0.22	0.22	0.33	0.33	0.22	0.11	0	0	0	0
Did not develop typhoid disease	10^3 (n=9)	Total number of stool culturing *S.* Typhi / total sent	0/9	1/8	0/6	0/8	0/9	0/7	0/9	0/9	0/8	0/9	0/8	0/9	1/8	0/9
		Total number of stool culturing *S.* Typhi / total sent	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.00
	10^4 (n=7)	Proportion of participants stool culture positive for *S.* Typhi	1/7	0/6	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/6	0/7	0/7
		Proportion of participants stool culture positive	0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

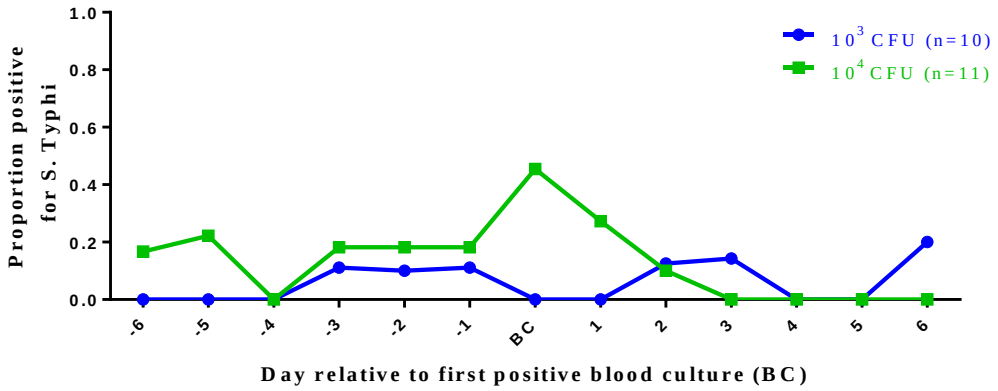
Figure 57: The proportion of participants with *S.* Typhi excretion detected in stool samples collected on each day following challenge on day 0 with either 10^3 or 10^4 CFU of *S.* Typhi, shown by those who were diagnosed with typhoid disease (typhoid) or not (no typhoid), in a human challenge model of typhoid disease



Excretion of *S.* Typhi in the stool occurred from 24 hours after challenge, probably reflecting passage of the organism through the gut (Figure 57). A higher proportion of participants who developed typhoid disease were stool culture positive in the first 3 days after challenge compared to those who did not (12/24 vs. 2/16). This equates to a positive predictive value of 85.7%.

Stool culture positivity tended to occur early in the post challenge period, resolve and then reoccur, often preceding blood culture positivity or occurring at the same time point (Figure 58). Following initiation of antibiotic treatment, no participant had further stools positive for *S.* Typhi at any sampling point.

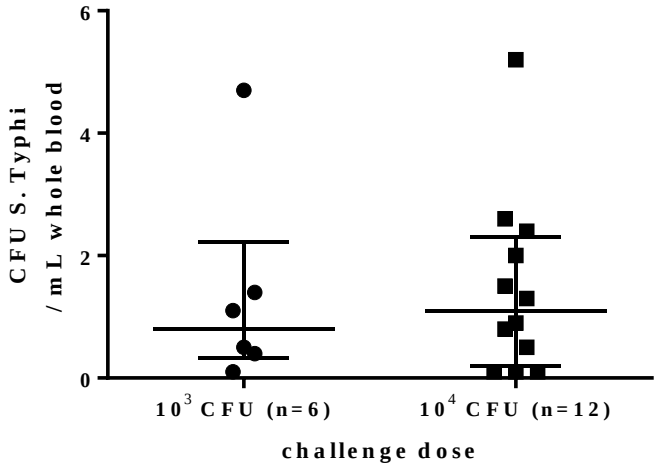
Figure 58: The temporal relationship of stool excretion of *S.* Typhi to *S.* Typhi bacteraemia for participants challenged with 10^3 or 10^4 CFU of *S.* Typhi, in a human challenge model of typhoid disease



7.3.3. Quantitative blood culture

Results of quantitative blood culture, performed for 6 of 11 participants and 12 of 13 participants diagnosed with typhoid disease following challenge with 10^3 CFU and 10^4 CFU of *S.* Typhi respectively are shown in Figure 58. The numbers of bacteria were generally low, with a median of 0.8 CFU/ml in those challenged with 10^3 CFU and 1.1 CFU/ml in those challenged with 10^4 CFU.

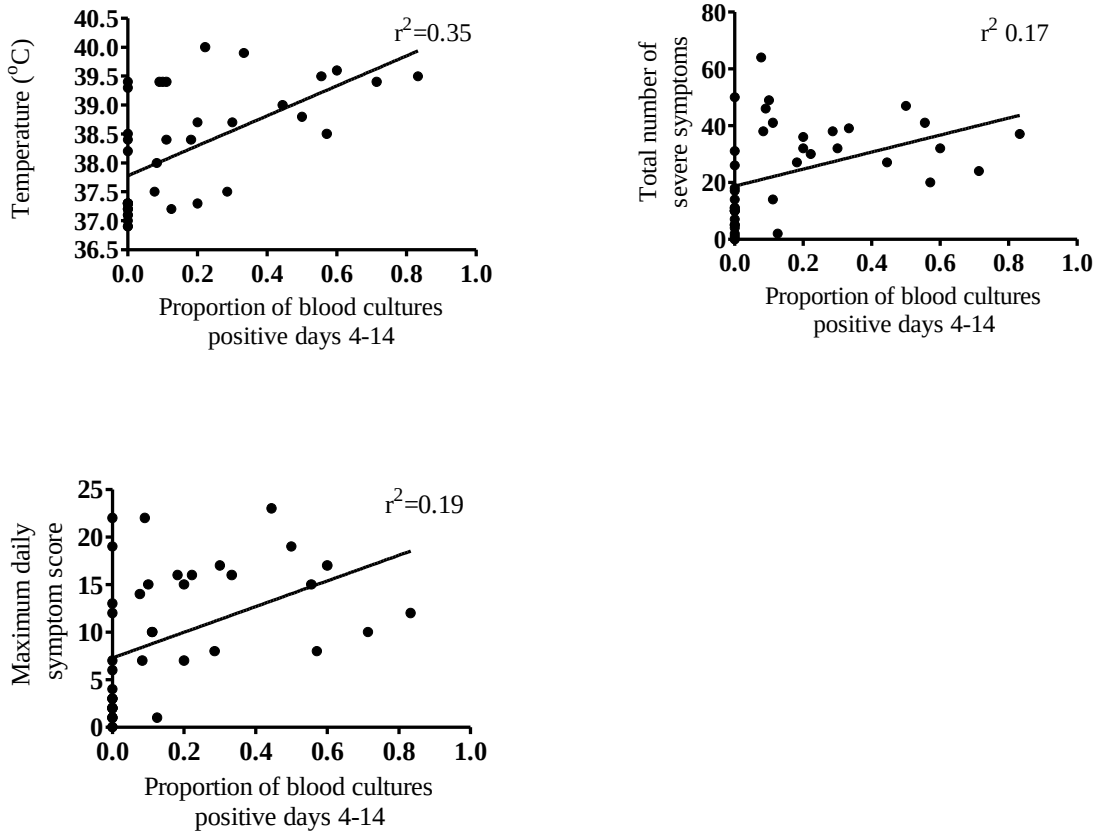
Figure 59: The median number and interquartile range of *S.* Typhi CFU isolated by quantitative blood culture in participants diagnosed with typhoid disease following challenge with 10^3 CFU of 10^4 CFU of *S.* Typhi in a human challenge model of typhoid disease



7.3.4. Correlation of blood culture results with clinical symptoms

There was a weak correlation between the proportion of an individual's blood cultures that were positive between days 4 and 14 after challenge and the maximum temperature (Figure 60). There was no correlation between the proportion of blood cultures positive between days 4 and 14 and the total number of severe symptoms reported or the maximum daily symptom score (the sum of mild, moderate, and severe symptoms per day, scored as 1,2 and 3 respectively) as shown in Figure 60.

Figure 60: Correlation between the proportion of blood cultures positive between days 4 and 14, and the maximum temperature, total number of symptoms and the maximum daily symptom score in participants challenged with *S.* Typhi in a human challenge model of typhoid disease



Four participants with *S.* Typhi bacteraemia had oral temperatures below 37.6°C. Features of these participants are outlined in Table 57. As well as having a minimal temperature response, participants 0005 and 0020 were asymptomatic. Systemic inflammation indicated by the maximum CRP was minimal in participant 0020. All demonstrated the characteristic drop in platelets seen in typhoid disease.

Table 57: Features of four participants who were afebrile (oral temperatures <36.6°C) despite *S.* Typhi bacteraemia in a human challenge model of typhoid disease

Participant number	Actual challenge dose (CFU)	Day(s) of *S.* Typhi bacteraemia	Day(s) stool culture positive	Oral temperature at diagnosis	Maximum oral temperature recorded	Total number of solicited symptoms reported on day of diagnosis	Total severity score of symptoms at typhoid diagnosis	Maximum CRP	Drop in platelet count (baseline/ lowest)
0005	1285	D11, D13	Nil	37.3	37.3	2	2	17	52 (220-163)
0014	1000	D12, D13	D8, D12	37.4	37.5	6	8	20.9	25 (184-159)
0020	705	D13	D5, D6	36.9	37.2	0	N/A	5.8	110 (219-104)
0040	20300	D8	D7, D8	37.5	37.5	7	14	58.8	112 (330-218)

7.4. Discussion

Diagnostic tools for typhoid disease that are both sensitive and specific are lacking.[139] Although isolation of *S.* Typhi from blood culture provides a definitive diagnosis, sensitivity is limited, with different studies quoting rates between 30% and 90%.[36,161-163] The overall sensitivity of blood culture in this study was 87.5%. This high sensitivity is likely to be related to the number of blood cultures taken, and the proximity of obtaining blood cultures to the onset of clinical illness in the study. The average participant had 16.5 cultures taken over the post-challenge follow up period significantly more than is likely to be performed in a clinical setting. Blood culture sensitivity is also enhanced if it is obtained at the beginning of illness onset,[162,163] as was achievable in this study with daily blood cultures obtained in the 14 days following challenge. This contrasts with the situation in resource–poor countries where most studies of blood culture have been performed. Delayed presentation is a frequent feature in resource-poor countries, and the number of blood cultures that can be performed is often limited by cost. Blood culture media[164] and the volume of blood cultured[86] are also known to influence the sensitivity of blood culture and may explain the difference in observed sensitivity.

The pathogenesis of typhoid disease is thought to include a brief primary bacteraemia following invasion across the gut wall.[84,85] This was not detected by blood culture in this study, suggesting that if it does occur then the bacterial load is extremely small, or that it is so brief that culture at 6, 12 and 24 hours after challenge is not sufficient to capture it. Detection of bacterial DNA by PCR may be more sensitive,[171,172] and may allow detection of this primary bacteraemia in samples that have been obtained from these participants. Following invasion and dissemination, *S.* Typhi is thought to incubate in the reticulo-endothelial system, before being released into the blood stream during a secondary symptomatic bacteraemia.[7] Incubation period is thought to be detected by virulence of the organism, the host response to infection and number of bacteria ingested. Bacteraemia was

detected in this study as early as 4 days after challenge, and as late as day 14, which was the latest point that blood cultures were obtained from participants. Blood cultures obtained in participants challenged with 10^4 CFU tended to be positive earlier on in the follow up period compared to those challenged with 10^3 CFU, supporting the idea that incubation period is partially dictated by the bacterial load ingested. The higher challenge dose was associated with a slightly higher rate of bacteraemia, concentrated over a narrower period. These dose-response data are in keeping with data presented on the symptom profile and the serological response to challenge, that also demonstrate an association between challenge dose and subsequent disease. Had blood cultures been obtained after day 14 it may have been possible to detect bacteraemia (and therefore diagnose typhoid disease) in a greater number of participants, with incubation periods as long as 56 days described in the Maryland studies.[73]

Stool culture is of limited use in diagnosis of acute typhoid disease, although it can indicate exposure in cases of fever of unknown origin and is important in the detection of the carrier status. Overall, 79% of participants diagnosed with typhoid disease in this study had a positive stool culture at some point. Single stool cultures on admission have been shown to detect *S*. Typhi in 13% to 14% of patients with typhoid disease[86] and multiple cultures, as was performed in this study, increase detection rates. For example, culturing three samples detected 31.6% of patients, compared to 13.4% from single culture.[13] The relatively high proportion of participants with typhoid disease that had positive stool samples in this study is therefore likely to be due to the large number of samples that were cultured for each individual.

S. Typhi was most frequently isolated in the stool either just before or at the same time as *S*. Typhi bacteraemia. Antibiotic therapy led to rapid clearance of *S*. Typhi from stool, suggesting that the risk of secondary transmission may be aborted by prompt treatment. It is known that patients with high bacterial load in their blood are more likely to excrete *S*. Typhi in their stools,[86] and the timing of positive cultures in this study is in keeping with

this. Several participants also excreted *S*. Typhi in their stools in the first few days following challenge, and this strongly predicted the later diagnosis of typhoid disease (positive predictive value, 85.7%). Detailed data on stool culture preformed prior to onset of clinical illness are not available, reflecting the unique setting of this study.

Quantitative blood culture identified a low bacterial load in the blood, in keeping with a study in Vietnam that found an average of 1.7 CFU/ mL of blood from samples taken during the first week of illness. This level of bacteraemia has also been reported in other enterobacterial bacteraemias, including fatal bacteraemia, although average counts tend to be higher than observed in this study.[471,472] This suggests that the absence of the typical picture of gram-negative septicaemia in patients with typhoid disease is not simply a factor of decreased bacterial load.[86]

Indeed, the clinical profile of typhoid disease seemed to have minimal relationship with the presence of bacteraemia, with the only slight correlation between bacteraemia and clinical symptoms being with the maximum oral temperature. The limited correlation between bacteraemia and symptoms is most clearly illustrated by the four participants that failed to mount a temperature response despite bacteraemia. This has been described previously in typhoid challenge studies.[87,246] Fevers of 38°C or more also occurred in participants without demonstrable bacteraemia, further supporting the idea that the clinical profile has limited correlation with bacteraemia.

7.5. Conclusion

Frequent blood and stool culture in participants in the challenge study led to a high rate of overall positivity. Excretion of *S*. Typhi in stool immediately after challenge is associated with subsequently developing typhoid disease. Quantitative blood culture shows a low bacterial load during acute typhoid disease. Bacteraemia does not closely correlate with clinical disease, and indeed silent bacteraemia with *S*. Typhi can occur. The full pathological mechanisms in typhoid disease remain to be understood.

8. Humoral response to *S.* Typhi challenge

8.1. Introduction

The relative importance of the B cell and T cell response to *S.* Typhi infection remains unclear. The mouse model of typhoid disease has been used to investigate the immune response, but has significant limitations that limit its applicability to humans.[42] Data from humans is principally from previous challenge studies, where strong antibody responses to the surface expressed antigens of *S.* Typhi, namely the virulence factor (Vi) polysaccharide, lipopolysaccharide (O) and flagellin (H) antigens, were detected.[87] Although these studies provided some insight into the antibody response to *S.* Typhi infection in humans, data are restricted to total antibody data, and do not provide information on the relative importance of the differential IgG, IgM and IgA response. Similarly, there are no data on terminally differentiated B cells, termed plasma cells, which produce antibody. This chapter will provide the first such data by describing the humoral immune response of participants in the human challenge model described in Chapter 4.

8.2. Material and methods

8.2.1. Serum samples

Serum from participants in the challenge study described in Chapter 4 were obtained at baseline, and days 4, 7, 10, 14, 28, 60 and 90 post-challenge and at 48 hours after the diagnosis of typhoid disease, where applicable. Serum was processed as detailed in Chapter 2.

8.2.2. Kinetics of the antibody response to the H, LPS and Vi antigen

A detailed kinetic profile of the antibody response in 9 participants was performed to assess critical time points for further assays. Three participants were selected from each of 3 groups. The groups were those who developed typhoid disease following challenge with 10^3 CFU of *S*. Typhi, those who developed typhoid disease following challenge with 10^4 CFU, and those who did not develop typhoid disease. Availability of sera at different time points varied by participant as blood sampling was dictated by if and when they were diagnosed with typhoid disease, whether a participant was available to attend for a visit, and the volume of blood obtained. Based on these results, 4 time points were selected for analysis on remaining participants. All sera was assayed using the ELISAs described in Chapter 2.

8.2.3. Antibody secreting cells (ASCs)

Peripheral blood mononuclear cells (PBMCs) were isolated from 25 ml of heparinised blood as outlined in Chapter 2. ASCs were assayed by *ex vivo* ELISPOT using the methods outlined in Chapter 2. IgG, IgM and IgA ASCs to the H, Vi and LPS antigens were assayed at baseline and days 7 and 9 post-challenge, along with 48 hours after typhoid diagnosis, where applicable.

8.2.4. Statistical analysis

ELISA units and plasma cell counts were log transformed to normalise the distribution of the data. Where the plasma cell count was zero, 0.25 was used to allow log transformation. Group comparisons are presented as geometric means with the 95% confidence interval (CI). Formal statistical testing of differences was not performed as the study was not powered for this endpoint.

8.3. Results

8.3.1. Detailed kinetic profile of the IgG, IgM and IgA antibody response to the H, LPS and Vi antigens of *S.* Typhi

The results for the detailed kinetic response to the H antigen are shown in Table 58 and Figure 61, for the LPS response in Table 59 and Figure 62, and for the Vi response in Table 60 and Figure 63.

Table 58: Detailed kinetics of the anti-H antibody concentrations (ELISA units/ ml) following challenge (Day 0) with *S.* Typhi for 9 participants, 3 of whom developed typhoid following challenge with 10^3 CFU, 3 of whom developed typhoid following challenge with 10^4 CFU, and 3 or whom did not develop typhoid, in a human challenge model of typhoid disease

Antibody	Group	Anti- H antibody concentration (ELISA units/ ml)													
		Day 0	Day 4	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 28	Day 60	Day 90
IgG	Developed typhoid-10^3 CFU	14.6	17.2			94.6		243.2			329.7		276.8	101.9	98.6
		26.9	30.0	28.7					73.6	157.3		301.6	673.4	185.7	120.9
		46.5	54.3		47.4		62.6				78.0		78.5	85.7	79.0
	Developed typhoid-10^4 CFU	55.3	50.3	51.3			44.3		48.8		78.2		89.6		65.8
		56.9	69.2		387.7		1408.0				1288.0		708.3	114.1	
		19.1	19.6	24.1		23.6		128.3					138.3	93.5	
	Did not develop typhoid	169.7	154.4	165.9					131.5	17.3			140.9	142.4	108.9
		49.3	46.6	40.1					41.8	41.0			38.1	48.5	44.6
		26.2	25.8	28.7					31.3	82.3			82.6	87.7	
IgM	Developed typhoid-10^3 CFU	14.2	13.6			20.3		20.9			27.3		18.6	11.5	14.2
		8.7	9.2	9.1					12.0	22.6		41.0	39.4	20.0	9.1
		11.6	12.5		9.8		16.2				13.8		21.8	21.9	11.1
	Developed typhoid-10^4 CFU	33.7	31.6	36.1			65.3		135.8		256.5		240.7		43.0
		13.9	13.1		22.2		34.6				45.2		28.5	12.4	
		7.1	6.5	7.5		18.9		228.7					100.2	23.3	
	Did not develop typhoid	15.3	13.4	18.6					12.8	9.2			17.3	17.6	14.7
		30.5	29.2	28.6					30.7	42.8			33.1	40.5	38.8
		18.7	16.1	17.4					19.6	15.4			13.2	17.5	
IgA	Developed typhoid-10^3 CFU	3.2	2.9			80.1		168.1			195.5		22.4	5.4	13.1
		8.8	10.8	10.5					21.2	29.8		43.3	35.5	12.6	19.8
		13.4	13.0		15.1		44.9				261.1		121.5	48.2	26.2
	Developed typhoid-10^4 CFU	21.4	25.5	23.6			40.6		102.0		307.3		96.0		18.6
		5.6	7.6		34.7		100.5				219.4		72.0	19.4	
		39.2	35.9	35.6		59.4		521.5					446.1	108.6	
	Did not develop typhoid	22.4	25.3	20.5					32.2	12.5			24.9	27.2	28.3
		21.7	17.7	20.7					20.3	19.1			25.3	24.3	24.2
		6.0	4.8	7.4					6.7	7.0			6.3	6.3	

Figure 61: Detailed kinetics of the anti-H antibody concentrations (ELISA units/ ml) following challenge (Day 0) with *S.* Typhi for 9 participants, 3 of whom developed typhoid following challenge with 10^3 CFU, 3 of whom developed typhoid following challenge with 10^4 CFU, and 3 or whom did not develop typhoid, in a human challenge model of typhoid disease

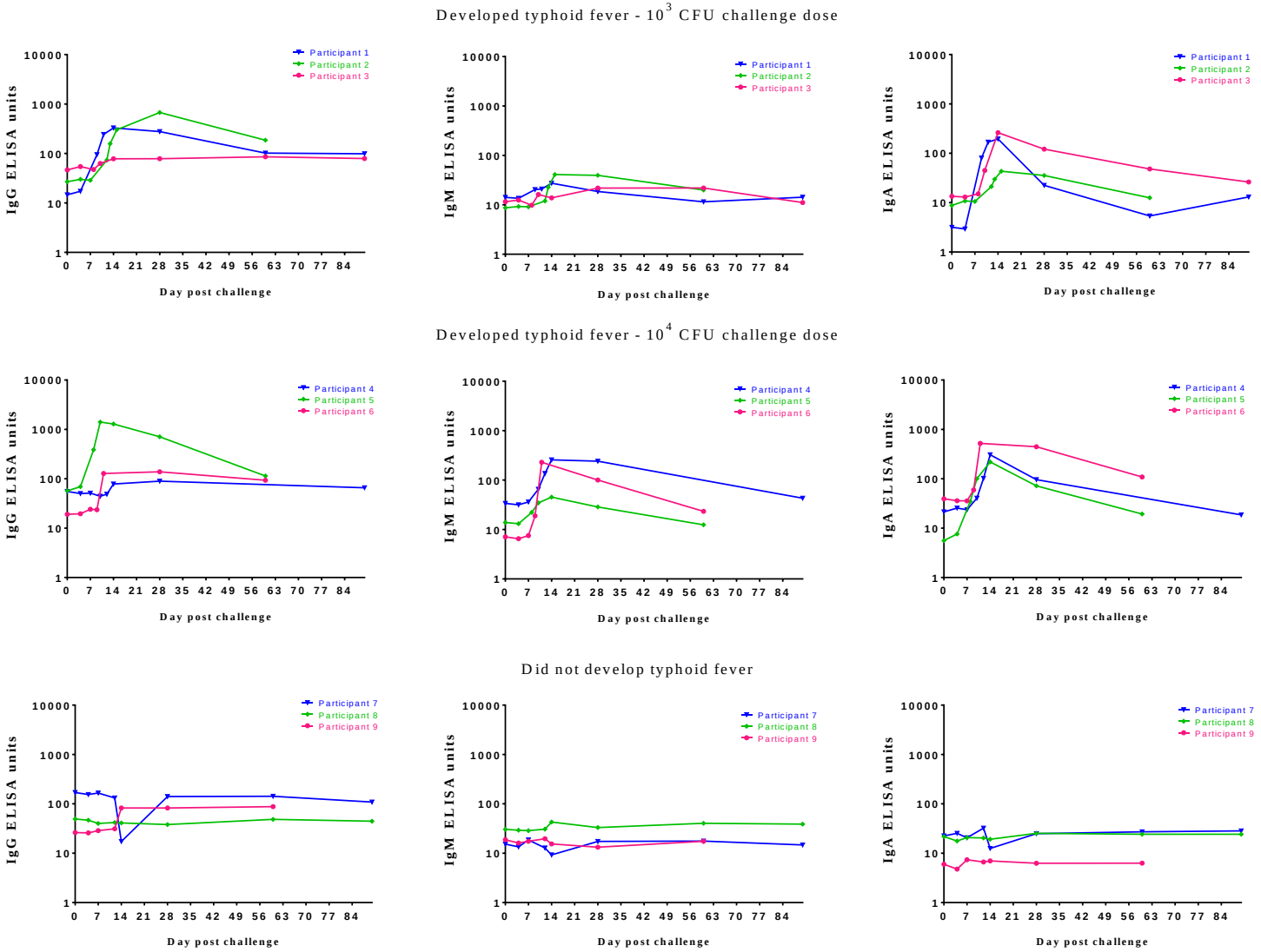


Table 59: Detailed kinetics of the anti-LPS antibody concentrations (ELISA units/ ml) following challenge (Day 0) with *S.* Typhi for 9 participants, 3 of whom developed typhoid following challenge with 10^3 CFU, 3 of whom developed typhoid following challenge with 10^4 CFU, and 3 or whom did not develop typhoid, in a human challenge model of typhoid disease

Antibody	Group	Anti- LPS antibody concentration (ELISA units/ml)													
		Day 0	Day 4	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 28	Day 60	Day 90
IgG	Developed typhoid-10^3	53.8	57.4			510.6		2023.2			3584.8		3588.5	1622.2	1094.4
		31.1	33.1	34.8					103.5	388.6		623.2	658.7	448.4	105.4
		7.3	8.2		8.2		15.0				132.6		169.1	128.8	
	Developed typhoid-10^4	8.3	10.0	40.7			46.1		36.7		101.4		149.2		140.1
		11.0	16.4		930.9		1262.2				844.8		643.0	219.8	
		134.4						310.0					396.5	270.3	
	Did not develop typhoid	46.3	48.5	43.3					44.0	9.3			39.3	42.7	34.6
		36.0	31.4	33.4					32.0	45.6			41.7	42.8	36.8
		11.3	11.6	13.3						13.2			10.8	10.7	
IgM	Developed typhoid-10^3	52.2	55.2			104.0		137.2			201.5		125.3	61.3	49.9
		40.7	39.0	38.7					43.5	68.6		175.7	153.1	82.3	60.2
		31.7	24.9		30.7		148.6				944.6		805.6	222.2	
	Developed typhoid-10^4	144.0	124.0	132.9			301.2		674.4		1144.3		1400.9		434.8
		37.7	43.9		135.1		208.8				340.9		202.4	87.4	
		197.2	192.2	232.4		327.0		1674.1					1503.1	377.1	
	Did not develop typhoid	90.9	86.3	83.7					88.5	58.9			77.7	88.7	107.5
		219.0	215.1	221.5					220.0	217.5			243.6	253.3	249.1
		65.2	68.6	64.5						60.9			61.2	58.9	
IgA	Developed typhoid-10^3	39.4	63.6			226.7		705.4			1706.0		626.9	132.7	125.1
		32.5	27.5	39.5					55.8	256.6		450.3	368.7	115.2	79.3
		13.2	11.0		11.0		10.9				406.6		127.8	21.8	
	Developed typhoid-10^4	21.4	25.5	23.6			139.2		1613.1		3982.8		1777.3		109.6
		13.1	12.9		823.6		1329.9				1478.7		293.8	60.1	
		41.3						935.3					628.7	98.5	
	Did not develop typhoid	20.5	19.0	17.4					20.7	70.4			16.9	17.1	20.0
		354.3	338.3	355.6					316.9	323.6			363.3	377.1	419.3
		13.2	11.0	11.0						10.9			12.3	12.9	

Figure 62: Detailed kinetics of the anti-LPS antibody concentrations (ELISA units/ ml) following challenge (Day 0) with *S.* Typhi for 9 participants, 3 of whom developed typhoid disease following challenge with 10^3 CFU, 3 of whom developed typhoid disease following 10^4 CFU, and 3 or whom did not develop typhoid, in a human challenge model of typhoid disease

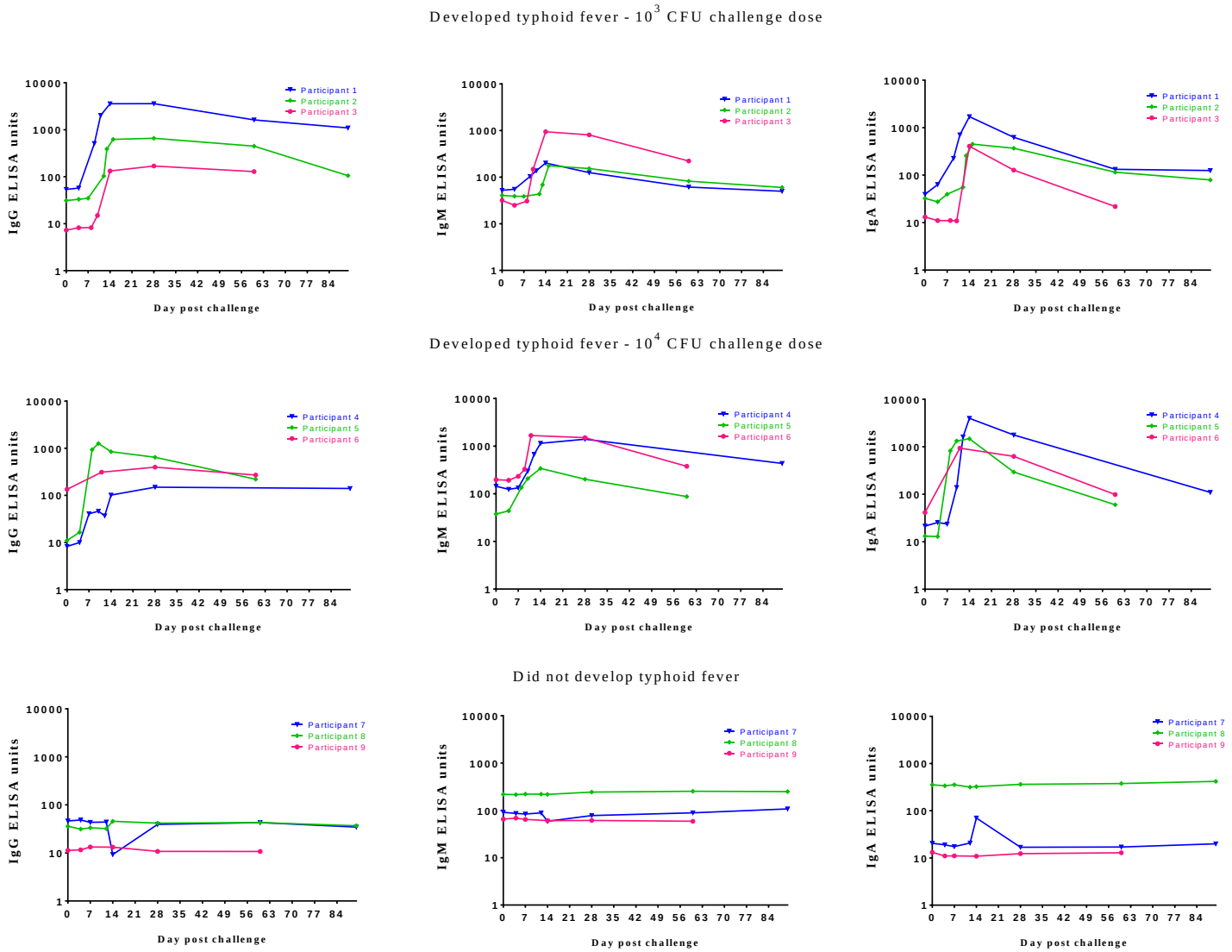
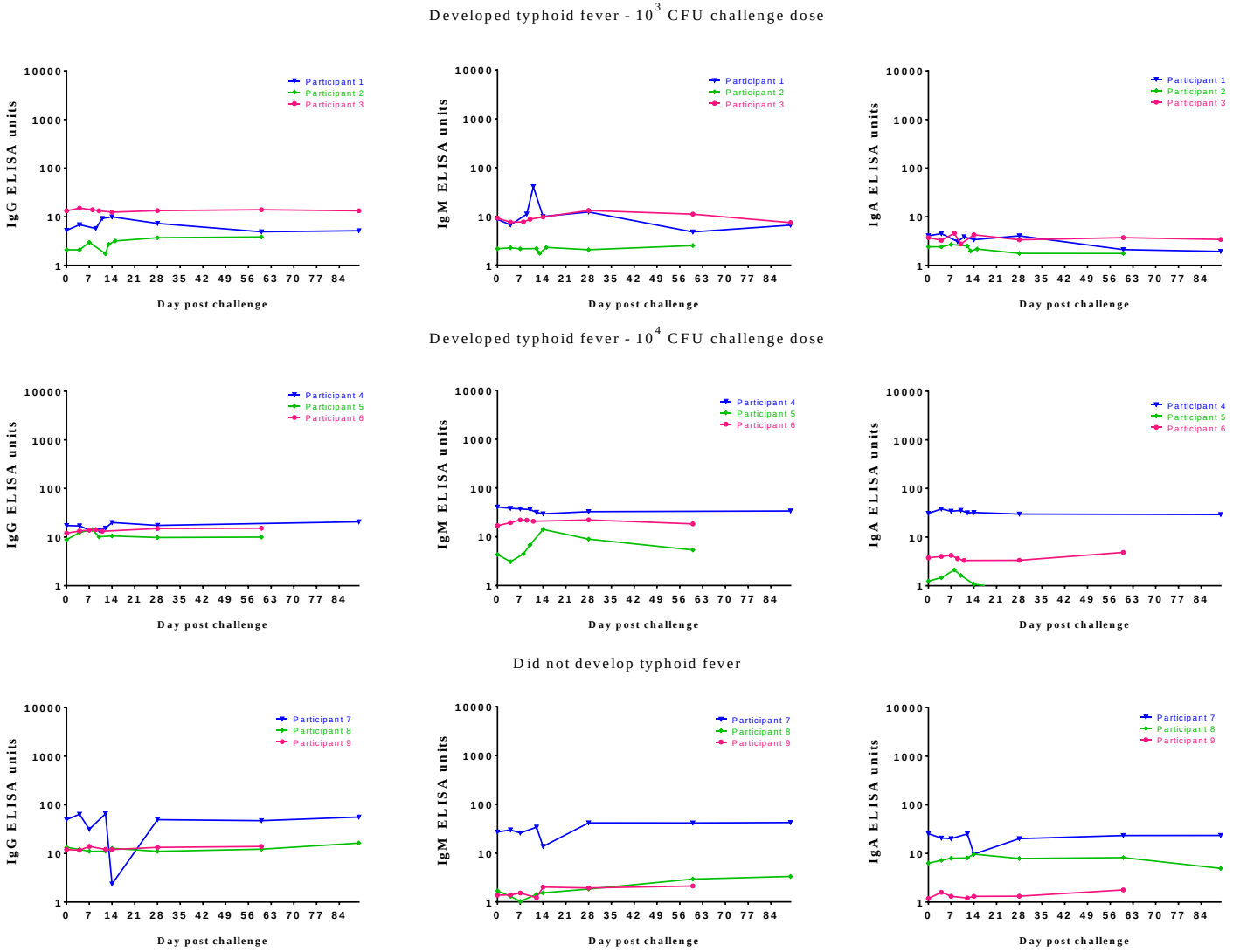


Table 60: Detailed kinetics of the anti-Vi antibody concentrations (ELISA units/ ml) following challenge (Day 0) with *S.* Typhi for 9 participants, 3 of whom developed typhoid following challenge with 10^3 CFU, 3 of whom developed typhoid following challenge with 10^4 CFU, and 3 or whom did not develop typhoid, in a human challenge model of typhoid disease

Antibody	Group	Anti –Vi antibody concentration (ELISA units/ml)													
		Day 0	Day 4	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 28	Day 60	Day 90
IgG	Developed typhoid-10^3	5.3	6.9			5.7		9.2			9.9		7.3	4.9	5.2
		2.1	2.1	3.0					1.7	2.7		3.2	3.7	3.8	7.2
		13.2	15.0		13.9		13.2				12.4		13.4	13.9	13.2
	Developed typhoid-10^4	17.3	17.0	14.0			14.0		15.3		20.0		17.4		20.7
		8.9	12.4		14.3		10.2				10.6		9.8	10.0	
		12.1	13.4	13.9		14.2		13.2					15.0	15.3	
	Did not develop typhoid	49.6	64.0	31.4					65.4	2.4			49.4	47.3	56.0
		13.4	12.2	11.0					11.1	12.8			11.1	12.3	16.4
		12.1	11.7	13.9					12.1	12.1			13.4	13.9	
IgM	Developed typhoid-10^3	8.7	6.7			11.1		40.6			9.9		12.3	4.8	6.6
		2.2	2.3	2.2					2.2	1.8		2.3	2.1	2.5	4.2
		9.2	7.6		7.7		8.7				9.7		13.2	11.1	7.5
	Developed typhoid-10^4	40.7	38.3	37.2			36.1		31.7		29.6		32.7		33.8
		4.3	3.1		4.5		6.8				14.2		9.0	5.4	
		16.9	19.5	22.1		21.8		20.8					22.2	18.4	
	Did not develop typhoid	27.2	29.9	25.9					34.0	13.7			41.6	41.6	42.4
		1.7	1.3	1.0					1.4	1.5			1.8	2.9	3.3
		1.4	1.4	1.5					1.2	2.0			1.9	2.1	
IgA	Developed typhoid-10^3	4.0	4.5			3.1		3.9			3.4		4.0	2.1	1.9
		2.4	2.4	2.7					2.5	2.0		2.2	1.8	1.8	2.0
		3.7	3.3		4.6		2.8				4.3		3.4	3.7	3.4
	Developed typhoid-10^4	30.9	37.8	34.1			35.4		31.6		32.0		29.8		29.1
		1.2	1.5		2.1		1.6				1.1		0.8	1.0	
		3.7	4.0	4.2		3.6		3.3					3.3	4.8	
	Did not develop typhoid	25.5	20.8	20.2					25.3	9.7			20.2	23.4	23.5
		6.3	7.2	8.0					8.1	9.7			7.9	8.2	4.9
		1.2	1.6	1.3					1.2	1.3			1.3	1.8	

Figure 63: Detailed kinetics of the anti-Vi antibody concentrations (ELISA units/ ml) following challenge (Day 0) with *S.* Typhi for 9 participants, 3 of whom developed typhoid following challenge with 10^3 CFU, 3 of whom developed typhoid following challenge with 10^4 CFU, and 3 or whom did not develop typhoid disease, in a human challenge model of typhoid disease



Antibody to the H and LPS antigens rose from day 4, and preceded the diagnosis of typhoid disease. Peak levels were observed at either day 14 or day 28. There was little variation between values observed at day 60 and day 90 post-challenge. The responses to the Vi antigen were minimal in all participants. On the basis of these results, remaining ELISA assays were performed at days 0 (baseline), 14, 28 and 60 post-challenge.

8.3.2. Antibody responses to the H, LPS and Vi antigens in 40 participants challenged with *S.* Typhi Quailes strain

The antibody responses to the H antigen of participants challenged with *S*. Typhi are shown in Table 61. The antibody response to the LPS antigen is shown in Table 62 and to the Vi antigen in Table 63. Participants were grouped according to challenge dose received (10^3 CFU of 10^4 CFU) and if typhoid disease was diagnosed post challenge or not.

Table 61: The geometric mean IgG, IgM and IgA response, with 95% CI, to the H antigen at baseline, day 14, day 28 and day 60 in 40 participants who did and did not develop typhoid disease following challenge with either 10^3 or 10^4 CFU of *S.* Typhi in a human challenge model of typhoid disease

Antibody	Time point	Geometric mean antibody response to the H antigen (95% CI)			
		Developed typhoid disease		Did not develop typhoid disease	
		10^3 CFU challenge dose (n=11)	10^4 CFU challenge dose (n=13)	10^3 CFU challenge dose (n=9)	10^4 CFU challenge dose (n=7)
IgG	Baseline	27.76 (15.48-49.78)	38.83 (24.94-60.46)	46.19 (27.44-77.73)	33.35 (22.99-48.39)
	Day 14	117.8 (53.87-257.6)	335.3 (128.4-875.4)	52.23 (32.12-84.94)	41.96 (26.95-65.31)
	Day 28	150.6 (82.61-274.7)	286.5 (123.2-665.8)	61.79 (34.64-110.2)	44.74 (28.62-69.94)
	Day 60	81.94 (48.80-137.6)	219.8 (88.19-547.8)	70.30 (29.41-168.1)	43.55 (26.92-70.46)
IgM	Baseline	15.55 (11.42-21.18)	19.22 (12.26-30.14)	22.36 (13.69-36.54)	16.22 (8.213-32.05)
	Day 14	50.47 (22.67-112.4)	170.1 (88.61-326.7)	28.58 (16.80-48.62)	16.90 (10.68-26.74)
	Day 28	83.87 (31.37-224.2)	127.7 (70.11-232.5)	36.93 (17.71-77.03)	26.31 (11.05-62.65)
	Day 60	49.51 (17.30-141.7)	40.21 (19.99-80.88)	33.03 (16.47-66.26)	26.08 (16.39-41.49)
IgA	Baseline	7.295 (4.794-11.10)	8.959 (5.702-14.08)	9.577 (5.104-17.97)	8.351 (2.057-33.91)
	Day 14	90.26 (35.17-231.6)	192.3 (86.96-425.3)	11.70 (7.188-19.03)	11.14 (3.414-36.37)
	Day 28	46.64 (22.10-98.41)	61.83 (27.13-140.9)	16.24 (9.938-26.53)	13.63 (4.261-43.62)
	Day 60	13.84 (6.842-28.00)	18.94 (9.916-36.16)	12.70 (5.253-30.70)	11.92 (3.740-37.99)

Table 62: The geometric mean IgG, IgM and IgA response, with 95% CI, to the LPS antigen at baseline, day 14, day 28 and day 60 in 40 participants who did and did not develop typhoid disease following challenge with either 10^3 or 10^4 CFU of *S.* Typhi in a human challenge model of typhoid disease

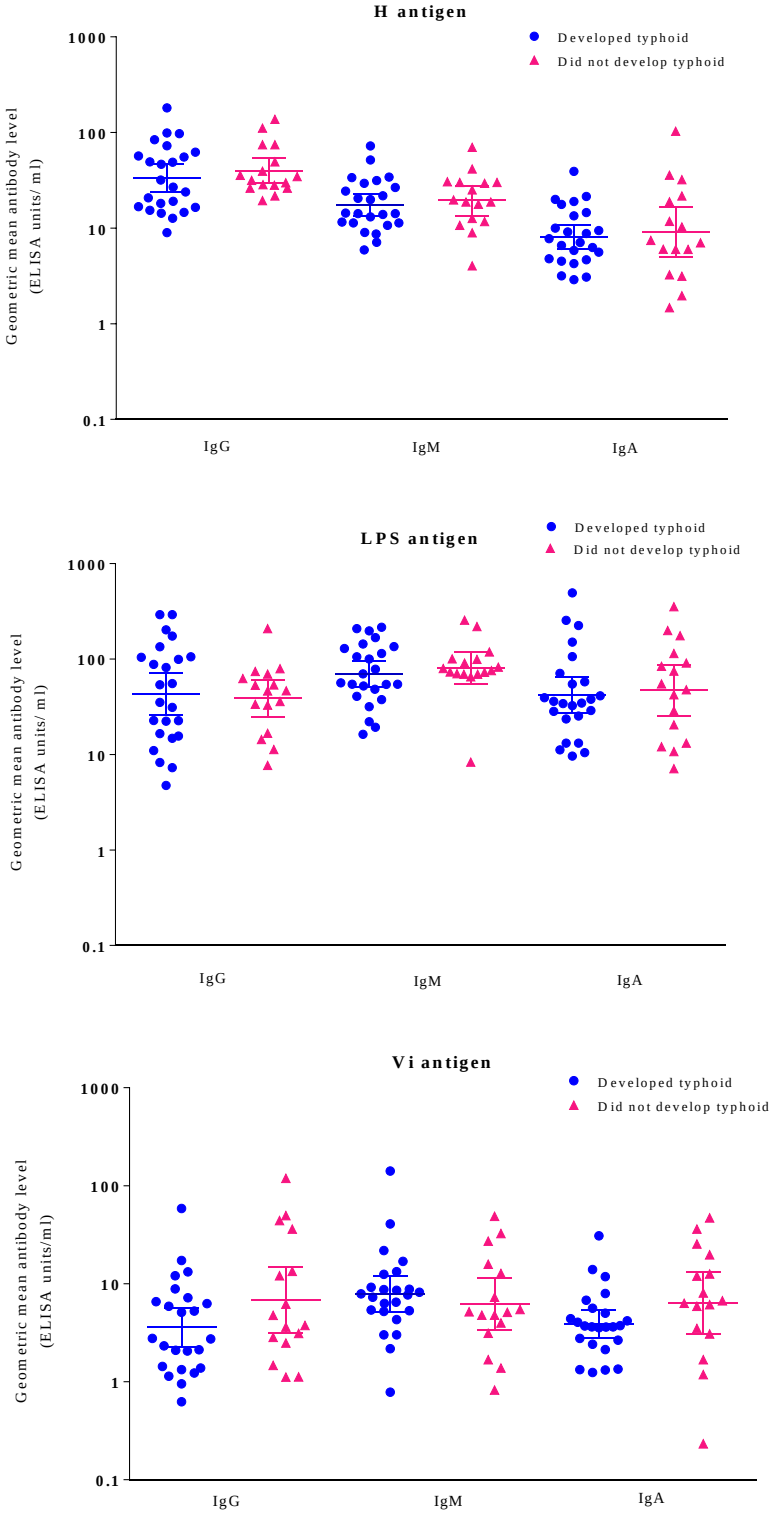
Antibody	Time point	Geometric mean antibody response to the LPS antigen (95% CI)			
		Developed typhoid disease		Did not develop typhoid disease	
		10^3 CFU challenge dose (n=11)	10^4 CFU challenge dose (n=13)	10^3 CFU challenge dose (n=9)	10^4 CFU challenge dose (n=7)
IgG	Baseline	33.71 (13.58-83.70)	52.36 (27.32-100.4)	34.57 (16.48-72.49)	45.16 (24.04-84.82)
	Day 14	235.4 (65.76-842.4)	590.0 (277.6-1254)	30.76 (13.88-68.17)	48.02 (27.45-84.01)
	Day 28	329.8 (114.5-950.4)	580.2 (324.3-1038)	55.95 (22.80-137.3)	47.40 (25.21-89.13)
	Day 60	237.5 (71.66-787.3)	439.4 (216.4-892.0)	70.53 (25.93-191.9)	49.11 (25.63-94.07)
IgM	Baseline	51.39 (32.85-80.40)	90.90 (57.89-142.7)	107.1 (73.92-155.1)	55.32 (25.30-121.0)
	Day 14	214.2 (90.83-505.1)	581.7 (304.8-1110)	111.9 (58.62-213.4)	61.36 (43.80-85.94)
	Day 28	392.6 (191.6-804.4)	541.0 (323.9-903.5)	154.7 (68.93-347.2)	95.44 (45.88-198.6)
	Day 60	172.7 (73.42-406.3)	202.7 (141.7-290.0)	156.1 (78.68-309.7)	75.45 (56.31-101.1)
IgA	Baseline	39.50 (19.28-80.92)	43.89 (23.29-82.73)	60.41 (26.30-138.8)	34.63 (10.96-109.5)
	Day 14	424.9 (146.5-1232)	994.2 (510.2-1937)	88.24 (48.59-160.3)	40.96 (15.11-111.0)
	Day 28	329.4 (159.2-681.6)	484.6 (243.3-965.3)	69.79 (34.01-143.2)	52.75 (17.17-162.1)
	Day 60	102.8 (42.81-246.8)	131.8 (66.01-263.3)	44.00 (6.796-284.8)	37.66 (13.83-102.5)

Table 63: The geometric mean IgG, IgM and IgA response, with 95% CI, to the Vi antigen at baseline, day 14, day 28 and day 60 in 40 participants who did and did not develop typhoid disease, following challenge with either 10^3 or 10^4 CFU of *S.* Typhi in a human challenge model of typhoid disease

Antibody	Time point	Geometric mean antibody response to the Vi antigen (95% CI)			
		Developed typhoid disease		Did not develop typhoid disease	
		10^3 CFU challenge dose (n=11)	10^4 CFU challenge dose (n=13)	10^3 CFU challenge dose (n=9)	10^4 CFU challenge dose (n=7)
IgG	Baseline	2.979 (1.574-5.640)	4.241 (2.048-8.779)	9.640 (2.331-39.87)	4.440 (2.703-7.293)
	Day 14	3.175 (1.421-7.094)	4.743 (2.497-9.009)	5.378 (1.621-17.84)	4.878 (3.063-7.769)
	Day 28	3.551 (1.681-7.505)	5.036 (2.475-10.25)	8.600 (1.873-39.50)	5.062 (3.120-8.213)
	Day 60	3.198 (1.395-7.335)	4.997 (2.358-10.59)	15.53 (2.364-102.0)	5.500 (3.093-9.780)
IgM	Baseline	8.958 (4.290-18.70)	7.037 (3.989-12.41)	9.356 (3.883-22.54)	3.677 (1.500-9.012)
	Day 14	11.48 (5.429-24.26)	9.531 (5.465-16.62)	7.809 (3.393-17.97)	3.930 (1.642-9.403)
	Day 28	10.41 (5.062-21.42)	8.793 (5.747-13.45)	11.20 (4.399-28.51)	4.736 (2.513-8.922)
	Day 60	8.297 (3.551-19.39)	8.758 (5.008-15.32)	16.22 (4.772-55.14)	7.618 (2.708-21.43)
IgA	Baseline	3.975 (2.960-5.338)	3.830 (2.099-6.991)	12.80 (6.378-25.71)	2.620 (0.7446-9.216)
	Day 14	4.888 (3.488-6.850)	4.686 (2.570-8.545)	12.12 (6.436-22.82)	2.948 (1.239-7.012)
	Day 28	4.696 (3.412-6.463)	3.754 (1.993-7.070)	13.21 (6.579-26.51)	3.199 (1.088-9.406)
	Day 60	3.587 (2.461-5.228)	3.918 (2.299-6.676)	15.09 (6.484-35.11)	4.195 (1.526-11.54)

Baseline antibody levels varied between participants, but did not consistently differ between those who did and did not develop typhoid, as shown in Figure 64.

Figure 64: Baseline IgG, IgM and IgA antibody concentrations to H, LPS and Vi antigens in participants who did and did not develop typhoid disease following challenge with *S.* Typhi in the development of a human challenge model



One participant had a much higher baseline anti-Vi IgG antibody level than other participants, and did not develop typhoid disease. However, this participant was

symptomatic and stool culture positive at day 14, and showed a rise in anti—H and anti-LPS antibody levels consistent with that seen in participants developing typhoid disease, and therefore may have gone on to meet the diagnostic threshold with a longer follow up period. There were no other notably outlying baseline values.

Changes in the geometric mean antibody levels following challenge (Day 0) are shown in Figure 65, Figure 66 and Figure 67.

Figure 65: Changes in geometric mean antibody levels to the H antigen following challenge (day 0) in participants challenged with 10^3 or 10^4 CFU *S.* Typhi, shown by those who did (typhoid) and did not (no typhoid) develop typhoid disease

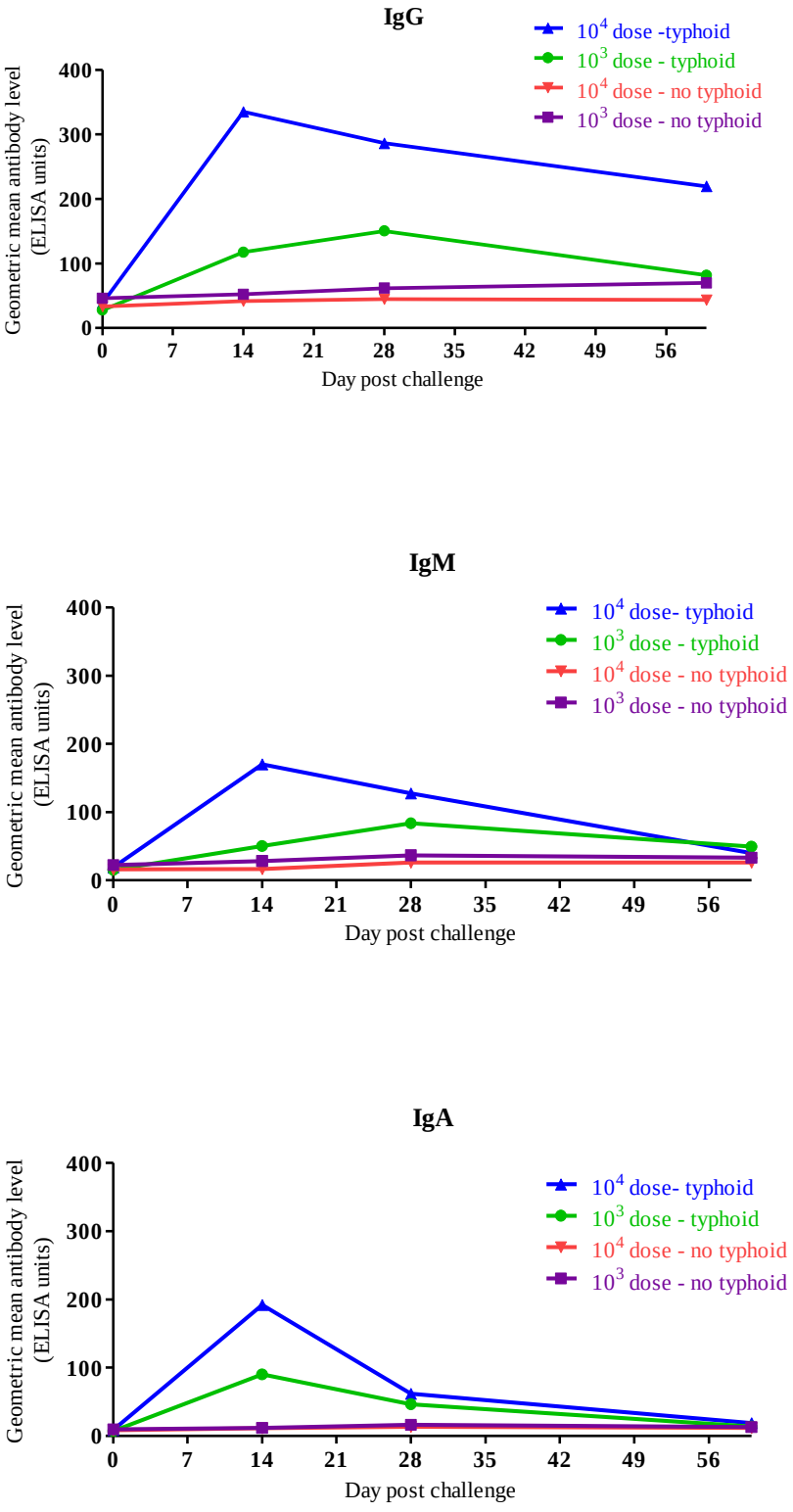


Figure 66: Changes in geometric mean antibody levels to the LPS antigen following challenge (day 0) in participants challenged with 10^3 or 10^4 CFU *S.* Typhi, shown by those who did (typhoid) and did not (no typhoid) develop typhoid disease

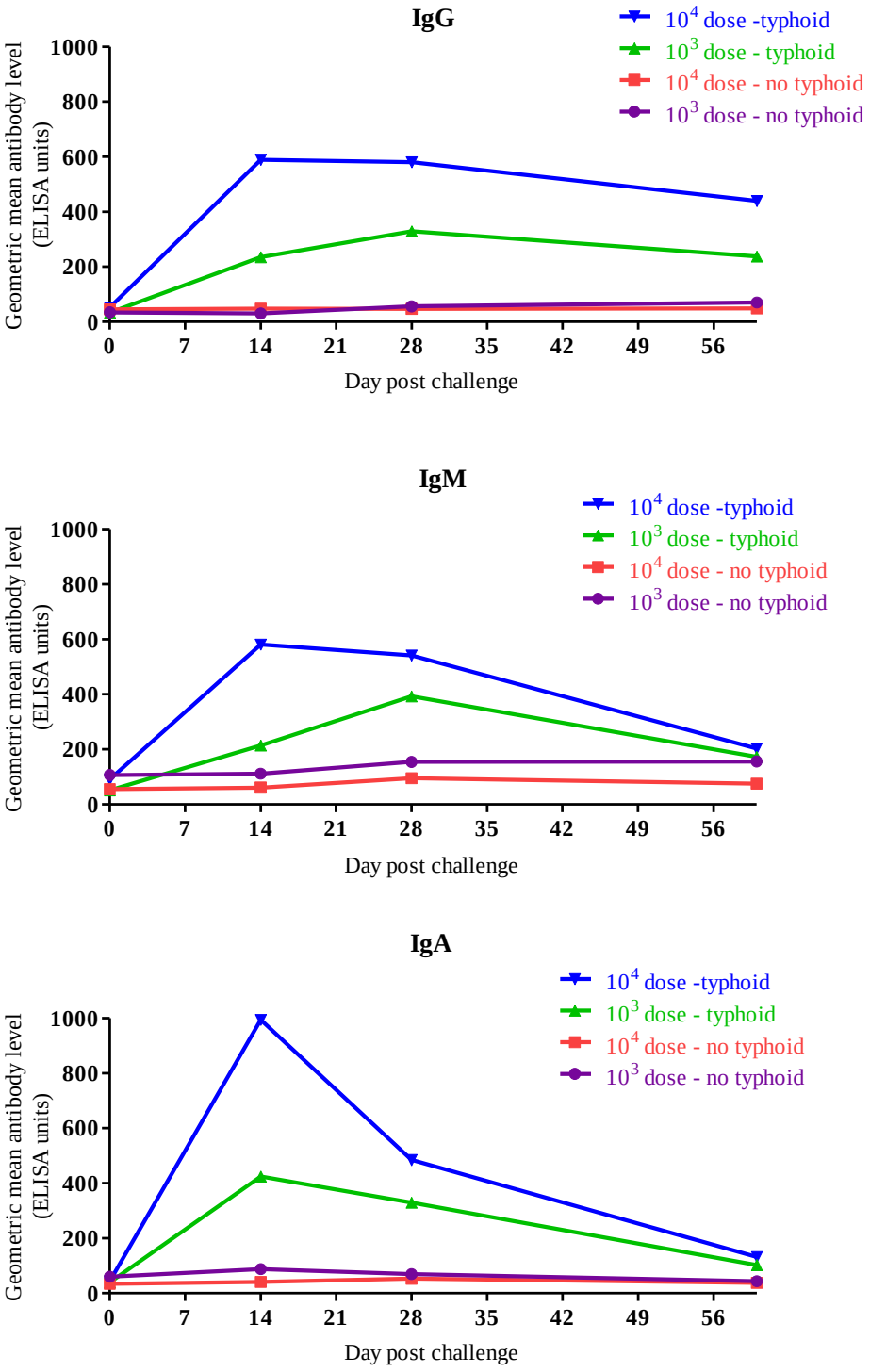
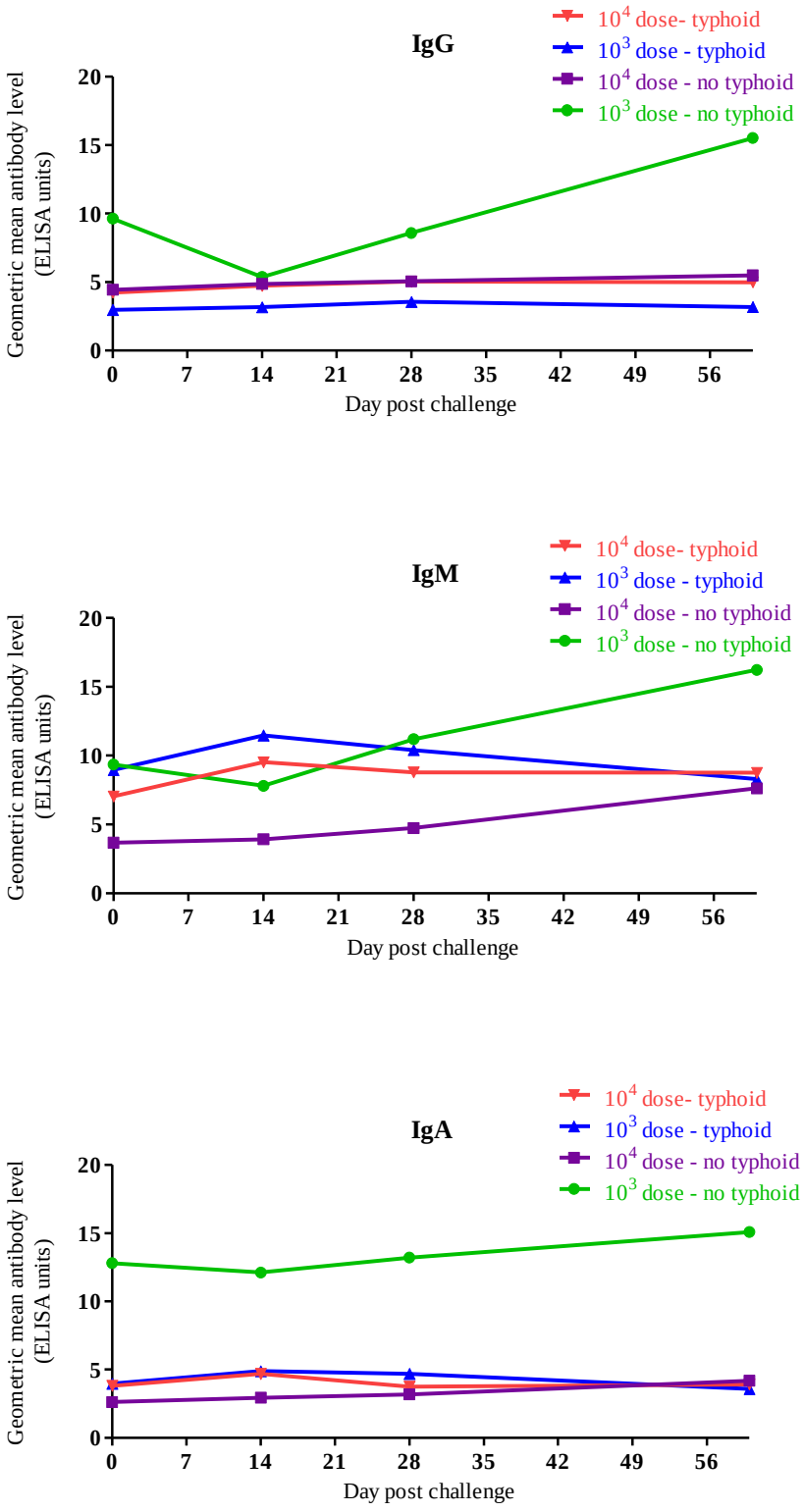


Figure 67: Changes in geometric mean antibody levels to the Vi antigen following challenge (day 0) in participants challenged with 10^3 or 10^4 CFU *S.* Typhi, shown by those who did (typhoid) and did not (no typhoid) develop typhoid disease



In those who developed typhoid disease, there was a marked rise in all antibody classes to the H and LPS antigens, in contrast to those who did not develop typhoid disease, in whom responses were not demonstrated. There was no demonstrable response to the Vi antigen. The higher Vi antibody levels in the group who did not develop typhoid following challenge with the 10^3 CFU dose is explained by one participant who had much higher baseline IgG and IgA anti-Vi antibody levels that also increased by day 60.

The IgG and IgM antibody levels to the H and LPS antigens peaked earlier (day 14) in those receiving 10^4 CFU, compared to those receiving 10^3 CFU (day 28). The IgG response to these antigens is also longer lasting with the higher challenge dose compared to the lower challenge dose. There was a dose-response relationship to the anti-H and anti-LPS IgG, and IgA response, shown by the higher maximum fold rises over baseline (Table 64) in those receiving a challenge dose of 10^4 CFU compared to those who were challenged with 10^3 CFU. The IgA response shows both the highest maximum fold rise, and the greatest dose response relationship. IgM fold rises were approximately equal for the 2 challenge doses. These findings suggest a greater antigenic stimulus following challenge with the higher challenge dose compared to the lower challenge dose.

Table 64: Maximum fold rises over baseline for anti-H and anti- LPS antibody in participants who developed typhoid disease following challenge with either 10^3 CFU or 10^4 CFU of *S.* Typhi Quailes strain in the development of a human challenge model of typhoid disease

Challenge dose (CFU of *S.* Typhi)	Maximum fold rise over baseline					
	Anti- H antibody			Anti- LPS antibody		
	IgG	IgM	IgA	IgG	IgM	IgA
10^3 (n=11)	5.4	5.4	12.4	9.8	7.6	10.8
10^4 (n=13)	8.6	6.6	21.5	11.3	6.4	22.7

The proportion of participants demonstrating a fourfold rise over baseline in antibody level to the H, LPS and Vi antigens is shown in Table 65, Table 66, and Table 67 respectively.

Table 65: Proportion of participants demonstrating a fourfold rise in antibody level to the H antigen over baseline by either day 14 or day 28 following challenge at Day 0 with either 10^3 or 10^4 CFU of *S.* Typhi, shown by those who did (typhoid) and did not (no typhoid) develop typhoid disease

Antibody	Time point	Proportion of participants with a fourfold rise in antibody level over baseline (%)			
		Developed typhoid disease		Did not develop typhoid disease	
		10^3 CFU challenge dose (n=11)	10^4 CFU challenge dose (n=13)	10^3 CFU challenge dose (n=9)	10^4 CFU challenge dose (n=7)
IgG	Day 14	5/11 (45.5)	10/13 (76.9)	0/9 (0.0)	0/7 (0.0)
	Day 28	7/11 (63.6)	10/13 (76.9)	0/9 (0.0)	0/7 (0.0)
	Any time point	7/11 (63.6)	11/13 (84.6)	0/9 (0.0)	0/7 (0.0)
IgM	Day 14	4/11 (36.4)	7/13 (53.8)	1/9 (11.1)	0/7 (0.0)
	Day 28	6/11 (54.5)	8/13 (61.5)	1/9 (11.1)	1/7 (14.3)
	Any time point	7/11 (63.6)	8/13 (61.5)	1/9 (11.1)	1/7 (14.3)
IgA	Day 14	8/11 (72.7)	12/13 (92.3)	1/9 (11.1)	1/7 (14.3)
	Day 28	8/11 (72.7)	9/13 (69.2)	1/9 (11.1)	1/7 (14.3)
	Any time point	9/11 (81.8)	12/13 (92.3)	1/9 (11.1)	1/7 (14.3)

Table 66: Proportion of participants demonstrating a fourfold rise in antibody level to the LPS antigen over baseline by either day 14 or day 28 following challenge at Day 0 with either 10^3 or 10^4 CFU of *S.* Typhi, shown by those who did (typhoid) and did not (no typhoid) develop typhoid disease

Antibody	Time point	Proportion of participants with a fourfold rise in antibody level over baseline (%)			
		Developed typhoid disease		Did not develop typhoid disease	
		10^3 CFU challenge dose (n=11)	10^4 CFU challenge dose (n=13)	10^3 CFU challenge dose (n=9)	10^4 CFU challenge dose (n=7)
IgG	Day 14	6/11 (54.5)	10/13 (76.9)	0/9 (0.0)	0/7 (0.0)
	Day 28	7/11 (63.6)	10/13 (76.9)	1/9 (11.1)	0/7 (0.0)
	Any time point	7/11 (63.6)	11/13 (84.6)	1/9 (11.1)	0/7 (0.0)
IgM	Day 14	6/11 (54.5)	8/13 (61.5)	1/9 (11.1)	0/7 (0.0)
	Day 28	7/11 (63.6)	8/13 (61.5)	1/9 (11.1)	1/7 (14.3)
	Any time point	8/11 (72.7)	9/13 (69.2)	1/9 (11.1)	1/7 (14.3)
IgA	Day 14	7/11 (63.6)	11/13 (84.6)	1/9 (11.1)	1/7 (14.3)
	Day 28	8/11 (72.7)	9/13 (69.2)	1/9 (11.1)	1/7 (14.3)
	Any time point	9/11 (81.8)	11/13 (84.6)	2/9 (22.2)	1/7 (14.3)

Table 67: Proportion of participants demonstrating a fourfold rise in antibody level to the Vi antigen over baseline by either day 14 or day 28 following challenge at Day 0 with either 10^3 or 10^4 CFU of *S.* Typhi, shown by those who did (typhoid) and did not (no typhoid) develop typhoid disease

Antibody	Time point	Proportion of participants with a fourfold rise in antibody level over baseline			
		Developed typhoid disease		Did not develop typhoid disease	
		10^3 CFU challenge dose (n=11)	10^4 CFU challenge dose (n=13)	10^3 CFU challenge dose (n=9)	10^4 CFU challenge dose (n=7)
IgG	Day 14	0/11	0/13	0/9	0/7
	Day 28	0/11	0/13	0/9	0/7
	Any time point	0/11	0/13	0/9	0/7
IgM	Day 14	1/11	0/13	0/9	0/7
	Day 28	0/11	0/13	0/9	0/7
	Any time point	1/11	0/13	0/9	0/7
IgA	Day 14	0/11	0/13	0/9	0/7
	Day 28	0/11	0/13	0/9	0/7
	Any time point	0/11	0/13	0/9	0/7

Fourfold rise in antibody levels to the H and LPS antigens occurred in the majority of participants developing typhoid disease, but very few of these participants had a fourfold rise in Vi antibody levels. The proportion demonstrating a fourfold rise in anti-H and anti-LPS antibodies was slightly higher at day 28 for those challenged with 10^3 CFU compared to day 14 except for the IgA response (Figure 68 and Figure 69). For those challenged with 10^4 CFU of *S.* Typhi, the proportion of participants with a fourfold rise was approximately

the same at day 14 and day 28, except for IgA responses which were higher at day 14. A four-fold rise in the IgA responses to the H antigen was the most sensitive marker of diagnosis, with 83% of participants diagnosed with typhoid disease demonstrating a four-fold rise by day 14. The one participant who did not develop typhoid disease but demonstrated a four-fold rise in IgM and IgA was symptomatic and stool culture positive at day 14, and may have gone on to be diagnosed with typhoid disease had they not been started on antibiotics as per protocol. Hence, a fourfold rise in anti-H antibody was also highly specific as a diagnostic threshold. Similarly, a four-fold rise in the IgA response to the LPS antigen was the most sensitive of the LPS responses in identifying those with typhoid disease, with 75% of participants having a fourfold rise by day 14. There was however no antibody threshold that would have correctly diagnosed people with typhoid disease based on a single assay.

Figure 68: The percentage of participants demonstrating a fourfold rise in antibody level to the H antigen over baseline by either day 14 or day 28 following challenge at Day 0 with either 10^3 or 10^4 CFU of *S.* Typhi, shown by those who did (typhoid) and did not (no typhoid) develop typhoid disease

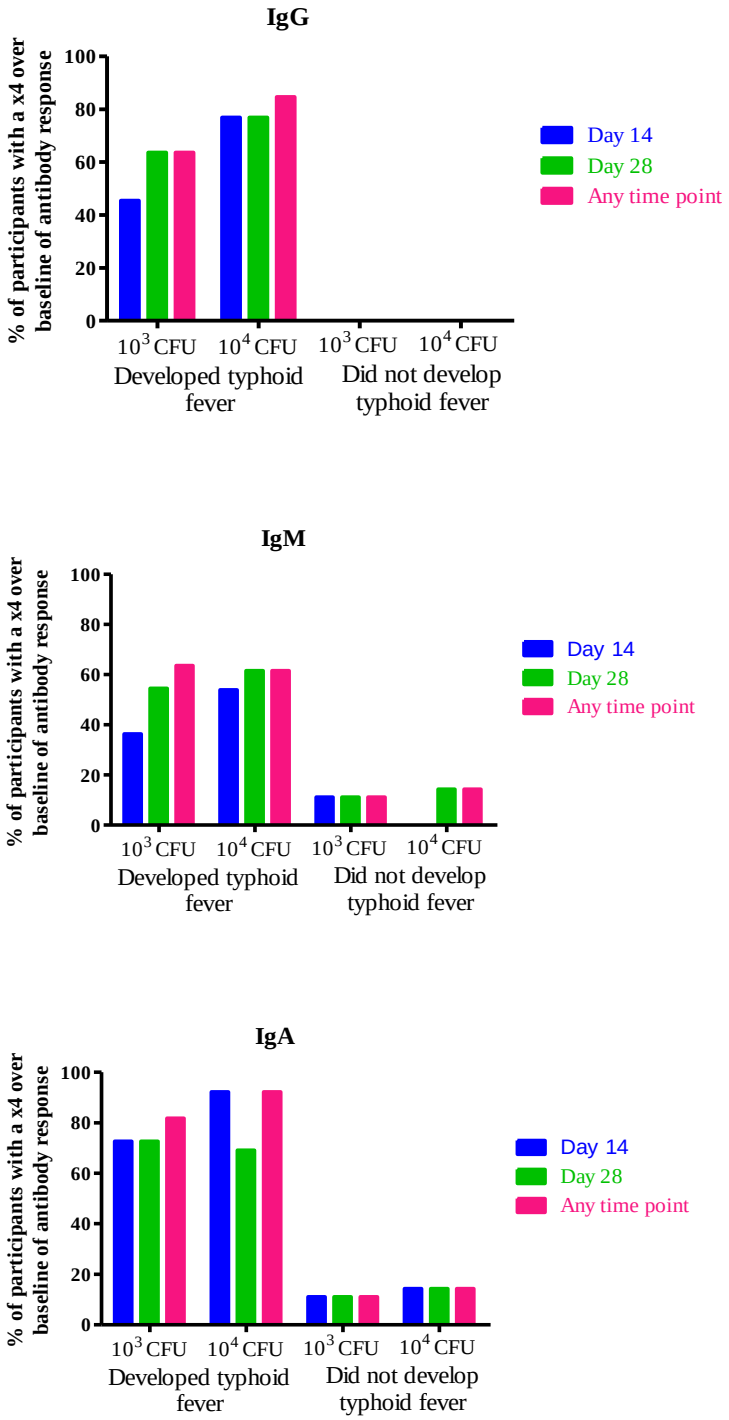
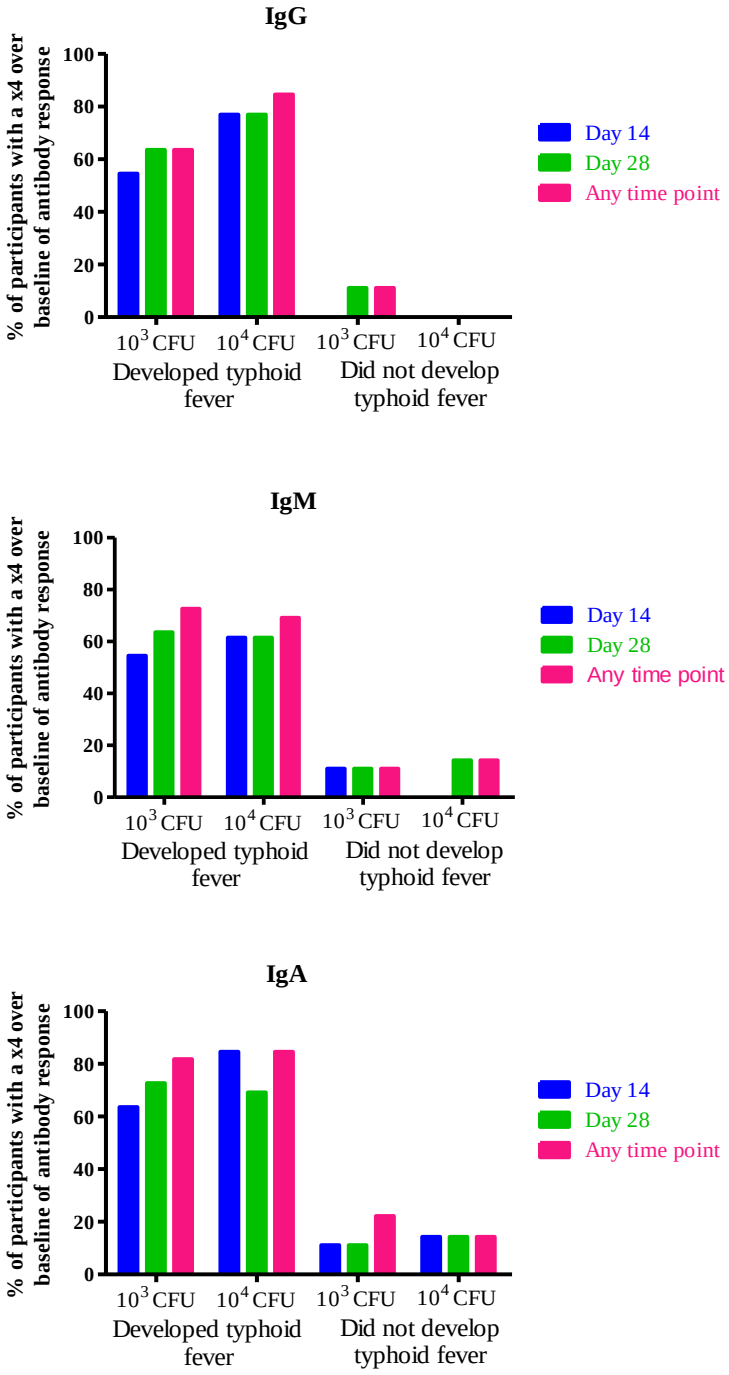


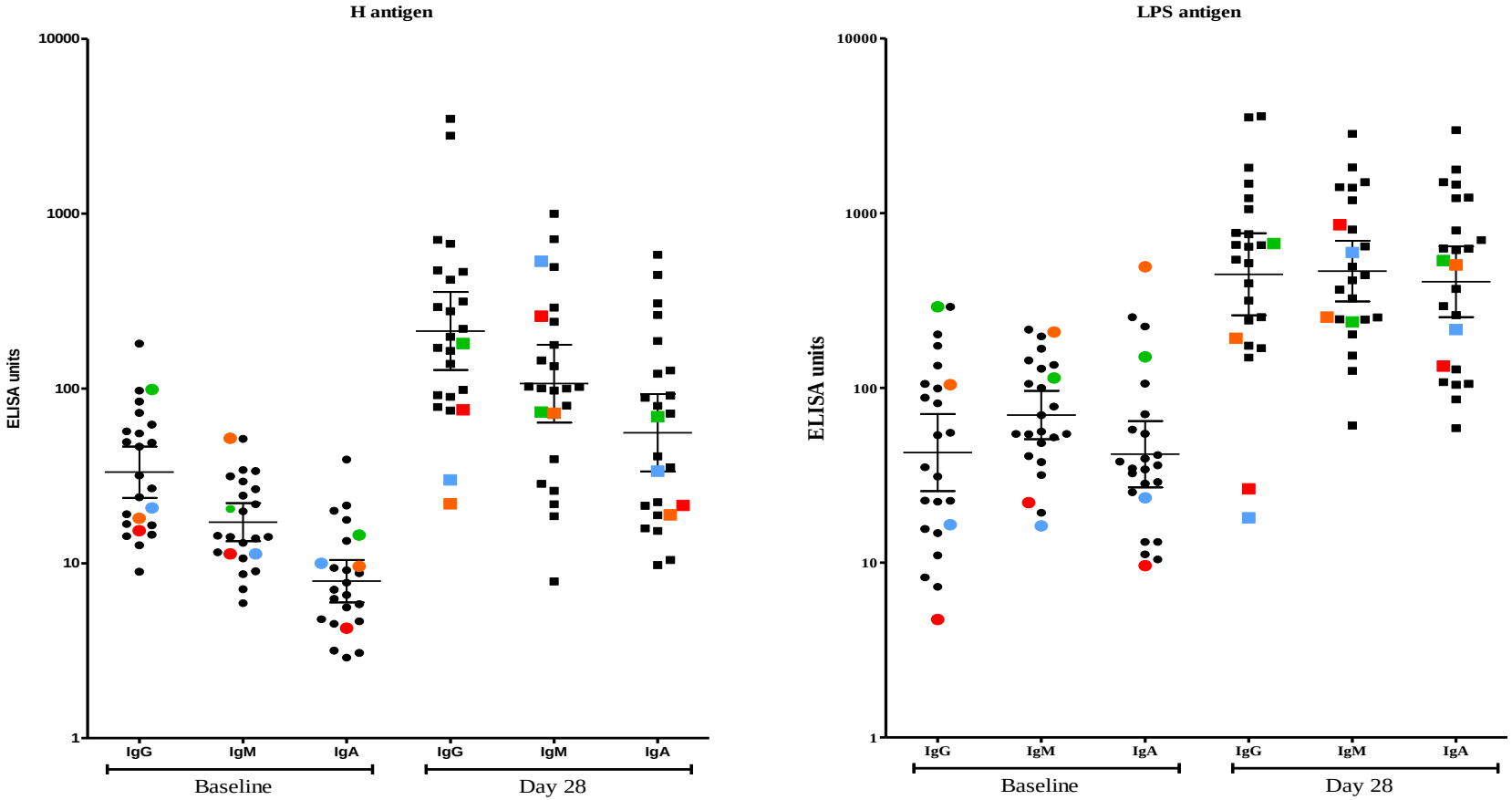
Figure 69: The percentage of participants demonstrating a fourfold rise in antibody level to the LPS antigen over baseline by either day 14 or day 28 following challenge at Day 0 with either 10^3 or 10^4 CFU of *S.* Typhi, shown by those who did (typhoid) and did not (no typhoid) develop typhoid disease



8.3.3. Antibody responses in asymptomatic *S.* Typhi bacteraemia

Four participants with *S*. Typhi bacteraemia did not mount a concurrent febrile response (as described in 7.3.4). The antibody response of these participants to the LPS and H antigens of *S*. Typhi are shown in Figure 70. Participant 0005 (green symbols) had a high baseline level of anti-H and anti-LPS IgG and IgA that rose only modestly by 28 days after challenge. Participant 0040 (orange symbols) had a high baseline anti-H IgM level and anti-LPS IgM and IgA level, and very little response by 28 days after challenge. Participant 0020 (blue symbols) who was the least symptomatic of the 4 had low or normal antibody levels to both antigens, and demonstrated little IgG increase by day 28, but a normal IgM and IgA response. Participant 0014 (red symbols) also had low- normal baseline antibody levels to the H antigen, and low antibody to the LPS antigen, all of which were boosted in response to infection.

Figure 70: Class antibody responses to the H and LPS antigens of *S.* Typhi at baseline and day 28 for 4 participants in whom bacteraemia was detected in the absence of a febrile response. Results for participants 0005 (green), 0014 (red), 0020 (blue) and 0040 (orange) are shown relative to other participants challenged with *S.* Typhi (black). The geometric mean and 95% confidence interval for all participants is shown.



8.3.4. Plasma cell responses to the H, LPS and Vi antigens in all challenged participants

The geometric mean cell counts and 95% confidence intervals for cells responding to the H antigen are shown in Table 68, to LPS antigen in Table 69, and to the Vi antigen in Table 70. Data points were limited by the blood volume required, and the time required to perform the assay meaning that it could not performed in all those with typhoid disease, and hence the confidence intervals are large.

Table 68: The IgG, IgM and IgA plasma cell response to the H antigen at baseline, day 7 and day 9 after challenge, and 48 hours after typhoid disease was diagnosed (where applicable) in 40 participants who did or did not develop typhoid disease following challenge with either 10^3 or 10^4 CFU of *S.* Typhi

Antibody	Time point		Geometric mean plasma cell count/10^6 PBMC to the H antigen (95% CI)			
			Developed typhoid disease		Did not develop typhoid disease	
			10^3 CFU challenge dose (n=11)	10^4 CFU challenge dose (n=13)	10^3 CFU challenge dose (n=9)	10^4 CFU challenge dose (n=7)
IgG	Baseline	Number of samples	11	9	7	11
		Geometric mean cell count (95% CI)	1.0 (0.4-2.5)	0.8 (0.4-1.4)	0.8 (0.3-2.3)	0.4 (0.2-0.7)
	Day 7	Number of samples	7	8	9	7
		Geometric mean cell count (95% CI)	1.6 (0.2-10.8)	3.0 (1.2-7.3)	1.0 (0.4-2.6)	1.4 (0.4-4.8)
	Day 9	Number of samples	5	4	9	5
		Geometric mean cell count (95% CI)	0.8 (0.2-3.2)	2.6 (0.1-60.8)	0.6 (0.2-1.6)	2.9 (1.9-4.4)
	TD 48	Number of samples	11	9		
		Geometric mean cell count (95% CI)	3.1 (0.8-12.3)	5.9 (2.0-17.6)		
IgM	Baseline	Number of samples	11	11	9	7
		Geometric mean cell count (95% CI)	0.5 (0.3-1.0)	1.0 (0.5-2.1)	0.5 (0.3-1.1)	0.7 (0.3-2.1)
	Day 7	Number of samples	7.0	8.0	9.0	7.0
		Geometric mean cell count (95% CI)	2.6 (0.4-17.2)	11.2 (2.8-44.5)	0.4 (0.2-0.7)	1.0 (0.3-2.9)
	Day 9	Number of samples	5.0	4.0	9.0	5.0
		Geometric mean cell count (95% CI)	1.0 (0.1-10.5)	54.7 (6.9-435.3)	1.1 (0.4-2.7)	0.8 (0.1-5.5)
	TD 48	Number of samples	11	9		
		Geometric mean cell count (95% CI)	33.8 (13.2-86.5)	95.8 (39.1-234.3)		
IgA	Baseline	Number of samples	11	11	9	7
		Geometric mean cell count (95% CI)	0.8 (0.4-2.0)	1.6 (0.6-3.9)	0.7 (0.3-1.7)	0.7 (0.1-3.0)
	Day 7	Number of samples	7	8	9	7
		Geometric mean cell count (95% CI)	8.6 (3.2-23.3)	9.6 (5.1-18.2)	1.9 (0.7-5.1)	1.4 (0.4-4.6)
	Day 9	Number of samples	5	4	9	5
		Geometric mean cell count (95% CI)	3.0 (0.3-28.1)	28.9 (6.4-131.1)	2.1 (1.0-4.4)	1.8 (0.4-8.8)
	TD 48	Number of samples	11	9		
		Geometric mean cell count (95% CI)	5.3 (1.5-18.2)	54.4 (29.1-101.6)		

Table 69: The IgG, IgM and IgA plasma cell response to the LPS antigen at baseline, day 7 and day 9 after challenge, and 48 hours after typhoid disease was diagnosed (where applicable) in 40 participants who did or did not develop typhoid disease following challenge with either 10^3 or 10^4 CFU of *S.* Typhi

Antibody	Time point		Geometric mean plasma cell count /10^6 PBMC to the LPS antigen (95% CI)			
			Developed typhoid disease		Did not develop typhoid disease	
			10^3 CFU challenge dose (n=11)	10^4 CFU challenge dose (n=13)	10^3 CFU challenge dose (n=9)	10^4 CFU challenge dose (n=7)
IgG	Baseline	Number of samples	11	11	9	7
		Geometric mean cell count (95% CI)	1.4 (0.5-3.7)	1.3 (0.6-2.9)	0.8 (0.3-2.0)	0.5 (0.2-1.2)
	Day 7	Number of samples	7	8	9	8
		Geometric mean cell count (95% CI)	1.7 (0.2-12.9)	3.6 (1.2-10.6)	0.6 (0.2-1.6)	1.8 (0.8-4.4)
	Day 9	Number of samples	5	4	9	5
		Geometric mean cell count (95% CI)	2.0 (0.2-17.1)	8.3 (0.1-515.0)	0.4 (0.2-0.9)	1.6 (0.9-3.0)
	TD 48	Number of samples	11	9		
		Geometric mean cell count (95% CI)	10.8 (2.9-40.3)	9.7 (1.7-54.0)		
IgM	Baseline	Number of samples	11	11	9	7
		Geometric mean cell count (95% CI)	0.7 (0.3-1.4)	0.8 (0.3-2.3)	0.5 (0.2-1.3)	1.9 (0.7-5.2)
	Day 7	Number of samples	7	8	9	7
		Geometric mean cell count (95% CI)	1.7 (0.3-11.8)	7.8 (2.1-28.5)	0.6 (0.3-1.3)	2.2 (0.8-6.1)
	Day 9	Number of samples	5	4	9	5
		Geometric mean cell count (95% CI)	1.0 (0.2-6.2)	43.8 (2.5-777.4)	0.8 (0.4-1.8)	0.9 (0.2-4.1)
	TD 48	Number of samples	11	9		
		Geometric mean cell count (95% CI)	32.4 (13.2-79.2)	53.9 (18.9-153.5)		
IgA	Baseline	Number of samples	11	11	9	7
		Geometric mean cell count (95% CI)	0.9 (0.3-2.5)	2.9 (0.8-9.8)	1.4 (0.6-3.1)	1.4 (0.3-6.9)
	Day 7	Number of samples	7	8	9	7
		Geometric mean cell count (95% CI)	5.7 (1.0-31.7)	8.7 (4.1-18.6)	1.8 (0.5-6.3)	3.9 (1.9-8.0)
	Day 9	Number of samples	5	4	9	5
		Geometric mean cell count (95% CI)	5.8 (1.1-29.8)	44.5 (5.5-362.5)	1.2 (0.4-3.5)	3.7 (2.2-6.4)
	TD 48	Number of samples	11	9		
		Geometric mean cell count (95% CI)	22.4 (5.8-87.3)	53.7 (18.5-155.6)		

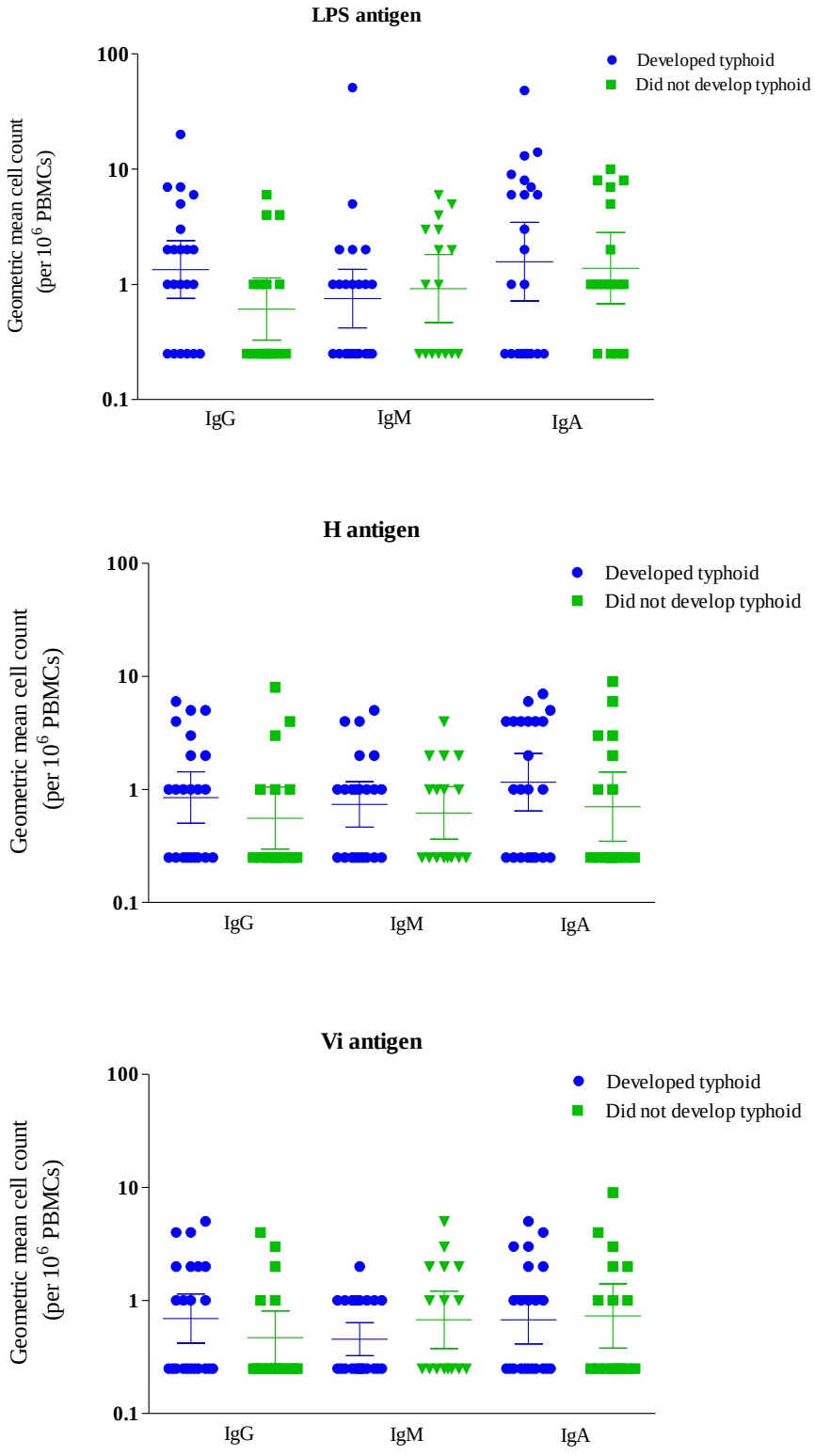
Table 70: The IgG, IgM and IgA plasma cell response to the Vi antigen at baseline, day 7 and day 9 after challenge, and 48 hours after typhoid disease was diagnosed (where applicable) in 40 participants who did or did not develop typhoid disease following challenge with either 10^3 or 10^4 CFU of *S.* Typhi

Antibody	Time point		Geometric mean plasma cell count /10^6 PBMC to the Vi antigen (95% CI)			
			Developed typhoid disease		Developed typhoid disease	
			10^3 CFU challenge dose (n=11)	10^4 CFU challenge dose (n=13)	10^3 CFU challenge dose (n=9)	10^4 CFU challenge dose (n=7)
IgG		Number of samples	11	11	9	7
		Geometric mean cell count (95% CI)	1.0 (0.4-2.3)	0.5 (0.3-0.9)	0.7 (0.3-1.7)	0.3 (0.2-0.5)
	Day 7	Number of samples	7	8	9	7
		Geometric mean cell count (95% CI)	1.0 (0.3-3.7)	0.9 (0.3-2.5)	1.0 (0.4-2.4)	0.9 (0.3-2.6)
	Day 9	Number of samples	5	4	9	5
		Geometric mean cell count (95% CI)	0.6 (0.1-3.1)	0.5 (0.0-5.7)	0.5 (0.2-1.3)	0.6 (0.1-3.0)
	TD 48	Number of samples	11	9		
		Geometric mean cell count (95% CI)	0.8 (0.3-2.0)	0.9 (0.3-3.4)		
IgM	Baseline	Number of samples	11	11	9	7
		Geometric mean cell count (95% CI)	0.6 (0.3-1.0)	0.4 (0.2-0.6)	0.7 (0.3-1.5)	0.7 (0.2-2.2)
	Day 7	Number of samples	7	8	9	7
		Geometric mean cell count (95% CI)	0.8 (0.2-3.3)	0.9 (0.3-2.9)	0.4 (0.2-1.1)	0.6 (0.2-1.9)
	Day 9	Number of samples	5	4	9	5
		Geometric mean cell count (95% CI)	0.4 (0.1-1.2)	0.8 (0.0-32.1)	0.4 (0.2-0.8)	0.4 (0.1-1.6)
	TD 48	Number of samples	11	9		
		Geometric mean cell count (95% CI)	1.6 (0.8-3.3)	1.7 (0.5-6.1)		
IgA	Baseline	Number of samples	11	11	9	7
		Geometric mean cell count (95% CI)	0.4 (0.2-0.8)	1.1 (0.5-2.2)	0.5 (0.3-1.0)	1.2 (0.3-4.9)
	Day 7	Number of samples	7	8	9	7
		Geometric mean cell count (95% CI)	1.5 (0.5-4.7)	2.4 (0.9-5.9)	1.1 (0.3-3.7)	1.3 (0.3-5.9)
	Day 9	Number of samples	5	4	9	5
		Geometric mean cell count (95% CI)	0.4 (0.1-2.0)	2.8 (0.7-11.7)	1.0 (0.4-2.4)	1.6 (0.4-7.1)
	TD +48	Number of samples	11	9		
		Geometric mean cell count (95% CI)	1.3 (0.6-2.5)	1.4 (0.5-3.4)		

There was no difference in the IgG, IgM and IgA secreting plasma cell numbers at baseline between those who did and did not develop typhoid, as shown in Figure 64. Several

participants had cells detectable at baseline indicating that plasma cells actively secreting antibody were present prior to challenge. This did not affect the outcome of challenge.

Figure 71: The IgG, IgM and IgA plasma cell response to the LPS, H and Vi antigen at baseline for participants who did and did not develop typhoid disease in the development of a human challenge model of typhoid disease



Changes in geometric mean plasma cell counts following challenge (Day 0) are shown in Figure 72, Figure 73 and Figure 74. The cell counts at 48 hours after typhoid diagnosis (TD^{+48}) are plotted for reference, but the actual timing of this assay varied from day 7 to day 16 post challenge depending on when the diagnostic threshold for typhoid disease was reached.

Figure 72: Geometric mean plasma cell count/10^6 PBMCs to the H antigen in participants challenged with at day 0 (D0) day 7 (D7), day 9 (D9) relative to the day of challenge with 10^3 CFU or 10^4 CFU shown by those who did (typhoid) and did not (no typhoid) develop typhoid disease. Cell counts 48 hours after typhoid disease was diagnosed are plotted for reference.

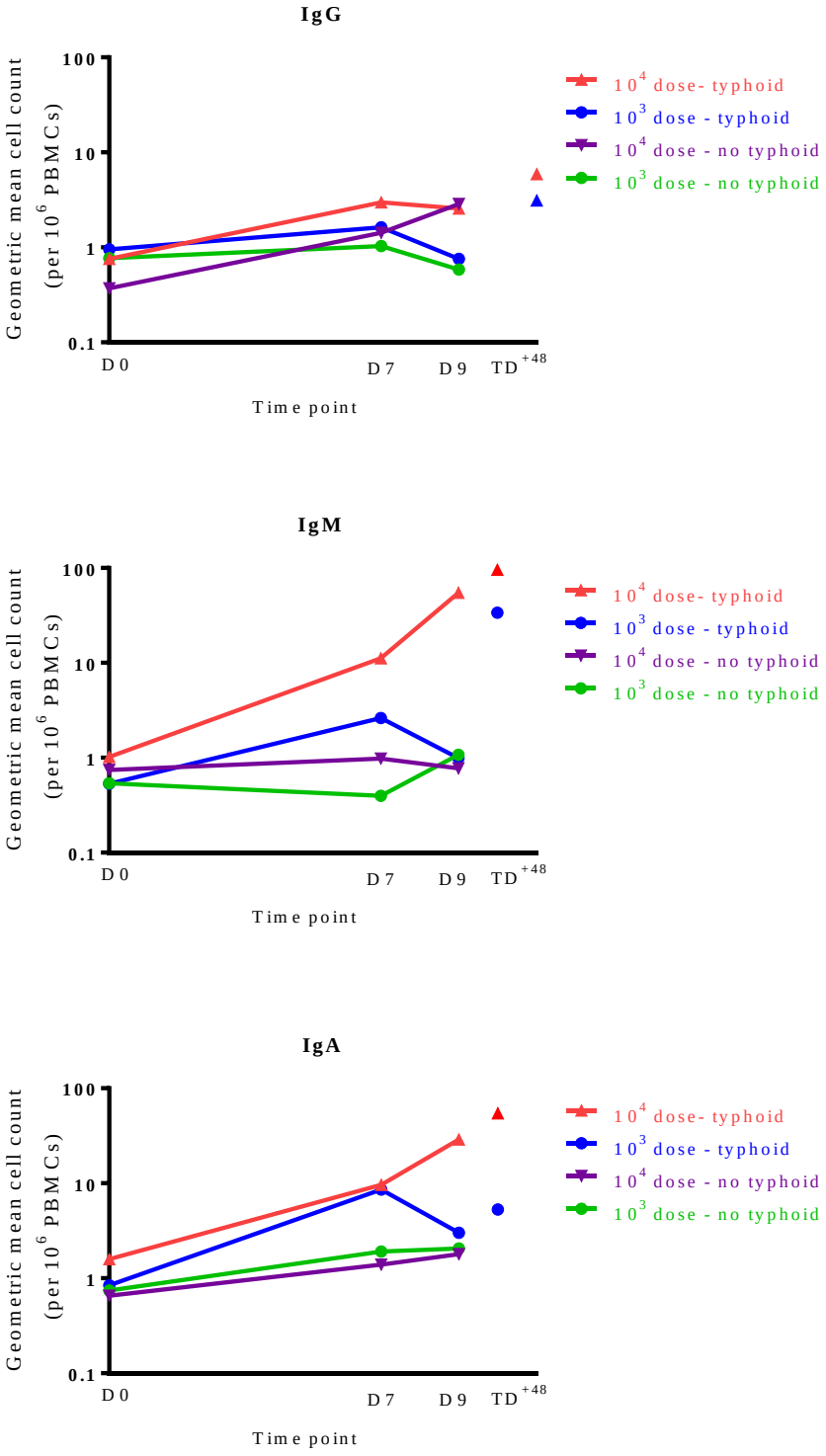


Figure 73: Geometric mean plasma cell count/10^6 PBMCs to the LPS antigen in participants challenged with at day 0 (D0) day 7 (D7), day 9 (D9) relative to the day of challenge with 10^3 CFU or 10^4 CFU shown by those who did (typhoid) and did not (no typhoid) develop typhoid disease. Cell counts 48 hours after typhoid disease was diagnosed are plotted for reference.

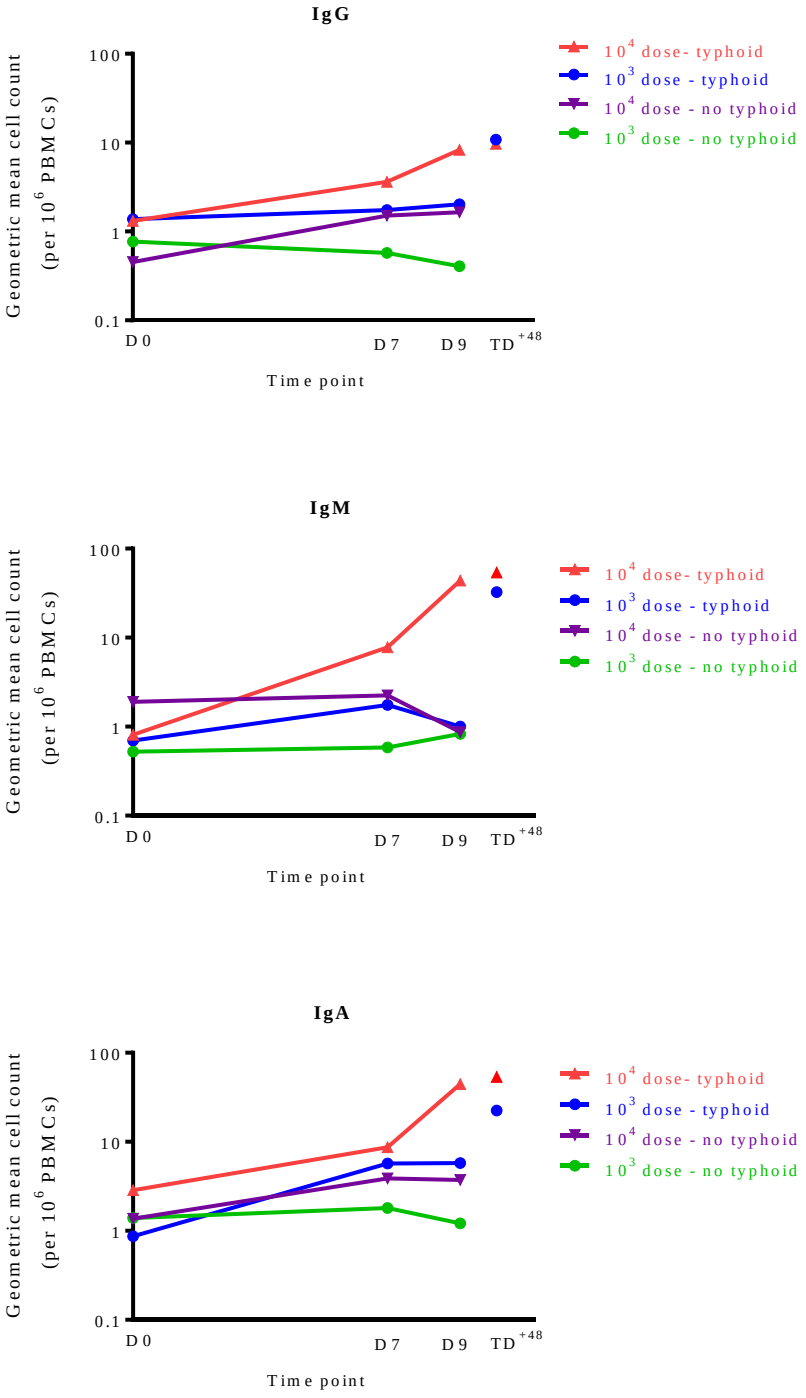
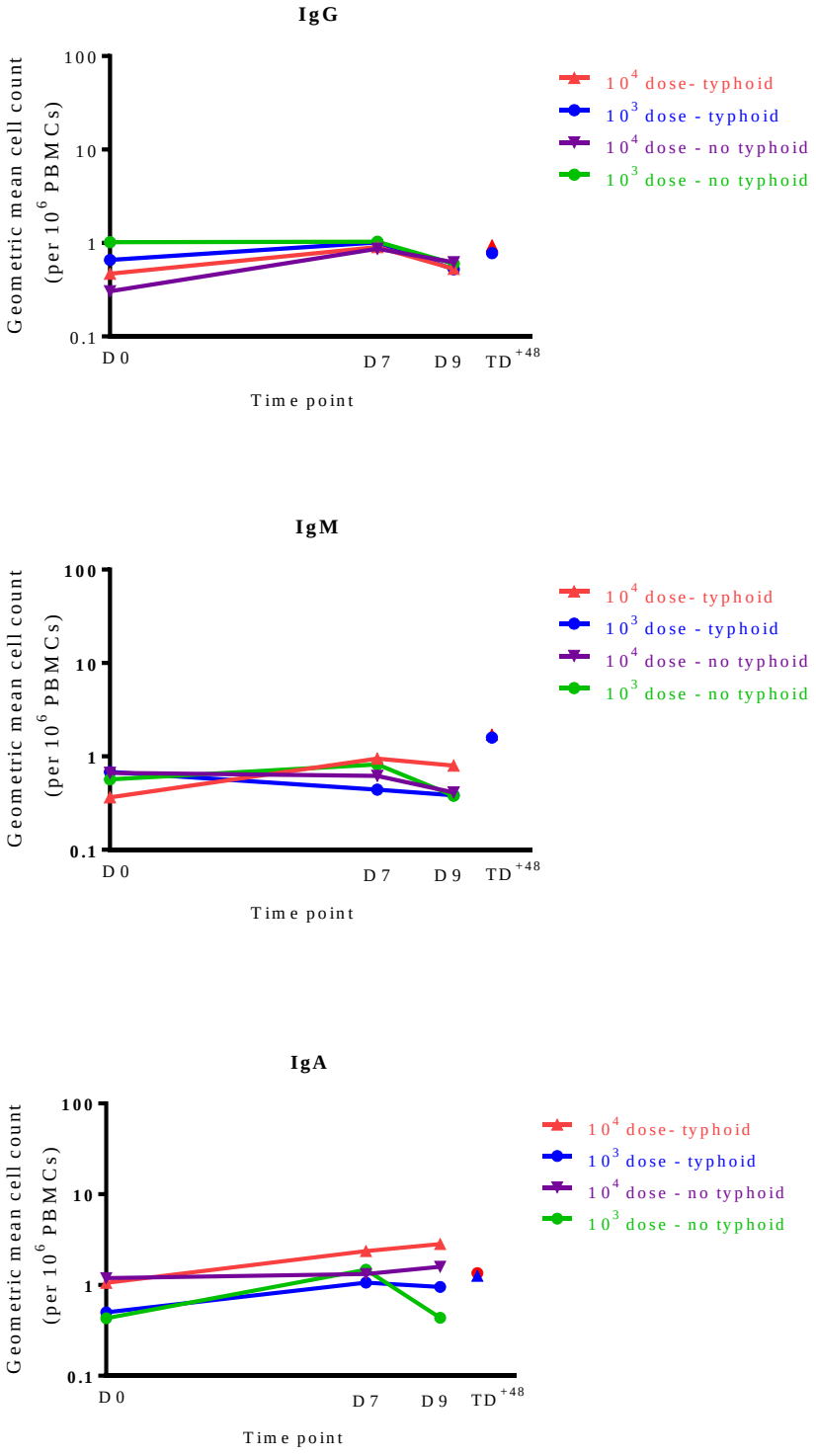
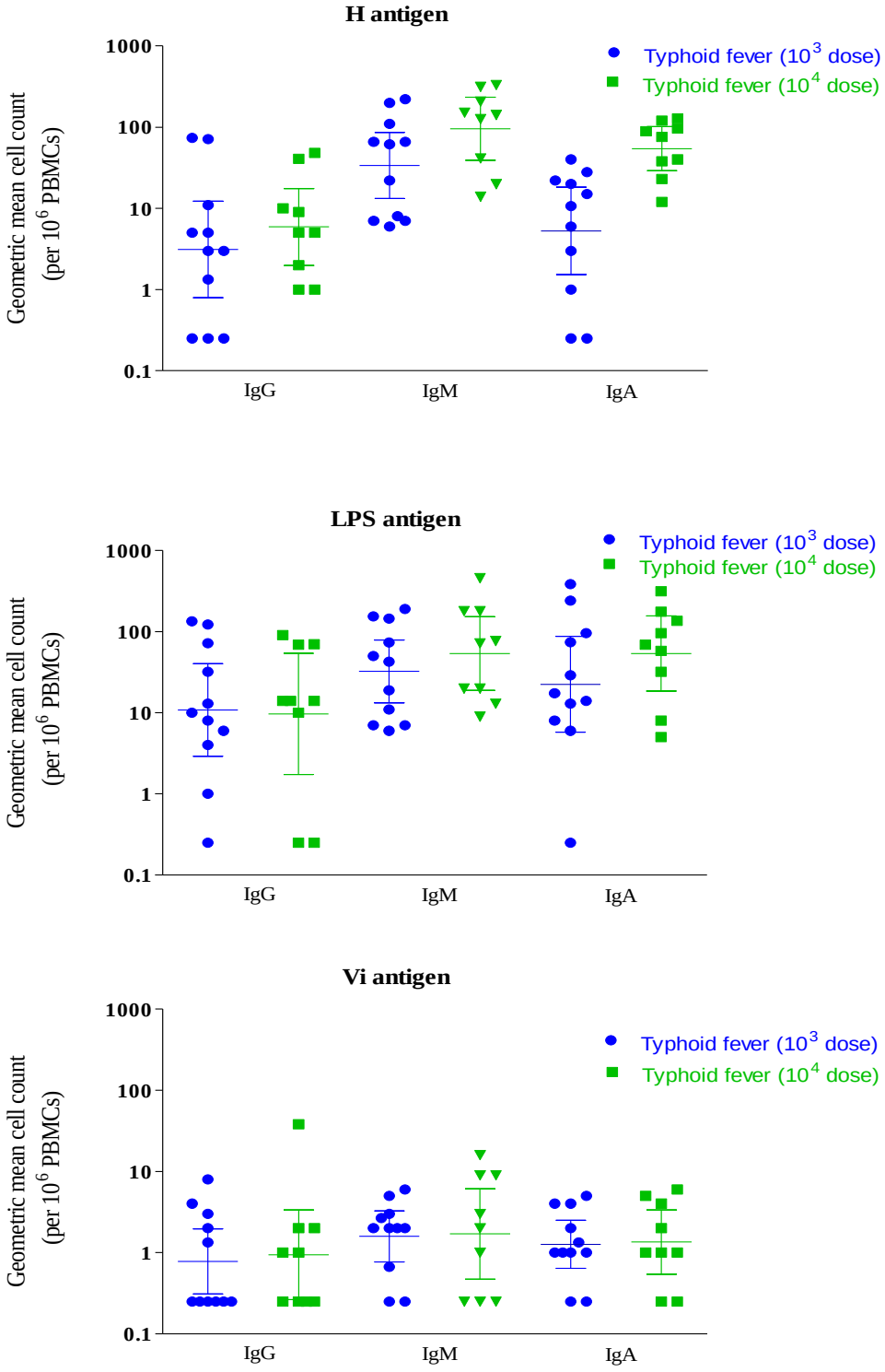


Figure 74: Geometric mean plasma cell count/10^6 PBMCs to the Vi antigen in participants challenged with at day 0 (D0) day 7 (D7), day 9 (D9) relative to the day of challenge with 10^3 CFU or 10^4 CFU shown by those who did (typhoid) and did not (no typhoid) develop typhoid disease. Cell counts 48 hours after typhoid disease was diagnosed are plotted for reference.



The greatest changes in cell numbers were seen with the H and LPS antigens in those who developed typhoid disease. Cell numbers in those responding to the Vi antigen were low at all time points in all participants. There was no apparent change in cell numbers in those who did not develop typhoid disease. Of the ASCs involved in the response to the H and LPS antigens, the majority secreted IgM and IgA, consistent with a primary mucosal immune response. Detailed kinetic description is not possible with the limited number of time points available, but there was a general trend for the cell numbers to peak at day 7 in those developing typhoid disease following challenge with the 10^3 CFU dose of *S.* Typhi, whereas the peak was later (day 9) in those who developed typhoid disease following challenge at the 10^4 CFU dose. There was a trend suggesting a dose–response effect, with higher cell numbers in those developing typhoid disease after the 10^4 CFU challenge dose compared to after the 10^3 challenge dose, although confidence intervals were overlapping. This was most obvious 48 hours after typhoid disease diagnosis when cell numbers were at their highest, as shown in Figure 75. These data correspond to the findings with antibody responses.

Figure 75: Geometric mean plasma cell counts/10^6 PBMCs to the H, LPS and Vi antigens measured 48 hours after the diagnosis of typhoid disease in participants challenged with either 10^3 or 10^4 CFU of *S.* Typhi in the development of a human challenge model of typhoid disease, showing a trend for higher cell counts following challenge with the higher dose.



8.4. Discussion

This chapter presents the first data on the immunoglobulin class response and plasma cell response to *S.* Typhi infection. Humoral responses to *S.* Typhi are likely to be of critical importance, both in protecting against infection and overcoming established infection. Humoral response data to typhoid disease from humans is however limited, and complicated by pre-existing immunity in residents of endemic countries.

8.4.1. Baseline antibody and plasma cell levels

Baseline antibody levels and plasma cell frequency specific for the H, LPS and Vi antigens were variable between participants, but were generally low, and consistent in range and geometric mean between those who did and did not develop typhoid disease. Baseline antibody titres are known to be variable, especially in settings with chronic exposure to *S.* Typhi. For example, high titres to the H and O antigens in the background population have been found in 7% and 1% respectively in Vietnam[278] where typhoid disease is endemic. Plasma cell and antibody responses seen at baseline may be due to cross-reactive antibody, which has been frequently demonstrated with the Widal test, and can originate from both infectious and non-infectious illness.[142] There are 78 *Salmonella* serovars classified by their O antigen as being in the antigenically related group D along with *S.* Typhi[40] and infection with any of these serovars can give a positive O titre.[473] Although assayed differently, very high baseline antibody levels were frequently detected in the Maryland challenge studies, in contrast to the findings presented here, notably in participants with previous military service and older participants, probably related to compulsory vaccination during service and to natural exposure from when disease incidence in the USA was higher.[87] The population in this challenge study have not received typhoid vaccines, or lived in typhoid endemic countries and are unlikely to be exposed to *S.* Typhi within the UK, and hence are more immunologically naïve population than previously studied in Maryland.

One participant had high anti-Vi antibody response at baseline. This may have resulted from previous vaccination. This raises the issue of whether baseline immunity should be checked, with the exclusion of those who are pre-immune. This was employed in the challenge studies of *Campylobacter* species, where potential participants were screened using an in-house immunoassay, and those with immune response excluded, in order to try and increase the attack rate.[416] Pre-existing immunity may be especially relevant in the field of typhoid where history of vaccination is often unreliable. Conversely, in the absence of a validated test, participants may be unnecessarily excluded and the results may be biased.

Antibodies against all 3 antigens studied here have been suggested as being a correlate of protection against *S*. Typhi infection.[73,304,339] Previous typhoid challenge studies showed a correlation between baseline anti-H antibodies and to a lesser extent anti-O antibodies and subsequent protection.[87] The enteric coated Ty21a vaccine showed a close correlation between the seroconversion rate to the O antigen and the protective efficacy in field trials.[304] However, this correlation did not exist for the formulation based on gelatine capsule/ sodium bicarbonate, weakening the biological plausibility of this as a correlate of protection.[304] The data presented here do not support or refute the role of any of these antibodies as a correlate of protection, with no differences in the baseline antibody response between those who did and did not develop typhoid disease, although baseline levels were generally low.

8.4.2. Kinetics of the humoral immune response

Antibody levels and plasma cell frequency to the H and LPS antigens rose in the first week after challenge in those who developed typhoid disease, with antibody peaking between 14 and 28 days after challenge. This is consistent with observations in previous typhoid challenge studies.[87] The response to the H and LPS antigens was comparable in this study, in contrast to previous findings where the anti-H response predominated. [87] Participants in Maryland studies had high baseline anti-H antibody levels compared to the population in

this study, and the observed greater response to the H antigen may have been due to boosting of pre-existing antibody by challenge. These data presented here are the first to look at antibody class response, and showed that the response was dominated by IgA and IgM plasma cells, and that the greatest fold rise in antibody occurred in the IgA class. These findings are consistent with a primary mucosal immune response. The short lived plasma cell responses are also indicative of an innate mucosal response but no inference can be made about induction of immune memory from this data.

Participants who did not develop typhoid disease had no demonstrable change in either antibody or plasma cell response, suggesting that the humoral immune system was not exposed to *S*. Typhi. This may indicate that the control of *S*. Typhi infection occurs prior to bacterial invasion across the gut wall or is dominated by innate immune responses.

There was a dose-response relationship between the challenge dose and degree of the plasma cell and antibody response, as well as the longevity of the IgG response to the H and LPS antigens. The higher challenge dose appears to have provided greater antigenic stimulus to the immune system compared to the low challenge dose. There is no data available from previous challenge studies on the variability of antibody response with different challenge doses, and this study therefore provides the first evidence a dose-response pattern to the humoral immune response to *S*. Typhi.

8.4.3. Diagnostic thresholds

Antibody responses in those who developed typhoid disease were marked, with the majority developing at least a fourfold rise in levels. In previous typhoid challenge studies, around 70% of participants had a fourfold rise in antibody levels to the O and H antigens and the findings in participants in this study are consistent with that.[12] The Widal test, based on the detection of a fourfold rise in agglutinating antibody to the LPS and H antigens has been used as the basis of diagnostic tests, but lacks sensitivity and specificity.[158] ELISA assays has been shown to be more reliable,[474-476] and while there is no Widal test

data available for the participants in the challenge study, the data presented show greater sensitivity and specificity for a fourfold rise detected by ELISA than is widely reported for the Widal test.[142] However the requirement for paired sera, and the finding that the antibody response starts prior to clinical illness limits the usefulness of the detection of fourfold rises in a clinical setting.[148] There was no single cut off value for participants in this study that would have accurately differentiated those with typhoid disease from those without. This limitation would be more pronounced in an endemic setting where chronic exposure, sub-clinical infection and vaccination to *S.* Typhi and other antigenically similar microorganisms are likely to lead to a high antibody titre in the absence of a history of typhoid disease,[139] especially in older individuals.[12] Indeed, in endemic regions, setting a limit below which typhoid disease was unlikely may be more useful than a non-specific positive result.[146]

New serological tests have been developed that aim to retain the ability to give rapid results with minimal expertise and equipment, but with improved sensitivity and specificity.[141] These tests have focused on detecting an IgM response to improve diagnostic accuracy,[143,477,478] but field trials have suggested there remains a lack of sensitivity and specificity.[158,159,479] ELISA detection of the anti- LPS IgM response has previously been shown to be more sensitive and specific than the IgA response,[474] but others have found salivary IgA,[480] plasma IgA and IgA antibody in lymphocyte supernatant specimen responses[481] to *S.* Typhi antigens to be highly sensitive and specific for the diagnosis of typhoid disease. This corresponds with the data presented here, where the greatest differentiation between those with and without typhoid disease was seen for IgA responses.

8.4.4. Antibody responses in asymptomatic *S.* Typhi bacteraemia

Asymptomatic *S.* Typhi bacteraemia has been described in this *S.* Typhi challenge study as well as previous challenge studies.[246] It has been theorised that the absence of symptoms may be due to tolerance to LPS.[246] Only 2 of the 4 participants in this study had elevated

anti-LPS titres at baseline, and the least symptomatic of the 4 had baseline anti-LPS levels far below average for the cohort. It has also been suggested that participants may be asymptomatic if the bacteria are within the intracellular space[246], but the strong antibody responses of 2 participants with low baseline titres suggest that at some point during their illness exposure to the humoral immune system must have occurred. Hence the mechanisms of asymptomatic *S*. Typhi bacteraemia remain elusive.

8.5. Conclusion

This detailed description of the humoral response to typhoid disease contributes to the understanding of the immunological response to infection. While strong antibody and plasma cell responses were seen in those who were diagnosed with typhoid disease, there were minimal responses in those who did not develop illness, suggesting that infection is controlled before systemic invasion. Antibody and plasma cell responses were dose dependent, suggesting that the antigenic stimulus to the immune system was greater in those ingesting a higher number of organisms. The IgA response to both LPS and H antigens was most discriminatory between those who did and did not develop typhoid disease, and may be useful as a diagnostic marker of typhoid disease.

9. Challenge model validation using Ty21a as a positive control

9.1. Introduction

Challenge models have previously allowed the direct appraisal of vaccine efficacy, safety and tolerability using a smaller sample size compared to field trials. Previously, enteric challenge models have been used to successfully appraise vaccines against typhoid,[114,267,279] cholera,[393,482,483] *Shigella sp.,* [484,485] *E. coli* [388,389] and *C. jejuni.* [416] Vaccine-challenge studies are not without their own limitations however; inclusion of a positive control arm in the model by using a vaccine of known efficacy in both challenge models and in the field provides additional confidence in the findings of vaccine-challenge studies and (potentially) confirms model validity.

9.1.1. Potential limitations of vaccine-challenge studies

When theoretically useful vaccines fail to demonstrate efficacy in vaccine-challenge studies, discerning the cause of failure is often problematic. Failure may be due to features of the vaccine such as the formulation used or the dose, or due to features of the model design such as the type or quantity of challenge strain used or the definition of disease endpoints. This dilemma was illustrated by the study of a hybrid *E. coli-S. flexneri* vaccine which, although immunogenic, was not efficacious against challenge with *S. flexneri.*[486] It was unclear whether this was due to insufficient vaccine dose, excessive interval between vaccine doses, the use of too large a challenge dose overwhelming any vaccine-derived protection or an excessive interval between vaccination and challenge.[486] To assess this, and avoid dismissing a potential effective vaccine, further challenge experiments were conducted. These included three rather than two doses of vaccine, reducing the dosing interval from four weeks to one week, and decreasing the time from vaccine to challenge from 8 weeks to 4 weeks were investigated.[486] Despite these modifications, attack rates in

vaccinees and controls were similar (47% and 39% respectively), and it was concluded that the vaccine lacked efficacy for unknown reasons.

Even when efficacy is demonstrated in vaccine-challenge experiments, this may not correspond to protection in target populations. Despite providing 95% protection against moderate or severe cholera when tested in a challenge model, a single dose of the live cholera vaccine CVD103-HgR failed to demonstrate protective efficacy in Indonesia.[487] These findings demonstrate that although challenge models can facilitate significant development in vaccines, they cannot fully replicate disease in the local population in an endemic field setting.

9.1.2. Ty21a vaccine as a positive control

Inclusion of a positive control vaccine arm to vaccine-challenge studies provides evidence of the scientific validity of the model. An ideal positive control vaccine would be safe and well tolerated, and be effective at preventing both disease induced in a challenge setting and in the field. If the positive control vaccine failed to protect in a vaccine-challenge study it could be reasonably concluded that the model design itself was the limiting factor, and that any novel vaccine simultaneously tested should not be automatically rejected.

Ty21a vaccine has the potential to act as a positive control in a typhoid challenge study. Ty21a is a live, oral vaccine, attenuated by chemical mutation of *S.* Typhi strain Ty2,[300] and is the first and (to-date) only licensed oral vaccine for typhoid disease. It has an excellent safety record, and during its development, demonstrated efficacy in both vaccine-challenge models and a variety of field settings.[488]

Following the demonstration that intra-peritoneal injection of Ty21a protected against challenge with wild-type Ty2 in mice,[300] the protective efficacy of Ty21a was investigated in human challenge studies.[267] Five to eight doses of Ty21a (each containing 3-10 $x10^{10}$ CFU) were given in 45ml of milk at an interval of three to four days over a four week period. Vaccine preparations grown in media with and without exogenous galactose were

compared.[267] To enhance the survival of Ty21a, participants ingested two grams of sodium bicarbonate in 60 ml of water prior to vaccination in an attempt to neutralise stomach acidity. One hundred men received vaccine grown with exogenous galactose, and 56 men received vaccine grown without. Protective efficacy against challenge with 10^5 CFU *S.* Typhi, 5 to 9 weeks following vaccination, was compared with unvaccinated challenged controls. The vaccine grown in galactose had an overall protective efficacy of 87%, and also decreased excretion of *S.* Typhi after challenge.[267] Vaccine grown without galactose was less effective, decreasing the attack rate in vaccinees by approximately half. No data was given on the effect of the number of doses of Ty21a on subsequent protective efficacy. Strain growth with galactose allowed the attenuated bacteria to take up galactose to produce a smooth lipopolysaccharide capsule that was more immunogenic than the capsule that resulted from growth without galactose.[489] Correspondingly, the antibody response to the O (lipopolysaccharide) antigen was greater in those receiving vaccine grown with galactose.[85] The largest serological response was seen against the H antigen, occurring in 51- 60% of participants vaccinated.[267,490] Approximately one third of participants excreted the vaccine strain in their stool for up to 3 days post immunisation, and reversion to wild-type during gastro-intestinal passage was not detected in the 958 stool isolates tested.[46] Colonisation of the small intestine by Ty21a was demonstrated in nine participants using nasogastric aspirates obtained six to eight hours after immunisation.

On the strength of these findings, Ty21a went on to be assessed in field trials. Subsequently, it became evident that the 87% protective efficacy observed in the challenge model was higher than that observed in field-trials performed with Ty21a in Chile[303,317,373,399,400], and Indonesia[401], although comparable to the efficacy in Egypt, which exceeded 90%.[302] A recent meta-analysis has found an overall protective efficacy of 51% for Ty21a vaccination.[488] Reasons for the overall lower protective efficacy in field trials compared to the challenge model may include the higher number of doses used in the

model compared to field trials, the altered vaccine formulation and the proximity of infection exposure to vaccination.

By demonstrating efficacy of Ty21a in the current challenge model of typhoid infection, scientific validity of the model will be confirmed, as well as providing a benchmark for potential field efficacy for any candidate vaccines tested in the model.

9.2. Methods

9.2.1. Study design

The study (OVG 2011/02) was a single centre, randomised, double-blind placebo-controlled study, comparing the efficacy of an investigational oral attenuated live oral vaccine M01ZH09 with placebo using a human challenge model, with the Ty21a vaccine as a positive control.[491] Only data pertaining to the use of Ty21a as a positive control will be presented.

9.2.2. Study organisation and approvals

The study was conducted in accordance with Good Clinical Practice (GCP) regulations and with the principles set out in the Declaration of Helsinki. The University of Oxford was the study Sponsor. Approvals for the study were sought and given by Oxfordshire Research Ethics committee, Oxford University Hospitals NHS Trust, and the MHRA. Clinical monitoring was provided for the University of Oxford's Clinical Trials and Research Governance department.

9.2.3. Recruitment, inclusion and exclusion criteria

Recruitment methods, inclusion and exclusion criteria and consent procedures used were the same as those used in establishing the challenge model, described in Chapter 4. In addition, participants were temporarily excluded if they had received a live vaccine in the 3 weeks prior to vaccination or a killed vaccine in the one week before, or had received

antibiotic treatment in the 7 days prior to vaccination. Participants were additionally consented for the risks of vaccination (anaphylaxis, allergy).

9.2.4. Randomisation and blinding

Participants satisfying the inclusion and exclusion criteria were randomised sequentially in a 1:2 ratio to either the open, un-blinded Ty21a positive control arm or the double blinded placebo/ investigational arm of the study. The randomisation sequence was generated by an independent statistician at the Centre for Statistics in Medicine (University of Oxford) and was of variable block size, and the allocation was placed in a sealed envelope, labelled with the randomisation number. Within the double-blinded arm, participants received a novel, investigational, oral vaccine, M01ZH09 or placebo. Allocation was in a 1:1 ratio and was effected through packing concealment.

9.2.5. Vaccine

The Ty21a (Crucell UK Ltd, Bradford, UK) vaccine was supplied in enteric-coated gelatine capsules packaged in foil blister packs and was given according to the manufacturer's instructions.[492] Participants were nil-by-mouth for one hour prior to, and one hour after swallowing the vaccine capsule whole with a cold or lukewarm drink as necessary. Each dose contained not less than $2x10^9$ viable cells of Ty21a. Three doses of the vaccine were given at 48 hour intervals. Vaccination with Ty21a was completed 28 days prior to challenge.

9.2.6. Challenge procedures and follow up

Participants were challenged 28 days after receiving the vaccine with 1-5 x 10^4 CFU suspended in sodium bicarbonate, prepared as outlined in Chapter 4. Participants were followed up daily for the 14 days after challenge except on days 2 and 4 when participants were reviewed by phone. Challenge procedures, follow up, diagnostic definitions and treatment were as described in Chapter 4.

9.2.7. Statistics

Analysis was based on a per-protocol population, consisting of those who underwent vaccination, challenge and follow up for 14 days after challenge without significant protocol deviation in the judgement of investigators. Attack rates were calculated for each vaccine group (Ty21a, placebo or M01ZH09) and were the percentage of those in each group who developed typhoid disease following challenge. Protective efficacy was calculated using the following formula:

> Protective efficacy = (attack rate in placebo group – attack rate in vaccinated)/ attack rate in placebo x 100

9.3. Results

9.3.1. Recruitment

Following an initial brief telephone screening procedure, 178 participants attended screening visits where informed, written consent was obtained, and inclusion and exclusion criteria were assessed, from whom 99 participants were randomised 1:1:1 to each of the 3 vaccine arms. Of these, 30 participants in each of the placebo and Ty21a arms and 32 of those receiving the investigational vaccine underwent challenge, of whom all except one in the investigational arm completed the 14 day post-challenge follow-up period. Data presented here is from those in the placebo and Ty21a arms.

9.3.2. Baseline characteristics

Baseline characteristics were similar between those in the Ty21a and placebo groups (Table 71).

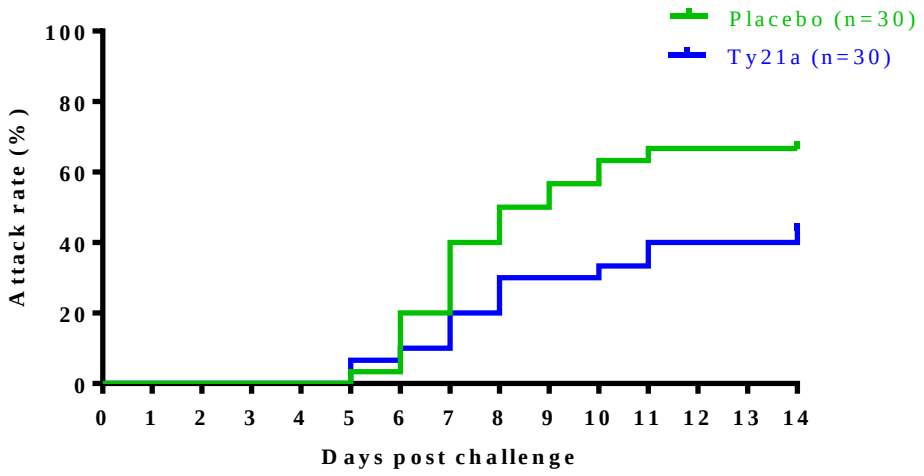
Table 71: Baseline characteristics of participants receiving either Ty21a or placebo in a vaccine-challenge study of typhoid disease

		Study vaccination arm	
		Ty21a	**Placebo**
Gender	No. of males (%)	23 (69.7)	19 (57.6)
	No. of females (%)	10 (30.3)	14 (42.4)
Age (median)		23 (19-59)	25 (19-55)
Ethnicity	Caucasian (%)	28 (84.8)	31 (93.9)
Cigarettes smoked per day (mean)		2.8	2.4
Alcohol intake per weak (mean)		9.3	10.3

9.3.3. Outcome from challenge with *S.* Typhi

Participants were challenged with a median dose of $1.83x10^4$ CFU (range $1.46\text{-}2.66x10^4$ CFU) of *S*. Typhi Quailes strain. Over 14 days of follow up, 13 of the 30 of the participants challenged after vaccination with Ty21a developed typhoid disease (attack rate 43.3%; Figure 76). Of the 30 participants challenged after placebo vaccination, 20 developed typhoid disease (attack rate 66.7%). Hence, protective efficacy of the Ty21a vaccine in the challenge model was 35.1% (p=0.052). Incubation periods were similar in those participants who developed typhoid disease in the Ty21a and placebo vaccinated groups (median values, 7.5 days and 7 days respectively).

Figure 76: Time to infection curve for participants vaccinated with either Ty21a or placebo, who subsequently developed typhoid disease following challenge with 10^4 CFU of *S.* Typhi in a vaccine-challenge study of typhoid disease



Criteria by which participants were diagnosed are shown in Table 72.

Table 72: The number of participants vaccinated with either Ty21a or placebo that were diagnosed with typhoid disease by each of the diagnostic criteria following challenge with *S.* Typhi in a vaccine-challenge study of typhoid disease

Vaccine	Diagnostic criteria for typhoid disease			
	Number of participants with bacteraemia after day 5 with objective sign of typhoid disease (%)	Number of participants with bacteraemia after day 7 (%)	Number of participants with temperature >38°C for 12 hours	Total number of participants diagnosed with typhoid disease
Ty21a	5 (38.5)	4 (30.8)	4 (30.8%)	13 (43.3)
Placebo	7 (35.0)	4 (20.0)	9 (45.0)	20 (66.7)

A greater proportion of the placebo vaccinated participants was diagnosed on the basis of having a sustained fever compared to the Ty21a vaccinated participants (45 vs. 30.8%).

9.4. Discussion

Prior vaccination of participants with Ty21a provided protective efficacy of 35.1% against subsequent challenge. This provides evidence of the scientific validity of the model in the appraisal of vaccine candidates.

The efficacy demonstrated in this study is lower than that shown in previous challenge models of typhoid disease, where protective efficacy of Ty21a was 87%,[267] but similar to that observed in some field trials. For example, 3 doses of enteric-coated Ty21a capsules conferred 42% vaccine efficacy over 30 months in Indonesia.[401] A meta-analysis of typhoid vaccines calculated the efficacy of Ty21a in gelatine capsules, as was used in this study, as being 25%.[488] Other field trials have shown higher levels of protective efficacy, with early trials in Egypt demonstrating protective efficacy of 95%[302] and trials in Chile later showed only 67% efficacy.[303] Reasons for the marked variation in the efficacy of Ty21a include formulation,[303] the number of doses,[400] the genetic makeup of the populations,[493] circulating strain variation,[11] and differences in the background rate of infection and immunity in the two populations.[399]

One notable difference between this study and previous studies of Ty21a efficacy against challenge with *S.* Typhi is the dose of vaccine used. Earlier studies used 5 to 8 doses, each containing 3-10x10^{10} viable bacteria, a significantly higher number of doses and number of bacteria per dose than are now used in commercial preparations. The increased efficacy of Ty21a with increasing doses has been shown in field studies, where doubling the dose from one to two doses increased protection from 29% to 59%.[317] A later trial showed that 3 and 4 doses resulted in incremental levels of protection.[400] Current vaccine guidelines recommend using 3 (UK and Europe) or 4 doses (North America),[492,494] and the study presented here was in line with these recommendations.

Different formulations of Ty21a are also known to influence efficacy. For example, liquid formulations have been shown to be more effective than enteric capsule formulations in

Chile (76.9 vs. 33.2%).[399] The differences in efficacy were even more marked in younger children aged 5-9 years, in whom the enteric coated formulation failed to demonstrate any notable efficacy.[399] The reasons for the superior efficacy of the liquid formulation is thought to be due to both exposure to the tonsils and other gut associated lymphoid tissue to a higher number of viable organisms.[399,401] In the original studies of Ty21a in a challenge model, Ty21a was given as a liquid in 45 mL of milk, 5 minutes after 2 g of sodium bicarbonate had been swallowed. An important additional consideration is that efficacy in the challenge study was assessed in the near term (challenge was performed 5 to 9 weeks after vaccination), whereas the field studies performed compared protective efficacy up to 5 years after vaccination.[317]

The burden of disease in different settings may also impact on the reported efficacy of Ty21a. A higher incidence of typhoid disease in a population may reflect a higher intensity of transmission, with a higher number of infecting organisms[401] which in turn may lead to lower vaccine efficacy. For example, the background incidence of typhoid disease in Egypt when Ty21a field trials demonstrated 95% efficacy was half that experienced in Chile where efficacy was 67%.[303] The lower efficacy of Ty21a in Chile may have resulted from the protective effect of the vaccine being overwhelmed by the higher exposure.[11] This hypothesis was supported by findings of later efficacy study in Indonesia where the disease burden was over six times that of Chile at 1206/100,000 cases per year.[401] The reported efficacy was 53% for the liquid formulation, lower than the 76.9% reported in Chile.[401]

Differences in baseline immunity in vaccinees between the previous Maryland challenge studies and the current study may also have contributed to different rates of protective efficacy of Ty21a against *S*. Typhi challenge observed. The original challenge studies enrolled a significant number of people who had been in the military, where the use of typhoid vaccines was routine. Military veterans were shown to be relatively protected against disease, and to have high baseline antibody titres.[87] Although the number of

veterans in the Ty21a studies is not known, it is plausible that for some vaccinees Ty21a acted to boost pre-existing immunity, increasing reported vaccine efficacy.

9.5. Conclusion

The licenced, live, oral, attenuated vaccine Ty21a has previously demonstrated efficacy in both human challenge models of *S*. Typhi and field trials. Inclusion of Ty21a as a positive control when using a human challenge model of *S*. Typhi to appraise novel typhoid vaccines helps validate the model design, overcoming some of the limitations of human challenge models in the appraisal of vaccines. Efficacy of Ty21a in the current typhoid challenge model was 35%, similar to that observed in several endemic settings.

10. Reported perceptions of participants in the challenge study

10.1. Introduction

Human challenge studies aim to provide detailed data on disease pathogenesis and immune-protection;[370] healthy participants in such studies provide a means to this end. The success of such studies is entirely dependent on the willing participation of healthy volunteers to ensure timely, scientifically valid and cost-efficient studies.[495] However little is known about what motivates healthy volunteers to participate in these studies or about their actual experiences when they do so. In particular, there are no data pertaining to participants in human challenge studies. To ensure the future success of human challenge studies, an understanding of participant motivators and experience is needed. This chapter will review these issues and present the questionnaire reported views, and experiences of participants in the typhoid challenge study.

10.1.1. Motivations for participation

Challenge studies conducted with scientific rigor can contribute greatly to the knowledge of infectious disease pathogenesis and immune protection.[369,424] Healthy participants in challenge studies experience discomfort, inconvenience and a degree of risk.[496] This contrasts with therapeutic trials in which participants may gain therapeutic benefit, or help contribute to the knowledge of a disease that directly affects them.[497] What motivates volunteers to participate in challenge studies is not known. Wider evidence for the motivations of healthy volunteers in any type of research study is also limited. However, non–medical psychological research on 'obedience to authority' should alert us to the subtle, even insidious, ways in which truly voluntary consent can be eroded by the context in which an experiment is located.[498]

Financial reward for participation in challenge studies has been reported since 1851, when Walter Reed offered $100 in gold, with an additional $100 for the next of kin in the event of a participant's death in studies of yellow fever.[499] Financial compensation is an important motivator for enrolment,[396,496,500-503] and has been described as 'essential' for phase 1 trials,[504] which can be logically extrapolated to challenge studies. However, payment of volunteers remains ethically fraught. Clear guidelines on payment still do not exist and are rarely detailed in study information or in the scientific literature.[499,505] Although participants' payment is reviewed by ethics committees, they too are uncertain as to what constitutes appropriate payment of volunteers,[506] and may be poor at judging payments offered.[507]

Objections to volunteer payment have been widely reported, but are theoretical in nature. One concern is that the potential for financial gain will act as an inducement to participate against a person's better judgement, causing them to negate the risks, and thereby undermining the voluntary nature of informed consent.[508-510] Although a weak inverse correlation between the importance of financial compensation and income has been shown for healthy volunteers,[496] evidence that financial compensation leads to people ignoring the risks of studies is lacking. In a study of payment and risk perception, higher payments were actually associated with an increased perception of risk, and led to a more careful review of study information by participants.[511] This was supported by a further study that presented different risks and compensation levels for theoretical studies to different individuals, and failed to demonstrate that risk perception was moderated by financial compensation offered.[512] Others object that financial compensation leads to injustice by attracting poorer members of society and disproportionally burdening them.[510,513,514] Further concerns are expressed that financial compensation may induce volunteers to lie about their health status,[509,515] but evidence for this is again lacking. Others have argued, conversely, that concerns over inducement might be unduly paternalistic,[516] and may negate autonomous decisions.[517] Inducements to change our behaviour are part of normal life, for example special offers on bought goods, but are not met with moral objection.[516]

Many of these debates have assumed that financial compensation is the sole motivation for participation.[499,518] However, this is not supported by evidence.[519] This is illustrated in the context of early challenge studies, both of *S.* Typhi[394] and other enteric pathogens,[386,395] which were undertaken using male volunteers, who were inmates at the Maryland House of Correction. Although concerns regarding the vulnerability of prisoners to exploitation now prevent their use in most research,[396] their testimony provides interesting insights into possible motivators in the absence of external rewards such as financial compensation. In these fully informed consent was obtained following at least two explanations of the study.[87] During the entire study course, only one participant requested early withdrawal from the study.[175] Prisoners repeatedly reported altruistic motivations for their involvement, such as wanting to give back to, and contribute to society,[397,520,521] as well as non-altruistic motivations such as the opportunity to do something different, providing a break from the routine of prison life[397] and obtaining a thorough medical examination.[522]

Similar motivations have been reported by trial participants outside of a prison setting. Whilst financial motivations may be important, or even the principal reason[496,501-503] for participating, altruistic motivations such as contributing to science and society are repeatedly reported as also being one of the most important motivations for participation.[396,523-525] Financial reward has been shown to be not sufficient in itself as a reason for participating,[396,501] and healthy volunteers have stated that they would participate in interventional studies without any financial compensation.[496] Other, non-altruistic, non-financial motivations are also a reason to participate[526], and include medical care,[500,527] the opportunity to meet people,[500] to do something different and curiosity.[496]

10.1.2. Participant experiences of clinical trials

Informed, voluntary consent for participation in clinical trials is at the heart of ethical guidelines for research.[528-530] In practice, processes for obtaining informed consent are driven by ethical theory and policy, rather than being evidence based.[531,532] Although the

general population have a generally positive attitude towards clinical trial research,[523,533] the understanding of research purposes, procedures such as randomisation and risk is generally poor,[533-535] with many perceiving all clinical trials as highly risky,[496,536] irrespective of the information provided. Despite this, where participants' opinions have been sought, most report participation as a positive experience,[523] and indicate that they would participate again.[496]

Contact with study staff and other volunteers is an important aspect of the study experience for many volunteers.[519,537] Personal contact was reported to be the most pleasant aspect of participation by 30% of volunteers in one study.[500] Conversely, staff inattention to participant experience can lead to negative perceptions of the experience as a whole.[396] This aspect of study experience may be of relevance not only to ensuring participant wellbeing, but because word–of-mouth is a significant source of volunteer enrolment.[496] However, detailed appraisals as to how the demands of clinical trials such as traveling to clinic visits, providing samples, undergoing examinations and completing trial associated paperwork, and side effects compare to what participants expect is lacking. There are, more specifically, no such data on experiences of human challenge studies.

10.2. Methods

10.2.1. Questionnaire development

The aim of the questionnaire was to explore possible motivations for, and reported experiences of, participating in the 'Understanding typhoid disease' challenge study. Participants in this study are compensated with a total of £3275 for the entire 3 year period of the study, paid on a pro-rata basis such that £2775 was paid on completion of the post challenge 14 day follow up period. A published questionnaire that had been used for the appraisal of the motivations and experiences of healthy volunteers in phase I studies was used as the basis of the questionnaire.[496] Additional questions were based on anecdotal

issues raised by participants during the study and informal discussion with study staff. The questionnaire was piloted by asking 3 study participants and 5 non-study participants to complete and comment on the questionnaire, followed by discussion regarding their interpretation of the questions and suggestions for improvement. The revised questionnaire was agreed with the study team and consisted of 49 questions (see Appendix).

Participants' motivations were assessed by asking participants to rate on a five-point scale the importance of each of the desire to participate in the clinical trial; the desire to contribute to the progress of medicine; the desire to find out about their own health; the desire to obtain financial compensation; and their curiosity about typhoid disease in their decision to participate. The same five factors where then ranked by relative importance to each other in the decision to participate. Baseline knowledge of typhoid disease, clinical trials, and controlled human infection studies was assessed by choosing one of four options on a four–point scale. Whether the participant had consulted other people prior to deciding to enrol, the expressed opinions of others and reasons for advised non-participation was asked to appraise the decision making process.

To appraise the participant's perception of the consent process, ease of understanding of the information presented in the information booklet and verbally, overall satisfaction with the information provided, and any other information that the participant would have liked was enquired about. To see how the actual experience of the study compared to that perceived following the consent process, participants were asked how their experience of having typhoid disease (if relevant), the overall inconvenience of the study, the daily blood tests, and the stool collection compared to what the participant expected. Overall satisfaction with the study was ranked for convenience on a five-point scale.

The influence of compensation on the decision to participate was assessed by asking if the participant would have taken part if there had been no compensation, or if participants had only been reimbursed direct costs. Opinions on the amount of compensation were appraised by asking how much compensation would be required for participation in a

similar study in the future, the cost of participation, the amount of time off work or study that participation had required, and overall feelings about the amount of compensation received.

The perception of risk and influence of compensation on the perception of risk was assessed by asking overall how risky participants felt the study was in advance of participating, and the influence the compensation had had on the level of risk the participant was willing to take. Perceived risks by close contacts, the participants' perceived risk of infection transmission, life threatening illness, hospitalisation and chronic carriage were all asked about.

The overall experience was appraised by inquiring whether the participant would recommend participation to a friend or relative, or participate again in the study.

Respondent characteristics were assessed by establishing previous clinical trial experience, employment status, annual income bracket, level of education, living arrangements, support during the study, and year of birth.

10.2.2. Questionnaire administration

Thirty-six of the forty-one participants in the typhoid challenge study were invited via email to complete a self-administered, anonymous, on-line questionnaire regarding the motivations and experiences of participation in the 18 months following study enrolment. The remaining 5 participants had been lost to follow up since the study. A reminder e-mail was sent two weeks after the initial invitation. Paper versions of the questionnaire were available on request. Participants were offered the opportunity to be entered into a draw for one of ten vouchers for an online store.

10.2.3. Questionnaire approvals

Ethical approval was granted by Oxfordshire Research Ethics Committee.

10.2.4. Statistical analysis

Data were coded and analysed using 'Eval & Go' online survey software. Further data interrogation was performed using Microsoft Excel and Graphpad Prism, as before. Descriptive statistics were presented as frequencies and percentages for categorical variables.

10.3. Results

10.3.1. Population characteristics

Thirty-one of the thirty-six contactable participants completed the questionnaire (response rate 86%). One participant only completed 12% of the questionnaire and was therefore excluded. Only 5 of contactable participants did not respond. As the questionnaire was anonymous, demographics for these participants cannot be given. The 5 participants lost to follow had an average age of 22.9 years, 4 were male, 4 were students, and 1 was in employment at the time of study participation. Baseline characteristics of respondents are shown in Table 73. The gender distribution of respondents was similar to that in the challenge study as a whole, which included 27 males and 12 females (65.8% and 29.2% respectively). Other demographic variables were not collected from all participants in the challenge study. Of the 31 participants completing the questionnaire, 26 participants requested to be entered into the voucher draw.

Table 73: Baseline characteristics of participants answering a questionnaire on motivations and experiences of taking part in a human challenge study of *S.* Typhi

Characteristic		Proportion of respondents, n=30 (%)
Gender	Male	21 (70.0%)
	Female	9 (30.0%)
Employment status	Student	9 (30)
	Employed	16 (53.3)
	Self-employed	3 (10)
	Unemployed	2 (6.7)
Annual income	<£10,000	8 (26.7)
	£10,000-15,000	1 (3.3)
	£15,001-20,000	6 (20)
	£20,001-25,000	2 (6.7)
	£25,001-30,000	7 (23.3%)
	£30,001-40,000	5 (16.7%)
	>£40,000	1 (3.3%)
Highest level of education	Higher degree	7 (24.1)
	Bachelor's degree	14 (48.3%)
	A-level or equivalent	4 (13.8)
	GCSE or equivalent	4 (13.8%)
Previous trial experience	Only this trial	14 (46.7%)
	1 previous trial	9 (30%)
	2 previous trials	4 (13.3%)
	3 or more previous trials	3 (10%)
Living arrangements at the time of the study	Living with relatives/family	6 (20%)
	Living alone	5 (16.6%)
	Living with partner	8 (26.67%)
	Living in university room	1 (3.33%)
	Living in a shared house	10 (30%)
	Temporarily staying with friends	0 (0%)

The median year of birth was 1983. As with the original study, males predominated with 21 male and 9 female respondents. Education levels, income and employment were higher than would be expected in the general population. Education levels were high, with just over two-thirds (21 participants) having a bachelor's degree or higher. Participants reported hearing about the study from a variety of sources, with the commonest source being via

another participant (8 participants). A minority of participants were living alone at the time of the study (4 participants,), with most participants living either with a partner (8 participants 26.7%) or family (6 participants). The majority of the participants were in employment (19 of 30), with only 2 participants unemployed. Students represented a significant proportion of participants (9 participants). The most frequently reported income bracket was less than £10,000 with just half of participants earning £20,000 or more.

Eleven participants either had no-one or people only for emergencies living with them. Overall, almost everyone had some support besides the study team, with only one participant reporting that they had no-one that they could contact in an emergency, with a further 6 participants only having support for emergencies.

Previous experience and knowledge of clinical trials was high, with over half of participants (16 participants) reporting previous clinical trial experience, and 3 participants reporting participation in 3 or more clinical trials previously. The majority of participants answered that they knew something about clinical trials (25 participants), with 6 participants knowing 'quite a bit' and a further 6 'a lot' about them. Specific knowledge of human challenge studies was slightly lower than that of clinical trials in general but remained high, with only 8 participants reporting knowing nothing about them. The majority of participants (24 participants,) reported knowing 'a little bit' about typhoid disease, with only one participant responding that they had never heard of it.

10.3.2. Motivations for participation, and perceptions of financial compensation

Wanting to obtain the financial compensation for participating in the study was an important motivator (Table 74). Twenty-two respondents reported that it was extremely important, 6 responded that it was important, and no respondent said it was extremely unimportant.

Table 74: The reported importance on a five point scale of potential motivators for participation in a human challenge model of typhoid disease, reported by previous participants responding to a questionnaire

Motivator	Number of participants reporting each category of importance				
	5	**4**	**3**	**2**	**1**
	Extremely important				Of no importance
Curiosity about typhoid disease	2	11	9	7	2
Wanting to be part of a clinical trial	9	13	5	2	2
Wanting to contribute to the progress of medicine	10	13	7	1	0
Wanting to find about own health	2	12	9	5	3
Wanting to receive financial compensation	22	6	2	1	0

Financial compensation was not the only important motivator however with respondents ranking other potential motivators directly inquired about in the questionnaire as important, as shown in Table 74. When asked to rank the relative importance of these factors, financial compensation was the principal motivator for 17 of 30 participants; wanting to contribute to the progress of medicine was the principal motivator for 6 participants, and the second overall most important motivator; curiosity about typhoid disease, and wanting to participate in a clinical trial were ranked overall third and fourth most important; and wanting to find out about own health was the least important. This order of importance did not alter when students and unemployed participants were analysed separately, with financial compensation being the principal motivator for 6 of 11 of students/ unemployed participants, and 11 of the 19 of those in employment. The

overall importance attributed to financial reward did not correlate with income bracket (r^2=0.02).

Only one participant stated that they would have taken part without any financial compensation, although this response and others might reflect the 'social desirability' of being seen to act for the most noble of reasons. A further nine participants said they were undecided. Reimbursement for direct costs only did not increase the reported willingness to participate beyond that for no compensation, with 24 participants responding that they would not participate with this method of payment, and only one participant definitely willing to do so. The minimum amount needed to participate in a similar trial in the future participants was said by participants to be less than they received for this trial, with the most common response being the £1500 to £2000 payment bracket (9 participants), and the majority (22 participants) requiring less than £2500. Only one participant reported requiring more than was offered in the challenge trial. Correspondingly, 11 participants felt the compensation was generous given what was involved, and 18 participants reporting that they thought it was fair. Only one participant reporting that it was too little given what was involved.

10.3.3. Decision to participate

Before consenting, 18 participants claimed to have asked someone else's opinion about participating. The opinions of those asked was reported as being mixed, with 23 of 27 respondents who asked the opinion of others reporting that there had been at least some negative views expressed. The commonest reported concern of those participants consulted was risk to the participant themselves followed by the risk of spread to others.

The level of perceived risk was reported as being moderate or less by all participants, with the commonest reported perception being that they thought overall the study was slightly risky (15 participants). The responses to specific risks inquired about in the questionnaire are shown in Table 75.

Table 75: The reported concern on a five point scale of potential concerns for participants in a human challenge model of typhoid disease, reported by previous participants responding to a questionnaire

Concern	Number of participants reporting each category of concern				
	5	**4**	**3**	**2**	**1**
	Extremely concerned				Of no concern
Developing a life threatening complication	0	3	0	13	14
Being hospitalised	0	2	0	10	15
Long term complication like chronic carriage	1	3	0	12	12
Transmission of *S.* Typhi to someone else	0	5	0	9	9

Whilst the financial compensation made no difference to the level of risk that participants reported to be willing to accept in a third of cases (10 participants) the majority were slightly more likely to accept risk (8 participants) or much more likely accept the risk (11 participants) because of the financial compensation. The influence of compensation on attitude towards risk did not correlate with income bracket (r^2=0.00). Only one participant reported not considering the risks at all after seeing the amount of compensation offered.

10.3.4. Experiences of the challenge trial

Overall participants expressed a high level of satisfaction with the information presented. Written information was described as very easy to understand by 18 participants, and easy to understand by 12 respondents. Verbal information was reported by 24 participants as being very easy to understand and as easy to understand by 6 participants. No-one reported finding either source of information very difficult to understand. One participant would

have liked more information of the overall safety of trials conducted at The University of Oxford, and one would have liked a study nurse to discuss the risks with concerned colleagues.

The majority of participants appeared to have a positive experience of taking part in the challenge study; 27 of the participants would definitely participate again, with only 2 stating they definitely would not. Similarly, 23 participants would recommend participation to others, whilst 6 answering that they may recommend participation, depending on many things.

The amount of time taken off work or study to attend study visits was small, with majority of participants reporting that they took an hour a day (8 participants,) or a few hours a day (8 participants) off, and a significant number reporting not taking any time off (7 participants). The reported amount of time taken off due to typhoid disease was also small, with 4 participants reporting taking no time off at all, and 14 taking off less than four days. Direct costs of participation were reported as being over £500 for 16 of 30 participants, with 9 participants reporting costs of £500 to £1000, 3 participants reporting costs of £1000 to £1500, 3 participants reporting costs of £1500 to £2000, and one participant reporting that it had cost in excess of £4000.

Overall 17 of the respondents reported developing typhoid disease during the study. Aspects of the study surveyed were reported to be as expected by the majority of participants, although some participants found aspects of the study much worse than expected.

Table 76: The reported experience of different aspects of the study compared to expected by participants in a human challenge study of typhoid disease responding to a questionnaire

Aspect of study	Reported actual experience, relative to expected				
	Much worse	A little bit worse	As expected	A Little bit easier	A lot easier
Overall inconvenience	0	7	15	3	5
Daily blood tests	3	3	15	7	1
Daily stool sampling	3	6	17	3	1
Typhoid disease (experience by 17/30 respondents)	2	3	5	2	5

The experience of typhoid disease showed most variability compared to what participants expected, with only 5 of those with typhoid reporting that it was as expected. Around half of participants reported other aspects of the study as expected. A small minority reported the daily blood tests and stool collection to be much worse than expected (3 participants each).

10.4. Discussion

The majority of those participating in the trial aimed at establishing a human challenge model of typhoid disease, reported in Chapter 4, responded to the questionnaire appraising their motivations and experiences of the study, with 30 of the original 41 participants represented. The educational level, employment rates and experience of clinical trials was all high in respondents to this study. This suggests that participants were of an unusual social demographic, and previous participation in trials may suggest a particular interest in the field of medical research and/ or willingness to tolerate medical intervention. An increase in translational research has meant an increase in the need for healthy volunteers willing to participate in clinical trials.[509] Healthy volunteers are less motivated than participants in patient based trials,[536] presumably because they stand to gain little individual

benefit. Although clinically significant adverse events in such trials are rare,[538] where things do go wrong they are well publicised,[539] and may result in an unfavourable view of clinical trials by the general population. Studies of infectious diseases in humans are also not without historical controversy.[522] Understanding the motivations of those that do participate is therefore important. In this study, financial compensation was an important motivator for almost all participants, as has been reported in other studies.[396,496,500-503] However, financial compensation was not the sole motivator, with altruistic motivations also important for the majority of participants, in keeping with the testimony of participants in previous challenge studies[87] and of participants in non-challenge trials.[396,496,501] Wanting to receive a medical check-up was the least important to participants of the motivations given. The low importance of this may be due to the availability of universal care under the National Health Service within the UK, in contrast to the location of other studies looking at participant motivations.[509,527]

Wanting to receive financial compensation for participating is understandable given the burden of human challenge studies on individual participants. It has been shown that even in therapeutic trials, drug side-effects, blood sampling, completion of paperwork and travel to study visits is difficult for participants.[525,540] Although the majority of participants in this study had some social support during the study period, a significant number lived alone and only had support in case of emergency, possibly increasing the burden experienced during participation. It is perhaps surprising therefore that one third of participants was willing to consider participation without financial reward, although similar findings have been reported by others.[496] Whether this would have been true in practice is difficult to say as respondents to questionnaires such as this one may report what they imagine are socially acceptable answers.

Dissatisfaction with the level of compensation has previously been linked with study intensity, with participants in studies requiring suspension of normal life for several days being the least satisfied with the compensation received.[500] Participants in this study were

largely satisfied with the amount received and the majority felt it was fair given what was expected of them. Participation is also not without cost to the individual,[505] and in this study, just under half of participants reported that their involvement in the study had cost them £500 or more. Even if participation is purely altruistic, this does not dictate that participants should suffer financially. Compensation payments may also be advantageous to study investigators, as it has been shown to increase patient follow up,[541] thereby contributing to the scientific integrity and safety of studies.

Different approaches to determining amounts of compensation have been suggested, but none is without limitation.[542] A lower limit for payment to prevent 'worker exploitation' has been advocated, reasoning that the poor may be disproportionately vulnerable to inducement by financial compensation, especially if compensation levels are low.[542] The market model allows the market to decide what the reimbursement should be; offer too little and recruitment fails or is slowed down.[542] The wage payment model recognises being a normal healthy volunteer as the equivalent of unskilled labour and pays an allowance based around this with additional compensation for discomfort.[542] The reimbursement model allows payment only for the costs directly incurred by the volunteer. Where study participants' opinions have been sought, the majority agree with the wage payment model for normal healthy adult volunteers.[396,542] Participants in this study were no more likely to participate on a reimbursement only basis than if there was no financial compensation at all. It was interesting to note that participants would participate in a future similar study for less compensation than was received in this study. When individuals with clinical trials experience were asked how much compensation would have to be offered for them to participate in a malaria challenge study and there was a range of answers (median $1500, low $250, high $10,000),[543] and the findings in this study are similar.

Ethical objections to participant compensation frequently orientate around the potential exploitation of vulnerable individuals.[508-510] This study population was highly educated, largely employed and a significant number had previous clinical trial experience on which

to draw in making their decision to participate. Financial compensation did not lead participants to ignore potential risks of participation. Only one participant stated they did not consider risk, even though participants knew that the study had been through ethical review and was conducted under the auspices of a university. Other studies have also found that participants do not negate risk when offered financial reward, and the threshold at which payment becomes inductive is very high, if it exists at all. [512,544] Indeed, if anything, participants perceived too much risk, with the majority indicating that they thought the study moderately risky, despite the ethical requirement for the study to have only minimal risk. One study examining perceptions of potential participants in a clinical trial showed that the perceived risk of severe complication was at least moderate in over half of the sample,[536] a finding that would support the idea that participants over estimate risk. Nor did participants consider the risks alone, with the majority discussing their involvement with someone else, the majority of whom expressed views against participation.

Participants in this study were largely satisfied with the information provided prior to consent, and had found it easy to understand. Consent documents for all trials are appraised by ethics committees but are only a small part of the consent process, with verbal information having been shown to be a large component.[531] Despite clear legal requirements for informed consent, participant understanding of studies following consent can be poor.[533,534] The high level of satisfaction with the information provided in this study is therefore reassuring. That said, some participants reported finding different aspects of the trial worse than expected, possibly reflecting limited understanding of the nature of the trial. There is no standard way of testing informed consent. Validated questionnaires aimed at testing informed consent in therapeutic clinical trials have been developed,[532] but are not validated in human challenge studies. Overall, the study experience appeared to be positive in the majority of participants, with a high number expressing willingness to participate again, and to recommend participation to others.

Although this study provides some interesting insights into the views and experiences of participants in the challenge study there are several limitations. First, the number of respondents was small. Use of the questionnaire in participants in vaccine-challenge studies using the challenge model will provide more data, helping to overcome this limitation in the future. Second, actual motivations and experiences may be different from those reported due to recall bias and participants wanting to report what they may see as socially desirable characteristics. Third, questionnaire completion was 18 months after challenge, and this may have led to recall bias. Future use of the questionnaire could include administration to participants at various points of the study, including before and after consent, prior to challenge, at diagnosis and at time points following treatment and disease resolution. Forth, the questionnaire was designed to solicit answers to particular topics of interest to the investigators, and may not reflect factors that were important to participants.

10.5. Conclusion

Human challenge studies can at first sight appear to contradict the very essence of the duties of a doctor. However scientifically valid, carefully conducted studies of minimal risk can be ethically conducted in fully informed, consenting participants. Although financial compensation is an important motivator for such participants, it is not the sole motivator. Concerns have been expressed that financial compensation leads participants to negate risk, but there is little evidence that this occurred in the *S*. Typhi challenge study.

11.Discussion

This thesis describes the development of a human challenge model of typhoid disease that can be used in the appraisal of novel vaccine candidates. Infections caused by *S.* Typhi cause a considerable world-wide burden of disease.[2] Although moderately efficacious vaccines against typhoid disease are available,[545] they have not been widely deployed and none are suitable for young children. Novel vaccines are needed, and there are several promising strategies on the horizon.[337,342,354] However, as a human restricted pathogen, clinically relevant models for appraising these vaccines and improving our understanding of the disease are lacking.[84] A controlled human infection model of typhoid disease was previously developed at the University of Maryland and has provided much of the current understanding of disease pathology[73,114] as well as providing support for the Ty21a vaccine to be developed.[267] The development of a 21st century human challenge model of typhoid disease described in this thesis will further the understanding of typhoid disease and its prevention.

Previous human challenge models of typhoid disease used freshly harvested *S.* Typhi Quailes strain given in milk.[73,87] Use of this same strain in the challenge model described in this thesis was advantageous in that it is a wild type strain, for which considerable safety data was available. However, this strain was poorly characterised, and the previous reliance on freshly harvested organisms and the use of a milk buffer for challenge had required the simultaneous challenge of a large number of participants. Chapter 4 describes the careful characterisation and manufacture of the Quailes strain to GMP standards. This has provided a reliable source of the Quailes strain, manufactured to a level suitable for human ingestion, and of known bacterial specification, that can be consistently prepared in sodium bicarbonate to a pre-specified dose prior to participant challenge.

This strain was used successfully, and safely, to challenge healthy adult participants in an outpatient setting. An attack rate of 65% was achieved using 10^4 CFU of *S.* Typhi Quailes

strain given with a sodium bicarbonate buffer. The use of a sodium bicarbonate buffer allowed disease to be induced at a lower challenge dose that was previously described in the Maryland challenge studies.[73] Given the small sample size used in establishing the model, the reported attack rates were strongly influenced by disease endpoint definitions and the post challenge follow up period. Alternative approaches would include having a disease endpoint more akin to a clinical trial setting whereby only febrile patients that are confirmed bacteraemic are considered true cases. Extending the follow up period to 3 or 4 weeks may also allow demonstration of a higher attack rate at any given dose, especially at lower challenge doses, as incubation period has been shown to be inversely related to challenge dose in this work and previous challenge studies.[73] An attack rate of 65% will allow the model to be used for typhoid vaccine appraisal in the future with a modest sample size. The limitation of using this attack rate is that it may overwhelm vaccine protective efficacy, which has previously been shown in typhoid challenge studies at a 50% attack rate.[73] This model may therefore represent a relatively stringent method for testing future vaccine efficacy, but is advantageous in that it is practical and affordable to use. The clinical response to challenge, described in Chapter 5, is consistent with the profile of typhoid disease reported from both endemic[7,25] and non-endemic settings,[445,455] and provides reassurance that the challenge model is representative of real-life typhoid disease. Clinical illness was well tolerated by participants. In particular there were no medically significant events that raised concerns about the safety of the model. This work also establishes methodologies that are relevant to future studies of related enteric pathogens. For example, a human challenge model of *S.* Paratyphi A infection, which is increasingly common worldwide[2,5,546-549] and for which there is no licenced vaccine, could facilitate progress against this important pathogen.

Typhoid disease in the challenge model was defined by either the presence of *S.* Typhi bacteraemia or the presence of a sustained temperature for 12 hours. This definition is less stringent than had previously been used in the Maryland typhoid challenge studies, which

was based on a sustained temperature for 24 hours alone.[73,390] Naturally occurring typhoid disease is known to be of variable severity, but previous typhoid challenge studies failed to identify such variation, possibly due to the stringent case definition used.[390] The model described here was able to demonstrate variation in severity, and as such is probably more reflective of naturally occurring disease. This variation included four participants with *S.* Typhi bacteraemia in whom no significant febrile response was detected and who were minimally symptomatic. Asymptomatic *S.* Typhi bacteraemia was described in previous typhoid challenge studies and suggest that *S.* Typhi is sometimes able to avert the inflammatory-mediated immune response that is normally seen in response to Gram negative bacteraemia.[246] Conversely some participants in this study had significant temperature responses, usually associated with symptoms of typhoid disease, but were not bacteraemic. Even where bacteraemia occurred, quantitative blood culture demonstrated low bacterial loads. These data correspond with previous data suggesting that the clinical features of typhoid disease are not simply predicted by the presence of Gram negative bacteraemia,[86,246] and that the host immune and inflammatory response is important in dictating disease manifestations.

Despite *S.* Typhi being a largely intra-cellular infection, humoral immunity is thought to play a significant role in the response to infection.[42] In Chapter 8, significant antibody and plasma cell responses to the H and LPS antigens of *S.* Typhi were demonstrated in participants that were diagnosed with typhoid disease, but there were no significant response to the Vi antigen. This is in keeping with data from historical challenge studies of typhoid disease.[73,87] No such response was demonstrated in participants who did not develop typhoid disease, suggesting that the humoral immune system of these participants was not exposed to these antigens. Although there was some variation in baseline antibody and plasma cell responses to *S.* Typhi antigens, there were no consistent differences between those who did and did not develop typhoid disease. This is contrary to findings from previous challenge studies where baseline antibody levels to H and LPS antigens

showed a degree of correlation with subsequent protection against disease following challenge.[73,87] It was suggested that this correlation in previous studies was due to previous immunisation during military service.[87] The absence of this correlation in these studies would support this, and suggest that participants in the challenge model described in this thesis were more antigenically naïve compared to previous participants.

Future studies into the cell-mediated immune response to challenge in these participants, along with further humoral immune studies including antibody assay in saliva and stool, and lymphocyte suspension assays and antigen arrays will provide further insight into the immunobiology of typhoid disease. It is hoped that by linking the immune response data to clinical data, immune correlates of protection may yet be identified. Further use of the model will provide more data with which to investigate immune correlates. Immune correlates of protection would greatly facilitate future field studies of typhoid vaccines by allowing the identification of correlates in vaccinated participants to serve as a proxy for vaccine efficacy.

Data in this thesis suggest there may be a dose-response relationship between the challenge dose of *S.* Typhi and the clinical, microbiological and humoral immune response. These are the first data that have suggested this. Human challenge models provide a unique opportunity to appraise the response to a carefully controlled infection challenge dose and the finding of a possible dose response relationship highlights this. Further appraisal of the genomic, cytokine and microbiological response using samples obtained in the challenge studies will allow further insights in to this response to be gained.

Although challenge studies provide a uniquely controlled setting in which vaccines can be appraised, they are not without limitation. For example, the use of a high challenge dose in a model may overwhelm vaccine protection,[73,388] resulting in the apparent inefficacy of the vaccine. The use of Ty21a vaccine as a positive control in the challenge model was described in Chapter 9. The demonstration of protective efficacy of Ty21a in the model, in keeping with field trials of Ty21a from some endemic settings provides assurance that the

model will be able to be used for the appraisal of novel vaccines. However, the protective efficacy of Ty21a in the model was at the lower end of efficacy demonstrated in field trials, and may reflect the use of a relatively large challenge dose in this study.

The success of human challenge studies relies on the dedicated participation of healthy adult volunteers. These volunteers stand to gain little from participation, in contrast to participants in therapeutic studies. Understanding their motivations for enrolment and experiences of being in the challenge study is important for the future success of these studies. Financial compensation was a strong motivator for participants in the challenge study described in this thesis, but was not the sole motivator, and there was no evidence that the financial compensation had acted as undue inducement. Similar findings have been found in other studies of healthy participants.[396,500,501,519,550] Appraisal of participants in future challenge studies will strengthen these data.

Findings from the challenge study are likely to be highly dependent on the challenge dose, disease end point definitions and follow up periods used. Future work could explore the influence of these factors further. Having established the safety of the challenge model as it stands, it would be reasonable to extend the follow up period to 3 weeks, as there was no association between longer incubation period and disease severity. The effect of challenge doses either side of those used in this study, i.e. 10^2 CFU and 10^5 CFU would also allow further characterisation of the dose response phenomenon that was suggested by this work. Alternative diagnostic endpoints could also be explored. Of particular interest would be the natural course of disease in patients with asymptomatic bacteraemia. In a carefully controlled clinical setting, these patients could be safely followed up without initiating antibiotics unless clinical illness developed. The Vi polysaccharide vaccine and the Vi conjugate vaccine are further typhoid vaccination approaches that could be assessed in the model. This would provide further data on protective immunity and the use of the model in vaccine appraisal.

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Appendix

Inclusion and exclusion criteria for participation in *S.* Typhi challenge studies

To enrol in the typhoid challenge studies, participants must satisfy all of the following criteria to be eligible:

- Participant is willing and able to give informed consent for participation after the nature of the study has been explained;
- Male or Female, aged 18- 65 years inclusive;
- In good health as determined by:
    a) Medical history
    b) history-directed physical examination
    c) Clinical judgment of the investigator
- Female participants of child bearing potential must be willing to ensure that they or their partner use effective contraception from one month prior to challenge and continue to do so until a negative stool sample has been obtained at least 3 months after completion of antibiotic treatment.
- Able and willing (in the Investigator's opinion) to comply with all study requirements, including capacity for good personal hygiene.
- Willing to allow his or her General Practitioner and consultant, if appropriate, to be notified of participation in the study.
- Willing to allow the Health Protection Unit to be informed of participation in the study.
- For those involved in provision of health or social care to vulnerable groups only – willing to allow his or her employer to be notified of participation in the study

- Willing to give his or her contacts (defined as someone who is likely to have been exposed to the excreta of a challenged participant, usually a household or sexual contact) letters informing them of the participants involvement in the study and offering the contacts screening for *Salmonella* Typhi carriage.
- Agree to refrain from blood donation in the future unless the participant does not develop typhoid fever.
- Be willing to have 24 hour contact with study staff during the four weeks post challenge.

Participation was excluded by the following:

- Are unwilling or unable to give written informed consent to participate in the study;
- Have previously received any typhoid vaccine
- Have previously been resident in a typhoid endemic country for >6 months
- Have previously been diagnosed with laboratory confirmed typhoid
- Have a known or suspected autoimmune disease or impairment /alteration of immune function resulting from (for example):
    1. Congenital or acquired immunodeficiency (including IgA deficiency)
    2. Receipt of immunosuppressive therapy such as anti-cancer chemotherapy or radiation therapy within the preceding 6 months or long-term systemic corticosteroid therapy
- Have a suspected or known HIV infection or HIV related disease
- History of significant cardiovascular disease (including congenital heart disease, previous myocardial infarction, valvular heart disease (or history of rheumatic fever), previous bacterial endocarditis, history of cardiac surgery (including pacemaker insertion), personal or family history of cardiomyopathy or sudden adult death.)

- History of significant respiratory disease (e.g. uncontrolled asthma, chronic obstructive pulmonary disease)
- History of significant endocrine disorder (e.g., diabetes mellitus, Addison's disease)
- History of significant renal or bladder disease (including history of renal calculi)
- History of biliary tract disease (including biliary colic and gallstones (including asymptomatic gallstones detected by ultrasound).
- History of significant gastrointestinal disease (including hepatitis B or C, (including positive serum hepatitis B surface antigen or hepatitis C antibody), inflammatory bowel disease, abdominal surgery, coeliac disease, liver disease, or requirement for antacids, H2-receptor antagonists, proton pump inhibitors, or laxatives)
- History of significant neurological disease (including history of seizures and myasthenia gravis)
- History of significant metabolic disease (e.g. glucose-6-phosphate dehydrogenase deficiency)
- History of significant haematological diagnosis (e.g. bleeding diathesis, sickle cell disease)
- History of psychiatric illness requiring hospitalization, current known or suspected drug abuse or alcohol abuse (defined by an alcohol intake of greater than 42 units per week).
- Moderate or severe depression or anxiety as classified by the Hospital Anxiety and Depression Score at challenge that is deemed clinically significant by the Chief Investigator or Consultant Physician. If elevated scores are due to temporary significant life events, the questionnaire may be repeated after resolution of the event with a view to inclusion if normal.

- History of significant infectious disease (e.g., previous or current schistosomiasis, positive syphilis serology (determined by non-treponemal test), stool examination positive for an enteric pathogen)
- History of cancer (except squamous cell or basal cell carcinoma of the skin and cervical carcinoma in situ).
- Presence of implants or prosthesis (e.g. artificial joints, pacemakers)
- Any clinically significant abnormal finding on biochemistry or haematology blood tests or urine analysis as assessed using Table 4.
- Contra-indication to ciprofloxacin, β-lactam antibiotics, or trimethoprim/sulfamethoxazole therapy
- Female participant who is pregnant, lactating or who is unwilling to ensure that they or their partner use effective contraception one month prior to challenge and continue to do so until a negative stool sample, a minimum of 3 months after completion of antibiotic treatment, has been obtained
- Current occupation involving clinical or social work with direct contact with young children (defined as those attending pre-school groups or nursery or aged under 2 years) or highly susceptible patients or persons in whom typhoid infection would have particularly serious consequences (unless willing not to work until demonstrated to not be infected with *Salmonella* Typhi in accordance with guidance from the Health Protection Agency).
- Current occupation as a commercial food handler (involving preparing or serving unwrapped foods not subjected to further heating).
- Household contact with a young child (defined as those attending pre-school groups, nursery or those aged less than 2 years)
- Household contact who is immunocompromised (e.g., AIDS, chemotherapy*)*

- Scheduled elective surgery or other procedures requiring general anaesthesia during the study.
- Participants who have participated in another research study involving an investigational product that might affect risk of typhoid infection or compromise the integrity of the study within the 30 days prior to enrolment (e.g. significant volumes of blood already taken in previous study), as assessed by both participant questioning and the TOPS Over Volunteering Prevention System database.
- Have received blood, blood products and/or plasma derivatives or any parenteral immunoglobulin preparation in the past 3 months
- Any other significant disease or disorder which, in the opinion of the Investigator, may either put the participants at risk because of participation in the study, or may influence the result of the study, or the participant's ability to participate in the study.

The following temporary exclusion criteria also applied:

- Have experienced significant acute or chronic infection within the previous 7 days or have experienced fever (>37.5°C) within the previous 3 days.
- History of any antibiotic therapy during the 14 days before challenge
- Febrile illness (oral temperature >37.5°C) on the day of inclusion.
- Any systemic corticosteroid (or equivalent) treatment in the 14 days prior to challenge, or for more than seven consecutive days within the past 3 months.
- Significant blood donation (e.g. to the National Blood Transfusion Service) in the 3 months immediately prior to the date of challenge.

Questionnaire regarding the participant experience of typhoid challenge studies

The questionnaire given to participants was completed on-line. The wording of the questionnaire was as follows:

First, may we take this opportunity to thank you for participating in the 'Understanding Typhoid Disease (typhoid challenge) study' that we have been conducting. The following brief questionnaire asks for your views and experiences of that participation. Please answer frankly; we want to hear your honest views! Your answers will be kept confidential and will not influence your on-going participation in the study.

Please indicate how important the following factors were in your decision to take part in this study.

1. I was curious about typhoid fever


Strongly disagree


Strongly agree

1 ○ 2 ○ 3 ○ 4 ○ 5 ○

2. I wanted to be part of a clinical trial


Strongly disagree


Strongly agree

1 ○ 2 ○ 3 ○ 4 ○ 5 ○

3. I wanted to contribute to the progress of medicine


Strongly disagree


Strongly agree

1 ○ 2 ○ 3 ○ 4 ○ 5 ○

4. I wanted to find out more about my own health

✖ Strongly disagree ✔ Strongly agree

1 ○ 2 ○ 3 ○ 4 ○ 5 ○

5. I wanted to receive the financial compensation for participating

✖ Strongly disagree ✔ Strongly agree

1 ○ 2 ○ 3 ○ 4 ○ 5 ○

6. How would you rank the importance of these factors in your decision to participate?

Move the responses so that the most important factor for you is first and least important last.

[ ] I was curious about typhoid fever

[ ] I wanted to part of a clinical trial

[ ] I wanted to contribute to the progress of medicine

[ ] I wanted to find out more about my own health

[ ] I wanted to receive the financial compensation for participating

7. Before reading about the study, what was your knowledge of typhoid fever?

○ I had never heard of it ○ I knew a little bit about it ○ I knew quite a bit about it ○ I knew a lot about it

8. Before this study, what was your knowledge of clinical trials?

○ I knew nothing about them ○ I knew a little bit about them

○ I knew quite a bit about them ○ I knew a lot about them

9. Before this study, what was your knowledge of human challenge trials?

○ I knew nothing about them ○ I knew a little bit about them

○ I knew quite a bit about them ○ I knew a lot about them

10. Before participating, did you ask someone else's opinion about taking part?

☐ No (go to question 11) ☐ Yes, family ☐ Yes, partner ☐ Yes, friend ☐ Yes, my GP / other doctor or health professional ☐ Other

11. How did the people you talked to about the study react regarding your participation?

○ All said it was a good idea ○ Almost all said it was a good idea

○ Some said it was a good idea, some said it was a bad idea

○ Almost all said it was a bad idea ○ All said it was a bad idea

12. If people recommended that you should not participate, what reasons did they give?

☐ Risk to myself ☐ Risk of spread to others ☐ Missing work/ study

☐ 'Guinea pig' role ☐ Not applicable ☐ Other

13. How easy or difficult was it to understand the information booklet given to you before you signed the consent form?

○ It was very easy to understand ○ It was easy to understand

○ It was difficult to understand ○ It was very difficult to understand

14. How easy or difficult was it to understand the verbal information given to you in person at the initial screening visit?

○ It was very easy to understand ○ It was easy to understand

○ It was difficult to understand ○ It was very difficult to understand

15. On the scale below, how would you rate your satisfaction with the information provided before the study?

Very dissatisfied Very satisfied

1 ○ 2 ○ 3 ○ 4 ○ 5 ○

16. Is there any other information you would have liked before you decided to take part in the study?

17. Did you develop typhoid fever following challenge?

○ No (go to question 19) ○ Yes ○ Don't know

18. Compared to what I expected, typhoid fever was:

If you did not develop typhoid fever, go straight to question 19 ○ Much worse ○ A little bit worse ○ As I expected ○ A little bit easier

○ A lot easier

19. Compared to what I expected, the overall inconvenience of the study was:

○ Much worse ○ A little bit worse ○ As I expected ○ A little bit easier

○ A lot easier

20. Compared to what I expected, the daily blood tests were:

○ Much worse ○ A little bit worse ○ As I expected ○ A little bit easier

○ A lot easier

21. Compared to what I expected, the daily stool specimen collection was:

○ Much worse ○ A little bit worse ○ As I expected ○ A little bit easier

○ A lot easier

22. On the scale below, how would you rate your satisfaction with the care provided by study staff?

Very dissatisfied Very satisfied

1 ○ 2 ○ 3 ○ 4 ○ 5 ○

The following questions are regarding your actual experience during the challenge phase of the study. Please indicate on the scale given to what extent you agree with the following statements.

If you developed typhoid fever, please answer all the questions.

23. Would you have participated if there was no financial compensation for participation in this study?

○ Yes ○ No ○ I am undecided

24. What is the minimum amount of compensation that would need to be offered for you to take part in a similar trial in the future?

○ Less than £500 ○ £500- £1000 ○ £1000-£1500 ○ £1500- £2000 ○ £2000-£2500 ○ £2500-£3000 ○ £3000-£3500 ○ £3500-£4000 ○ More than £4000

25. How much do you think your involvement in the trial cost you (e.g. travel expenses, loss of income)?

○ Less than £500 ○ £500- £1000 ○ £1000-£1500 ○ £1500- £2000 ○ £2000-£2500 ○ £2500-£3000 ○ £3000-£3500 ○ £3500-£4000 ○ More than £4000

26. If you were only reimbursed for costs for which you had receipt (such as travel expenses), rather than for your time and inconvenience, would you have participated in this trial?

○ No ○ Yes ○ I am undecided

27. How much time did you take off work / study to attend study visits over the challenge phase of the study?

○ None ○ One hour a day ○ A few hours a day ○ 1- 5 days ○ 6-10 days ○ 11-15 days

28. How much time did you take off work / study due to being unwell with typhoid fever?

○ None ○ One half day ○ 1 day ○ 2 days ○ 3 days ○ 4 days

○ More than 4 days

29. How do you feel about the compensation received?

☐ I think it was a fair amount given what was involved ☐ I think it was generous amount given what was involved ☐ I think it was too little given what was involved

30. Please indicate how you spent the compensation received

○ Savings ○ Paying off costs already incurred (e.g. loans, mortgage) ○ Charity donation ○ Something special for myself (e.g. a holiday) ○ Something special for other people ○ Nothing special, just costs of everyday life ○ A one off investment in something material (e.g. a new car, home improvements) ○ Other

31. Do you have any other comments about the amount of compensation received?

32. When you decided to take part in the study, how risky did you think the study was?

○ Not at all risky ○ Slightly risky ○ Moderately risky ○ Very risky ○ Extremely risky

33. Did the financial compensation offered influence your attitude to the risk of being in the study?

☐ I didn't consider the risks involved in the study after I saw the amount of compensation offered

☐ The compensation offered made me a lot less likely to accept the risks of the study

☐ The compensation offered made me a little less likely to accept the risks of being in the study

☐ The compensation offered made no difference to how I viewed the risks of being in the study

☐ The compensation offered made me slightly more likely to accept the risks of the study ☐ The compensation offered made me much more likely to accept the risks of being in the study

34. How concerned or unconcerned were YOUR CLOSE CONTACTS that even with the suggested precautions (hand washing, etc.) you may transmit typhoid fever to someone else?

Completely unconcerned Very concerned

0 ○ 1 ○ 2 ○ 3 ○ 4 ○

35. How concerned or unconcerned WERE YOU that even with the suggested precautions (hand washing, etc.) you may transmit typhoid fever to someone else?

Completely unconcerned Very concerned

0 1 ○ 2 ○ 3 ○ ◉ 4 ○

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36. How concerned or unconcerned were you that you could develop a life threatening complication during the study?

Completely unconcerned Very concerned

0 ○ 1 ○ 2 ○ 3 ○ 4 ○

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37. How concerned or unconcerned were you that you would be hospitalised during the study?

Completely unconcerned Very concerned

0 ○ 1 ○ 2 ○ 3 ○ 4 ○

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38. How concerned or unconcerned were you that you would develop a long -term complication like chronic carriage as a result of the study?

Completely unconcerned Very concerned

0 ○ 1 ○ 2 ○ 3 ○ 4 ○

Please answer the following questions regarding your experiences and thoughts having completed the challenge phase of the study.

39. Would you, or would you not, recommend participation in this study to a close friend or relative?

○ Yes, probably ○ Maybe, it would depend on many things ○ Probably not

◉ No, not under any circumstances

40. Would you take part in the study again, or not?

○ No ○ Yes ○ Don't know

The following questions ask about your personal circumstances, so that we can understand how this relates to your reasons for taking part in this study and your experiences in this study. Please answer them as correct at the time that you took part in the challenge phase of the study. All answers are confidential.

41. How did you hear about the study?

☐ Direct email ☐ E mail link via email newsletter ☐ Poster ☐ Letter in the post ☐ Stand at University of Oxford fresher's fare ☐ Stand at Oxford Brookes University fresher's fare ☐ Advert in printed newsletter ☐ Via another participant ☐ Via someone who had not participated ☐ Other

42. How many clinical trials have you participated in (including this trial)

○ One (this study) ○ Two ○ Three ○ Four or more

43. What is your employment status?

○ Student ○ Employed by someone else ○ Self-employed ○ Unemployed ○ Other

44. What is your annual income?

○ less than £10,000 ○ £10,000 to £15,000 ○ £15,000 to £20,000 ○ £20,000 to £25,000 ○ £25,000 to £30,000 ○ £30,000 to £40,000 ○ more that £40,000

45. What is your highest level of education?

○ Secondary school/ GCSE ○ A level or equivalent ○ Bachelor's degree ○ Higher degree

46. At the time of the study, where were you living?

○ Living with relatives/family ○ Living alone ○ Living with partner ○ Living in university room ○ Living in a shared house ○ Temporally staying with friends ○ Other [ ]

47. During the challenge phase of the study, were you living with anyone who was able to give you practical support (such as making you a meal, driving you to an appointment)?

☐ No one ☐ Friends/ housemates ☐ Partner ☐ Relative ☐ There were people for emergencies, but not other support ☐ Other [ ]

48. During the challenge phase of the study, were you able to access support from anyone who you were not living with?

☐ No one ☐ Friends/ housemates ☐ Partner ☐ Relative ☐ There were people for emergencies, but not other support ☐ Other [ ]

49. What is your year of birth?

50. If you wish to be entered into the prize draw to win one of 10 Amazon gift vouchers, please enter your name and contact number here:

Thank you for completing this survey.