

**APPLICATION OF A CHALLENGE MODEL
TO ASSESS THE PROTECTIVE EFFICACY OF
ORAL TYPHOID VACCINES IN HUMANS**

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The work described in this thesis is one result of a large and highly collaborative typhoid programme initiated at the Oxford Vaccine Group in 2010. The studies detailed herein have attracted the advice, support, enthusiasm, patience and dogged determination of a large number of colleagues, collaborators and interested onlookers.

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Human infection by *Salmonella* Typhi has been occurring for the last 50,000 years and still accounts for ~22million new cases each year worldwide. Through faeco-oral transmission, this human-restricted infection disproportionately affects the most impoverished sections of endemic communities where adequate sanitation infrastructure and effective vaccination approaches are lacking. Development of new control measures to accurately measure the burden of disease and to prevent infection with new vaccine candidates are hindered by an incomplete understanding of host-pathogen interactions and of what constitutes a protective human response after exposure.

In this thesis I describe the practical application of a recently developed human challenge model of typhoid infection in assessing new control measures, including the evaluation of the oral single-dose vaccine candidate, M01ZH09. In performing a large, double-blind, placebo-controlled study, I was able to measure the direct protective efficacy (PE) of vaccination with either M01ZH09 or 3-dose Ty21a by performing human challenge with 10^4 CFU *Salmonella* Typhi, Quail's strain, 28-days later.

Using clinical and microbiological definitions to confirm typhoid diagnosis during a 14-day period after ingestion, I found insignificant levels of protection afforded by a single dose of M01ZH09 (12.9%), and a low PE after Ty21a vaccination (35%), demonstrating the stringency of the model and the endpoints used. Many additional insights into pathogen dynamics and host responses were found highlighting several important characteristics of oral vaccination. M01ZH09 was highly immunogenic, and both active vaccines significantly reduced bacterial burden (bacteraemia and stool shedding) while having no effect on symptomatic severity of infection in those diagnosed.

M01ZH09 receipt resulted in a significantly longer incubation period, suggesting underlying protective responses were being generated. Further findings included the first objective demonstration of primary bacteraemia occurring after typhoid exposure, and frequent asymptomatic infection or stool shedding in those exposed but remaining well. Overall, these data also demonstrated significant protective effects against challenge by anti-Vi antibody status and age at baseline. Taking these factors into account, M01ZH09 and Ty21a vaccination did convey an overall protective advantage against developing typhoid infection, each reducing the risk of diagnosis by ~two-fold during the challenge period.

DECLARATION AND CONTRIBUTION

The work presented in this thesis was performed by the author and these data and analyses have not been submitted for any degree elsewhere and are solely the authors own work.

Due to the complex nature of the underpinning research programme to provide these data, which included the design, approval and completion of two research studies, numerous colleagues provided considerable additional support. These include research nurses (Kathryn Haworth, Anna Peters, Louise Willis, Lily Norman, Sarah Kelly, Maria Moore, and Rachel Wendler) and clinical research fellows (Claire Waddington, Cathy Jeppesen, and Christopher Green) who assisted in providing continual clinical and safety support to the participants during the trials.

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Chapter 1. HISTORICAL CONTEXT

1.1 EMERGENCE AS A PATHOGEN

Salmonella are ubiquitous. Widespread in the environment and food chain, in addition to being effective commensals, these motile, Gram-negative, facultatively anaerobic bacilli are also capable of causing disease in humans and animals. Host adaptation varies markedly with some species able to cause infection in multiple hosts, including domesticated livestock and humans, while others are more selective, for example, *S. Arizona* in reptiles.¹

Salmonella enterica subspecies *enterica* serovar Typhi (*S. Typhi*) is almost unique amongst the 2,541 serotypes of *Salmonella* species known, in solely causing human disease; **Typhoid Fever**. *Salmonella* Paratyphi A and *S. Sendai* are also human-restricted, arising from a common ancestral variant.² The evolutionary degradation undergone to attain this degree of host-restriction is demonstrated by the fact that, though infection may be produced in 'our' closest extant relative (the chimpanzee, *Pan troglodytes*) following deliberate exposure, many of the key pathologic and symptomatic features of typhoid infection are not reproduced.³

Accurate classification of *Salmonella* is complex; genetic sequencing and DNA-DNA hybridisation experiments have divided the genus into two species, *S. enterica* and *S. bongori*. Within *Salmonella enterica* there are 6 subspecies, including *enterica* (I), *Salmae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV) and *indicae* (VI).⁴ *S. bongori* was formerly subspecies V; there is also a subspecies VII, although this is only identifiable by multilocus enzyme electrophoresis.⁵ Further discrimination of *Salmonella* species may be performed using surface-expressed lipopolysaccharide (somatic or 'O', indicating serotype) and flagellin ('H', indicating serovar) antigens and the Vi capsule, according to the Kauffman-White classification.⁶ Uniquely to *Salmonella*, flagellin presents in two antigenically distinct phases regulated through expression of the *fliC* and *fljB* loci.⁴ Ninety-nine per cent of isolates pathogenic to humans are contained within *S. enterica* subspecies *enterica*.⁷

Complete genome sequences for many (>25) *Salmonella* serotypes are available, containing ~4.7-4.9 megabases and ~5000 gene-encoding regions. Comparison with other Enterobacteriaceae has identified close genetic similarity between *S. enterica* species (*S. Typhimurium*) and *Escherichia coli*; amino acid sequences of eight enzymes demonstrate 94-95% homology.⁸ This similarity, which is further supported by rates of genomic substitution and 16sRNA divergence, suggests that *S. enterica* and *E. coli* last shared a common ancestor approximately 100-130 million years ago.^{8,9}

The host-restriction demonstrated by *S. Typhi* suggests that divergence from a common ancestor has occurred since *Homo sapiens* evolved from the higher primates.¹⁰ This was confirmed initially by MLST and subsequently by whole genome sequencing, indicating that divergence events occurred between 15 and 150,000 years ago.^{10,11} Since then there has been very little further adaptive selection; rather, the *S. Typhi* genome appears to have decrementally lost both genes (42 deletion events) and gene function (55 non-sense

SNPs) resulting in pseudogene accumulation (92 in addition to the 180 contained in the last common ancestor).¹¹ *S. Typhi* has therefore evolved into its human-specific niche through gradual genetic drift and loss of gene function rather than adaptive selection.

These findings complement the current understanding of *S. Typhi* pathogenesis, in which the human gallbladder acts as an ecological niche for bacterial survival. Small populations of bacteria cause occasional asymptomatic human carriage (~5% of those surviving acute infection) with subsequent intermittent shedding of less-fit isolates in faeces. These ‘less-fit’ isolates are transmitted to close human contacts resulting in symptomatic infection and therefore a potential evolutionary cul-de-sac.¹¹ This purposeful life cycle suggests that typhoid biology fits with the ‘source-sink’ model of evolutionary genetics (Figure 1-1).¹¹⁻¹⁴

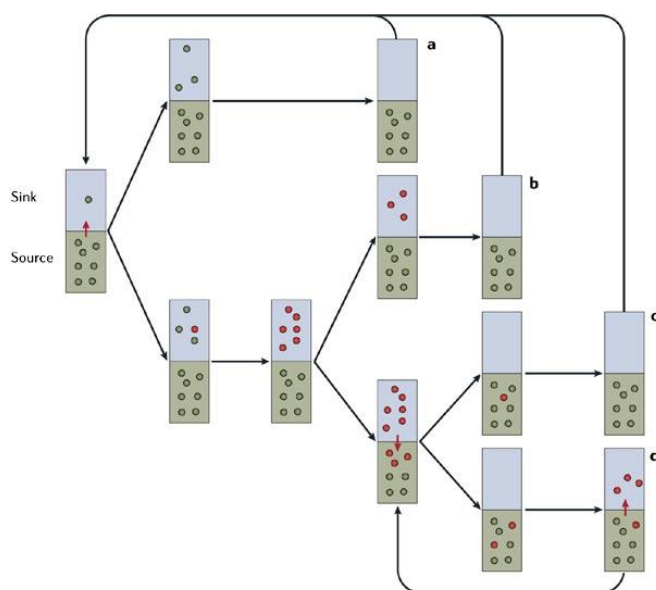


Figure 1-1. Examples of source-sink scenarios demonstrating the potential mechanisms of genetic evolution of virulence mechanisms in bacteria, including *Salmonella Typhi*.

Salmonella Typhi (Green dots) may fit best with ‘b’, in which there is no reinvasion of the source (or the reservoir habitat, i.e. the gallbladder) by sink organisms. Once ‘in the sink’ (the virulence habitat, i.e. the GI tract of acutely infected individuals), organisms may acquire or exchange virulence factors with other bacteria conferring enhanced pathogenicity (in the case of *S. Typhi*, SPI’s 1-7 for example), antibiotic resistance (e.g. *incHI1* plasmid and MDR)⁵ etc., to become ‘pathoadaptive’ mutants (Red dots). These pathoadaptive mutants are evolutionarily short-lived and found (almost) exclusively in acutely infected hosts. Figure from Sokurenko and colleagues,¹² with permission, Nature Publishing Group.

That *S. Typhi* may have evolved into its new niche at this particular stage of human evolution is unsurprising given more recent understanding of transmission dynamics within populations. During the late-Mesolithic period, existing small groups of hunter-gatherers probably experienced long periods of geographic and social isolation. In an anthropomorphic interpretation, it made sense for bacteria to remain quiescent and to survive until further spread to non-immune hosts was possible. When contact between social groups did occur, *S. Typhi* took advantage in being transmitted through faceo-oral spread such as might occur more readily in communal cooking or feasting, resulting in occasional disease outbreaks.^{13,16} This ‘occasional virulence’ pattern is the hallmark of human-typhoid interaction, and remains characteristic of outbreaks still occurring in the early 21st century;^{17,18} most cases in resource-affluent settings occur in returning travellers who have shared food or drink with family members living in endemic regions. Subsequent domiciliation and development of farming techniques in the early-Neolithic period perpetuated *S. Typhi* transmissibility through local environmental contamination, ensuring a continual cycle of carriage and infection and giving rise to ideal conditions for a long-term coexistence.

1.2 PLAGUE POTENTIAL: THE FALL OF ATHENS

Reports of probable human infection with *S. Typhi* date back to antiquity. These include an apparent early account of an epidemic which had major repercussions on the development of western civilisation.¹⁹ In *History of the Peloponnesian War* (421-404 B.C.), Athenian historian Thucydides describes the fall of his City-state to the Spartans. As a result of superior opposition land-forces, many Athenians had fled into the capital greatly increasing its population.²⁰ Possibly originating in Ethiopia, Libya or Egypt, an unknown illness was imported into the besieged city through the arrival of ships into the Port of

Piraeus.²¹ At least 25% of Athens population (~75-100,000 individuals) succumbed to this infection during the next 4-years.²² Thucydides himself became sick but survived and thus left a vivid first-hand written account;²² the Athenian ruler and general Pericles was less fortunate.²⁰

Despite also infecting the inhabitant animal population, other features described have often implicated *S. Typhi* as a cause of this almost apocalyptic event.^{23,24} These include the indiscriminate infection of a high proportion of physicians and caregivers (suggesting nosocomial transmission)²⁵ and vivid descriptions of the clinical syndrome. Thucydides description included fever, headache (“*violent heats in the head*”), cough, retching/vomiting and malaise and the clear description of a 7-8 day incubation period. Patient demise was characterised by “*violent ulceration*” of the bowel, severe diarrhoea, and, frequently, death.^{21,22} It is therefore unsurprising that the foreign mercenaries vital for Athenian success refused to come - apparently put off by the burning funereal pyres!

Numerous aetiologies for this epidemic have been debated, based both on the detailed accounts by Thucydides and interpretations of the epidemiological data.^{21,23,25-32} While most authors concede that louse-borne typhus (*Rickettsia prowazekii*) provides the most likely unifying diagnosis,^{22,33} this lively debate was re-opened in 2004 as a result of the XXVIII Olympiad. During the construction of a new metro station in Kerameikos, a mass grave was uncovered and dated to 430-426 B.C. by accompanying votive pottery (Figure 1-2).¹⁹ Investigators seeking to solve the millennia-long mystery examined DNA extracted from the dental pulp of three teeth. Of seven possible pathogen sequences used (including *Y. pestis*, *R. prowazekii*, *B. anthracis*, *M. tuberculosis*, Cowpox virus, *B. henselae*, and *S. Typhi*) amplifications were achieved with primers for the *osmC*, *clyA* and *narG* genes thus confirming ... *S. Typhi* infection!^{24,34} The debate has been inflamed, however, with scientific criticism of the methodology used and lack of phylogenetic corroboration

of the results.³⁵ Counter-attacks have included the suggestion that *S. Typhi* release may have been a deliberate attempt at bioterrorism by the Spartans.³⁶



Figure 1-2. The archaeological site at Kerameikos in modern day Athens.

Looking towards the outer zone, beyond the city walls (approximately in-line with the row of trees), the 'plague pit' was found at a site near the building with the red sign in the centre of the photo (courtesy of D. M. Darton).

1.3 EARLY ILLNESS DESCRIPTIONS

Early descriptions of febrile illnesses more in keeping with contemporary typhoid infection were made in both western and eastern cultures. Hippocrates (460-377 B.C.), a contemporary of Thucydides, described seasonal cases of **continuous** type fever characterised by diarrhoea and systemic symptoms including rash and in some cases neurological deterioration.³⁷ Some years later, Zhang Zhongjing (150-219 A.D.) wrote extensively describing many causes of fever and epidemic disease affecting his fellow clan members which included typhoid infection.³⁸ Zhang was an early proponent of medical treatment (rather than acupuncture) targeted specifically at the pattern of infection and described the use of cold (water) therapy for treatment. This regained popularity for typhoid fever many years later, remaining the mainstay of treatment until the advent of antibiotics in the 1950's.^{39,40}

The first true clinical description of typhoid (the '**putrid** fever') is attributed to the celebrated neuroanatomist Thomas Willis (1621-1675).^{41,42} Willis described the distinguishing features and clinical course of typhoid (as distinct from the '**continued** fevers') in his treatise *Diatribae Duae Medico-Philosophicae* in 1659.^{43,44} This work (translated and abbreviated 1685) includes several revealing case descriptions detailing, for example, the characteristic diurnal fever pattern.⁴⁵ Willis' account also includes suggestions for treatment involving frequent bloodlettings and some cautionary dietary advice: "Men very often incur a Relapfe, to wit, by an over-hafty eating of Flefh or ftrong Food ..."[p576] This could also be the first recorded comment on the frequency of typhoid reinfection due to the incomplete protection afforded by natural infection.

1.4 TYPHOID = TYPHUS-LIKE

Encompassing Willis' description, the profuse fever literature of the mid-17th century is complicated.⁴⁶ Reasons for this are a source of debate in their own right, and include the ease with which early physicians could identify febrile patients, the multiple (as then) unknown underlying disease pathologies, and often serious fatal outcomes.^{46,47} Key was the difficulty in providing a framework encompassing multiple aetiologies in diverse geographic settings with the background progressive shift towards germ theory.

Typhoid derives its names from the Greek word *typhos* (τυφος). English translations of *typhos* include smoky, hazy or pungent odour; common modern interpretations for its name include description of the clinical stupor found in severe infection (often resulting in subsequent coma and death), or possible early understanding of the association between infection and the rotting odours emanating from blocked or open sewerage systems.⁴⁰ Common differential diagnoses for typhoid in the 17th and 18th century are still

true today, and include typhus and malaria as well as a countless other non-specific causes of fever.

Based on the work of Willis, the various causes of fever became characterised by adjectives including putrid, nervous, hectic and puerperal⁴⁸, and broadly divided into two groups: the **periodic** or **continued** fevers⁴⁹. While the **periodic** fevers (which included **intermittent** and **remittent** fevers) were associated with warmer climates, marshes and appeared seasonally, outbreaks of contagious **continued** fevers were common in army camps, on-board ships and in gaols during the eighteenth century.⁴⁹ The Edinburgh physician Cullen distinguished the **continued** fevers by the presence of delirium, stupor, brain or nervous disturbance as **typhus** or a short-lived illness with a strong pulse as **synocha**.⁴⁹ Cullen and Bretonneau thought that the illness was a result of inflammation occurring throughout the body and particularly in the stomach and intestines, the latter terming it **dothiéntenteritis** (gastroenteritis)⁴⁹.

1.5 PATHOLOGICAL DISTINCTION

The specific term '**typhoid fever**' was first used by the French physician Pierre Charles Alexandre Louis (1787-1872), to describe a disease entity that was clinically similar yet pathologically different to (louse-borne) typhus infection. Collecting over two-thousand case histories and autopsy reports from the wards of La Charité Hospital in Paris, Louis published his book¹ in 1829.⁵⁰ Comparing the post-mortem findings of 138 patients with fever and gastroenteritis symptoms, Louis identified typhoid as a unique pathological process characterised by lesions visible in the Peyer's patches, mesenteric glands and spleen at autopsy.^{50,51} He also suggested that typhus and typhoid may be differentiated clinically, with signs of Rose spots, diarrhoea, intestinal perforation and haemorrhage

¹ '*Recherches anatomiques, pathologiques, et thérapeutiques sur la maladie connue sous les noms de gastroenterite, fièvre putride, adymanique, typhoïde, comparée avec les maladies aiguës les plus ordinaires*'

favouring the latter.^{52,53} Further observations included the high proportion of Parisian newcomers affected by typhoid, who Louis surmised must be more susceptible to infection having not been exposed or infected previously.⁵⁴ Louis was as a highly influential teacher, whose students included William Wood Gerhard and William Budd.^{49,55}

In performing his work in Paris, Louis concluded that the descriptions of '**continued**' fever available in the literature at the time, which described large outbreaks of illness occurring in various cities in Ireland and Great Britain, formed one illness syndrome. In finding no stomach lesions he therefore named the disease typhoid, after typhus, and surmised it was part of the same disease process.⁴⁹

The Frenchman's work was not well received in Great Britain, as the pathological findings described were seldom found on post-mortem, probably because typhus was the more commonly seen infection. In contrast, his theories were well received in North America where typhus was a rarely seen.⁵³ Gerhard returned to Pennsylvania in 1833 and confirmed Louis' findings in fatal cases of febrile illness in newly arriving Irish immigrants, sailors returning from North Carolina with **remittent** (or autumnal) fever and conclusively in an 1836 outbreak of typhus.^{49,53} This outbreak, which began in a crowded section of Philadelphia housing many Irish immigrants, was characterised by frequent misdiagnoses of pneumonia, presence of rash but absence of small bowel lesions.^{53,56} Gerhard concluded that this was not typhoid but typhus, as seen during the large outbreaks affecting Great Britain during the mid-19th century.

Gerhard's findings were slow to be accepted in North America and Great Britain, however, where diagnostic confusion continued into the 1850's.⁵³ In England, Edward Jenner published his seminal work distinguishing typhus, typhoid and relapsing fever in 1850.⁵⁷



Figure 1-3. Figure from Gerhard's 1837 manuscript describing the outbreak of Typhus seen in Philadelphia.

The figure emphasizes the invariable 'measles-like' rash seen in typhus but not found in Dothienenteritis (or typhoid).⁵⁶ In the outbreak, the rash was found in 32/36 'white' patients; those without either had already died or had a more mild severity illness⁴⁹. With permission Wolters Kluwer Health.

Over three years (1847-49) Jenner meticulously studied cases presenting to the London Fever Hospital. He provided a careful description of the distinguishing rashes: Rose spots seen in typhoid which follow on from a characteristic tint - "*that of the skin of a person soon after leaving a hot-bath*",⁵⁷ and the three-stage Mulberry rash seen with typhus. Jenner also traced each patient back to their household residence demonstrating that typhoid cases and typhus cases came from different geographic locations, and finding that an epidemic increase in one disease type had no effect on the other.⁵⁷ Jenner concluded by suggesting that while they were separate diseases, both typhoid and typhus were contagious, however the latter a lot more so.⁵⁷

1.6 TRANSMISSION

William Budd identified the human-human transmissibility of typhoid following an outbreak in Cowbridge, Devon. While demonstrating conclusively that contacts including family members became secondarily infected, he was unsuccessful in getting his findings published until 1859, almost twenty years after the outbreak occurred.^{55,58} Budd was a proponent of the **contagionist** theory and met with strong opposition from those who believed that disease was caused by miasma (bad air or drainage) or even spontaneously.⁵⁵ Budd continued and demonstrated that the disease was transmitted by

faecally-contaminated water or milk. He further evolved methods for preventing infection through disinfection and was a vocal proponent on the use of clean water and the importance of a functioning sewerage system.^{55,59,60} Budd died in 1880, the same year that Erberth described the discovery of the causative agent that he had been so correct in predicting.

1.7 FINDING BACTERIA

Eberth identified the causative agent of typhoid infection in 1880 while professor of pathological anatomy in Zurich.^{61,62} He visualised poorly staining bacterial rods in the lymph nodes and spleens of patients with the now characteristic pathological features of typhoid infection (*Typhus abdominalis*).⁶¹ Earlier that year, Edwin Klebs had made a similar discovery in a series of twenty-four fatal cases, in which he described thread-like short rods (*schistomycose*) at various sites including the Peyer's patches and mesenteric lymph nodes.^{37,63,64} Although less definite in their causal relationship, he suggested the name *Bacillus typhosus* and went on to attempt culture of the organism in the laboratory.⁶⁵ In 1881, Robert Koch published a series of photomicrographs demonstrating the same bacteria,⁶⁶ while Georg T. A. Gaffky successfully managed to culture the bacteria from mesenteric lymph nodes using solid gelatine culture media.⁶⁷ Further advances were made by Pfeiffer (1885) and Hueppe (1886) in finding bacteria in stool and urine, respectively.^{37,68} Direct culture of bacteria from blood was not performed successfully until 1886, when culture from post-mortem blood was performed successfully by Fraenkel and Simmonds.³⁷ Successful culture of bacteria from a live patient blood sample was not achieved until 1887 when Vilchur succeeded, but only once in 35 attempts.³⁷

1.8 NO TURNING BACK?

The last years of the 19th century saw major advances being made in elucidating the cause of typhoid fever which were quickly followed by discoveries in prevention, firstly by recognition of the importance of hygiene and subsequently through development of vaccination. The potential public health impact of these prevention measures was vividly portrayed during the major wars of the late 19th/early 20th century. With the characteristic dogmatic determination of the Victorian age, improvements in societal living standards and hygiene had almost excluded typhoid from being a long-term western problem.

This Victorian enlightenment paradoxically hampered further major progress in typhoid elimination. Firstly, scientific advancement did not cover all aspects of disease control; for example, until the advent of antibiotics to treat infection in 1948, the only therapy/treatment available was targeted at symptom (predominantly fever) control rather than cure. Secondly, as a problem most scientists and practitioners considered to be 'solved' at the start of the 20th century, almost no further clinical research activity took place for a further 50 years.

Only since the advent of global travel for non-warfare purposes has research activity been hurriedly resumed, based on late realisation that antibiotic treatment may not be the elixir hoped for, and that instituting Budd's principles of 'hygiene' could be a delicate, expensive and often only temporary solution. As a result of this stagnation, typhoid at the beginning of the 21st century is therefore much more of an elusive, complex and politically-charged threat that it was 100 years ago.

Chapter 2. TYPHOID IN THE 21ST CENTURY

2.1 A MODEL INFECTION

A major barrier to understanding the immunobiology of *S. Typhi*-host interactions, in addition to stalling the successful development of diagnostic, treatment and vaccination control measures, has been the lack of success in creating a true small animal model of clinical typhoid infection. Edsall and colleagues described the key elements for such a model in 1960 as: “...infection *via* the alimentary tract, invasion of the blood stream, and a marked, extensive and sustained enteritis.”³ Despite apparent initial success in infecting fasted laboratory animals following oral challenge (Erberth and Klebs in 1880),³⁷ successfully reproducing the major clinical features of malaise, diarrhoea, fever, increasing Widal titres and alterations in the Peyer’s patches, was only achieved by infecting chimpanzees in 1904.⁶⁹

2.1.1 PRIMATE MODELS

Based on these early findings and corroborating data from Metchnikoff and Besredka,^{70,71} a further programme of primate studies was undertaken at the Walter Reed Army

Institute of Research (WRAIR) in the 1950-60's.^{3,72-75} Challenge strains used included Ty2 and, latterly, strain 2593, both of which had previously demonstrated similar O agglutination titres and virulence in mice.³ Chimpanzees were challenged using bananas inoculated with doses of between $0.4-0.8 \times 10^9$ CFU of Ty2 or $4-100 \times 10^9$ CFU of strain 2593, which appeared to require higher doses to cause infection. Of note, chimpanzees were frequently infected with other parasites at baseline and 26% had detectable O antibodies prior challenge, albeit at low levels.

Following challenge, most chimpanzees were stool culture positive within 24 hours, while development of fever or positive blood cultures occurred after 4-7 days. Stool and blood cultures were found to be intermittently positive during the febrile period; blood culture positivity coincided with increase in recorded temperatures which maximally reached 3-5.7°F (approximately 3°C) above normal. Specific O and H antibodies were found from day 6 onwards in all chimpanzees, but interestingly Vi antibody only developed in about 1/3 of the animals. Clinical symptoms in untreated animals generally subsided within 2 weeks.

Another interesting finding was the observation that afebrile, '*inapparent*' infections occurred at all doses but were more common with lower challenge inocula and sometimes only manifest by increases in antibody titres.

While the findings of the primate models confirmed the previous work, several notable differences with human infection remained. These included shorter incubation periods and a milder, shorter duration of clinical illness. Investigators also suggested that despite the similarity in pathological features, there was an absence of Peyer's patch ulceration and thus no episodes of bowel perforation were seen. Many features were shared with human infection however, including the spectrum of infection (which included chronic carriage) and infection severity, and the development of O and H antibody responses.⁷²

2.1.2 MOUSE MODELS

Mice are inherently resistant to oral challenge with *S. Typhi*. Only when co-administered with hog gastric mucin to overwhelm host phagocyte response,^{75,76} or when given in massive doses can lethal infection be achieved.⁷⁷ Challenge with non-typhoidal *Salmonella* (NTS) serovars including *S. Enteritidis* or *S. Typhimurium* can, however, produce lethal infections and are therefore used as surrogates. The murine-NTS model has been extensively used to investigate mechanisms specific to *Salmonella* infection but also as a more 'generic' model of enteric infection or Gram-negative sepsis;⁷⁸ much of what is currently known regarding innate and adaptive immune responses to infection and of bacterial mechanisms of pathogenesis are therefore based on this model.^{75,79}

A major limitation to using a murine model to study typhoid is this requirement to use *Salmonella Typhimurium* to produce clinical infection. In addition to genetic variability there are also key differences in expression of virulence factors including the Vi capsule and cytolethal distending toxin B.^{80,81} Investigators have sought to overcome these limitations by both rendering mice more susceptible to *S. Typhi* specifically, and by altering the *S. Typhimurium* infecting strain. For example, a recombinant *S. Typhimurium* strain (Vi4072) expressing Vi has been shown to be able to induce antibody responses following oral challenge.⁸²

Only certain strains of mouse are susceptible to *S. Typhimurium* (for example, those with mutation of *Nramp1*), including CL57/BL6 and BALB/C laboratory mice.⁸³ Recent efforts to increase the susceptibility of mice to *S. Typhi* infection specifically have included the development of a TLR11 knockout murine model.⁷⁸ TLR11^{-/-} backcrossed CL57/BL6 mice were found to be more susceptible to *S. Typhimurium* infection and could be infected by oral challenge with *S. Typhi*. Furthermore, mice immunised with killed whole-cell bacteria could be protected.⁷⁸ While this presents exciting progress in the development of a small

animal model, shortfalls include the high level of mucosal destruction and bloody diarrhoea seen and induction of an IL-12 response, both of which are not characteristic of human infection.⁸⁴

An alternative approach has been to use humanised mouse models, which have the advantage of less animal-to-animal variability, however they remain expensive.⁸⁴⁻⁸⁶ A further clear limitation to these newer murine models is the requirement for a highly immunodeficient background and therefore the relevance to human infection maybe further removed. Some authors suggest that using strains more resistant to infection may actually reflect human disease more closely.⁸⁷

2.2 CURRENT UNDERSTANDING OF *S. TYPHI* PATHOGENESIS

2.2.1 TRANSMISSION AND DOSE

As observed by Budd in 1859, *Salmonella Typhi* is transmitted between humans by ingestion of food or water contaminated with human waste; as a human-restricted infection therefore, contamination always comes from faecal or, less commonly, urinary carriers/shedders. After shedding, bacteria may survive for several days in the environment or longer in certain foodstuffs such as contaminated eggs or frozen oysters.⁸⁸

The infectious dose required to cause human infection in a natural setting is not known, however based on the human challenge studies performed in Maryland (see: 3.1.1), at least 10⁵ organisms (*S. Typhi*, Quail's strain) were required to induce infection when given in milk.^{89,90} Indirect assumptions regarding natural exposure may be made based on the duration of the apparent incubation period. Using this measure, Glynn and colleagues were unable to demonstrate a relationship between exposure dose and severity of

clinical infection in natural outbreak settings,⁹¹ however, no correlation was found in retrospective analysis of Maryland challenge study participants.⁹⁰ This lack of relationship between exposure dose and clinical disease severity may be due to the indiscriminate protection afforded by the acidic gastric environment in healthy adults under normal circumstances. To recreate clinical infection, historical human (and presumably chimpanzee) experimental challenges probably used inocula many fold higher than those encountered in natural exposures.

2.2.2 GASTRIC ACID SURVIVAL

A major factor impeding the development of enteric infection in many individuals is the broad, non-specific protection offered by the normal acidic gastric environment.⁹² *Salmonella* sp. including *S. Typhi* are more susceptible to low pH conditions than *Shigella* and many types of *E. coli* species.⁹³ Deliberate artificial suppression of gastric acid has been performed in several enteric challenge studies in order to allow a lower, more 'physiological' challenge dose to be used, and to produce higher attack rates allowing smaller numbers of participants to be exposed.^{94,95} Methods of artificial suppression vary and range from variable periods of prior fasting to use of sodium bicarbonate or suppression by use of PPIs (famotidine *H. pylori* challenge)[reviewed in ref.⁹⁶].

2.2.3 INTESTINAL COMPETITION

The contribution of commensal gut flora in altering the risk of colonisation or invasion once *S. Typhi* bacteria successfully negotiate the acidic gastric environment is unknown, but may be vitally important in affecting susceptibility to infection (or altering host responses to oral vaccines).^{97,98} In addition, local species may exert control over potential pathogens by both direct (for example, production of specific toxins or alteration of environmental conditions) and indirect (for example, stimulation of host immunity

through production of antimicrobial peptides or increased production of sIgA via MyD88 bacterial signalling) methods. One important mechanism for controlling *Salmonella* survival in the intestine may be through the regulation of iron availability.⁹⁹ In the normal host environment lipocalin-2 binds the bacterial siderophore, enterobactin. *Salmonella* spp. however have developed an alternative siderophore, salmochelin, which is not blocked by lipocalin and thus are able to bypass this defence mechanism.¹⁰⁰

2.2.4 PEYER'S PATCHES AND INVASION

Systemic invasion by *S. Typhi* occurs in the terminal ileum by translocation via the M (microfold) cells overlying Peyer's patches. The normal function of M cells includes sampling antigens passing through the ileum (immunosurveillance), however this mechanism has been exploited by multiple enteric pathogens resulting in host invasion.¹⁰¹

S. Typhimurium, for example, secretes specific factors to promote the differentiation of FAE enterocytes into M cells to increase the site available for host invasion.^{102,103}

Additional routes of entry from the intestine may include direct invasion across the epithelial surface (paracellular) or uptake by specialised phagocytic cells.¹⁰⁴

Bacterial invasion of intestinal cells is achieved through flagellin and T3SS-1 which are under the control of *tviA* and *RcsB* mediated expression of the *tviBCDEvexABCDE* operon.¹⁰⁵ Whilst in the intestine *Salmonella Typhi* require motility to both attach to and invade intestinal cells;¹⁰⁶ therefore, while in the intestine full suppression of *tviA* expression ensures that flagellin is produced and the bacteria remain invasive. After invasion the low osmolarity results in rapid up-regulation of *tviA* expression, which, with *RcsB* induces the expression of Vi capsule, while also repressing flagellin production and T3SS-1 expression.¹⁰⁷ Through this mechanism it may take as little as two hours for bacteria to invade intestinal epithelia and reach the lamina propria layer.¹⁰⁸ Vi expression could also serve to 'hide' the pathogen associated molecular patterns (PAMPs; including

flagellin and LPS) that would result in a major inflammatory response and neutrophil recruitment as seen in NTS infection.

This evasion of the innate immune system explains the lack of a host inflammatory response following bacterial invasion and allows subsequent dissemination of bacteria to reticuloendothelial tissues in the relative absence of any external signs of infection, correlating clinically to the incubation period.

2.2.5 SYSTEMIC DISTRIBUTION

Once invasion has occurred, bacteria disseminate to diverse compartments of the reticulo-endothelial system (RES). While the mechanism for this distribution is not well understood it may include spread by the lymphatic system and/or intracellular transport within macrophages or other cells of the immune system.⁸³ Internalisation of *Salmonella* by macrophages resident in the lamina propria occurs through a variety of mechanisms including phagocytosis, active invasion using T3SS-1 or by T3SS-1 independent invasion using fimbriae or other adhesion molecules on the bacterial surface.

Once internalised, *Salmonella* reside within a modified phagosome (*Salmonella*-containing vacuole, SCV), where they are able to survive and replicate whilst avoiding the host response mechanisms; systemic virulence and survival within macrophages relies on the T3SS-2 in *S. Typhimurium* infections but is less important in *S. Typhi*.^{83,109} Bacteria are only able to survive in human macrophages; manipulation of a specific T3SS effector protein (GtgE) resulted in the ability of *S. Typhi* to survive and grow in mouse macrophages by preventing the RAB32-BLOC3-dependent delivery of antimicrobial proteins to the SCV.^{110,111}

2.2.6 SECONDARY BACTERAEMIA

Secondary dissemination of bacteria from the reticuloendothelial system (RES) tissues occurs via the bloodstream; the factors responsible for release are not known but could include bacterial density, virulence factors or interference by the host response.¹¹² This period of ‘maturation’ or quiescence within the RES ultimately determines the length of the incubation period and is thought to be dependent on the ingested exposure dose (see: 2.2.1).⁹⁰ A peculiar feature of typhoid infection found in many studies, has been the extremely low numbers of bacteria present in the blood at clinical disease onset. In addition, at least 50% of viable bacteria present in the bloodstream may still be contained with macrophages or other phagocytic cells.¹¹³

The onset of secondary bacteraemia generally corresponds to the onset of clinical symptoms. While the precise mechanism of the host response and symptom causation is unknown, inflammatory mechanisms including IL-6 release may be important. Recent discovery of a unique *S. Typhi* virulence factor, A₂B₅ typhoid toxin (CdtB), may explain why the features of human typhoid infection differ from other closely related *Salmonella* species and are so difficult to replicate in NTS/animal models.⁸¹

2.2.7 GALLBLADDER COLONISATION

A key feature of typhoid infection is its propensity to colonise the gallbladder resulting in chronic, asymptomatic carriage. The role of flagellin (in particular FlhC) and outer-membrane protein C are thought to be critical for the attachment and colonisation of bacteria to cholesterol-coated surfaces, increased by the presence of gallstones.¹¹⁴ Overexpression of surface fimbriae can mask these surface components and negatively affects attachment.¹¹⁵ Additionally, it has also been recently demonstrated that *S. Typhi*

may also cause chronic gallbladder infection through gallstone independent mechanisms.¹¹⁶

2.3 HUMAN IMMUNE RESPONSES TO S. TYPHI

Relatively little is known regarding the human immune response to natural *Salmonella* Typhi exposure or infection. What is known is extrapolated from animal models (see: 2.1), responses to specific surface-expressed antigens in isolation or as combinations presented in vaccine formulations (see: 2.7), or from endemic settings, where individuals are likely repeatedly exposed to *S. Typhi* or *S. Typhi*-like antigens often in combination with other enteric pathogens (reviewed in refs.^{79,83,117-120}).

2.3.1 PROTECTION FOLLOWING WILD-TYPE INFECTION

That humans are able to develop protection following natural exposure is predicted by the epidemiology of infection in endemic areas (see: 2.6.2). Two recent modelling studies have suggested that humans living in typhoid endemic areas probably require at least three episodes of exposure to develop long-lasting immunity to natural infection.^{121,122}

This estimation is supported by data from the early typhoid challenge studies performed in Maryland. Re-challenge of 22 previously infected participants with 10⁵CFU up to 12 months after initial exposure, for example, resulted in an attack rate of 23%, only slightly lower than the 30% found in a parallel naïve challenged cohort.¹²³ A report of two large successive outbreaks occurring 3 months apart in Egypt in the 1950s further confirmed the protection provided by a single episode was at best incomplete.¹²⁴

The nature of “protection” to infection is poorly understood and is likely to be multifactorial, requiring both immunological and non-immunological mechanisms such as features of genetic susceptibility, gastric acidity levels and gut microbiome

composition.¹⁰⁰ In addition *S. Typhi* has adapted to deliberately subvert or escape various of the usual immunologically protective mechanisms.^{83,105}

2.3.2 INNATE RESPONSES

Innate immune responses to *Salmonella* infection occur early after ingestion, and have been documented within 48-hours following intragastric challenge with *S. Typhimurium*.¹¹⁹ Following invasion of the Peyer's patches, activation of pathogen recognition receptors (TLR and NLR) by an array of pathogen associated molecular patterns, including T₃SS, flagella, fimbriae, LPS/Vi antigens and bacterial DNA, results in release of pro-inflammatory mediators (including IFN γ , IL-1 β , IL-6 and TNF α) and chemokine receptor up-regulation (including CXCR2 and CCR2).^{79,83,119} This leads to the rapid influx of neutrophils and macrophages,^{83,125} which, at least in NTS infection, results in significant local inflammation and collateral tissue damage. Attraction of inflammatory mediators and initiation of effector responses is amplified by antigen-independent stimulation of antigen experienced T-cells, particularly by IL-18 or IL-23,¹¹⁷ leading to enhanced production of IFN γ , IL-17 and IL-22 and increases in local Th17 cells, $\gamma\delta$ T-cells and natural killer T (NKT) cells. After engulfment, bacterial killing is mediated through reactive oxygen species.¹²⁵ Expression of the Vi capsule by *S. Typhi* limits or subverts several of these responses, however, including recognition of LPS by TLR4 and chemotactic migration of neutrophils;^{126,127} altering gene regulation to reduce expression of the T₃SS-1 and therefore limit inflammation at the site of invasion has also recently been demonstrated.¹²⁸

2.3.3 CELL-MEDIATED IMMUNITY

Induction of cell-mediated immunity (CMI) is likely to be of central importance in the control of *Salmonella* infection, however CMI does not make a major contribution to early

infection. In T-cell deficient mice at least, early growth of bacteria is limited through alternative mechanisms.⁷⁹ Initial evidence for the role of CMI was provided by the early Maryland challenge studies, and possibly accounts for the lack of correlation between humoral responses to oral vaccination and subsequent protection from infection.¹¹⁸

T-cells are activated by MHCII and I presentation of antigen by dendritic cells (DCs) to CD4⁺ and CD8⁺ T-cells. DCs play an important role in immunosurveillance of the normal gut flora and potential food allergens.¹²⁵ Evidence for the importance of this process is supported by the observation that genetic variation in MHC classes II and III loci convey enhanced susceptibility or resistance to typhoid fever in an endemic setting.¹²⁹

Immunisation with attenuated oral vaccines (including Ty21a) results in sensitised lymphocytes being found in the peripheral blood.¹¹⁸ These PBMCs have been found to display enhanced lymphoproliferative responses and Th1-type cytokine secretion patterns to *in vitro* *S. Typhi* antigen (including flagellin) stimulation. These Th1-type responses were found to convey enhanced resistance to infection by augmentation of macrophage activity in addition to enhancement of antigen presentation; findings further supported by HLA haplotype and TNF α polymorphism associations with either protection or susceptibility to infection.^{130,131}

Cytotoxic (CD8⁺) T-cell responses are likely to be crucial for containing intracellular *Salmonella* infection. Demonstration of its importance includes the ability of classical MHC class 1a-restricted CD8⁺ cytotoxic effector T-cells collected from vaccinated individuals to lyse *S. Typhi* infected targets.¹¹⁸ An additional effector mechanism is through the production of cytokines (IFN γ in particular) in response to soluble antigens and infected target cells; this has been demonstrated in vaccine recipients through use of an IFN γ ELISpot assay and flow cytometry techniques.¹³² Generation of IFN γ -producing CD4⁺ and CD8⁺ T-cells and antigen-specific CD4⁺ and CD8⁺ memory T-cells after Ty21a vaccination

was confirmed by Lundin and colleagues. They demonstrated that peak responses were seen 7-14 days after vaccination and that the highest levels of IFN γ were produced by PBMCs expressing the gut homing marker integrin β_7 .¹³³ Further differentiation of gut-homing cells found has identified multiple subsets of central memory (T_{CM} , CD45RO⁺CD62L⁺) and effector memory (T_{EM} , CD45RO⁺CD62L⁺) cells. Studies of CVD909 vaccine responses demonstrated production of a specific subset of T_{EM} cells able to co-express CD45RA (T_{EMRA}), which had not previously been recognised as playing a part in response to bacterial infection in humans.¹³⁴

While many of these CMI responses have been demonstrated in Ty21a and other live-attenuated oral vaccine recipients, the relative contribution either to the protection of vaccine recipients from infection or whether they are also seen following exposure to wild-type *S. Typhi* bacteria remains to be investigated.

2.3.4 HUMORAL RESPONSES

The evidence for contribution of a B-cell response to protection from *S. Typhi* is variable. In addition to production of antibodies, which may be important for extracellular bacterial killing, B-cells may play role in antigen presentation and in the development of the effector T-cell response.⁷⁹ This is supported by the observation that passive transfer of a large amount of immune serum to Igh-6^{-/-} mice prior to challenge does not restore complete protection.⁷⁹ Most data exploring the link between humoral immune responses and protection focuses on antibody generation to the surface expressed antigens Vi, LPS and flagellin following vaccination and measurement in peripheral blood.

The protective nature of anti-Vi antibody is demonstrated by the efficacy of ViPS vaccination (see: 2.7.4).^{135,136} Only ~30% of those recovering from bacteraemic infection eventually produce anti-Vi IgG however,^{137,138} and therefore its role in protection after

natural exposure is unclear. The common identification of high levels of anti-Vi antibody in carriers (~80%) but their frequent lack of preceding clinical illness may suggest at least a disease-moderating effect.^{14,139}

Anti-flagellin antibodies are more frequently seen in convalescence, are long-lived, and form part of the diagnostic Widal reaction, however their role in mounting future protective responses is unknown.^{140,141} The early challenge studies performed in Maryland demonstrated a correlation between H antibody titre and protection,⁸⁹ and the more effective whole cell vaccines used in field trials were also those which induced the greatest anti-H response (see: 2.7.2).¹⁴¹

Anti-LPS (or O-polysaccharide) responses are seen in response to oral vaccination with Ty21a, and have been widely used as an endpoint in vaccine development studies.^{120,141} Evidence for this is based on the association found between Ty21a dose, anti-LPS titres and subsequent protection from infection seen in early field studies (see: 2.7.3).¹⁴² A series of studies performed by Kantele and colleagues, demonstrated that measurement of plasma B-cell response by ELISpot correlated more closely with oral vaccine dose.^{143,144} Detection of IgA secreting gut-homing ASCs in the peripheral blood is a useful, albeit indirect, marker of intestinal secretory antibody levels and appears to depend on oral rather than parenteral exposure.¹⁴⁵ The role of secretory IgA in protection against mucosally-invasive pathogen has been relatively little explored, not least due to technical issues with accurate measurement in the laboratory, however is likely to be a significant factor in determining protection.^{120,146}

Other antibody responses such as those to outer membrane proteins (e.g., OmpC or OmpS) and heat shock proteins (e.g., GroEL) are also likely to play some role in protection, and form the basis for the development of novel vaccines.^{118,147} Identification

of seroprotective antigens may be more effectively achieved through the use of high-throughput antigen microarrays.¹⁴⁸

Antibody responses measurable to the surface expressed antigens do not imply protection however, and individuals may still be susceptible to infection in particular in areas with high exposure intensities. Antibody quantitation also does not imply functionality; this lack of correlation may be a problem more specific to *S. Typhi* than NTS or *S. Paratyphi* serovars.¹⁴⁹ A study performed in Kathmandu (pre-vaccine era) demonstrated no association between *S. Typhi* serum bactericidal activity and anti-Vi antibody positivity, whereas a positive correlation was found with increasing participant age.¹⁵⁰ Given the epidemiological observation that typhoid infection is less frequent in older age groups, these findings may imply that it is increasing functionality of the antibody response rather than titre that is protective. How antibodies may play a direct role in controlling a predominantly intracellular pathogen still requires a full explanation.

2.4 CLINICAL PRESENTATION

The clinical presentation of patients with typhoid infection is highly variable both in severity and in the symptoms reported, and is heavily dependent on the population being studied (Table 2-1).¹⁵¹ Much of this variation is affected by delay in seeking healthcare, accuracy in making the diagnosis and the age group being studied. Young children in particular may present with a non-specific illness and the diagnosis is often missed.¹⁵² In contrast returning travellers are, in general, exposed to lower infectious doses, have no pre-existing immunity (unless vaccinated) and are able to access medical care more quickly and easily.¹⁵¹ Interestingly, the severity of infection in travellers with vaccine failure is similar to that in those never vaccinated.¹⁵³ The clinical presentation of *S. Typhi*

and *S. Paratyphi* are ostensibly indistinguishable and, from the limited data available, rates of complications appear to be similar between the two infections.^{154,155}

2.4.1 ACUTE INFECTION

Following ingestion, a period of incubation ensues prior to the onset of clinical typhoid symptoms, lasting 7-14 days (range 3-60 days).¹⁵¹ Initial symptoms frequently include headache and malaise, and diarrhoea is often an early symptom especially amongst travellers (Table 2-1, Supplementary Table 1).¹⁵³

Traditionally, at least some 'fever' has been a requirement for a diagnosis to be made, likely producing reporting bias in the literature. Temperature increases happen progressively with a characteristic saw-tooth rise reflecting a diurnal variation reaching maximal temperatures in the late afternoon or early evening.^{151,156} Temperatures peak at 39-41°C and rigors are an uncommon feature, although chills are commonly reported (Table 2-1). Other symptoms frequently reported include abdominal discomfort, distension and/or abnormal bowel movements. Patients may experience either constipation and/or diarrhoea; the frequency of each is probably affected by patient age, HIV status and possibly infection with *S. Paratyphi* as opposed to *S. Typhi*.¹¹²

Specific clinical signs indicating typhoid infection are almost never found in practice. Rose spots, a fine, blanching, evanescent maculopapular rash with lesions of 2-4mm, are found in 5-30% of cases and seen more easily in white-skinned individuals on the trunk. They occur at or immediately prior to diagnosis but are frequently missed however; whether they preferentially indicate a diagnosis of typhoid vs. paratyphoid remains uncertain.^{157,158} Relative bradycardia is found in many other disease processes, as first reported by Faget in Yellow Fever,¹⁵⁹ and has been reported with variable low frequency for typhoid.¹⁶⁰

SETTING	Study No.	Study size	Per cent reporting symptom												
			Fever	Diarrhoea	Headache	Vomiting	Nausea	Abdominal pain	Cough	Joint or muscle pain	Chills or rigors	Constipation	Anorexia	Intestinal bleeding	Jaundice
ENDEMIC	18	119	90	32	95	58	91	95	46	79	78	34	90		
	23	60	100	45	40	55		38	35		65	12	97	2	5
	20	422	100	24	38	21		19	23		36	2	26	3	
	25	53	100	43	77	22		53		39	73	13	69		
	11	33	94	18	21	24		27	39		36				3
	17	40	100	20	18	20		11	7	2		9			
	6	189	92	10	58	27		76				5		1	
	16	30	100	27	90	23	27	37				33			
	15	46	100	28	83	22		26				13			
	3	106	100	27	59	28		20							
	4	46	96	20		17			39						0
	7	1650		37				40	14			9			2
	13	130	100	30				30	28			7			
	14	70	97	49	50	71		54							
	9	14	100	43		71			57						
	8	152		57		17		24							
24	82	100		33						41					
TRAVELLER	1	19	100	68	58	47	42	32	26	21	21	16			
	2	26	100	62	77	27		58	42				15	8	0
	12	135	100	53	30	48		29	19		25	4	18		
	21	22	100	50	82	23	23	36		27	50		36		
	10	61	100	59	62	38		44	31	34		2			
	19	24	100	75	13	4		46	42			4			
	5	17	100	91				41							
	22	78	100	27				31							
Mean endemic			98	32	55	34	59	39	32	40	55	14	71	2	2
Min endemic			92	10	18	17	27	11	7	2	36	2	26	1	0
Max endemic			100	68	90	71	42	76	57	39	73	33	69	3	3
Mean traveller			100	61	54	31	33	40	32	27	32	6	23	8	0
Min traveller			100	27	13	4	23	29	19	27	25	2	15	8	0
Max traveller			100	91	82	48	23	46	42	34	50	4	36	0	0
Mean TOTAL			99	42	55	33	46	39	32	34	47	12	50	3	2
Min TOTAL			92	10	13	4	23	11	7	2	21	2	15	1	0
Max TOTAL			100	91	90	71	42	76	57	39	73	33	69	8	3

Table 2-1. A summary of the frequency with which 13 common typhoid symptoms are reported in case series from the published literature since the year 2000.

Case series are ordered by those from endemic regions ('endemic') and those occurring in returning overseas travellers ('traveller') and are arranged in order of symptom report completeness. Further detail regarding studies included is given in Supplementary Table 1. Symptom shade indicates the approximate frequency of the symptom; white cells indicate no data available.

2.4.2 CHRONIC CARRIAGE

The association between *Salmonella* Typhi and the gallbladder niche was made soon after its initial discovery by Erberth; in 1890, Gilbert and Girode demonstrated the presence of *S. Typhi* in the gallbladders of patients with acute cholecystitis.³⁷ It was subsequently recognised by Budd and others that, in addition to causing acute local infection, *S. Typhi* could be carried asymptotically for long periods of time (>50 years has been reported) and possibly indefinitely.^{60,161,162} Current UK public health definitions for convalescent carriage encompass those excreting *S. Typhi* in stool (after 2 courses of appropriate antibiotic therapy) for less than 12 months, while chronic carriers are those who continue to excrete bacteria for 12 months or more.¹⁶³

It is difficult to estimate how commonly chronic carriage occurs following, although rates of carriage in endemic areas are thought to be approximately 3-5%.^{14,164} Carriage is known to be more common in those with cholelithiasis and therefore increases with age, female gender,¹⁶⁵ and body habitus, although normal epithelial tissue may also act as an ecological niche.¹¹⁶ Carriers are therefore often only incidentally detected as a result of cholecystectomy;¹⁶⁶ 25% give no history of typhoid infection.¹¹² Difficulty in detecting carriage, given the asymptomatic, intermittent nature of bacterial shedding is a significant problem, both in investigating typhoid outbreak sources and in assessing the efficacy of vaccine programmes. Individual detection of chronic carriers may be improved through using a string test to sample duodenal contents,¹⁶⁷ although this of limited use in population level studies.¹⁴ Carriers may also be detected by serology using anti-Vi detection by ELISA,¹³⁸ although results are obfuscated by previous ViPS vaccination and inter-laboratory assay variation.

Aside from potential onwards transmission of infection, asymptomatic carriers pose two further important public health concerns. Firstly, is the relationship between chronic

gallbladder infection and subsequent development of gallbladder carcinoma, the risk of which is more than a confounding effect by the presence of gallstones.¹⁶⁸⁻¹⁷⁰ Secondly, several historical observations and contemporary modelling studies have highlighted the potential increasing importance of carriers in disseminating infection as the overall incidence of typhoid decreases.^{121,166,171} This is of concern if Vi vaccination schedules are to be rolled out, as non-sterilising vaccines may have no effect on (or actually increase) the pool of asymptomatic shedders in the populations targeted.¹²²

2.4.3 SEVERE INFECTION

Severe or fatal typhoid infection mainly occurs in endemic settings or in immigrants from those regions, but rarely in returning short-term travellers.^{151,172} Rates of severe infection in endemic areas range from 10-20% of cases with contributory factors including increasing patient age, longer illness duration and infection by ciprofloxacin intermediate susceptibility (but not MDR) strains.^{173,174} In general, severe or complicated infection is seen after at least 2 weeks of symptomatic illness,^{112,175} although there may be exceptions even with appropriate antimicrobial treatment.¹⁷⁶

Abdominal perforation as a result of chronic intestinal ulceration with or without haemorrhage is the most commonly seen severe complication (1-3%),^{177,178} resulting in death in up to 40% of cases (reviewed in ref.¹⁷⁸).^{174,178-180} Survival is affected by various considerations including delayed diagnosis, inappropriate treatment, gender, HIV co-infection and delayed or inadequate surgical intervention.^{174,176,179,181,182} Data suggests, however, that rates of perforation were not greatly affected by the historical introduction of antibiotics.^{174,179} A very high rate of perforation (43%) was reported during a recent epidemic in Uganda, however microbiological confirmation of infection was only available in 27/577 cases reported.¹⁸³ While associated with a high attributable mortality (94%), this study also highlights the complexity of case reporting and diagnostic

confirmation in resource-limited settings. A recent systematic review demonstrated a significantly higher case fatality rate following perforation occurring in Africa compared to Asia (CFR rate [95%CI]: 0.20 [0.17-0.22] vs. 0.11 [0.08-0.13]).¹⁷⁸

While many studies reporting typhoid incidence fail to report associated mortality rates, the overall mortality rate following typhoid infection is estimated to be approximately 1%.¹⁸⁴ Rates are considerably higher for sub-Saharan Africa and South Asia; 7.2 and 3.9/100,000 person years, respectively.¹⁸⁵ This high rate in Africa and its association with drug-resistant strains is especially concerning.¹⁸⁶

2.5 DIAGNOSIS

2.5.1 CLINICAL DIAGNOSIS

Making a clinical diagnosis of typhoid infection is notoriously difficult. In a sick returning traveller, corroborating travel and vaccination history may support the clinical decision-making regarding therapy, however early presentation and self-medication may mask other positive symptom details. Despite this complexity, algorithms have been produced using a 'syndromic' approach,^{175,187} or computer-aided decision-making.^{188,189} In addition, the World Health Organization has proposed standardized definitions for categorising of cases in surveillance reporting.¹⁹⁰ This approach has been found to out-perform serological assays in sensitivity for diagnosing possible cases (82.6%), but are poorly specific (36.3%) and require the patient to be febrile for at least 3-days.¹⁹¹⁻¹⁹³

Routine laboratory blood results are also frequently unhelpful although may support a clinical hunch. Full blood count findings including lymphopaenia and thrombocytopenia are non-specific, although there is some limited data supporting 'aneosinophilia' as being suggestive of typhoid.^{187,194,195} During the early disease course, several authors have

suggested that leucopaenia with a relative lymphocytosis and neutropaenia may be predictive.¹⁹⁵⁻¹⁹⁷ Some degree of liver function derangement occurs in approximately 50% of patients, however severe liver abnormality accompanied by jaundice is uncommon and may be due to coexisting aetiologies.¹⁹⁸⁻²⁰⁰

2.5.2 MICROBIOLOGICAL DIAGNOSIS

Laboratory confirmation to ensure an accurate diagnosis of typhoid infection requires the isolation (or detection) of the causative organism from a sterile site. Microbiological culture of bone marrow aspirate remains the gold-standard test,¹⁹² as it harbours 10-fold more bacteria than can be detected in blood.^{113,201} The procedure is often impractical and uncomfortable for the patient, however it may still retain an important role both to diagnose those who have been heavily antibiotic exposed, or in whom the procedure may find an alternative (often haematological) diagnosis.^{192,202-206} Sterile culture due to pre-hospital antibiotic exposure is a particular issue in endemic settings where it may be ubiquitous.^{204,207-209}

Blood culture is technically easier to perform and therefore frequently acts as the ‘*de facto*’ reference-standard. Culture facilities and trained personnel are still required however, and the test is less sensitive due to the few bacteria present.^{202,209,210} Data regarding the level of bacteraemia in human infection are limited; those available relate to patients with at least 4-days fever presenting in an endemic area.^{204,211} Wain and colleagues found a median of 0.3CFU/mL in peripheral blood (range, 0.1-399) compared to 9CFU/mL (range 0.1-1,580) in bone marrow samples, at least half of which were contained within circulating macrophages.²⁰⁴ Maybe surprisingly, qualitative blood cultures are more likely to be positive earlier in the illness course and numbers of bacteria may be higher in non-immune or less antibiotic-exposed populations.^{208,209} The sensitivity of ‘routinely’ performed blood cultures in those with acute infection is often reported as

40-80%, although contributory factors include volume of blood sampled (ideally >10mL), antibiotic exposure and delay between sample collecting and culture initiation.^{175,187,192,208}

A key advantage to performing culture over other methods remains the availability of the bacterial isolate for further testing including antimicrobial susceptibility testing.^{192,210,212}

Although non-sterile, stool is also often collected during the routine diagnostic work-up of patients with suspected typhoid infection. To optimise detection of *S. Typhi*, stool is frequently cultured in selenite enrichment broth with or without the addition of mannitol.^{202,213} In a non-sick individual a positive result may reflect chronic carriage rather than acute infection; in a symptomatic patient stool cultures often become positive immediately prior to the onset of infection and may remain positive longer than either blood or bone marrow.²⁰⁸ Sensitivity in acute infection is approximately 30% but may reach 60% in children.^{112,209} Several studies have demonstrated that when performed in combination, culture of blood, bone marrow and either stool or duodenal aspirate, may make the diagnosis in >90% of patients.^{208,214,215}

2.5.3 SEROLOGICAL DIAGNOSIS

Use of serum agglutination to diagnose typhoid infection was discovered in 1896 by Fernand Widal.^{216,217} Utilising visible agglutination to lipopolysaccharide (LPS; O) and flagella (H) antigens, this test ideally requires paired serum samples (taken in the acute and convalescent phase) to be tested to demonstrate a 4x fold rise in antibody titre.^{192,216}

Antibodies to these components are detectable at 6-8 and 10-12 days following infection, respectively. Major limitations to interpretation of single or paired results include lack of sensitivity and specificity due to, for example, prior antibiotic treatment or false-positivity in other infections including malaria and scrub typhus or recent vaccinations.^{216,218-220}

Although cheap, the Widal test is therefore of little value in diagnosing acute infection in

endemic settings, but is still commonly used with the premise that, if the baseline population titre is known, cut-off diagnostic thresholds may be set.^{192,221-223}

2.6 GLOBAL BURDEN OF INFECTION

Accurate estimates or calculations of the likely true burden caused by enteric fever are important, not least to allow for rational decisions to be made regarding vaccine implementation and to provide further incentive for affected areas to improve provision of clean water and sanitation facilities. Disease burden figures regarding enteric infection are complicated by the lack of useful surveillance tools and the paucity of field data from many endemic regions.²²⁴ Current estimates for the global burden of disease are based on few studies (13-22 over the last 25 years), of which fewer still contain primary surveillance data. Many contain figures derived from vaccine study control groups, and is therefore targeted towards specific, limited age groups. These figures are also frequently associated with improved outcomes due to additional interventions or enhanced case-management. Equally, both types of studies (surveillance or vaccine) are also more likely to have been performed in high-incidence settings, and therefore these data may be further difficult to generalise.²²⁴

2.6.1 DISEASE ESTIMATES AND GEOGRAPHIC DISTRIBUTION

Using adjustment for blood culture sensitivity, in 2000 Crump *et al* estimated 21.7 million new typhoid and 5.4 million new paratyphoid infections occurred each year globally resulting in 215,000 deaths (assuming a conservative case-fatality rate of 1%), mostly from *S. Typhi* infection.¹⁸⁴ Updated analyses, albeit with few additional data, confirm these figures estimating 26.9 million typhoid infections occur annually (interquartile range 18.3 - 35.7 million).¹⁸⁵ More specific community prevalence studies using blood-culture diagnostics often suggest that many enteric fever diagnoses are still not being accurately

captured using public health reporting systems.²²⁴ The burden of disease caused by enteric fever continues to predominantly affect those living in South East Asia, in particular children and younger adults (*see below*),²²⁵ although there are few accurate prevalence data available for Africa and South America.²²⁴ Recently reported notable outbreaks have occurred in Zimbabwe, Zambia, Fiji and the Philippines (Figure 2-1).¹⁸⁶

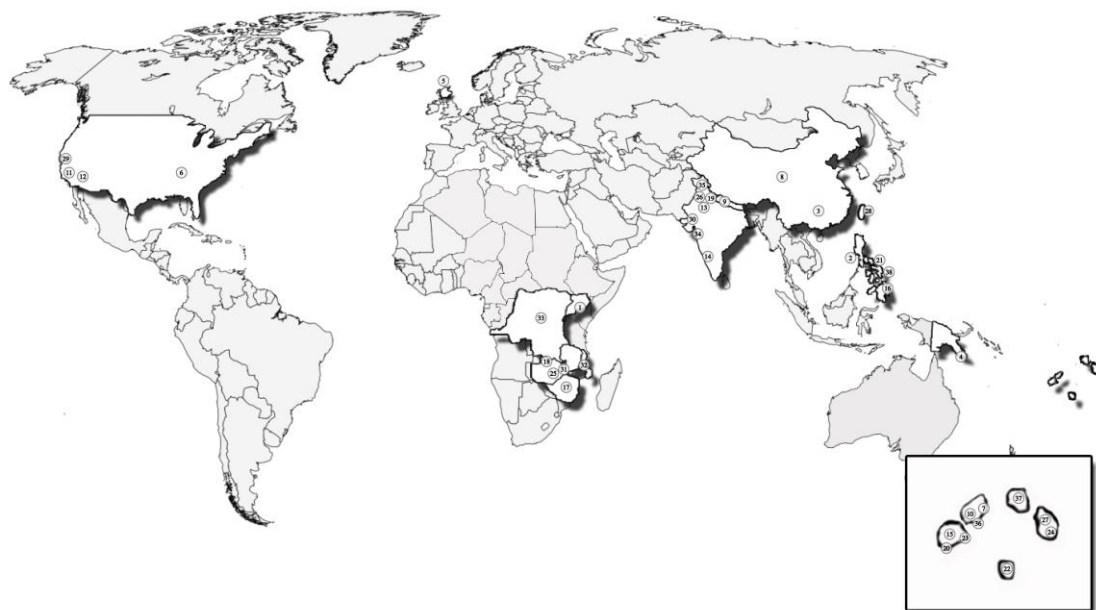


Figure 2-1. A map of recent typhoid fever outbreaks.

Outbreaks reported by the infectious diseases list-serve ProMED mail in the last 5 years (August 2008 and August 2013). From Darton and colleagues.¹⁸⁶

2.6.2 AGE/SEX DISTRIBUTION

In endemic settings young children and adolescents between 1 and 15-years of age are disproportionately affected by enteric fever.^{225,226} Traditionally, the burden of disease in infants was considered to be relatively low due non-specific presentation, difficulty obtaining confirmatory culture material, and the stringent (often 3 day) fever requirement in most clinical definitions.²²⁷ More recent data have demonstrated that approximately 25% of the disease burden may occur in those under 2 years old, and that illness may be severe and associated with significant mortality.^{182,228,229} Some studies report a comparatively higher risk of paratyphoid fever in this age group,²³⁰ while others

suggest that paratyphoid risk is higher in older age groups related to the apparent predominant transmission outside of the household setting.¹⁵⁴

Several studies have demonstrated no sex-bias in apparent exposure to typhoid, as ascertained using community seroprevalence surveys. Whether this is also true for presentation with clinical illness is less certain. In several settings apparently higher rates of infection among males have been reported. In a more detailed study looking at year- and age-specific risks for specific infectious diseases in Brazil, males had a higher risk of typhoid only in the 10-19 age category (at puberty).²³¹ Many other studies confirm this lack of gender association.^{154,230} Interestingly however, several studies have found an association between male gender, severe infection and both hospitalisation and mortality,^{173,178} which also appears to be specific to Asia rather than Africa or the rest of the world.

Age and sex effects are likely to be combinatorial with cultural factors and geographic region. In Kathmandu for example, no difference in age was found in *S. Paratyphi* cases, whilst males experiencing *S. Typhi* were significantly younger than their female counterparts (16 vs. 21.5 years),²³²; the reverse was seen in Brazil.²³¹

2.7 PREVENTION BY VACCINATION

2.7.1 EARLY SUCCESSES

Shortly after the successful culture of pure *S. Typhi* from the spleens of typhoid patients was made by Gaffky in 1881,⁶⁷ attempts were started to produce an attenuated live vaccine.^{233,234} By 1886 Fraenkel and Simmonds had achieved bacterial attenuation by passage through rabbits and mice and successfully demonstrated protection from re-infection with the wild strain.²³⁴ During a subsequent typhoid outbreak in 1893 and with

the success of heat-killed vaccines for cholera prevention, Fraenkel used a heat-killed typhoid vaccine given in suspension to ‘treat’ 57 typhoid patients.²³⁵ This success apparently inspired Pfeiffer and Kolle to perform human immunisation in 1896, using a heat-killed whole cell vaccine.²³⁶ Serum from the 2 subjects collected 11 days later was used successfully to provide passive protection in guinea pigs, and demonstrated agglutinating properties using the Widal’s recently discovered reaction.^{217,233,234}

At the same time (although a matter of great controversy),^{237,238} Almroth Wright gave a heat-killed typhoid vaccine preparation to a horse and subsequently two British Army officers, as part of his on-going investigations into the use of calcium chloride in this case for its anti-inflammatory effects.^{233,234} One of the officers was subsequently given a non-attenuated typhoid strain, and was apparently protected. Publishing the results of his experiments in the *Lancet*, within two weeks of their completion and two months prior to Pfeiffer and Kolles publication, allowed Wright to ‘claim priority’.²³⁷

Heat-killed vaccines were widely used for the next 60 years before evidence for efficacy was confirmed.²³⁹ Typhoid vaccination was initially offered to the British army in India and during deployment in the Boer War, where, among others, Sir Arthur Conan Doyle argued for their compulsory use.^{240,241} While typhoid vaccination was widely promoted, notably by Wright, Osler and Conan Doyle in Britain there was also fierce opposition to its use, for both societal and scientific reasons.^{234,242-244} Typhoid vaccination was made compulsory in the US army in 1911, German army in 1914 and British army (for enlisted troops but not officers) in 1915.^{234,244} The decision was certainly spurred on by the major epidemics seen: official German statistics report 116,000 soldiers were affected by typhoid during WWI, 8,000 of whom died during the first (pre-vaccine introduction) year of the war.²⁴⁴ Many other factors may have contributed to the dramatic fall in typhoid cases during the war and peacetime stages of the early 20th century however, not least radical improvements

in food and water quality and generally increasing awareness about bacterial disease causation. It was not until the 1960's that firm evidence of the benefit of typhoid vaccination was produced.

2.7.2 WHOLE CELL VACCINE

Whole cell vaccines (WCVs) to prevent typhoid infection have been produced by various means, including inactivation through acetone ('K' vaccine) or alcohol treatment, or heat-killed and phenol preserved ('L' vaccine). A series of WHO-sponsored studies performed in the 1950-60's prospectively assessed the protective efficacy (PE) of these WCVs. In 10 trials, over 1.5 million individuals were involved in these studies in a total of 21 vaccine arms.²⁴⁵ In summary, efficacy of the L formulation ranged from 51-81% depending on the region.^{239,246-248} Two doses were found to be more effective than one,²⁴⁹ and protection persisted for up to 5 years.^{248,250} The K vaccine was found to be more efficacious (PE, 66-94%),²⁴⁸ but was associated with more frequent and severe adverse events attributed to retention of the Vi-capsule during manufacture,^{234,251} and was also more expensive to prepare.^{239,246-248}

Meta-analysis suggest that the cumulative 3-year efficacy of WCV in preventing typhoid is 73% (95%CI, 65-80%).²⁵⁰ Use in vaccine programmes, for example in Thailand, where it was introduced into the national EPI schedule for 7-12 year old children from 1977, resulted in a 93% decline in typhoid admissions to hospital with similar effects on overall incidence over an 8-year period.^{252,253} Despite this apparent efficacy, WCV continued to be associated with high rates of local and systematic reactogenicity, which eventually led to cessation of the Thailand programme in the early 1990's.²⁵² While WCV is still licensed, it is no longer manufactured or used in national prevention programmes and recommendations suggest that less-reactogenic alternatives be used.²⁵⁴

2.7.3 TY21A

Increasing recognition of antibiotic-resistant *S. Typhi* strains in the 1970's coupled with the recognition that WCV implementation was unlikely to be feasible at a large scale, led to a resurgence of efforts to develop an efficacious, non-reactogenic oral vaccine.^{142,234,255}

First described by Germanier and Fuerer in 1975, *S. Typhi* Ty21a was derived by random chemical mutagenesis from the parent Ty2 strain using nitrosoguanidine.²⁵⁶ As a 'galE mutant', Ty21a is deficient in (UDP)-galactose-4-epimerase,^{256,257} leading to defective synthesis of the polysaccharide component of LPS.²⁵⁸ In the absence of galactose, Ty21a does not express smooth O-antigen and is not immunogenic; it may also be distinguished morphologically from the parent Ty2 strain (Figure 2-2). In the presence of galactose, LPS is expressed normally, but galactose-1-phosphate and UDP-galactose accumulate resulting in cell lysis, and supposed virulence attenuation.^{234,259} More recently, however, it has been demonstrated that *galE* mutation alone is insufficient to attenuate the strain, at least in mice, and that there are in fact many other contributory mutations.²⁶⁰ Whole genome sequencing has recently demonstrated that Ty21a has 679 SNPs relative to the parent Ty2 strain,²⁶¹ including mutations in each of the steps involved in Vi synthesis.^{258,262}



Figure 2-2. Morphological appearances of *S. Typhi* Ty21a and Ty2.

Ty21a appears as small blue-grey colonies and Ty2 as large yellow colonies when grown on BTB-gal agar from Kopecko and colleagues (ref. from R. Germanier).²⁵⁸ With permission Elsevier Ltd.

Further mutations found include alteration of *mpoS* and *ilvD*.^{258,263} As a result of these mutations Ty21a is less able to survive starvation states or to resist environmental stress, such as occurs in the small bowel.²⁶⁴

Preliminary studies demonstrating the efficacy of Ty21a vaccine were performed in North American volunteers using the human challenge model in Maryland (see: 3.1.1).¹⁴² With 5- to 8-doses (equivalent to 3-10¹⁰ live organisms) no significant side effects were reported, and a PE of 87% was found using a challenge dose that resulted in 53% of non-vaccinated volunteers developing infection (reviewed in ref.⁸⁹).^{257,265}

Following the successes of these studies, an initial field trial was performed in Egyptian schoolchildren in Alexandria using a 3-dose alternate day schedule.^{266,267} Over 36-months, 96% PE was demonstrated against blood culture positive infection ('confirmed' cases) or confirmed and positive stool plus either high O or H antibody titre ($\geq 1:400$) ('probable') cases.^{257,267}

Subsequent large-scale field trials (involving >300,000 participants) to assess lyophilised, enteric coated, gel or liquid formulations of Ty21a at different intervals and schedules were performed in the higher incidence settings of Santiago, Chile and Indonesia.²⁶⁸⁻²⁷¹ The studies performed in Chile demonstrated that 3-doses of Ty21a given over the course of a week conveyed a 67% PE which lasted for ≥ 7 -years.^{142,270,272} Also of note, was the apparent herd protection effect demonstrated in neighbouring placebo control groups in Chile,¹⁴² and the suggestion that those vaccinated may have had lower rates of faecal *S. Typhi* excretion.¹⁴¹

Rates of protection were lower in a subsequent Indonesian study involving 20,543 participants.²⁷¹ PE over 30 months of follow-up was 53% and 42 % for 3-doses of the liquid or enteric-coated formulation, respectively.²⁷¹ Of note, neither was found to protect

against *S. Paratyphi A* infection. Reasons for this poorer performance are relevant, and thought to relate to the much higher force of infection (attack rate and presumed higher infectious inoculum): 1,206 in Indonesia,²⁷¹ 103 in Chile,²⁶⁹ and 49/100,000 cases per year in Egypt.²⁶⁷ This difference in efficacy was not seen in all groups, however, and therefore additional factors such as vaccine variability may also have been important.²⁷¹

Although varied in time and place performed, vaccine formulations used and underlying rate of infection, meta-analysis of the five Ty21a field trials demonstrates a cumulative protective efficacy of 51% (95%CI, 35-63).²⁵⁰ While application of meta-analysis to this highly heterogeneous dataset has drawn heavy criticism,^{273,274} these data underscore the repeatedly poor performance seen when oral vaccines are utilised in endemic settings.²⁷⁵

The efficacy of Ty21a in preventing infection in non-immune individuals such as travellers is not well determined,²⁵¹ despite the early demonstration of efficacy in challenge study participants. Important data suggest that vaccines containing fewer viable bacteria (3-doses of 10⁹ Ty21a were studied) do not result in any measurable immune response in individuals not previously exposed to infection or vaccination or in young children in endemic settings.^{146,276} Studies of sick travellers seeking healthcare in Nepal, suggest that Ty21a may be almost ineffective in protecting this group in particular,¹⁵⁸ and several studies have shown compliance with vaccine schedules to be poor.^{277,278} Recent development of a room stable preparation may improve this, at least in travellers.²⁷⁹

A major improvement of Ty21a over its WCV predecessors is its safety; post-marketing surveillance has identified only mild and infrequent adverse events. From 1990 to 2000 >38 million people were vaccinated with Ty21a which resulted in 743 spontaneous reports of adverse events (incidence of 0.002%).²⁵⁹ In addition there has been no evidence of reversion to virulence in either the master or working seed lot over time,²⁵⁸ and in total >200 million doses have been given during the last 25 years.^{258,261}

How immunologic protection by Ty21a is mediated is unknown, and there is no widely accepted correlate for protection following vaccination.¹¹⁸ Oral Ty21a vaccination elicits some modest anti-LPS and H IgG serum antibody response, intestinal secretory IgA, and both CD4⁺ and CD8⁺ T-cell responses.^{118,280-282} In addition there is some evidence for cross-protective immune responses to *S. Paratyphi* A and B following Ty21a vaccination, consistent with scattered reports of fewer paratyphoid clinical cases during periods of more intense Ty21a prescribing.^{153,283}

2.7.4 VI-POLYSACCHARIDE

The surface capsular polysaccharide of *S. Typhi* is thought to be one of the major typhoid virulence factors, hence its abbreviation 'Vi'.²³³ First recognised by Felix and Pitt in 1932, Vi is almost invariably expressed by clinical isolates and is therefore key to Kaufmann-White identification in the laboratory.⁶ Thought to be essential for bacterial survival in the blood, through avoidance of complement-mediated lysis and phagocytosis (and recently demonstrated avoidance of neutrophils),^{126,259} Vi-expressing strains were found to have a lower LD₅₀ in mice and produce higher attack rates in humans.²⁸⁴⁻²⁸⁶ Additionally, passive transfer of Vi antibodies (of either animal or human origin) conferred protection against lethal challenge with Vi-expressing strains in laboratory mice.²⁸⁶ Initial studies exploring the role of Vi antibody in prevention of infection were fraught with difficulties, however, due to degradation of Vi during the preparation process and the use of impure antigens in insensitive assays.²⁸⁶

There was evidence that vaccination with Vi could provide clinical protection, however, which included superior efficacy of the Vi-expressing acetone/alcohol-inactivated WCV preparation,¹⁴² and detection of very high anti-Vi antibody titres in chronic carriers, which fitted with the pattern of re-exposure infection risk.

Subsequent more sensitive methods for generating Vi proved effective in providing safe, more immunologically active vaccine supplies.²⁸⁷ The efficacy of Vi vaccination was confirmed in field trials performed in Nepal and South Africa in the mid-1980s.^{135,136} In both studies, the levels of immunogenicity seen with either 25 or 50µg doses were identical, whereas the higher dose was associated with more erythema and pain at the injection site.²⁸⁸ In South Africa, the cumulative vaccine efficacy over a 3-year period for blood-culture conformed cases was 55% (95%CI, 30-71) while efficacy in Nepal (for clinically suspected and blood-culture confirmed cases) over 20 months was 77% (59-87).^{135,289}

Despite apparently good protection and immunogenicity persisting for at least 2-years, responses were not boosted by subsequent doses of Vi polysaccharide being given as characteristic of a TI-2 antigen.^{233,290} Similarly, the vaccine is unlikely to be immunogenic in young infants and it is therefore licensed for those aged 2 and over,²⁸⁸ although the level of protection in 2-5 year olds is unclear from the studies performed.²⁹¹

Two subsequent large studies performed in South-western China in the late 1990s, evaluated the efficacy of a locally produced vaccine containing 30µg Vi polysaccharide.²⁹² This followed on in a programme that had previously used WCV and Ty21a, which were discontinued due to high rates of reactogenicity and cost, respectively.²⁵² In the two studies, 81,000 then 131,000 mainly school-aged children were vaccinated, with resulting protective efficacies of 71% over 12 months and 69% (95%CI, 28-87) over 19 months of follow-up.²⁹² As a result of these highly successful studies, 10 million doses of the vaccine were distributed in 1997. As found in South Africa however, long-term follow-up of protection demonstrated that by year 3 it had fallen to borderline significance (cumulative PE, 74% [31-90]; Year 3 PE, 51% [-95-88]).²⁹³

Despite clear recommendations regarding the introduction of typhoid vaccines in communities and populations at high-risk by the WHO,²⁵⁴ there has been almost no

implementation in vaccine programmes.^{252,294} Reasons for this are diverse but include logistic and programmatic issues in addition to specific questions such as whether Vi vaccination is effective in the high-risk 2-5 year age group.^{294,295}

To further directly address these questions of feasibility and efficacy, a large cluster-randomised study was performed in Eastern Kolkata.^{295,296} Overall protective efficacy (Typherix containing 25µg Vi polysaccharide) against febrile, blood-culture confirmed typhoid fever was 61% (41-75).²⁹⁵ PE was highest in the youngest age group vaccinated (2-5yrs, 80% [53-91]), falling in the older 5-14.9 (56% [18-77]) and >15 (46% [-43-79]) year age groups.²⁹⁵ This is contrary to the field trial findings of WCV and oral Ty21a vaccination, in which older age groups demonstrated more benefit.²⁵⁵ A further important finding from this study was the apparent demonstration of an indirect herd protection effect. Those individuals not receiving vaccine but living in clusters where it was dispensed had a significant level of protection against developing infection (44% [2-69]).²⁹⁵ This finding would have a major effect on the impact of typhoid vaccine introduction on transmission and in the calculation of the cost-effectiveness for such programmes.^{121,122} Disappointingly, the findings of indirect herd protection, significant protection in the 2-5 year age group or an overall protective effect were not replicated in a subsequent field trial performed in Karachi.²⁹⁷ Reasons for these divergent findings may include different vaccine target populations (all vs. children only, who may not be responsible for the bulk of shedding/transmitting infection) and differing migration patterns into the study area,^{122,297} although the parallel immunogenicity study findings in Pakistan are more in keeping with previously available datasets.^{295,297-300}

Correlates of protection resulting from Vi vaccination have been suggested, based on studies performed with both polysaccharide and Vi-conjugate vaccines (*described further below*). Analysis of immunogenicity data from the South African study,¹³⁶ demonstrated

that a serum anti-Vi antibody titre in the range 0.6-1.2 μ g/mL (as measured by radio-immunoassay) conveyed a significant level of protection to vaccine recipients compared to controls until 3 years after vaccination.²⁸⁹ From this range a slightly arbitrary 1 μ g/mL was suggested as a possible conservative cut-off level.²⁸⁹ Further follow-up of these participants 10-years later demonstrated that 58% in both vaccine and control arms had a protective level (\geq 1 μ g/mL), suggesting that gradual exposure to *S. Typhi* (or the identical *Citrobacter freundii*) polysaccharide antigen through childhood conveys eventual protection from infection.³⁰¹ Further refinement of this estimate using data from the rEPA-Vi studies and two methods (percentile distribution and relative risk estimation) supported these data suggesting a protective threshold of 1.4 μ g/mL or 10 μ g/mL Vi IgG measured 4-years or 6-months after vaccination, respectively.³⁰²

2.7.5 SHORTFALLS OF THE CURRENTLY LICENSED VACCINES

While each of the currently available licensed vaccines appears to offer similar moderate levels of protection against typhoid, there are several important caveats to making this comparison. Firstly, no direct comparative trials have been performed to assess this accurately. Secondly, while previous attempts to make comparisons using meta-analyses and systematic review has been performed, comparison of these data in this way is highly limited due to the variable vaccines, formulations, populations, age-groups and background infection prevalence in each of the different settings. Thirdly, the majority of prospective studies performed so far have assessed vaccine efficacy in endemic settings and in young children; the applicability of the findings to other age groups and non-endemic settings is therefore uncertain. The only exceptions are those using WCV and Ty21a, which were performed in healthy adult volunteers challenge studies at the University of Maryland. Thus, none of the currently available vaccines has proven

effectiveness in preventing typhoid infection in healthy non-previously exposed adults at the doses or using the schedules currently approved by the licensing authorities.²⁵¹

Specific reasons for the limited use of these vaccines in national control programmes include the limited support provided by major donors (e.g., GAVI and UNICEF). Concerns included programmatic utility, high cost, lower efficacy in young children, the short duration of protection and requirement for revaccination (Table 2-2).

Vaccine	Advantages	Disadvantages
Vi-PS	<ul style="list-style-type: none"> Single dose Suitable for mass campaigns Monitoring coverage is easier Cheaper (~\$0.50 only) Multiple manufacturers 	<ul style="list-style-type: none"> Protection is short-lived (< 3 years) Injection waste management needed Injection safety issues Cannot be given to children ≤ 2 years Does not elicit immunologic memory Injectable, requires trained personnel to administer Hypo-responsiveness may occur in some subjects re-immunized after 3 years
Ty21a	<ul style="list-style-type: none"> Oral administration; needs no special training to administer Capsule, easy to handle No injection equipment required No injection waste and no injection safety issues Can easily be administered in a settings such as schools, military bases, refugee camps etc. No problem with hypo-responsiveness upon re-vaccination Partial cross protection against <i>Salmonella</i> Paratyphi B (but not against <i>S. Paratyphi</i> A) Longer duration of protection and less frequent re-immunization 	<ul style="list-style-type: none"> Campaigns are challenging as 3 doses must be given on alternate days (4 dose in US) Protection is 5-7 years More expensive (~ \$ 1.25 per capsule) Cannot be given to children ≤ 5 years (capsule), and children ≤ 2 years (liquid formulation) Monitoring coverage can be complicated

Table 2-2. A summary of the advantages and disadvantages of ViPS and Ty21a typhoid vaccines.
Adapted from Martin, ref.³⁰³.

Further issues have arisen over the last few years relating to vaccine availability. Initially there was global shortage of Vi vaccine (Typherix, GSK) caused by a delay in new manufacturing facility completion, and a subsequent decision to focus on EPI schedule

vaccines.^{304,305} A short time later, testing of an alternative Vi vaccine (Typhim, Sanofi Pasteur MSD) identified that there was insufficient antigen in several of the released batches and 16 batches (~88% of stock) were recalled.³⁰⁵⁻³⁰⁷ While there were no specific safety issues, those who had received vaccines from the affected batches were advised to seek alternatives. Typhoid vaccine shortage was further compounded by news that the main manufacturing facility for Ty21a (Vivotif, Crucell) in Europe was to close, with no current plans to restart production elsewhere apparently due to the long-term strategy of its parent company (Johnson & Johnson).^{308,309}

2.7.6 DEVELOPMENT OF NEWER TYPHOID VACCINES

Acknowledging these shortfalls, there has been considerable renewed effort to develop vaccines with improved efficacy, in particular for young children as part of routine immunisation or EPI schedules. Manufacture of new vaccines will also preferably be based in an endemic region; this has been spurred on by WHO pre-qualification of the existing typhoid Vi vaccine (Typhim, Sanofi Pasteur), which allows procurement by UN agencies and is a prerequisite for GAVI Alliance support.

The main approaches being taken include the development of Vi vaccine conjugates, live-attenuated oral vaccines (summarised in Table 2-3), and vaccines targeting a broader spectrum of pathogens, in particular combination vaccines for enteric fever. There have been several recent reviews regarding the development of novel vaccines for typhoid and the current developments are therefore summarised briefly below.^{303,310}

2.8 VI-CONJUGATE VACCINES

A key limitation to the use of a TI-2 antigen such as the repeating epitope of the *S. Typhi* Vi capsular polysaccharide, is the lack of an immune response amenable to boosting by

provision of additional doses or in those younger than two years of age. These properties would allow it to be included in the routine childhood immunisation schedules.³¹¹

Vaccine	Description	Sponsor – stage of development
Vi-conjugate vaccines - injectable		
Vi-rEPA	<i>S. Typhi</i> Vi conjugated to recombinant <i>P. aeruginosa</i> exoproteinA	NIH, US, transferred to Lanzhou Institute (China National Biot. Group), ³⁰³ China – phase III (licensure applied for)
Vi-tetanus toxoid	<i>S. Typhi</i> Vi conjugated to tetanus toxoid	Bio-Med (licensed in India, 2009) – phase III Bharat Biotech (licensed in India, 2013) – phase III
Vi-diphtheria toxoid	<i>S. Typhi</i> Vi conjugated to diphtheria toxoid	Shantha Biotech – clinical programme commencing? Transferred from IVI to manufacturers in: S. Korea (SK Chemicals) – phase I Indonesia (PT BioFarma) – phase 1
Vi-CRM ₁₉₇	<i>Citrobacter</i> Vi conjugated to nontoxic mutant of diphtheria toxin	NVGH transferred to Biological E (India) – phase I (4Q2015)
Unknown		Finlay Institute, Cuba – near licensure EuBiologics, S. Korea – pre-clinical Incepta, Bangladesh – pre-clinical
O-polysaccharide conjugate - injectable		
	<i>S. Typhi</i> O-specific polysaccharide conjugated to diphtheria toxin	IVI- preclinical
Live-attenuated vaccines - oral		
CVD908-htrA	Ty2 parent with mutations in <i>aroC</i> , <i>aroD</i> , and <i>htrA</i> genes	University of Maryland -?
CVD909	Ty2 parent with mutations in <i>aroC</i> , <i>aroD</i> , and <i>htrA</i> genes; constitutive Vi expression	University of Maryland with Bharat biotech – phase II (monovalent and combination with CVD1902 for <i>S. Paratyphi</i>)
Ty800	Ty2 parent with mutations in <i>PhoP/PhoQ</i> genes ³¹²	Avant Immunotherapeutics – phase II
Mo1ZH09	Ty2 parent with mutations in <i>ssaV</i> and <i>aroC</i>	Emergent BioSolutions – phase IIa/b

Table 2-3. Summary of novel vaccine approaches currently under investigation.

Adapted from Martin, ref.³⁰³ and Coalition against Typhoid data.

Early results demonstrated that native Vi was superior over lower molecular weight derivatives, and that conjugation to Vi using tetanus toxoid was technically complex due to the size and rigidity of the Vi molecule, and its relative insolubility.³¹¹

2.8.1 VI-REPA

Work performed at the NIH demonstrated the immunogenicity and safety of two Vi-conjugate constructs in small animal models and human volunteers (reviewed in ref.³¹³).³¹¹ As alternatives to the larger more traditional conjugate proteins, the B subunit of the heat-labile cholera-like enterotoxin (LT-B) of *Escherichia coli* or the recombinant exoprotein A (rEPA) of *Pseudomonas aeruginosa* were used as Vi carriers. Both constructs yielded higher anti-Vi IgG antibody titres than Vi polysaccharide alone and were well tolerated. Vi-rEPA was selected for further clinical evaluation based on its (non-significant) higher antibody responses.

The safety and immunogenicity of the Vi-rEPA vaccine was subsequently assessed in Vietnamese adults and children (≥ 2 years),³¹⁴ demonstrating that the vaccines were safe and immunogenic in an endemic population, and also that a novel method of conjugation produced a more immunogenic vaccine formulation. The response was also amenable to boosting 6-weeks after primary vaccination.

The pivotal double-blind efficacy study of rEPA-Vi was performed in 2001, also in Vietnam.³¹⁵ In this study ~6,000 2-5 year old children received either 2 doses of Vi-rEPA vaccine or placebo given 6-weeks apart. The calculated PE against febrile, blood-culture confirmed typhoid infection was 91.5% (77.1-99.6); even a single dose of the vaccine demonstrated 88% PE over the 27-month follow-up period.³¹⁶ Over the full 46-month follow-up period, Vi-rEPA PE was 89% (76-97).³¹⁷ Further studies were performed to demonstrate that the vaccine did not interfere with EPI scheduled vaccinations,³¹⁸ and long-term follow-up of the original immunogenicity study suggested that protective levels of antibody were still detectable 10-years later.³¹³ A major limitation to use of the rEPA carrier protein is that it is not approved by European or US regulatory agencies, and there are no existing vaccines using this carrier.³¹³

2.8.2 OTHER VI-CONJUGATES IN DEVELOPMENT

Several other companies and collaborations are in the process of developing alternative Vi-conjugate vaccines using more traditional carrier proteins. The development of these vaccines is briefly summarised in Table 2-3.

2.8.3 O CONJUGATES

In part to forestall the emergence of Vi-negative strains following selective Vi vaccination, there has been renewed interest in the development of vaccines using the *S. Typhi* O₉ polysaccharide component, which, together with Lipid A, forms surface expressed lipopolysaccharide.^{310,319} These constructs would also have the theoretical advantage of protecting against non-typhoid serovars, in particular *S. Enteritidis*, which could be especially important in high HIV settings, such as sub-Saharan Africa.³²⁰

2.9 LIVE ORAL VACCINE CANDIDATES

There are several reasons why the goal of achieving protection using an orally delivered vaccine has continued to be desirable, both scientific and logistic.

Firstly, after invasion *Salmonella* sp. are able to target, invade and survive within mammalian cells including those of the immune system. This direct interaction makes *Salmonella* sp. highly immunogenic in the correct host environment, which makes them ideal candidates for antigen delivery, both of native bacterial components and as 'foreign' antigen delivery systems.^{321,322} Multiple efforts to attenuate *Salmonellae* (in order to prevent infection developing) by introduction of specific genetic mutations have been made. These have targeted bacterial components including biochemical pathways, global regulatory systems, stress proteins, other regulatory genes and putative virulence components.^{141,321} Whilst many of these vaccine/delivery candidate had been evaluated in

animal models, until relatively recently few had been tried in the disease relevant host (i.e. humans).

Secondly, while the mechanisms of individual protection following receipt of one of the currently licensed vaccines are not clearly understood, a major advantage of oral vaccination is the induction of CMI and mucosal immune responses in addition to generation of non-Vi antibody. Vaccination with Ty21a is thought to reduce the shedding of bacteria in stool,¹⁴¹ and therefore effective oral vaccination may reduce transmission of infection to the community in addition to reducing the risk of long-term carriage.

Thirdly, the practicalities and delivery costs of administering a single dose oral vaccine to a target population are more favourable than that of an IM injection. An intramuscular vaccine requires needles and syringes for delivery with the associated expense and logistics of disposal of contaminated sharps material. Additionally, programmatic introduction of a vaccine would require fitting in to or re-arrangement of the EPI schedule if young infants were to be targeted. For older children at school or in containment of an outbreak, dispensing of a single oral dose of vaccine would be cheaper and logistically easier to arrange.

Finally, incorporating its role as a potential antigen delivery system, would allow the use of an effective vaccine for typhoid to also immunise populations against multiple other enteric pathogens.^{75,322} This may be achieved mechanistically by engineering bacteria to contain expression plasmids or by delivery of eukaryote genes, expression of which is triggered by the intracellular mammalian environment (DNA vaccines).^{75,322,323} Proof of these concepts has been performed with a number of enteric and mucosal antigens, including *Streptococcus pneumoniae*,³²⁴ *Vibrio cholerae*, *Plasmodium falciparum*, *E. coli*, and Hepatitis B antigens [reviewed in ref.³²⁵].

Some of the most promising novel oral typhoid vaccine candidates are summarised briefly below (and reviewed in ref.³²⁶).

2.9.1 CVD908 & CVD908-HTRA

One mechanism for achieving pathogen attenuation has been to target the aromatic hydrocarbon acid biosynthesis pathway. *Aro* deletion, auxotrophic mutants of *S. Typhimurium* and of *S. Typhi* have demonstrated safety and immunogenicity in mice, cattle and in humans as both vectors and vaccines.³²⁷ CVD908 and CVD906 were designed as double $\Delta aroC\Delta aroD$ mutants of the parent wild-type strains *S. Typhi* Ty2 and ISP1820, respectively.^{328,329} Despite being safe and immunogenic in initial studies,³²⁸ several participants developed vaccinaemia after CVD908 administration and CVD906 caused fever in recipients.³²⁹ Further attenuation was performed by deletion of the heat-shock protein *htrA* which resulted in safety and immunogenicity, albeit slightly less so than the *htrA*⁺ strains.³³⁰

A further phase II study was performed using a commercially prepared live lyophilised version of the vaccine.³²⁷ In 80 healthy adult US volunteers receiving 2 different doses of vaccine, a wide range of immunogenic responses including anti-LPS and anti-H humoral and CMI responses were demonstrated after a single dose.^{326,327}

2.9.2 CVD909

A noticeable feature in the previous live oral vaccine studies was the failure to generate serum anti-Vi or Vi ASC responses;³²⁷ a rational next step was therefore to engineer a vaccine strain which constitutively expressed Vi. This had been attempted previously with a Vi-expressing Ty21a strain, although when studied in humans it failed to generate an anti-Vi serum, ASC or secretory antibody response.³³¹ CVD908 was further engineered to constitutively express Vi, and designated CVD909.^{326,332,333}

Initial human studies demonstrated that CVD909 was well tolerated and that reinstatement of Vi expression did not restore natural virulence. While anti-Vi ASC responses were seen, there was no apparent generation of an anti-Vi response.³³³ Subsequent studies demonstrated that a serum anti-Vi response could be generated following CVD909 priming and Vi vaccine boosting,³³⁴ and that this response was bactericidal to both *S. Paratyphi* A and B, in addition to *S. Typhi*.³³⁵

2.9.3 Mo1ZH9

An alternative strategy to further attenuate the *aro* mutants, so as to avoid the undesirable vaccinaemia associated with CVD908 and reactogenicity with CVD906, was to target the specific *Salmonella* pathogenicity island 2 (SPI-2).³³⁶ SPI-2 encodes a type-III secretion system (TTSS), which is required for the transfer of virulence-associated effector proteins across the host cell membrane. Both SPI-1 and -2 are distinct to *Salmonella*; SPI-2 is activated intracellularly and is essential for replication within phagocytic cells such as macrophages.³³⁷ There are at least 10 effector molecules involved in intracellular SPI-2 employment, at least in *S. Typhimurium*.^{336,337} One of the key integral effector genes is *ssaV*, as the *ssaV* protein forms part of the secreton, the needle-like organelle that exports proteins across the inner and outer bacterial cell membranes.³³⁶ Mutations in *ssaV* render bacteria unable to secrete SPI-2 effectors, and hence it was selected as a possible attenuation target.³³⁶

Two potential vaccine candidates demonstrating potential immunogenicity include *Salmonella enterica* serovar Typhi (Ty2 *aroC ssaV*, designated ZH9) and serovar Typhimurium (TML *aroC ssaV*, designated WT05).³³⁶ The *S. Typhi* ZH9 construct was formed by introduction of the pYCVC21 plasmid containing the 4.8kb *HindIII* fragment (encoding the mutated *aroC* gene) into the Ty2 serovar by electrocorporation.³³⁸ Selection of the mutations was performed by growth in ampicillin (to which ZH9 is

resistant) and 5% sucrose, and subsequently by inability to grow in the absence of supplemental aromatic compounds.³³⁶ Resulting isolates were confirmed by PCR and gene sequence analysis, from which one (*S. Typhi* Ty2 Δ aroC) DTY8 was selected for *ssaV* gene mutation. Mutation of the *ssaV* gene was performed by inverse PCR introduction of a 1,893-bp deletion into *ssaV* open-reading frame contained with the plasmid pTYSV21 to form pYDSV214. This plasmid construct was introduced into DTY8 by electrocorporation. Selection of the desired mutants was performed by PCR identification of ampicillin-sensitive, sucrose-resistant strains containing the *ssaV* deletion. From the 5 isolates identified, one, *S. Typhi* (Ty2 Δ aroC Δ ssaV) ZH9 was selected for further development and assessment. To-date there has been 6 clinical trials to evaluate Mo1ZH09 in humans.³³⁹

A first dose-escalation study (**MS01.01**), using a frozen formulation with doses of 10^{7-9} CFU, demonstrated no evidence of positive blood or urine cultures, or febrile episodes in 9 participants.³³⁶ Several participants experienced mild gastrointestinal symptoms, although overall the vaccine was well tolerated and was immunogenic in 7/9 participants. A further larger study was performed (**MS01.03**) in which 48/60 participants received active vaccination with $5 \times 10^{7-9}$ CFU Mo1ZH09.³⁴⁰ This study confirmed the tolerability and safety of the vaccine and, in addition, confirmed that (ASC and serum anti-LPS IgG) immune responses were seen in the majority of those given the highest (5×10^9 CFU) dose. Importantly, this study also confirmed that the vaccine strain did not persist long term; stool cultures performed at 3 and 6 months were negative.³³⁹

Further studies were performed to assess the tolerability of a lyophilised freeze-dried formulation of Mo1ZH09 in two differing presentations (**MS01.04**).³⁴¹ In the first, participants consumed 100mL 2%(w/v) sodium bicarbonate solution 5-20 minutes prior to receiving the vaccine in a further 50mL sodium bicarbonate solution (using Evian™ mineral water). In the second, participants received the vaccine in 150mL 1.75%(w/v)

sodium bicarbonate solution, which also contained 1.1%(w/v) ascorbic acid and 0.02%(w/v) aspartame (in tap water).³³⁹ In an open-label randomised trial, 5×10^9 CFU were given to 32 participants in each of the presentations. Both were well tolerated with mild gastrointestinal side effects reported by only a few participants. Immune responses demonstrated broad humoral, mucosal and cellular responses to both preparations in most participants. In particular, seroconversion (>4-fold increase in serum anti-LPS IgG) was seen in 81 and 53% of participants given Mo1HZ09 with presentation 1 or 2, by Day 28 after vaccination, respectively. This was superior to any other single-dose typhoid vaccine response. Furthermore, ELISPOT measurement of anti-LPS specific IgA secreting plasma cells, which had been reported as correlating with field trial efficacy,¹⁴³ demonstrated equivalency with both 3-dose Ty21a and CVD-908*htrA* vaccination.

These original studies had been performed in the UK (London) and US, and so to assess safety and immunogenicity of the vaccine in an endemic setting the subsequent two studies were performed in Vietnam (Ho Chi Minh City).³⁴² In the initial exploratory study (**MS01.07**, *unpublished material*), 16/27 adult participants received active vaccination with a single dose of freeze-dried Mo1ZH09.³³⁹ Vaccine was generally well tolerated and safe with no episodes of bacteraemia or urine excretion reported. In total, 69% (11/16) of the participants developed an immune response defined in the primary immunogenicity endpoint as either $\geq 4 \text{ASC}/10^6$ PBMCs secreting anti-LPS IgA at Day 7 and/or a ≥ 4 fold increase in serum anti-LPS IgG titre between baseline and Day 28.

A further larger study was performed in a population of paediatric participants aged 5-14 years, of whom 71% were 5-10 years old (**MS01.08**).^{339,342} Of 151 children enrolled, 101 received active vaccine. There was no difference in the AE reporting rate between vaccine and control groups and the vaccine was well tolerated with no SAEs or bacteraemia. Vaccine shedding was found in 51% of participants, which lasted ≤ 3 days.

Mo1ZH09 was also found to be immunogenic in this population, with an 81% (95%CI, 68-89) difference in response rate between vaccine and placebo arms being reported (serum anti-LPS IgG at Day 14/28 and/or anti-LPS IgA at Day 7/14). Of the 28 participants whose vaccine responses were also assessed by anti-LPS IgA ELISPOT, 100% had a detectable immune response.³⁴²

The most recent study performed was a phase II multi-centre, double-blind, placebo controlled dose escalation study to evaluate the proposed drug potency specification for Mo1ZH09, now being manufactured at a new facility in Germany (IDT Biologika).³³⁹ Four different dose levels were used ranging from 5×10^9 to 1.7×10^{10} , and, in total, 150 healthy adult volunteers were enrolled (**MS01.13**).³⁴³ There were no SAEs or bacteraemias reported in the study; one participant had transient low-grade fever and nausea appeared to be more commonly reported by participants in active vaccine groups (19% vs. 14%).³⁴³ At the highest dose level, 5 participants shed vaccine strain *S. Typhi* in stool samples beyond day 7: 2 to day 11, 1 to day 14 and 2 to day 17.³³⁹ All were asymptomatic. At the 7.5×10^9 CFU dose, 95% (88-100) had an immune response consisting of ≥ 4 ASCs/10⁶ PBMCs above baseline by anti-LPS IgA ELISPOT at day 7 or ≥ 4 -fold rise in serum anti-LPS IgG at Day 28.

2.10 SUMMARY

Eradication of *S. Typhi*, as a human-restricted pathogen with no environmental reservoir, should be eminently achievable. Despite only partial understanding of infection pathogenesis, major reductions in disease burden have been achieved through improvements in living standards in affluent countries. Recent advancements in diagnostics and vaccine development may now provide a means to redress this imbalance in resource-limited settings, thus enabling control and eventual eradication of this human blight.

Chapter 3. HUMAN CHALLENGE STUDIES AND THEIR ROLE IN VACCINE DEVELOPMENT

3.1 HISTORICAL CHALLENGES

As a method of clinical research, deliberate exposure of human subjects to known (or putative) disease causing agents has been in existence for nearly 300 years. Following the introduction of variolation to the English royal court by Mary Montagu in 1721, studies of its safety were performed in orphaned children and inmates at Newgate Prison.³⁴⁴⁻³⁴⁷

Edward Jenner famously demonstrated the safety and effectiveness of vaccination as a less harmful alternative in 1876 when, six weeks after vaccination with cowpox pus, a neighbour's son (James Phelps) was deliberately exposed to small pox material by variolation.³⁴⁷ The lack of systemic reactogenicity paved the way for further studies and the field of vaccinology.³⁴⁶ Challenge studies have been applied to a wide range of research questions, including confirming the aetiological agents for particular disease

processes, identifying specific virulence factors and attempting to better understand the immunological response to infection (Table 3-1, and reviewed in ref.⁹⁶).

Unfortunately, deliberate exposure of humans to infectious agents with known high pathogenicity or long-term sequelae risk has also been performed for more malevolent reasons and with little ethical restriction, both in war and peacetime settings.³⁴⁸ These include those performed in concentration camps by doctors working for the 3rd Reich during WWII, research performed at Unit 731 by the Japanese in Mongolia (1937-1945), and many others.³⁴⁹⁻³⁵¹ At the other extreme, there are also many famous examples of researchers deliberately exposing either their own family members or themselves, not infrequently leading to major discoveries and Nobel Prizes.³⁵²⁻³⁵⁵

Two centres making major positive contributions through the use of human challenge studies include the Common Cold Unit based at Salisbury (between 1946-1989) and the University of Maryland School of Medicine, which performed human typhoid challenge studies between 1952 and 1974.^{356,357}

3.1.1 THE MARYLAND TYPHOID CHALLENGE STUDIES

Human typhoid challenge studies were initiated in Maryland in 1959, using consenting adult volunteers from the Maryland House of Corrections (reviewed in refs.^{89,282}).^{285,357-359}

The rationale for performing these studies using human volunteers was clearly stated:

*“Since no adequate laboratory or animal model was (or is) available to predict vaccine efficacy...”*³⁵⁸

In total, 1886 adults were successfully and safely involved in the typhoid challenge studies performed in Maryland.³⁶⁰ Initial attempts to recreate the clinical pattern of infection using 6,000CFU of a Ty2 strain originally isolated during the Spanish-American War (1898) failed.³⁵⁷ Later studies used a strain, then recently isolated from extracted gallbladder of a known chronic carrier, Mrs Quailles.

Question	Example(s)	References
Elucidate pathogenesis	Enterotoxigenic <i>Escherichia coli</i> (ETEC) – confirmation of aetiology and identification of pathogenic mechanism	361
	Enteropathogenic <i>Escherichia coli</i> (EPEC) – confirmation of pathogenicity by non-enterotoxigenic mechanism	362
	Confirmation that Norwalk viruses cause gastroenteritis	363
	<i>Helicobacter pylori</i> – self administration	352,355
	<i>Neisseria gonorrhoea</i> – determination of infectious dose, identification of virulence factors	364,365
	Influenza, Respiratory Syncytial Virus, Rhinovirus	366,367
Identify host susceptibility factors	<i>Vibrio cholerae</i> – hypochlorhydria and blood group O	368,369
	Norovirus – glycan expression at mucosal surfaces	370
Estimate infective inoculum (i.e. dose escalation)	<i>Cryptosporidium parvum</i>	371
	<i>Vibrio cholera</i>	372
	<i>Shigella flexneri</i> 2a, <i>S. dysenteriae</i> 1, <i>S. sonnei</i>	373,374
	<i>Salmonella</i> Typhi	90,358
	<i>Necator americanus</i> (hookworm)	375
Assess infection-derived immunity	<i>Cryptosporidium parvum</i>	376
	Dengue	377
	<i>Vibrio cholerae</i>	368,378
	Enterotoxigenic <i>Escherichia coli</i> (ETEC)	95
	<i>Salmonella</i> Typhi	379
	<i>Streptococcus pneumoniae</i> (carriage)	380-382
Characterise immune response	<i>Vibrio cholerae</i>	372,383
	<i>Shigella flexneri</i> 2a	384
	<i>Plasmodium falciparum</i> malaria (cell-mediated immunity)	385
	<i>Streptococcus pneumoniae</i> (carriage)	386

Question	Example(s)	References
Measure vaccine efficacy	<i>Vibrio cholera</i> – CVD 103-HgR	383,387
	<i>Salmonella</i> Typhi (e.g., Ty21a)	257,284
	Norovirus – intranasal VLP (with chitosan and MPLA)	388
	<i>Francisella tularensis</i> (tularemia)	389
	<i>Bacille Calmette Guerin</i> (BCG – tuberculosis)	390
	<i>Plasmodium falciparum</i> malaria	391-393
	Dengue (live-attenuated tetravalent vaccine)	394
Identify correlates of protection	Influenza	395,396
	Dengue – sustained interferon- γ levels were associated with protection against fever and/or viraemia	377
	Norovirus – pre-challenge serum antibody correlated with resistance to diarrhoea and shedding	397
Assess therapeutic intervention/benefit	Influenza – pre-existing cytotoxic T-cells and CD4 T-helper cells were shown to be protective	398-400
	Influenza – therapeutics: Amantadine, Interferon prophylaxis, Zanamivir, Oseltamivir	401-404
	RSV – therapeutics (siRNA taken forward to several studies in lung transplant recipients)	405
	Malaria - therapeutics	406
	<i>Necator americanus</i> (hookworm)- asthma	407
	Malaria – malariotherapy for neurosyphilis [review]	408
Assess public health intervention	Influenza transmission	409,410
	Rhinovirus transmission	411,412
	Adenovirus transmission	413

Table 3-1. A summary of the various research questions which human infection ('challenge') studies have been used to address.

Amongst the major findings from the studies conducted at Maryland, investigators determined the apparent site of bacterial invasion,²⁸⁵ the role of chloramphenicol in successfully treating infections,⁴¹⁴ and an apparent protective effect of serum anti-flagellin antibodies.^{282,360,415} They also extensively explored the role of endotoxin tolerance,^{416,417} and even identified potential biomarkers of infection.^{418,419}

The main purpose of initiating the typhoid challenge programme at Maryland was to actively investigate the efficacy of vaccines in a highly controlled setting. For example, 3-doses K or L WCVs were compared with a single dose of purified Vi. After challenge with 10^5 CFU, attack rates were 28% in unvaccinated participants and 9, 7, and 18% in the K, L and Vi groups, respectively.²⁸⁵ The protective efficacy of 63% and 71% demonstrated for K and L vaccination was equivalent to that found in field trials.^{141,282} Importantly, earlier studies in which a higher 10^7 CFU challenge dose had been used did not show any protection from vaccination highlighting the importance of exposure levels in overriding any potentially protective vaccine response.^{89,282,359}

The efficacy of oral Ty21a vaccine was also assessed in three human challenge studies reported in 1977.²⁵⁷ Between 5 and 8 doses of vaccine were given in milk at 3-4 day intervals, each dose preceded by ingestion of 2g sodium bicarbonate in 60mL water. In the participants receiving Ty21a grown in galactose-free media, a protective efficacy of 87% was demonstrated with additional effects including reduced shedding of challenge strain in stool.²⁵⁷ These data were similar to the those found in the subsequent field trial performed in Egypt (see: 2.7.3), and thus not only paved the way to vaccine licensure and subsequent widespread introduction, but also proved to be pivotal in pursuing oral typhoid vaccine research.^{89,420}

3.1.2 TERMINATION OF THE MARYLAND PROGRAMME

The Prison Volunteer Research Unit at the University of Maryland closed in 1974. The predominant reason for closure was concern for the inherently coercive nature of recruiting study subjects in the prison environment.³⁵⁷ Since the earliest challenge studies, specific vulnerable groups, including children and adults in residential care and soldiers, had been repeatedly targeted for challenge study enrolment.^{347,348,421,422} The Maryland challenge studies were ethically progressive, in that studies were explained to prisoners and those who volunteered underwent a consent process. Named in a lawsuit brought by the American Civil Liberties Union, not only was there no evidence of any prisoner ever being coerced into participation, but the presiding judge also praised the studies for their public health impact and high level of ethical standards.³⁵⁷ Although still a highly emotive issue, it is interesting that 40 years later, there is currently a move to re-involve prisoners in medical research, reflecting updated bioethical principles.^{423,424}

Given the limitations of currently available typhoid vaccines and increasing threat of pan-antibiotic resistant strain emergence, attempts were made to restart the Maryland typhoid challenge programme.^{420,425} Despite FDA approval, these were ultimately unsuccessful mostly due to the cost and logistics required to ‘hotel’ study participants until they were declared non-infectious.⁴²⁵

3.2 CURRENT REGULATORY CONSIDERATIONS

Given their historical and unique contribution to understanding the pathogenesis of and immune responses to infection and potential application to diagnostic, treatment and vaccine/prevention development, more recent guidance has been provided by the Academy of Medical Sciences as to the considerations involved in performing human challenge studies.⁴²⁶ In addition to the design and ethical (aside from the scientific)

considerations in performing such work, there are also major differences in regulatory terminology and approach to supporting such studies.^{96,427} In brief, challenge agents used in the US require Investigational new drug (IND) status in being pharmacologically active agents.⁴²⁸ In contrast, challenge agents used in the UK are not classed as pharmaceutical products under the European Clinical Trials Directive (ECTD 2001/20/EC) and therefore regulatory approval (from the MHRA) is not currently required prior to their use.^{426,429} Deliberate attenuation or genetic alteration of strains requires additional approvals for Genetically Modified Organism (GMO) release in most regions. Interpretation of the ECTD is currently delegated to each EU competent authority and therefore the MHRA current interpretation is not replicated in other jurisdictions nor is it immutable.

3.3 ROLE IN VACCINE DEVELOPMENT

Human challenge studies continue to have a major and increasingly important role in the development and assessment of new vaccine candidates. Not least, the process of specific vaccination and then challenge, either with homologous or heterologous organism strains/species/serotypes etc., allows investigators to directly measure the protective efficacy of vaccine candidates against the infection of interest. Measures of vaccine efficacy have been found to relate closely to those obtained in subsequent field trials for many infections since the Maryland Ty21a efficacy was reproduced in Egypt (see: 2.7.3). The malaria vaccine candidate RTS,S for example, demonstrated efficacy of approximately 30-50% (sterile protection) in healthy adult volunteer studies,^{392,430,431} a level of protection subsequently borne out in phase III field-trials.⁴³¹⁻⁴³³

Human challenge studies have a wide range of useful applications to the process of vaccine development aside from recreating possible field efficacy, examples of which are given in Table 3-2. Assessment of protective efficacy is a key driver, however, not least for

cost-, time-, and labour-saving prior to instituting large-scale phase II studies. This issue is particularly relevant to typhoid and other infections rife in endemic settings (such as malaria or tuberculosis) for two important reasons. Firstly, background subclinical exposure and immunity may reduce the apparent effect of vaccination. Secondly, without accurate diagnostic tests available, ‘disease’ incidence may be under- or overestimated, and the definitions of what constitutes ‘disease’ may not be clearly defined (many typhoid studies require a fever $>38^{\circ}\text{C}$ for 3 days duration, however this may miss mild or subclinical cases or may include other causes of persistent fever unless confirmed microbiologically). Both of these factors, together with the relatively poor performance of typhoid vaccines historically, mean that sample sizes for phase III studies may require $>10,000$ participants depending on disease incidence and possibly baseline antibody (anti-Vi, to exclude carriers) status. As seen in a recent (phase IIb) tuberculosis vaccine study, the infrastructure, population cooperation and size of such trials mean that there is relatively little global capacity for multiple studies to be performed.^{434,435}

Application

Provide a direct figure of vaccine protective efficacy
 (De-)escalate putative vaccine candidates
 Provide a ‘standard measure’ in which to perform comparative efficacy studies
 Provide investor/scientific reassurance going into subsequent phase testing
 Identify factors affecting disease susceptibility
 Identify correlates/surrogates of infection/protection (biomarkers)
 Identify new antigens/attenuated organisms for use in vaccine development
 Assess vaccine efficacy against endpoints difficult to measure in the field including for example, stool shedding, ‘objective’ symptom benefit, inflammatory markers
 Bridging studies – to bridge results from one setting, population or infection to another without needing to redo the studies

Table 3-2. Potential applications for challenge studies to vaccine development.

3.4 DEVELOPMENT OF A HUMAN TYPHOID CHALLENGE MODEL IN OXFORD

With the specific purpose of accelerating the development of diagnostics and vaccines to prevent typhoid infection a new human challenge model was established in Oxford in

2011. The model was developed with the Maryland *Salmonella* Typhi, Quail's strain, which was transferred to the United Kingdom and manufactured according to GMP standards. With the appropriate regulatory approvals in place, a preliminary dose-level escalation study was performed from February to October 2011 (OVG 2009/10).⁴³⁶ In addition to identification of a challenge dose for use in the vaccine-challenge study (see: 5.4.2), it also served to demonstrate the feasibility and safety of conducting such a complex trial using an ambulant outpatient population.¹⁸⁶

A major difference from the historical Maryland studies was the use of a sodium bicarbonate buffer solution, to neutralise stomach acid before ingestion and to create the suspension for bacterial ingestion. This likely contributed to the requirement for a lower challenge dose than was required in the historical studies and produced a smoother, more predictable pattern of infection, as seen for other enteric challenge organisms.^{94,95} Both features would be advantageous for use in vaccine efficacy assessment.

Chapter 4. RATIONALE AND AIMS

4.1 RATIONALE FOR A THESIS

Humans have been affected by typhoid fever for thousands of years. While improvements in living conditions have rendered typhoid obsolete for those living in affluent areas, this simple, treatable infection causes a significant burden of disease in impoverished communities. Effective control could lead to eradication, however understanding of bacterial pathogenesis and immune control is lacking and has limited the development of adequate diagnostics and vaccines.

Creation of a safe, ambulatory, outpatient model of human typhoid infection has provided the opportunity to accelerate the development of new control measures, including the selection of promising vaccine candidates. In addition to evaluating the effectiveness of vaccines, thus permitting selection of only the most suitable or efficacious candidates for subsequent phase III field trials, these studies provide a unique opportunity to evaluate the precise impact of vaccines on infection at the individual level and to identify and validate novel biomarkers of infection or infection severity, in addition to potential immunological correlates predictive of subsequent protection. Translating

these data could directly improve the care of patients and affected communities through the development and assessment of new measures to control and prevent infection.

4.2 AIM & OBJECTIVES

This thesis aims to describe the application of a human challenge study to improving typhoid control measures and specifically the evaluation of a promising novel vaccine candidate, Mo1ZH09. The objectives of this thesis are:

1. To design and get approvals for a vaccine study that includes a human challenge component to directly measure the degree of protection afforded by vaccination.
2. To develop and validate additional diagnostic assays to assist in determining the study endpoints.
3. To describe the recruitment and logistics required to perform a vaccine-challenge study in the local Oxford population.
4. To detail the humoral immune responses to vaccination with a novel single dose oral vaccine, Mo1ZH09, in comparison to placebo and using the comparator vaccine licenced 3-dose Ty21a.
5. To detail the human response to typhoid challenge and its moderation by previous live-attenuated oral vaccination.
6. To describe in detail the bacterial dynamics occurring after exposure and during the development of typhoid infection.
7. To evaluate the protective efficacy of the vaccines used in the study, examining the effect of endpoint definition and determining the factors responsible for the protection/non-protection of participants to typhoid infection.

Chapter 5. CLINICAL TRIAL OVG 2011/02:

DESIGN AND METHODOLOGY

5.1 STUDY OVERVIEW AND SET-UP

5.1.1 STUDY SUMMARY

OVG2011/02 was a single centre, randomised, double-blind, placebo-controlled study of the single-dose live-attenuated oral vaccine candidate, M01ZH09, in a healthy volunteer challenge model of typhoid infection using 3-doses of open-label Ty21a as a positive control.⁴³⁷

After recruitment, 99 participants were randomised (2:1) to a blinded or unblinded 'positive comparator' study arm. Within the blinded arm, participants were randomly allocated (1:1) to receive M01ZH09 or placebo vaccine, concealment of which was effected through use of identical packaging and a coded labelling system. M01ZH09 and placebo were given as a single oral dose 28-days prior to challenge. Open-label oral Ty21a vaccine was given in a 3-dose schedule administered on alternate days, with the final dose being given 28-days prior to challenge.

To assess the protection afforded by vaccination, all eligible participants were challenged with 1.5×10^4 CFU *Salmonella* Typhi, Quailles strain, 28-days later (Day 0; Figure 5-1). Participants were reviewed at frequent intervals during the 2-week follow-up period and at reduced intervals thereafter.

At the pre-defined endpoint for Typhoid Diagnosis or Day 14, measurement of the differential attack rates in each of the three vaccine/placebo groups during the 2-week period was used to calculate the protective efficacy of vaccination.

5.1.2 PRIMARY OBJECTIVE

To determine the relative protective effect of M01ZH09 vaccine compared to placebo in a healthy adult typhoid challenge model, using licensed Ty21a vaccine as a positive control.

5.1.3 FURTHER OBJECTIVES

Further study objectives relevant to this thesis included:

- Describing host clinical, inflammatory and immune responses to typhoid challenge in participants in each vaccine group;
- Assessment of the safety and tolerability of M01ZH09;
- Development of novel methods for diagnosing typhoid infection;
- Confirmation of the scientific integrity of the human challenge model, by assessment of the protective efficacy afforded by licensed Ty21a vaccine.

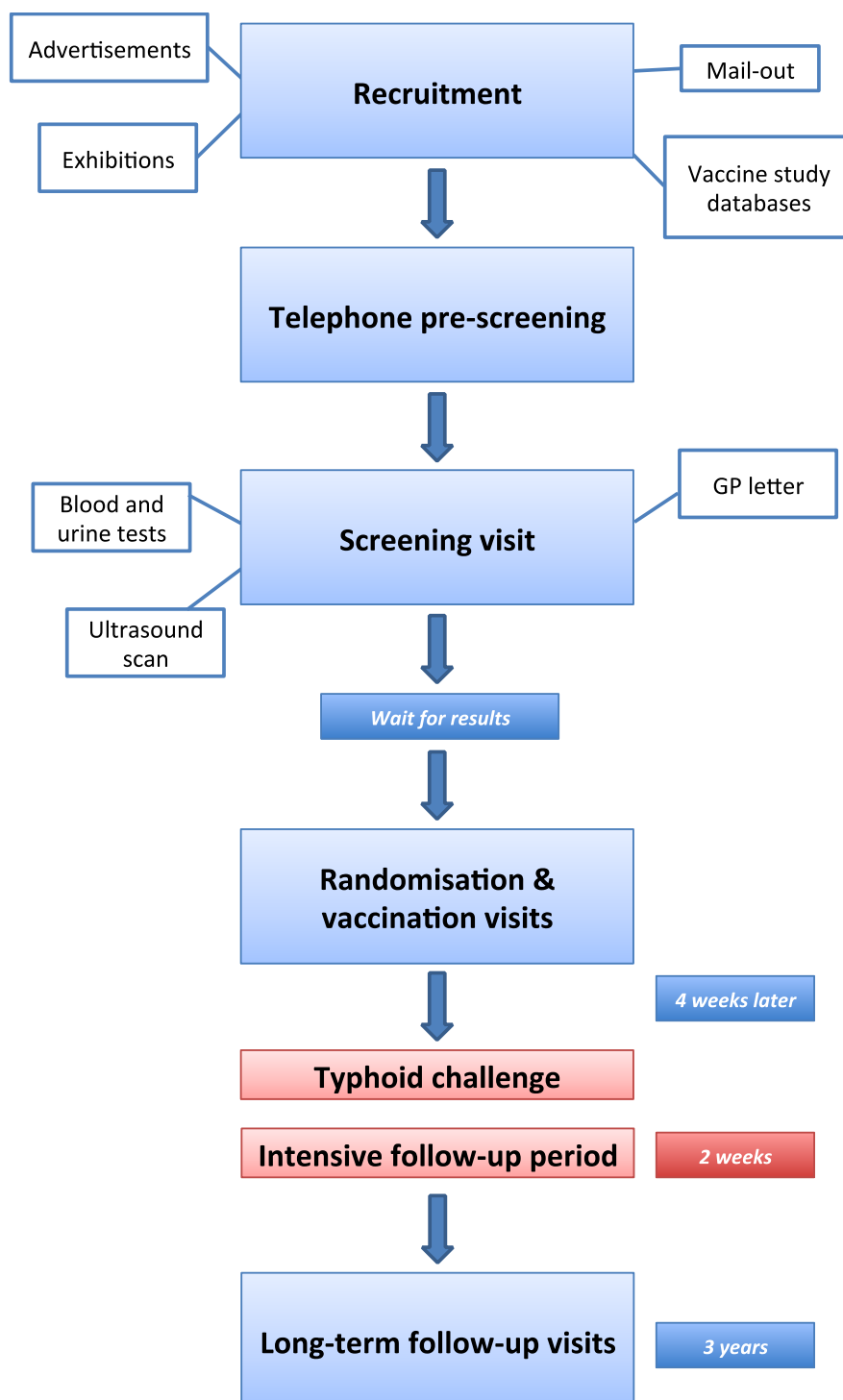


Figure 5-1. Overview of the clinical trial design for a study to assess the protective efficacy of vaccination by human typhoid challenge.

5.1.4 REGULATORY APPROVALS

This study was conducted in accordance with the UK *Medicines for Human Use (Clinical Trials) Regulations 2004* and subsequent amendment,^{438,439} and registered with the European Medicines Agency's European Clinical Trials Database (EudraCT; 24 Jan 2011,

2011-000381-35). The Department for Environment, Food and Rural Affairs approved the 'Deliberate release' of M01ZHo9 vaccine as a genetically modified organism (DEFRA; 9 Mar 2011, 10/R40/01), following a meeting of ACRE (Advisory Committee on Releases to the Environment; 10 Feb 2011, ACRE/11/P4). The clinical trial was performed in accordance with the principles of the International Conference of Harmonisation Good Clinical Practice 1996 guidelines and was in keeping with the principles of the Declaration of Helsinki.^{440,441} Ethical approval for the study was provided by NRES Committee South Central – Oxford A (18 Aug 2011, 11/SC/0302). The clinical trial was accepted for portfolio adoption by the NIHR Clinical Research Network (11099) and listed on both the UKCRN portfolio and clinicaltrials.gov (NCT01405521).

5.1.5 STUDY SETTING

The clinical trial was performed on-site at the Centre for Clinical Vaccinology and Tropical Medicine at the Churchill Hospital in Oxford. The UK is non-endemic for enteric fever, and the vast majority (97% in 2012) of the approximately 500 cases reported each year are travel-related. In the UK, *S. Typhi* accounts for just over 50% of all enteric fever cases reported.⁴⁴² The rate of typhoid fever notification in Oxfordshire is low (0.5 per 100,000 in 2012) and is all travel-related.⁴⁴³

5.2 PARTICIPANTS

Healthy adult volunteers aged 18-60 years of age who were in good health and able to provide written informed consent were eligible to take part in the study.

5.2.1 RECRUITMENT

To streamline the screening process so that the full complexities of the study and procedures involved were only detailed to those eligible to participate, a 3-stage recruitment procedure was used:

1. Volunteers were approached using a variety of pre-approved advertising and promotional materials by methods including direct mail-out through the electoral roll, using CCVTM and OVC volunteer databases, poster campaigns, local newspaper advertising and canvassing at university (Oxford and Oxford Brookes) fresher's fairs.
2. Once an expression of interest was received, volunteers were sent more detailed information regarding the study (the Participant Information Sheet). A member of the study team then contacted any potential participants by telephone to provide further study information and to ensure understanding of the essential inclusion and exclusion criteria.
3. Potential participants still interested in taking part were then invited to the CCVTM for a face-to-face screening interview and, if consented to proceed, an assessment of eligibility was performed as detailed below (taking ~2hrs per volunteer).

5.2.2 CONSENT

At the screening visit full details regarding the study and study-related procedures were discussed with the volunteers, who were required to have read the Information Booklet prior to attendance. Full details of the consent process maybe found elsewhere,⁴³⁷ however the risks detailed included those of study participation, including vaccination, typhoid infection (chronic carrier state, relapse, severe illness, and mood alteration), daily phlebotomy, antibiotic treatment, and the risks to others of potential infection. Emphasis was placed on the likelihood of study fatigue due to the numerous scheduled and unscheduled visit requirements and on the potential antibiotic-related side effects based on previous experiences from the preliminary dose-escalation study.⁴³⁶

Important additional features of the consent process were the requirements for potential volunteers to:

- Supply the details for a 24-hour emergency contact, signed by the 24-hr contact that they were informed of the study and the potential to be contacted if need be;
- Inform their close-contacts of their involvement in the study, providing them with a means to contact us should they have concerns or opt to be screened for infection;
- Agree to return for antibiotic treatment even if withdrawing from the study;
- Consent to being registered with TOPS registry.⁴⁴⁴

Due to the intensity and perceived risk involved in taking part in a challenge study and to ensure that participants had further opportunities to ask questions, a repeat consent procedure was performed immediately prior to vaccination (i.e., at ‘enrolment’).

5.2.3 SCREENING

With provision of written informed consent, screening procedures were performed to minimise the risks of vaccination and challenge both to the individual, their close-contacts and others. The aim was also to recruit only those individuals who were likely to be typhoid-antigen naïve; i.e. those who had not received any previous typhoid vaccination, had typhoid infection or had lived in endemic setting for a prolonged period and therefore potentially been exposed to infection through contaminated water or food. These procedures are described briefly below.⁴³⁷

After ensuring study eligibility, further screening procedures were performed including eliciting a complete medical history and physical examination, which included a urine pregnancy test, 12-lead electrocardiogram, assessment of anxiety and depression (using a HADs questionnaire),^{445,446} and baseline blood tests. To exclude individuals at increased risk of becoming typhoid carriers a gallbladder ultrasound scan was also performed (Radiology Department, Churchill Hospital).

Key features of completing the screening process and ensuring ‘good health’ included receiving agreement from the participant’s general practitioner regarding eligibility for

the study (using the same questions answered by the volunteer at screening), normal baseline laboratory blood parameters, while also ensuring participant availability for the duration of the vaccine/challenge period at least.

5.2.4 INCLUSION/EXCLUSION CRITERIA

Study inclusion and exclusion criteria were extensive and broadly similar to those used for the initial dose-escalation study.⁴³⁶ Additional criteria were added to address the use of a GMO IMP, which included prohibition of travel outside of England for 21-days following vaccination.

Other exclusion criteria aimed to identify individuals who:

- Had previously been exposed to typhoid antigens (e.g., exclusion of any previously vaccinated, infected or potentially infected individuals), which included individuals who had resided for >6 months in a typhoid-endemic area.
- Had any underlying medical condition that may make them more susceptible to infection or respond to challenge in an atypical or more severe fashion (e.g., any participant taking acid-suppressing medication for a history of reflux disease).
- Were at risk of not attending for follow-up at the required time points, for example, those with active or a history of hospital-treated depression, alcohol or drug misuse.
- Were hypersensitive to any of the vaccine components or proposed antibiotics.
- Were at an increased risk of transmitting the infection to vulnerable groups through occupational or close household contact; specifically to:
 - Young children (those attending pre-school groups, nursery or <2 years old),
 - Debilitated or immunocompromised individuals, including the elderly.
- Were at an increased risk of transmitting the infection to the public by occupation as a commercial food handler.

- Were at risk in taking part in the trial due to concomitant study participation or recent blood donation.

Temporary exclusion criteria were instituted prior to vaccination and challenge visits to ensure that participants were well and 'immunologically normal' at these time-points; for example, they had not received recent vaccinations and or antibiotic treatment.

5.2.5 VISIT SCHEDULE

After satisfactory completion of screening procedures, participants were invited to a first vaccination visit. Subsequent visits were scheduled at pre-arranged time intervals. The number of visits attended by each participant varied depending on which vaccination group they were allocated to (those in the open-label vaccine arm attended 3 times for dosing to take place; those in the blinded arm attended once) and whether or not they developed typhoid infection during the 2-week follow-up period (which necessitated additional visits to ensure participant safety and additional sample collections).

In total a maximum of 36 visits were possible over the full 3-year period; the procedures performed at each visit are summarised below. In general, interactions with participants at each visit were similar and focused on obtaining up-to-date symptom and medical information and collection of samples according to the pre-arranged schedule.

5.2.6 PARTICIPANT REIMBURSEMENT

An important factor in recruiting healthy participants to an intensive clinical study is the offer, scale and nature of financial reimbursement.^{447,448} During the consent process study investigators highlighted that reimbursement offered was to compensate participants for their time and effort in taking part in the study, for travel to visits and for their inconvenience. Using guidance provided by the RCP London and the NRES,^{449,450} a tariff was designed for participant reimbursement, which was performed on a *pro rata*

basis. The total compensation paid for completion of all study procedures over the full 3-year period was ~£3340.

Previous data collected demonstrates that while volunteers may be initially enticed by the perceived financial reward, during the course of the research a relationship is built with the study team and many participants develop a more altruistic attitude.⁴⁵¹ This was high-lighted during a BBC news report filmed in relation to the study.⁴⁵²

	Screening	Vaccinations (Day -28)	Post-vaccination Visit a (Day -21)	Post-vaccination Visit B (Day -14)	Day 0 (Visit 1)	Days 1-14 (V 2-13)	At typhoid diagnosis (TD) or at Day 14	Days 21, 28, 60, 90, 180 (V 14-18)	1, 2, 3 years (V 19-21)
Consent	X	X ^A							
Medical exam	X	X			X	X	X		
Blood sample ^B	X	X	X	X	X	X	X	X	X
Urine sample	X				X	X	X	X	X
Saliva sample		X	X	X	X	X	X	X	
Pregnancy test ^C	X	X			X		X ^D		
Stool sample		X ^E	X ^E	X ^E	X	X	X	X ^F	X
Abdominal ultrasound	X								
ECG	X								
Psychological assessment	X	X			X	Day 7 & 14			
Vaccination(s)		X							
S. Typhi challenge					X				
Diary card review			X			X	X	X	
Antibiotic treatment							X		
Telephone call/text					X ^G	X ^G	X ^H		

Table 5-1. Summary clinical study procedures performed during the vaccine-challenge study (OVG 2012/02).

A, Informed continued consent; B, see Table 6-1; C, for females of child bearing potential; D, prior to antibiotics; E, coproantibodies and faecal microbiome only; F, plus screening for chronic carriage/clearance; G, twice-daily, unless visit scheduled; H, until completion of antibiotic treatment.

5.3 VACCINATION

5.3.1 ENROLMENT, RANDOMISATION, ALLOCATION AND BLINDING

A volunteer was considered enrolled on successful completion of screening and at allocation to a vaccine group. Allocation was performed using a randomisation sequence provided by the Centre for Statistics in Medicine. The sequence was generated with variable block size and provided in sealed sequentially numbered envelopes by the unblinded study statistician (LMY); the code given in each envelope allocated participants (2:1) to either the unblinded or blinded vaccine arm.

Participants allocated to the blinded arm received either M01HZ09 vaccine or placebo. No members of the study team or the participant were aware of group allocation, which was continued through use of packaging and labelling concealment, i.e. the study was double-blind. On allocation to the unblinded arm all further vaccination procedures were performed in an open fashion, with all participants receiving 3-doses of the comparator, Ty21a. All vaccines were packaged, labelled and shipped by Aptuit (Deeside, UK) according to the agreed schedule.

5.3.2 UNBLINDING PROCEDURES

Vaccine allocation was revealed to participants 28-days following challenge by one of three dedicated study staff members. All other investigators remained blinded until the last participant had reached this time point, in accordance with the statistical analysis plan. In case of emergency, code-break envelopes were kept securely at the study site so that they could be accessed urgently.

5.3.3 SIMILARITY OF INTERVENTIONS

Vaccines given to individuals in the blinded arm (M01ZH09 or placebo) were identical in appearance of packaging and reconstituted vaccine. There was no difference in taste or smell of the reconstituted vaccine, which had initially been raised as a concern by the

vaccine developers (Emergent). As an additional precaution, however, participants were asked not to comment on any features that might reveal any difference between vaccine or placebo, to ensure that concealment was continued.

5.3.4 M01ZH09

M10ZH09 is a candidate oral vaccine for the prevention of typhoid fever (see: 2.9.3). Its active ingredient is live-attenuated *Salmonella* Typhi, strain (Ty2 *aroC ssaV*) ZH9, which is given with a sodium bicarbonate solution as a single oral dose (1×10^{10} CFU, range 0.5- 1.7×10^{10} CFU per vial). To ensure that an equal dose of live *S. Typhi* ZH9 vaccine strain bacteria were given to each allocated recipient, monthly assessment of vaccine viability was performed by the manufacturer (IDT Biologika, Germany). Viability of the vaccine strain batch (M01ZH09_0041010) was used to calibrate the volume of vaccine/placebo used in each preparation thus ensuring that the number of viable cells ingested remained consistent (see: 8.5.1).

5.3.5 PLACEBO

Placebo vaccine consisted of excipients only, lacking the active agent (*S. Typhi*, ZH9). It was administered with bicarbonate solution in the same manner as M01ZH09.

All blinded arm vaccinations were stored and dispensed in secure rooms in the outpatient clinical area (in accordance with DEFRA guidance). After vaccination all contaminated clinical material was autoclave sterilized prior to disposal in accordance with local GMO waste SOPs.

5.3.6 TY21A

Individuals allocated to the open-label arm received 3-doses of Ty21a vaccine on alternate days (Day -32, -30 and -28) in accordance with national recommendations.^{453,454} Enteric-

coated gelatin capsules were used as supplied by the manufacturer (Crucell UK Ltd, High Wycombe). Each capsule contained not less than 2×10^9 CFU *S. Typhi* strain Ty21a.⁴⁵⁵

Participants were required to return to the CCVTM on each day of the vaccine schedule in order for ingestion of each vaccine dose to be witnessed by a study team member.

5.4 CHALLENGE PROCEDURE

On successful completion of vaccination and the post-vaccination visits participants were eligible to enter the *S. Typhi* challenge stage of the study. The procedures used were identical to those used to develop the challenge model and determine the dose required to achieve an attack rate of 60-75% of volunteers, as described previously.^{436,451} They are therefore only summarised briefly below.

5.4.1 CHALLENGE STRAIN AND MANUFACTURE

Initial attempts to cause clinical typhoid infection in Maryland by challenging consenting prisoners with either Vi-expressing (Zermatt, Ty21V) or non-Vi-expressing (0-901, Ty2W) strains of *S. Typhi* were largely unsuccessful.^{89,285} Clinical infection was seen, however, following challenge with a more recently isolated strain, cultured from the recently excised gallbladder of a Mrs Quail (in 1958).^{89,285,359} The Quail strain had demonstrated natural virulence by its transmission and successful infection of several family members, prior to its selection by the investigators in Maryland. During the course of the Maryland programme the Quail strain was given to ~762 volunteers, providing a wealth of resource regarding its clinical and biological characteristics and supporting its safe administration to challenge volunteers.^{89,282} This strain was therefore selected for establishment of the Oxford programme, during which further characterisation including whole genome sequencing was performed.⁴³⁶ Important features of the Quail strain (phage type D-1) include its expression of key virulence determinants including Vi

capsule.^{285,436} Genetic sequencing of the GMP-manufactured lots in comparison with an original archived strain confirmed that no additional mutations or adaptation to the genome had occurred during the passages involved in the manufacturing process, and its similarity to other sequenced strains in circulation (Figure 5-2).

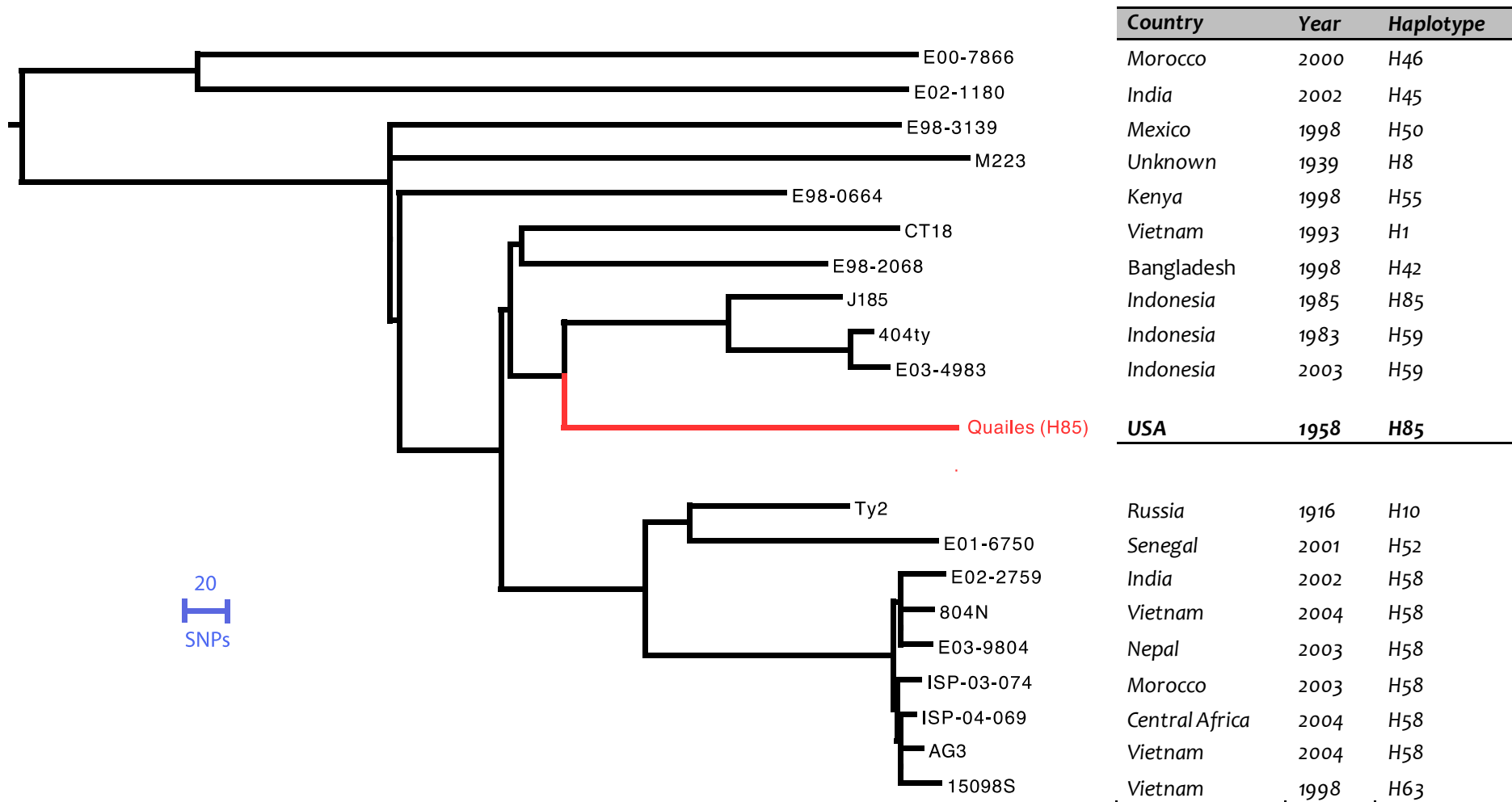


Figure 5-2. Relationship of the *S. Typhi*, Quailles strain, challenge agent to other known typhoid disease-causing strains.

Maximum Likelihood tree generated using SNPs from whole genome sequencing of *S. Typhi* isolates. From ref.11. The length of the scale bar indicates the estimated number of SNPs determined from the rate of substitution per variable site. From supplementary material ref.⁴³⁶.

5.4.2 IDENTIFICATION OF CHALLENGE DOSE

To establish an ambulant outpatient model of human *S. Typhi* challenge administered in a sodium bicarbonate solution, a preliminary dose-escalation study was performed (OVG2009/10; OxREC A, 10/H0604/53; UKCRN, 9297). From February 2010 cohorts of 20 healthy adult volunteers were challenged with increasing dose levels of *S. Typhi*, Quailles strain. Using a dose-escalation algorithm, the primary objective of the study was to identify the challenge dose resulting in an attack rate of between 60-75%. Following a starting dose of 10^3 CFU, which resulted in an attack rate of 55%, the dose of 10^4 CFU was found to result in infection in 65% of challenge participants.⁴³⁶ This dose was therefore selected to assess vaccine protection in this study.

5.4.3 CONFIRMATION OF CHALLENGE DOSE GIVEN

Calculation of the actual challenge dose given during each (set of) challenge(s) during the preliminary dose-escalation study and the subsequent vaccine-challenge study was performed in two ways. Firstly by direct plating of aliquots from the challenge inoculum given (contained in sodium bicarbonate solution, indirect method), and secondly by culturing aliquots from the remaining working cell bank vials (direct method).⁴³⁶ Culture was performed in triplicate using tryptone soya agar (Oxoid), and colonies were counted after 24-hour incubation.

5.4.4 CHALLENGE

On the day of challenge, 28-days after (the final dose of) vaccine ingestion, participants attended the CCVTM after fasting for >90-minutes. Temporary exclusion criteria to ingestion of the challenge agent included fever or acute gastrointestinal illness within the preceding 24-hours, and antibiotic therapy in the preceding 14 days. At challenge, a 120mL oral NaHCO_3 solution was ingested followed 2-minutes later by the challenge dose suspended in 30mL NaHCO_3 .

Following challenge, participants were fasted and observed for a further 90-minutes prior to departure from the centre.

5.4.5 POST-CHALLENGE ASSESSMENT

Participants were assessed in person by the clinical team each day after challenge for 14-days, with the exception of Days 2 and 4, when a telephone review was performed. If participants felt unwell on these days, then they would be reviewed in person.

From challenge until Day 28, solicited and unsolicited symptoms in addition to twice-daily temperatures and medication history were recorded using a diary card. Diary cards were checked at each review to ensure accurate completion. Solicited symptoms included frequency and severity grading of headache, arthralgia, myalgia, abdominal pain, nausea/vomiting, cough, constipation, diarrhoea and rash. Participants were specifically required not take antipyretic treatment until antibiotics had been initiated. All other medications were discussed prior to use with a study doctor. Pulse, temperature, blood pressure recordings and blood, stool and saliva samples were collected at each clinic visit (Table 5-1).

5.4.6 ILLNESS DEFINITIONS

To determine the primary objective and initiate antibiotic treatment, a predefined illness definition for the development of typhoid infection (**'Typhoid Diagnosis', TD**) was used. This definition was based on data from the Maryland studies,^{89,257} a retrospective analysis of the Maryland studies exploring different illness definitions,⁹⁰ and data from the preliminary dose-escalation study.⁴³⁶

Once participants met either of the TD criteria a full clinical review was performed and antibiotic (and symptomatic) treatment was initiated at a **'typhoid diagnosis visit'**. After

diagnosis, participants followed the TD visit schedule in place of the arranged routine visits (Table 6-1).

5.4.7 TYPHOID DIAGNOSIS

A typhoid diagnosis was made if either a microbiological and/or a clinical definition for confirmed typhoid infection was met (Table 5-2). This definition was also used as the basis for notifying cases to the Health Protection Unit (HPU). Details of all participants diagnosed with typhoid and/or with *Salmonella* Typhi cultured from one or more stool specimens were passed to the Thames Valley HPU.

Microbiological definition
<p>A. A blood culture positive with <i>Salmonella</i> Typhi was identified from Day 5 onwards, with the presence of one or more objective clinical signs of typhoid infection, or,</p> <p>B. A blood culture positive with <i>Salmonella</i> Typhi was identified from Day 7, with or without objective clinical signs of typhoid infection,</p>
Clinical definition
<p>C. The participant developed an oral temperature $\geq 38^{\circ}\text{C}$, persisting continuously for at least 12-hours in the absence of anti-pyretic medication, occurring from 72-hours after challenge.</p>

Table 5-2. Diagnostic definitions for typhoid infection.

These definitions were used in the vaccine-challenge study (OVG2012/02) and were identical to those used in determining the attack rates in the preliminary dose-escalation study. The day of blood culture positivity was determined here as the day that the sample was drawn to perform the subsequent culture.

Should participants have been unable to tolerate challenge or any symptoms experienced during the challenge follow-up period, the protocol allowed for antibiotics to be initiated at the investigators discretion. Participants treated under such circumstances would have been excluded from the *per protocol* population and therefore not included in calculation of vaccine efficacy.

5.4.8 SEVERE TYPHOID DEFINITION

In addition, severe typhoid fever was defined as a participant meeting the criteria for TD (as above), with the addition of one or more of the following features:

- Oral temperature $\geq 40^{\circ}\text{C}$,
- Systolic blood pressure $\leq 85\text{mmHg}$,
- Significant lethargy or confusion,
- Gastrointestinal bleeding or suspected/confirmed perforation,
- Any Grade 3 or above laboratory abnormality.

Participants meeting the criteria for severe typhoid infection or any of the following were specifically assessed for consideration for hospital admission and inpatient treatment:

- Failure of symptoms to improve within 72-hours of starting antibiotic treatment,
- Inability to tolerate oral antibiotics,
- Dehydration/hypotension requiring intravenous fluid therapy,
- Unanticipated concern regarding the participant's home circumstances.

5.4.9 TREATMENT

A list of study medications to assist with tolerating symptoms and for typhoid treatment was in place, and included analgesia, anti-emetics, laxatives, and antihistamines, but excluded anti-pyretics (paracetamol, NSAIDs) until the TD/Day 14 visit was performed. All participants received treatment for typhoid with either a first- (ciprofloxacin 500mg bd for 14 days) or second-line antibiotic (azithromycin 500mg od for 14 days).

Adherence to treatment was confirmed at each visit and by telephone contact. In addition, all participants were screened for stool shedding to confirm typhoid clearance a minimum of two weeks following completion of the antibiotic course.

5.4.10 CLINICAL DATA COLLECTION

All data gathered by diary cards, Case Report Forms and from the OUH Laboratories was transferred or uploaded to an online database (OpenClinica Community edition v3.1.3-beta). Following data monitoring and quality assurance assessment they were locked and exported to Microsoft Excel (v14.4.2). In this format datasets were collated and analysed using SPSS Statistics (IBM, v22) and GraphPad Prism (v6.0e).

5.4.11 ADVERSE EVENT REPORTING AND SAFETY OVERSIGHT

All adverse events were assessed and attributed according standard Oxford Vaccine Group standard operating procedures. Regular meetings were held with the DMSC and the Trial Steering Committee to whom regular reports regarding SAE and SARs were sent, in addition to the Sponsor (CTRG, Oxford University) and vaccine manufacturer (Emergent BioSolutions).

5.5 TRIAL-RELATED STATISTICAL METHODS

5.5.1 STATISTICAL HYPOTHESIS

In attempting to determine the relative protective efficacy of M01ZH09 vaccination compared to placebo, the statistical null and alternate hypotheses were:

$$H_0 = TD_{\text{Placebo}} = TD_{\text{M01ZH09}}$$

$$H_1 = TD_{\text{Placebo}} \neq TD_{\text{M01ZH09}}$$

Where TD_{Placebo} or TD_{M01ZH09} is the proportion of participants diagnosed with typhoid and receiving placebo or M01ZH09, respectively.

5.5.2 SAMPLE SIZE CONSIDERATIONS

To determine the number of participants required for the study the following considerations were taken into account.

1. The attack rate determined in the preliminary dose-escalation study was found to be 65% (95% CI, 41-84) after challenge with 1.5×10^4 CFU in a final group size of 20 individuals. This attack rate was assumed for the vaccine-challenge study.
2. A correlate of protection for typhoid fever has not been determined, however a 4-fold or greater rise in anti-typhoid specific LPS or flagellin antibodies has been used as a measure of immunogenicity in naïve subjects and as a presumed protective threshold previous studies of M01ZH09. Using this endpoint, a response rate of approximately 75% to vaccination with M01ZH09 has been found.³⁴⁰
3. A participant dropout rate of ~10% between enrolment (randomisation) and completion of the challenge phase (Day 14) was assumed, based on previous local experience of healthy adult studies.

Taking these considerations into account, a sample size of 28 participants per group would allow us to demonstrate the protective efficacy of 70% of M01HZ09 vaccination over placebo with 90% power ($1-\beta$) and 5% error (α ; Table 5-3).

Similarly a sample size of 30 participants would still be able to demonstrate a significant effect of vaccination (5% error) if the level of protective efficacy measured was at least 80% and if the attack rate in the placebo arm fell to 50% (which was still within the confidence intervals calculated for the true attack rate for the dose-escalation study) but with lower power (80%). This level of protection and resultant attack rate was broadly similar to that seen during the studies evaluating Ty21a efficacy during the Maryland studies. Combining data from two Ty21a trials, Gilman and colleagues demonstrated a 53% attack rate in 43 controls and 7% in 28 vaccine recipients after challenge with 10^5 CFU

Salmonella Typhi.²⁵⁷ This resulted in a protective efficacy of 86.8% for Ty21a, albeit after 5-8 doses of vaccine.

In the current study we therefore aimed to enrol 33 participants per arm (99 in total), with the assumption that data from 60 or more participants from each of the Mo1ZH09 and placebo arms would be available for statistical analysis.

<i>Salmonella</i> Typhi attack rate		Sample size requirement per group				
Mo1ZH09	Placebo	1- β : α :	90% 5%	90% 1%	80% 5%	80% 1%
10%	30%		92	126	71	102
10%	40%		48	66	38	54
10%	50%		30	41	24	34
10%	60%		21	28	17	24
10%	65%		18	24	13	19
10%	70%		15	20	12	17
10%	80%		11	15	9	13
10%	90%		8	11	7	10
10%	100%		6	8	5	7
20%	40%		118	164	91	131
20%	50%		57	79	45	64
20%	60%		34	47	27	38
20%	65%		28	37	22	30
20%	70%		22	31	18	25
20%	80%		16	21	13	18
20%	90%		11	15	9	13
20%	100%		8	11	7	9
30%	60%		62	86	48	69
30%	65%		47	64	37	50
30%	70%		36	49	28	40
30%	80%		22	31	18	25
30%	90%		15	20	12	17
30%	100%		10	14	9	12

Table 5-3. Vaccine-challenge study sample size calculation table.

This table demonstrates the effect of various attack rates in the Mo1ZH09/placebo groups on the sample size requirement and power to reject the null hypothesis. Highlighted rows demonstrate the sample size requirement were the attack rate in the placebo group to equal that found in the dose-escalation study, i.e. 65%.⁴³⁶

5.5.3 ANALYSIS OF PRIMARY STUDY OBJECTIVE

The per protocol (PP) population was used to determine the primary objective of the study and was defined as all participants who had undergone both vaccination and challenge, and who remained under observation until Day 14. The protective effect of

active vaccination with M01ZH09 or Ty21a (and 95% exact confidence intervals) was calculated by:

$$PE = 100 \times (AR_{\text{placebo}} - AR_{\text{vaccine}}) / AR_{\text{placebo}} = 100 \times (1 - AR_{\text{vaccine}} / AR_{\text{placebo}}),$$

where PE was the protective effect and AR was attack rate. The attack rate in Ty21a recipients was compared to that in placebo recipients to confirm integrity of the challenge model in comparison to previously performed challenge and field studies.

5.5.4 INTERIM ANALYSIS

Two interim analyses of the study attack rates were discussed in closed DSMC meetings, to ensure that the assumed attack rate in the placebo-vaccinated group was being met. This was to confirm that the study objective was not futile, should the attack rate have been lower than expected and therefore the assumed power to detect a benefit from vaccination not reached.

Initial interim analysis was performed after the first 30 participants had been vaccinated and completed challenge in March 2013. The decision from the DMSC was to continue the study. Further data analysis was performed by the unblinded study statistician (LMY) and discussed with the DMSC after completion of 66 challenges; again the advice was to continue with vaccinations and challenges.

5.5.5 ANALYSIS OF OTHER TRIAL ENDPOINTS

Secondary endpoints were explored using either intention to treat (all vaccine recipients) or PP populations. Prior to analysis, data distributions were analysed and transformed to assume a normal distribution where relevant. Proportions and percentages are presented with exact 95%CI intervals while continuous data is presented with 95%CI if normally distributed or median and interquartile range (or range) if not. Comparisons between

groups are made with the statistical tests stated with corrections made for multiple comparisons. When multiple comparisons were made, data pertaining to participants allocated to either active vaccine arm were compared to the placebo group as the control group and not between active vaccine arms.

Chapter 6. LABORATORY METHODS

6.1 GENERAL LABORATORY METHODS

6.1.1 SAMPLE COLLECTION

Blood was collected from participants by standard venepuncture by priority of clinical samples before blood for immunologic and other experimental laboratory assays. The volume of blood collected in total during the study was designed to not exceed recommendations for blood donors to the National Transfusion Service, i.e. maximum of 3 blood donations (or 1410mL) per year (Table 6-1). In addition, full blood count results were actively checked to ensure that participants were not becoming anaemic.

Stool samples were self-collected by participants prior to study visits, with the date and time of collection recorded. Following collection, samples were kept cold where possible, and transported to the centre using insulated bags and ice packs. Samples were ideally delivered within 6-hours of being produced, and were kept refrigerated in the laboratory until processed.

6.1.2 LABELLING AND TRANSPORT OF LABORATORY SAMPLES

All samples collected prior to 28-days following challenge were treated as ‘High Risk’ and therefore labelled appropriately (e.g., with ‘Danger of Infection’ labels), and handled in CL3 facilities according to standard local practices. Samples were transported to laboratories in double packaging in containers identified as containing ‘High-risk’ material.

6.2 ROUTINE LABORATORY METHODS (OUH)

Clinical trial material collected primarily for participant safety and for fulfilment of the primary endpoint criteria were transported to the OUH Laboratories at the John Radcliffe Hospital for further processing and reporting from an accredited laboratory.

6.2.1 HAEMATOLOGY & BIOCHEMISTRY

Samples for routine laboratory tests including full blood count, urea and electrolytes and liver function tests were collected in the relevant EDTA or serum separation tubes prior to further processing. This was performed in the clinical laboratory department as routine testing in accordance with local protocols. Results were made available via the hospital intranet reporting system.

Table 6-1. Blood sampling schedule for participants enrolled into the vaccine-challenge study (OVG2012/02).

Blue shading, assays or tests performed in the Oxford University Hospitals NHS Trust laboratories. CRP, C-reactive protein; U&E, urea and electrolytes (including sodium, potassium and creatinine).

A, screening tests performed within 90-days of first vaccine receipt; B, Ty21a recipients only; C, M01ZH09 and placebo recipients only; D, FG visits and blood collections only performed in those assigned to this subgroup (see text); E, Typhoid diagnosis visits were performed on fulfillment of either of the pre-defined criteria (Table 5-2)– blood was collected prior to ingestion of the first antibiotic dose.

Investigation		Blood culture	Culture-PCR	Bacterial quantification	Full blood count	CRP	U&E	LFT	Antibodies	Serum bactericidal assay	Antibody-secreting cells	Cell mediated immunity	ALS assay	Cytokines	Functional genomics	
Volume (mL)		10	5	10	1	2			5	3	25	30-80	5	2	3	
Visit	Time														TOTAL	
Screening ^A					3	2			5							10
Vaccination	Day -32 ^B								5	3	25	75	5	2	3	118
	Day -30															0
	Day -28 ^C								5	3	25	75	5	2	3	118
FG-1 ^D	Day -30														3	3
FG-2	Day -28														3	3
FG-3	Day -26														3	3
FG-4	Day -24														3	3
Va	Day -21								5	3	25			2	3	38
Vb	Day -14								5	3		35		2	3	48
1	Day 0				1	2			5	3		75	5	2	3	96
1	12hrs		5											2	3	10
2	24hrs		5						5	3				2	3	18
3	D3		5											2	3	10
4	D5	10	5		1	2			5	3				2	3	31
5	D6	10	5											2	3	20
6	D7	10	5						5		25	30	5	2	3	85
7	D8	10	5		1	2									3	21
8	D9	10	5												3	18
9	D10	10	5		1	2			5	3			5	2	3	36

Investigation		BC	PCR	QBC	FBC	CRP	U&E	LFT	ABs	SBA	ASC	CMI	ALS	Cyto	FG	
10	D11	10	5												3	18
11	D12	10	5		1		2								3	21
12	D13	10	5												3	18
13	D14	10	5		1		2		5	3		45	5	2	3	81
14	D21								5	3					3	11
15	D28								5	3		75	5	2	3	93
TD ^E	Hr 0	10	5	10	1		2		5	3			5		3	44
TD+6	Hr 6	10	5												3	18
TD+12	Hr 12	10	5												3	18
TD+24	Hr 24	10	5		1		2								3	21
TD+48	Hr 48	10	5		1		2		5	3	25	40	5	2	3	101
TD+72	Hr 72	10	5												3	18
TD+96	Hr 96	10	5		1		2		5	3		40	5	2	3	76
Total in 28 days																
If <u>NO</u> typhoid diagnosis																804
If typhoid diagnosed at day 14 & FG subgroup (i.e. maximum volume)																117
16	D60								5	3		45		2	3	58
17	D90								5	3		45		2	3	58
18	D180								5	3		80		2	3	93
19	D365								5	3		80		2	3	93
Total in 1 year																1419
20	Yr2								5	3		80		10	3	101
21	Yr3								5	3		80		10	3	101
Total in 3 years																1621

6.2.2 QUALITATIVE BLOOD CULTURE

Blood taken for culture was collected with additional care to avoid contaminating flora, by using 1.5mL 2%-chlorhexidine gluconate to disinfect the skin (ChloraPrep, CareFusion, UK) prior to venepuncture. Using a non-touch technique, 10mL venous blood was inoculated into a single aerobic culture vial (BACTEC Plus, BD&Co, Oxford). Note that 10mL blood was collected for culture at all time points before Day 14, however 5mL was collected at the typhoid diagnosis time point only in order reduce/minimise the volume of blood taken from potentially acutely sick individuals.

After collection and transport, qualitative culture was performed according to local procedures.^{6,456,457} On arrival inoculated vials were incubated using an automated culture system (BD, BACTEC FX), which detects organism growth through CO₂ production. Reaction between a dye in the media and CO₂ produced alters the level of fluorescent light absorbed measured by photo detectors in the machine. Negative samples were cultured for 5-days prior to being reported as 'no growth' and discarded.

On detection of growth, Gram's stain was performed; on confirmation of Gram-negative rods by direct light microscopy, further subculture was performed as described below. In addition the result was communicated to a member of the study team (on-call medic) as indicating probable typhoid infection, in this particular context. Subculture was performed as for routine identification and growth of suspected coliform bacteria. One drop of blood/media broth was directly inoculated onto blood (Oxoid) and chocolate agar (BD) and incubated overnight at 37°C anaerobically or aerobically, respectively.

A further ~10µL of the blood/media broth was mixed with 1mL peptone water (Oxoid) and used to inoculate further media including MacConkey (Oxoid) and chromogenic (CHROMagar Orientation; CHROMagar Microbiology) agar plates, which were incubated

at 37°C overnight. Further agar plates (Iso-Sensitest and Wilkins-Chargren anaerobic media) were inoculated with the peptone/broth suspension to perform simultaneous antibiotic susceptibility testing, as described below (see: 6.2.5).

6.2.3 QUALITATIVE STOOL CULTURE

Stool was submitted for standard microbiological culture to the OUH microbiology laboratory, which was performed according to local procedures and based on national guidance.⁴⁵⁸ In brief, selenite broth was inoculated with ~1g faeces and mixed by vortex. Agar plates including XLD were either directly inoculated with 100µL of the suspension, or after 18-24 hours incubation at 37°C, when chromogenic agar (Salmonella Plus agar, E&O laboratories) for the detection of *Salmonella* sp. including *S. Typhi*, was inoculated.

All cultures were incubated and kept for 1-week until being discarded and being reported as 'no growth'.

6.2.4 SALMONELLA TYPHI IDENTIFICATION

With confirmation of possible *Salmonella* growth (i.e. red or black colonies seen on XLD media or mauve colonies on chromogenic agar), further biochemical and serological identification steps were performed in accordance with local and national standard procedures.^{6,456} One colony was picked for subculture and mixed with 5mL normal saline prior to inoculating an API20E strip, Columbia blood (for serological testing) and MacConkey agar (to ensure homogeneity and purity) plates. Inoculation of the API20E strip (bioMérieux) was performed according to manufacturer's instructions prior to humid incubation overnight at 37°C. Strips were read at 24-hours using the API database (APIweb™).

With confirmation of *Salmonella* identification by API profile, serological testing was performed using 'O' and 'H' antigen specific sera following the Kauffmann-White scheme by slide agglutination.⁶ Briefly, colonies were selected and emulsified in normal saline before an equal volume (2 μ L) of antisera was added and mixed. Positive agglutination was identified by the formation of grainy white particles within 20-seconds of antisera addition, visible against a dark background. When required, unmasking of the O antigen (by the Vi capsule) was performed by boiling a dense solution for 30-minutes before repeating the agglutination procedure. All agglutinations were controlled for using a negative reaction.

One sample of each isolate was kept, subcultured on a nutrient agar slope and stored by freezing to allow future investigations to be performed.

6.2.5 ANTIBIOTIC SUSCEPTIBILITY TESTING

Antibiotic susceptibility testing was performed on at least one isolate from each typhoid-diagnosed participant according to standard local procedures. The disk sensitivity test (DST) method was used to determine the effect on organism growth of a standard antibiotic panel including: ampicillin (10 μ g), trimethoprim (125 μ g), chloramphenicol (10 μ g), ertapenem (10 μ g), ceftriaxone (30 μ g), and nalidixic acid (30 μ g; all BD). A ciprofloxacin Etest (bioMérieux) was also performed on all isolates to measure the MIC directly.

Susceptibility tests were performed and read according to standard protocols using Iso-Sensitest agar (Oxoid).⁴⁵⁹ After overnight culture on Iso-Sensitest agar, zones of inhibition were measured to the nearest millimetre and susceptibility or resistance was inferred using standard interpretation.⁴⁵⁹

6.3 STUDY SPECIFIC LABORATORY METHODS (OVGL)

6.3.1 BUFFERS

3% Ox bile Tryptone Soy Broth

30g Tryptone Soy Broth (Oxoid) powder and 30g ox-gall powder (Oxgall, BD) were weighed in a fume cupboard and dissolved in 1L dH₂O by continuous mixing before autoclaving prior to use.

Tris EDTA (50mM pH8.0 Tris & 2mM EDTA)

5mL of 0.5M TRIS solution was added to 0.2mL 0.5M EDTA solution and 45mL of bottled dH₂O. After thorough mixing by vortex in a 50mL falcon tube, the solution was filtered using a 0.2µm filter and syringe.

50x TAE

242.2g TRIS base and 37.2g EDTA powder were dissolved in 750mL dH₂O. pH was adjusted to 8.0 using acetic acid and the solution made up to 1L using dH₂O once all chemicals were in solution.

10x Phosphate Buffered Saline (PBS)

Sodium chloride (80g/L), sodium phosphate dibasic heptahydrate (20.6g/L), potassium phosphate monobasic (3.14g/L) and potassium chloride (1.6g/L) were mixed in 800mL dH₂O and titrated to a pH in the range 7.2-7.4, by the addition of NaOH/HCl as appropriate. After dissolution the volume was made up to 1L using dH₂O.

Ro media

Ro media was prepared by the addition of 2mM L-glutamine (Sigma-Aldrich) and 0.5mL penicillin-streptomycin (=0.25g) solutions to 500mL RPMI-1640 (Sigma-Aldrich; containing phenol red indicator and 25mM hepes modification), mixed and stored at 4°C prior to use.

R10 media

R10 media was prepared by the addition of 50mL NBBS (new born bovine serum, Sigma-Aldrich) to 450mL Ro, mixed and stored at 4°C prior to use.

6.3.2 HANDLING AND BLINDING OF OVGL SAMPLES

Initial sample processing was performed using participant numbers, however, when required, further laboratory analyses were performed using pre-allocated blinded random laboratory numbers provided by CSM. At processing all experimental laboratory samples were stored in duplicate in 2 identical aliquots. The selection and labelling of samples for further assays was performed by a minimum of 2 laboratory staff to minimise error and ensure consistency. Plasma cell ELISpot assays were performed in real-time and samples were therefore not blinded. Blinded laboratory staff performed plate spot counting.

6.3.3 QUANTITATIVE BLOOD CULTURE

Blood was collected for quantitative culture at typhoid diagnosis and at some additional time points in a small subset of individuals. In brief, peripheral venous blood was inoculated into an Isolator 10mL microbial tube (Alere Limited) and delivered immediately (within 4-hours) to the OVG laboratory. The Isolator tube contains ingredients including 2-5% saponin and 1-2% sodium polyanetholsulphonate to ensure cell lysis and avoid coagulation, respectively.⁴⁶⁰

On arrival the sample was centrifuged at 3000rcf for 30-minutes (without brake) prior to aspiration of the supernatant and plating of the pellet directly onto two dry XLD agar plates (Oxoid). Inoculated plates were incubated at 37°C in an aerobic incubator for 24-hours before colony counting was performed. Bacterial quantity was calculated by:

$$\text{BL (CFU)} = \text{Total count (n)} / \text{volume of blood used (mL)}.$$

6.3.4 QUANTITATIVE STOOL CULTURE

In order to quantify the number of bacterial present in stool samples the following methods were used. In brief, 1g of stool was pre-incubated with 5mL selenite F broth by vortexing and leaving at RT for 20-minutes, prior to plating 1.2mL (3x0.4mL) onto three XLD plates (Oxoid). The number of colonies was counted after 24-48 hours of incubation at 37°C in an aerobic incubator.

6.3.5 SERUM SEPARATION

Serum was collected from whole blood using serum separation tubes and allowing the samples to clot for a minimum of 30-minutes at RT (or 24-hours at 4°C) prior to centrifugation at 3000rcf for 10-minutes. The sera supernatant was then aspirated and divided equally into 2 cryovials prior to storage at -80°C until further use.

6.3.6 LPS ELISA

An ELISA protocol for the detection of IgG, IgA or IgM antibody specific to *S. Typhi* lipopolysaccharide (LPS) was developed based on an original protocol transferred from Emergent Biosolutions,⁴⁶¹ and initially successfully used to measure the antibody response to *S. Typhi* challenge as previously described.⁴³⁶

Briefly, 96-well plates (F96 Maxisorp Nunc-immuno plates; Thermo Fisher Scientific) were coated with 100µL/well of 15µg/mL LPS (*Salmonella typhosa*, Sigma), sealed and

incubated overnight at 4°C and a maximum of 7-days prior to assay performance. Before use plates were washed five times (BIO-TEK EL-405 plate washer, BioTek UK) with wash buffer (PBS+0.05% Tween®20), blocked with 300µL/well of blocking buffer (PBS+1%BSA, Bovine serum albumin) and incubated at RT for 1-hour.

After 5 further washes, 200µL/well of test and known standard concentration (nominal concentration of 30,000 units/mL, Emergent Biosolutions) sera were added in duplicate and two-fold serial diluted from 1/100 to 1/12800 with PBS/BSA. High, medium and low levels of quality control (QC) sera were also prepared using standard sera to nominal concentrations of 48, 26 and 14 units/mL, respectively, and 100ul/well was added to the plate (for plate layout see Table 6-2). PBS/BSA alone was used in the blank control wells and plates were incubated at RT for 1-hour.

	Standard		QC & Control		Test Sera 1		Test Sera 2		Test Sera 3		Test Sera 4	
	1	2	3	4	5	6	7	8	9	10	11	12
A	1/100	1/100	High QC	High QC	1/100	1/100	1/100	1/100	1/100	1/100	1/100	1/100
B	1/200	1/200	Med QC	Med QC	1/200	1/200	1/200	1/200	1/200	1/200	1/200	1/200
C	1/400	1/400	Low QC	Low QC	1/400	1/400	1/400	1/400	1/400	1/400	1/400	1/400
D	1/800	1/800	Blank	Blank	1/800	1/800	1/800	1/800	1/800	1/800	1/800	1/800
E	1/1600	1/1600	Blank	Blank	1/1600	1/1600	1/1600	1/1600	1/1600	1/1600	1/1600	1/1600
F	1/3200	1/3200	Blank	Blank	1/3200	1/3200	1/3200	1/3200	1/3200	1/3200	1/3200	1/3200
G	1/6400	1/6400	Blank	Blank	1/6400	1/6400	1/6400	1/6400	1/6400	1/6400	1/6400	1/6400
H	1/12800	1/12800	Blank	Blank	1/12800	1/12800	1/12800	1/12800	1/12800	1/12800	1/12800	1/12800

Table 6-2. LPS ELISA plate layout plan.

QC, quality control; Blank, PBS/BSA only.

After further washing, 100µL/well of goat anti-human HRP (horse radish peroxidase) conjugate antibody was added in concentrations of 1:20,000 for anti-IgG and 1:5,000 for anti-human IgA and IgM (AbD Serotec). Plates were further incubated for 1 hour at RT, prior to the addition of substrate for development. After further washing 100µL of TMB (3,3',5,5'-Tetramethylbenzidine, Sigma) brought to RT was added to each well. Plates were incubated for 20-25 minutes at RT out of direct light, before the reaction was

terminated using 50µL/well of 2M sulphuric acid (H₂SO₄; VWR International). Optical density values were read at 450nm using an automated plate reader (MultiscanEX plate reader; Thermo Fisher) and used to calculate the concentration of antibody per well. The lower limit of detection for the assay is calculated as 0.276, 0.283 or 0.317 ELISA units for the IgG, IgM and IgA assays, respectively.

6.3.7 H ELISA

Based on an ELISA previously developed for the measurement of the IgG, IgM and IgA antibody response to the *S. Typhi* H (flagellin) antigen at the Oxford Vaccine Group,⁴³⁶ a modified assay was developed to measure the responses to vaccine and challenge in this study. The major development was use of a purified H antigen preparation, initially prepared in Oxford by culture and shear-centrifugation of *S. Typhi*, Quail's strain, and then purified by Dr. Raphael Simon at the University of Maryland.

In brief, 96-well plates were coated with 100µL/well diluted stock H-antigen at concentration of 1µg/mL and incubated overnight at 4°C. Plates were washed and blocked for 1 hour as for the LPS ELISA, prior to the addition of test and standard sera. As previously, these were added in duplicate and two-fold serially diluted (range 1:100 to 1:12,800) using PBS/BSA. High, medium, medium/low and low QCs were prepared by serial dilution of the high QC wells starting with a concentration of 1:200 or 1:100 for the IgG and IgA, or IgM assays, respectively. Plates were incubated for 90-minutes at RT.

Goat anti-human IgG, IgA and IgM antibodies conjugated to HRP (Bio-Rad) diluted in PBS/BSA were added in 100µL/well at concentrations of 1:20,000, 1:10,000 and 1:10,000, respectively, and plates were incubated for a further hour at RT. Substrate (TMB) and sulphuric acid were added as for the LPS ELISA, with final incubation times between 24-

40 minutes in duration. The lower limit of detection was calculated to be 0.092, 0.167 and 0.178 ELISA units for the IgG, IgM and IgA assays, respectively.

6.3.8 VI ELISA

Assessment of Vi antibody levels by ELISA was performed using a commercially available kit from The Binding Site Ltd (VaccZyme™ Human anti-*Salmonella* Typhi Vi IgG Enzyme Immunoassay Kit) as per the manufacturer's instructions.

6.3.9 PREPARATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCs)

To perform cellular assays, venous blood was collected into 10mL EDTA tubes (ethylenediaminetetraacetic acid; Vacutainer, BD) from which PBMCs were separated using standard methods. Briefly, blood was diluted 1:2 with R0 media prior to density gradient separation over Lymphoprep (Axis-Shield). After washing twice and re-suspending in fresh R0, cell density was estimated by counting a 10µL aliquot taken from a 150µL suspension (containing 50µL cell suspension, 50µL PBS and 50µL 0.4% trypan blue stain, Sigma-Aldrich) using a haemocytometer. Mean cell counts were used to calculate the total cell number in the suspension, prior to dilution to a final concentration of 2.5×10^6 cells/mL in R10 solution.

6.3.10 ELISPOT

ELISpot (enzyme-linked immunosorbent spot) assays were performed to detect and quantify the number of typhoid antigen specific antibody-secreting cells (ASCs) before and after vaccination. The method used here was initially developed and used during the preliminary dose-escalation study, and is described briefly below.

96-well plates (Milipore) were prepared by coating with antigens diluted in carbonate coating buffer (Sigma) to a working concentration as follows: *S. Typhi* LPS (Sigma),

10.0µg/mL; *S. Typhi* Vi antigen (OVGL supply), 2.0µg/mL; *S. Typhi* H antigen (OVGL supply), 10.0µg/mL; and pan anti-human IgG, IgM and IgA (Invitrogen), 20.0µg/mL.

Following overnight incubation at 4°C plates were washed and blocked with R10 media and further incubated for 1-hour at 37°C in 5%CO₂. After separation, fresh PBMCs were re-suspended to concentrations of 2.5 or 5.0x10⁶ cells/mL and added to the blocked ELISpot plate (for plate layout see Table 6-3).

	2.5x10 ⁵ cells/well						5.0x10 ⁵ cells/well					
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	LPS	LPS	LPS	Vi	Vi	Vi	LPS	LPS	LPS	Vi	Vi	Vi
C	αIgG	αIgM	αIgA	αIgG	αIgM	αIgA	αIgG	αIgM	αIgA	αIgG	αIgM	αIgA
D												
E				PanAb	PanAb	PanAb				PanAb	PanAb	PanAb
F	H	H	H	αIgG	αIgM	αIgA	H	H	H	αIgG	αIgM	αIgA
G	αIgG	αIgM	αIgA	Buffer	Buffer	Buffer	αIgG	αIgM	αIgA	Buffer	Buffer	Buffer
H				αIgG	αIgM	αIgA				αIgG	αIgM	αIgA

Table 6-3. Typhoid ELISpot plate layout plan.

PBMCs from one participant and one time point only were added to each plate and all wells, as cell counts allowed.

Plates and cells were incubated for a further 24-hours at 37°C in 5%CO₂ prior to development. ELISpot plates were developed after washing by the addition of 100µL alkaline-phosphatase goat anti-human IgG, IgM and IgA antibodies (diluted 1:5000 with filtered PBS/10%NBBS) to each well. After further incubation for 4-hours at RT, plates were washed prior to the addition of alkaline phosphatase development substrate (composed of colour development buffer diluted 1:25 with dH₂O and solutions A and B) in volumes of 50µL/well. The reaction was terminated in each well with the appearance of spots or after 10minutes development by quenching with 200µL/well dH₂O and 2 further rinsing steps. Plates were dried overnight in the drying oven prior to reading and

counting of these data. Spots were read and counted using an ELISpot automated reader (AID ELR03m) with AID ELISpot software (v4, Autoimmun Diagnosticka) as per local operating procedures.

6.4 GENERAL CONSIDERATIONS IN DATA ANALYSIS

Data was compiled using Microsoft Excel (V14.4.2) and analysed using GraphPad Prism (v6.0e) and SPSS Statistics (IBM, v22). In brief, data were cleaned and quality-assured prior to unblinding and statistical analysis, which was performed as described in each subsequent chapter. All statistical tests performed were 2-sided and p values less than 0.05 were considered significant.

Chapter 7. USE OF HUMAN TYPHOID CHALLENGE STUDIES TO ASSESS AND DEVELOP PCR-BASED DIAGNOSTICS

7.1 INTRODUCTION

A key limitation to improving the control of typhoid infection is the lack of ‘accurate’ diagnostic tests. In addition to confirming infection in individuals, accurate case discrimination and detection of the ‘true’ disease burden is important for evaluating the efficacy of disease prevention measures such as vaccination, and for advocating their requirement.

Diagnostic approaches for typhoid infection are broadly aimed either at directly detecting bacteria or bacterial products or measuring the host response in clinical samples.²¹⁰ As discussed (see: 2.5), while the few bacteria available in collected specimens reduce

bacterial culture sensitivity, the latter approach is frustrated by lack of knowledge regarding the host responses specific to acute typhoid infection.

The current reference standard for typhoid diagnosis is blood culture, which, if performed under ideal conditions may have a sensitivity of ~80%.^{175,187} Advantages to blood culture include 100% specificity and the end-availability of isolates to perform further testing. Limitations include lower sensitivity under non-ideal conditions, such as pre-treatment with antibiotics, cost and time-to-result.

7.1.1 PCR

Direct detection of pathogen DNA is a commonly used method to increase sensitivity, specificity and time-to-result in modern diagnostic laboratories.⁴⁶²⁻⁴⁶⁴ For bacterial infections in particular, this approach also identifies non-cultivable bacteria and is thereby not adversely affected by antibiotic pre-treatment or immune interference. Studies by Rubin and colleagues in the 1980's utilised a *viaB* probe cloned from *Citrobacter freundii* DNA to accurately identify isolates in the laboratory.^{465,466} Subsequent clinical studies were less successful, however, as the probe was unable to detect fewer than 500CFU/mL blood.^{467,468} An improvement in detection threshold was achieved by adaptations to the technique including bacterial concentration by centrifugation and overnight incubation, which resulted in a sensitivity of 42% in culture-confirmed typhoid cases using the equivalent of 2.5mL blood.⁴⁶⁷

Song and colleagues first described the use of PCR to detect *S. Typhi* flagellin sequences in 1993.⁴⁶⁸ *Flagellin* expression is monophasic in several *Salmonella* sp. including *S. Typhi* and the phase-1 antigen 'd' is found in found in many species. While the end regions of *fliC-d* (previously *H1-d*) are identical between species, there are two hypervariable regions (IV and VI) unique to *S. Typhi* and similar to *S. Muenchen*.⁴⁶⁹ One-stage PCR amplification

of the *fliC-d* gene was unsuccessful when performed on clinical isolates (0/12 were positive), however, nested-PCR using a second set of primers detected *S. Typhi* in 11/12 patient specimens.⁴⁶⁸

These initial findings highlight the two main obstacles to use of PCR for *S. Typhi* detection. Firstly, that conventional one-stage PCR was insufficiently sensitive to detect DNA in clinical samples, requiring a second round of amplification.⁴⁷⁰⁻⁴⁷³ Secondly, use of nested-PCR to detect *S. Typhi* is prone to contamination or false-positive amplification and has proven to be a significant problem.^{474,475}

Subsequent emergence and spread of *S. Paratyphi* and Vi-negative strains resulted in efforts to multiplex arrays capable of detecting several pathogens simultaneously, although few of these have demonstrated effectiveness in detecting *S. Typhi* DNA from clinical material.^{203,476-478} In further attempts to overcome the methodological variability of previous studies, real-time PCR has highlighted the difficulty of amplifying very low DNA copy numbers and therefore its continued inferiority to standard bacterial culture.⁴⁷⁷

7.1.2 PRE-CULTURE

One approach to increase PCR sensitivity is to pre-incubate clinical material prior to performing DNA extraction.⁴⁷⁹⁻⁴⁸² Disadvantages to this method include loss of the ability to accurately quantify bacterial numbers in the blood directly, prolonging assay time and the requirement for microbiological culture facilities.

Pioneering work by Conradi in 1906 suggested that ox-bile could be used as a selective media to improve the culture detection of *S. Typhi*.^{483,484} Subsequent work suggested that ox-bile reduced coagulation and serum complement killing activity and caused selective lysis of human rather than *Salmonella* cells.^{485,486} Release of viable bacteria from the blood intracellular compartment has been shown to produce an almost 2x fold rise in

bacterial numbers.²⁰⁴ While broths containing 10% and more of ox-bile have been shown to be at best bacteriostatic to *S. Typhi* growth, recent work identified that, at an optimal concentration of ~2.4% and with 5-hour incubation, a significant increase in bacterial numbers may be achieved (Table 7-1).^{487,488} The addition of micrococcal endonuclease during the extraction process to remove remaining human DNA further improved assay sensitivity.⁴⁸⁹

Incubation time (hours)	CFU
0	3
1	4
2	17
3	105
4	209
5	4461

Table 7-1. Bacterial growth rate in 2.4% Ox-bile/TSB media from 20mLs blood.
Mean of 3 experiments. Adapted from Zhou and colleagues.²⁰³

7.1.3 LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP)

A more recent technique for DNA amplification that has the potential to exceed PCR sensitivity is Loop-mediated isothermal AMPlification ('LAMP').⁴⁹⁰⁻⁴⁹⁴ While not without limitations, chiefly the inability to multiplex the assay, LAMP may be more suited as a resource-limited technology due the non-requirement of thermocycling equipment. The basic LAMP assay uses 4 specifically designed primers to target 6 distinct, short gene target sequences and is performed using a DNA polymerase enzyme with strand displacement activity at a constant temperature (Figure 7-1).⁴⁹² With appropriate primer design, this technique should therefore be highly specific and capable of greater amplification than a standard PCR reaction. Sensitivity of the technique may be further enhanced by addition of two loop primers.⁴⁹⁵

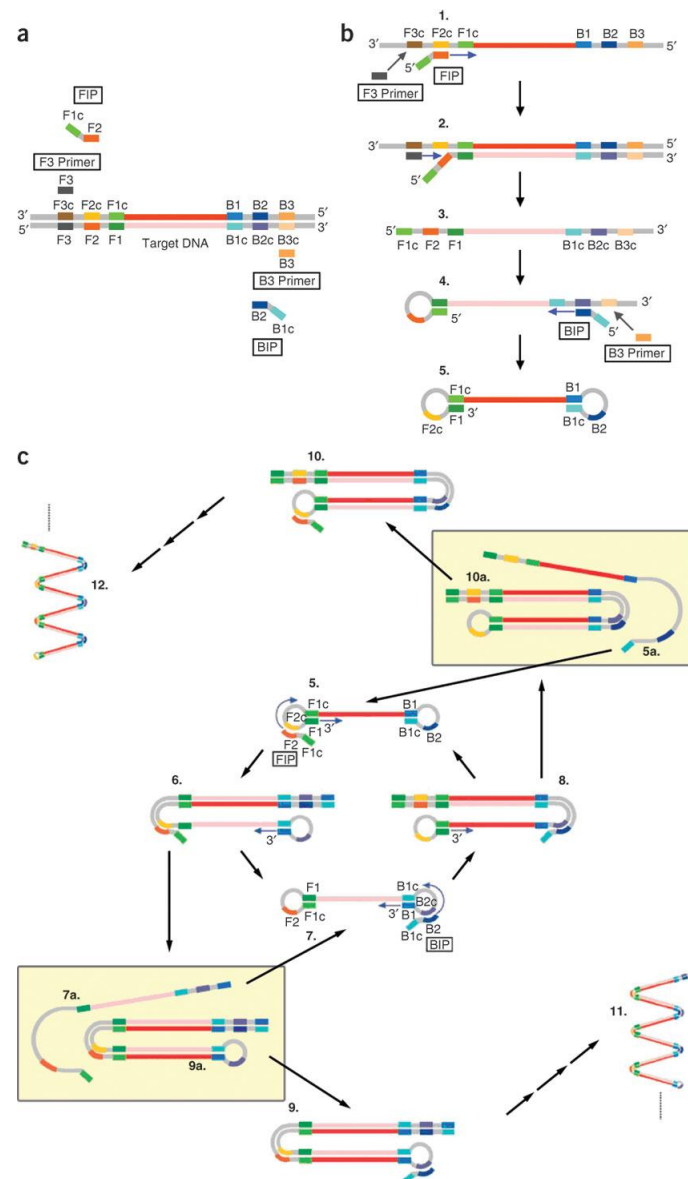


Figure 7-1. A diagram to demonstrate the central stages of loop-mediated isothermal amplification (LAMP).

From Tomita and colleagues,⁴⁹⁶ with permission Nature Publishing Group.

a) Primer design. The 6 distinct short gene target sequences from the 5' end are F3, F2, F1, B1c, B2c and B3c, 'c' indicating the complementary sequence. In the LAMP method, two inner primers (FIP and BIP) and two outer primers (F3 and B3) are used; the inner primers being composed of hybrid F1c and F2 or B1c and B2 sequences, respectively.

b) Starting structure and production of loops. DNA synthesis is initiated by annealing of the F2 (FIP primer) to the F2c DNA sequence (or BIP to B2c) resulting in elongation (1). Strand displacement occurs due to binding of the outer F3 primer to the F3c region and subsequent DNA synthesis (2). Separation of the elongated strand results in the formation of a loop structure at the 3' end (3,4). Using this single-strand DNA as a template, the BIP and B3 primers generate an equivalent loop at the other end, creating a dumbbell-like structure (5).

c) Cycling amplification. From the resulting dumbbell-like template, self-primed DNA synthesis is initiated at F1 (3' end) with annealing of FIP to F2c region in the loop (5,6). This process continues resulting in the formation of shorter and more elongated structures in both orientations (7-12). The addition of loop primers to complement the region between F2 and F1 (or B1 and B2) results in amplification in the F2 and F1 or B1 to B2 direction of the stem-loops not already targeted by the inner primer pair (not shown).

7.1.4 AIM & OBJECTIVES

This chapter explores the use of a human typhoid challenge model to evaluate and develop potentially sensitive PCR-based diagnostic assays. Few human challenge studies have been used for the development or assessment of novel diagnostic tests, and yet they provide ideal preliminary test conditions given that the pathogen, exposure dose, pre-treatment status and corroborating laboratory data are available.

Objectives:

1. Assess the diagnostic accuracy of a culture-PCR (PCR) assay targeting the *S. Typhi* *fliC-d* gene using the predefined study endpoints for typhoid diagnosis and compare to positive blood culture as the reference standard.
2. Optimise a culture-LAMP assay for the detection of *S. Typhi* *fliC-d* DNA and evaluate its performance as a proof-of-concept for diagnosis of typhoid infection.

7.2 METHODS

7.2.1 STUDY DESCRIPTION

Clinical samples were collected from 41 participants taking part in the preliminary dose-escalation study, as previously described (see: 3.4).⁴³⁶ In general, study procedures after challenge were identical to those performed for the subsequent vaccine-challenge study, with the exception that blood cultures were collected daily from Day 0 onwards.

7.2.2 REFERENCE METHODS AND DEFINITIONS

Blood and stool culture were performed as described (see: 6.2.2). Typhoid was diagnosed (TD) if predefined clinical (oral temperature $\geq 38^{\circ}\text{C}$ for ≥ 12 -hours) and/or microbiological (≥ 1 Gram-negative blood culture) criteria were reached. All PCR and blood

culture data were collected prospectively during the study, while the LAMP optimisation experiments were performed after study completion.

7.2.3 PARTICIPANT 'PCR' SAMPLE COLLECTION

After challenge, 5mL peripheral venous blood was collected into heparinised falcon tubes according to the schedule shown below (Table 7-2). Liquid culture was performed as soon as practicable and within 24-hours of sample collection. Note that only 5mL blood was collected for routine blood culture at the TD visit, whereas 10mL were collected at all other time-points. Sampling was performed between 6-hours following challenge and Day 14 or the TD+96hour visit (whichever was later; Table 7-2).

Days/hrs after challenge							Typhoid Diagnosis							
	0	0/6	0/12	1	1/12	2-14	0	+6	+12	+24	+36	+48	+72	+96
Visit no.	1	1	1	2	2	3-15	0	+6	+12	+24	+36	+48	+72	+96
Blood culture	-	10	10	10	10	10	5	10	10	10	10	10	10	10
QBC	-	-	-	-	-	-	10	-	-	-	-	-	-	-
Culture-PCR	-	5	5	5	5	5	5	5	5	5	5	5	5	5

Table 7-2. Assay schedule (with volumes of blood collected) for laboratory microbiology tests performed during the preliminary dose-escalation study.

QBC, quantitative blood culture was performed at point of typhoid diagnosis only. Typhoid diagnosis schedule could start at any point between Days 5 and 14 if criteria for diagnosis were met (see text).

7.2.4 OX-BILE/TSB CULTURE

Ox-bile/TSB culture was performed as previously described.^{203,488,489} In brief, 1.5µL micrococcal endonuclease (New England Biolab) was added to 20mL 3% (w/v) ox-bile/TSB solution in a 50mL falcon tube (Figure 7-2). The collected 'PCR' sample was added to the culture media and incubated at 220RPM for 5 hours at 37°C. Next, bacteria were concentrated by centrifugation at 5000RPM for 20min and the supernatant removed. DNA was extracted from the bacterial pellet using a standard kit (UltraClean™

BloodSpin™ Kits) according to the manufacturer's instructions, except that elution of the final DNA-containing pellet was performed using 100µL pre-heated 'Buffer 5' prior to incubation (65°C for 5min) and the final extraction step.

After completion, DNA samples were labelled and kept at -20°C until amplification by PCR or LAMP as described below. All experiments were performed in a dedicated clean-hood, regularly disinfected by UV light and sterile conditions were maintained throughout.

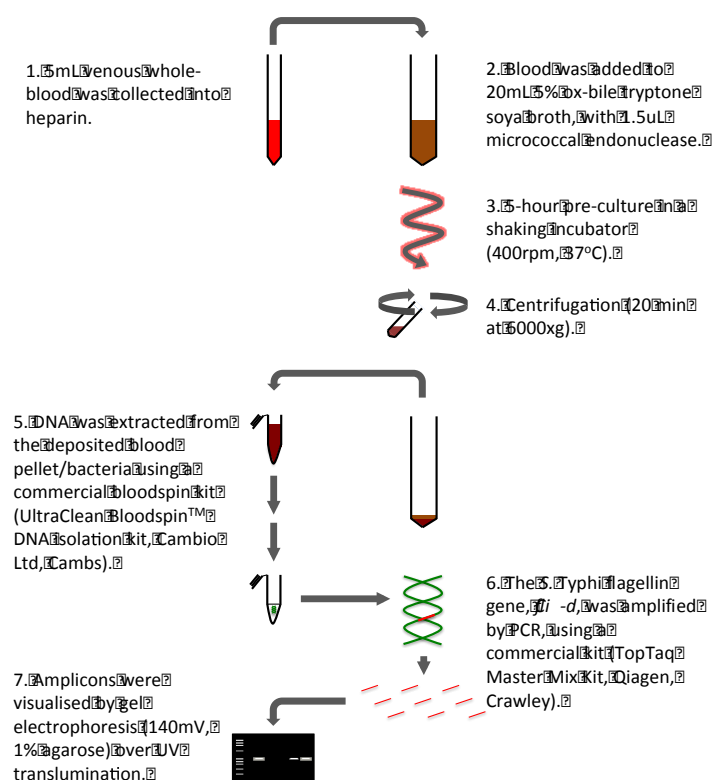


Figure 7-2. Schematic describing the ox-bile culture-PCR assay methodology.

7.2.5 PCR AMPLIFICATION

PCR primers were obtained to complement the *S. Typhi* phase-1 *flagellin C 'd'* (*fliC-d*, GenBank L21912)⁴⁶⁹ gene sequence as described: H-for, ACTCAGGCTTCCCCTAACGC; and Hd-rev, GGCTAGTATTGTCCTTATCGG (Sigma-Aldrich; Figure 7-3).⁴⁷⁶

Amplification reactions were performed using TopTaq PCR master mix (Qiagen) in 50µL volumes. Each reaction contained: 25µL master mix, 5µL Coral load, 2x5µL H-for and Hd-rev, and 10µL DNA template. Distilled H₂O and/or non-study DNA extracted from healthy

donor blood was used as negative control, while genomic DNA extracted from *S. Typhi*, Quail's strain culture was used as a positive control template.

DNA amplification was performed using standard thermocycler equipment at: 95°C for 5min followed by 35 cycles of 93°C for 30s, 55°C/30s, and 72°C/40s and terminating with 1 cycle of 72°C for 5min. The specific target PCR products appeared at 763bp when separated by 1% (w/v) ethidium bromide gel electrophoresis and visualized using UV light transillumination. Bands were categorised as either being present (positive) or absent (negative).

1	ATGGCACAAG	TCATTAATAC	AAACAGCCTG	TCGCTGTTGA	CCCAGAATAA	CCTGAACAAA
61	TCCAGTCCG	CACTGGGCAC	TGCTATCGAG	CGTTTGTCTT	CCGGTCTGCG	TATCAACAGC
121	GCGAAAGACG	ATGCGGCAGG	ACAGGCGATT	GCTAACCGTT	TTACCGCGAA	CATCAAAGGT
181	CTG <u>ACTCAGG</u>	<u>CTTCCCCTAA</u>	<u>CGCTAACGAC</u>	<u>GGTATCTCCA</u>	<u>TTGCCGAGAC</u>	<u>CACTGAAGGC</u>
241	GCGCTGAACG	AAATCAACAA	CAACCTGCAG	CGTGTGCGTG	AACTGGCGGT	TCAGTCTGCG
301	AATGGTACTA	ACTCCCAGTC	TGACCTCGAC	TCCATCCAGG	CTGAAATCAC	CCAGCGCCTG
361	AACGAAATCG	ACCGTGTATC	CGGCCAGACT	CAGTTCAACG	GCGTGAAAGT	CCTGGCGCAG
421	GACAACACCC	TGACCATCCA	GGTTGGTGCC	AACGACGGTG	AAACTATCGA	TATTGATTTA
481	AAAGAAATCA	GCTCTAAAAC	ACTGGGACTT	GATAAGCTTA	ATGTCCAAGA	TGCCTACACC
541	CCGAAAGAAA	CTGCTGTAAC	CGTTGATAAA	ACTACCTATA	AAAATGGTAC	AGATCCTATT
601	ACAGCCCAGA	GCAATACTGA	TATCCAAACT	GCAATTGGCG	GTGGTGCAAC	GGGGGTTACT
661	GGGGCTGATA	TCAAATTTAA	AGATGGTCAA	TACTATTTAG	ATGTTAAAGG	CGGTGCTTCT
721	GCTGGTGTTT	ATAAAGCCAC	TTATGATGAA	ACTACAAAGA	AAGTTAATAT	TGATACGACT
781	GATAAAACTC	CGTTGGCAAC	TGCGGAAGCT	ACAGCTATTC	GGGGAACGGC	CACTATAACC
841	CACAACCAAA	TTGCTGAAGT	AACAA <u>AAGAG</u>	<u>GGTGTGATA</u>	<u>CGAC</u> CACAGT	TGCGGCTCAA
901	CTTGCTGCAG	CAGGGGTAC	TGGCG <u>CCGAT</u>	<u>AAGGACAATA</u>	<u>CTAGCC</u> TTGT	AAAATATCG
961	TTTGAGGATA	AAAACGGTAA	GGTTATTGAT	GGTGGCTATG	CAGTGAAAAT	GGGCGACGAT
1021	TTCTATGCCG	CTACATATGA	TGAGAAAACA	GGTGCAATTA	CTGCTAAAAC	CAC TACTTAT

Figure 7-3. *Salmonella Typhi* flagellin C 'd' gene sequence

Primer sequences used for PCR (red) and nested-PCR (blue) amplification reactions are highlighted.

7.2.6 NESTED-PCR

To resolve ambiguous results, for example where contaminating human DNA produced non-discrete bands, a second nested-PCR amplification step was also performed as required. Nested-PCR primers encoding complimentary sequences within the original *flagellin* sequence were used: Inter-F, 5'-TCTCCATTGCGCAGACCA; and Inter-R, 5'-GTCGTATCAACACCCTCTT (Sigma-Aldrich; Figure 7-3). Amplification reactions were performed in 50µL volumes containing 25µL master mix and Coral load (as above) but

with 0.5µL of each primer, 18µL dH₂O and 1µL of the initial PCR amplification template. A shorter amplification time was used: 15 cycles of 93°C/30s, 55°C/30, 72°C/40s terminating with 72°C/5min as before. Visualisation of the product was performed as described above.

7.2.7 LAMP AMPLIFICATION

The LAMP method was performed as originally described by Notomi and colleagues,^{491,496} and according to the kit manufacturers instructions (Loopamp DNA Amplification Kit, Eiken Chemical Co. Ltd., Japan). Primers were designed using the *S. Typhi flicD* gene sequence as described in Table 7-3.

To perform the assay a master mix was prepared on ice using 2x reaction mix (RM, including deoxynucleotide triphosphate substrates and MgSO₄), primer mix (PM, either with or without additional loop primers, at the concentrations given in Table 7-3), *Bacillus stearothermophilus* (Bst) DNA polymerase (8units/uL), and dH₂O to a total volume of 23µL.

Primer	Sequence (5'-)	Concentration
FIP	CACACGCTGCAGGTTGTTGTTGTCTCCATTGCGCAGACCA	40 pmol
BIP	GTGAACTGGC GGTTCAGTCT GCGGCGCGTG GGTGATTTTCAG	40 pmol
F3	CTTCCCGTAA CGCTAACGAC	5 pmol
B3	AGTCTGGCCG GATACACG	5 pmol
Loop-F (LF)	G TTCAGCGCG CCTTC	20 pmol
Loop-B (LB)	G TACTAACTC CCAGTCTGACCTC	20 pmol

Table 7-3. LAMP primer sequences used.

Sequences consist of inner (FIP and BIP) and outer (F3 and B3) pairs amplifying 6 regions of the *fliC*D sequence and an additional loop primer pair (LF and LB). Primers were designed using the online proprietary software PrimerExplorer (V3, Fujitsu Ltd., Japan; <http://primerexplorer.jp/e/>) and used at the final concentrations stated as recommended in the manufacturers instructions.

Prepared master mix was mixed only briefly by vortex (3 x 1s) to prevent inactivation of polymerase. To initiate the reaction, 2µL of DNA template or control was added to each reaction tube, prior to gentle mixing by tapping, and subsequent spinning down by microfuge. Amplification temperature and duration conditions were optimised between 60-65°C for 30-60min, as described below (see: 7.4.1). *Bst* polymerase was inactivated to terminate the reaction by incubation at 80°C for 5 minutes. The amplified product was visualised by 2% EtBr (w/v) agarose gel electrophoresis, as described previously, and by visual inspection for turbidity. LAMP results were categorised as either positive or negative based on the appearance of a band/smear on the electrophoresis gel.

7.2.8 REPORTING AND STATISTICAL ANALYSIS

Data were reported according to the STARD criteria.⁴⁹⁷ The diagnostic accuracy of PCR or blood culture in comparison to TD are reported using sensitivity, specificity, positive and negative likelihood ratios, positive and negative predictive values and diagnostic odds ratio, each with the 95% normal confidence interval. The statistical significance of the differences in sensitivities of PCR and blood culture was assessed by discordant pairs analysis using a two-tailed binomial test. Data were analysed using GraphPad Prism (v6.0e).

7.3 PCR RESULTS

7.3.1 PRELIMINARY PCR RESULTS

Prior to investigating clinical samples, assay performance and laboratory sensitivity was evaluated using *S. Typhi*-negative whole blood samples containing known ('spiked') concentrations of *S. Typhi* DNA added after incubation. The time from initiation of incubation to availability of electrophoresis results was 8-hours.

Experiments demonstrated that assay sensitivity was relatively dependent on the amount of DNA present prior to amplification. Sensitivity of PCR amplification was at least 0.015pg/ μ L (Figure 7-4), equivalent to a DNA starting concentration of 30 bacteria per reaction (each bacterium containing ~5fg DNA)^{482,498} or ≥ 6 CFU/mL in the original starting blood volume, assuming that no subsequent multiplication had taken place during ox-bile/TSB incubation (for example, bacteria were non-viable after antibiotic treatment).

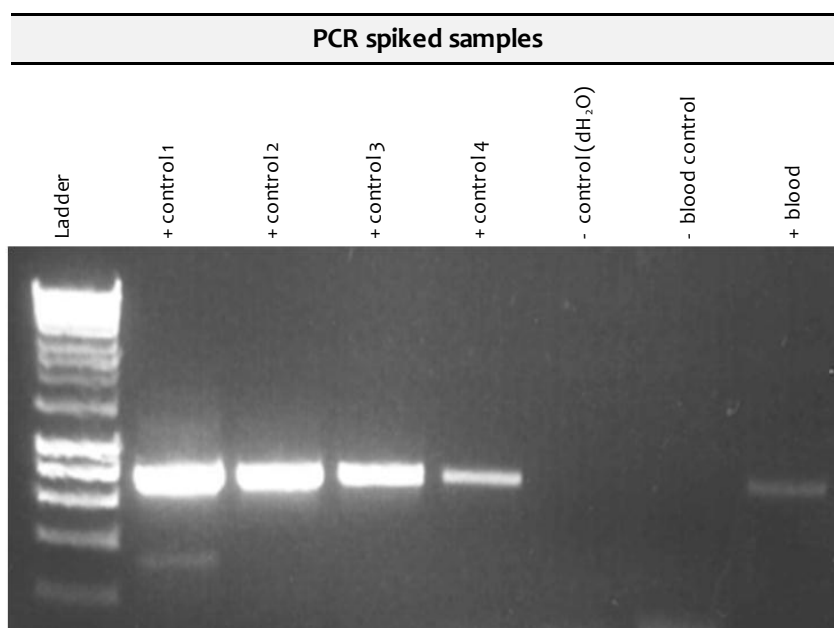


Figure 7-4. *Salmonella Typhi* fliC-D amplicons (763bp).

Lanes: molecular weight ladder; +control 1,2,3,4: positive spiked controls (15, 1.5, 0.15 and 0.015pg/ μ L, respectively); - control (distilled H₂O); - blood (non-typhoid clinical sample); + blood (typhoid study clinical sample).

In addition, *S. Typhi* *fliC-d* DNA could be amplified and detected from clinical material using the method described (+blood lane, Figure 7-4), without requirement for a second amplification stage. Occasionally (~4/41 participants) non-discrete bands were obtained; Figure 7-5 demonstrates band resolution using the nested primer set.

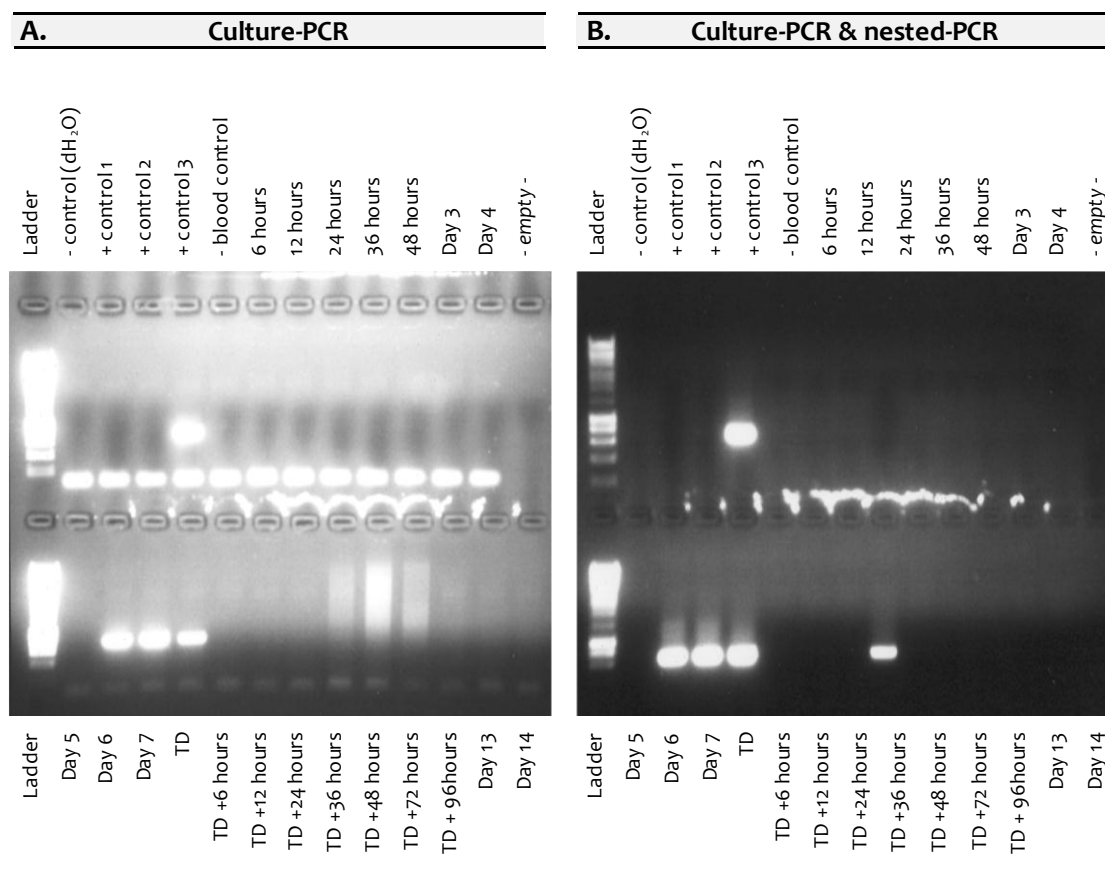


Figure 7-5. Culture-PCR time course for an individual participant who was diagnosed with typhoid infection on Day 8 (TD).

Results at TD+36, +48 and +72 hours were ambiguous (A.), and therefore the time course was repeated using nested primers (B.). This confirmed the TD+36 hour result as being a true positive.

7.3.2 PERFORMANCE IN A CHALLENGE STUDY

Study participant demographic profile and challenge outcome have been reported previously.⁴³⁶ In brief, 41 participants were safely challenged with either 10^3 or 10^4 CFU *S. Typhi*, Quail's strain. Attack rates were 55% and 65% for each dose respectively, and the median day of illness onset was Day 7. Overall, blood culture confirmation of diagnosis

was obtained in 21/25 (84%) participants challenged, with or without additional clinical features of infection.

In total, 684 samples were collected for culture-PCR from 41 challenge study participants, 24 of whom were diagnosed with typhoid fever (TD), 1 who was treated based on symptoms alone (here included with the TD group), and 16 who were treated with antibiotics at day 14 but did not develop infection (nTD).

Of these, 57/684 (8.3%) samples were positive by the PCR assay (Table 7-4). From Day 3 onwards, TD and nTD participants produced 11.6% and 4.2% of the positive results, respectively. After the initiation of antibiotics, 7/24 TD participants had 9 further positive results. Of these, 5/9 were from samples collected within 6-hours of starting antibiotic treatment.

Challenge outcome	Time point after challenge, n (%)					Total
	Day 0 to Day 3	Day 3 onwards				
nTD (n=16)	3/57 (5.3)	8/189 (4.2)				11/246 (4.6)
TD (n=25)	6/92 (6.5)	40/346 (11.6)				46/438 (10.5)
<i>Day relative to TD</i>		>-3	-3 to -1	-1 to 1	1 to 3	>3
		3/65 (4.6)	18/46 (39.1)	15/43 (34.9)	1/86 (1.2)	3/72 (4.2)
Total (n=41)	9/149 (6.0)	48/535 (9.0)				57/684 (8.3)

Table 7-4. Number (%) of culture-PCR positive samples identified during the preliminary dose-escalation study (OVG 2010/9) according to challenge outcome and day of sample collection.

Samples from participants diagnosed with typhoid are further described by day relative to typhoid diagnosis (and antibiotic initiation) in 48-hour blocks. nTD, non-typhoid diagnosed; TD, typhoid diagnosed.

By comparison, 'routine' blood culture yielded 53/674 (7.9%) positive samples. Positive PCR results were evident earlier in the challenge course, however the overall maximal day of blood culture and PCR positivity was Day 6 (Figure 7-6). Interestingly, positive PCR

results were also obtained from participants who remained undiagnosed after challenge; this was not seen with blood culture as it formed part of the definition.

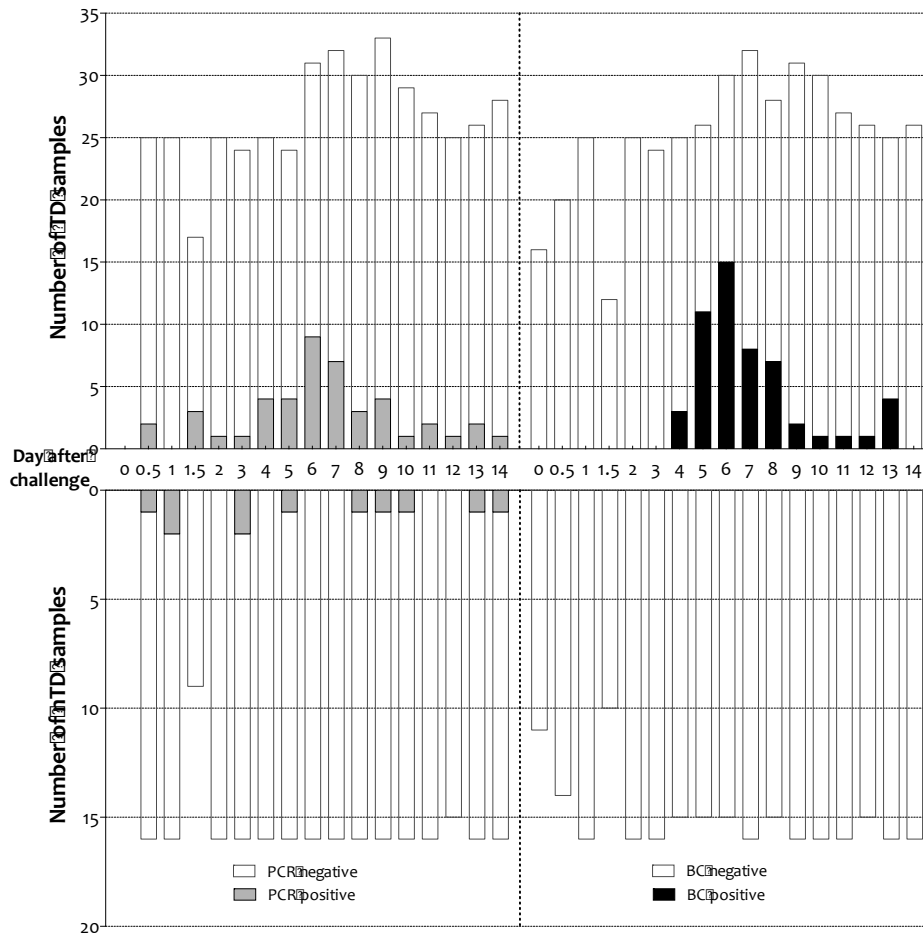


Figure 7-6. Number of positive culture-PCR and blood culture samples collected after challenge by typhoid outcome.

TD, typhoid diagnosed; nTD, non-typhoid diagnosed. The 6 and 12-hour positive results have been pooled into the 0.5 day group. The maximum number of TD samples/day exceeds the number of TD participants as more than one sample was collected per day following initiation of antibiotic treatment. PCR, culture-PCR assay; BC, blood culture.

7.3.3 PRIMARY DNA-EMIA

The earliest sample positive by PCR was collected at 6-hours and a further 2 participants were positive by 12-hours after challenge. In total, 9 positive samples from 7 participants were obtained ≤ 48 -hours after *S. Typhi* ingestion with samples from a further 2 participants positive by 72-hours (Table 7-4 & Figure 7-6). While early positive results were occasionally found in participants who also demonstrated shedding of *S. Typhi* in the

stool ($n=4/41$, presumed to be transit of the challenge agent, example shown in Figure 7-7), participants with positive early stool culture results were no more likely to have an early positive PCR result. Similarly there was no predictive value in a positive PCR result ≤ 72 -hours after challenge and subsequent development of typhoid infection (DOR [95%CI], 0.57 [0.12–2.71]) or development of *S. Typhi* bacteraemia (0.50 [0.10–2.44]).

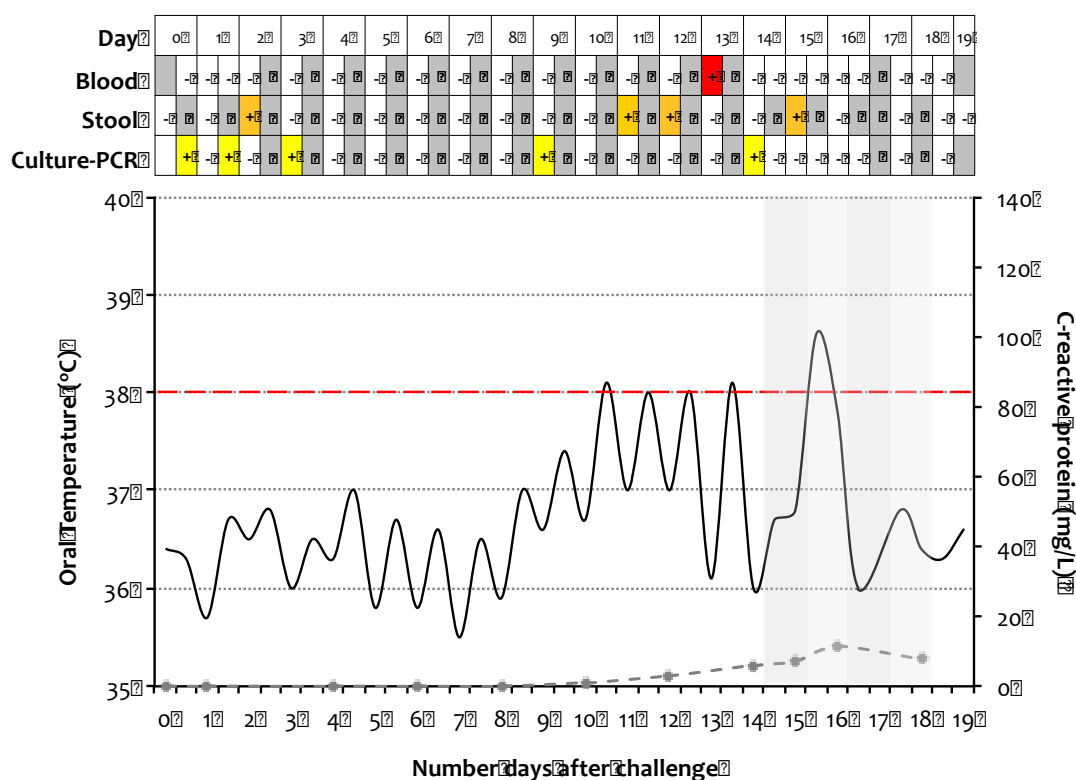


Figure 7-7. Example of a challenge study participant who had several early positive culture-PCR results in addition to an early positive stool culture result.

Positive culture-PCR result, yellow squares; positive stool culture result, orange squares. This participant was subsequently diagnosed with typhoid infection based on both microbiological and clinical criteria. Red square, positive blood culture; grey squares, no sample collected; black line, oral temperature; dashed grey line, C-reactive protein level; shaded area, antibiotic treatment initiated.

7.3.4 CONFIRMATION OF TYPHOID DIAGNOSIS

Of the 24 participants diagnosed with typhoid infection (TD) plus one participant treated for severe symptoms without meeting the predefined criteria, 21/25 were bacteraemic with or without also reaching the temperature threshold ($\geq 38^{\circ}\text{C}$ for ≥ 12 hours; Figure 7-8). While the PCR and microbiology results concurred in TD participants with bacteraemia

plus fever, there was some discrepancy of results in participants who were diagnosed based on positive blood culture or temperature criteria alone

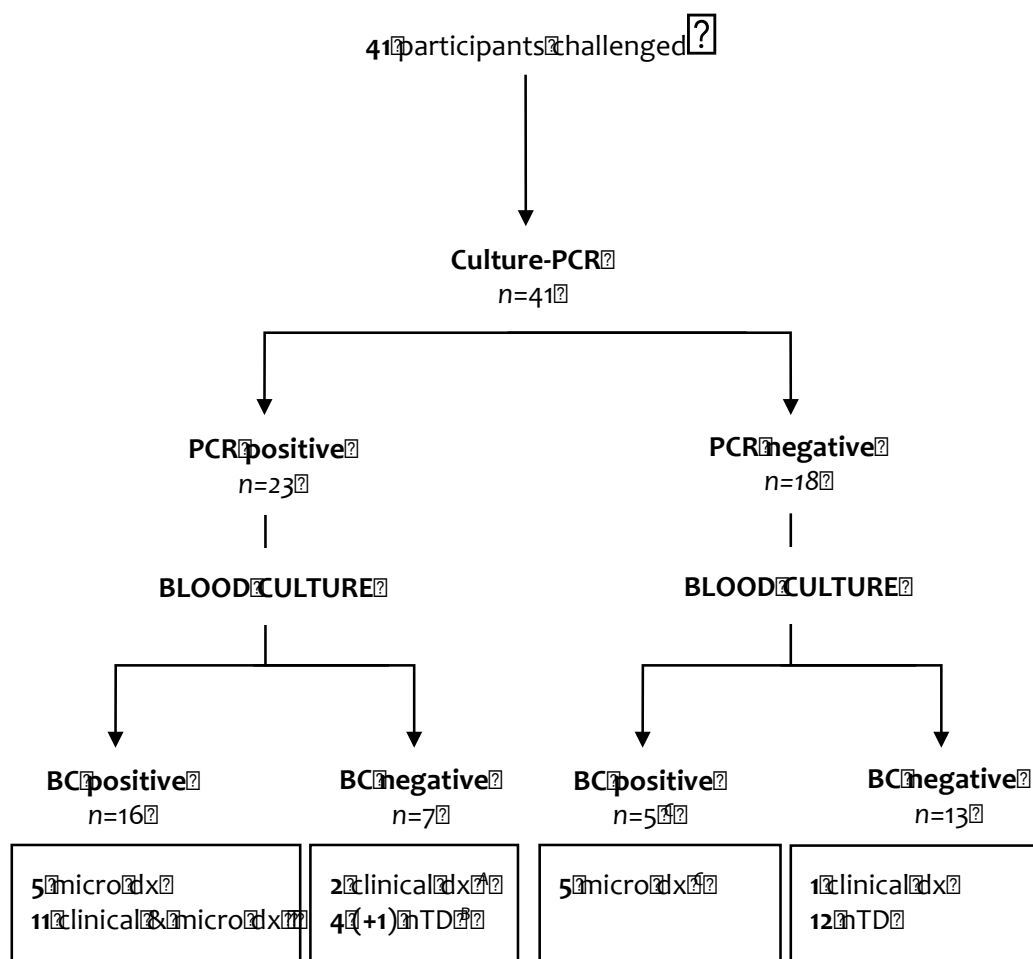


Figure 7-8. STARD flowchart describing culture-PCR results in comparison with blood culture (reference standard) for diagnosis of participants after challenge.

nTD, non-typhoid diagnosed; clinical, sustaining an oral temperature of $\geq 38^{\circ}\text{C}$ for ≥ 12 hours; microbiological, having ≥ 1 positive blood culture result; dx, diagnosis. Superscript letters are described in text.

Three participants reached the clinical definition for typhoid without becoming bacteraemic, i.e., there was no blood culture confirmation of the diagnosis. Of these, 2/3 participants had positive PCR results to support the clinical diagnosis (A, in Figure 7-8; see example in Figure 7-9). In addition, the participant treated based on symptoms only without meeting either of the required criteria for a ‘diagnosis’ to be made, was also found to have positive PCR and stool culture results (B; Figure 7-16).

There were 5 participants who were blood culture positive but remained PCR negative. In general, they had fewer positive blood cultures (mean, 2.0 vs. 2.6; C, in Figure 7-8), although this difference was non-significant (95%CI, -2.2-1.1), and they were no less symptomatic.

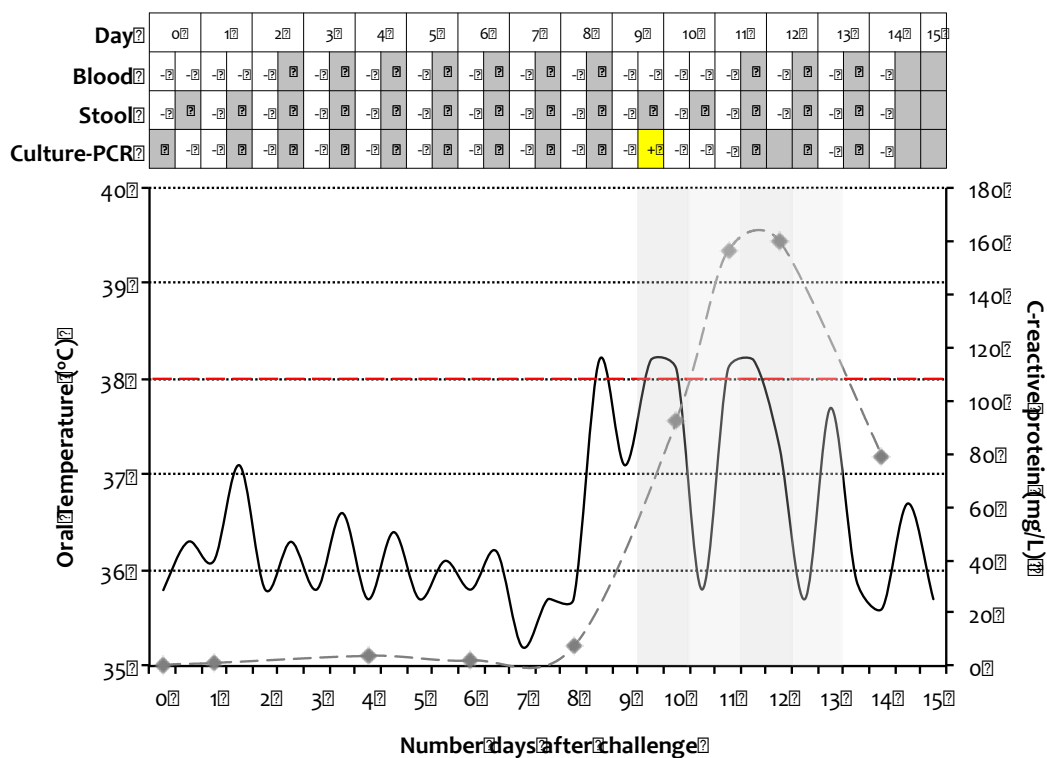


Figure 7-9. Example of a challenge study participant who was diagnosed with typhoid infection based on clinical criteria (oral temperature $\geq 38^{\circ}\text{C}$ for ≥ 12 hours) on Day 9 after challenge. The only laboratory confirmation of the diagnosis was by culture-PCR (yellow square).

Despite also forming part of the TD definition therefore, use of blood culture alone as a reference standard appeared to miss some typhoid cases. Assuming that the predefined TD criteria were a better reference standard, PCR demonstrated a sensitivity and specificity of 75.0 and 55.6%, respectively (Table 7-5). Routine blood culture was more sensitive and specific, 87.5 and 100% respectively, with the caveat of bacteraemia forming part of the TD definition.

		Challenge outcome			Challenge outcome				
		TD	nTD	total	TD	nTD	total		
PCR result	pos	18	7	25	Blood culture result	pos	21	N/A*	21
	neg	6	10	16		neg	3	17	20
	total	24	17	41		total	24	17	41
		PCR		Blood culture					
Sensitivity	0.75	[0.53 – 0.90]	0.86	[0.68 – 0.97]					
Specificity	0.56	[0.31 – 0.78]	1.00*	[0.80 – 1.00]					
LR+	1.69	[0.96 – 2.97]	N/A						
LR-	0.45	[0.20 – 1.01]	0.12	[0.04 – 0.36]					
PPV	0.69	[0.48 – 0.86]	1.00	[0.84 – 1.00]					
NPV	0.53	[0.35 – 0.85]	0.85	[0.62 – 0.97]					
DOR	4.3	[1.1 – 16.3]	215	[10.4 – 4448]					

Table 7-5. Contingency tables displaying estimates [95% CIs] of the sensitivity and specificity for culture-PCR and routine blood culture for diagnosing participants with typhoid infection during a challenge study.

*Note that bacteraemia was one of the diagnostic criteria. TD, typhoid diagnosed; nTD, non-typhoid diagnosed; LR, likelihood ratio; PPV, positive predictive value; NPV, negative predictive value; DOR, diagnostic odds ratio.

Discordant pairs analysis identified 12 mismatching PCR and blood culture results, and confirmed that there was no significant difference between PCR and blood culture sensitivity if used alone to diagnose infection ($p=0.77$, binomial test; Table 7-6).

		Diagnostic test results		
		Blood culture		
		Pos	Neg	
PCR result	pos	16	7	23
	neg	5	13	18
	total	26	15	41

Table 7-6. Discordant pairs analysis comparing blood culture to culture-PCR sensitivity for the diagnosis of typhoid infection during a human typhoid challenge study.

7.3.5 (A) SYMPTOMATIC DNA-EMIA

In those participants who remained non-typhoid diagnosed throughout the 14-day observation period (nTD), 5/17 had ≥ 1 positive PCR results (B, in Figure 7-8). Further investigation of participants' clinical features demonstrated that several had features in keeping with development of typhoid infection in this controlled setting (Table 7-7).

Pt Id.	Day	Blood/stool	Oral temperature, °C	Symptoms recorded
438*	10	-/-	36.0 (38.3 in evening)	Generally unwell, loss of appetite and muscle pain
140	8	-/-	36.6	Mild generally unwell, abdominal pain and diarrhoea (most symptomatic day)
	13	-/-	36.4 (35.2 previous evening)	None recorded
740	3	-/-	35.7	Mild constipation only
607	3	-/-	36.7	Mild headache, generally unwell and cough (most symptomatic day)
	5	-/-	36.5	Mild cough only
191	9	-/-	36.6	Mild cough only

Table 7-7. A table describing the microbiological and clinical features of five participants with positive culture-PCR results who remained non-typhoid diagnosed after challenge.

Pt id., participant identifier; Day, number of days after challenge that the positive sample was collected on; Blood/stool, blood and stool culture results from samples collected contemporaneously; -, negative result. *see example below.

These included a single elevated temperature reading within a 12-hour window of the positive PCR result (Pt 438, Figure 7-10 & Table 7-7), and maximal symptom reporting on the day of the positive result (Pts 438, 140 & 607, Table 7-7). The remaining 2/5 participants were only mildly symptomatic, reporting mild severity constipation and cough only on the day of the positive result. They were slightly more symptomatic than other nTD participants, reporting 10 and 11 symptoms each compared to an overall mean of 7.4 per participant during the 14-day period, however symptom reporting was highly variable (range 0-18/14-day period).

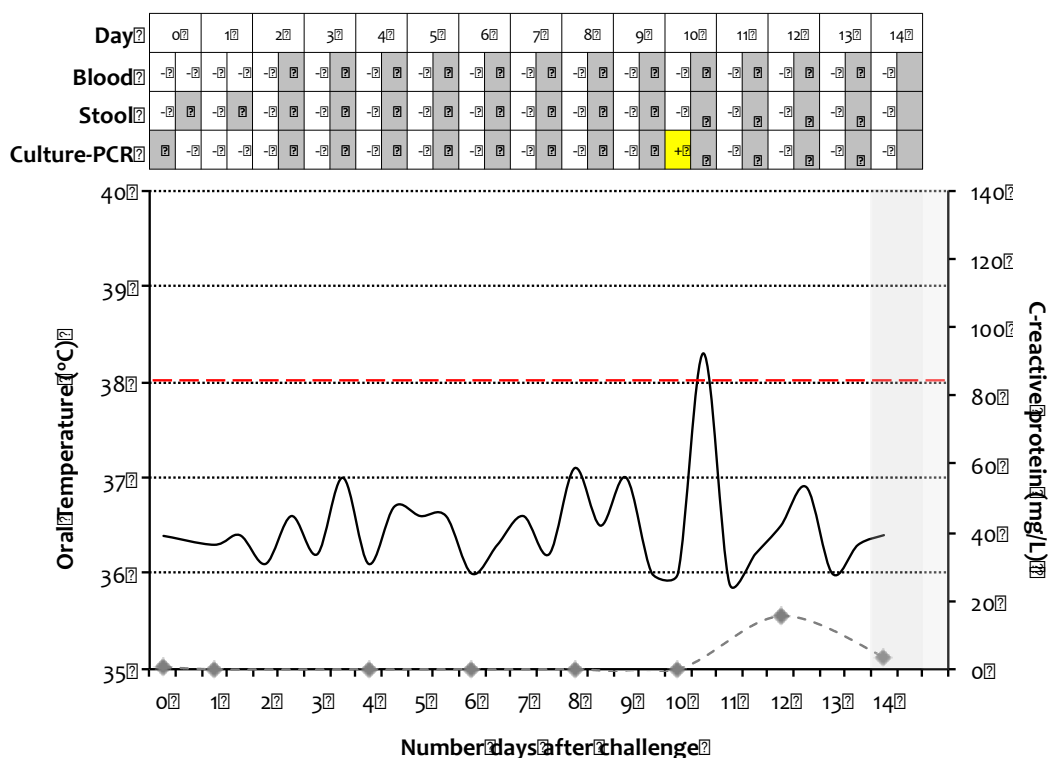


Figure 7-10. Example of a participant's timecourse who remained non-typhoid diagnosed but had a positive culture-PCR result (yellow square) on Day 10.

Pt Id. 438 in Table 7-7. This occurred on the maximal symptomatic day with an elevated recorded temperature (38.3°C, black line) that evening and a subsequent modest increase in CRP levels (dashed grey line).

7.4 CULTURE-LAMP

7.4.1 LAMP OPTIMISATION RESULTS

A successful LAMP reaction results in amplified DNA structures of various lengths that are seen as a dense ladder-type band by gel electrophoresis or as turbidity when inspected under a bright light (Figure 7-11). Initial experiments were performed to assess whether altering the ratio of DNA template to distilled water (dH₂O) or S₃ (x10 buffer solution) resulted in any obvious alteration in assay sensitivity and whether using 1 or 2% EtBr resulted in clearer bands being produced. While some increase in signal was seen using 5µL vs. 1µL positive control results were not affected by using additional S₃ rather than dH₂O; use of 2% EtBr in the gel did result in a more distinct banding pattern and was therefore used subsequent experiments (Figure 7-12).

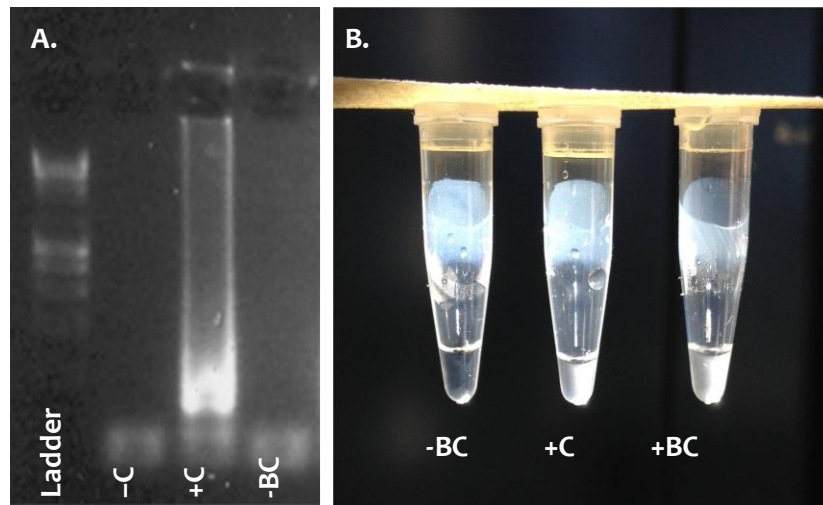
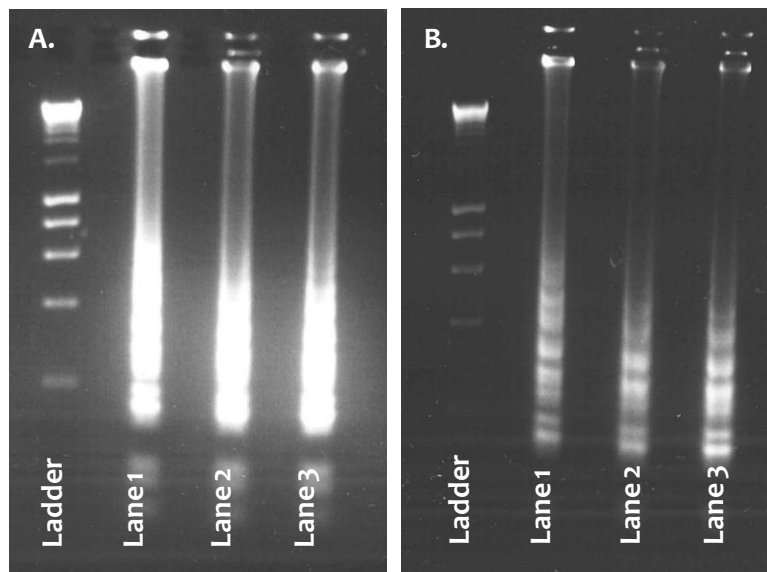


Figure 7-11. Examples of positive and negative LAMP assay results.

Using A, agarose gel electrophoresis with 1% ethidium bromide incorporation to detect the band (lane +C), and B, visible detection of turbidity (in +C and +BC tubes) caused by accumulation of magnesium pyrophosphate as a by-product of the amplification reaction. -C, dH₂O alone; +C, dH₂O spiked with *S. Typhi*, Quail's strain DNA; -BC, negative blood control (non-typhoid clinical sample); +BC, positive blood (typhoid clinical sample).



µLs	Lane 1	Lane 2	Lane 3
Reaction mix	12.5	12.5	12.5
Bst polymerase	1	1	1
Primer mix	1.3	1.3	1.3
DNA template	5	5	1
dH ₂ O	5.2	0	0
S ₃ (buffer)	0	5.2	9.2

Figure 7-12. Optimisation of LAMP assay sensitivity and gel run conditions.

Using A, 1% EtBr, or B, 2% EtBr agarose gels. Corresponding reaction mixtures for each lane are given in the table.

Optimal results were obtained when all reagents were kept on ice and the processes were performed without delay; at RT or with delay, a noticeable increase in false positivity was seen. Conversely, once the LAMP reaction had been initiated alteration of incubation temperature in the range of 60-65°C, appeared to have no effect on assay performance (*data not shown*).

7.4.2 ADDITION OF LOOP PRIMERS

Initial experiments were performed using 4 primers only (FIP, BIP, F3 and B3), thus further experiments were performed to assess whether the addition of loop primers (LF and LB) increased assay sensitivity. Time-course experiments were performed to investigate different incubation durations at 63°C, with and without the addition of loop primers (Figure 7-13). Reactions were terminated by incubating tubes at 80°C for 5 min.

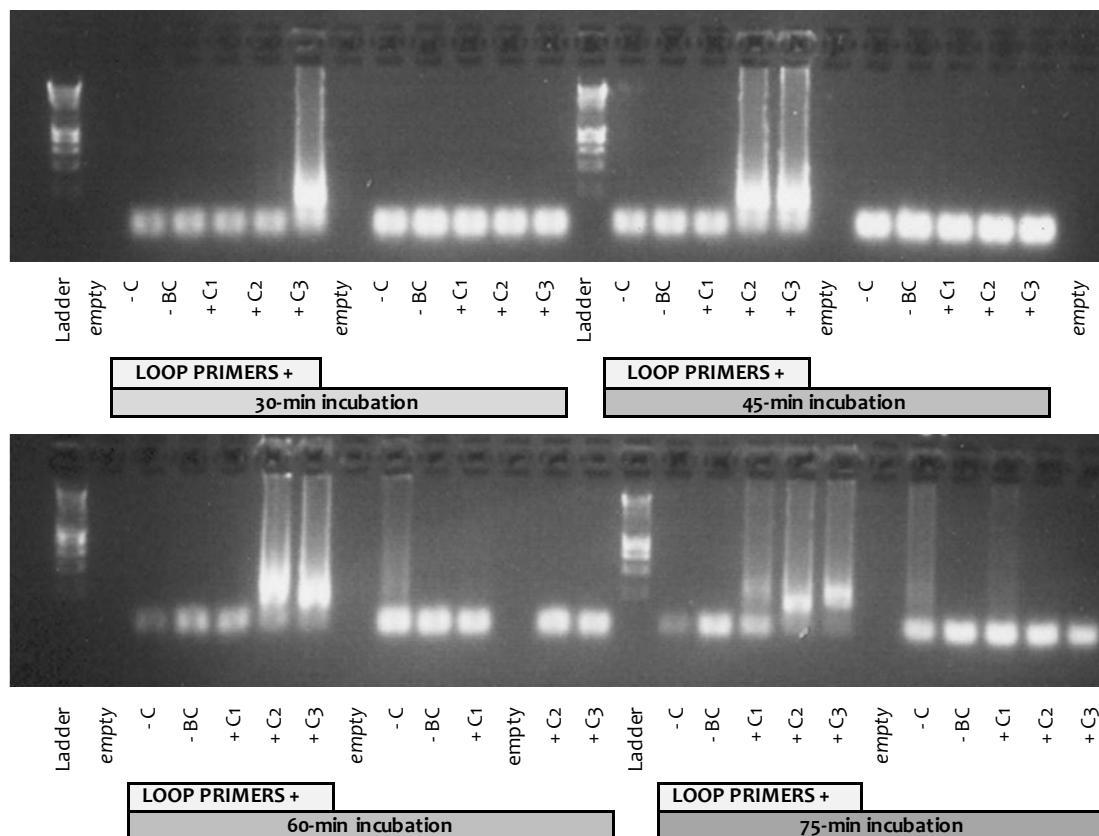


Figure 7-13. Example LAMP timecourse experiment demonstrating the effect of incubation time and loop primer (LF and LB) addition on assay sensitivity.

-C and -BC, as previous; + C[x], dH₂O plus (1) 0.01, (2) 0.15, or (3) 1.5 µg/µL genomic *S. Typhi* DNA.

These data demonstrate that the addition of loop primers results in enhanced sensitivity of the assay for detecting *S. Typhi fliC-d* DNA. Increasing the duration of incubation also increased assay sensitivity, although after 60 minutes false positive results were seen in the non-loop primer reactions. The sensitivity of LAMP was ~10x fold greater than PCR, as positive results were obtained using DNA quantities of $\geq 0.015\mu\text{g}$, equivalent to a starting number of 0.6CFU/mL blood, assuming no subsequent bacterial multiplication had occurred.

Using these optimisation data, subsequent LAMP experiments were adjusted so that incubation was limited to $\leq 45\text{min}$ and were performed with the additional primers.

7.4.3 FINDINGS USING CHALLENGE STUDY SAMPLES

To assess whether LAMP may be used to more sensitively detect low levels of genetic material in ox-bile/TSB cultured DNA extracts, samples obtained from selected challenge study participants were used. In addition to PCR, LAMP was performed using the optimisation conditions described above.

In four participants whose samples were assessed by LAMP, the assay appeared to demonstrate a high level of sensitivity for detecting *S. Typhi fliC-d* DNA (examples in Figure 7-14 & Figure 7-15), in particular after challenge and around typhoid diagnosis. As with the comparison of PCR and blood culture positivity, however, LAMP results were frequently discrepant with both other assays, even when participants had high levels of bacteraemia. The participant in Figure 7-15, had a quantitative bacterial load of 4.7CFU/mL at point of typhoid diagnosis, for example.

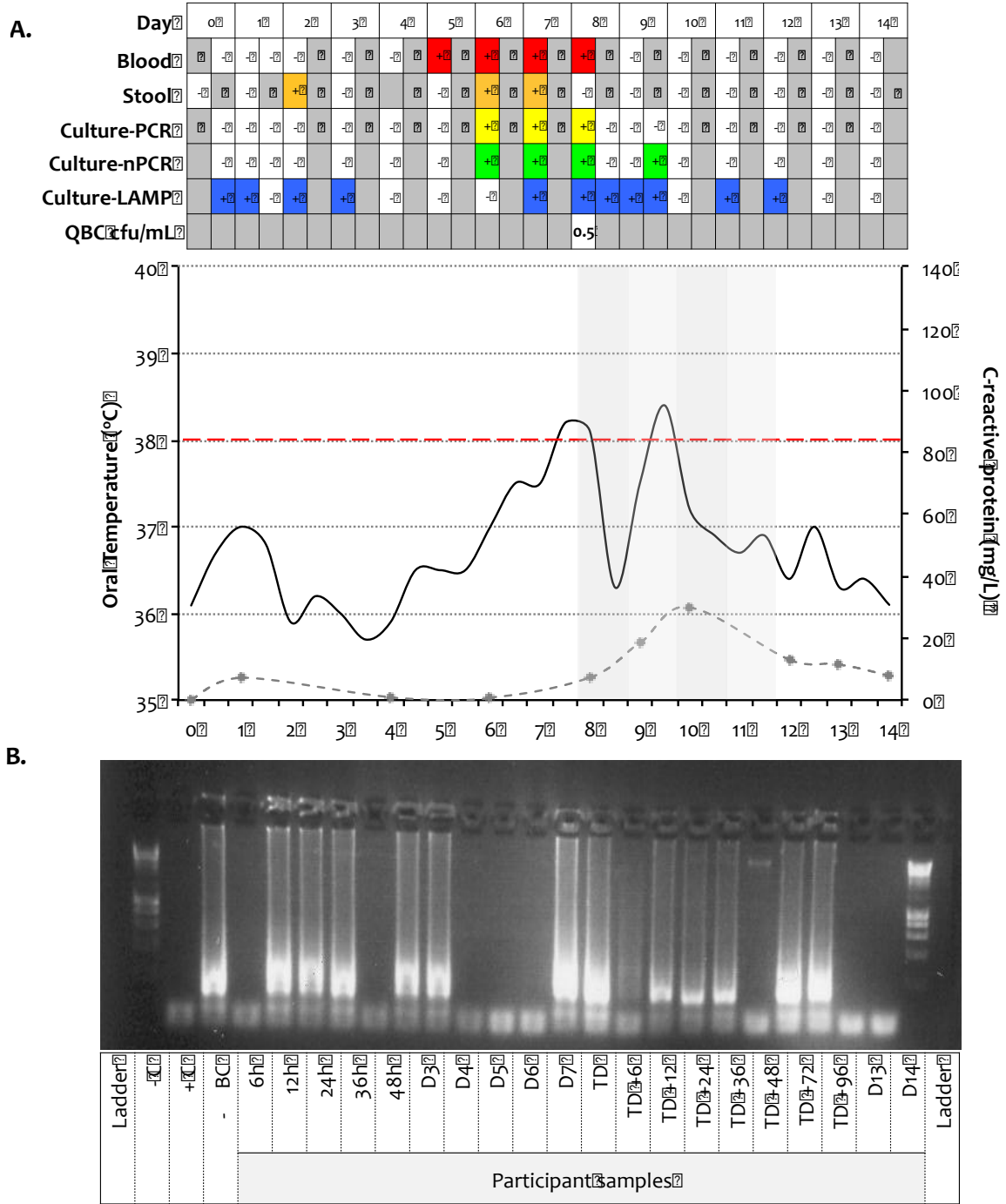


Figure 7-14. Example time course following participant challenge with 1655CFU *S. Typhi*, Quailles strain.

Demonstrating A, summary of the diagnostic laboratory results (see also Figure 7-5) and B, the culture-LAMP agarose gel electrophoresis results. nPCR, nested PCR; QBC, quantitative blood culture (performed at TD only); - C, + C and -BC, as previous; TD, typhoid diagnosis.

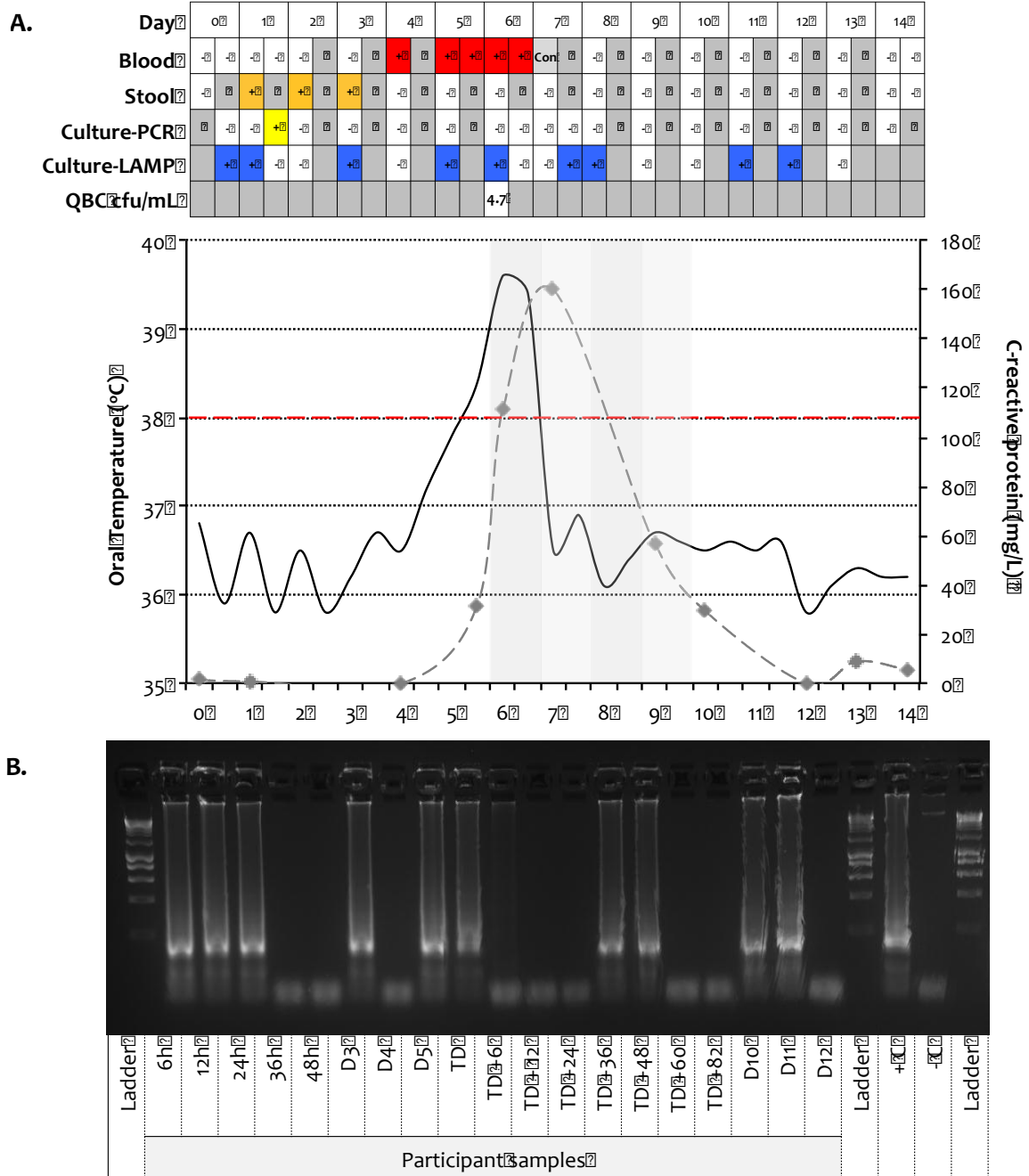


Figure 7-15. Example time course of a challenge study participant with apparently discordant blood and stool culture, and ox-bile culture PCR/LAMP results.

A, participant laboratory diagnostic results and B, culture-LAMP agarose gel electrophoresis results. Con, blood culture was contaminated by skin flora.

7.5 DISCUSSION

7.5.1 NATURE OF THE PROBLEM

Patients clinically infected with typhoid are often difficult to diagnose. Difficulties arise due to the non-specific nature of presenting symptoms, overlap in presentation and epidemiology with other common febrile conditions and the lack of resources in areas where infection predominates. Nevertheless, accurate and rapid diagnosis could be life saving. Early institution of appropriate antibiotic treatment and infection control measures could both save the individuals life and prevent onward transmission of bacteria.

The gold standard for typhoid diagnosis is microbiological culture of material obtained from a normally sterile site. Due to the idiosyncrasies of *S. Typhi* dynamics bacteria are most concentrated in bone marrow while being relatively scarce in the blood stream, even in highly symptomatic patients. Aside from the inconvenience of obtaining bone marrow aspirate, culture may take several days and requires laboratory resources, infection control precautions and expertise in confirming and analysing the microbiological data.

For many other problematic infections, whether due to low pathogen quantities or poor organism viability, for example, due to the activity of pre-administered antibiotics, detection of DNA by PCR has become a standard approach. Potential benefits include the rapid availability of results to guide management in addition to increased test sensitivity and specificity; both highly desirous to improved management of typhoid-infected patients. Previous studies have failed to demonstrate these benefits, in particular in using clinical material rather than isolate collections. A key role of a controlled human infection model could be to provide clinical material from individuals both with known infection

status, but also with known time from organism exposure, illness onset, symptom profile and antibiotic treatment course. Comparing data from diagnostic assays performed in parallel against clinical challenge outcome data provides a powerful tool to evaluate new approaches, as demonstrated in Figure 7-16.

These data presented here, using optimal blood culture (volume, automated culture system, non-antibiotic exposed etc.) as a reference standard, accurately describe the sensitivity of a novel culture-PCR approach for the detection of *S. Typhi* DNA. They suggest that, albeit using half the volume of blood, that culture-PCR may be almost as sensitive as routine bacterial culture for typhoid diagnosis. Comparative specificity was complicated by use of bacteraemia as one of the diagnostic endpoints, however, many previous studies support PCR as being highly specific assuming correct primer selection and moderate laboratory technique.

7.5.2 NOVEL FINDINGS

Interestingly, while there was significant overlap in culture/PCR confirmed cases, culture-PCR also identified or confirmed a number of clinical suspected/diagnosed cases who remained negative by routine blood culture diagnosis (Figure 7-16). Reasons for this in this highly controlled setting are few, as participants were selected for non-immunity and were not antibiotic-exposed. Bacterial characteristics resulting in non-growth may include delay in delivery of the sample to the lab resulting in non-viability or dormancy or the stochastic nature of sampling blood containing very few bacteria. A key further finding was the identification of 'asymptomatic DNAemia', presumably as a feature of non-viable bacterial DNA following host control of the infection. Alternative explanations may include laboratory contamination, however, all positive samples were re-run to confirm the results and precautions were taken to process the samples under clean conditions. Of note, PCR-positive blood-culture negative patients have been identified in previous

studies; a study in Cambodia identified a subpopulation among children <16 years old presenting with fever who were culture-negative but positive using a real-time PCR assay. Of note, this subpopulation was younger, had a shorter duration of illness prior to presentation and presented with fewer features characteristic of typhoid infection. ‘Incomplete immunity’ and earlier presentation with illness are common to both patient/participant groups.⁴⁹⁹

A further finding of interest is the identification of primary circulation of bacteria (or at least DNA) shortly after pathogen exposure (challenge). The existence of an episode of primary bacteraemia as the means for bacterial dissemination to reticulo-endothelial organs has long been hypothesized but these data uniquely provide some confirmatory evidence. These data were not confirmed by blood culture, suggesting that bacteria were non-viable. That culture-PCR is in fact detecting bacterial remnants from a successful innate immune response in preventing infection may therefore be a plausible alternative explanation, however, the relative paucity of symptoms, fever or other features of an inflammatory response are less supportive. Regardless of its origin, early presence of bacterial products including DNA (whether viable or not), may provide some evidence as to the relatively atypical presentation of patients with typhoidal bacteraemia. Patients (and participants in this study) were relatively asymptomatic given the presence of Gram-negative bacteria in the blood. Early exposure to bacteria may induce some degree of tolerance such that subsequent exposure during the second ‘true’ bacteraemic phase results in less of a host inflammatory response.⁵⁰⁰ More recent data suggests responses to response to LPS re-exposure, at least, could be driven by common polymorphisms in TNFR2-encoding haplotypes.⁵⁰¹

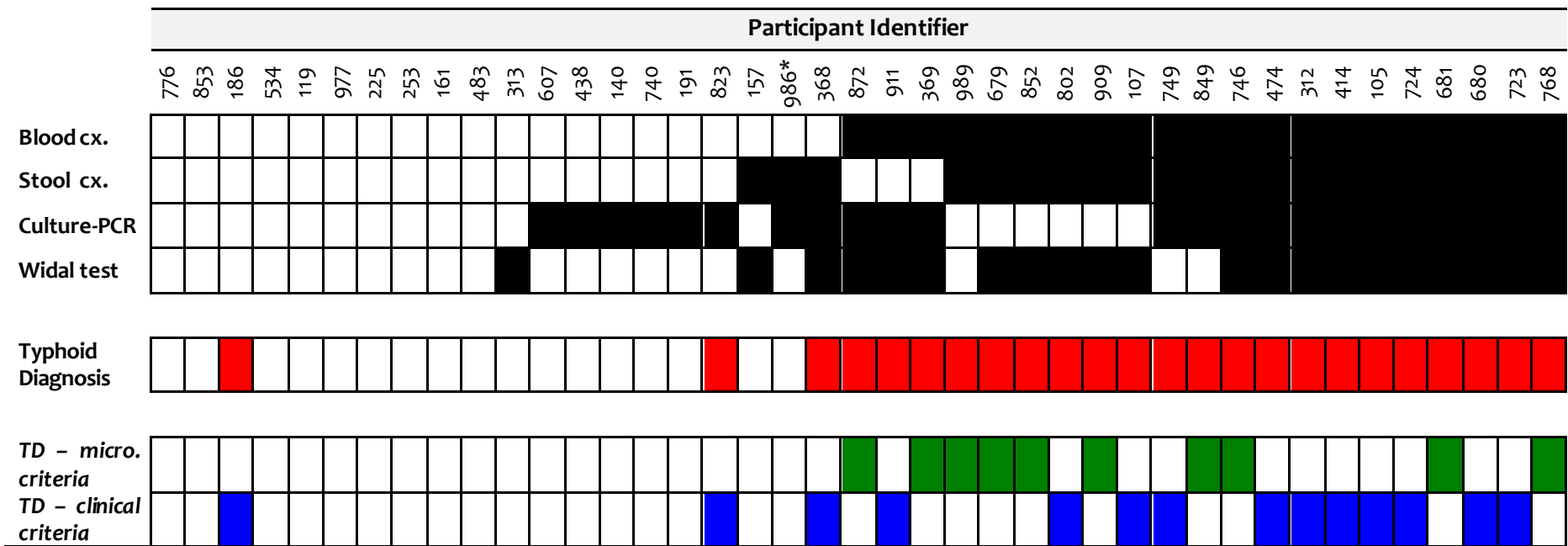


Figure 7-16. A figure demonstrating individual challenge study participants' laboratory diagnostic results and challenge outcome. With filled squares representing a positive result. A positive Widal slide agglutination test was defined as a 4-fold rise in either O or H agglutination titres between samples collected at Day 14 (or Typhoid diagnosis, if applicable) and Day 28 after challenge. The reason for reaching the typhoid diagnosis endpoint was identified by a study physician at the TD visit, and based on either microbiological (=micro.) or clinical criteria. Some participants reached both criteria (see Section 7.2.2) and therefore solely the indication for starting antibiotics is used here.

*Participant 986 was treated based on symptoms experienced without meeting either of the formal study definitions for having typhoid infection.

Cx, culture.

7.5.3 LIMITATIONS

PCR is now a relatively commonly performed technique in most diagnostic laboratories, including those in less well-resourced settings. Unfortunately the pre-culture step, which is vital to increase assay sensitivity, does not yet render the procedure beyond cross-infection (or cross-contamination) risk. The highly selective nature of ox-bile, while suitable for *S. Typhi* and other bile resistant organisms, means that this assay is of unlikely applicability to most settings where clinical material is scant and *S. Typhi* is not the predominant pathogen.²⁰² Alternative lysing agents have been proposed, including saponin (used for bacterial blood quantification in this study) and digitonin, which may produce cell lysis without loss of bacterial viability.^{502,503} As with all PCR, detection of DNA does not equate to detection of viable bacteria, and therefore extrapolation of results to bacterial behaviour must remain circumspect. These data do suggest that it may be a highly useful research tool, both to more accurately categorise or confirm clinical diagnoses where other methods have failed, and as a benchmark for the discovery and development of other diagnostic tools.

As mentioned above, a major limitation to the interpretation of the comparative blood culture data, is the difference in volumes of blood used. A lower volume was used to reduce the risk of exsanguination to the participants but also as a pragmatic approach in order to develop an assay that may be acceptable in other non-research settings.

7.5.4 TOO SENSITIVE?

Methods to further increase the sensitivity of the assay using the volume of blood available and to develop the assay for use in settings where thermocycling facilities were not available prompted the exploratory development of a culture-LAMP assay. These initial results suggest that the culture-LAMP assay shows high sensitivity in detecting *S.*

Typhi DNA from clinical specimens. Using spiked samples, Francois *et al* demonstrated a detection threshold of 1.5-2CFU/mL; in experiments performed to-date, a sensitivity of 2-3CFU/reaction has been found.⁴⁹⁴ This work supports LAMP as being almost 10-fold more sensitive than PCR. While sensitivity was apparently improved during optimisation experiments, there was concern that non-specific reactions were observed, possibly in part due to the non-specific cross-binding of the 6 primers. This effect appeared to be exacerbated by prolonging the reaction time. Future work will aim to optimise primer selection and assess whether this sensitive method may be sufficient to detect bacterial DNA without the pre-culture step used here.

7.5.5 SUMMARY

Despite relative limitations to their use in clinical settings, culture-PCR has provided interesting and unique data in the context of this highly controlled clinical trial regarding pathogen dynamics and potential diagnostic approaches. While the method holds potential for providing additional diagnostic information in highly endemic areas or if selective typhoid identification is required, for example in the context of a vaccine field-trial, a more realistic application may be in comparing bacterial dynamics and confirming challenge outcomes in the absence of alternative supporting diagnostic data.

Chapter 8. RECRUITMENT, VACCINATION AND

CHALLENGE

8.1 INTRODUCTION

This chapter will describe the overall performance of procedures used in performing a study of typhoid vaccine efficacy by oral challenge with wild-type *S. Typhi*; the first regulator-approved study involving human challenge with a containment level (CL) 3 pathogen in the UK. In addition to allowing subsequent interpretation and generalisation of vaccine and challenge study results, they are also of potential interest to anyone involved in designing similar future studies.

Aside from the logistic complexity involved in recruiting and enrolling a large number of healthy, typhoid-naïve adult volunteers, further trial methodology issues included optimal vaccine administration, follow-up of participants and challenge with the pre-determined dose of *S. Typhi*, Quail's strain. The aim of this chapter is therefore to detail each of these study processes to allow accurate interpretation of the clinical trial dataset.

8.2 METHODS

Potential participants were recruited using the methods agreed by the ethics approvals (NRES Committee South Central – Oxford A, 11/SC/0302) and described previously (see: 5.2.1). Following the initial approach of volunteers, a two-stage screening and recruitment process was used. Following enrolment into the study (which occurred on randomisation to a vaccine arm) participants followed the schedules and procedures as described in section 5.2.5 .

8.3 RECRUITMENT

With the necessary study approvals in place, participant recruitment procedures commenced in November 2011.

8.3.1 RECRUITMENT METHODS

A range of recruitment methods were used ranging from targeted approaches to individuals previously providing consent to be included on the Oxford Vaccine Centre healthy volunteers database, to full-page adverts in the Oxford United football programme and county-wide newspapers. Although difficult to estimate, >17,000 people are likely to have seen advertisements offering participation in the study (Table 8-1.).

Specific considerations in recruiting study volunteers in Oxford include the large, generally well-informed student population of Oxford and Oxford Brookes University. Each institution has different measures in place for approaching the student body, in addition to variations in term dates and exam schedules.

Recruitment approach	Details
Electoral roll	10 separate mail-outs to 16,611 addresses around Oxfordshire
Posters	271 locations including shops, colleges and libraries
E-mails	Sent to 107 college common room presidents and university department administrators for further dissemination
Weekly information	Advertised in Daily Info around Oxford
Newspapers	Newsquest Digital (including Herald series, Oxford Times, Oxford Mail and featured jobs) and South West Jobs newspaper (to South Oxfordshire)
Football programmes	Oxford United matches on 2 match days and supporters list serve email

Table 8-1. Description of some of the varied strategies used to recruit participants to this vaccine-challenge study.

8.3.2 RECRUITMENT RESULTS

In reply to the advertising 710/1371 (52%) favourable replies were received. Of these potential participants, 154/710 (21.7%) were invited for a consent/screening visit, following which 10 declined further participation and 45 were excluded by one or more screening procedures (Table 8-2).

Reason for exclusion	Number excluded	
	Prior to screening visit	At/after screening visit
Health Concerns	40	26
Not available for all study visits	26	1
Out of age range	3	0
Previous typhoid vaccination	73	7
Study enrolment complete	92	7
Taking disallowed medication	8	0
Unknown	3	0
Other (see below)	42	4
TOTAL	287	45

Table 8-2. Reasons underlying potential volunteer screening failures.

Participants were excluded either during the initial telephone screening call or at/after the CCVTM screening visit. Other includes: contact with young children (3), contact with vulnerable individuals (9), food-related occupation (5), previously resident in typhoid-endemic area for >6 months (15), unable to contact (6) & unknown (8).

8.3.3 PARTICIPANT ENROLMENT

Ninety-nine participants successfully completed all screening procedures and were enrolled by mid-June (Figure 8-2). A further 7 participants completed screening procedures but after completion of study enrolment; these participants were held in reserve in case of a >10% dropout rate was encountered prior to completing the course of vaccination before challenge.

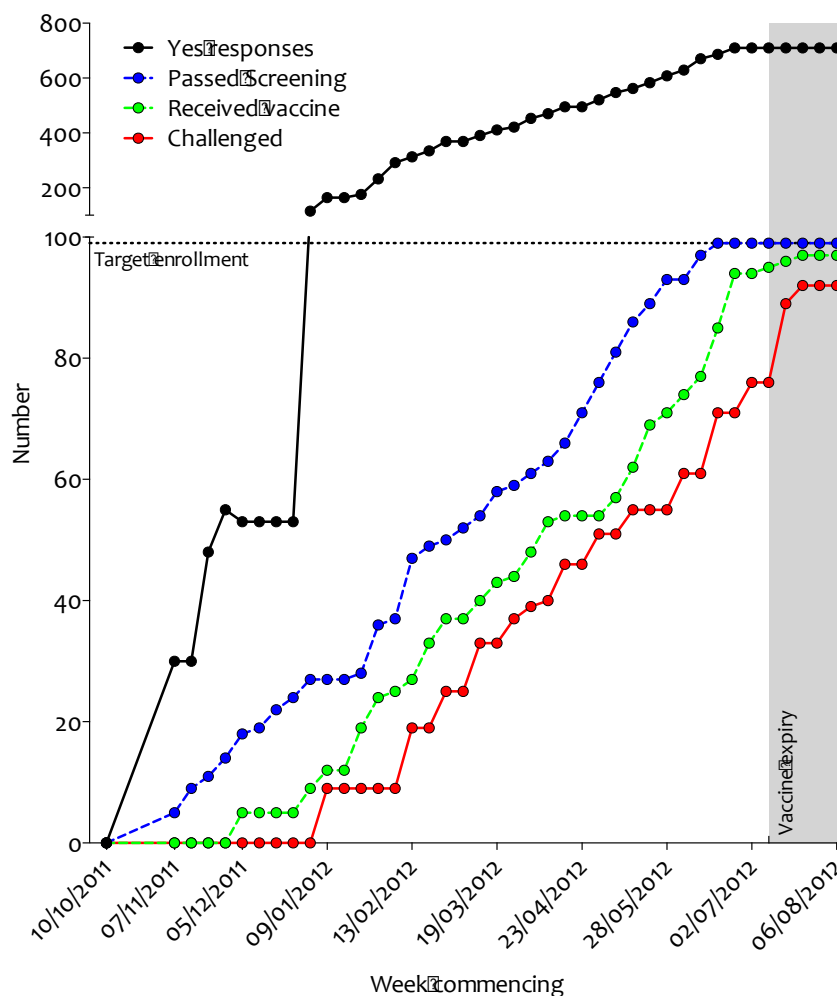


Figure 8-1. Study timecourse between completion of regulatory approvals (7 Oct 2011) and cumulative recruitment response, successful screening visits, vaccinations and challenge. M01ZH09 vaccine expiry date was 09 Jul 2011; those given after this date were Ty21a open-label vaccinations only.

8.4 PARTICIPANT DEMOGRAPHICS

The majority of those enrolled were males (65%) in their mid-20's and of self-declared white British ethnicity (89%; Table 8-3). Many were students or in professional employment. Participant occupations ranged widely, and included professional musicians, business consultants, plumbers and life models.

Previous travel to an endemic area was not an exclusion criteria, however participants were required to have been resident <6-months. Twenty-nine participants reported a relevant travel history, i.e. had travelled to a country where typhoid is considered endemic which was defined as country for which the UK National Travel Health Network and Centre (NaTHNaC) recommends typhoid vaccination. The most frequent countries visited were Thailand ($n=9$), South Africa (6) and the North African region (6). Duration of previous exposure ranged markedly, and, although not noted in detail, individual trips ranged from short excursions to 6-months and took place between 6-months and 25-years prior to study enrolment. These included individuals who had worked in refugee camps (Kenya and Tanzania), had expatriate parents or had been backpacking as students. Additional effort was made to ensure that a previous vaccine record was available from these individuals; many had travelled prior to the advent of typhoid vaccine introduction for travel-use in the late 1980's.

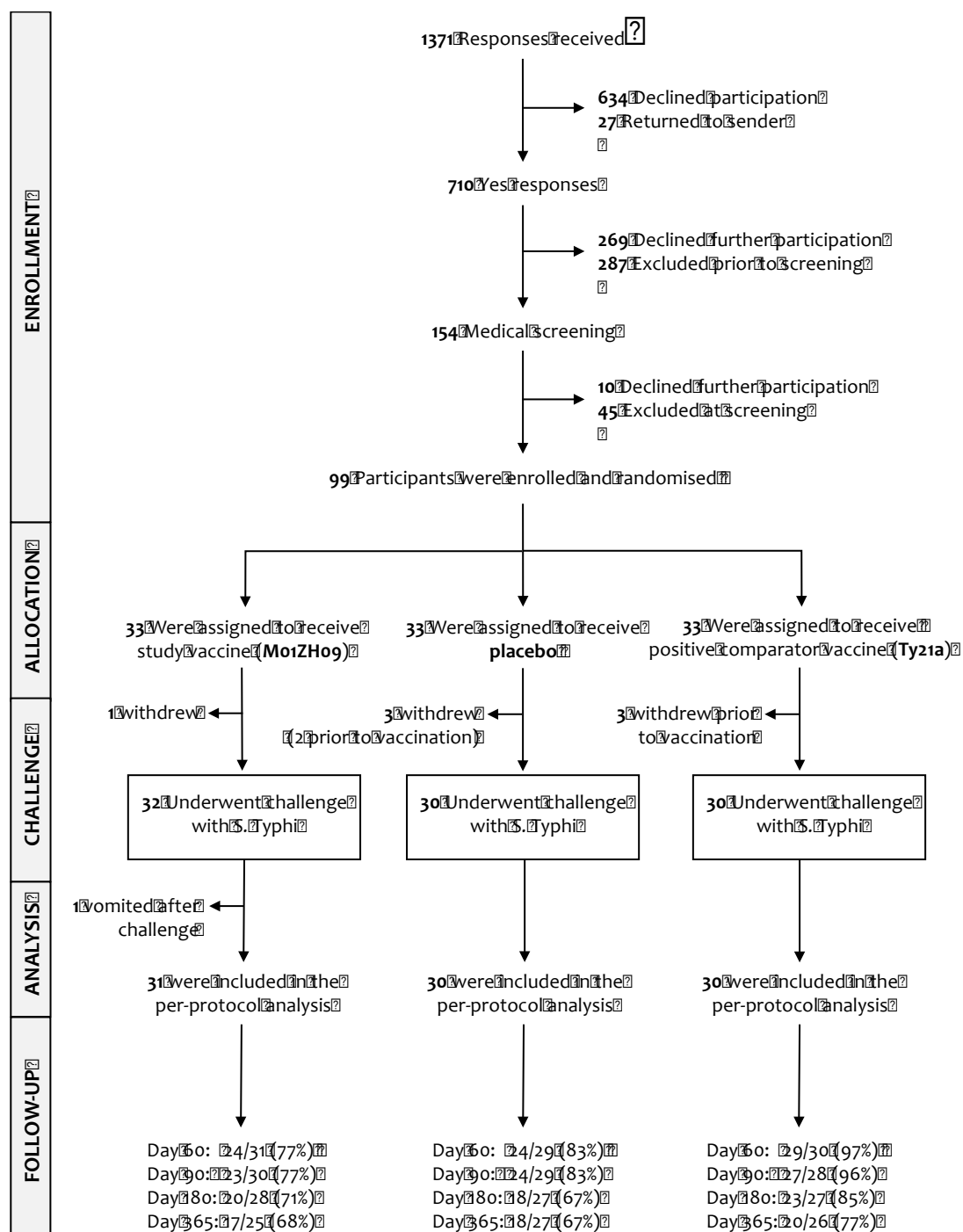


Figure 8-2. CONSORT flow chart describing volunteer recruitment and disposition for the vaccine-challenge study, OVG2011/02.

8.4.1 GROUP MATCHING

While participants to the three groups were well matched overall, participants in the placebo group tended to be younger and fewer had a history of travel to an endemic region (Table 8-3).

Sixty-five participants reported previous medical or surgical histories; however, 24 only reported current medical problems. These included asthma ($n=7$), hayfever (3), eczema/psoriasis (2) and assorted musculoskeletal problems (3). Of note, two participants had past medical histories including depression, both of whom were currently 'stable' on treatment, and 2 participants reported active irritable bowel syndrome.

	M01ZH09 (n=33)	Placebo (n=33)	Ty21a (n=33)
Male sex (%)	66.7	57.6	69.7
Age years, median (range)	24 (19-60)	23 (19-59)	25 (19-55)
Ethnicity (%)			
White British	87.9	93.9	84.8
Other White	6.1	6.1	6.1
Mixed	-	-	6.1
Mixed Caribbean	-	-	3.0
White Irish	3.0	-	-
Chinese	3.0	-	-
Employment status (%)			
Employed	31.2	45.5	48.4
Student	43.8	45.5	45.2
Self-employed	6.2	3	6.5
Unemployed	12.5	6.1	0
Retired	6.2	0	0
Alcohol consumption, any (%)	81.8	90.9	97
Tobacco smoker, any (%)	37.5	36.4	29.1
Other drug use (%)	6.5	3	9.1
Previous travel to endemic area (%)	31.2	24.2	33.3

Table 8-3. Participant demographic profile by vaccine group allocation.

8.4.2 BASELINE CLINICAL FINDINGS

There were no significant differences in the clinical examination findings from individuals allocated to any of the three groups either at the screening visit or at the baseline pre-vaccination visit, although measured pulse rates tended to be faster at the later visit (mean [95%CI]: 68.0 [64.1-71.9] and 73.8 [69.4-78.2], respectively; Table 8-4).

	Time point	MoiZHog	Placebo	Ty21a
Temperature, °C	Screening	ND	ND	ND
	Pre-vaccine	36.4 (36.2-36.5)	36.51 (36.4-36.7)	36.4 (36.2-36.6)
	Day 3	-	-	36.4 (36.2-36.6)
	Day 5	-	-	36.3 (36.1-36.4)
	Day 7 (Va)	36.2 (36.1-36.3)	36.3 (36.1-36.5)	36.2 (36.1-36.4)
	Day 14 (Vb)	36.1 (35.9-36.4)	36.3 (36.1-36.4)	36.2 (36.0-36.3)
Pulse, b.p.m.	Screening	66.9 (62.9-70.9)	69.4 (65.2-73.5)	67.7 (64.4-71.2)
	Pre-vaccine	73.6 (69.7-77.4)	73.9 (69.7-78.1)	74.6 (69.8-79.4)
	Day 3	-	-	76.3 (72.0-80.5)
	Day 5	-	-	74.2 (69.7-78.7)
	Day 7 (Va)	73.7 (69.9-77.5)	73.8 (68.8-78.9)	75.1 (70.6-79.5)
	Day 14 (Vb)	71.3 (72.9-81.5)	74.9 (70.0-79.8)	76.5 (71.3-81.6)
Systolic blood pressure, mmHg	Screening	129.5 (124.2-134.8)	127.1 (122.3-131.9)	131.1 (127.2-135.1)
	Pre-vaccine	129.5 (124.7-134.3)	129.4 (126.0-132.9)	129.8 (124.9-134.6)
	Day 3	-	-	126.3 (121.5-131.1)
	Day 5	-	-	127.7 (123.1-132.4)
	Day 7 (Va)	127.0 (121.9-132.2)	126.5 (122.3-130.6)	128.1 (123.5-132.7)
	Day 14 (Vb)	130.5 (126.2-134.7)	126.1 (120.6-131.7)	131.1 (126.9-135.2)
Diastolic blood pressure, mmHg	Screening	80.2 (76.4-83.9)	79.4 (76.1-82.7)	79.7 (76.5-82.9)
	Pre-vaccine	78.1 (75.0-81.2)	80.9 (78.0-83.8)	79.5 (75.9-83.0)
	Day 3	-	-	77.9 (74.7-81.2)
	Day 5	-	-	80.4 (76.7-84.1)
	Day 7 (Va)	78.4 (74.4-82.3)	78.3 (75.3-81.4)	81.9 (78.6-85.2)
	Day 14 (Vb)	76.1 (73.6-80.1)	77.9 (74.7-81.1)	78.5 (75.1-81.9)

Table 8-4. Clinical examination findings for ITT participant population enrolled to each vaccine group.

Data presented as mean (95%CI). Clinical findings were recorded at the screening visit (performed within 3 months prior to vaccination), pre-vaccination (Day 0) and at four post-vaccination visits (Day 3, 5, 7 and 14). ND, not done at this visit; -, participants in this group not seen on this day.

Electrocardiogram findings reflected those expected in a young healthy cohort; 6 participants had borderline left ventricular hypertrophy and/or left axis deviation while 26 participants had resting pulse rates <60b.p.m.

8.4.3 BASELINE LABORATORY RESULTS

Laboratory blood tests performed at the initial screening visits reflected those of a healthy westemised mainly male adult population and demonstrated no significant differences between vaccine groups (Table 8-5).

	Mo1ZH09	Placebo	Ty21a
Haematology			
Haemoglobin, g/dL	14.7 (14.4-15.1)	14.3 (13.8-14.9)	14.7 (14.3-15.0)
MCV, fl	92.6 (91.1-94.1)	90.9 (89.4-92.3)	91.2 (89.8-92.6)
Platelets, x10 ⁹ /L	259.4 (238.1-280.7)	259.1 (238.9-279.3)	248.5 (232.2-264.8)
White cells, x10 ⁹ /L	6.8 (6.2-7.4)	6.6 (6.2-7.0)	6.9 (6.3-7.5)
Neutrophils, x10 ⁹ /L	4.4 (3.8-4.9)	3.9 (3.6-4.2)	4.1 (3.6-4.6)
Lymphocytes, x10 ⁹ /L	1.8 (1.6-1.9)	1.9 (1.7-2.1)	2.1 (1.9-2.3)
Monocytes, x10 ⁹ /L	0.5 (0.5-0.6)	0.6 (0.5-0.6)	0.5 (0.5-0.6)
Eosinophils, x10 ⁹ /L	0.2 (0.1-0.2)	0.2 (0.1-0.2)	0.2 (0.1-0.2)
ESR, mm/hour	3.4 (2.0-4.8)	3.0 (1.8-4.2)	4.3 (2.1-6.4)
Biochemistry			
Sodium, mmol/L	141.0 (140.4-141.6)	141.0 (140.3-141.7)	141.1 (140.5-141.6)
Potassium, mmol/L	3.9 (3.7-4.1)	3.8 (3.7-3.9)	3.8 (3.7-4.0)
Creatinine, µmol/L	75.1 (70.0-80.2)	76.5 (71.3-81.6)	74.1 (70.1-78.1)
Urea, mmol/L	5.0 (4.5-5.4)	4.8 (4.4-5.1)	4.9 (4.5-5.3)
Amylase, IU/L	59.5 (52.3-66.7)	54.1 (49.5-58.6)	60.2 (53.0 (67.4)
Albumin, g/L	47.5 (46.6-48.5)	46.8 (46.0-47.7)	47.3 (46.7-47.9)
Bilirubin, µmol/L	11.6 (9.3-13.9)	11.1 (9.3-12.9)	12.0 (10.4-13.6)
ALT, IU/L	23.6 (20.6-26.6)	22.8 (17.6-27.9)	26.2 (21.1-31.4)
ALP, IU/L	157.5 (146.1-169.0)	154.5 (140.0-169.1)	152.4 (136.8-168.1)
CRP, mg/L	1.4 (0.3-2.4)	1.8 (1.1-2.5)	1.2 (0.1-2.4)
Immunology			
IgA, g/L	2.2 (1.9-2.5)	2.0 (1.8-2.2)	2.3 (2.0-2.6)

Table 8-5. Baseline blood parameters for the ITT participant population according to vaccine allocation.

Data presented as mean (95%CI). Blood was collected at each participant screening visit within 3 months prior to enrolment. No participants tested positive for anti-endomysial antibody (not included). MCV, mean cell volume; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; ALT, alanine aminotransferase; ALP, alkaline phosphatase.

8.5 VACCINATION

The required 99 participants were enrolled within eight months such that all participants had been screened, consented and vaccinated by the time of Mo1ZH09 expiry in July 2012 (Figure 8-1). At enrolment, participants were randomised to one of the three vaccine groups; of the 99 enrolled, 94 completed the required vaccine schedules (5 participants withdrew prior to receiving vaccines).

Of the 5 participants who withdrew prior to vaccine receipt, 4/5 (80%) were female with a mean age of 23.8-years. Reasons for withdrawal all related to non-adherence to study-related procedures; in general work or social commitments had altered such that they were now unable to take part either due to perceived or protocol defined transmission risk or time commitments. One participant was admitted to hospital for acute psychiatric treatment and thus was no longer eligible for study participation. Of these, 3/5 had been assigned to receive open-label Ty21a vaccination and two to receive blinded vaccine (both placebo).

8.5.1 VACCINE VIABILITY AND DOSING

To ensure satisfactory viability of the investigational live vaccine strain (Mo1ZH09, lot 0041010), the manufacturers (IDT Biologika, Germany) performed 4-weekly survival assays using aliquots from the original parent vaccine lot. The measured viable cell count was used to titrate the volume of Mo1ZH09 given, such that each individual received 1.0×10^{10} CFU live bacteria. Despite an apparent initial increase in strain viability (November to December 2011), vaccine viability fell overall during the study period as expected, which required an increased volume of vaccine to be dosed (Figure 8-3).

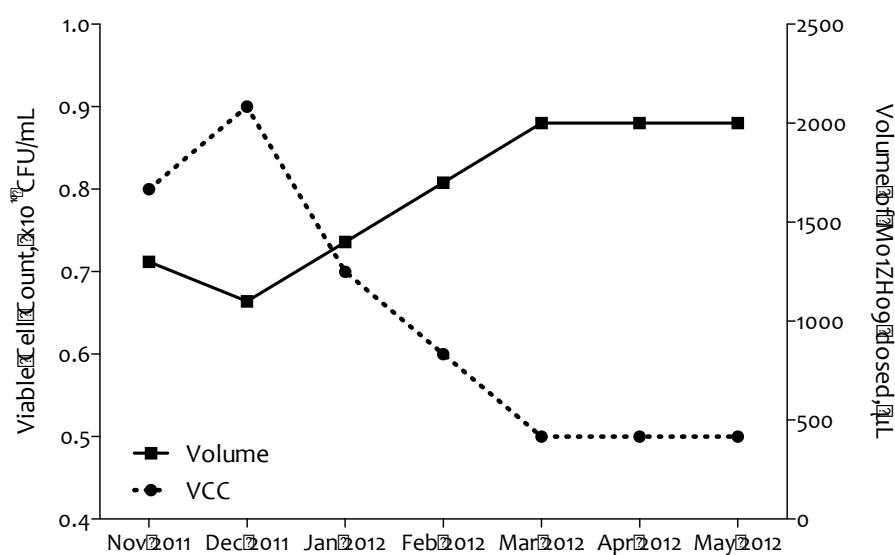


Figure 8-3. Mo1ZH09 vaccine viability during the dosing period of the clinical study. VCC, viable cell count.

8.5.2 VACCINE ALLOCATION AND SCHEDULE COMPLIANCE

All vaccinations were performed in accordance with the allocation schedule provided and within the vaccine expiry dates; vaccinations were given between 05 December 2011 and 23 July 2012. The final participant in the blinded (M01HZ09 or placebo) arm was vaccinated on 6 July 2012, three days prior to the expiry date (9 July 2012) approved in the MHRA CTA.

Participant adherence to vaccine ingestion timings and requirements was extremely high; only 1/63 (1.5%) participant in the blinded arm was given the vaccine whilst un-fasted. The majority of the unblinded vaccine doses (26/30) were also given to fasted participants, despite there being no requirement to be fasted prior to Ty21a ingestion.⁴⁵⁵ Of the 30 Ty21a vaccine schedules completed only one participant did not adhere to the required alternate day schedule, taking doses on Day 1, 3, and 4 instead.

8.6 CHALLENGE

Twenty-eight days after receiving the final vaccination, participants were asked to attend the study site to undergo challenge with *Salmonella* Typhi, Quail's strain at a target dose of 1.5×10^4 CFU. The importance of challenge dose preparation has been highlighted by retrospective assessment of the Maryland data, which demonstrated the ability of higher doses to overwhelm potentially beneficial vaccine responses.⁸⁹ Data from the preliminary dose-escalation study demonstrated the clear dose-response relationship that could be seen when sodium bicarbonate was used to eradicate the effects of stomach acidity, even within each dose level given.⁴³⁶ This was most sensitively seen in measuring time-to-diagnosis, which takes other 'non-challenge' factors (such as stomach acidity, innate immune responses etc.) into account and at lower challenge doses (Figure 8-4).

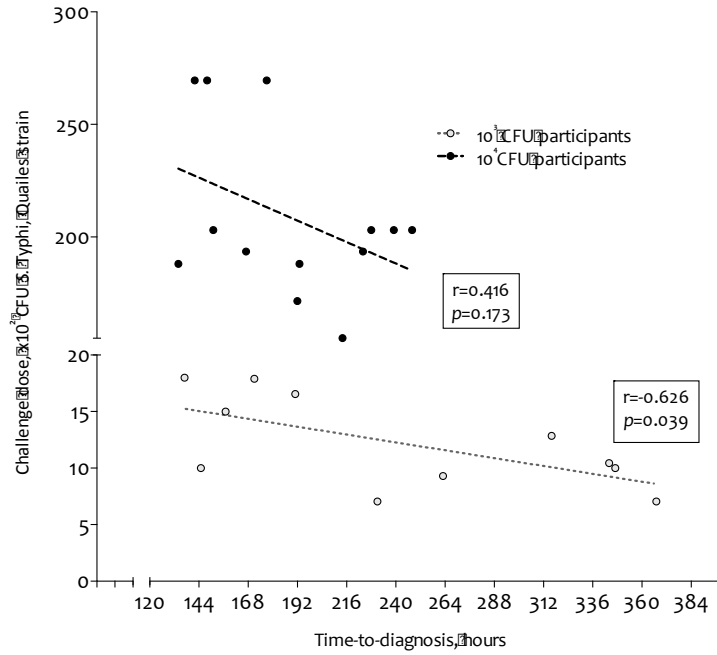


Figure 8-4. Data from the preliminary dose-escalation study demonstrating the effect of challenge dose on time-to-diagnosis.

Ninety-two participants remained eligible to participate in the challenge phase of the study, and were challenged in groups of up to 10 participants at a time (Figure 8-5).

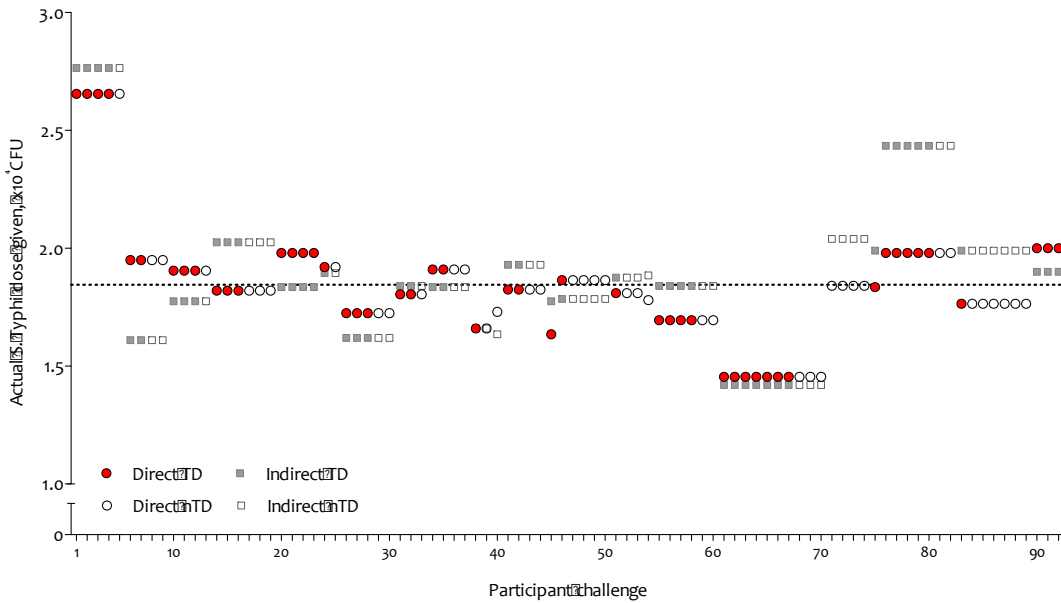


Figure 8-5. Challenge doses administered to 92 participants taking part in the challenge stage of the vaccine-challenge study, according to method used and subsequent challenge outcome. TD, participants subsequently typhoid diagnosed; nTD, participants not subsequently typhoid diagnosed; Dotted line=mean overall challenge dose administered (direct method, $=1.84 \times 10^4$ CFU).

8.6.1 VACCINATION TO CHALLENGE INTERVAL

The target day for challenge was 28 days after receiving the final dose of vaccine. For logistic reasons and participant availability, however, challenges were performed within a 21-33 day interval (Figure 8-6). Overall, 65/92 (70.7%) and 85/92 (92.4%) of challenges were performed within either a 3- or 5-day window, respectively. There was no significant difference in the vaccine-challenge interval between the three vaccine groups (one-way ANOVA, $p=0.872$).

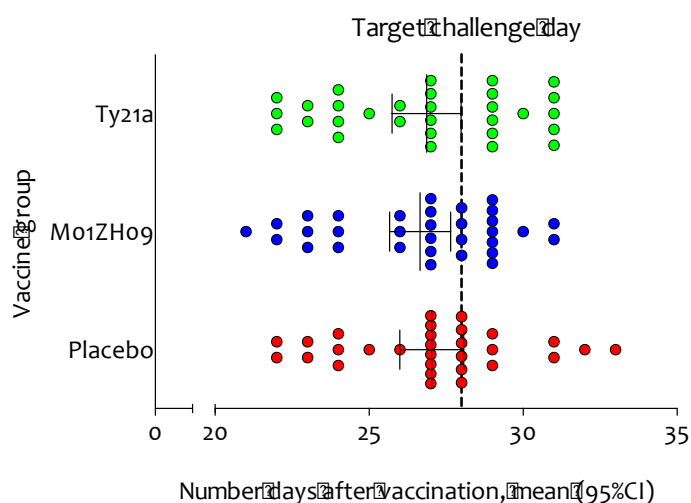


Figure 8-6. Individual and mean (95%) time intervals between (final dose of) vaccination and challenge administration by assigned vaccine group.
Dashed line marks the target interval of 28 days.

8.6.2 CHALLENGE DOSE PREPARATION

Both direct quantification of bacterial numbers in the challenge vial used for dose preparation and indirect quantification using an aliquot from the suspension ingested by the participant was performed ('direct' and 'indirect' method). Challenge doses given ranged from $1.46\text{-}2.66 \times 10^4$ CFU *S. Typhi* bacteria, remaining in the target range ($1\text{-}5 \times 10^4$ CFU) throughout the study period. There was no significant difference in the dose administered to either of the three vaccine groups (ANOVA, $p=0.619$, Figure 8-7). All challenges were performed between 08:55 and 10:34am and all participants had fasted.

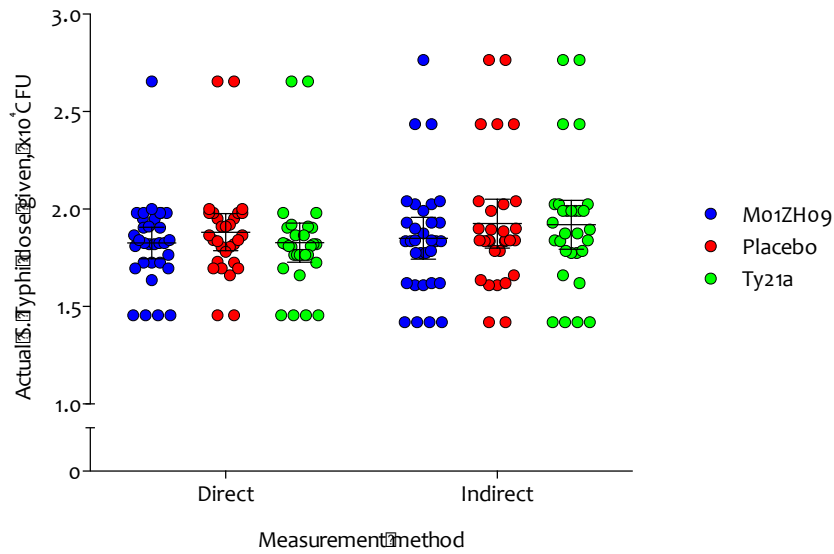


Figure 8-7. Typhoid challenge doses administered (individual, mean and 95% CI) according to vaccine allocation and by two methods of measurement, direct and indirect.

8.7 STUDY RETENTION

Despite the high proportion of participants remaining in the study to undertake the challenge phase, retention fell following the Day 28 visit (Figure 8-8). By one year, 67% participants returned for their follow-up visit.

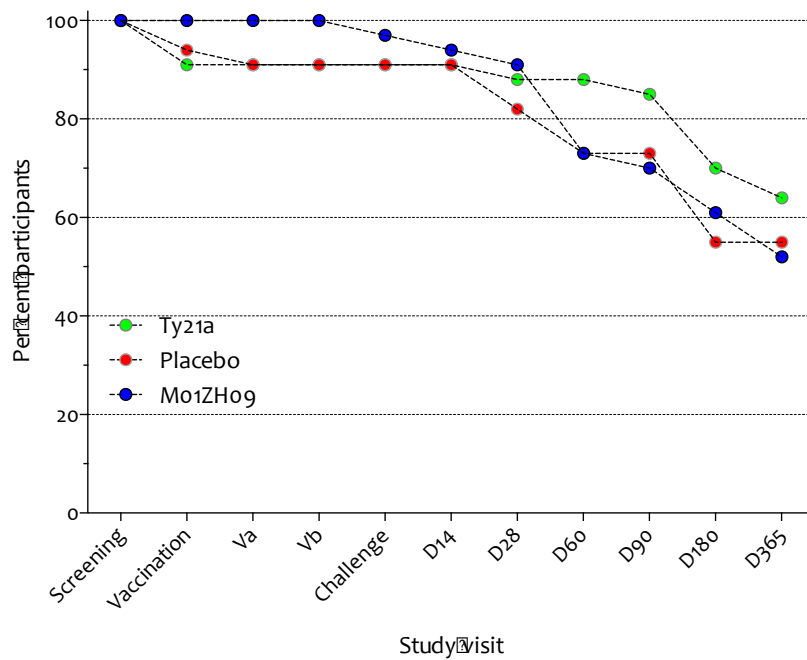


Figure 8-8. Study participant retention during the vaccine-challenge study by vaccine allocation and last study visit attended.

8.8 DISCUSSION

These data describe the recruitment, demographic and clinical trial related procedures of the largest single, randomised control trial involving a challenge agent ever conducted, and the first regulator-approved study involving challenge with a CL₃ pathogen in the United Kingdom.

8.8.1 RESPONSE TO RECRUITMENT

Given the vaccine expiry date approved by the MHRA, screening, consenting and vaccinating sufficient volunteers within the 8-month time-frame required considerable organisational effort. The local Oxford population is well sensitised to participation in clinical trials, and challenge studies (malaria, influenza, BCG etc.) are also performed intermittently. There was an enthusiastic response to advertising material therefore, however this required additional work in fully explaining the study to those ultimately ineligible to participate. Each participant screening visit took approximately 1.5-2hours, required both a doctor and nurse to be available, following which the participant was often required to return to attend an ultrasound screening appointment in the Radiology Department. Given the time and requirements asked of participants, even prior to study enrolment, the amount of reimbursement paid likely attracted most participants to volunteer. A deliberate decision was made to not advertise the amount of reimbursement offered (~£3300) in order to minimise the number of non-genuine expression of interests, although this proved relatively ineffectual mostly due to word-of-mouth interest.

Methods of recruitment that had a high-uptake (interest:participation ratio) included contacting those registered on the Oxford Vaccine Centre database and targeting poster advertising material to other biomedical research institutes. Using these methods alone

could have resulted in a 'less' representative study population, however, in that many participants came from a well-informed biomedical science background. While a large proportion of students participated in the study, this likely resulted in the relatively poor long-term retention of study participants, as many had left the area and were unwilling to return for the financial reimbursement offered for later visit attendance.

The 2-stage screening system of contacting volunteers by telephone prior to arranging a formal study visit worked well, with many ineligible potential participants being excluded at this stage. In particular, the number of individuals expressing interest despite having previously received typhoid vaccination was high. Making this even clearer in future studies would be useful.

8.8.2 PARTICIPANT PROFILES

Given the inclusion/exclusion criteria implemented, the baseline demographic profile of study participants was as expected the majority being males of white British ethnicity. The wide age range of those taking part and the variety of participant occupations was unexpected. Anecdotally, many 'older' volunteers expressed interest as they had not previously been approached for clinical trial participation or for altruistic reasons. In general, these 'older' participants were highly reliable (for visit attendance and paperwork completion, for example) and provide an interesting additional dimension to examining host vaccine/challenge responses. Participants were well matched between each vaccine group allowing meaningful comparisons to be made in subsequent chapters.

8.8.3 VACCINATIONS

Vaccination schedules were tightly adhered to with almost no deviation from protocol requirements. Vaccine viability fell over the course of the study although remained within

expected and acceptable limits equivalent to those of licensed Ty21a and other experimental live-attenuated vaccine candidates (*verbal communication, M. M. Levine & S. Lockhart, 2012*). As described previously, the level and number of viable bacteria in the vaccine are key drivers for vaccine immunogenicity (see: 2.7.3), and therefore it proved vital to have regular viability results to alter dosing accordingly. Whether an increase in the overall number of bacteria (live+dead) has an effect on overall immunogenicity or response is not currently known.

8.8.4 CHALLENGES

The logistic practicalities of bringing in groups of study participants to be challenged together resulted in a relatively broad vaccine-challenge interval (range 21-33 days), although >90% were challenged within a 28 ± 5 day time-frame. The vaccine-challenge interval was no different between either of the vaccine groups, however subsequent assessment of vaccine immunogenicity and effectiveness will take this into account. The challenge doses given remained within the required dose target range, and in fact were remarkably consistent throughout the study.

8.8.5 SUMMARY

Using a wide range of methods a suitable well-matched study population could be identified and recruited from the local Oxford population over a relatively short time frame. Vaccines and challenges were administered to participants as required by the protocol although the time interval between the two procedures in some cases ranged quite widely. After completion of the main study participant retention fell quickly, likely due to the average demographic of study participants and waning pecuniary interest.

Chapter 9. HUMORAL RESPONSES TO VACCINATION WITH THE NOVEL SINGLE DOSE VACCINE M01ZH09 IN COMPARISON TO PLACEBO AND LICENSED TY21A ADMINISTERED IN 3-DOSES

9.1 INTRODUCTION

In studies performed to-date, the oral vaccine candidate M01ZH09 has been well tolerated and has demonstrated significant ‘immunogenicity’ after a single dose (see: 2.9.3). These studies, performed in the USA, UK and Vietnam (in adults and children) have used seroconversion or plasma cell response to LPS antigen as the predominant determinant of immunogenicity.^{336,340-343} While immune responses to live-attenuated

vaccines are broad, involving every component of innate, humoral and cell-mediated immunity (CMI), selection of meaningful, easy-to-measure indicators of response in peripheral blood provides some ability to compare immunogenicity and identify correlates of protection. Humoral responses to the *S. Typhi* surface-exposed antigens, LPS, flagellin and Vi, have traditionally been used for this purpose.

In the absence of an accepted/proven correlate of protection, reasons for choosing LPS response as a marker of immunogenicity include data provided by field-use of Ty21a in Chile and subsequent Ty21a dose-response laboratory studies.^{142,143} While efficacy of the ViPS vaccine supports Vi antibody status as a protective marker, lack of a Vi response to Ty21a (Vi) suggests that alternative humoral or other immunological responses are important. Field and previous human challenge studies evaluating whole cell vaccines demonstrated a role for anti-flagellin responses, but not LPS or Vi.^{141,504} In contrast, the protection generated by live oral vaccination appears to be related to mucosal IgA and CMI responses to flagellin or LPS, but not Vi. In the Chilean field-studies, two Ty21a formulations were used (enteric-coated capsules or vaccine plus NaHCO₃ in a gelatin capsule) and given in 1-3 doses to 15-19 year olds.¹⁴² Immunogenicity data demonstrated dose-responses in seroconversion rate and subsequent protection from infection (Table 9-1). Notably, the increased seroconversion to the NaHCO₃/gelatin formulation did not predict its subsequent low efficacy.

Formulation	No. of doses	Seroconversion rates (%)	Vaccine efficacy (%) in controlled field trials
Enteric-coated capsules	3	61/96 (64)	67*
	2	22/50 (44)	53 [†]
	1	9/50 (18)	24 [†]
Vaccine plus NaHCO ₃ in gelatin capsules	3	99/195 (50)	19*

Table 9-1. Rates of seroconversion of IgG antibody to *S. Typhi* LPS determined by ELISA and corresponding efficacy found during two Chilean field trials.

From Levine and colleagues.¹⁴² 36-month follow-up data from two field sites were included: *Area Occidente and [†]Area Norte, both Santiago, Chile.

The rationale for measuring ASC responses as a marker of oral vaccine response, was that migrating sensitized gut-homing lymphocytes could be collected from the peripheral blood, following antigen exposure in intestinal Peyer's patches. This might, in particular, provide some indication of the mucosal secretory IgA responses.⁵⁰⁵ In a series of laboratory studies, Kantele demonstrated significant differences in ASC magnitude and persistence to different dose schedules and formulations of Ty21a vaccine.¹⁴³⁻¹⁴⁵ This was in contrast to serological responses (measured by EIA), which were relatively unremarkable. ASC responses were greater and more persistent after 3-doses (compared to 2 or 1) and enteric-coated capsules were less immunogenic than a lyophilised vaccine suspension (both contained 10^9 bacteria).¹⁴³

In studies performed to-date, there has been no direct comparison between the tolerability or immune responses seen following Ty21a or M01ZH09 vaccination. Available evidence suggests that M01ZH09 should be at least as immunogenic after a single dose of 1×10^{10} bacteria, although there are clear differences between the two vaccines, at least in the currently available formulations.

This chapter describes the comparative tolerability of each vaccine schedule with a placebo-vaccinated group, in addition to the nature and magnitude of the humoral and ASC immune responses at time points after vaccination. This will allow the vaccine responses measured to be placed in context of the currently available literature. Furthermore, the outcome following subsequent standardised challenge will provide direct information as to the usefulness of these putative immunological measures as correlates of protection.

9.2 METHODS

9.2.1 BASELINE ASSESMENT AND VACCINATION

Baseline assessments and vaccination were performed as described in section 5.3 .

9.2.2 VACCINE TOLERABILITY

Vaccine tolerability was assessed by collection of diary card data for 7-days after first vaccine receipt and clinical assessment and evaluation at the 7- and 14-day follow-up visits, Visits A and B. Details of the procedures performed are described in section 5.2.5 . To limit the number of comparisons made, only symptoms reported with $\geq 10\%$ difference in frequency or ≥ 0.1 difference in severity scores compared to the placebo group are described.

9.2.3 VACCINE IMMUNOGENICITY

To assess the immunogenicity of vaccines and placebo given, samples were collected at baseline and Day 28 for assay by ELISA and at baseline and Day 7 for enumeration of the ASC response. Visits, sample collection and assays were performed as described in Chapter 5&6.

9.2.4 STATISTICAL CONSIDERATIONS

Comparison of symptomatic data was selectively performed using either unpaired T-tests or Fisher's exact test to assess the difference in proportions of symptoms or symptom severity, respectively, compared to placebo recipients.

Serum ELISA data was \log_{10} -transformed to assume a normal distribution, with a nominal value of 3.7EU/mL (LLOD/2) being assigned to non-measurable anti-Vi IgG titres. Baseline comparisons between groups were performed by ANOVA. To assess for significant increases after vaccine, repeated measures two-way ANOVA with Bonferroni's multiple comparison correction was used. Assessment of seroconversion at Day 28 was performed using two thresholds; either ≥ 4 fold increase, which was used in earlier M01ZH09 studies (see: 9.9.5), or ≥ 1.5 or ≥ 1.7 fold increases (for IgG/IgA, respectively), which have been used in the more recent literature. To evaluate differences in response, contingency tables and Fisher's exact tests were used.

Antibody-secreting (plasma) cell counts were \log_{10} -transformed prior to analysis, with zero counts given a nominal value of 0.25. Comparisons were made as for the ELISA data, with differences between groups evaluated by ANOVA and between time points by repeated measures two-way ANOVA with Bonferroni's correction.

Further exploratory analyses were performed using \log_{10} -transformed data; interactions between continuous variables were assessed by Pearson's correlation, and grouped data by unpaired T-tests as described.

9.3 VACCINE-ASSOCIATED SOLICITED SYMPTOMS

Diary card data were available for 29, 32 and 30 participants receiving placebo, M01ZH09 or Ty21a vaccine, respectively. Overall, 347 solicited symptoms were reported by 91 participants (mean [range]: 3.81 [0-27] symptoms each) during the 7-day post-vaccination period.

Vaccines were generally well tolerated, although those receiving placebo vaccination had an unexpected relatively high rate of symptom reporting. On average, 15% of participants

in this group reported at least one symptom each day in comparison to 16% or 11% receiving either of the active vaccines, M01ZH09 and Ty21a, respectively. Participants allocated to placebo, M01ZH09 and Ty21a vaccine groups reported a mean (95%CI) of 4.55 (2.23-6.87), 3.81 (2.07-5.56) and 3.10 (0.94-5.26) symptoms each ($p=0.609$, ANOVA). There was no trend between the number of days elapsed since vaccination and symptom frequency or severity.

Overall the types of symptoms reported by each group were broadly similar, with 'headache' and 'feeling generally unwell' predominating in all three groups (Figure 9-1.).

Average maximum symptom severity scores were also broadly similar overall for participants allocated to either of the three vaccine groups; mean (95%CI) severity grades were 2.76 (1.55-3.97), 2.31 (1.30-3.33) and 2.07 (0.80-3.33) for placebo, M01ZH09 or Ty21a recipients, respectively ($p=0.692$, ANOVA).

9.3.1 M01ZH09 VACCINE RECIPIENTS

There was a $\geq 10\%$ difference in the percentage of participants reporting four symptoms. More M01ZH09 recipients reported 'cough' (OR [95%CI]: 2.08 [0.55-7.84], $p=0.343$, Fisher's Exact test) compared to placebo, while fewer reported 'headache' (0.32 [0.11-0.92], $p=0.041$), 'loss of appetite' (0.45 [0.12-1.73], $p=0.323$), and 'myalgia' (0.09 [0.00-1.70], $p=0.045$; Table 9-2.).

A ≥ 0.1 difference in mean (maximum) symptom score was found for three symptoms: 'cough' (difference between means [95%CI]: 0.14 [-0.14-0.42], $p=0.313$, unpaired T-test) and 'diarrhoea' (0.18 [-0.08-0.44], $p=0.176$) were more severe, whereas 'myalgia' (-0.21 [-0.41- -0.01], $p=0.041$) was less severe in M01ZH09 recipients.

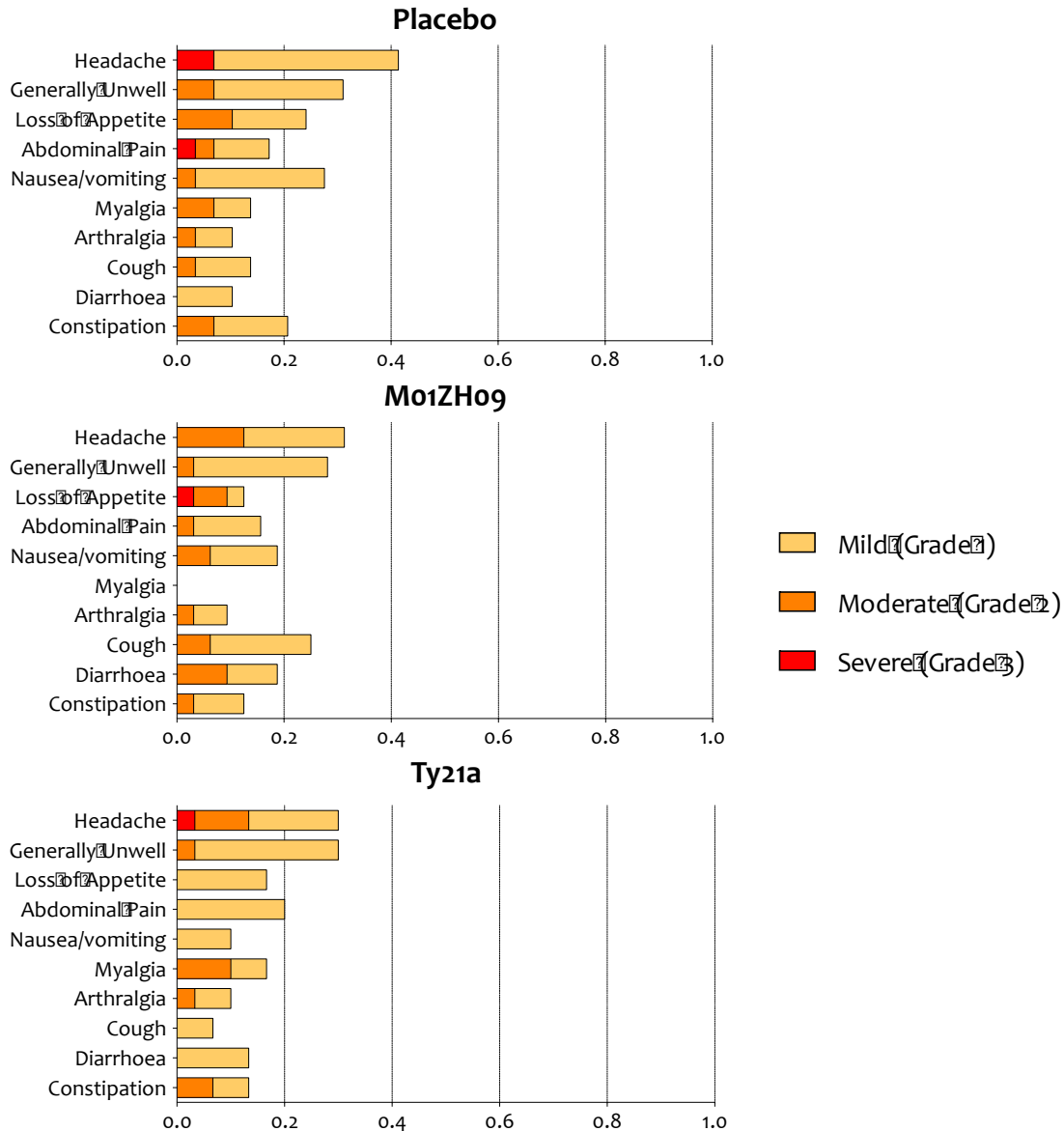


Figure 9-1. Proportion of participants reporting each solicited symptom during the 7-days after receipt of (first) vaccine according to vaccine allocation.

Maximum severity score per participant for each symptom was used and graded according to criteria detailed in Supplementary Table 2.

9.3.2 TY21A VACCINE RECIPIENTS

Symptoms reported by Ty21a recipients were broadly similar to the other groups, although upper gastrointestinal symptoms were less frequently reported and of milder severity. Two symptoms were reported with $\geq 10\%$ difference between Ty21a and placebo groups: ‘headache’ (0.3 [0.10-0.89], $p=0.037$) and ‘nausea/vomiting’ (0.29 [0.07-1.24], $p=0.104$) were both less frequent in Ty21a recipients.

Symptom severity was similar to that reported by placebo recipients; 3 symptoms were reported with ≥ 0.1 difference in severity. ‘Loss of appetite’ (-0.18 [-0.46-0.10], $p=0.212$), ‘nausea/vomiting’ (-0.21 [-0.44-0.01], $p=0.070$) and ‘cough’ (-0.11 [-0.30- 0.09], $p=0.283$) were all reported as less severe in Ty21a recipients compared to placebo, although the differences were all non-significant.

Symptom recorded		M01ZH09	Placebo	Ty21a
Headache	% (95% CI)	31.3 (15.2-47.3) *[30]	41.4 (23.5-59.3)	30.0 (13.3-46.7) *
	Mean severity	0.44	0.57	0.48
Generally unwell	% (95% CI)	28.1 (12.5-43.7) [NA]	31.0 (14.2-47.9)	30.0 (13.3-46.7)
	Mean severity	0.31	0.39	0.34
Loss of appetite	% (95% CI)	12.5 (1.0-24.0) [1]	24.1 (8.6-39.7)	16.7 (3.1-30.2)
	Mean severity	0.25	0.29	0.17
Abdominal pain	% (95% CI)	15.6 (3.0-28.2) [22]	17.2 (3.5-31.0)	20.0 (5.4-34.6)
	Mean severity	0.19	0.28	0.21
Nausea/vomiting	% (95% CI)	18.8 (5.2-32.3) [14]	27.6 (11.3-43.9)	10.0 (-0.9-20.9)
	Mean severity	0.25	0.32	0.10
Myalgia	% (95% CI)	0.0 (0-0) *[10]	13.8 (1.2-26.3)	16.7 (3.1-30.2)
	Mean severity	0 *	0.21	0.28
Arthralgia	% (95% CI)	9.4 (-0.7-19.5) [2]	10.3 (-0.7-21.4)	10.0 (-0.9-20.9)
	Mean severity	0.13	0.14	0.14
Cough	% (95% CI)	25.0 (10.0-40.0) [3]	13.8 (1.2-26.3)	6.7 (-2.4-15.7)
	Mean severity	0.31	0.18	0.07
Diarrhoea	% (95% CI)	18.8 (5.2-32.3) [12]	10.3 (-0.7-21.4)	13.3 (1.0-25.7)
	Mean severity	0.28	0.11	0.14
Constipation	% (95% CI)	12.5 (1.0-24.0) [0]	20.7 (5.9-35.4)	13.3 (1.0-25.7)
	Mean severity	0.16	0.21	0.21

Table 9-2. Percentage of study participants recording solicited symptom and mean symptom severity during 7 days following receipt of first vaccine dose by vaccine allocation.

For comparison, in the M01ZH09 column [X] is the percentage of participants reporting the symptom for all previous M01ZH09 trials ($n=347$), as reported in the Investigators Brochure.³³⁹ NA, not available; *, significant difference to placebo ($p<0.05$, see text).

9.3.3 CLINICAL FINDINGS AFTER VACCINATION

Clinical examination findings in each of the three vaccine groups were similar at the 7- and 14-day post-vaccination follow-up visits (Va and Vb), and were not significantly different from the pre-vaccination visit (Table 8-4). Nor was any significant perturbation found in

the oral temperature measurements recorded during 7-days after vaccination, although there was some downward trend (Figure 9-2). Of note, no fever was recorded by any participants; the maximum temperature recorded was 37.3°C.

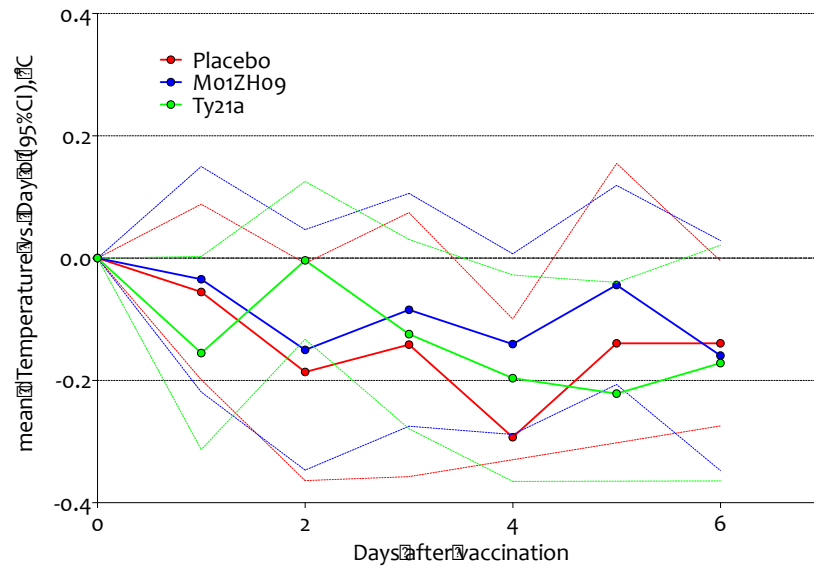


Figure 9-2. Mean changes (dotted lines, 95%CI) in oral temperature measurements compared to baseline recordings during 7-days after vaccination according to vaccine allocation.

9.4 NON-SOLICITED SYMPTOMS AND POST-VACCINATION EVENTS

Of 94 participants vaccinated, only two reported adverse events not captured by solicited symptom recording. Both events occurred in the period between vaccination and challenge and are described below:

1. A participant with a pre-existing history of migraines reported the onset of a new migraine (headache, generally unwell, loss of appetite etc.) shortly after the vaccination visit had occurred. They had been allocated to placebo vaccination.
2. The second participant developed clinical features in keeping with tonsillitis 2 weeks after vaccination with Ty21a (outside of vaccine event reporting window). The participant was also reviewed by their General Practitioner and started on a course of phenoxymethylpenicillin following which symptoms resolved.

Both of these events were attributed to previous vaccine/placebo receipt. There were no vaccine-related SAEs reported in this study.

9.4.1 POST-VACCINATION LABORATORY TESTS AND STOOL SHEDDING

Assessment of blood haematology and biochemical parameters were not performed between vaccination and challenge, however there were no significant differences between the groups at the D0 challenge visit (Supplementary Table 10 & Supplementary Table 11).

Similarly, stool was not collected to detect shedding of the vaccine, as these data have been reported in detail previously (e.g., Lyon and colleagues).³⁴³ All participants had a negative bacterial stool culture prior to challenge, with one interesting exception. One participant had a positive stool culture with growth of a *Salmonella* species detected prior to challenge and at the 24-hour visit (Day 1). This participant was in the unblinded Ty21a vaccine group, and, aside from recently eating a chicken kebab had no other immediately relevant history of travel etc. Initial testing confirmed it was a Vi-negative strain and biochemical typing gave a 67% probability of a *Salmonella* strain. Results from the HPA confirmed that it was neither the Ty21a nor M01ZH09 strain but a group 4 *Salmonella* sp., which was not further typeable. Further investigation revealed that the participant worked as a pet shop owner and had regular contact with reptiles. He/she also confessed to poor hand-hygiene, which was rapidly addressed.

9.5 SERUM ANTIBODY RESPONSES

9.5.1 ANTI-LPS ANTIBODY RESPONSES

No significant differences in IgG antibody titres were found between groups prior to vaccination ($p=0.948$, ANOVA; Supplementary Table 6). Significant increases in anti-LPS IgG were seen after vaccination with M01ZH09 (mean increase \log_{10} titre [95%CI], 0.428 [0.292-0.564]) and Ty21a (0.149 [0.006-0.291]) but not placebo (-0.011 [-0.151-0.129]; Figure 9-3a). Using a protective immune response (PIR) threshold of ≥ 1.7 fold-rise to assess response, significantly more M01ZH09 recipients responded (50%) compared to those in the placebo (6.7%, $p<0.001$, Fisher's exact test). In contrast, there was no significant difference in response rate between Ty21a (24.1%) and placebo groups ($p=0.063$).

Baseline anti-LPS IgA measurement was not different between vaccine groups ($p=0.793$). 28-days after vaccination, significant increases in antibody titres were seen in M01ZH09 recipients (0.253 [0.171-0.334]) but not in those allocated to Ty21a (0.080 [-0.006-0.165]) or placebo (0 [-0.084-0.084]; Figure 9-3b). Using a PIR threshold of ≥ 1.5 fold-rise, there were significantly more responders in the M01ZH09 group (59.4%) than in either of the placebo (3.3%, $p<0.0001$) or Ty21a groups (24.1%, $p=0.009$).

There were no significant differences in baseline anti-LPS IgM titres ($p=0.809$). After vaccination significant increases were seen in M01ZH09 (0.220 [0.152-0.288]) but not Ty21a (0.040 [-0.032-0.111]) or placebo recipients (-0.013 [-0.084-0.057]; Figure 9-3c). Significantly more M01ZH09 vaccine recipients (40.6%) had a PIR (≥ 1.7 fold-rise in IgM titre) compared to either placebo (0%, $p<0.0001$) or Ty21a (3.4%, $p<0.0001$) groups, respectively.

9.5.2 ANTI-FLAGELLIN ANTIBODY RESPONSES

Baseline anti-H IgG antibody titres were not significantly different between the three vaccine groups ($p=0.751$; Supplementary Table 6). After vaccination, participant anti-H IgG titres increased significantly in M01ZH09 recipients (0.442 [0.328-0.557]) but remained unchanged in the placebo (0.017 [-0.101-0.135]) and Ty21a (0.022 [-0.098-0.143]) groups (Figure 9-4a).

Anti-H IgA titres were significantly different at baseline; participants in the Ty21a group had significantly higher levels than those participants in either placebo or M01ZH09 groups ($p=0.0003$). After vaccination, antibody titres in placebo and Ty21a recipients remained unchanged (-0.017 [-0.127-0.092] and -0.018 [-0.129-0.094], respectively), but increased significantly in M01ZH09 recipients (0.422 [0.316-0.528]; Figure 9-4b).

Baseline anti-H IgM titres were also different at baseline; participants receiving M01ZH09 had significantly lower titres than other recipients ($p=0.022$). Significant responses to vaccination were found in participants receiving M01ZH09 (1.46 [1.22-1.70]), while those in the placebo and Ty21a groups remained unchanged (-0.086 [-0.334-0.163] and 0.115 [-0.138-0.368], respectively; Figure 9-4c).

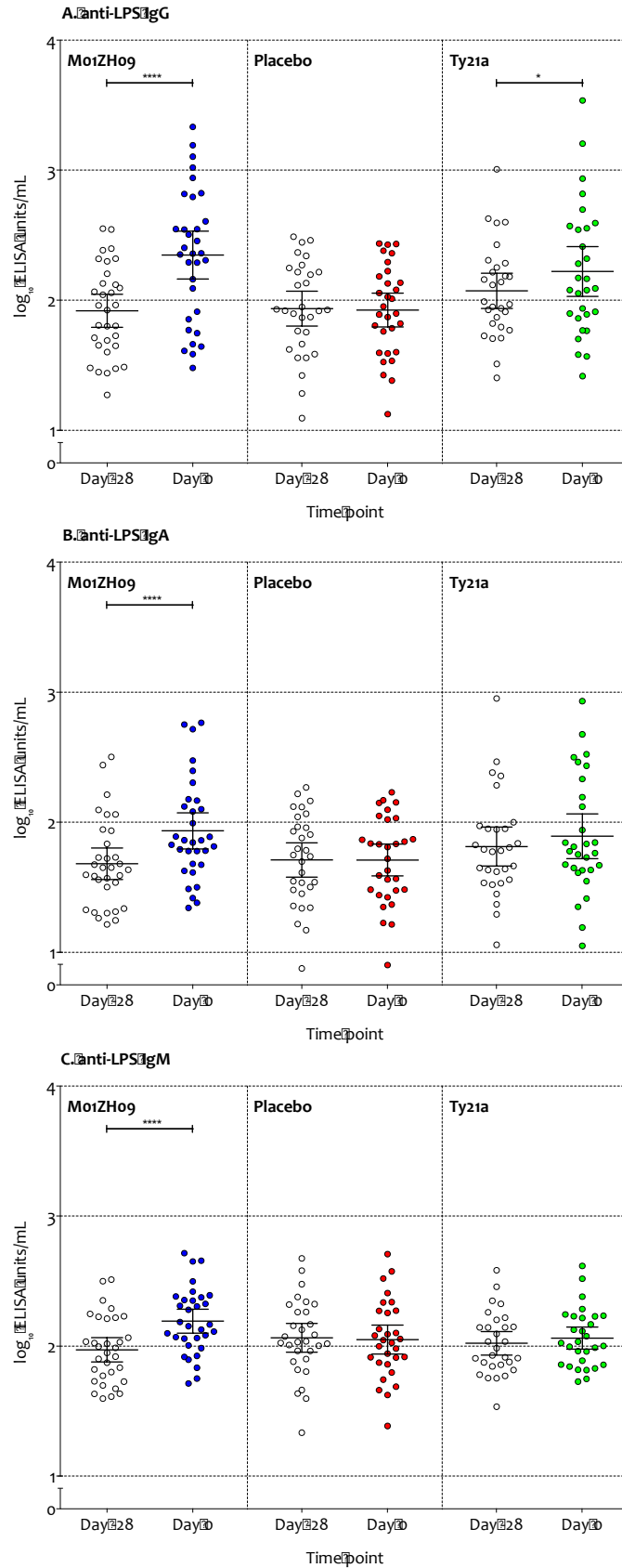


Figure 9-3. Individual, mean (95%CI) serum anti-LPS antibody responses before and 28-days after vaccination according to vaccine group allocation.

Significant differences were assessed by two-way repeated measures ANOVA with Bonferroni's correction. * $p < 0.05$; **** $p < 0.0001$.

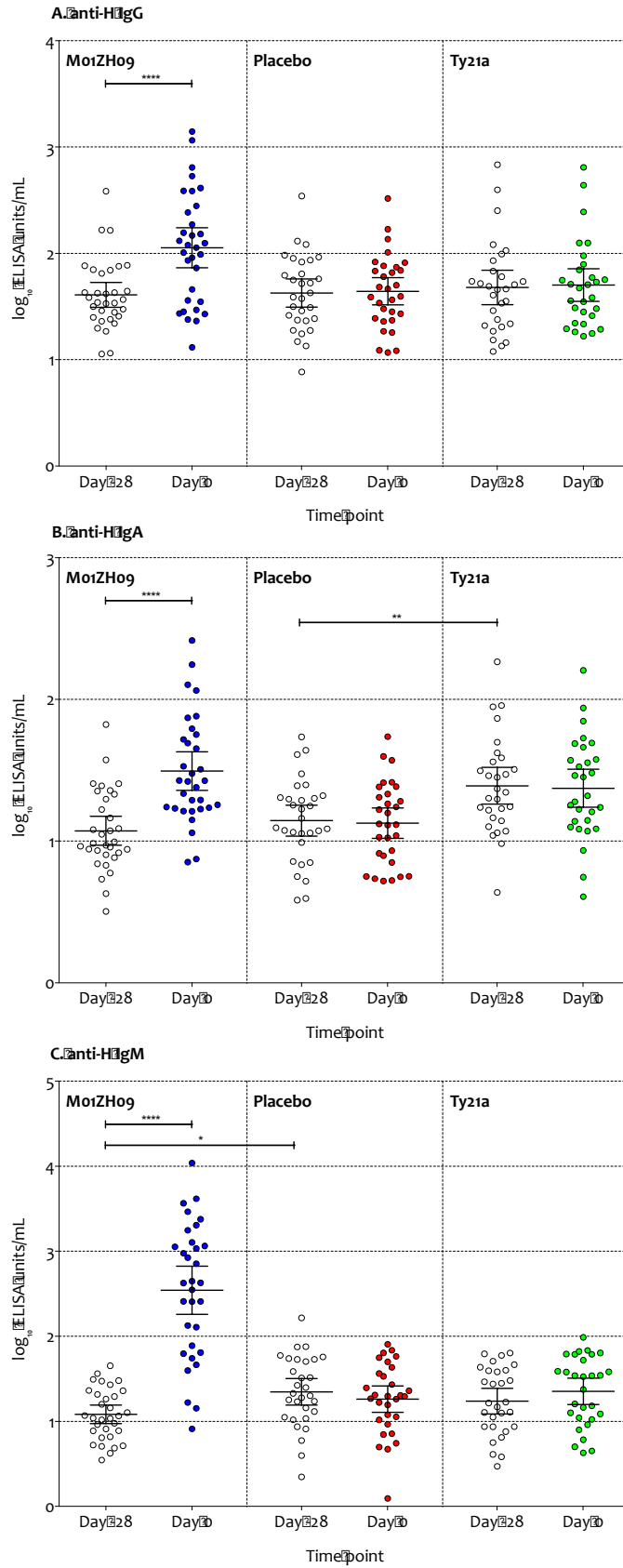


Figure 9-4. Individual, mean (95%CI) serum anti-H antibody responses before and 28-days after vaccination according to vaccine group allocation.

Differences between baseline titres assessed by ANOVA; Increases between time points assessed by two-way repeated measures ANOVA with Bonferroni's correction. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

9.5.3 ANTI-VI ANTIBODY RESPONSES

Levels of anti-Vi IgG antibodies were measured at baseline and 28-days after vaccination.

Note that neither vaccine strain expresses Vi capsule.

A higher than expected proportion of participants had anti-Vi IgG antibody titres detected at baseline: 12/30 (40%), 6/32 (19%), and 8/29 (28%) participants in the placebo, M01ZH09 and Ty21a vaccine arms, respectively, had levels detected above the limit-of-detection (Figure 9-5).

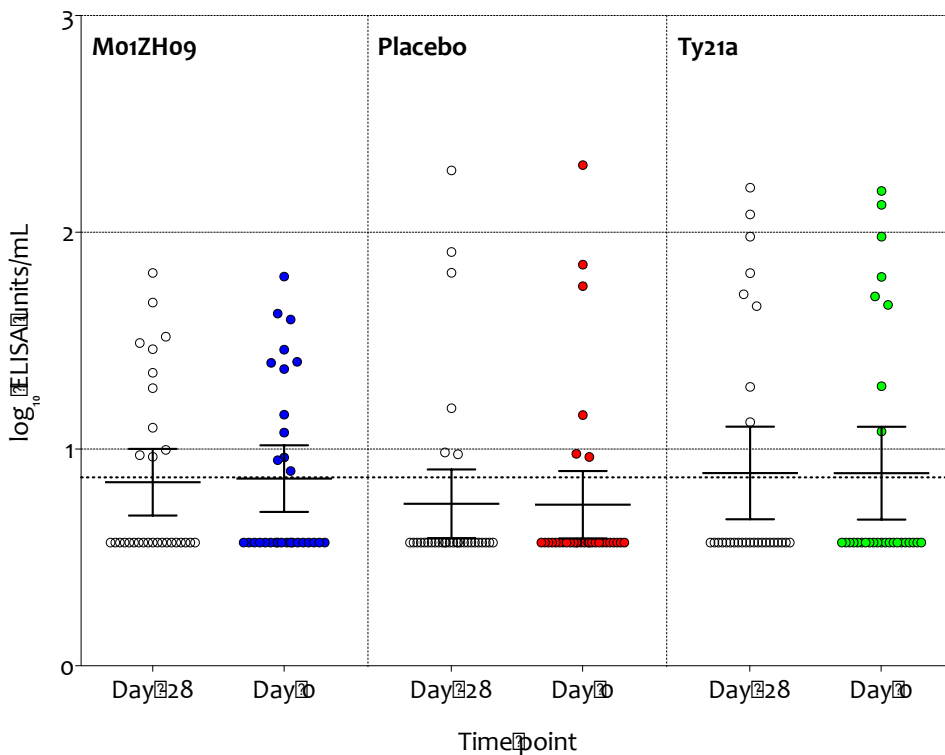


Figure 9-5. Individual, mean (95%CI) serum anti-Vi antibody responses before and 28-days after vaccination with M01ZH09, placebo or Ty21a.

There was no significant difference between baseline levels of anti-Vi IgG antibody by vaccine group ($p=0.452$). Using a putative threshold of ≥ 10 EU/mL for protection,³⁰² 4, 8 and 8 participants had a pre-vaccination level conveying possible protection from infection, in the M01Z98, placebo and Ty21a groups, respectively. The proportions of ‘protected’ participants were not significantly different in any group compared to any

other ($p=NS$). Overall, anti-Vi antibody titres were not significantly affected by vaccination with Mo1ZH09, Ty21a or placebo (Table 9-3).

	Mo1ZH09 (n=32)		Placebo (n=30)		Ty21a (n=29)	
	GMT, EU/mL (95%CI)	% ≥ 10 EU/mL (95%CI)	GMT, EU/mL (95%CI)	% ≥ 10 EU/mL (95%CI)	GMT, EU/mL (95%CI)	% ≥ 10 EU/mL (95%CI)
Pre-vaccine	5.6 (3.9-8.1)	12.5 (9-16.5)	7.2 (5.1-10.2)	26.7 (14.4-19.2)	7.6 (4.7-12.7)	27.6 (14.9-19.7)
Pre-challenge	5.5 (3.9-7.9)	""	7.5 (5.3-10.6)	30 (15.3-19.4)	7.7 (4.7-12.7)	""

Table 9-3. Anti-Vi antibody titre summary from samples collected pre-vaccination, pre-challenge and at Day 28 after challenge.

GMT, geometric mean titre (95%CI); EU/mL, ELISA units per millilitre.

9.6 ANTIBODY-SECRETING CELLS

Vaccine immunogenicity was further compared by assessment of the number of antibody-secreting (plasma) cells before and 7-days after vaccination. For Ty21a recipients this corresponded to 11 days after ingesting the first dose. The number of anti-LPS, -flagellin and -Vi, IgG, IgA and IgM ASC/ 10^6 PBMC was measured by ELISpot as described in section 6.3.10. As per previously performed studies of Mo1ZH09 immunogenicity,^{342,343} a positive ELISpot response was defined as ≥ 4 spot-forming cells (ASC) per 10^6 PBMC at Day 7.

9.6.1 BACKGROUND ANTI-LPS ASC RESPONSES

A high proportion of participants with ≥ 4 anti-LPS secreting cells was found at baseline; in total 24%, 43% and 27% of participants had pre-vaccine responses of ≥ 4 spots for IgG, IgA and IgM, respectively. Significant increases in anti-LPS IgG, IgA and IgM were seen in low background responders (< 4 SFC) only (Figure 9-6).

While there were no significant differences found in ASC counts at Day 7, the high background subset was excluded from the further analysis presented below to maintain consistency with previous Mo1ZH09 immunogenicity studies. There were no significant

differences in the distribution of these high background responders between each of the vaccine groups; the final numbers included in the analysis are shown in Supplementary Table 7.

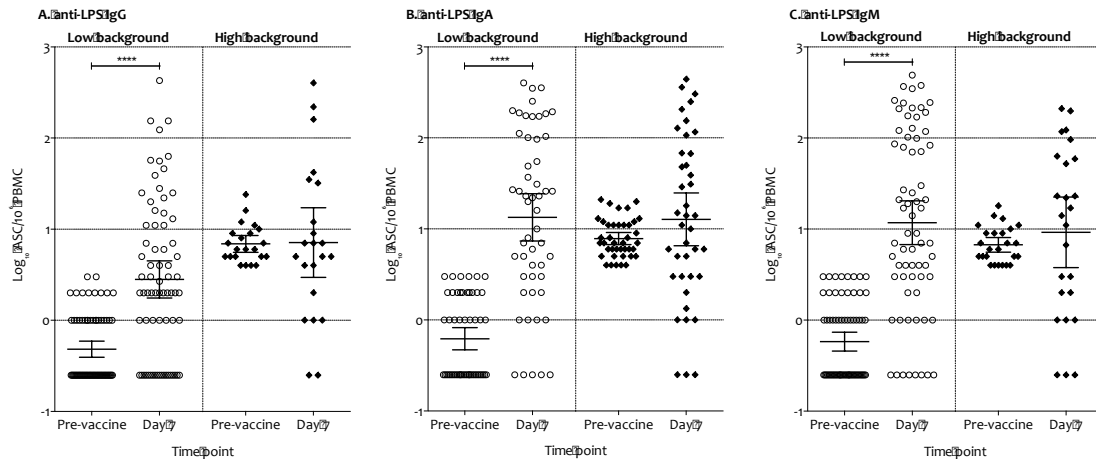


Figure 9-6. Effect of background anti-LPS antibody-secreting cell numbers on subsequent responses after vaccination by antibody isotype.

Bars represent mean, 95%CI. Differences between time points assessed by two-way repeated measures ANOVA with Bonferroni's correction. **** $p < 0.0001$.

9.6.2 POST-VACCINATION ANTI-LPS ASC RESPONSES

7-days after vaccination significant increases in IgG-secreting plasma cells were seen in M01ZH09 (mean [95%CI] increase \log_{10} ASC count: 1.381 [1.016-1.746]) and Ty21a (0.544 [0.140-0.949]) but not placebo recipients (0.231 [-0.173-0.636]). ASC numbers were significantly higher at Day 7 in M01ZH09 recipients compared to placebo (Figure 9-7a).

Significant increases in IgA ASC numbers were seen by Day 7 in both active (M01ZH09, 2.083 [1.696-2.469]; Ty21a, 1.201 [0.774-1.629]) but not placebo (0.309 [-0.175-0.794]) groups compared to baseline. Day 7 ASC numbers were significantly higher in both M01ZH09 and Ty21a recipients compared to placebo (Figure 9-7b).

Similarly significant increases in IgM-secreting cells were found in both active vaccine groups (M01ZH09, 2.227 [1.896-2.558]; Ty21a, 1.018 [0.627-1.410]), but not placebo

recipients (0.339 [-0.044-0.721]) compared to baseline (Figure 9-7c). Cell counts at 7-days were significantly higher in M01ZH09 and Ty21a recipients compared to placebo.

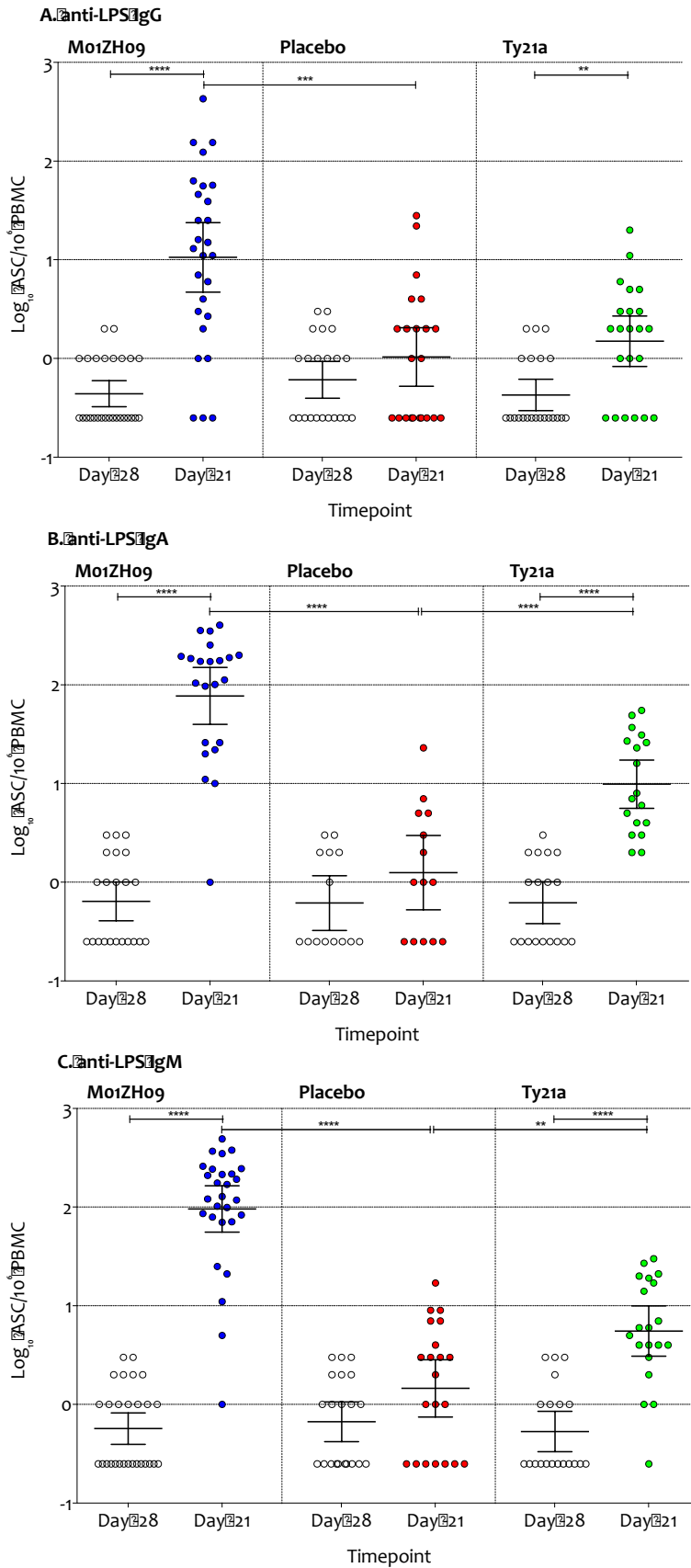


Figure 9-7. Individual, mean (95%CI) anti-LPS ASC responses before and 7 days after vaccination with according to vaccine allocation.

High-background responders (≥ 4 SFC/10⁶ PBMC) are excluded. Differences assessed by two-way repeated measures ANOVA with Bonferroni's correction. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

9.6.3 BACKGROUND ANTI-FLAGELLIN ASC RESPONSES

In general baseline anti-H responses were of a lower magnitude than those seen for anti-LPS ASC. There were significant increases in numbers seen after vaccination in all low background responders and in the anti-H IgM ASC high background responses (Figure 9-8). Fewer sample numbers were affected, however, and the decision was made not to exclude any data from the subsequent analyses performed.

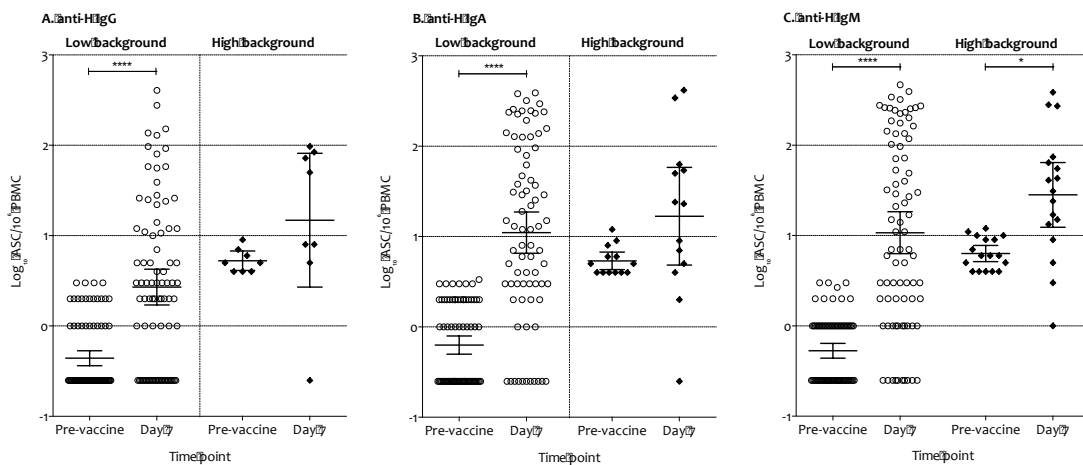


Figure 9-8. Effect of background anti-H antibody-secreting cell numbers on subsequent responses after vaccination by antibody isotype (mean [95%CI]).

Differences between time points were assessed by two-way repeated measures ANOVA with Bonferroni's correction. * $p < 0.05$; **** $p < 0.0001$.

9.6.4 POST-VACCINATION ANTI-FLAGELLIN ASC RESPONSES

There were no significant differences in anti-H ASC responses between groups at baseline, however significant responses were seen by Day 7 following vaccination.

Significant increases in anti-H IgG ASC were seen compared to pre-vaccination numbers, in both vaccine groups, both of which were significantly higher than placebo recipients (Supplementary Table 7 & Figure 9-9A). Significant increases were also seen in anti-H IgA and IgM ASC numbers. Day 7 counts were significantly high in both active vaccine groups compared to placebo (Figure 9-9b&c).

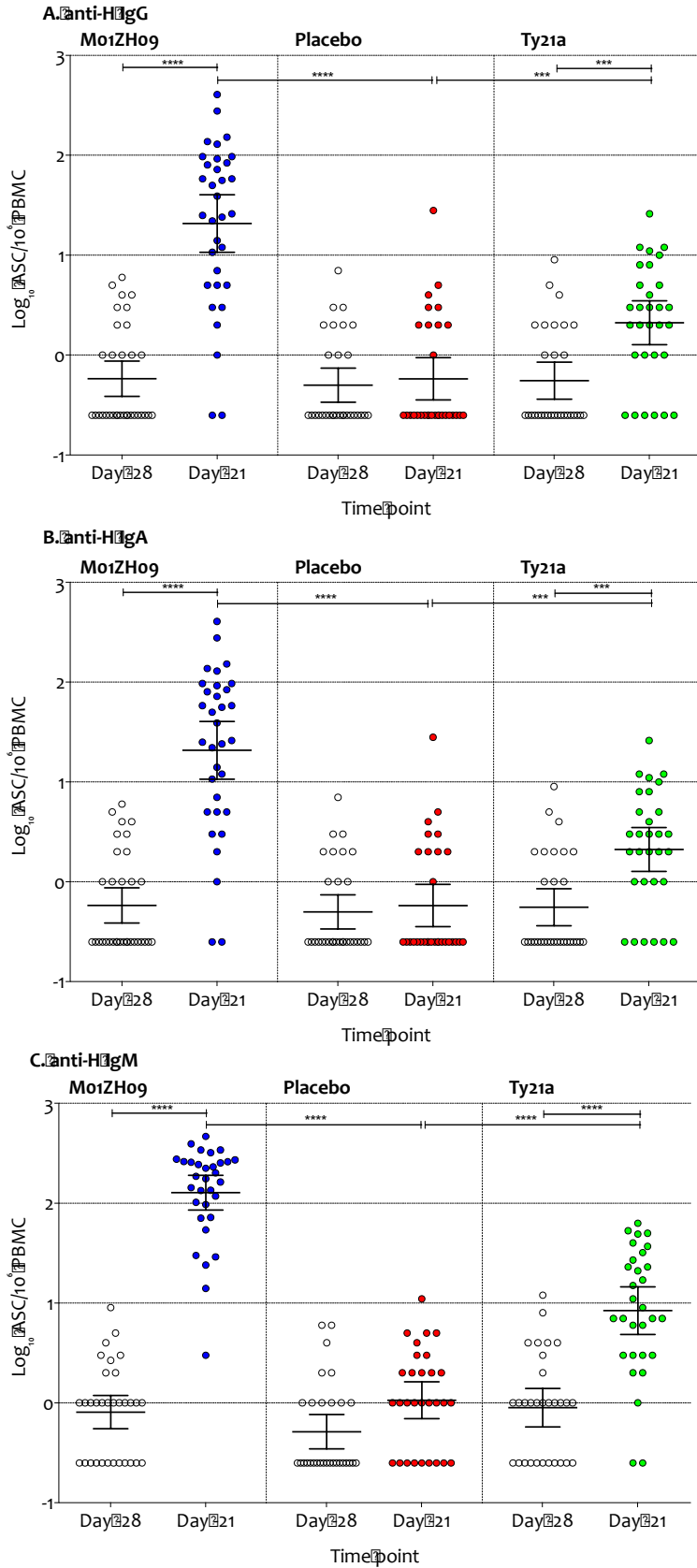


Figure 9-9. Individual, mean (95%CI) anti-H (flagellin) ASC responses before and 7 days after vaccination according to vaccine allocation.

Differences assessed by two-way repeated measures ANOVA with Bonferroni's correction. *** $p < 0.001$; **** $p < 0.0001$.

9.6.5 POST-VACCINATION ANTI-VI ASC RESPONSES

In general anti-Vi ASC responses were unremarkable. Baseline counts were not significantly different between either of the active vaccine groups and placebo, and were not significantly affected by vaccination (Figure 9-10).

9.7 CORRELATION OF ASC COUNTS AND ANTIBODY LEVELS

Significant associations were found between log-transformed numbers of ASC counted at Day 7 and anti-LPS and –H antibody titres measured at Day 28 (Table 9-4). These relationships did not hold true when explored by vaccine allocation; significant associations remained for M01ZH09 recipients and anti-LPS IgG, IgA and anti-H IgM responses only, although the strength of correlation remained low.

Antigen/isotype	M01ZH09		Placebo		Ty21a		COMBINED	
	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>
LPS IgG	***	0.576	0.082	0.323	0.104	0.308	****	0.505
LPS IgA	0.278	0.198	0.914	0.021	0.548	0.116	*	0.244
LPS IgM	*	0.365	0.764	0.057	0.741	0.064	**	0.279
H IgG	0.072	0.323	0.844	-0.038	0.245	0.227	****	0.414
H IgA	0.112	0.287	0.997	0.00	0.843	-0.039	***	0.381
H IgM	**	0.452	0.429	0.150	0.539	0.119	****	0.674

Table 9-4. A summary of the associations between log₁₀-transformed ASC counts measured at Day 7 and subsequent antibody titres measured at Day 28 according to vaccine allocation.

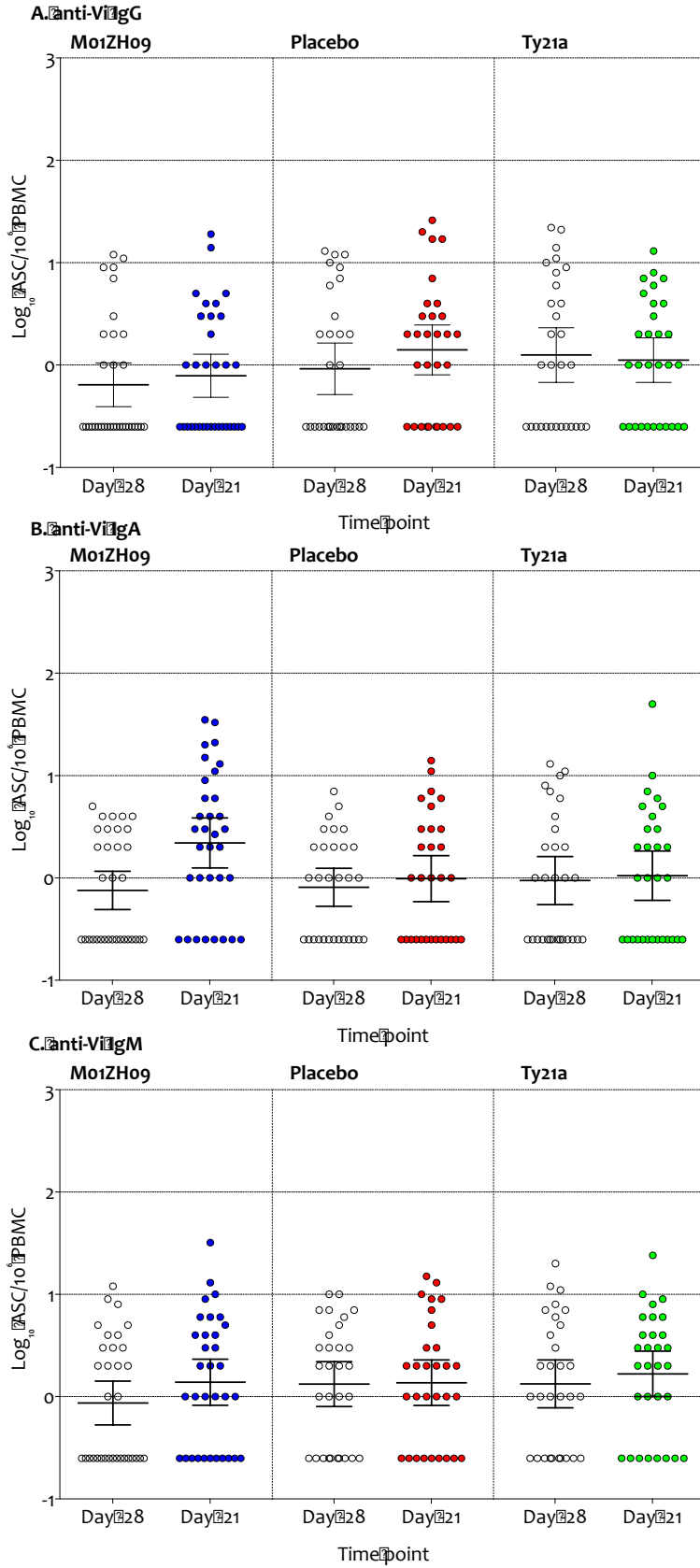


Figure 9-10. Individual, mean (95%CI) anti-VI ASC responses before and 7 days after vaccination according to vaccine allocation.

9.8 FACTORS AFFECTING POST-VACCINATION IMMUNE RESPONSES

9.8.1 VACCINE DOSE

To assess whether dose of M01ZH09 received corresponded to greater or less immunogenic responses, vaccine dose was correlated with anti-LPS and anti-H ASC counts or antibody titres at Day 7 or Day 28 respectively. No correlations between exposure dose and immune responses were found with the exception of Day 7 anti-LPS IgG secreting plasma cells, which were significantly associated with dose given ($p=0.005$, $r=0.502$; Pearson's correlation).

9.8.2 OTHER FACTORS

Exploratory analyses were performed to investigate whether other factors including participant gender, age or previous travel to endemic settings influenced the immunological parameters already described. No further significant associations were found.

9.9 DISCUSSION

Human challenge studies provide a unique opportunity to directly assess existing and novel vaccines in their ability to prevent infection. Key to placing these data in the context of previous studies and for translating apparent efficacy into future populations is the initial stage of demonstrating at least 'adequate' or comparable immune responses to those found previously. One advantage of this setting includes the ability to directly test (or discover) putative correlates of protection.

No previous studies have compared the tolerability and immunogenicity of M01ZH09 with Ty21a; while there are clear differences in formulation and delivery, these data provide some opportunity to make these comparisons.

9.9.1 TOLERABILITY AND SYMPTOM REPORTING

Overall a high background rate of symptom reporting was found which included participants allocated to the placebo vaccine group. This was somewhat unexpected but may be for several reasons, including encouragement to report all symptoms experienced or the perceived 'high-risk' nature of participation in a human challenge study. That symptom reporting rates were also high in placebo recipients in this carefully screened population may, at least, demonstrate the effectiveness of vaccine allocation concealment. Comparison of symptom reporting data is problematic between studies and populations; however, rates reported were similar to those previously published.^{339,343} In the most recent M01ZH09 vaccine dose-escalation study performed by Lyon and colleagues, rates of symptom reporting by a placebo-recipient healthy North American population were similar to those found here.³⁴³ The exceptions were 'headache' and 'nausea/vomiting', which were both more commonly reported in this study. Anecdotally, many of the early symptoms reported were felt by the participants to be related to sodium bicarbonate ingestion.

Both active vaccines were well tolerated by study participants; those symptoms experienced were predominantly mild in nature and short-lived. Of note was the relatively commonly experienced symptom of cough; while not often asked for specifically in previous studies, it was included here to maintain consistency with post-challenge symptom reporting. We found that cough was frequently reported after M01ZH09 ingestion and was described as being dry and tickly in nature.

9.9.2 IMMUNOGENICITY

Oral ingestion of both active vaccines resulted in significant activation of humoral immune responses. While correlates of protection against typhoid remain to be found, there is an array of evidence supporting both antibody and ASC responses to lipooligosaccharide (LPS), flagellin and/or Vi polysaccharide antigens as being markers of protection after vaccination.

9.9.3 ANTIBODY TITRES

Background levels of anti-LPS antibody were relatively high in this carefully selected study population. There may be many reasons for this, which, in the relevant setting, could include on-going exposure to *S. Typhi* bacteria, cross-reactive antibodies from other infectious/non-infectious causes or cross-reactivity with other Group D *Salmonella* species.⁵⁰⁶ This high rate of background LPS/O reactivity underlies some of the shortcomings of the Widal test as a useful diagnostic tool.¹⁸⁶ Baseline anti-LPS titres found in this study were similar between each vaccine group and to those found in the preliminary dose-escalation study.^{436,451}

Evidence for the importance of the anti-LPS IgG response to Ty21a in particular, comes from the Chilean field-studies, which demonstrated a dose-response effect and subsequent relation to field-efficacy.^{142,268,269} Anti-LPS IgG responses are now used as a key measure of oral typhoid vaccine immunogenicity.¹⁴¹ In the current study, significant increases in anti-LPS antibody were seen in both M01ZH09 and Ty21a vaccine recipients; differences were seen in the anti-LPS IgA responses between the two groups, although this may be due to the time points chosen and the relative short durability of this isotype (*see below*).

There is also considerable evidence to support the role of antibody responses to flagellin as being a possible marker of protection from infection. The early large-scale field trials of parenteral whole cell vaccines demonstrated that the most effective formulations were able to generate the highest levels of anti-H antibody. These findings were supported by data from the Maryland challenge studies, which also demonstrated a protective effect against challenge with the Quail strain.⁸⁹ Further evidence for the importance of the anti-H response is from a field-trial of a flagellin-negative vaccine which failed to demonstrate any protection against infection.⁵⁰⁴

In this study, a single-dose of M01ZH09 resulted in high levels of anti-H antibodies of all three isotypes; responses were also seen to Ty21a vaccination, although to a slightly less extent. Of note, the Ty21a group had significantly higher levels of IgA pre-vaccination; although the effects of this on subsequent vaccine response are not known, it may conceivably have reduced the response to this antigen.

The role of Vi antibody in preventing typhoid infection is demonstrated by the effectiveness of ViPS and, subsequently, the Vi rEPA vaccines. Given the stringent exclusion criteria used and the thoroughness in checking participant eligibility, specifically with the aim of excluding previously vaccinated volunteers, it was somewhat unexpected to find such a high proportion of participants with detectable levels of anti-Vi IgG at baseline. While the level of protection of anti-Vi is not known for an adult population, in a recent repeat retrospective analysis of field-trial data by Szu and colleagues,³⁰² a protective threshold of 10µg/mL one year after vaccination has been suggested. With the caveat that the original assay used to measure the anti-Vi responses in the South African studies is unlikely to be comparable to that used in this study,^{289,301} using this level, 22% of participants recruited may expect to be protected from challenge, even prior to receiving vaccination. Reasons for this may clearly include failure to detect previously vaccinated

(or chronically typhoid infected) volunteers at screening, over-sensitivity of the assay used or cross-reactivity related to *Citrobacter freundii* exposure (strain 5396/38 expresses an antigenically indistinguishable Vi capsule).¹⁴⁴ The serological assay used in our study is commercially available from Binding-Site and was developed in collaboration with the National Institute for Biological Standards and Control (NIBSC) and the NIH; the previous issues of LPS contamination of Vi, which has historically been problematic in accurately measuring anti-Vi levels, should now have been addressed (*personal communication: Sjoerd Rijpkema, NIBSC*). Neither Ty21a nor M01ZH09 expresses Vi capsular polysaccharide, not least as a safety measure to prevent vaccinaemia and invasive infection following ingestion. Therefore finding no response was to be expected.

9.9.4 ANTIBODY-SECRETING CELL RESPONSE

Reflecting the serological data, high numbers of anti-LPS secreting plasma cells were detected in some participants at baseline. To ensure the comparability of data to those previously published and to accurately measure the anti-LPS ASC response in a true 'naïve' population the decision was made to exclude the 'high background' responders from analyses. Comparison of these two subsets failed to demonstrate an appreciable difference in Day 7 responses, however, such was the magnitude of increase seen. Vaccination with M01ZH09 or Ty21a resulted in a significant increase in anti-LPS ASC numbers of all isotypes; however, significant differences with the Day 7 placebo anti-LPS IgG counts were only found in M01ZH09 recipients. This may be in part to the timing of sample collection (*see below*). Several authors have suggested that anti-LPS ASC counts maybe a more sensitive marker of host response to orally ingested vaccines compared to crude antibody titres. The rationale for this is that, if performed soon after vaccination, gut-homing activated IgA-secreting lymphocytes may be detected in the peripheral

blood. These data suggest that significant numbers of this cell-type are detectable in both M01ZH09 and Ty21a recipients.

Background anti-H ASC numbers were lower than anti-LPS secretory cells therefore all data were included in the analyses. Both active vaccines generated significant responses by Day 7, and counts were significantly higher for all isotypes compared to placebo controls. As expected, there were no significant differences found in anti-Vi secreting plasma cells after vaccination.

9.9.5 COMPARISON WITH PREVIOUS STUDIES

While comparison of immunologic assays is complicated by population characteristics and inter-laboratory variation in techniques, it may be useful to place these data in the context of previous M01ZH09 evaluations. Review of the literature demonstrates the range of selective definitions used to assess M01ZH09 immunogenicity, in addition to the effect of dose increases and different participant age groups and ethnicities (Table 9-5). Comparison with previous studies suggests that, despite the apparently high levels of immunogenicity seen in this study, the proportion of responders found is relatively modest.

The most comparable previous study is that mentioned above by Lyon and colleagues, in which escalating vaccine doses were assessed in North American healthy volunteers.³⁴³ While immune response rates in the current study are lower, the confidence intervals overlap with the proportions of responders found in that study. In contrast, the lower confidence interval found here does fall below the 50% threshold, which has been used in previous M01ZH09 evaluations as a level of acceptable immunogenicity.³³⁹

Study / population (country)	Group (n): Vaccine dose, CFU	S. Typhi ELISA		S. Typhi ELISpot	Difference compared to placebo [96%CI] ^A	Reference
		Anti-LPS IgG seroconversion, % [95%CI]	Anti-LPS IgA seroconversion, % [95%CI]	Anti-LPS IgG seroconversion, % [95%CI]		
MS01.03 / 60 healthy adults (US)		≥4x rise over baseline to Day 28		≥4x increase over baseline to Day 7		Kirkpatrick, 2006 ³⁴⁰
	Low (20): 5x10 ⁷	19 [4-46]	ND	56 [30-80]	NR	
	Mid (20): 5x10 ⁸ High (20): 5x10 ⁹	13 [2-38] 50 [24-75]		50 [25-75] 94 [70-100]		
MS01.04 / 32 healthy adults (US)		≥4x rise over baseline to Day 28		≥4x increase over baseline to Day 7 ^B		Kirkpatrick, 2005 ³⁴¹
	Evian ^C (16): 5x10 ⁹ Tap (16): 5x10 ⁹	81 [54, 96] 53 [27, 79]	ND	88 [62, 98] 93 [68, 100]	[70-100] ^D [68-100]	
MS01.07 / 27 healthy adults (Vietnam)		≥4x rise over baseline to Day 28		≥4x increase over baseline to Day 7		IB ³³⁹
	Vaccine (16): 5x10 ⁹ Placebo (11)	19 [4-46] 0	ND	73 [45-93] 0	73 [37-90]	
MS01.08 / 151 healthy children, 5-14 years (Vietnam)		≥1.7x rise over baseline to Day 14 or Day 28	≥1.5x rise over baseline to Day 7 or Day 14	≥4x increase over baseline to Day 7		Tran Tinh Hien, 2010 ³⁴²
	Vaccine (101): 5x10 ⁹ Placebo (50)	92 [85-97] 16 [7-29]	95 [89-98] 4 [1-14]	100 [88-100] ^E 0 [0-23]	81 [68, 89]	

MS01.13 (IDT^F) / 187 healthy adults (US)	$\geq 1.7x$ rise over baseline to Day 14 or Day 28	$\geq 1.5x$ rise over baseline to Day 7 or Day 14	$\geq 4x$ increase over baseline to Day 7	Lyon, 2010 ³⁴³
Vaccine (37): 5×10^9	62 [47-78]	84 [72, 96]	ND	70 [54-87]
Vaccine (38): 7.5×10^9	79 [66-92]	92 [84, 100]	92 [84-100]	76 [61-91]
Vaccine (36): 1.1×10^{10}	86 [75-97] ^G	83 [71, 96]	ND	73 [57-88]
Vaccine (39): 1.7×10^{10}	85 [73-96]	97 [93, 100]	ND	81 [68-94]
Placebo (37)	14 [3-25]	3 [0-8]	ND	NR
OVG 2011/02 / 91 healthy adults (UK challenge)	$\geq 1.7x$ rise over baseline to Day 28	$\geq 1.5x$ rise to Day 28	$\geq 4x$ increase over baseline to Day 7	This study
Vaccine (32): 1×10^{10}	50 [33-67] ^H	59 [42-76]	95 [76-100]	62 [38-76]
Placebo (30)	7 [-2-16]	3.3 [-3-10]	33 [12-61]	
Ty21a (29): 3-dose	24 [9-40]	24 [9-40]	76 [50-93]	13 [-10-35] ^I

Table 9-5. Comparison of immunogenicity endpoints between the current study (OVG2011/02) and those previously performed.

Confidence intervals for the differences in proportion of responders compared to placebo were calculated using a method described by Newcombe, based on the original Wilson procedure.^{507,508} ND, not done; NR, not relevant.

A, Note response rate is defined differently for each study depending on the immunology assays performed and a priori response criteria.

B, Anti-S. Typhi LPS IgA responses also assessed by antibody-in-lymphocyte supernatant (ALS) technique, which demonstrated good correlation with ELISpot ASC counts at Day 7.⁵⁰⁹

C, Two presentations were assessed comparing preparation using mineral water vs. normal tap water.

D, Compared to an assumed control response rate of 50%.

E, 29 and 15 participants ≥ 11 years old were eligible for the ELISpot from the M01ZH09 and placebo group respectively.

F, Previously made at Eurogenetech, Belgium.

G, A 'positive immune response' using a \geq rise over baseline to Day 28 was found in 37% [22-52] of participants at this dose.

H, $\geq 4x$ rise: 25% [12-43].

I, Response was determined by an increase in anti-LPS IgG antibody titre ≥ 1.7 over baseline to Day 28 AND/OR an increase in anti-LPS IgA antibody titre ≥ 1.5 over baseline to Day 28 AND/OR an increase in number of anti-LPS IgA SFU of ≥ 4 per 10^6 PBMCs (with high background responders excluded).

9.9.6 FACTORS AFFECTING IMMUNOGENICITY

Exploratory analysis was performed to examine other factors relating to antibody and ASC production. A strong correlation was found between Day 7 ASC numbers and Day 28 antibody titres but only for anti-LPS IgG and IgM and for anti-H IgM in M01ZH09 recipients. The association between anti-LPS IgG ASC and antibody titres and lack of an association with IgA ASC/antibody on Day 7 after vaccination has previously been described by Kantele.¹⁴³ These data support those observations, which, if found to be a relevant correlate of protection, may provide a logistically more useful time point for performing follow-up assessment. Interestingly our data also support a further previously described relationship between vaccination dose (over the range 5×10^9 - 1.7×10^{10}) and IgG seroconversion. While no relationship was found with antibody levels within the narrower dose-range used in this study, a significant association was found between vaccine dose and the number of anti-LPS IgG ASC at Day 7. While all participants received an M01ZH09 dose calculated to deliver the same number of live bacteria, presumably the total number of cells present increased, which may suggest that bacterial viability, *per se*, may be less important for antigen presentation in this setting. Both of these findings are limited by lack of available and/or widely accepted functional assays (*see below*).

9.9.7 LIMITATIONS

There are several limitations to these data. Firstly the time points chosen for sample collection and analysis were based primarily based on optimising assessment of M01ZH09 vaccine responses. Samples were collected at Day 7 following M01ZH09/placebo vaccination, but at a time point that corresponded to 11-days after Ty21a vaccination. This may therefore limit the comparisons that may be made between the immunogenicity of each vaccine. In his early studies, Kantele demonstrated that the peak anti-LPS ASC

responses are seen between 5 and 7 days after receipt of the first Ty21a dose; by Day 11 they are much reduced (Figure 9-11).¹⁴³

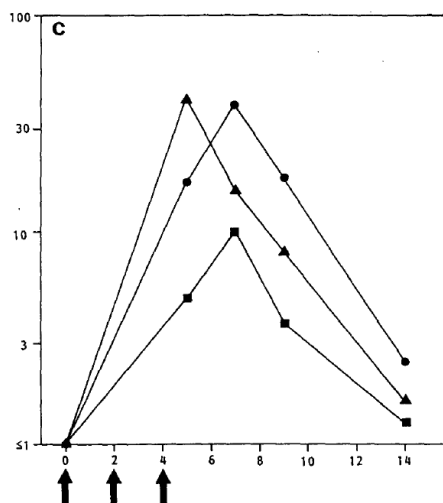


Figure 9-11. Anti-LPS ASC responses following 3-doses of oral Ty21a.

Ty21a was administered on alternate days (arrows); From Kantele and colleagues.¹⁴³ X-axis, time after vaccination (days). Y-axis, anti-Salmonella O-9,12 ASC/10⁶ PBMC. Circles, IgA; Squares, IgG; Triangles, IgM. With permission Elsevier Ltd.

While participants were scheduled to attend 7-days after receipt of vaccination, there was some variability in actual visit attendance dates (median, Day 7; range Day 5-15; mean day, M01ZH09, 6.8; Placebo, 7.0; Ty21a, 8.0). To address this issue, further exploratory analysis was performed to assess the peak and rate of decay of the ASC response. Using time series analysis, ASC counts appeared to be consistently lower in Ty21a vaccine recipients, suggesting that the optimal point for measuring Ty21a ASC responses had been missed (Figure 9-12). Note also that there was a wider spread of actual attendance days for Ty21a recipients.

Further limitations include the absence of functional antibody data (such as serum bactericidal or opsonophagocytosis assays), and data related to mucosal and cell-mediated immune responses, all of which are known to play an integral part in the response to *S. Typhi* infection. Available evidence suggests that these responses are likely

to play a major role in the development of immunity following live-oral vaccination in particular.

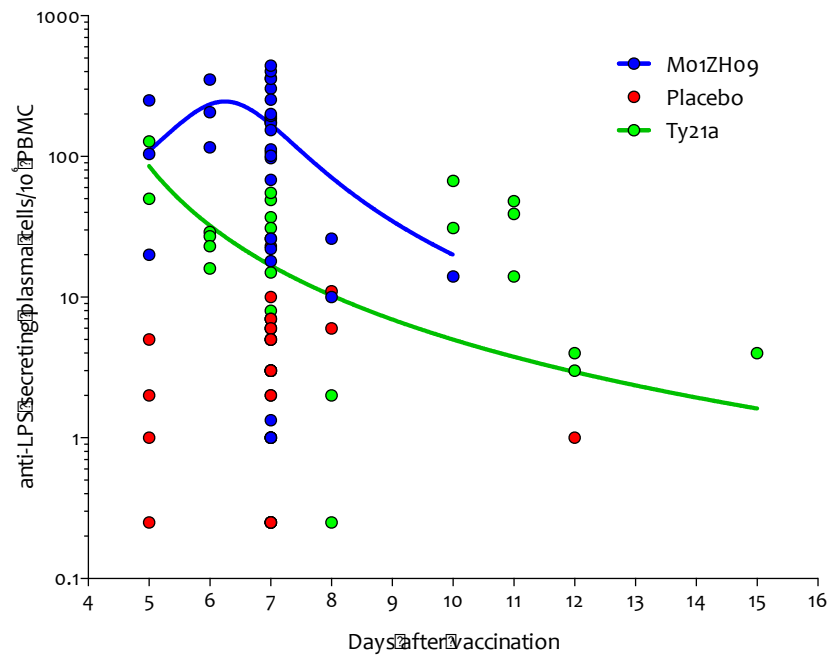


Figure 9-12. Anti-LPS IgA ASC decay after vaccination according to vaccine group by number of days after (last dose of) vaccine receipt. Includes both high and low background responders. Curve indicates line of best-fit.

9.9.8 SUMMARY

In summary, a single dose of the investigational vaccine Mo1ZH09 was well tolerated in comparison to placebo and generated significant immune responses. While the proportion of responders was lower than that seen in previous studies, this detailed set of immunological data provides evidence for a host response, which may or may not protect against exposure to the infectious agent and development of typhoid fever. These data may be used to directly evaluate putative correlates of protection after *S. Typhi* challenge.

Chapter 10. HUMAN RESPONSES TO *SALMONELLA* TYPHI INFECTION AND MODERATION BY ATTENUATED ORAL VACCINATION

10.1 INTRODUCTION

How humans respond to exposure and subsequent infection by *Salmonella* Typhi is not well understood. Together with endemic-setting or returning-traveller case series and the large historic vaccine field trials, challenge studies have informed much of what is known about how humans respond symptomatically and physiologically to typhoid infection. While data from these studies has sought to clarify the entity of typhoid fever, difficulties include the degree of background exposure and the immune statuses of populations studied, lateness or bias in healthcare presentation and accurate diagnosis for confirmation of the underlying aetiology. Detection of subclinical disease, which may

develop into chronic carriage and which may be of vital importance in transmission risk, is also mostly overlooked, particularly in the absence of intense active surveillance or use of more sensitive diagnostics.^{112,122,193} Accurate characterisation of exposure/infection responses in a challenge study setting may overcome or at least define these features more accurately, such as the protection afforded by a previous military service history in the Maryland studies.^{89,90} Challenge studies also allow the full time course of infection and illness evolution to be described from exposure to resolution with treatment, albeit sometimes at a relatively early stage of infection and without progression to the carriage state.

In addition to providing further information regarding the natural history of infection and indications of the underlying host immunobiological response to infection, description of early disease and its evolution is vital to inform the development of clinical diagnostic algorithms. Further translational applications include, for example, the identification of early features predicting severe infection outcomes which may be used to guide treatment stratification or clinical management decisions.

Aside from elucidating the clinical features of infection, performing challenge in the context of a vaccine study provides the opportunity to directly assess the protective efficacy of the vaccines being investigated. While this is generally assessed during the course of phase III studies performed in endemic settings, the financial, time and population resources required may be inhibitory to all but a selected few candidate vaccines. The effect of vaccination in preventing infection may also be subtler than a binary outcome variable of infection or not. Vaccines may alter disease presentation, the dynamics of infection, infection severity or transmission risk in a manner that may not be detected in large-scale field trial assessment. Human challenge models provide a uniform,

well-characterised population in which to closely measure the comparative differences seen both in those developing infection, or not, after exposure at the individual level.

This chapter will detail the human symptomatic and physiological response to *S. Typhi* after challenge in healthy volunteers and describe how clinical illness is altered in the context of previous oral vaccination. Responses were determined using self-reported solicited and non-solicited symptom records, clinical examination findings and description of the perturbations found in haematological and biochemical laboratory blood parameters.

10.2 METHODS

10.2.1 CHALLENGE AND FOLLOW-UP

Full methods for the challenge procedure have been described in Chapter 5. Briefly, after fasting overnight participants ingested 120mLs NaHCO_3 solution and waited 1 minute prior to ingesting a typhoid suspension (1.5×10^4 *S. Typhi*, Quail's strain, in 30mLs NaHCO_3). After a 90-minute observation period, participants were then free to leave the study site.

Participants were reviewed in person each day after challenge in the clinic (except for days 2 and 4) when routine observations (temperature, heart rate and blood pressure measurement) were performed and recorded. Participants noted symptoms experienced each day (to Day 21) using a diary card in which they recorded solicited, non-solicited and self-taken temperatures twice-daily. Symptoms, symptom severity (graded according to Supplementary Table 2 to 0-5) and recorded temperatures were confirmed at each clinic visit.

10.2.2 TYPHOID DIAGNOSIS

If either of the predefined criteria for diagnosis were reached (Table 5-2), participants were reviewed at a ‘typhoid diagnosis’ visit and antibiotic and additional symptomatic treatments were supplied as previously described (see: 5.4.9). Diagnosed participants were subsequently reviewed according to an altered ‘TD visit’ schedule (at 6, 12, 24, 48, 72 and 96-hours), at which additional observations and blood samples were collected.

10.2.3 LABORATORY BLOOD TESTS

Blood was collected for routine laboratory blood tests (full blood count, urea & electrolytes and liver function tests including C-reactive protein) before challenge (‘baseline’ or Day 0) and at later time points as described (Table 6-1). Results were checked in real-time using an intranet reporting system, with abnormal tests being repeated as clinically indicated. For analyses, data were uploaded to an online database (OpenClinica Community edition v3.1.3-beta) before being collated, as previously described (see: 5.4.10).

10.2.4 STATISTICAL CONSIDERATIONS

The analysis performed in this chapter are described by challenge outcome (typhoid diagnosed, ‘TD’, or non-typhoid diagnosed, ‘nTD’) and further stratified by placebo or vaccine (M01ZH09 or Ty21a) receipt.

Prior to analysis the distribution of each data set was evaluated. Symptom data (number, number of severe, mean severity scores and duration of symptoms) were non-parametrically distributed (positively skewed) and therefore described by median and IQR. Comparison of symptom data by challenge outcome was made by Mann-Whitney tests (MW); between-group comparisons (active vaccines to placebo) were made using Kruskal-Wallis tests with Dunn’s multiple comparison correction.

Clinical observations and laboratory data were parametrically distributed and are described by mean and exact 95% confidence intervals. Comparisons by outcome were made with unpaired T tests and between each vaccine group and placebo by ANOVA with Bonferroni's correction. To limit the data described, only symptoms reported with a \sim 20% difference compared to placebo vaccine recipient frequency are reported. A statistical significance threshold of 5% was assumed.

10.3 INFECTION RATE

Of 91 participants challenged, 49 satisfied the diagnostic criteria for typhoid infection within 14-days of challenge. 23, 11 and 15 reached the pre-defined clinical (A, in Table 10-1), microbiological (B), or microbiological plus symptoms before Day 7 (C) criteria for typhoid diagnosis (TD) to be made. An additional two participants were not diagnosed with typhoid until completing the 14-day follow-up period; they were diagnosed based on subsequently available microbiology results despite already starting antibiotic treatment ('*' in Table 10-1).

10.4 TOLERABILITY AND SYMPTOMATIC RESPONSE

In general *S. Typhi* challenge was well tolerated. One participant vomited within 2-hours of receiving the challenge dose and was therefore treated with antibiotics early without meeting either of the predefined study endpoints (see: 10.12.1). Therefore 91/92 (98.9%) participants completed the 2-week challenge period and are included in the *per protocol* population analyses presented here.

Overall, these 92 participants recorded 2068 solicited symptoms (median [IQR]: 20.5 [5.3-32.8] symptoms) during the 21-days data were collected for after challenge.

Outcome		Typhoid Diagnosed				nTD
		A. BC from Day 5 & clinical signs	B. BC from Day 7	C. Temperature $\geq 38^{\circ}\text{C}$ ≥ 12 hours	ALL	Day 14
Placebo (n=30)	N(%)	7 (23.3)	4 (13.3)	9 (30.0)	20 (66.7)	10 (33.3)
	Median IP, hours [range]	176.2 [147.5-193.7]	219.8 [174.4-272.0]	181.6 [120.7-263.1]	180.5 [120.7-272.0]	337.1 [336.0-338.0]
M01ZH09 (n=31)	N(%)	3 (9.7)	5 (16.1)	10 (32.3)	18 (58.1)	13 (41.9)
	Median IP, hours [range]	191.6 [156.8-200.4]	277.2 [200.8-342.5]	239.2 [197.7-334.9]	235.1 [156.8-342.5]	337.6 [340.7-334.6]
Ty21a (n=30)	N(%)	5 (16.7)	4 (13.3)	4 (13.3)	13 (43.3)	17 (56.7)
	Median IP, hours [range]	171.8 [169.1-193.7]	257.0 [205-336.2]	139.4 [119.9-263.4]	192.5 [119.9-336.2]	336.6 [335.6-342.3]
ALL GROUPS (n=91)	N(%)	15 (16.5)	13* (14.3)	23 (56.1)	51 (56.0)	40 (44.0)
	Median IP, hours [range]	176.2 [147.5-200.4]	243.2 [174.4-342.5]	216 [119.9-334.9]	197.7 [119.9-342.5]	337.2 [334.6-342.3]

Table 10-1. Number (%) of participants fulfilling specific criteria for initiation of antibiotic treatment according to vaccine group allocation.

Also shown are associated median [range] incubation periods between challenge and antibiotics being initiated. BC, positive blood culture result; nTD, non-typhoid diagnosed; IP, incubation period. *, includes 2 nTD participants started on antibiotics at Day 14 with a subsequently received positive blood culture result (referred to in text).

10.4.1 EARLY SYMPTOMS

Most participants experienced no immediate symptoms aside from commenting on the saltiness of the sodium bicarbonate pre-treatment solution. Frequently participants complained of mild/moderate bloating and ‘wind’ while several also experienced symptoms of acid reflux within 6-hours of challenge ingestion.

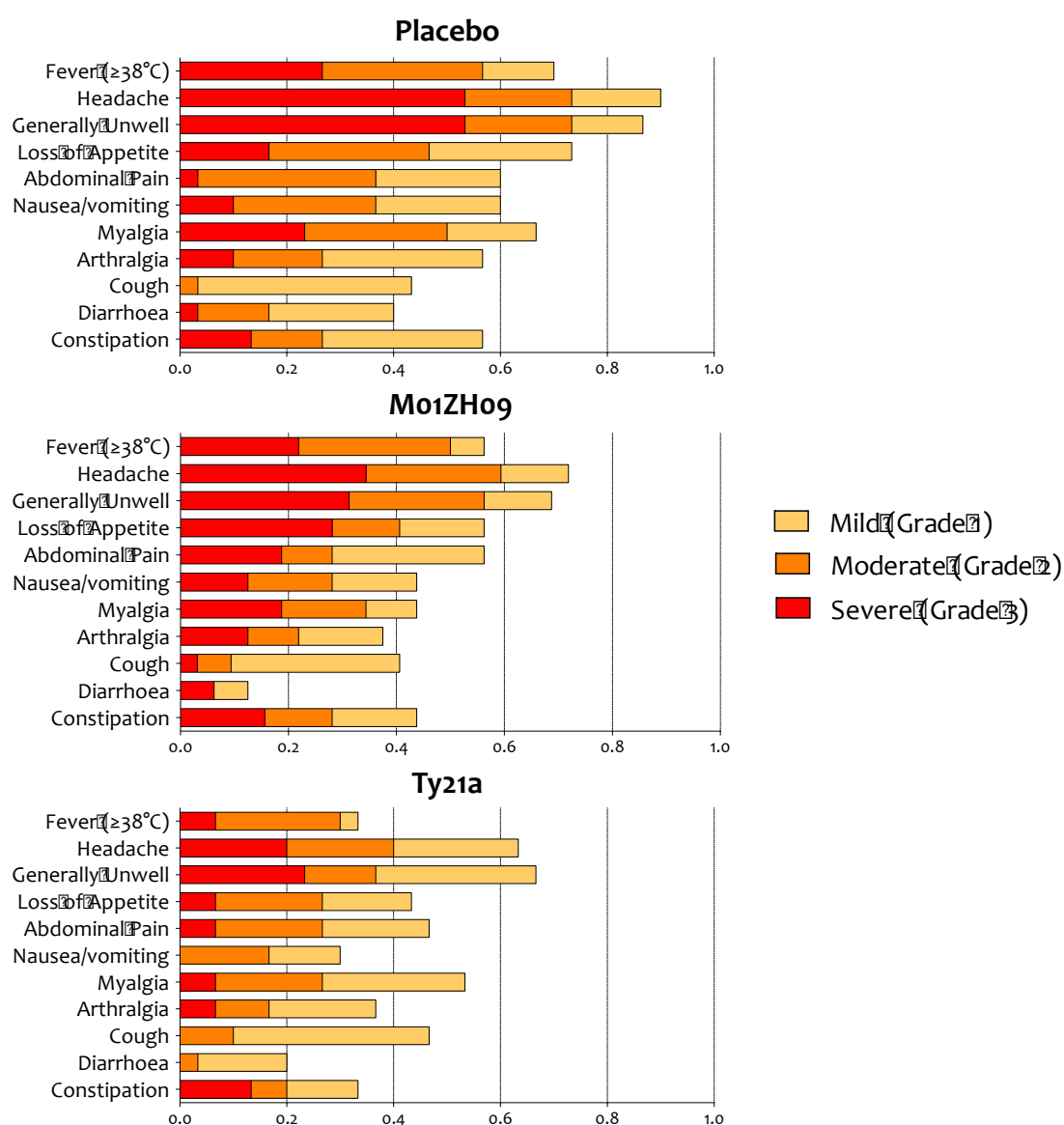


Figure 10-1. Proportion of participants reporting each solicited symptom during the 21-days after challenge according to vaccine group allocation.

Maximum severity score per participant for each symptom was used and graded according to criteria detailed in Supplementary Table 2: fever thresholds are Grade 1: $38.0\text{--}38.4^{\circ}\text{C}$; Grade 2: $38.5\text{--}38.9^{\circ}\text{C}$; Grade 3: $39.0\text{--}40.0^{\circ}\text{C}$; Grade 4: $>40.0^{\circ}\text{C}$.

Overall there was a relatively higher rate of symptom reporting during the earlier part of the incubation period: 20%, 28% and 17% of participants in the placebo, Mo1ZH09 and Ty21a groups, respectively, reported ≥ 1 symptom within 24-hours of challenge (Figure 10-2). Symptoms predominantly consisted of feeling generally unwell. Interestingly, early symptom reporting (on Day 0) was more common by those subsequently diagnosed with typhoid infection (TD, 75%) compared to those not diagnosed (nTD, 15%). Reporting ≥ 1 symptom on Day 0 was significantly associated with later typhoid diagnosis (OR [95%CI]: 17.1 [5.8-49.8], $p < 0.0001$, chi-squared test).

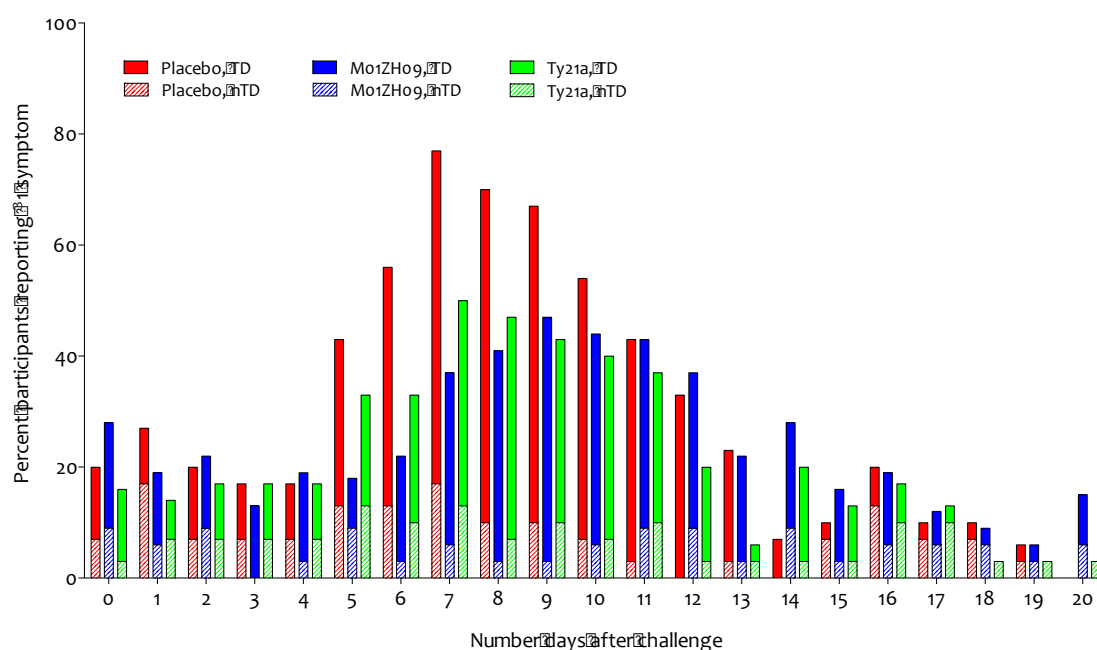


Figure 10-2. Per cent of symptomatic participants per day after challenge, grouped by vaccine allocation and subsequent challenge outcome.

TD, typhoid diagnosed; nTD, non-typhoid diagnosed.

Day 3 after challenge was associated with the fewest symptomatic participants overall (16/91, 16.5%), a level not reached again until Day 15 by which stage antibiotics had been initiated in all participants. In TD participants a subsequent substantial increase in symptom reporting occurred from Day 4, concurrent with the earliest initial increases in oral temperature measurements. TD placebo recipients reported significantly more

symptoms (median [IQR]: 29.0 [21.0-45.0]) than those not developing infection (5.0 [0.0-16.0], $p < 0.0001$, MW).

10.4.2 PLACEBO VACCINE RECIPIENTS

After challenge, 93% of placebo recipients reported at least one symptom; the median [IQR] number reported during the 21-day follow-up period was 27.0 [13.8-37.5] (Table 10-2), of which 12.6% were severe (\geq grade 3, Figure 10-1.).

	Mo1ZH09	Placebo	Ty21a	* p
TD, n	18	20	13	
Proportion (95%CI)	0.89 (0.65-0.99)	0.95 (0.75-1.00)	1.00 (0.75-1.00)	
Total number	24.5 [12.5-41.5]	30.0 [21.3-42.8]	26.0 [19.0-46.0]	
Number severe	2.5 [0-11.3]	5.0 [2.0-7.0]	2.0 [0-7.0]	
Duration, days	5.0 [3.8-10.0]	7.0 [5.0-8.0]	6.0 [5.3-8.5]	
Severity, grade	1.56 [1.32-2.24]	1.55 [1.33-1.70]	1.33 [1.19-1.64]	
nTD, n	13	10	17	
Proportion (95%CI)	0.46 (0.19-0.75)	0.90 (0.56-1.00)	0.59 (0.33-0.82)	
Total number	2.0 [0.5-15.0]	10.0 [3.0-20.3]	2.0 [0-7.0] *	0.050
Number severe	0 [0-0.5]	0 [0-0]	0 [0.0]	
Duration, days	2.0 [0-4.0]	3.0 [0.8-6.8]	0 [0-2.0]	
Severity, grade	1.33 [1.13-2.13]	1.00 [1.00-1.14]	1.00 [1.00-1.52]	
ALL to Day 14	31	30	30	
Proportion (95%CI)	0.84 (0.66-0.95)	0.93 (0.78-0.99)	0.77 (0.58-0.90)	
Total number	15.0 [11.5-22.7]	26.5 [19.0-32.7]	12.5 [9.8-24.0] *	0.038
Number severe	0 [0-3.0]	2.0 [0-6.3]	0 [0-2.3] *	0.034
Duration, days	4.0 [1.3-7.5]	6.0 [3.8-8.0]	4.0 [0-7.3]	
Severity, grade	1.46 [1.17-2.00]	1.47 [1.13-1.64]	1.25 [1.00-1.52]	
ALL to Day 21	31	30	30	
Proportion (95%CI)	0.84 (0.66-0.95)	0.93 (0.78-0.99)	0.77 (0.58-0.90)	
Total number	20.5 [2.8-31.0]	27.0 [13.8-37.5]	14.5 [2.3-29.0] *	0.061
Number severe	0.5 [0-5.8]	2.0 [0-6.0]	0 [0-2] *	0.029
Duration, days	4.0 [1.3-8.0]	7.0 [4.0-8.3]	4.5 [0-8.0]	
Severity, grade	1.45 [1.15-2.00]	1.41 [1.14-1.64]	1.24 [1.00-1.50]	

Table 10-2. A summary of the symptoms reported by typhoid-diagnosed (TD) and non-typhoid diagnosed (nTD) participants in the 14-days and (bottom) 21-days after challenge, according to vaccine group allocation.

Figures given are median [IQR] unless stated otherwise. Between group comparisons performed using Kruskal-Wallis tests with Dunn's correction; significant values only are given in column * p.

An increase in symptom reporting was observed from 4-days after challenge (Figure 10-2). Symptoms reported during the early course of infection were, in general, more systemic and less specific to gastrointestinal infection including 'headache', 'feeling generally unwell', 'loss of appetite' and 'myalgia' (Figure 10-3 & Supplementary Figure 2 & Supplementary Table 8). By Days 7 and 8, the maximal symptom reporting days, >50% of placebo recipients complained of these symptoms, which were accompanied in many cases by 'abdominal pain', 'nausea' or vomiting' and 'arthralgia'. 'Constipation' was more frequently reported than 'diarrhoea' in keeping with other adult populations studied (Supplementary Table 1).

Symptom reporting by placebo recipients developing typhoid infection ('TD placebo recipients') increased from Day 4. During the 14-day challenge period, TD placebo recipients reported a median of 30.0 solicited symptoms each of which 5.0 [2.0-7.0] were graded as severe (grade ≥ 3 ; Table 10-2). In the 95% of TD placebo recipients reporting symptoms, they lasted for a median [IQR] of 7.0 [5.0-8.0] days and were graded on average (median, [IQR]) as being 1.55 [1.33-1.70] in severity.

In contrast, non-typhoid diagnosed placebo recipients ('nTD placebo recipients') remained relatively less symptomatic throughout (Figure 10-3). Of 10 nTD placebo recipients, 9 reported symptoms between challenge and antibiotic initiation on Day 14. In addition to reporting fewer symptoms (10.0 [3.0-20.3]), they were also less severe (1.00 [1.00-1.14]) and of shorter duration compared to TD placebo recipients.

Of note, (blinded) nTD placebo recipients were relatively more symptomatic and reported an increasing number of symptoms between Day 4 and 8 after challenge, concurrent with the increase in symptom reporting by their typhoid-diagnosed counterparts. Also of note, was the apparent second peak in symptom reporting which occurred at Day 16 after challenge, two days after the initiation of antibiotic treatment.

Symptoms reported at this later time point included feeling ‘generally unwell’, ‘loss of appetite’, ‘headache’ and ‘cough’ (Figure 10-3).

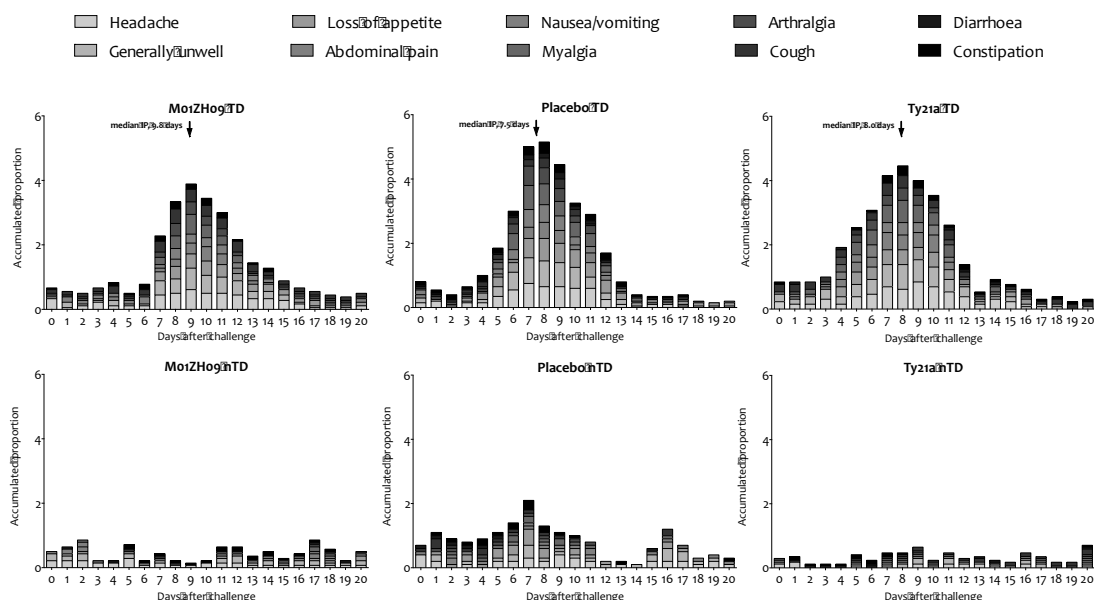


Figure 10-3. Accumulated proportions of participants reporting each of 10 solicited symptom by vaccine group allocation and typhoid challenge outcome.

Data reported between challenge (Day 0) and Day 21. Arrow indicates the median incubation period (day of typhoid diagnosis). TD, typhoid diagnosed; nTD, typhoid not diagnosed.

10.4.3 Mo1ZH09 VACCINE RECIPIENTS

Overall, 84% of Mo1ZH09 recipients reported a median [IQR] of 27.0 [13.8-37.5] solicited symptoms during the 21-days after challenge, of which 18.1% were severe (\geq grade 3).

In general, TD Mo1ZH09 recipients started to develop symptoms at Day 7, approximately 3-days after TD placebo recipients. Maximal symptom-reporting day did not occur until Day 9 (Figure 10-3). Proportions of TD Mo1ZH09 and TD placebo recipients reporting each of the solicited symptoms was broadly similar, although ~20% fewer Mo1ZH09 recipients reported ‘nausea/vomiting’, ‘myalgia’ and ‘diarrhoea’ (*not reported at all*), whereas ~20% more reported ‘cough’. TD Mo1ZH09 recipients reported a similar numbers, severity and duration of solicited symptoms compared to TD placebo recipients (Table 10-2)

Of note, not all participants diagnosed with typhoid reported symptoms during the 21-days post-challenge period. One placebo and two Mo1ZH09 recipients reported no symptoms during the study. The placebo recipient recorded a maximum temperature of 38°C and was blood culture positive at Day 6, while both Mo1ZH09 recipients were afebrile throughout and diagnosed based on blood culture (at Day 8 and 12) only.

Non-typhoid diagnosed Mo1ZH09 recipients ('nTD Mo1ZH09 recipients') were relatively asymptomatic throughout (Figure 10-3), although 46% reported at least one symptom (Table 10-2). Symptoms were of similar frequency and severity to nTD placebo recipients, although the numbers were too small to allow accurate comparisons to be made. There appeared to be some slight increase in symptom reporting around the time of antibiotic initiation, but this was less marked than for the placebo nTD group (Figure 10-3 and see below).

10.4.4 TY21A VACCINE RECIPIENTS

Ty21a recipients reported the fewest solicited symptoms after challenge; 77% reported a median [IQR] of 14.5 [2.3-29.0] solicited symptoms in the 21-day post-challenge period, of which 9.2% were severe (\geq grade 3). Ty21a recipients reported significantly fewer symptoms and fewer severe symptoms compared to placebo recipients during the 14 and 21-days after challenge (Table 10-2).

An increased frequency of symptom reporting was detected in TD Ty21a recipients from Day 4, as per the placebo group, and similarly maximal solicited symptom reporting occurred at Day 8. Symptoms recorded by ~20% fewer TD Ty21a recipients included 'nausea/vomiting', 'arthralgia', 'diarrhoea' and 'constipation', while ~20% more participants complained of 'cough' (Supplementary Table 8). The frequency, severity and

duration of symptoms reported by TD Ty21a recipients were very similar to typhoid-diagnosed participants in the other groups (Table 10-2).

Non-typhoid diagnosed Ty21a vaccine recipients were less symptomatic than nTD placebo recipients, with 59% reporting at least one symptom in the 14-days after challenge. Significantly fewer symptoms were reported compared to nTD placebo recipients during the 14-day follow-up period (Table 10-2).

10.5 OTHER FACTORS INFLUENCING SYMPTOMATIC RESPONSE

10.5.1 AGE AND SEX

Neither participant age nor gender had any significant effect on number of symptoms, symptomatic days or mean symptom severity scores recorded (*data not shown*).

10.5.2 TRAVEL TO ENDEMIC SETTING

Previous travel to an endemic setting for durations <6months was reported by 27/91(29.7%) of participants. There were no significant differences found in symptom reporting by those with or without a previous history of travel.

10.5.3 BASELINE ANTI-VI ANTIBODY

Anti-Vi IgG antibody was detected at baseline (>7.4 EU/mL) in 25/91(27.5%) of participants. There was no association found between symptoms reported and baseline Vi antibody status (assuming a protective threshold of ≥ 10 EU/mL) nor was there an association between the proportion of symptom-free participants and baseline Vi antibody status. There was also no effect seen when stratified by challenge outcome, i.e. Vi antibody positivity had no effect on symptoms reported within TD/nTD groups.

10.5.4 CLINICAL OR MICROBIOLOGICAL DIAGNOSIS

Participants diagnosed with typhoid based on clinical criteria (i.e. oral temperature $\geq 28^{\circ}\text{C}$ for ≥ 12 hours) recorded significantly more solicited symptoms (median [IQR]: Clinical, 33.0 [22.5-45.8]; Microbiological, 22.5 [13.0-32.8]), more symptomatic days (Clinical, 8.0 [5.0-10.0]; Microbiological, 5.0 [4.0-7.8]) and higher mean symptom severity scores (Clinical, 1.70 [1.55-1.86]; Microbiological, 1.27 [1.07-1.47]) compared to those diagnosed based on microbiological (blood culture) criteria (Figure 10-4).

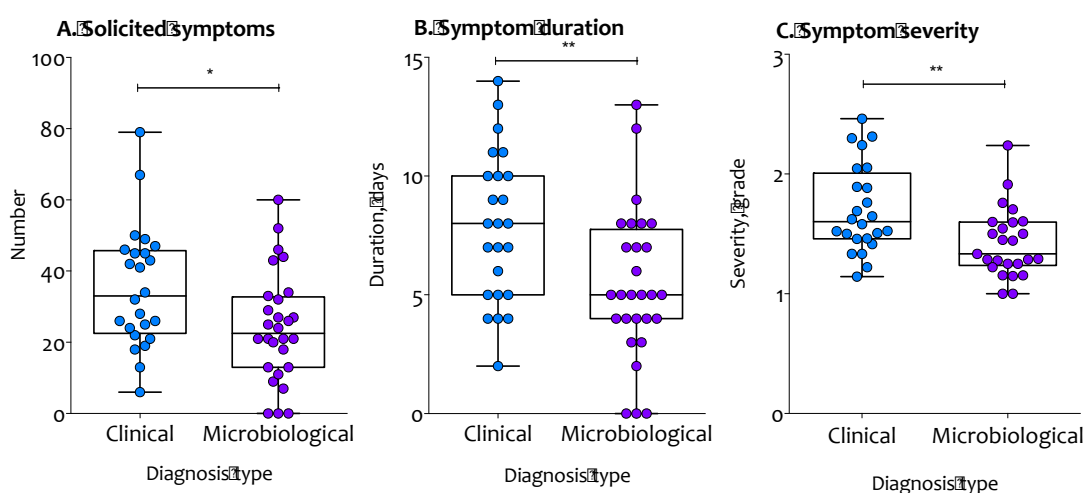


Figure 10-4. Associations between diagnostic criteria fulfilled for typhoid diagnosis and, A, number, B, duration, and C, severity of symptoms reported after challenge. Comparisons were made by Mann-Whitney Test. * $p < 0.05$; ** $p < 0.01$.

10.5.5 CHALLENGE DOSE

Overall there were no significant correlations between challenge dose received and number of solicited symptoms, symptomatic days or symptom severity. A difference was found, however, between the effects of dose on symptoms based on challenge outcome. Challenge dose received and number of symptomatic days correlated in nTD ($r = 0.366$, $p = 0.019$, Spearman's rank) but not TD participants ($r = -0.028$, $p = 0.845$). No correlations were found between dose and symptom number or severity. Further exploratory

analyses to assess dose-response did suggest a possible relationship between both \log_{10} (challenge dose) and symptom number or duration in nTD but not TD participants.

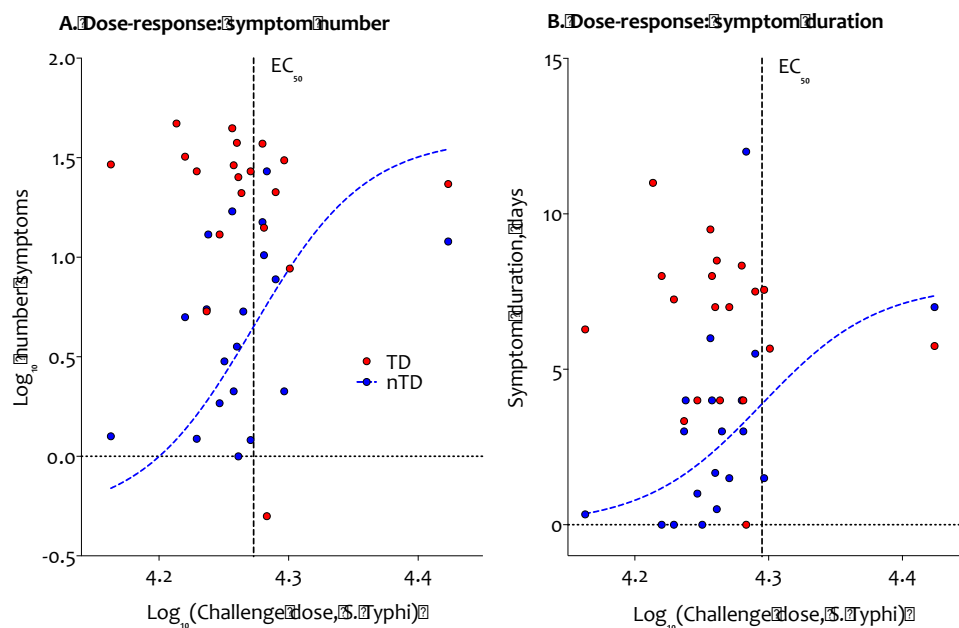


Figure 10-5. Relationship between challenge dose ingested and A, number of symptoms reported in 14-days, and B, symptom duration after challenge according to challenge outcome (TD or nTD). Dashed line, best-fit dose-response curve for nTD participants; EC₅₀ (unconstrained) for symptom number (A): 18,745CFU, and for duration (B): 19,712CFUS. Typhi.

10.6 CLINICAL EXAMINATION FINDINGS

Clinical measurements including peripheral pulse rate (HR), blood pressure and oral temperature were recorded from Day 1, 2 and 4 onwards (Supplementary Table 9).

10.6.1 EARLY FINDINGS

HR and systolic blood pressure (SBP) fell in most participants after the challenge visit, possibly related to initial challenge anxiety (Figure 10-6). By Day 3, the increase in HR from baseline tended to be higher in subsequent TD participants (mean [95%CI]: TD, 3.6bpm [0.0-7.2]; nTD, -1.6bpm [-6.4-3.2]; unpaired T-test, $p=0.08$). The earliest clinical evidence of infection was seen in placebo and Ty21a recipients who recorded increased temperatures from the evening of Day 4 onwards.

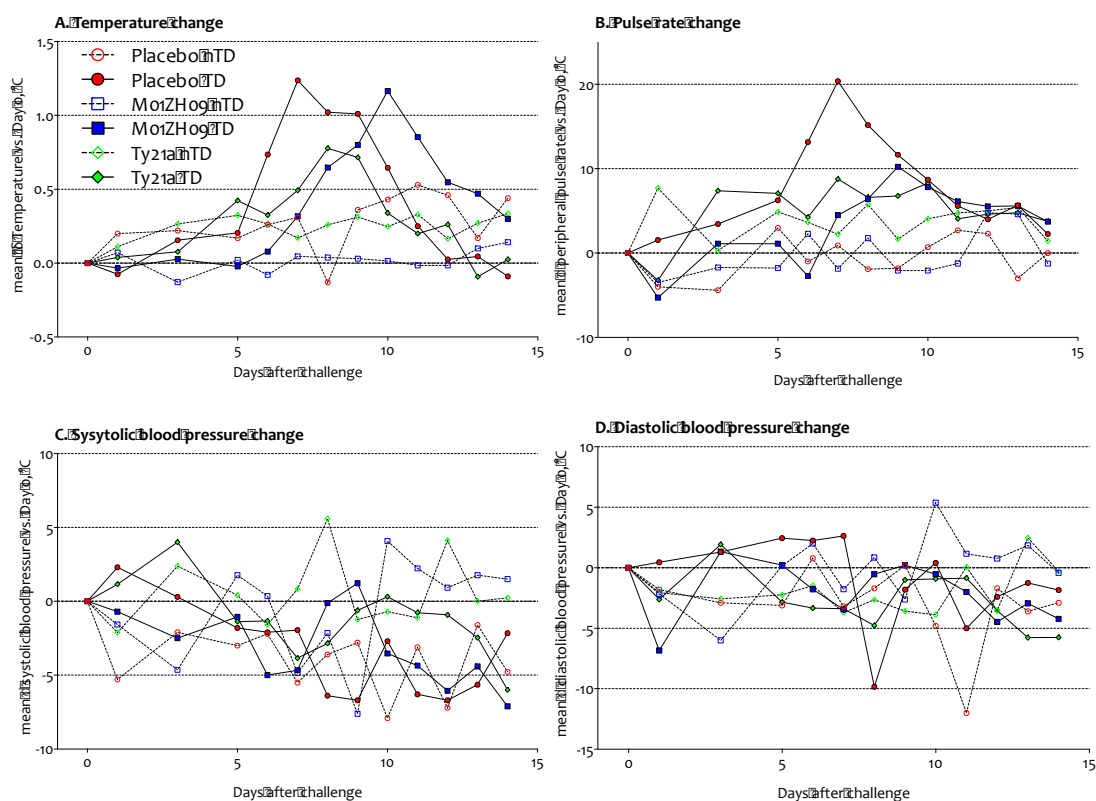


Figure 10-6. Mean changes in clinical examination findings recorded at each clinic visit compared to baseline recordings, according to vaccine group allocation and challenge outcome.

10.6.2 EXAMINATION FINDINGS IN TD PLACEBO RECIPIENTS

In TD placebo recipients, mean temperature measurements by 36-hours before diagnosis (36.8°C) had already exceeded the baseline upper 95%CI (36.3°C; Figure 10-7A). This increase was followed by a rise in both heart rate and systolic blood pressure at the TD-24 hour time point (Figure 10-7B&C). By the time of diagnosis, SBP had slightly fallen whereas mean HR and temperature had increased still further, suggesting febrile vasodilation and compensatory tachycardia.

After diagnosis and commencement of antibiotic (and paracetamol etc.) treatment, temperature and HR measurements fell initially (at TD+6 hours) but then increased subsequently from the +12 to +24 hour post-TD time points.

Temperature measurements at TD or at a matched Day 7 time point in nTD participants (commensurate with the median incubation period in TD placebo recipients) were

significantly higher compared to baseline for TD but not nTD participants (mean difference [95%CI], TD: +1.58°C [1.23-1.93]; nTD Day 7: +0.12°C [-0.31-0.55]; Figure 10-8A & Supplementary Table 9). Mean increases in heart rate were also significantly higher at diagnosis compared to baseline (TD: +15.1bpm [6.0-24.3]), whereas nTD HRs at the Day 7 were not significantly elevated (nTD: +6.6bpm [-4.9-18.2]). There was no significant difference seen in systolic blood pressure measured at typhoid diagnosis compared to baseline (TD: -7.5mmHg [-16.1-1.2], Figure 10-8B).

Of note, relative bradycardia was identified in 4/19 (21%) of TD placebo recipients, using the criteria suggested by Cunha,⁶⁰ i.e. temperature $\geq 38.9^{\circ}\text{C}$ with HR < 120bpm or $\geq 38.3^{\circ}\text{C}$ with HR of < 110bpm. This finding was present in all those with temperatures fulfilling the minimum threshold of $\geq 38.3^{\circ}\text{C}$. No dissociation between temperature and heart rate was found at the typhoid diagnosis visit specifically, however, and a significant positive correlation was found between the two measurements ($r=0.441$, $p=0.002$, Pearson's correlation).

Secondary survey performed at the TD visit was relatively unremarkable; probable rose spots were identified in 1/19 participant and 2/19 participants had abdominal discomfort on palpation. By Day 14 after challenge, there were no significant differences in temperature or heart rate compared to baseline measurements (Figure 10-8).

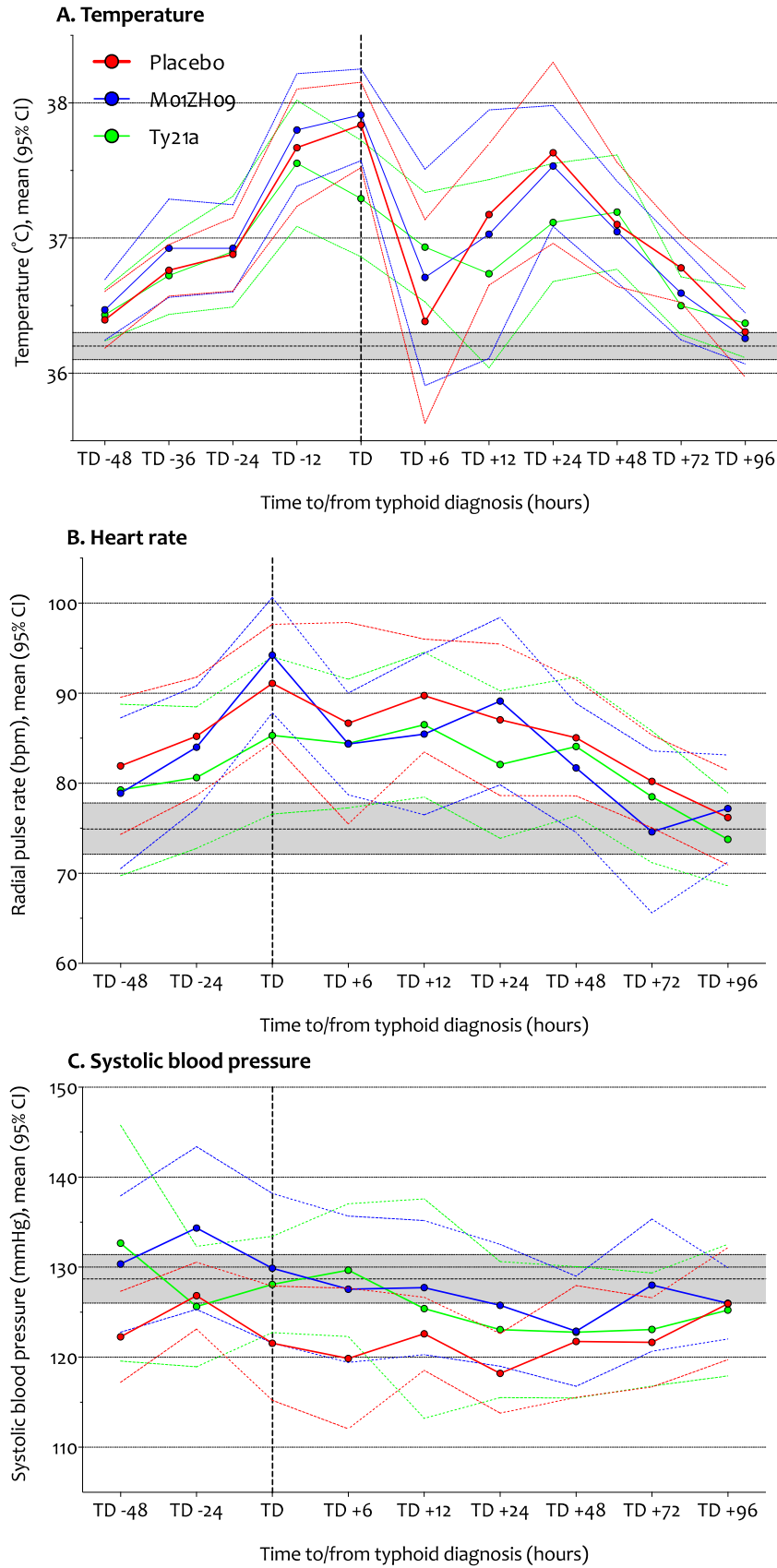


Figure 10-7. Clinical examination findings before, during and after typhoid diagnosis. Grey bar and dashed line indicates the mean (dotted lines, 95%CI) baseline (pre-challenge) recordings for all participants.

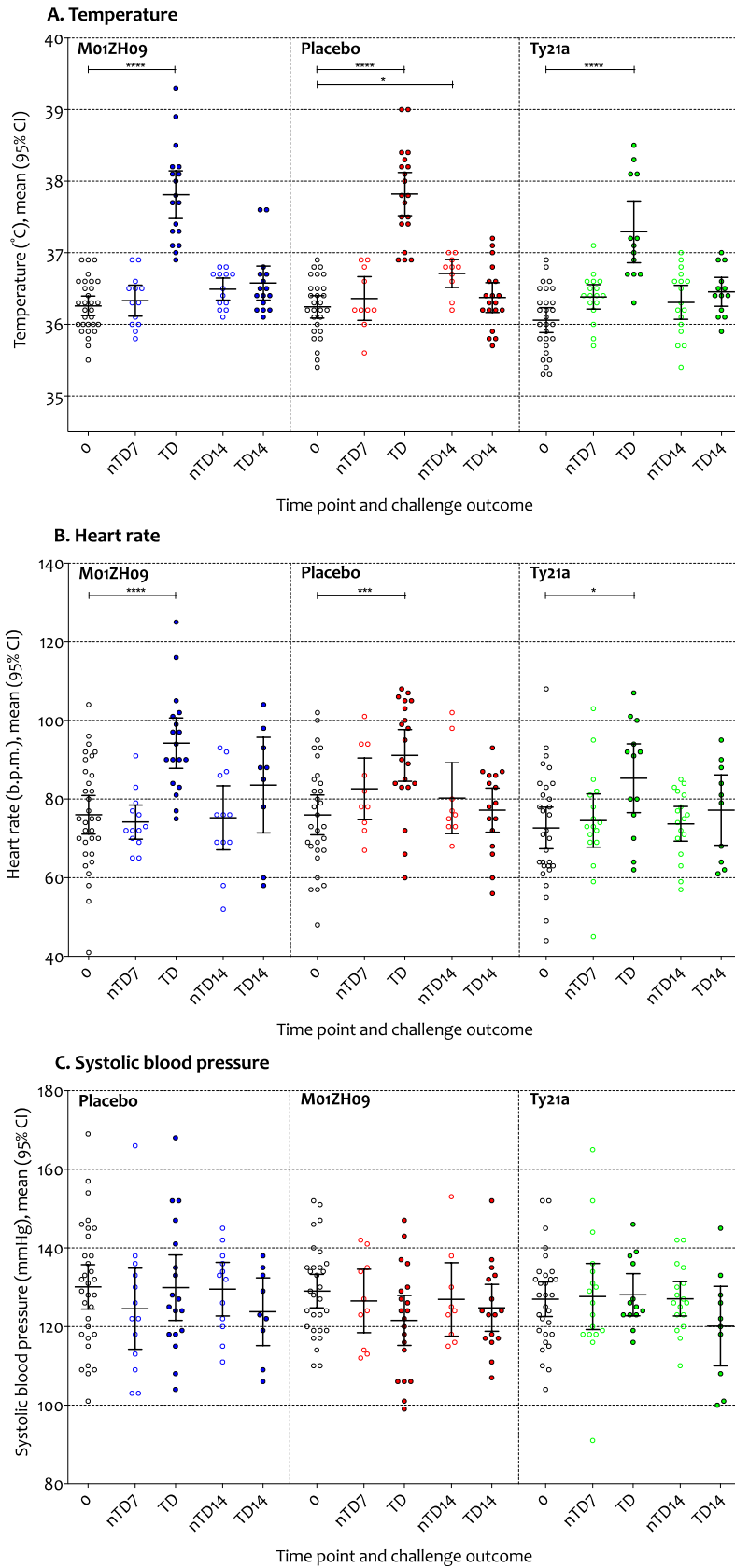


Figure 10-8. Clinical examination findings according to vaccine allocation and typhoid challenge outcome before and at time points after challenge.

'0', Day 0 (all participants). Comparisons between time points and Day 0 for each respective group made by ANOVA with Bonferroni's correction. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

10.6.3 EXAMINATION FINDINGS IN NTD PLACEBO RECIPIENTS

Examination findings in participants receiving placebo vaccination but not developing typhoid infection were unremarkable after challenge. By Day 7, there were no significant changes in temperature compared to baseline measurements, although by Day 14, recorded temperatures were significantly higher than baseline readings (nTD Day 14: +0.47°C [0.04-0.90]; Figure 10-8). Temperature changes were not accompanied by significant changes in heart rate or blood pressure readings, however, and the maximum temperature recorded prior to the start of antibiotics in this group (n=10) was 37.3°C.

10.6.4 EXAMINATION FINDINGS IN Mo1ZH09 RECIPIENTS

Overall there were no significant differences in the mean and maximum temperatures recorded by those receiving Mo1ZH09 vaccine compared to placebo. The delay in symptom reporting compared to placebo and Ty21a vaccinated groups was also reflected by a delay in temperature and heart rate increases in those Mo1ZH09 participants developing typhoid infection (Figure 10-6), with the maximum measurements recorded occurring 2-3 days after those in placebo recipients. Changes in temperature and heart rate measurements occurred at a similar time prior to typhoid diagnosis compared to TD placebo recipients, and these participants also had significantly increased temperature and heart rate recordings at diagnosis compared to baseline (Temp, TD: +1.55°C [1.22-1.88]; HR, TD: +18.2bpm [8.8-27.7]; Figure 10-8A&B). A similar secondary increase was seen in both temperature and HR measurements as for TD placebo recipients (Figure 10-7). By Day 14, temperature and HR observations had normalised in all but two participants likely due to the delay in illness onsets, as they were diagnosed Days 11 and 16.

Relative bradycardia was found in 5/16 (31%) of diagnosed participants (and 4/5 with temperatures $\geq 38.3^{\circ}\text{C}$); one participant meeting the temperature threshold (38.6°C) was tachycardic (125bpm). A rash consistent with rose spots was found in 4 TD M01ZH09 recipients (Figure 10-9), and cervical lymphadenopathy was noted in one participant.



Figure 10-9. Two examples of Rose spot-type rashes found on examination of study participants at typhoid diagnosis.

Left image: anterior left mediastinum demonstrates three faint pinkish-red spots (circled), which blanched on pressure and had disappeared within 1-2 hours; further spots were present on the back. Right image: more pronounced red lesions over hypothenar eminence and ulnar styloid region. Again these lesions blanched on pressure and the lower right lesion in the image was palpable. Both appeared and went within a few hours of typhoid diagnosis, and the participant would have been unaware of them had they not been found on examination.

The clinical measurements and findings in nTD M01ZH09 recipients remained constant throughout the challenge period; of note, there was no increase in temperature at the nTD Day 14 visit as was seen in placebo recipients (Figure 10-8A). The maximum temperature recorded in this group ($n=14$) during the challenge phase of the study was 37.5°C .

10.6.5 EXAMINATION FINDINGS IN TY21A RECIPIENTS

In general, less perturbation in clinical measurements was found in Ty21a recipients compared to those allocated to other groups.

TD Ty21a recipients started recording increased temperatures from 4.5 days after challenge, concordant with TD placebo recipients. While changes in temperature and

heart rate recordings were observed at 36- and 24-hours before diagnosis (as in the placebo recipients), by the time of diagnosis visit, the mean overall temperature had fallen in comparison to the TD-12 hour reading (Figure 10-7). While some secondary increases in temperatures were seen 24/48-hours after diagnosis, these were less marked than for other typhoid-diagnosed participants and were not accompanied by an increase in HR. Temperature and HR measurements at diagnosis were significantly higher than baseline (Temp: +1.24°C [0.85-1.63]; HR +12.6bpm [2.0-23.3]), however, they normalised by the Day 14 visit (Figure 10-8A&B).

Only two TD Ty21a participants reached the temperature ($\geq 38.3^{\circ}\text{C}$) threshold for assessment of relative bradycardia, and this finding was present in both (2/13). A new onset rash was documented at diagnosis in three Ty21a recipients.

Clinical observations made in nTD Ty21a recipients remained unremarkable during the period of follow-up (Figure 10-6).

10.7 OTHER FACTORS INFLUENCING CLINICAL FINDINGS

10.7.1 AGE AND SEX

Overall, age had no significant effect on the maximum change in temperature recorded compared to baseline (Day 0) during the 14-day post-challenge period, however, when stratified by challenge outcome, a significant age-related decline was found in nTD participants (nTD: $r=-0.452$, $p=0.003$, Spearman's rank). Maximum HR and SBP changes were not significantly affected, overall or when stratified by outcome. Participant gender had no significant effect on any measure studied (*data not shown*).

10.7.2 TRAVEL TO ENDEMIC SETTING

History of previous travel to an endemic setting (<6months) had no significant effect on clinical examination measurements (*data not shown*); maximum increases in HR tended to be higher in those with a previous travel history (mean difference [95%CI], +4.89 [-0.55-10.3], $p=0.078$, unpaired T test).

10.7.3 BASELINE ANTI-VI ANTIBODY

Presence of anti-Vi antibodies at baseline had no significant effect on the clinical measurements recorded after challenge (*data not shown*). This remained true when participants were stratified by challenge outcome, although the number of Vi+ TD participants was small ($n=7$).

10.7.4 BACTERAEMIA

Participants with bacteraemia had significantly higher maximum temperature and HR increases compared to those who remained non-bacteraemic (mean difference [95%CI], Temp: +1.01°C [0.65-1.38], $p<0.0001$; HR: +5.19bpm [0.3-10.1], $p=0.04$, unpaired T tests). No effect was seen on SBP. There were no clear relationships found between the (crude) number of positive blood cultures per participant and temperature, heart rate or systolic blood pressure recordings (*data not shown*).

10.7.5 CLINICAL OR MICROBIOLOGICAL DIAGNOSES

Participants who were diagnosed based on clinical (temperature $\geq 38^{\circ}\text{C}$ for ≥ 12 hours) rather than microbiological criteria had higher maximum temperatures recorded (+1.05°C [0.59-1.51], $p<0.0001$). While heart rate increases were similar in each group, the clinical diagnoses group ($n=24$) also had a significantly greater systolic blood pressure increase (+7.8 mmHg [1.6-14.1], $p=0.015$).

10.7.6 CHALLENGE DOSE

Challenge dose ingested had no significant effect on any of the three selected outcome measures, whether assessed overall or by typhoid challenge outcome (*data not shown*).

10.8 ALTERATIONS TO HAEMATOLOGY LABORATORY PARAMETERS

10.8.1 PLACEBO VACCINE RECIPIENTS

Alterations in haematology were seen in all challenged participants, but, in general, were more marked in placebo recipients (Figure 10-10 & Supplementary Table 10). A fall in haemoglobin was noted in all participants undergoing challenge, which appeared more marked in those developing infection (TD) than not. While not significantly different at diagnosis compared to baseline, by Day 14 this decrease was significant in TD placebo recipients (mean difference [95%CI]: -1.68g/dL [0.25-3.12], $p=0.018$, ANOVA with Bonferroni's correction). Platelet counts decreased approximately 48-hours prior to the development of typhoid infection, and while not significantly lower at diagnosis ($p=0.08$; Figure 10-11C), rebounded subsequently and were significantly increased by Day 14 ($+85.3 \times 10^9/L$ [24.1-146.0], $p=0.003$).

Although variable, TD placebo recipient WCCs remained statistically unchanged from baseline measurements throughout the course of challenge. This may have been due to sample size; when grouped across all vaccine groups, TD participants had a significantly decline in WCC between Day 0 and 14 ($-1.16 \times 10^9/L$ [-2.04- -0.28], $p=0.004$). Neutrophils numbers did alter significantly and were increased at diagnosis in TD placebo recipients ($+1.68 \times 10^9/L$ [0.67-2.70], $p=0.0004$) but returned to normal by Day 14. Conversely, lymphocyte counts decreased early and were significantly lower at diagnosis ($-1.06 \times 10^9/L$ [-1.49- -0.63], $p<0.0001$). Eosinophil counts also decreased early in the course of

challenge, were significantly lower at diagnosis ($-0.19 \times 10^9/L$ [-0.25 - -0.12], $p < 0.0001$) but had normalised by Day 14 (Figure 10-10). By Day 14, TD placebo recipients also had significant increases in Erythrocyte Sedimentation Rates recorded ($+9.4 \text{ mm/hr}$ [2.4 - 16.4], $p = 0.006$).

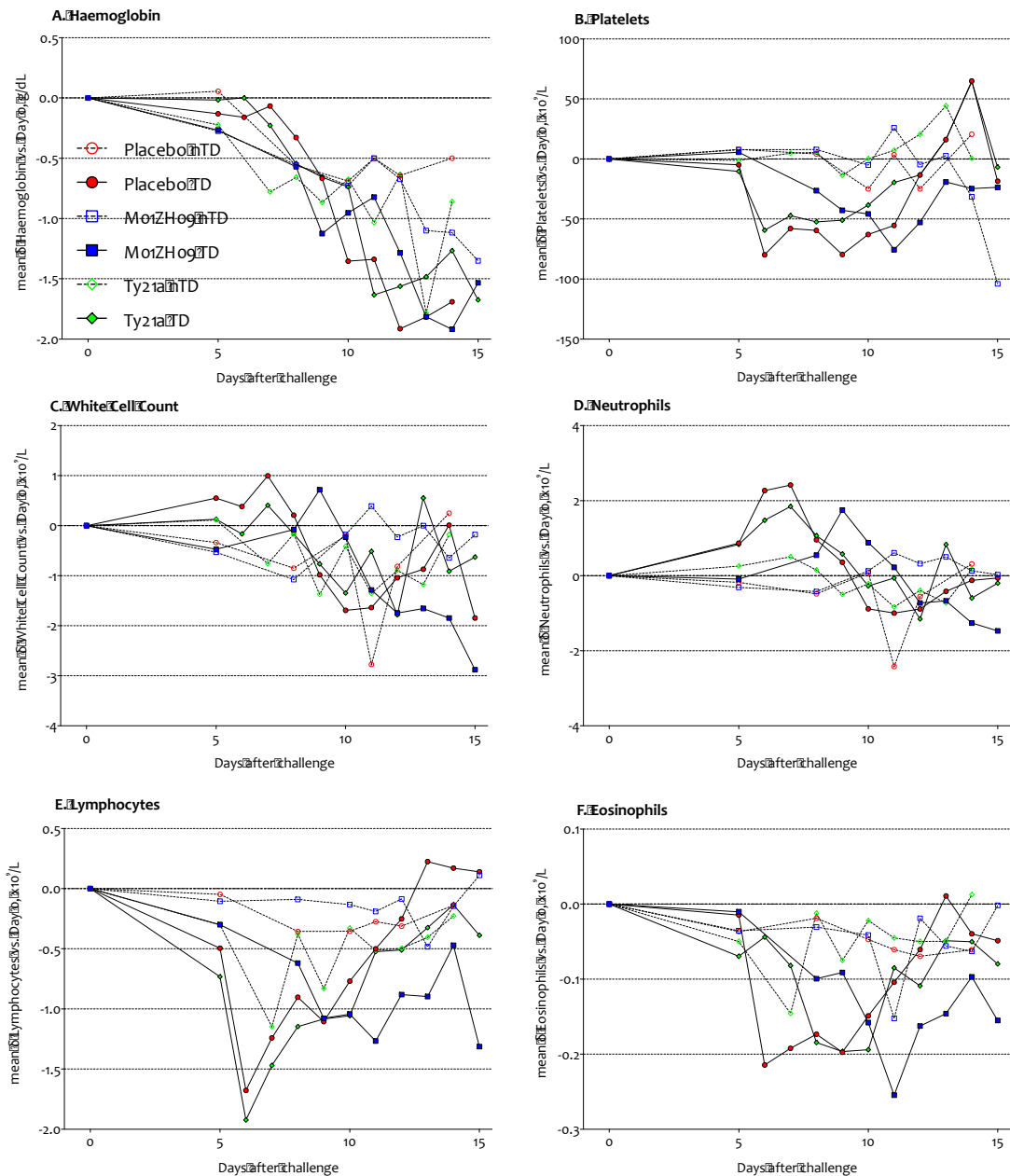


Figure 10-10. Illustrative mean changes in haematological blood parameters compared to individual baseline measurements according to vaccine allocation and challenge outcome by day after challenge.

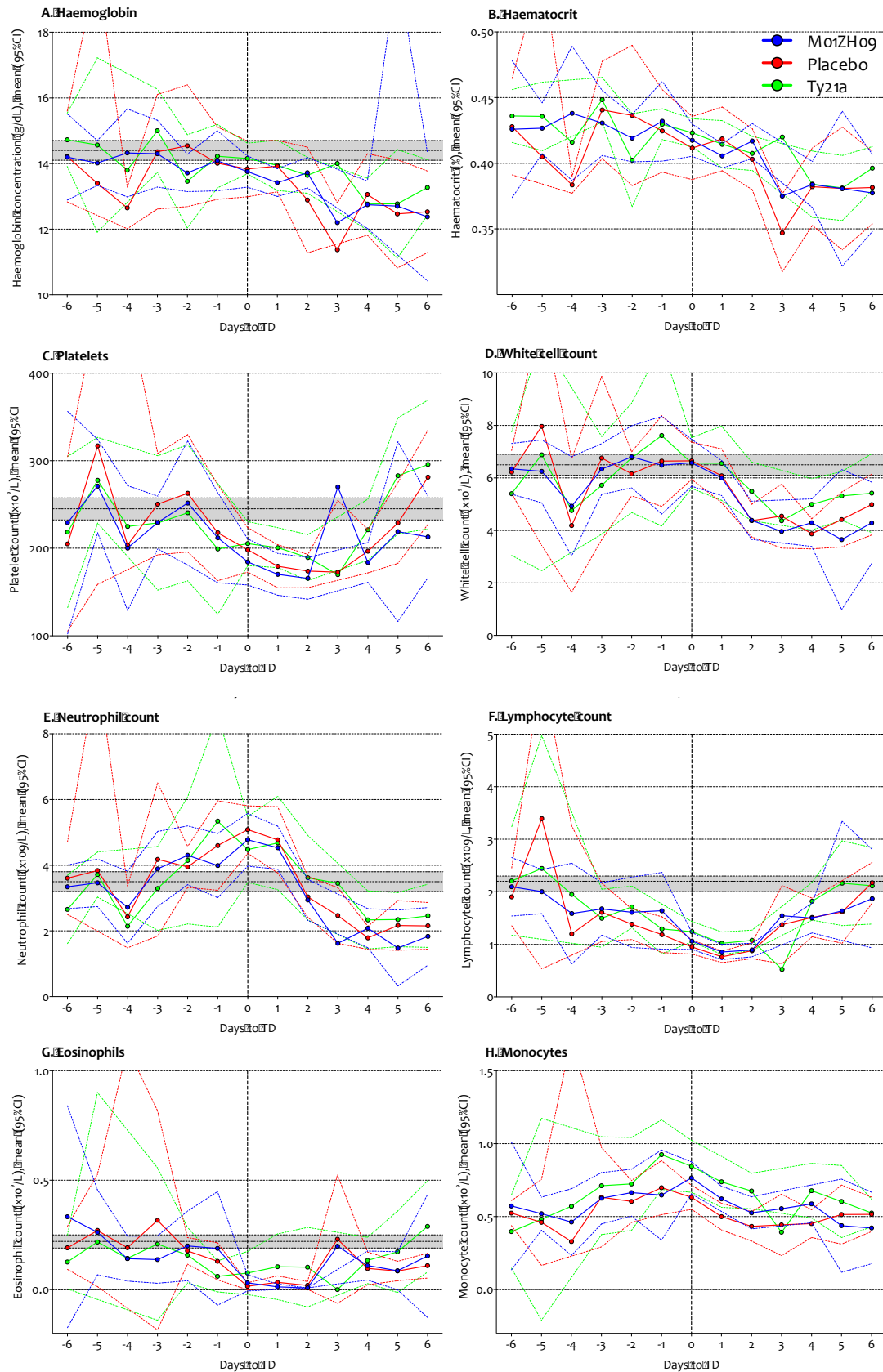


Figure 10-11. Changes in haematology blood parameters before, during and after typhoid diagnosis in those developing infection during the 14-day follow-up period.
Dashed line and grey shaded area, mean (95% CI) of ALL baseline measurements.

10.8.2 Mo1ZH09 VACCINE RECIPIENTS

Haemoglobin levels also decreased in all participants receiving Mo1ZH09 vaccine (Figure 10-10). By Day 14 the level had decreased significantly in typhoid-diagnosed participants (-1.56g/dL [-0.66 - -2.46], $p=0.0002$). Platelet counts were significantly lower at diagnosis ($-67.8\times 10^9/\text{L}$ [-109 - -26.7], $p=0.0004$) but had normalised by Day 14 in typhoid-diagnosed Mo1ZH09 recipients; nadir platelet count in Mo1ZH09 recipients occurred at Day 11, five days after the lowest levels in placebo (or Ty21a) recipients had been recorded (Figure 10-10B).

Typhoid-diagnosed Mo1ZH09 recipients demonstrated some biphasic response in total WCC after challenge, with levels initially increasing prior to a subsequent drop. This decrease was significant by TD+48 hours (Figure 10-11). Neutrophil counts also initially increased and then fell, with levels measured at Day 14 lower than those at baseline ($-1.47\times 10^9/\text{L}$ [-2.60 - -0.24], $p=0.0134$; Figure 10-10D). Also in common with placebo group findings, lymphocyte counts fell early after challenge, although only reached a nadir at Day 11 compared to Day 6 for placebo (and Ty21a) recipients; they had also decreased significantly by diagnosis ($-1.07\times 10^9/\text{L}$ [-0.67 - -1.46], $p<0.0001$) and remained low to Day 14. Eosinophil counts decreased rapidly in TD Mo1ZH09 recipients reaching a nadir at TD+48 hours; levels had fallen by diagnosis ($-0.19\times 10^9/\text{L}$ [-0.30 - -0.09], $p<0.0001$) and remained significantly depleted to Day 14. ESR levels in the Mo1ZH09 TD group increased significantly to Day 14 ($+9.3\text{mm/hr}$ [3.2 - 15.4], $p=0.0017$).

Haemoglobin levels in nTD Mo1ZH09 recipients also fell by Day 14, but not significantly (-1.01g/dL [-2.15 - 0.13], $p=0.099$). Total and differential white cell counts and ESRs remained unchanged in nTD Mo1ZH09 recipients throughout the challenge period.

10.8.3 TY21A VACCINE RECIPIENTS

Haemoglobin levels in all Ty21a recipients also decreased during challenge and were significantly lower in TD participants by Day 14 (-1.56g/dL [-2.6 -0.51], $p=0.0017$). Decreases in platelet counts were seen in TD Ty21a recipients, with nadir levels reached at Day 6. Platelet counts were not significantly decreased at diagnosis, however, and a subsequent reactive thrombocytosis was seen such that levels had increased significantly by Day 14 ($+73.2\times 10^9/\text{L}$ [10.4 -136], $p=0.0172$).

Changes in WCC after challenge were less marked in TD Ty21a recipients, with levels not changing significantly between baseline and diagnosis or Day 14. Neutrophil perturbations were more pronounced, however, with levels increasing significantly to diagnosis ($+1.1\times 10^9/\text{L}$ [0.05 -2.16], $p=0.038$). After diagnosis, neutrophil counts had normalised by Day 14. Lymphocyte levels in TD Ty21a recipients decreased significantly by diagnosis ($-1.22\times 10^9/\text{L}$ [-1.73 -0.70], $p<0.0001$). As for diagnosed participants in other groups, eosinophil counts were lower at diagnosis ($-0.17\times 10^9/\text{L}$ [-0.06 -0.28], $p=0.001$), however had returned to normal levels by Day 14. ESR levels were significantly increased by Day 14 in TD Ty21a recipients compared to baseline ($+15.1\text{mm/hr}$ [4.4 -25.9], $p=0.004$), however ESR data were only available for 4 participants at this particular time point.

Haematological parameters in Ty21a recipients not developing infection remained unchanged throughout; a significant decrease in haemoglobin being the only exception (-1.56g/dL [-2.61 -0.51], $p=0.002$).

10.9 BIOCHEMICAL CHANGES

As for the haematological changes, biochemical perturbations were mainly seen amongst those participants developing typhoid infection and were otherwise relatively unremarkable.

10.9.1 PLACEBO VACCINE RECIPIENTS

Blood sodium and urea levels decreased between challenge and diagnosis in TD placebo recipients (Sodium: -3.2mmol/L [-4.8 - -1.6], $p < 0.0001$; Urea: -1.1mmol/L [-0.2 - -1.3], $p = 0.01$), whereas potassium and creatinine were not significantly affected (Supplementary Table 11).

Liver function tests remained relatively unaffected in TD placebo recipients with one notable exception: alanine aminotransaminase (ALT) levels had increased significantly by Day 14 ($+126.4\text{IU/L}$ [44.7 - 208.0], $p = 0.0009$). C-reactive protein (CRP) levels increased in participants developing typhoid infection and were significantly higher at diagnosis ($+41.5\text{IU/L}$ [26.4 - 56.5], $p < 0.0001$), but not at points preceding this (Figure 10-13). Maximal CRP measurements were recorded at TD+24 hours in TD placebo recipients and, while recovering to normal levels by Day 14 ($+15.7\text{IU/L}$ [-2.0 - 33.5], $p = 0.098$), there was some lag in resolution persisting for 3-4 days after diagnosis/antibiotic initiation.

10.9.2 M01ZH09 VACCINE RECIPIENTS

Typhoid-diagnosed M01ZH09 recipients also had significant decreases in sodium levels by diagnosis (-3.3mmol/L [-5.2 - -1.5], $p < 0.0001$), however, these had normalised by Day 14. While potassium and urea levels were unchanged throughout, in contrast to the placebo (and Ty21a) groups, serum creatinine was significantly elevated at diagnosis ($+10.9\mu\text{mol/L}$ [2.1 - 19.7], $p = 0.01$).

Liver function tests remained largely unchanged in TD M01ZH09 recipients, with the exception of a significantly increased ALT levels by Day 14 (+34.8IU/L [15.3-54.3], $p=0.0001$). CRP levels were significantly elevated at diagnosis (+35.0mg/L [22.2-47.7], $p<0.0001$) and remained high at least to Day 14 (+29.0mg/L [15.9-42.0], $p<0.0001$), possibly due to the generally later infection onset. A similar delayed resolution in CRP levels was seen in TD M01ZH09 as for placebo recipients (Figure 10-13J).

10.9.3 TY21A VACCINE RECIPIENTS

Levels of electrolytes remained relatively unchanged throughout the challenge period although urea levels measured at diagnosis were lower than those at baseline (-1.16mmol/L [-2.1- -0.21], $p=0.012$). Liver function tests were similarly unaffected, although there was a significant increase in ALT by Day 14 (+152.7IU/L [111.2-194.2], $p<0.0001$). CRP levels were significantly increased at diagnosis (+25.6mg/L [17.0-36.1], $p<0.0001$) and remained elevated for several days, reaching maximal levels at the TD+48 hour time point.

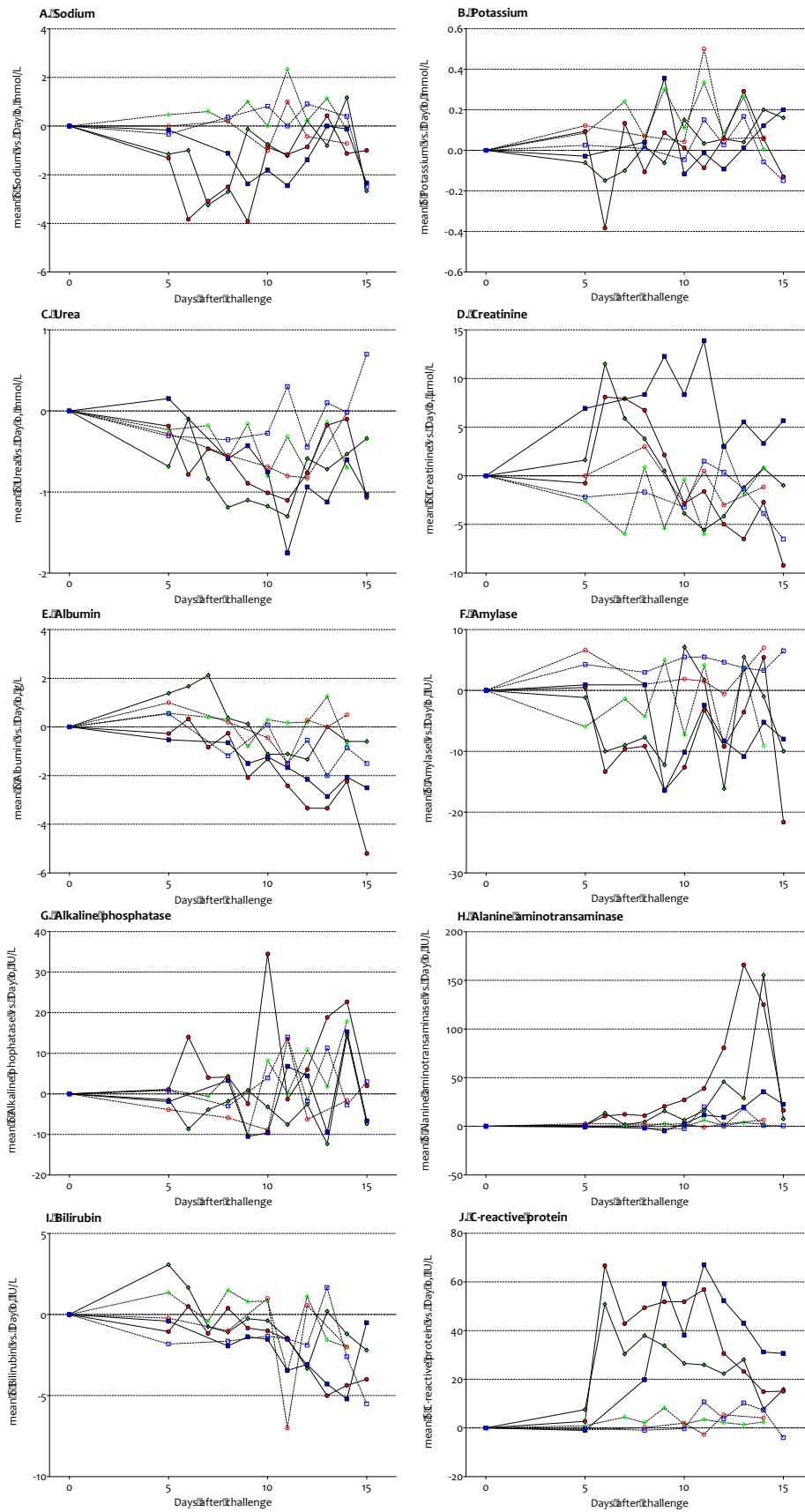


Figure 10-12. Mean changes in biochemistry blood parameters compared to individual baseline measurements according to vaccine allocation and challenge outcome by days after challenge. Legend as for Figure 10-10.

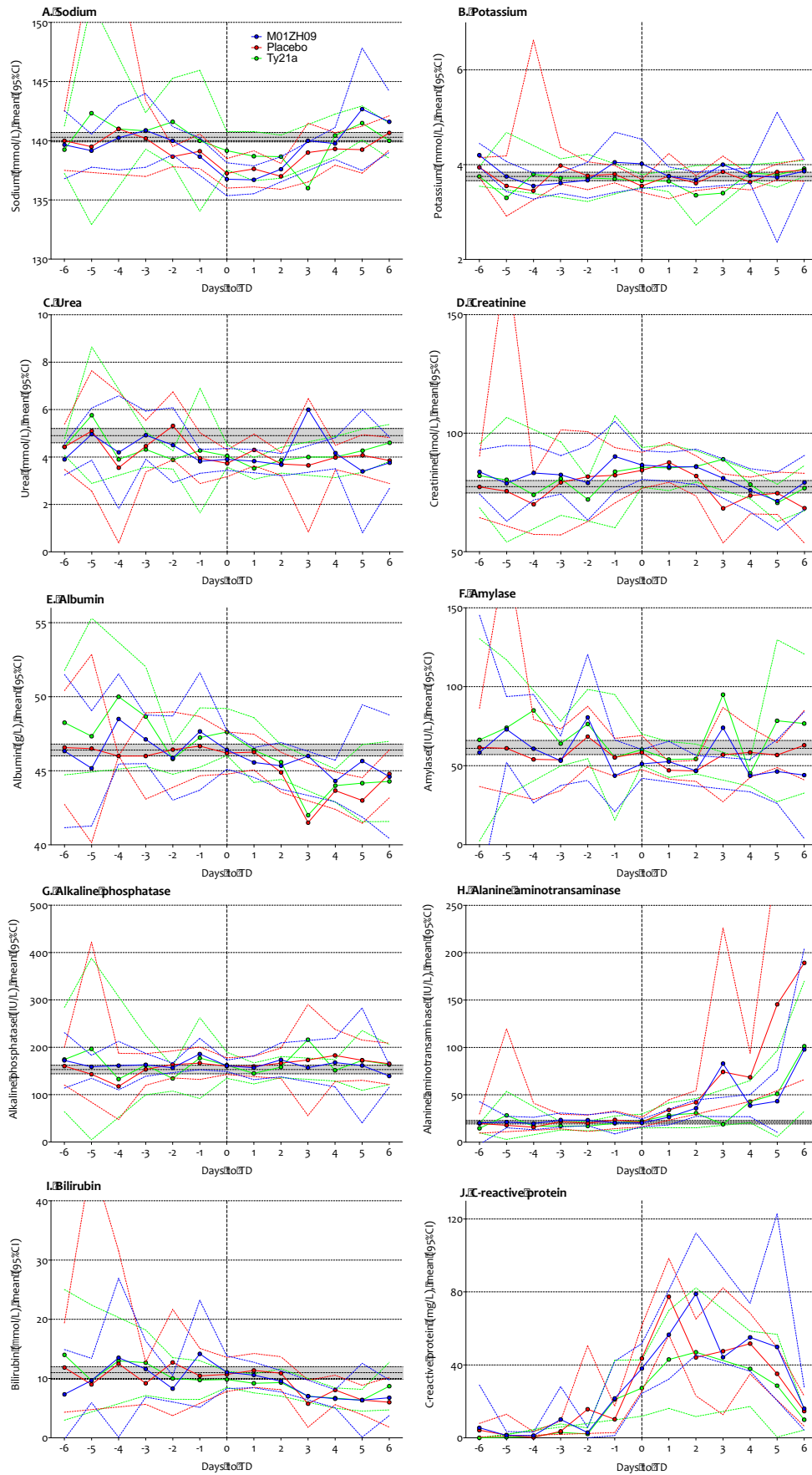


Figure 10-13. Changes in biochemistry blood parameters before, during and after typhoid diagnosis. Dashed line and grey shaded area, mean (95%CI) of ALL baseline measurements.

10.10 CHALLENGE SAFETY

Participant safety is an obvious important component of performing a challenge study in which individuals are left to develop symptomatic illness. Other specific safety features found in performing this challenge study are related below.

10.10.1 SEVERE INFECTION

An *a priori* definition for severe infection was used in order to detect participants with potentially more serious clinical disease (see: 5.4.8). The numbers and vaccine group allocation of participants fulfilling these definitions is given in Table 10-3, below.

Criteria	M01ZH09 (n=32)	Placebo (n=30)	Ty21a (n=30)	ALL (n=92)
Oral temperature $\geq 40^{\circ}\text{C}$	0	1	1	2
Systolic blood pressure $\leq 85\text{mmHg}$	0	0	0	0
Significant lethargy or confusion	0	0	0	0
Gastrointestinal bleeding or suspected/confirmed perforation	0	0	0	0
Any Grade 4 (grade 3) laboratory abnormality ^A	3 (19)	5 (22)	1 (16)	9 (57)
Anaemia	0 (3)	0 (4)	0 (10)	0 (17)
Neutropaenia	0 (1)	0 (3)	0 (0)	0 (4)
Thrombocytopenia	0 (2)	0 (1)	0 (0)	0 (3)
Hyperkalaemia ^B	2 (0)	1 (0)	0 (0)	3 (0)
Hypokalaemia	1 (3) ^C	3 ^D (3)	1 (1) ^E	5 (7)
Liver abnormality (ALT)	0 (0)	1 (1)	0 (1)	1 (2)
CRP	0 (10)	0 (10)	0 (4)	0 (24)
All severe infection	3	6	2	11

Table 10-3. Number and vaccine group allocation of participants fulfilling criteria for severe typhoid infection.

A, Definitions given in the appendix (Supplementary Table 3); B, 2 participants had single elevated readings with normal measurements one the day before and after while 1 participant (M01ZH09) had an elevated recording throughout; C, 1 abnormal pre-challenge; D, 2 abnormal pre-challenge; E, both values abnormal pre-challenge.

10.10.2 SAES

During the course of the study there were no SAEs reported that were attributed to either of the study vaccines given or as a result of undergoing challenge. A list of all SAEs reported to-date is given in Table 10-4.

Date reported	Criteria	Event	Vaccine related	Challenge related	Comment
10/02/2012	Medically important event	Mood alteration, depression symptoms	X	X	Possibly related to ciprofloxacin treatment (switched to azithromycin).
27/07/2012	In-patient hospitalisation or prolongation	Febrile illness	X	X	Abdominal pain, fever and diarrhoea. Resolved with antibiotics. All tests negative.
22/10/2012	In-patient hospitalisation or prolongation	Nasal fracture	X	X	Assault.
02/11/2012	In-patient hospitalisation or prolongation	Traumatic head injury	X	X	Rugby.
28/04/2014	Medically important event	Drug-induced liver injury	X	X	Thought to be omeprazole (initiated after challenge period).
27/05/2014	In-patient hospitalisation or prolongation	Fractured femur	X	X	
27/05/2014	Persistent or life-threatening disability	Malignant melanoma	X	X	

Table 10-4. Serious Adverse Events reported to-date (01/08/2014).

10.10.3 OTHER MEDICALLY IMPORTANT EVENTS

No participants required admission during the course of the study or required intravenous fluids or antibiotics. Four participants were switched from 1st (ciprofloxacin) to 2nd- line (azithromycin) antibiotic treatment, 3 for reasons of nausea/vomiting and diarrhoea within days of starting ciprofloxacin treatment and the fourth due to mood alteration symptoms (see SAEs above).

10.11 NON-SOLICITED SYMPTOMS AND OTHER ADVERSE EVENTS

Twenty events not captured by solicited symptom recording were reported after challenge. Eight occurred in the 14-days after challenge, and included: 3 symptomatic complaints, which were felt likely to be due to challenge (sweating [Grade 1], tiredness [2] and light-headedness/dizziness [2]); two sets of abnormal liver function tests (deranged ALT thought to be related to antibiotic treatment but relationship to challenge could not be excluded [3 & 4]) for which treatment was changed to azithromycin (included in Table 10-3, above); one insect bite (no further intervention needed); one newly found hypertension (referred to GP for further on-going management); and a 'chesty' cough (for which the GP prescribed antibiotic which the participant did not take on account of being enrolled in this trial).

Of the 12 events occurring after Day 14, one was thought possibly related to *S. Typhi* infection; a participant reported new onset abdominal symptoms including diarrhoea and stomach cramps [1] 22-days after challenge, and is under on-going investigation by their GP for malabsorption or possible coeliac disease. Two further events were thought to be possibly due antibiotic treatment specifically, including new onset depression at Day 21 ([4] see Table 10-4, above) and genital thrush at Day 32 ([2] treated successfully by GP). The remaining 9 events were unrelated, and included upper respiratory tract infections (n=2), minor trauma (2), constipation and cardiovascular complaints (2, hypertension and arrhythmia/headaches).

10.12 DISCUSSION

These data detail the clinical course of human participants undergoing challenge with virulent *Salmonella* Typhi bacteria. Challenge was well tolerated and a wide range of

responses were seen. Vaccination with oral live-attenuated vaccines one month prior to challenge resulted in marked changes in infection profile compared to those receiving placebo vaccine only.

10.12.1 SAFETY AND TOLERABILITY

Ninety-two participants were challenged during the 8-month period. One participant was treated shortly after challenge due to deliberate, self-induced vomiting. Despite a previous history of psychological problems, the participant was enrolled as they did not meet any of the definite exclusion criteria and the study procedures and risks involved had been explained in great detail. Following withdrawal, the participant was followed-up closely but there were no further complications. Of note, their HADS questionnaire did not indicate undue anxiety or depression symptoms at either of the occasions it was performed; recent stress or relationship problems have resulted in similar withdrawals from a malaria challenge study in Oxford and self-induced vomiting led to a participant being withdrawn from a norovirus challenge study.³⁸⁸

The remaining ninety-one participants tolerated challenge and the subsequent symptoms associated with typhoid infection well, as found in the preliminary dose-escalation study.⁴³⁶ There were no SAEs reported either to related vaccination or challenge, although one SAE and several adverse events did appear to be related to ciprofloxacin use. While the effects of ciprofloxacin on reducing seizure threshold are well known, caution to exclude even healthy potential volunteers with a history of depressive symptoms seems merited.⁵¹⁰ Of note, the adverse events that occurred took place early into treatment suggesting an idiosyncratic rather than dose-response effect.

10.12.2 ATTACK RATE

The challenge dose chosen for this study was selected based on findings from the preliminary dose-escalation study, as that which resulted in ‘typhoid diagnosis’ in 60-75% of participants. In twenty participants receiving 10^4 CFU, 13 (65%) developed infection using the same illness definitions.⁴³⁶ The actual median [IQR] dose received in that study was 1.98×10^4 CFU [1.88-2.16]; the challenge dose given in the current study was 1.83×10^4 [1.73-1.93] resulting in a 66% attack rate in placebo recipients. The model was therefore successfully reproduced and the desired exposure/attack rate achieved (see also section 13.1.4).

10.12.3 TYPHOID ILLNESS PROFILE

A wide range of symptoms were reported ranging from those who were highly symptomatic but did not meet criteria for diagnosis, to those who were completely asymptomatic but markedly bacteraemic and therefore did meet the diagnostic criteria. As expected, most participants developing typhoid infection reported many more symptoms than those remaining unaffected, and illness onset in placebo recipients occurred in keeping with the findings of the previous study (~days 5-6).⁴³⁶ This corresponds to an incubation period thought to be consistent with natural infection, albeit marginally on the short side (7-14 days; range 3-60 days).¹¹²

The symptom profile of challenged participants developing typhoid was broadly similar to that described for previous case series reported in returning-travellers and endemic settings (see: 2.4.1). Participants reported the anticipated (solicited) symptoms of typhoid more frequently with the exception of fever ($\geq 38^\circ\text{C}$) and diarrhoea, which were less commonly reported than in previous series combined or those in travellers only, respectively (Figure 10-14). Clearly much subjectivity underlies these data, however

afebrile infection may be less frequently detected in clinical settings (where presence of fever is a virtual necessity for typhoid ‘fever’ to be diagnosed).^{192,193} Alternatively had bacteraemic participants been allowed to remain untreated for longer, fever may have developed in a greater proportion of individuals. This would also render the observed incubation period more consistent with the later presentations in clinical settings. Sick returning travellers may have diarrhoea for a multitude of non-typhoidal reasons (other pathogens, different diets etc.); of note, constipation has actually been reported more frequently in the challenge studies performed so far.

		Per cent reporting symptom													
Setting/Study	N	Fever	Diarrhoea	Headache	Vomiting	Nausea	Abdominal pain	Cough	Joint or muscle pain	Chills or rigors	Constipation	Anorexia	Intestinal bleeding	Jaundice	
ENDEMIC	3242	98	32	55	34	59	39	32	40	55	14	71	2	2	
TRAVELLER	382	100	61	54	31	33	40	32	27	32	6	23	8	0	
CHALLENGE															
T1 – 10 ⁴ dose	11	85	46	100	-	92	92	62	92	-	62	100	NR	NR	
T2 – placebo	20	80	50	95	-	80	65	40	85	-	65	85	NR	NR	
T2 – M01ZH09	18	77	0	83	-	56	67	61	67	-	50	78	NR	NR	
T2 – Ty21a	13	69	23	100	-	54	77	69	100	-	46	69	NR	NR	

Figure 10-14. A summary of the frequency of 13 commonly reported symptoms during cases of typhoid infection by setting.

Shading of boxes reflects the frequency of reporting, with darker shades indicating more frequent reporting. Data for endemic and traveller case series are mean values for all studies reported in the literature since 2000, as described in section 2.4.1 and Supplementary Table 1, where the studies are also described. Challenge study data is from the initial dose-escalation study (using 10⁴ dosed participants only; ‘T1’) and for the current vaccine-challenge study (‘T2’), according to vaccine group allocation (‘nausea’ & ‘arthralgia’ data were used preferentially). NR, none recorded.

Although the numbers challenged in Oxford so far are small by comparison, symptoms reported appear to be relatively consistent in both typhoid studies performed to-date.⁴³⁶

Comparison of symptom progression in participants with typhoid between the Oxford and Maryland challenge models reveals an almost identical pattern of illness development.²⁸⁵ The earliest indication was a late-afternoon/early evening increase in recorded temperature soon followed by headache and non-specific abdominal

discomfort. Myalgia, anorexia and fatigue began 1-2 days later, and, as seen in Maryland following chloramphenicol treatment, sweating only started following antibiotic initiation.²⁸⁵

10.12.4 UNINFECTED PARTICIPANTS

Challenged participants not developing infection were, in general, considerably less symptomatic than their typhoid-diagnosed counterparts. A unique finding was the observation of an apparent dose-response relationship between challenge dose and symptoms reported in non-typhoid diagnosed participants. While challenge dose ingested is known to affect the incubation period prior to illness/symptom onset in those developing disease,⁹⁰ there is little evidence to support an association with symptom frequency, duration or severity once sick. A relationship in those apparently 'protected' from infection could suggest two further possibilities. Firstly that these individuals are mounting a physiologically protective response proportional to the degree of exposure, such as by generation of an innate or cytotoxic T-cell response (presuming no prior immunity and therefore absence of a humoral response at this stage), and that these individuals are therefore truly exposed and protected by some mechanism (as opposed to a gastric acid neutralisation of the challenge agent). Secondly that, in the absence of or prior to the onset of bacteraemia, a dose-response relationship may also be demonstrable in typhoid-diagnosed individuals preceding the development of bacteraemia. As observed in this study and those performed previously, and as evidenced by the *S. Typhimurium* model, once bacteraemic illness develops, symptoms are, in general, of similar severity and frequency. In the murine model, histologic changes and mortality rates are unaffected by challenge dose once bacteraemia is apparent.³⁵⁹

Rates of symptom reporting were higher after challenge than in the preceding post-vaccine period and, at least for placebo recipients, appeared to peak at two time points.

The first of these was between days 6-8, which coincided with the onset of symptoms in those developing infection. In addition to the subjective nature of symptom reporting, participants were also aware that illness was most likely to occur during the second week indicating possible reporting bias. Alternatively, this increase in reporting may reflect a signal of abortive infection in participants who were able to mount some degree of protective response, further supporting the hypothesis above. Retrospectively, there is some confirmation of this from the preliminary dose-escalation study, although only at the lower 10^3 CFU dose where peak symptom reporting in typhoid and non-typhoid diagnosed study participants coincided on Days 9 and 10.⁴⁵¹ Notably this increase wasn't seen in non-typhoid diagnosed (blinded) Mo1ZH09 vaccine recipients, additionally suggesting some symptom attenuation by vaccination. The second episode of increased symptom reporting occurred at Day 16, two days following the initiation of antibiotic treatment, and is discussed further below. This was not detected in the preliminary dose-escalation study as diary cards were only used to Day 15; capturing events occurring after initiation of antibiotics supports their extended use in these types of studies.

Approaches to corroborate these signals may include incorporating a placebo challenge population to get a true background symptom profile for comparison, or monitoring symptoms for a longer duration prior to challenge agent ingestion. While the former raises various ethical issues and would require a sizeable sample to account for inter-individual variability, the second approach is currently being included into future Oxford typhoid/paratyphoid challenge model design.

10.12.5 EFFICACY OF TREATMENT

Participants diagnosed with infection returned to normal health relatively quickly following the initiation of antibiotic treatment and additional symptomatic treatment including paracetamol and other analgesia. In Maryland, participants were symptomatic

and febrile for 3-5-days after the initiation of chloramphenicol treatment.²⁸⁵ The speed of action of ciprofloxacin compared to older antibiotics may in part be due to its immunomodulatory effects. Even relatively low concentrations of ciprofloxacin have been shown to inhibit pro-inflammatory cytokine synthesis, decreasing TNF α /IL12 and increasing IL-10, and protect mice from LPS challenge.⁵¹¹ Further non-antibacterial effects beneficial in typhoid may include stimulating G-CSF production thus enhancing haematopoietic recovery.⁵¹¹

Some lag in clinical and physiological recovery was observed in this study, however, and included an apparent secondary peak of temperature and heart rate increase 24-hours after diagnosis, with accompanying bouts of sweating. This was a phenomenon reminiscent of the early treatment studies performed in the early 1950's when closely monitored patients experienced marked fluctuations in temperature and haemodynamic instability following initiation of chloramphenicol treatment.^{512,513}

Reasons for 'recrudescence' in symptoms, clinical findings and delay in normalisation of CRP levels may include release of bacteria/bacterial products from intracellular compartments of the RES including LPS and other pro-inflammatory agents, or simply reflect the time taken for ciprofloxacin to reach a therapeutic and stable concentration in the tissues. While maximum serum concentrations (C_{max}) are reached 1-2 hours after ciprofloxacin ingestion, producing the rapid clinical improvement in symptoms, reaching a steady state tissue concentration takes 15-24 hours ($t_{1/2}$ =3-4hours).⁵¹⁴ This supports the frequent clinical observation that it often takes patients several days to defervesce following initiation of antibiotic treatment, and that this does not necessarily reflect underlying treatment failure or antibiotic resistance.¹⁹²

In future challenge studies, delay in temperature normalisation may be a useful endpoint to compare different antibiotic/therapeutic approaches. If release of bacteria/bacterial

products accounts for this finding, then it is interesting to speculate whether it may also account for the peak in symptoms seen in non-diagnosed participants 48-hours after antibiotic treatment was started. In addition, 3 participants who were undiagnosed by Day 14 and started on antibiotic treatment recorded temperatures of $>38^{\circ}\text{C}$ in the subsequent 36-hour period. Of these, 2 were M01ZH09 recipients and one was in the placebo group.

10.12.6 EFFECT OF VACCINATION

The clinical profile of infection was different in those participants receiving active vaccination in comparison to placebo and appeared to differ between Ty21a and M01ZH09 recipients. Ty21a recipients developed infection contemporaneously with placebo recipients relatively early after challenge, whereas M01ZH09 recipients developed infection several days later. Evidence for this included a delay in symptom and fever onset and also the timing of haematology and biochemical laboratory blood perturbations. Reasons for a delay in incubation period are speculative but could reflect the possible difference in humoral immune response generated, as described in the previous chapter, or suggest underlying differences in how other unmeasured protective immune responses (for example, innate or cell-mediated immunity) are generated.

It is important to note that, once illness had started, there was relatively little difference in the clinical profile despite prior vaccination receipt. This suggests that oral vaccination does not necessarily alter the clinical features of typhoid fever, although it could impact disease risk and timing of onset. A similar finding was made in the early Maryland studies of K and L whole cells vaccines; when disease occurred after vaccination, regardless of the challenge dose used, illness severity was similar.³⁵⁹

In this study, and as a basis for oral typhoid vaccine pre-clinical assessment, anti-LPS responses are used as a benchmark for comparative immunogenicity (Chapter 9).^{141,142} In later studies performed in Maryland, infusions of *S. Typhi* purified endotoxin, while resulting in increases in anti-LPS antibody titres, had no effect on the clinical profile of typhoid fever after challenge.³⁵⁹ Furthermore, volunteers could be rendered relatively immune to the effects of repeated endotoxin infusion (an effect also seen in several intravenous drug users), and still develop symptoms after challenge. Thus, the apparently successful generation of an anti-LPS response after vaccination may, alone, have little effect in either preventing infection or moderating the clinical course of illness following M01ZH09 receipt in this study.

Typhoid-diagnosed Ty21a recipients were possibly less unwell at diagnosis; average temperatures and heart rates were lower for example, although this may simply reflect a difference in diagnostic criteria fulfilment. Ty21a recipients were more likely to be diagnosed based on microbiological grounds (i.e. bacteraemia), and therefore could have been diagnosed earlier in the disease process, in comparison to those febrile participants who had to wait 12-hours until diagnosis was confirmed. This was supported by the observations made in assessing the contribution of diagnostic criteria to symptom reporting (see: 10.5.4), and clinical findings (10.7.5).

10.12.7 LABORATORY PERTURBATIONS

The data presented in this chapter further describe in detail the effects of *Salmonella* Typhi in altering human blood physiology. One of the most pronounced effects was the fall in haemoglobin and haematocrit. This occurred to such an extent as to be the most frequent cause of a Grade 3 laboratory abnormality (with the exception of CRP increase; Table 10-3). Clearly a major contributing factor to the development of anaemia in the context of this study may be the frequency and volumes of the bloods draws performed;

the maximum total volume drawn in participants developing typhoid infection at Day 14, by 28-days after challenge was 1117mL (Table 6-1). This is likely to have had at least a contributory if not causative effect on the haemoglobin depletion seen, however bacterial and host inflammatory factors are likely to also be important. Anaemia is a common finding in typhoid presentation in case series in both returning-travellers and in endemic settings,^{191,515,516} and among children in particular,¹⁷³ but may also reflect the indolent disease onset and later presentation compared to other febrile conditions.

The effect of anaemia (albeit iatrogenic) on susceptibility to or severity of bacterial infection is clearly of major importance in typhoid-endemic settings, as manipulation of iron handling is a major virulence determinant of *Salmonella* infection.^{173,517,518} From further work performed studying the effect of iron levels on susceptibility to typhoid and the host-bacterial interplay in controlling the free iron available in blood, hepcidin and related iron mediators have been assessed in samples collected from both the preliminary dose-escalation and the placebo recipient cohort from this study. Analyses demonstrate that reductions in serum iron and transferrin saturation are significantly greater in those developing typhoid, even taking relative blood loss into account. This suggests a direct effect by *Salmonella* Typhi in blocking iron uptake, thereby contributing to the fall in serum iron and haemoglobin depletion. Significant reductions were also seen in platelets and white cell subsets in those participants diagnosed with infection, which, together with the fact that bacteria are at least 10x-fold more abundant in the bone marrow, also supports additional indirect effects of *Salmonella* infection on erythro- and myelopoiesis.^{204,519}

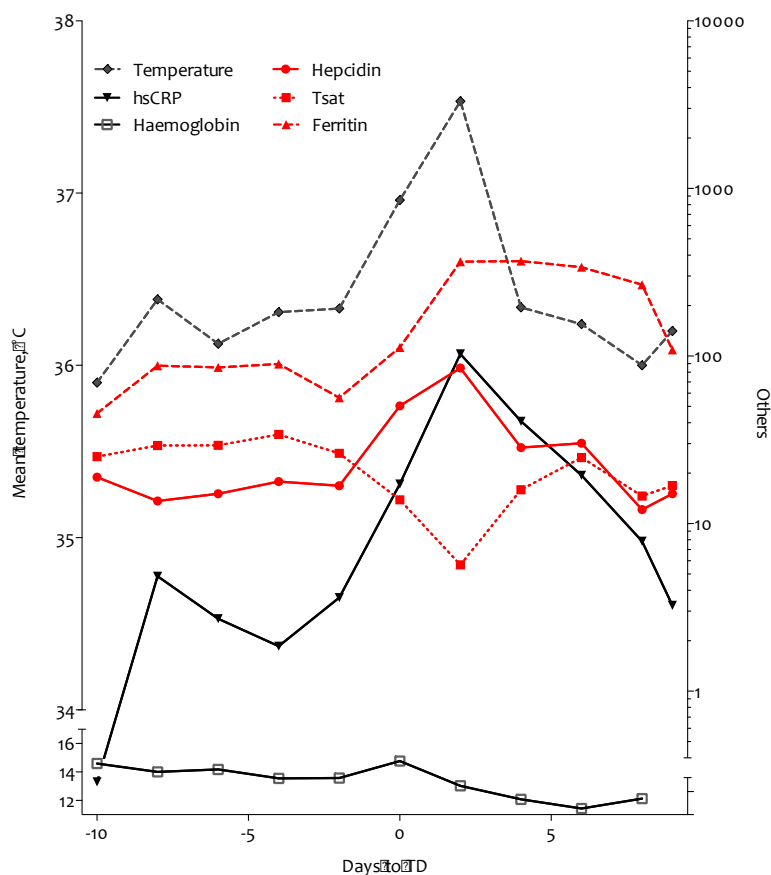


Figure 10-15. Summary of the alterations in iron parameters occurring during typhoid infection in challenge participants.

This figure demonstrates the factors contributing to the development of anaemia including acute infection, inflammation and blood loss through phlebotomy. Data is collated from the T1 dose-escalation study high dose (10^4 CFU) participants and is binned into 48-hour blocks. hsCRP, high sensitivity C-reactive protein; Tsat, transferrin saturation; TD, typhoid diagnosis. Darton and Armitage, manuscript in submission.

Reductions in platelet and white cell counts were most marked at or immediately after typhoid diagnosis. The mechanisms of thrombocytopenia and leucopenia are not clear in typhoid infection, although there have been multiple causes postulated in addition to bacterial suppression of bone marrow, which is not always involved.⁵¹⁹ These include platelet activation and sequestration by the spleen, peripheral autoimmune mechanisms or increased margination as a result of neutrophil activation.⁵¹⁹ Whether the more pronounced neutrophil response at diagnosis in Ty21 vaccine recipients is a marker of more effective underlying innate responses to vaccination requires further investigation and *in vitro* confirmation.

Further haematological findings of note include confirmation of a previous observation that the eosinophil fraction falls rapidly and several days prior to diagnosis being made. This may provide some supportive diagnostic or prognostic value to clinicians, at least in the context of future challenge studies,^{436,520} and will be interesting to assess in the context of *S. Paratyphi* challenge studies. Whether this finding is related to the frequent reporting of ‘cough’ amongst M01ZH09 vaccine recipients subsequently developing infection is interesting to speculate. Eosinophils are predominantly found at mucosal surfaces colonised by microorganisms, including those in the intestine.⁵²¹ Increased migration to this site as a result of recent ingestion of a live-attenuated vaccine strain may have served to enhance subsequent chemotaxis to this site in addition to respiratory burst and degranulation activity as part of an innate antibacterial response to local infection.^{521,522} Release of preformed mediators as part of this process would lead to systemic effects including cough, wheeze and itch.

Biochemical changes were less remarkable overall, although the relatively modest nature of the CRP rises in comparison to those found in clinical cases of bacteraemia due to other causes is interesting. Typhoid infection is known to result in relatively modest increases in pro-inflammatory cytokine production including IL-6, which is a principal driver of CRP synthesis in the liver.⁵²³ While the mechanisms of pro-inflammatory cytokine inhibition are unclear,⁵²⁴ data from endemic settings suggests higher IL-6 (and hence CRP) levels are linked to poorer disease outcomes.⁵²⁵ These relatively modest increases in cytokines have also been found in further work performed using samples from the preliminary dose-escalation study. While these effects may be compounded by the relatively short illness period, terminated (or reversed) by prompt institution of ciprofloxacin antibiotics, they mirror the relatively low peak temperatures measured in participants.

Both the clinical and laboratory data presented in this chapter support the assertion that while typhoid presentation appears to be relatively indolent, even in this immunologically-naïve population, multiple host-pathogen interactions are occurring in all those exposed.

10.12.8 SUMMARY

Typhoid challenge was successfully and safely performed 28-days after vaccination resulting in a range of clinical and sub-clinical illness in some of those exposed. The modifying effect of previous vaccination with two immunogenic, live-attenuated oral vaccines resulted in differences in illness onset and characteristics, while those not developing infection remained relatively asymptomatic. These findings support the role of challenge in accurately defining the nature of protection against clinical illness afforded by vaccination, and significantly contribute to our understanding of the clinical entity of typhoid fever.

Chapter 11. *S. TYPHI* BACTERIAL DYNAMICS

AFTER TYPHOID CHALLENGE AND DURING INFECTION AND ITS MODERATION BY LIVE- ATTENUATED ORAL VACCINATION

11.1 INTRODUCTION

Salmonella Typhi is special; compared to other Gram-negative enteric infections or causes of sepsis this bacterium has several peculiar features which have greatly complicated its control and which contribute to many of the complexities in accurately diagnosing infection or developing an effective vaccine.^{79,83,112,113,210,526} Despite its first successful culture from reticuloendothelial tissue, blood, stool and urine at the close of the 19th century,^{37,67,68} relatively few accurate data have been obtained since then to determine the location and activity of bacteria following exposure. This information is key to understanding the natural history of *S. Typhi* infection.

Controlled human infection studies provide an ideal setting to monitor host-pathogen interactions. This chapter details the stages at which bacteria (or bacterial DNA) are evident following a known exposure episode. In addition to informing on the basic biology of typhoid infection, these data have several important translational applications. These include identifying locations and time frames at which bacteria are present in order to develop novel diagnostic approaches or to assess the effects of antibiotics in reducing or altering the bacterial burden. Similarly the effect of vaccination in modifying bacterial burden through effecting host immune responses may be a useful tool by which to compare alternative vaccines or vaccine formulations. These applications further impact the means of assessing vaccine efficacy in field settings in addition to directly informing transmission-modelling estimates by providing key data on bacteraemia and stool shedding.

11.2 METHODS

11.2.1 QUALITATIVE BLOOD CULTURE

Routine blood cultures were performed according to the schedule and methods described in Table 6-1 and section 6.3.3, respectively. Note that 10mL blood was collected for culture at each time point **except** at typhoid diagnosis when 5mL was collected instead. This was to allow direct comparison of blood culture and PCR sensitivity at this time point, but also to avoid taking excessive blood at a time when participants were feeling least well. Blood for culture was collected only after Day 5 to exclude possible accidental detection of earlier primary bacteraemia (affecting diagnostic decision-making) and in order to minimise the total volume of blood collected from each participant.

11.2.2 QUANTITATIVE BLOOD CULTURE

Quantitative blood culture was performed using 10mL blood collected from participants at typhoid diagnosis according to the methods described (section 6.3.3). In addition, 10mL blood was collected from a randomly selected subset of volunteers (n=10) from challenge onwards to investigate longitudinal trends in bacterial load.

11.2.3 QUALITATIVE AND QUANTITATIVE STOOL CULTURES

Participant stool samples were collected at each visit after challenge, and processed for qualitative and quantitative culture, as previously described (sections 6.2.3 & 6.3.4).

11.2.4 DETECTION BY CULTURE-PCR

Blood was collected for culture-PCR according to the schedule described in Table 6-1. The assay was performed as described in Chapter 7.

11.2.5 STATISTICAL CONSIDERATIONS

Prior to analysis the frequency distributions of datasets were assessed. Numbers of qualitative blood and stool cultures and duration of bacteraemia were normally distributed while blood and stool quantification data was right skewed. Quantitative blood culture data was \log_{10} -transformed prior to analysis (with replacement of zero values with 0.05, LLOD/2). Analysis of quantitative stool culture data was mostly descriptive in nature (due to the few data points available and wide distributions) and is therefore presented as median [IQR].

11.3 QUALITATIVE BLOOD CULTURE

11.3.1 GENERAL DESCRIPTION

In total, 1050 blood cultures (BCs) were collected during the course of the study, i.e. approximately 11.4 BCs were performed for each challenged participant from Day 5 onwards (Table 11-1). Overall BC contamination by non-*S. Typhi* organisms (Gram-positive organisms including coagulase-negative *Staphylococci*, see table below) was low: 1.6% compared to 2.5% found in the previous dose-escalation challenge study.⁴³⁶

n (%)	MoiZH09	Placebo	Ty21a	ALL
S. Typhi				
Positive isolates	36 (9.7)	54 (15.6)	29 (8.7)	119 (11.3)
Bacteraemic participants	16	20	11	47
Index	2.25	2.70	2.63	-
Other organisms (contaminants)				
Coagulase negative <i>Staphylococcus</i> sp.	3	6	6	15 (1.4)
<i>Micrococcus</i> sp.	0	1	0	1
Diphtheroids	0	0	1	1
Negative (no growth 5 days)	331	285	295	911 (86.8)
Missing sample	0	0	1	1
Total sent	372	346	332	1050

Table 11-1. Summary of blood culture results obtained according to vaccine allocation.

Blood culture index is a crude measure of the mean number of positive blood culture per typhoid-diagnosed participant.

11.3.2 PLACEBO VACCINE RECIPIENTS

Salmonella Typhi was cultured from blood collected from placebo recipients from Day 5, with the greatest proportion of bacteraemic participants detected on Day 6 (Figure 11-1), and the last positive BC collected 11-days after challenge. The median [IQR] day of first positive BC collection was Day 6 [5-7.25].

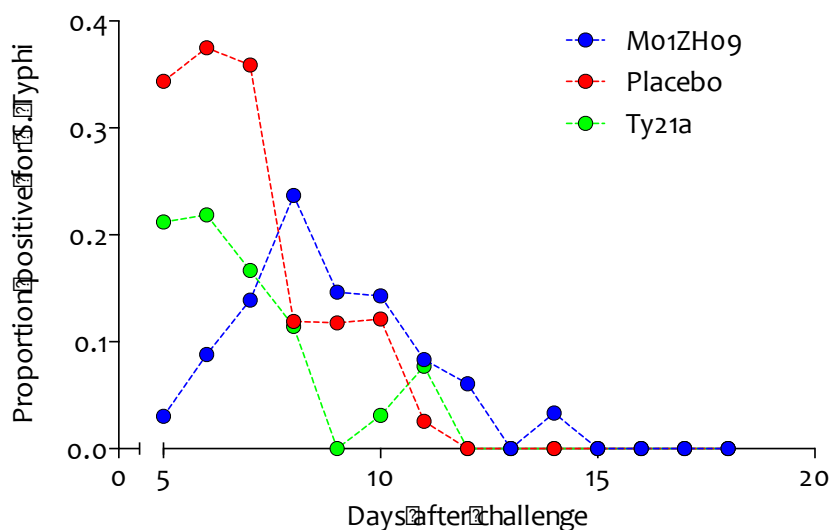


Figure 11-1. Proportion of positive blood cultures of those collected each day after challenge according to vaccine allocation.

Note that blood cultures were collected from Day 5 onwards. Final samples were collected: Placebo, Day 15; Ty21a, Day 17 and Mo1ZH09, Day 18.

Of those challenged, 20/30 (66.6%) placebo recipients developed *S. Typhi* bacteraemia (Table 11-1), and positive BCs were obtained from all diagnosed participants in this group. The mean (95%CI) number of positive BCs per bacteraemic participant was 2.70 (2.33-3.08); bacteraemia persisted for a mean (95%CI) of 1.05 (0.73-1.37) days.

As a surrogate of time-to-positivity, a commonly used indicator for bacterial load, bacterial fitness and a clinical outcome predictor in clinical settings,^{527,528} the median time [range] from first positive blood culture collection to initiation of antibiotic treatment was 1 [0-3] day (Figure 11-2).

11.3.3 Mo1ZH09 VACCINE RECIPIENTS

Positive BCs were detected from Day 5 onwards in Mo1ZH09 recipients (n=1 on Day 5), although in general, participants were bacteraemic later in the challenge period compared to either placebo or Ty21a recipients (Figure 11-3).

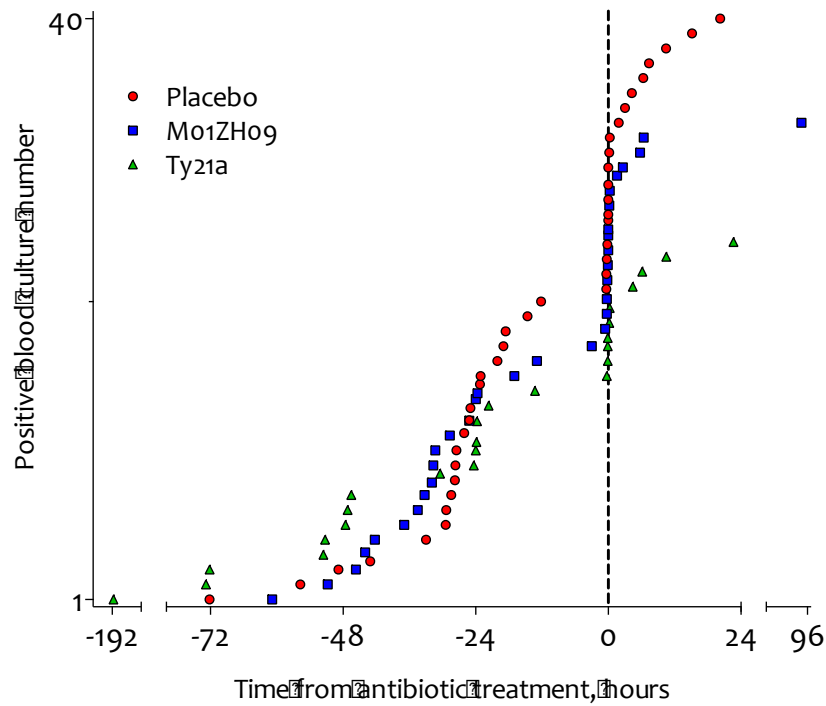


Figure 11-2. Time from positive blood culture to antibiotic initiation according to vaccine allocation.

The maximal proportion of bacteraemic participants was found on Day 8 and positive BCs were received throughout the 14-day period (Figure 11-1). The median [IQR] time from challenge to first positive BC was 8.5 [7-10] days. Of note, not all TD M01ZH09 participants were bacteraemic: BCs from 2/16 (12.5%) remained sterile throughout.

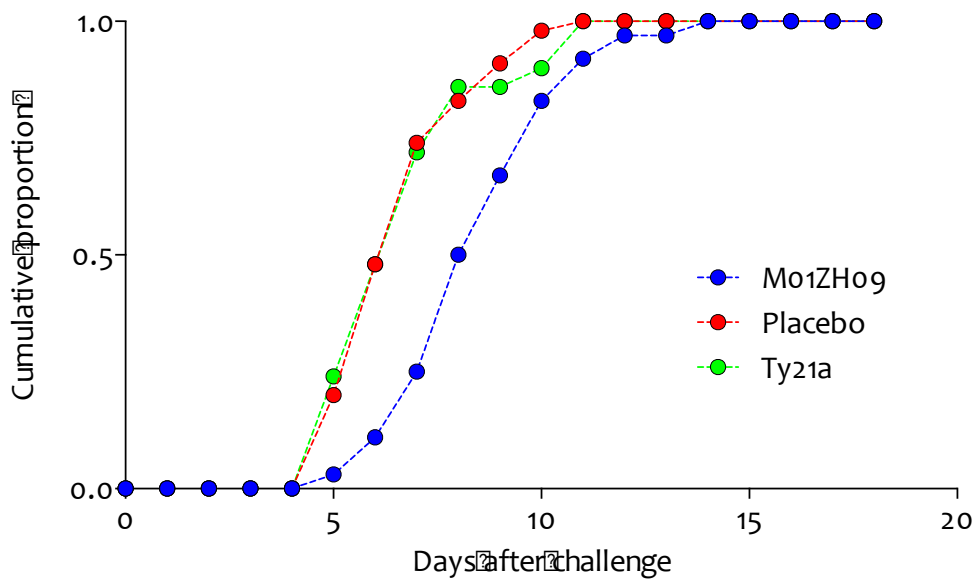


Figure 11-3. Cumulative proportion of positive blood cultures collected after challenge according to vaccine allocation.

There were no significant differences in the number of positive BCs collected from bacteraemic M01ZH09 recipients (mean [95%CI], 2.00 [1.32-2.68]) or in the mean duration of bacteraemia compared to placebo recipients (mean difference [95%CI], 0.20 [-0.72-1.12] days). The median time from first positive culture to antibiotic initiation was 1 day (range 0-3 days; Figure 11-2).

11.3.4 TY21A VACCINE RECIPIENTS

The majority of bacteraemia to occur in Ty21a recipients was also found during the early stage of the challenge period. Median [IQR] time from challenge to bacteraemia was 5 [5-6.5] days, while the maximal proportion of bacteraemic participants occurred on Day 6 (Figure 11-1) and the last positive BC was collected on Day 11 after challenge. Not all TD Ty21a recipients were bacteraemic, with BC from 2/11 (18.2%) participants remaining sterile throughout.

Bacteraemic Ty21a recipients had an average of 2.23 (1.48-2.98) positive BCs each and were culture positive for a similar duration compared to placebo recipients (mean difference [95%CI], 0.77 [-0.26-1.80] days). Median time from a positive blood culture result to antibiotic initiation was 2 days (range 0-7 days). One participant who was bacteraemic but asymptomatic on Day 6 (thus not meeting TD criteria) remained well and BC culture negative until Day 12. A repeat blood culture was positive at this stage and hence antibiotics were initiated accounting for the apparent 192-hour delay (Figure 11-2).

11.3.5 EFFECT OF ANTIBIOTICS

Antibiotic treatment (ciprofloxacin 500mg bd) was effective in rapidly sterilising participant blood cultures, with all but one participant becoming culture negative within 24-hours. Of note, more positive blood cultures were obtained after antibiotic initiation in placebo recipients than either of the active vaccine groups. The latest positive blood

culture was collected 89-hours after antibiotic initiation in a M01ZH09 vaccine recipient (shown in Figure 11-4). This individual had been diagnosed on Day 8 and had had 4 positive blood cultures previously. There was no suggestion of antibiotic non-compliance and other clinical indices (symptoms, temperature, CRP etc.) had improved.

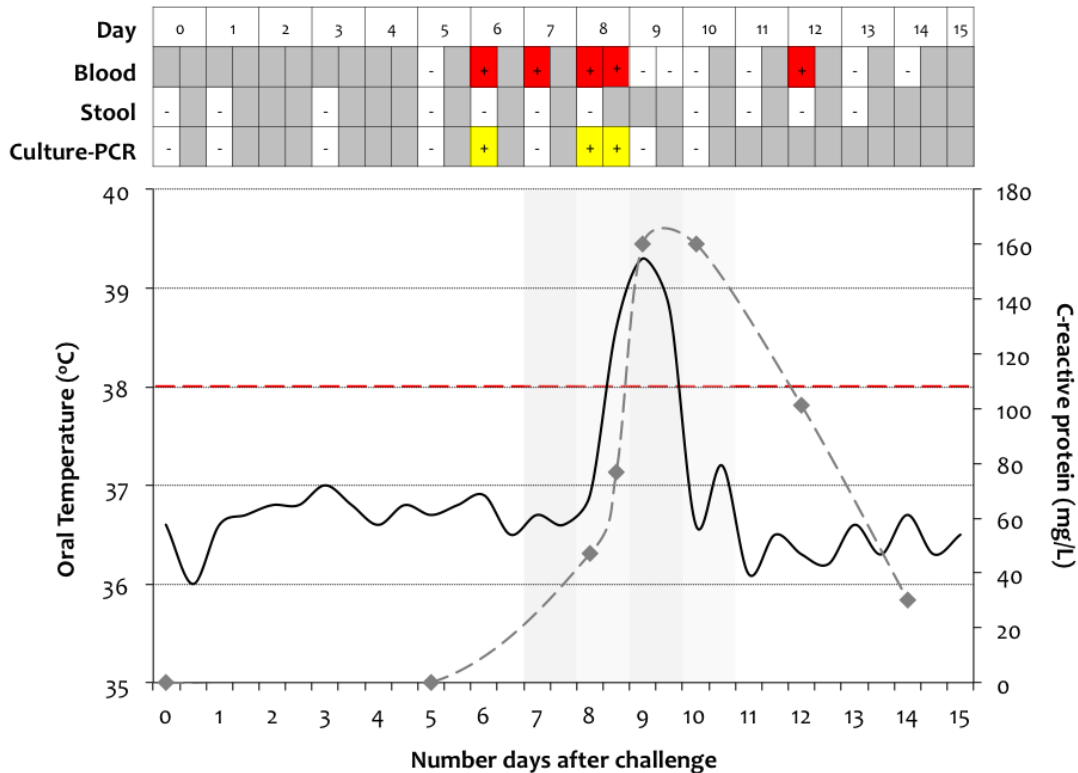


Figure 11-4. Example of a participant with prolonged period of bacteraemia detected following initiation of antibiotic treatment.

Red square, positive blood culture; grey squares, no sample collected; yellow square, positive culture-PCR result; black line, oral temperature; dashed grey line, C-reactive protein level; shaded area, antibiotic treatment initiated.

11.4 QUANTITATIVE BLOOD CULTURE

11.4.1 MAGNITUDE OF *S. TYPHI* BACTERAEMIA AT DIAGNOSIS

Quantitative blood culture (QBC) data were available for 41/51 TD participants. Those for whom data were not available comprised 4 placebo, 4 M01ZH09 and 2 Ty21a recipients; there were no distinguishing features for these participants, although 5 were diagnosed after 2200hrs. possibly indicating logistic difficulties in setting-up the culture overnight.

Bacterial counts were non-parametrically distributed such that the overall median [IQR] number of colonies was 0.4CFU/mL [0-1.37](n=41). Comparisons of \log_{10} -transformed bacterial loads demonstrated significantly higher counts in TD placebo than in TD Mo1ZH09 recipients (Figure 11-5).

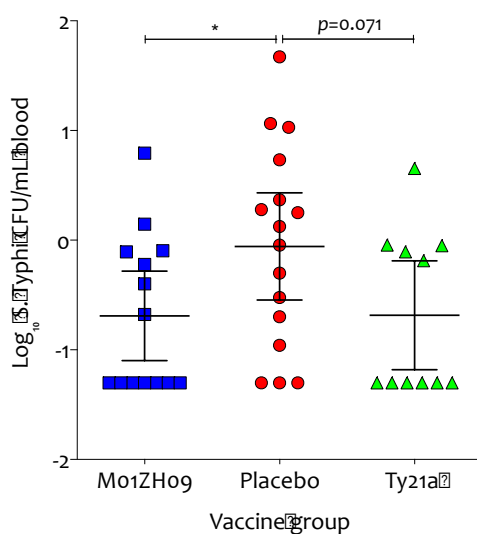


Figure 11-5. Blood quantification of *Salmonella Typhi* bacteria present at point of typhoid diagnosis according to vaccine group allocation.

QBC counts were \log_{10} -transformed with zero counts replaced with 0.05 (LLOD/2). Unpaired T test. * $p < 0.05$. Median[IQR] bacterial loads in CFU were: Placebo: 1.12 [0.13-4.63]; Mo1ZH09: 0.11 [0-0.89]; Ty21a 0.40 [0-1.37].

This was reflected in the number of ‘no-growth’ results found; 7/14(50%) vs. 3/16(19%) of 10mL blood cultures were negative in TD Mo1ZH09 or placebo recipients, respectively.

Bacterial counts in TD Ty21a recipients also tended to be lower than those of the placebo recipients (Figure 11-5), and 6/11(55%) cultures were sterile at diagnosis.

11.4.2 FACTORS ASSOCIATED WITH BACTERIAL LOAD AT DIAGNOSIS

There was a significant positive correlation between challenge dose ingested and measured bacterial load at typhoid diagnosis (Figure 11-6a). When explored as a dose-response relationship, only data for placebo recipients converged indicating an EC_{50} of 19,879CFU. There was also a significant inverse relationship between the time elapsed between challenge and typhoid diagnosis and QBC result (Figure 11-6b).

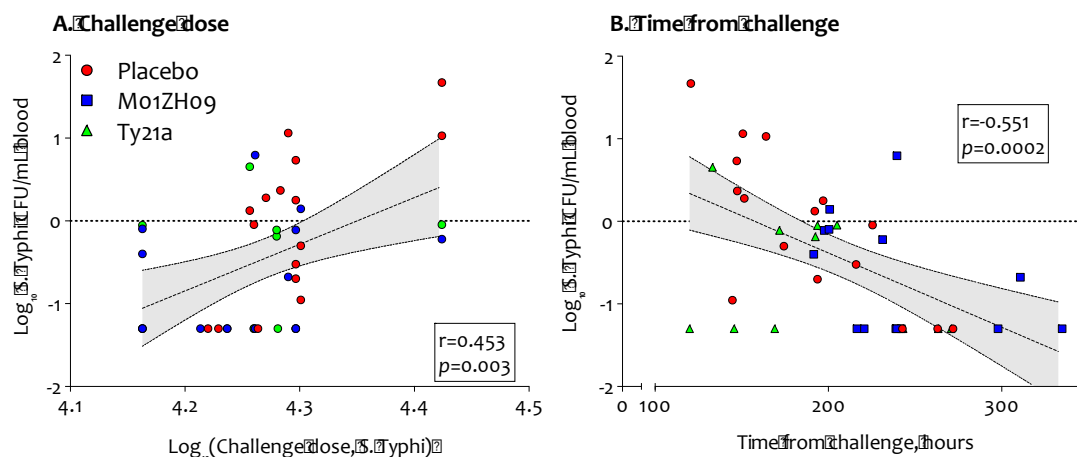


Figure 11-6. Relationship between *S. Typhi* bacterial load measured at TD and A, challenge dose or B, time from challenge to diagnosis according to vaccine allocation. Dashed lines represent best-fit (shaded area [95%CI]).

Two notable interactions were found between QBC and laboratory parameters. Firstly there was a positive correlation between bacterial load and maximum CRP measured (Figure 10-7a). Secondly there was a strong negative correlation between bacterial load and nadir platelet count (Figure 11-7b). No significant associations were found with nadir WCC, neutrophil or lymphocyte subsets or with maximum ALT measurements. No relationships were found between QBC at diagnosis and the number or severity of symptoms reported or temperature measurements (either maximum or recorded at diagnosis). This was in contrast to C-reactive protein level which correlated significantly with temperature measurement at diagnosis (Figure 11-7C).

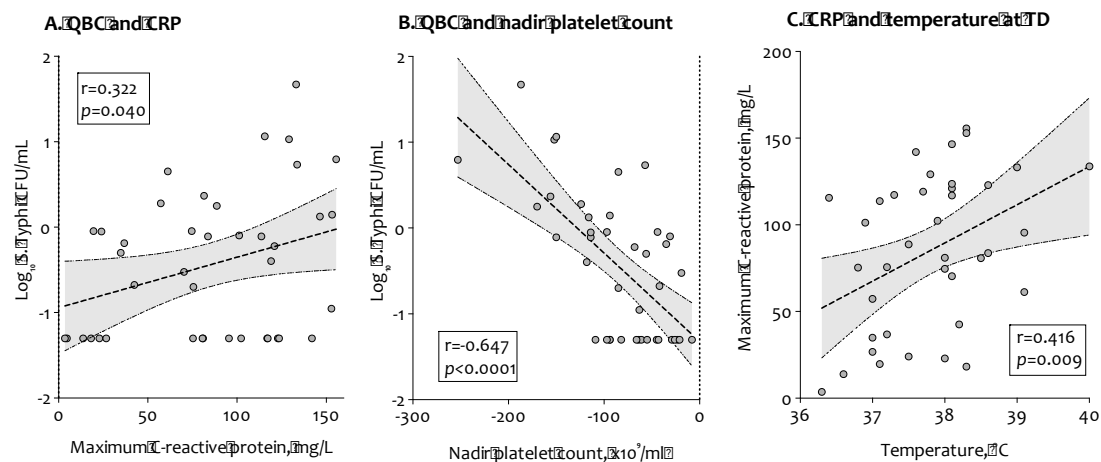


Figure 11-7. Interactions between *S. Typhi* bacterial load (QBC), laboratory and clinical parameters. Dashed lines represent best-fit (95%CI). Pearson's correlation.

11.4.3 TIME COURSE

Serial blood quantification was performed in a subset of study participants ($n=10$). Of these, 7/10 participants went on to develop infection in 5 of whom bacterial counts were measurable in the blood on at least one occasion after challenge (Figure 11-8). Bacteria were detectable in several participants during the 48-hours prior to diagnosis and, in general, remained at a relatively constant numbers until antibiotic treatment, which resulted in complete and rapid clearance. As previously, not all participants had a measurable level of bacteria at diagnosis; 3/7 10mL blood samples collected failed to grow any bacteria.

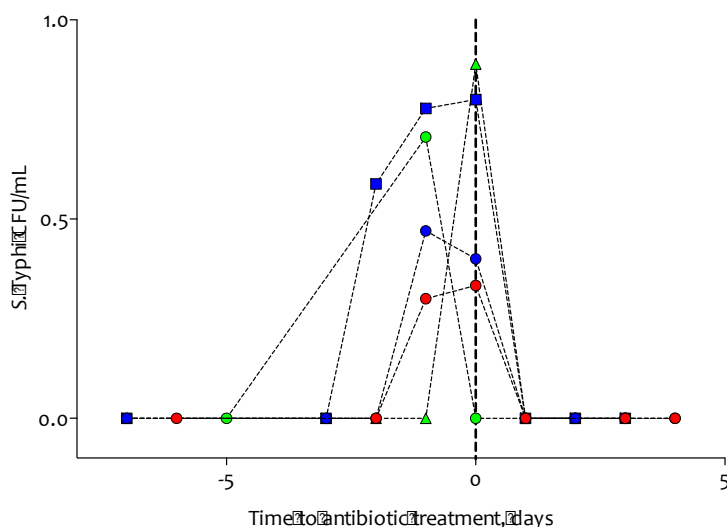


Figure 11-8. Serial bacterial load data for a selected subset of participants developing infection after challenge.

Data are grouped according to vaccine allocation (colours as previous) and participant identifier (symbol shape).

11.5 QUALITATIVE STOOL CULTURES

11.5.1 GENERAL DESCRIPTION

Salmonella Typhi was cultured from 149/1193 (12.5%) stool samples collected and from 57 participants during the course of the study to-date (Table 11-2). Overall, a mean (95%CI) of 1.60 (1.21-1.99) positive stool cultures (SCs) per individual challenged were found.

	Mo1ZH09	Placebo	Ty21a	All Groups
S. Typhi				
Number positive(%)	47 (11.5)	53 (14.1)	49 (11.9)	149 (12.5)
Number shedding	20	19	18	57
Index	2.35	2.79	2.72	-
Negative (no growth 5 days)	362	321	361	1044
Missing sample	1	1	1	3
Total sent	410	375	411	1196

Table 11-2. Summary of qualitative stool culture results collected according to vaccine group allocation.

Index is the mean number of positive S. Typhi stool culture results per participant with ≥ 1 positive result.

1035/1193 stool samples were collected during the challenge follow-up period (Table 11-3), i.e. between Day 0 and Day 18 (the last TD+96 hour visits for individuals diagnosed on Day 14). The mean number of samples submitted per individual in this time frame was 11.3, and although this number ranged widely (2-16), the median [IQR] number was 12 [10-13].

Group	Challenge outcome	Time point after challenge, n/N (%)		
		Day 0 to Day 3	Day 4 onwards	Total
Mo1ZH09	nTD	5/40 (13)	10/126 (8)	15/166 (9)
	TD	17/47 (36)	15/141 (11)	32/188 (17)
	ALL	22/87 (25)	25/267 (9)	47/354 (13)
Placebo	nTD	3/26 (12)	16/92 (17)	19/118 (16)
	TD	13/50 (26)	21/162 (13)	34/212 (16)
	ALL	16/76 (21)	37/254 (15)	53/330 (16)
Ty21a	nTD	6/44 (14)	10/151 (7)	16/195 (8)
	TD	13/38 (34)	20/118 (17)	33/156 (21)
	ALL	19/82 (23)	30/269 (11)	49/351 (14)
ALL	nTD	14/110 (13)	36/369 (10)	50/479 (10)
	TD	43/135 (32)	56/421 (13)	99/556 (18)
	ALL	57/245 (23)	92/790 (12)	149/1035 (14)

Table 11-3. Summary of stool culture results received from Day 0 (including pre-challenge) until completion of challenge, according to vaccine group allocation, challenge outcome and phase of shedding.

'Onwards'=from Day 4 (inclusive) to Day 18, day of last sample submission from individual still having challenge follow up visits (i.e. last TD+95hour visit).

11.5.2 EARLY SHEDDING

The earliest positive SCs were collected 12-hours after challenge: *S. Typhi* was detected in 2 participants' samples collected 14-hours after challenge. Of note, one of these participants also reported diarrhoea at the subsequent (Day 1) visit. As detailed previously (see: 9.4.1), one additional participant had non-typeable *Salmonella* cultured in stool collected at pre-challenge and D1.

Early shedding of *S. Typhi*, defined as a positive SC collected within 96-hours of challenge, was found in 55 stool samples collected from 43 participants (Figure 11-9). Note that although participants weren't seen on Day 2, stool samples could still be collected and were either delivered or kept refrigerated until their next visit. Early stool shedding was significantly more likely in those participants subsequently developing typhoid infection (odds ratio [95%CI]: 3.08 [1.30-7.29], $p=0.011$, chi-squared test).

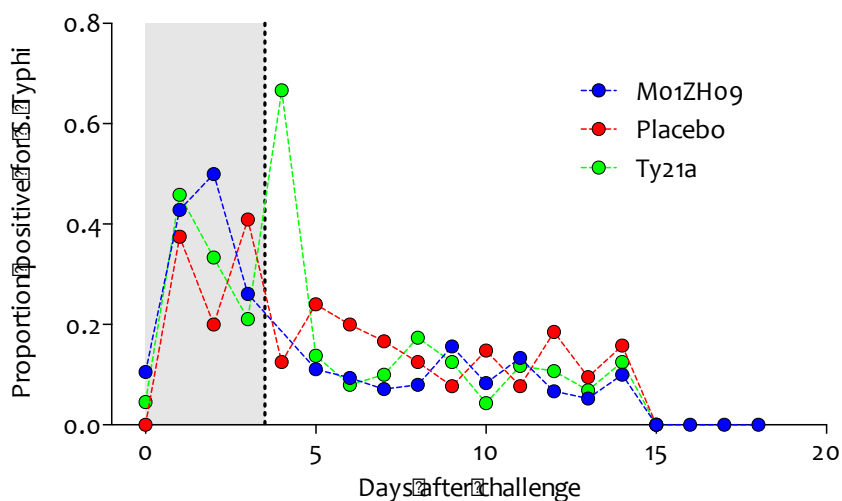


Figure 11-9. Proportion of positive stool culture of those collected each day after challenge according to vaccine allocation.

Shaded grey area indicates that considered to be early shedding related to challenge agent ingestion.

More positive SCs were obtained from those participants who had received active vaccination ~28-days previously (Figure 11-9 & Figure 11-10), although early stool shedding

was more frequent in Mo1ZH09 recipients (53%) than those belonging to either of the other two groups (45% each, $p=NS$, chi-squared).

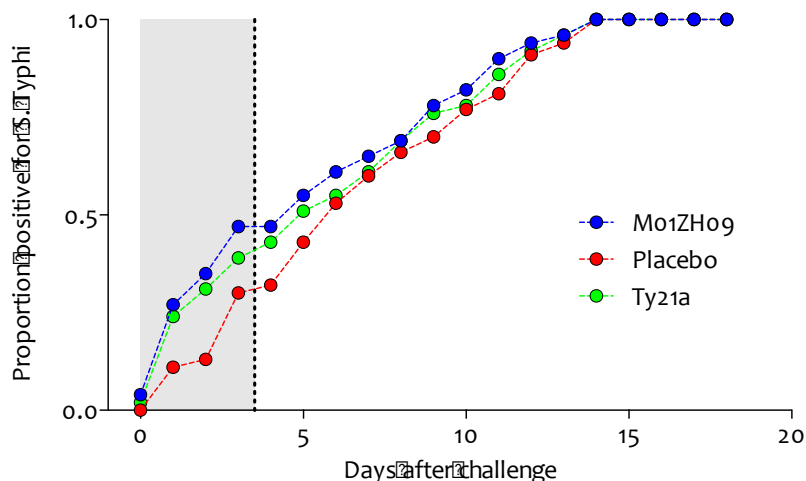


Figure 11-10. Cumulative proportion of positive stool cultures from those collected from challenge onwards according to vaccine group allocation.

11.5.3 LATER SHEDDING

After Day 3, *S. Typhi* was cultured in 92 samples from 41 study participants (12% culture-positivity rate; Table 11-3). Of interest, the participant with non-*S. Typhi* in stool cultures at baseline, became stool culture positive with *S. Typhi* on days 9 and 11 after challenge and also developed infection. While SC positivity rates in the early challenge period were similar between vaccine recipient groups and between subsequent challenge outcomes (TD or nTD), this pattern changed from Day 4 onwards. Among active vaccine recipients, the *S. Typhi* isolation rate was significantly lower in those not developing typhoid infection (OR [95%CI], 2.02 [1.14-3.61]), $p=0.016$, chi-squared), whereas among placebo recipients, rate of isolation were (non-significantly) higher in nTD participants (Table 11-3).

11.5.4 STOOL CULTURES AT TYPHOID DIAGNOSIS

S. Typhi was isolated from 27/51 (53%) TD compared to 14/41 (7%) non-diagnosed participants. SC positivity was frequently the earliest indication of infection developing (example shown in Figure 11-11). The median [IQR] interval between the first positive SC

and BC being collected was 1 [0-1.75] day ($n=24$), and between first positive SC and diagnosis being made was 2 [1-3] days ($n=26$).

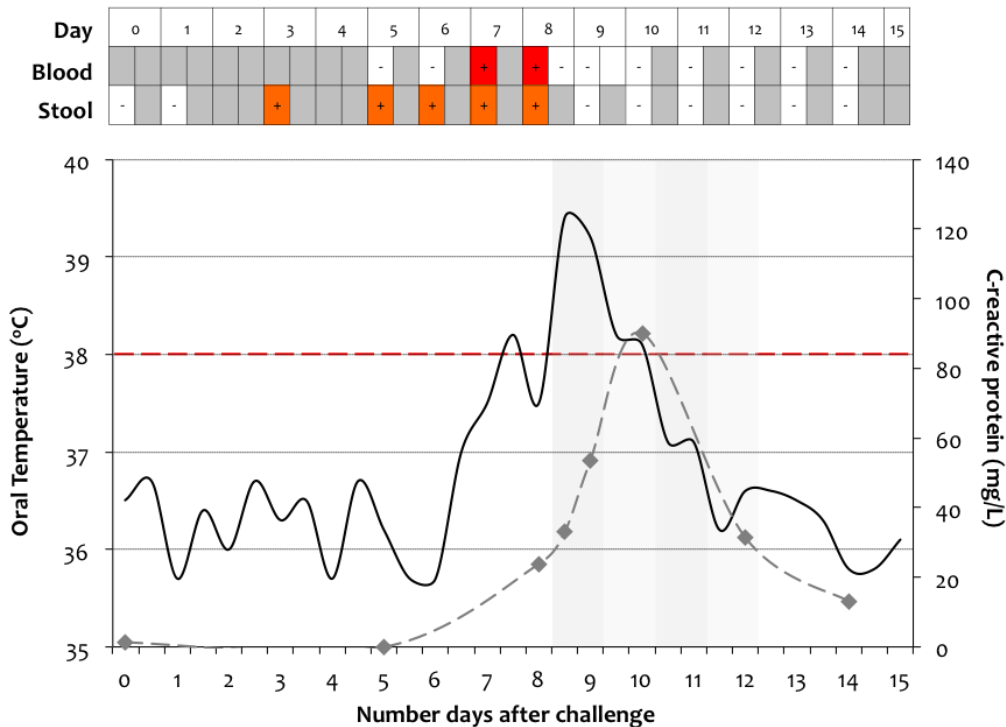


Figure 11-11. Example of a (placebo vaccinated) participant in whom positive stool cultures were the earliest indication of typhoid infection onset.

Red square, positive blood culture; orange square, positive stool culture; grey squares, no sample collected; black line, oral temperature; dashed grey line, C-reactive protein level; shaded area, antibiotic treatment initiated.

Within 96-hours of diagnosis, fewer TD Mo1ZH09 recipients had positive SC (6/18, 33%) than either TD placebo or Ty21a recipients (50% and 77%, respectively). The mean number per culture-positive participant was similar (~2), however, and there were no differences in the numbers of samples submitted - i.e. the difference could not be explained by constipation symptoms (Figure 11-12).

Diarrhoeal symptoms were not associated with increased odds of *S. Typhi* stool positivity with approximately equal symptom reporting rates found in those who did (26%) and did not (33%) report symptoms. With initiation of antibiotic therapy, all participants became culture negative within 1 day; in fact, only one stool culture collected after the first dose

of antibiotics had been taken was positive (115-minute interval between antibiotic initiation and sample collection).

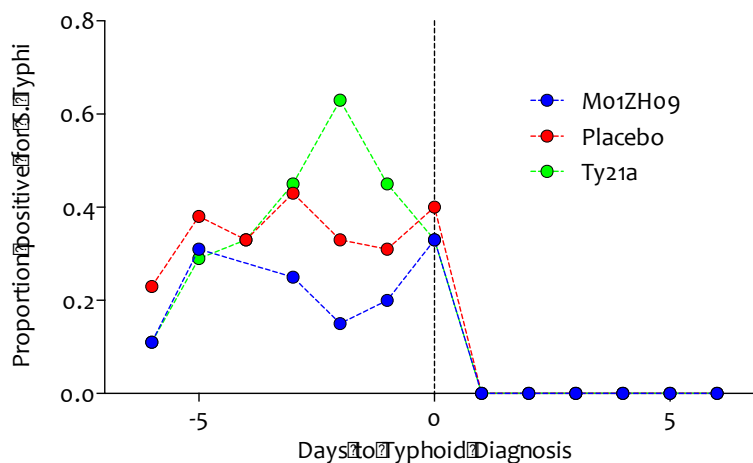


Figure 11-12. Proportion of positive stool samples submitted by participants developing typhoid infection after challenge according to vaccine allocation.

11.5.5 STOOL CULTURES IN NON-TYPHOID DIAGNOSED PARTICIPANTS

Although less frequent, *S. Typhi* was also cultured in stool samples collected from individuals not developing typhoid infection after Day 4. Overall, 14 participants demonstrated stool shedding without fulfilling criteria for TD, from whom 36 positive samples were collected (Table 11-3). All of these positive samples were collected during the second week of challenge, and, while the mean (95%CI) number of positive isolates per participant was 2.57 (1.38-3.76), 3 participants were ‘heavy’ shedders producing 6 positive samples each (one from each group; example in Figure 11-13).

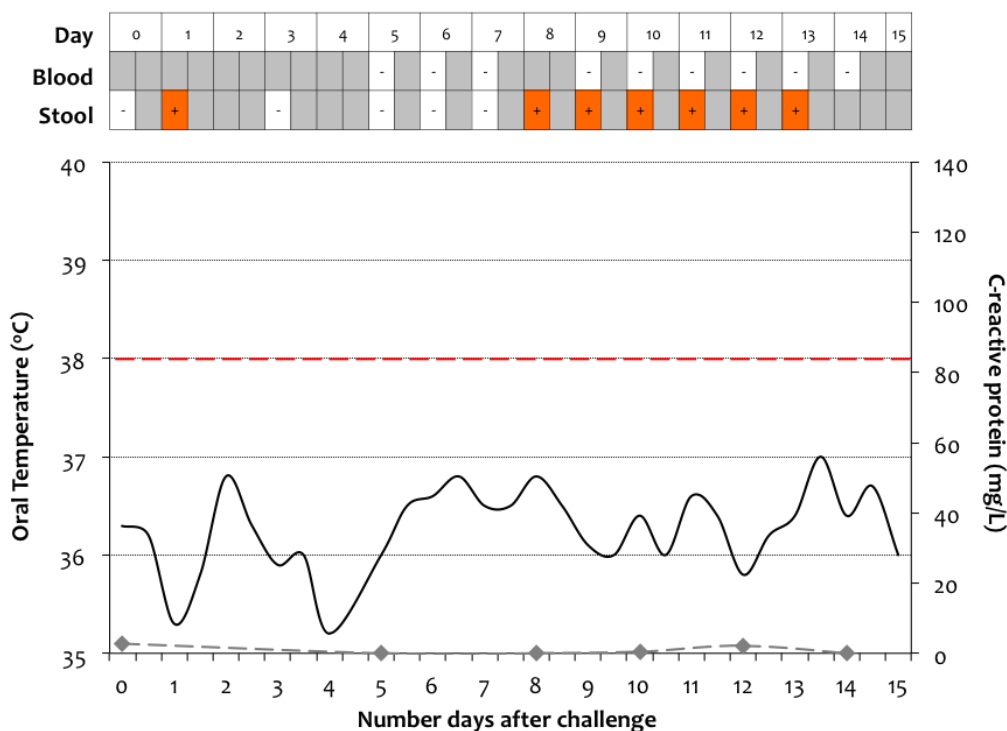


Figure 11-13. Example of a participant shedding *S. Typhi* during the second week of challenge in the absence of apparent infection.

Note normal CRP and unremarkable temperature; in addition serological markers tested at Day 28 (LPS/Vi antibodies by ELISA) failed to show seroconversion. This participant received M01ZH09 28-days prior to challenge. Orange square, positive stool culture; grey squares, no sample collected; black line, oral temperature; dashed grey line, C-reactive protein level. Antibiotic treatment was commenced on Day 14.

Comparison of these 14 participants to other non-typhoid diagnosed and non-stool culture positive individuals, demonstrated no significant differences in numbers of symptoms, temperature increases or maximum CRP values recorded (*data not shown*). In addition the 14 participants were split evenly between vaccine groups (placebo, $n=6$; M01ZH09 and Ty21a, $n=4$ each). Of note, only 2 participants had stool cultures positive on Day 14 alone, heralding possible subsequent diagnosis outside of the challenge frame. While one participant was asymptomatic and otherwise well, the second had a CRP of 46mg/L and increasing temperature recordings (Figure 11-14).

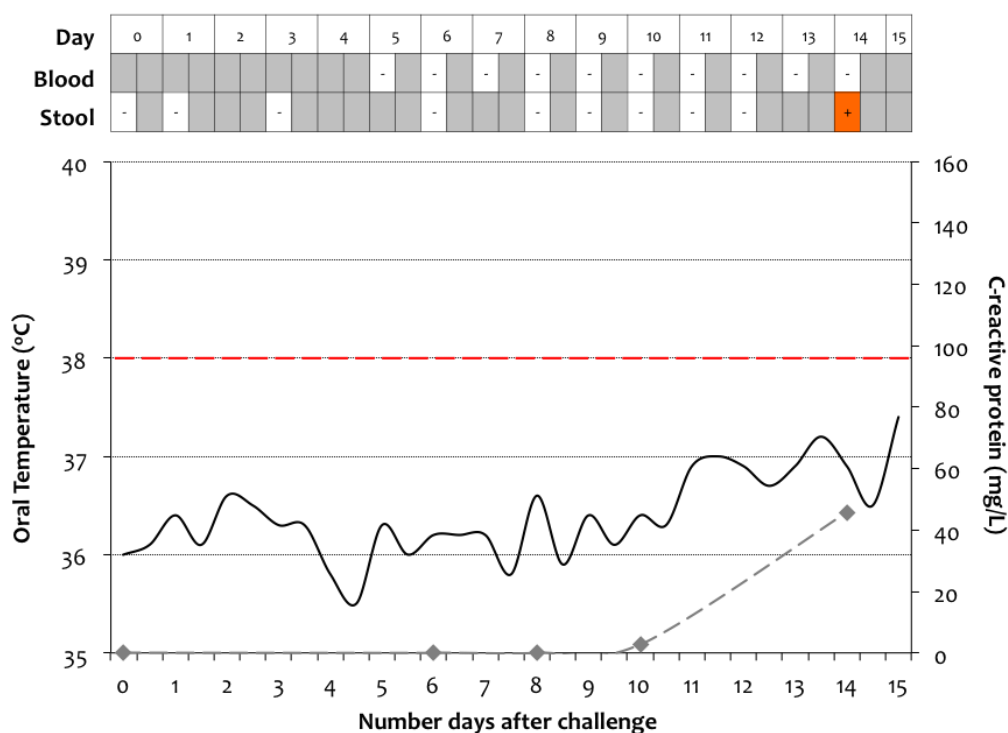


Figure 11-14. Example of a non-typhoid diagnosed participant (M01ZH09 recipient) with evidence of infection developing towards the end of the challenge period.

Note trend in increasing temperature measurements, elevated CRP and positive stool culture on Day 14. Orange square, positive stool culture; grey squares, no sample collected; black line, oral temperature; dashed grey line, C-reactive protein level. Antibiotic treatment was commenced on Day 14.

11.6 QUANTITATIVE STOOL CULTURE

904 stool samples were collected for quantification after challenge (mean, 9.9 samples per participant). *S. Typhi* was cultured and enumerable in 46 (5.1%) specimens collected from 28 participants. Bacterial shedding was non-normally distributed, with 37/46 (80%) of specimens having fewer than 250CFU/g (Figure 11-15). The overall median [IQR] quantity of bacteria found was 76 [16-194] CFU/g, and the median time to first positive culture was 5.5 [1-9.5] days.

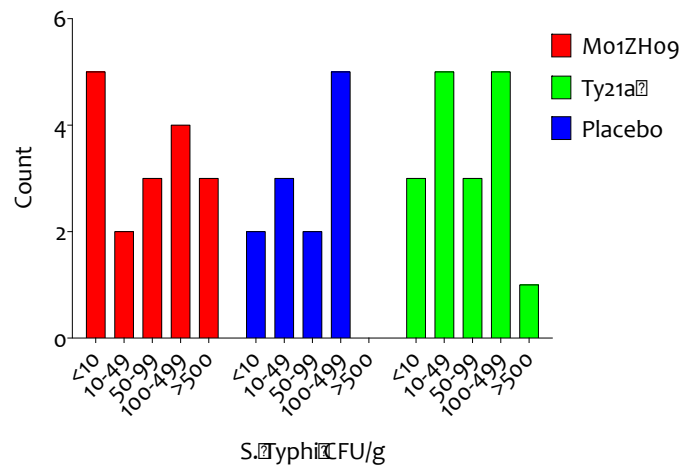


Figure 11-15. Distribution of stool bacterial quantification data according to vaccine allocation. Excludes ‘no growth’ samples.

11.6.1 EARLY SHEDDING

Positive quantitative stool cultures (QSC) were found from Day 1 after challenge, and overall, 20 participants had measurable shedding on either Day 1 ($n=13$) and/or Day 3 ($n=8$) after challenge. There was no significant change in numbers of bacteria detected at the two visits, although only one participant had positive stool cultures on both days (Day 1: 136, Day 3: 224CFU/g). Similarly there were no significant early differences in the numbers of bacteria measured in those participants subsequently developing typhoid, although the median [IQR] number was higher (TD: 116 [8-234]; nTD: 48 [8-64], $p=NS$), nor was there a significant difference in shedding between vaccine groups (Figure 11-16).

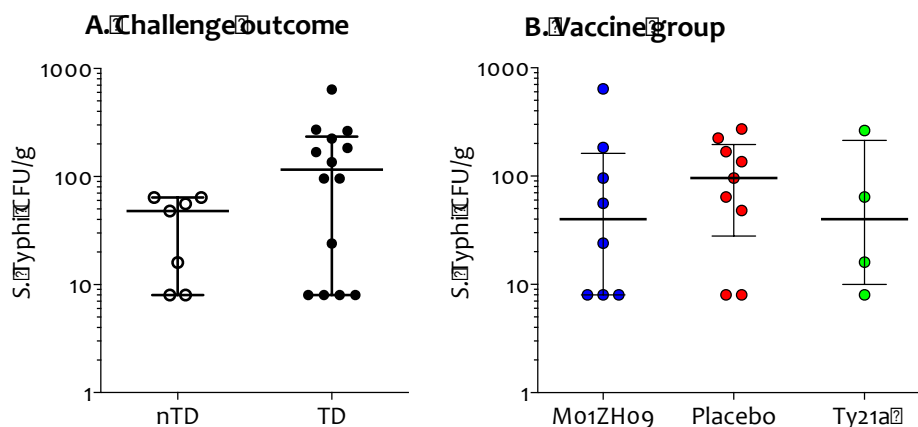


Figure 11-16. Quantified early shedding of *S. Typhi* bacteria in stool samples collected after challenge according to, A, subsequent challenge outcome, or B, vaccine allocation. Bars represent median, IQR.

11.6.2 LATER SHEDDING

After Day 3, quantification of bacterial shedding was possible in 15 participants and 25 stool samples submitted. Bacterial counts ranged widely; while 9/15 participants were subsequently diagnosed with typhoid, the numbers of bacteria shed were not significantly higher in this group compared to those remaining well.

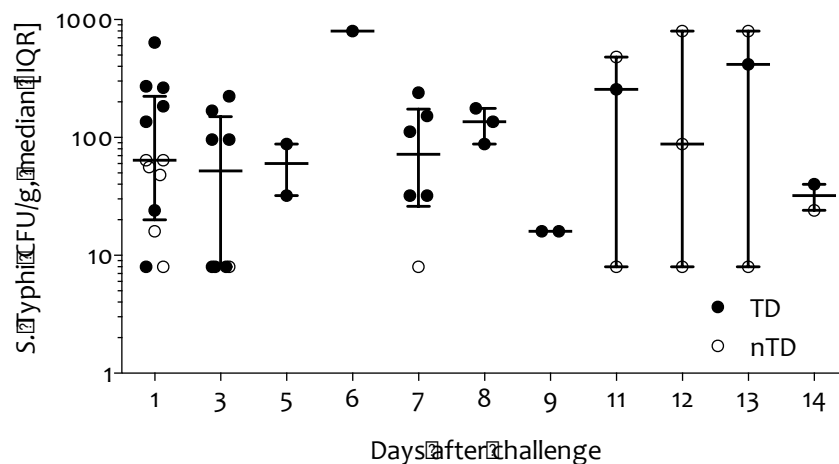


Figure 11-17. Quantification of bacterial shedding in stool samples collected from participants during the challenge period by outcome.

Bars represent median, IQR. TD, typhoid diagnosed; nTD, non-typhoid diagnosed.

When assessed by time to diagnosis, no clear pattern was apparent as only five participants had more than one data point available. Of these, bacterial shedding increased in 3/5 prior to diagnosis and antibiotic initiation but decreased in 2 (data not shown). All five were diagnosed based on development of bacteraemia.

11.7 DETECTION BY PCR

Based on its performance in the dose-level escalation study, the culture-PCR assay described in Chapter 7 was performed using blood collected from 12-hours after challenge onwards.

11.7.1 GENERAL DESCRIPTION

In total, 1180 samples were collected for culture-PCR assay from 89 participants during the course of the study (mean, 13.3/participant). Overall PCR sample positivity rate was 7.4% (87/1180; Table 11-4). As a comparison, the positivity rate in the previous (non-vaccine) dose-escalation study was 8.3%, however the proportion of culture-PCR positive samples collected from placebo recipients was higher (11.2% vs. 8.3%). As expected, the proportion of culture-PCR positive results was higher in participants diagnosed with typhoid, and in those receiving placebo rather than either active vaccine (Mo1ZH09, $p=0.002$; Ty21a, $p=0.002$, chi-squared test). As with the blood culture data, culture-PCR samples from Mo1ZH09 recipients were, in general, positive several days after those from either of the other two groups (Figure 11-18).

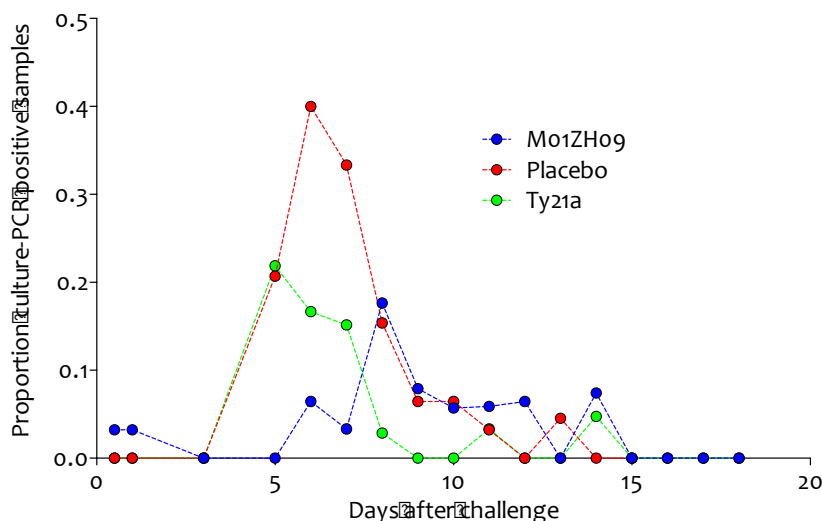


Figure 11-18. Proportion of positive culture-PCR samples collected each day after challenge according to vaccine allocation.

11.7.2 PRIMARY DNA-EMIA

Contrary to findings in the initial dose-escalation study (see: Table 7-4), positive PCR results in the early period (<3 days) after challenge were only found in two participants

(2/259 samples tested). Coincidentally both participants were in the M01ZH09 vaccine group; neither developed infection subsequently (Figure 11-19).

11.7.3 TYPHOID DIAGNOSIS

In keeping with the previous blood culture data, culture-PCR positivity rate increased from Day 5 onwards (note that participants were not sampled on Days 2 or 4) in the placebo and Ty21a vaccinated groups and Day 6 M01ZH09 recipients (Figure 11-19).

The earliest positive sample found in a participant subsequently developing infection, was 8 days prior to diagnosis (M10ZH09 recipient). This participant was also positive 5 and 2 days prior to and at typhoid diagnosis (* in Figure 11-20).

Group	Outcome	Time point after challenge, n(%)					Total
		Day 0-3	Day 3 onwards				
MoiZH09	ALL	2/62 (3.2)	20/354 (5.6)				22/416 (5.3)
	nTD	2/28 (7.1)	0/141 (0)				2/169 (1.2)
	TD	0/34 (0)	20/213 (9.4)				20/247 (8.1)
<i>Day relative to TD</i>		>-3	-3 to -1	-1 to 1	1 to 3	>3	
		2/50 (4)	5/33 (15.2)	13/67 (19.4)	0/34 (0)	0/29 (0)	
Placebo	ALL	0/58 (0)	45/327 (13.8)				45/385 (11.2)
	nTD	0/20 (0)	2/106 (1.9)				2/126 (1.6)
	TD	0/38 (0)	43/221 (19.5)				43/259 (16.6)
<i>Day relative to TD</i>		>-3	-3 to -1	-1 to 1	1 to 3	>3	
		1/21 (4.8)	3/26 (11.5)	35/77 (45.5)	1/39 (2.6)	3/58 (5.2)	
Ty21a	ALL	0/55 (0)	20/324 (5.6)				20/379 (5.3)
	nTD	0/29 (0)	0/168 (0)				0/197 (0)
	TD	0/26 (0)	20/156 (12.8)				20/182 (11.0)
<i>Day relative to TD</i>		>-3	-3 to -1	-1 to 1	1 to 3	>3	
		0/24 (0)	5/20 (25)	14/49 (28.6)	0/25 (0)	1/38 (2.6)	
TOTAL		2/175 (1.1)	85/1005 (8.5)				87/1180 (7.4)

Table 11-4. Number (%) of blood culture-PCR positive samples identified during the vaccine-challenge study according to vaccine allocation, challenge outcome and stage of challenge.

Samples collected from TD participants are further described by day relative to typhoid diagnosis (and antibiotic initiation) in 48-hour blocks. nTD, non-typhoid diagnosed; TD, typhoid diagnosed.

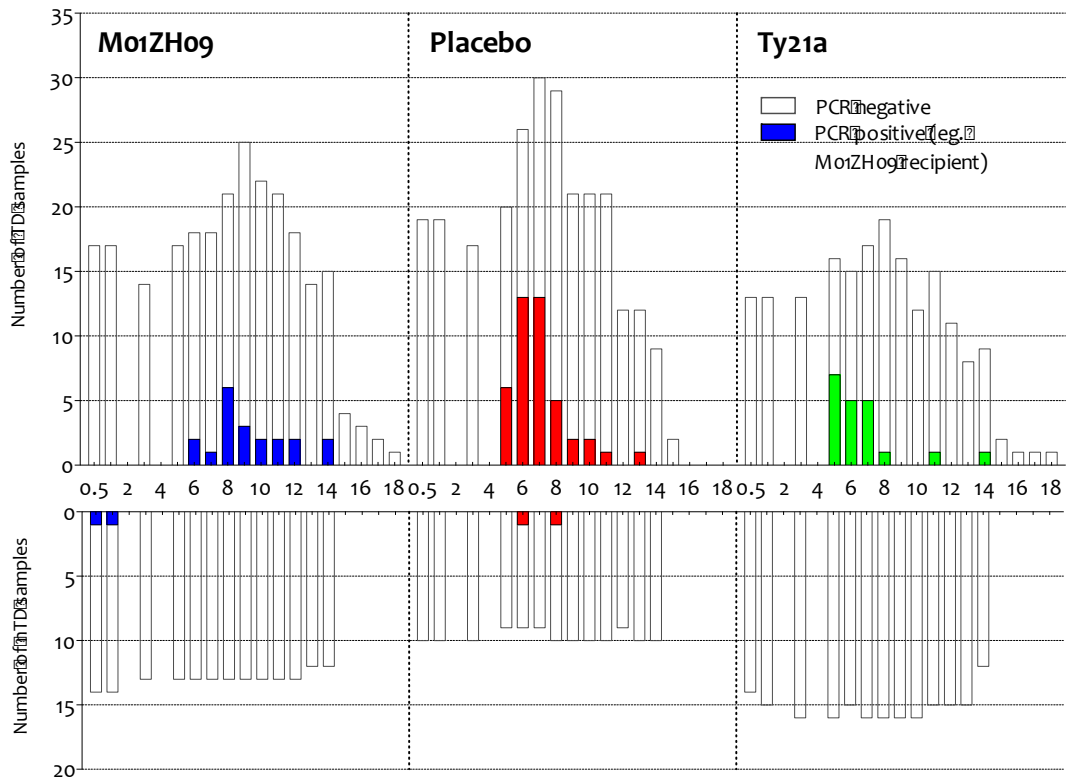


Figure 11-19. Number of culture-PCR samples collected after challenge by typhoid outcome and vaccine allocation.
X-axis is 0.5, 1 then consecutive days after challenge.

Typhoid diagnosis was confirmed by PCR in 75.5% of participants, ranging from 66.7% of those treated based on the clinical (temperature) definition to 86.7% of those diagnosed by blood culture before Day 7 (Table 11-5).

Antibiotic indication	Diagnostic test n/N (%)	
	Blood culture	Culture-PCR
TD		
BC before Day 7	15/15	13/15
BC after Day 7	13/13	10/13
High temp	17/21	14/21
ALL TD	45/49 (91.8)	37/49 (75.5)
nTD		
Symptoms	- withdrawn (1) -	
Day 14	0/38	3/38
ALL	45/88 (51.1)	40/88 (45.5)

Table 11-5. Summary of blood culture and culture-PCR diagnostic tests in confirming diagnoses of typhoid infection after challenge, according to primary antibiotic indication.

In addition to confirmation of bacteraemic diagnoses, an additional 2 non-bacteraemic TD participants had clinical diagnoses confirmed by positive PCR results (3 samples in total).

Few samples were found to be culture-PCR positive after antibiotic initiation, although, as for the blood culture data, placebo recipients appeared to remain culture-PCR positive for longer after the initiation of antibiotic treatment (Figure 11-20). Of note several participants were found to be culture-PCR positive at later time points (Day 4 and Day 8).

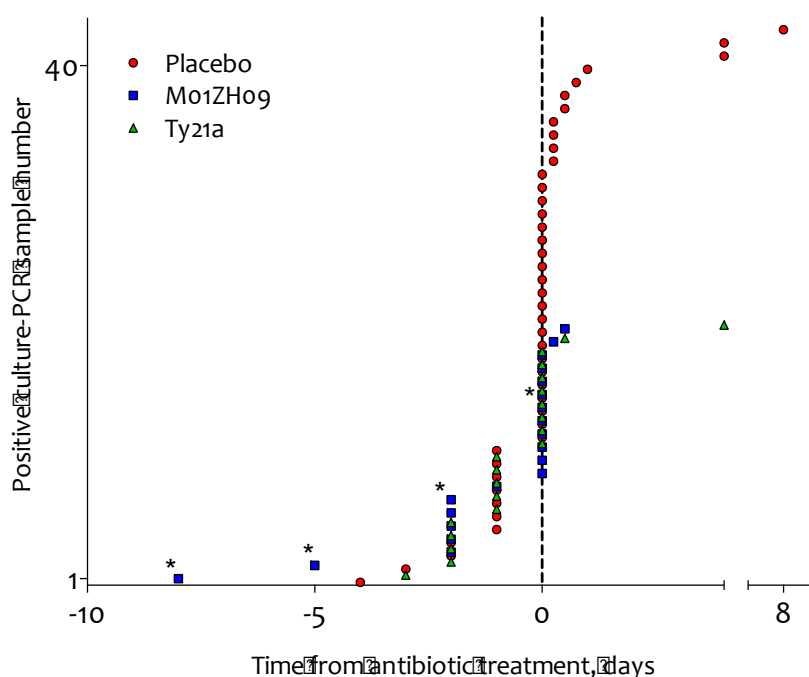


Figure 11-20. Time from positive culture-PCR result to antibiotic initiation for those developing typhoid infection according to vaccine group allocation.

11.7.4 ASYMPTOMATIC DNA-EMIA

In contrast to findings from the preliminary dose-level escalation study, few additional positive PCR results were found amongst those not diagnosed with infection ($n=3$; Table 11-5). One placebo recipient was PCR positive on Days 6 and 8; whilst all other microbiological and clinical parameters were unchanged at this time, notably the majority of the participant's symptoms were also reported on these days ("generally unwell", "loss of appetite", "nausea/vomiting" and "abdominal pain").

11.7.5 CULTURE-PCR TEST PERFORMANCE

Matched blood culture results were available for 857 time points (88 participants); BC and PCR positivity rate for these matched points was 13.4% (115/857) and 9.3% (80/857), respectively. Overall culture-PCR test performance was similar to that seen in the preliminary dose-escalation study when compared to the TD case definition as the reference standard (Table 7-5). Assuming that the positive culture-PCR results in non-diagnosed participants were false positive, the sensitivity and specificity for TD was higher than that found previously (sens/spec: this study, 0.76/0.92; preliminary dose-escalation study, 0.69/0.67).

		Challenge outcome					Challenge outcome		
		TD	nTD	total			TD	nTD	total
PCR result	pos	37	3	40	BC result	pos	45	N/A*	45
	neg	12	35	47		neg	4	38	42
	total	49	38	87		total	49	38	87

	PCR		Blood culture	
Sensitivity	0.76	[0.61-0.87]	0.92	[0.80-0.98]
Specificity	0.92	[0.79-0.98]	1.00	[0.91-1.00]
LR+	9.56	[3.19-28.67]		
LR-	0.27	[0.16-0.44]	0.08	[0.03-0.21]
PPV	0.93	[0.80-0.98]	1.00	[0.92-1.00]
NPV	0.74	[0.60-0.86]	0.90	[0.77-0.97]
DOR	35.8	[9.4-138.3]	778.6	[40.6-14921]

Table 11-6. Contingency tables displaying estimates [95% CIs] of the sensitivity and specificity for culture-PCR and routine blood culture for diagnosing participants with typhoid infection during the vaccine-challenge study.

*Note that bacteraemia was one of the diagnostic criteria. TD, typhoid diagnosed; nTD, non-typhoid diagnosed; BC, blood culture; LR, likelihood ratio; PPV, positive predictive value; NPV, negative predictive value; DOR, diagnostic odds ratio.

11.8 DISCUSSION

The data presented in this chapter provide a detailed overview of *S. Typhi* bacterial dynamics before, during and following typhoid infection, in addition to new data investigating the alteration in bacterial dynamics following vaccination with live-oral vaccination. These data are key to furthering knowledge regarding the natural history of typhoid infection, but also to inform on the potential effects of live-oral vaccination on shedding and transmission following introduction to an endemic setting.

11.8.1 EARLY INFECTION

The appearance of *S. Typhi* in the blood after a variable and sometimes prolonged incubation period accompanied by the onset of clinical symptoms and increasing fevers is the hallmark of typhoid fever.¹¹² Describing the evolution of an infection using a challenge model allows the key elements of time from exposure and infectious dose to be taken into account.⁹⁶

The first indication of host-bacterial interactions taking place was the excretion of *S. Typhi* in the stools of ~50% of individuals (placebo recipients) within 96-hours after challenge. This has been a common finding in previous human typhoid challenge studies, and has been associated, as confirmed here, with an increased risk of subsequent infection.^{284,285,436} While detection of shedding at this stage may solely reflect safe transit through the GI tract, the stool quantification data presented supports the hypothesis that active bacterial replication occurs in the gut soon after exposure. This was found to occur at high rates in some individuals with counts of up to 640CFU/g being retrieved; there was some suggestion that more bacterial replication may be occurring in those developing infection compared to those remaining uninfected, although the sample sizes were small.²⁸⁵

The site and timing of *S. Typhi* invasion and the establishment of systemic human infection are important questions that may be addressed by these challenge studies. Evidence from the Maryland studies suggested that invasion occurred in the duodenum and not the via the tonsillar/pharyngeal tissue, as infection did not develop after gargling (and expectorating) 10^9 CFU,²⁸⁵ although bacteria had previously been found in chimpanzee tonsillar tissue after oral challenge.⁷² Investigators also excluded the stomach as a site of invasion due to the lack of gastritis in comparison to *Shigella* infection in non-human primate and human challenge studies.²⁸⁵ Treatment of volunteers with chloramphenicol 24-hours after challenge, and subsequent development of typhoid 9-days after completing antibiotics in one individual, indicated that bacterial invasion/location to a secluded niche must occur within the first 24-hours after exposure.^{89,285}

Evidence of a primary DNA-emia occurring within 12-24 hours of challenge was provided in Chapter 7 and also (to a lesser extent) in this study, indicating that early invasion and low-level dissemination via the bloodstream is taking place. Alternative explanations for detection of DNA rather than viable bacteria could include bacterial degradation following an immune response at the intestinal mucosa, however, this time frame for invasion is consistent with the historical data. The association found here between challenge dose and time-to-infection and time-to-infection and quantitative bacterial load, suggests that a higher inoculum results in earlier invasion and a greater bacterial invasion. While it is most likely that bacterial replication and invasion occurred in the intestine, intensively-collected stool samples failed to culture bacteria in many individuals developing infection. Gastritis was a commonly reported feature after challenge in our volunteers, and the effects of NaHCO_3 could also conceivably have altered the gastric milieu sufficiently to allow early invasion to occur at this site. Further insights should be

provided by future studies in performing duodenal-string tests to sample the duodenal fluid at points after challenge and prior to typhoid onset.

11.8.2 BACTERAEMIA

The development of bacteraemia ~6-days after challenge, and with a median bacterial load (BL) of 1.12CFU/mL in placebo recipients at diagnosis, supports the challenge dose used (10^4 CFU) as being comparable to a relatively high-dose natural exposure and is more in keeping with the level of bacteraemia found in paediatric populations.^{91,113,204} Wain and colleagues, for example, found median *S. Typhi* bacterial loads of 1.5CFU/mL or 0.6CFU/mL in children under 15-years or adults, respectively, in a study performed in Vietnam.¹¹³ At this level, our findings suggest that the *S. Typhi* bacterial load distributions found in this study are comparable to other causes of Gram-negative bacteraemia; 50% had BLs <1CFU/mL while 72% were <10CFU/mL.⁵²⁹⁻⁵³¹ These higher levels of bacteraemia may be supported by the increased sensitivity of the culture-PCR assay for detecting DNA in the blood of participants diagnosed with typhoid (detection rate: 15.6%) even in comparison to the findings from the preliminary dose-escalation study (9.6%, see: 7.3.4).

As discussed by numerous previous authors,^{211,359,416,417,484,524} given that the burden of Gram-negative bacteria found in the circulation at peak symptom presentation is relatively similar to other causes of bacteraemia, reasons for the lack of overt sepsis in response to endotoxin/LPS during clinical presentation of patients with typhoid remain unclear.⁵³²

11.8.3 BACTERAEMIA AND ROLE IN SYMPTOM DEVELOPMENT

In many other infections, including those causing Gram-negative sepsis, infection severity and ensuing disease sequelae have been found to correlate with peripheral bacterial load measurements.^{211,462,531} *S. Typhi* rarely causes endotoxic shock, however, and is associated

with a significantly lower mortality (~1%) than for other causes of Gram-negative bacteraemia (~10-50%) irrespective of frequent delays in diagnosis.^{113,266,529,530,533} Despite speculation that this may be due to the cryptic intracellular nature of bacterial dispersion to the RES or abundance of intracellular bacteria at diagnosis (which may exceed 60% of the total burden),¹¹³ an association between BL and clinical severity of typhoid has still not been confirmed.^{90,417,436} Initial studies performed in Maryland and supported by more recent evidence, suggest that, either as a result of the initial primary bacteraemia or the sub-acute presentation of illness, some tolerance to LPS develops. This could result in a less dramatic response to the secondary bacteraemic phase of infection.^{211,359,416,501} Previous *in vitro* studies have demonstrated no difference in the nature of *S. Typhi* LPS compared to that of other enterobacteriaceae in cytotoxicity or ability to induce pro-inflammatory cytokines.

The findings here of correlations between BL and CRP, and CRP and fever, provide some confirmation that the link between bacterial burden and severity of illness may be the host inflammatory response. Notably, no association was found between BL and maximum recorded temperature, as was demonstrated in the preliminary dose-finding study.^{436,451} Furthermore, this host response appears to be relatively binary once bacteraemia has occurred, in that typhoid-diagnosed participants were found to be either symptomatic or non-symptomatic; this confirms previous observations of no association between BL and rates of complicated typhoid infection.²⁰⁴ The nature of these inflammatory responses will be dependent on the level of pro-inflammatory mediators present in the circulation,²¹¹ which, given the duration and complexity of bacterial passage after exposure, may only be indirectly related to the numbers of bacteria present. Furthermore, *S. Typhi* has been found to employ numerous mechanisms to deliberately down-regulate responses, possibly in order to facilitate persistence and transmission to further hosts.¹¹³ Whether *S. Typhi* LPS exposure is the trigger for

inflammatory responses and symptom development remains topical. Early challenge studies demonstrated that tolerating LPS had no effect on subsequent typhoid development, while others demonstrated that, by limulus assay, there was little or no evidence of endotoxin (LPS) in the blood of typhoid patients.^{211,359,416} This could suggest that alternative inflammatory mechanisms may be responsible for the clinical presenting features of typhoid. The recent discovery of a novel A₂B₅ CdtB toxin (with immunomodulatory activity) unique to the *S. Typhi* serovar may provide some further evidence for this,^{81,534} and there are plans to investigate its role using samples from this study. This also raises some speculation as to whether vaccines resulting in anti-LPS humoral responses, while having a beneficial effect on bacteraemic burden, are likely to impact clinical disease presentation.

11.8.4 MEASUREMENTS OF SEVERITY

As seen in the previous chapter, symptom reporting was a poor discriminator of illness severity. The correlation found between challenge dose (even within the single target dose used) and time-to-infection provides further support for using this parameter as an indicator of exposure dose in outbreak/challenge settings.^{90,91,436} While the correlation with BL at diagnosis provides new evidence for use of time-to-infection (or bacteraemia) as a possible surrogate marker of infection severity, these data suggest it may reflect the degree of end-organ involvement rather than overt clinical severity.²⁰⁴

The inverse correlation between BL and nadir platelet count support this measurement as useful indirect, albeit non-specific,⁵³⁵ marker of infection severity as has been shown in non-challenge studies performed in endemic settings previously.²⁰⁴ Thrombocytopenia in addition to hypofibrinogenaemia and other coagulation abnormalities have been identified in studies before;^{173,211} reduced platelet counts are thought to reflect either reduced production by the bone marrow or increased sequestration/destruction by an

enlarged spleen. Although hepatosplenomegaly was not found clinically in this study, possibly in part due to early recognition and treatment, a direct inverse relationship between bacterial quantity in the bone marrow and platelet count is supported by previous field data.²⁰⁴ Wain and colleagues also found an inverse correlation between bone marrow BL and peripheral white cell count inferring a directly inhibitive effect, however we were unable to demonstrate a relationship with peripheral WCC (or white cell subsets) and BL in this study.²⁰⁴

11.8.5 STOOL SHEDDING

S. Typhi were cultured from stool samples collected from 50% of placebo recipients developing typhoid infection within 96-hours prior to diagnosis, consistent with the higher diagnostic sensitivity of this test in children rather than adults.^{112,209} As shown by authors previously,¹¹³ we also found higher blood BLs in those participants who shed prior to diagnosis compared to those who did not. Shedding detected before the development of bacteraemic infection also supports the hypothesis of a second amplification cycle with bacterial replication re-occurring in the gut/gall-bladder prior to detection of the secondary bacteraemic episode, with or without reinvasion of Peyer's patches.

The frequency of stool shedding in the days preceding bacteraemia and diagnosis highlights this period as being high-risk for onward transmission to close family members or the community.⁴³⁶ In general, participants were asymptomatic 2-3 days before diagnosis, despite shedding *S. Typhi* in stool, sometimes in high quantities (up to 800CFU/g). This has important implications for typhoid transmission in endemic settings and affects diverse modelling assumptions, including the degree to which immunity may be boosted in close-contacts of sick patients, and the persistence of *S. Typhi* in contaminated environmental samples.^{121,122} A further finding of central importance to transmission modelling was the degree to which exposed but non-typhoid diagnosed

participants seemed to shed *S. Typhi* in stool samples during the second week of illness. This was a finding noted in the previous Maryland studies; participants challenged with the higher ID₅₀ dose (100,000CFU) in particular, frequently shed *S. Typhi* in stool commencing in the second week, persisting for 3-weeks then spontaneously abating and only occasionally continuing for longer than 6-weeks.²⁸⁵ This pattern was also seen in typhoid-diagnosed participants regardless of chloramphenicol treatment. While cessation of challenge exposure by fluoroquinolone treatment at 14-days may have curtailed disease development in our study, the findings are consistent with those from Maryland in which participants were followed untreated for considerably longer periods. In those studies the longest incubation prior to disease developing was 56-days.²⁸⁵ Reasons for detecting shedding in the second week only may include low stool culture sensitivity (due to low shedding quantity) during the first week of challenge follow-up, or inapparent silent infection occurring in those in whom shedding was detected. Both seem unlikely; the former given the intensity of sample collection and the several assays (quantitative, qualitative with and without selenite pre-culture) used to detect bacteria, and the latter due to the asymptomatic nature of participants and repeatedly negative optimal blood cultures and sensitive culture-PCR diagnostic assays.

Other explanations for sustained shedding in non-typhoid diagnosed individuals may include control of the secondary biliary/enteric replication cycle by local mucosal or CMI responses. This may result in sequestration of *S. Typhi* to the gallbladder from where replication and shedding into the gut lumen could occur. Of note, these individuals tended to have higher pre-challenge anti-LPS IgG responses compared to their typhoid-diagnosed stool culture positive counterparts, possibly also suggesting more effective humoral control of bacteraemia. Whether these participants would have gone on to develop chronic carriage had they not been treated is unknown, but further investigation of their mucosal and CMI responses and genetic susceptibility factors is merited. Similar

participants were followed-up long-term and without treatment in Maryland, and all managed to successfully clear the infection without recurrence or carriage developing.^{284,285}

11.8.6 EFFECT OF VACCINATION

Assessment of the effect of vaccination on the bacterial burden of infection following challenge has not previously been described, beyond the benefits of reducing shedding in stool after liberal Ty21a vaccination.^{123,257} Both vaccines used in this study had marked effects on bacterial dynamics occurring during the challenge and infection period. Major new findings from the data presented here include the effect of vaccination, with either Mo1ZH09 or Ty21a, on the bacterial load measured at diagnosis and the differential effect of the two active vaccines on the delay to bacteraemia.

The effect of reducing BL at diagnosis, while having little impact on the scale and range of clinical illness (symptoms and signs including fever) in those succumbing to infection, supports the importance of vaccine-derived immune responses in bacterial killing and control. The corollary of reductions in effect on end-organ involvement including platelet depletion and CRP increase are likely to also be beneficial to host outcomes, albeit not quantifiable in this setting.

For two vaccines that are orally ingested and are presumed to act through similar mechanisms the different effects on incubation period were unexpected. The duration of incubation periods during the earlier Ty21a studies performed in Maryland were not reported, suggesting no obvious difference in disease onset was found after vaccination.²⁵⁷ In this study both vaccines generated anti-LPS humoral antibody responses (Chapter 9), suggesting that the difference in anti-flagellin titres seen or some other, unmeasured, immune responses generated (including innate, mucosal or cell-

mediated immune responses) account for this difference in ability or inability to successfully control bacteraemic infection. Following the initiation of ciprofloxacin antibiotics, blood cultures from bacteraemic participants rapidly became sterile within a 24-hour period. Some delay was seen in placebo-recipients, possibly as a result of having a greater bacterial burden to clear or because they lacked the assistance of vaccine-derived immune responses to contain the infection more quickly.

Early collection and isolation of bacteria from stool samples after challenge indicated that participants receiving active vaccination have a higher rate of initial shedding after exposure. This may be the result of an effective underlying mucosal immune response as a result of oral vaccine ingestion or due to permissive alterations in the gut microbiome supporting *S. Typhi* replication.^{97,284,536} Whether this was an effect seen specifically in response to a live-attenuated vaccine or whether the same effect would have been caused by prior (recent) exposure to *S. Typhi* has significant public health implications. It suggests that repeatedly exposed individuals in endemic settings may amplify transmission to those less frequently exposed (and possibly less immune). Although limited in sample size, quantification data suggested that the numbers of bacteria being shed at these early time points were lower in those receiving active vaccination, supporting the possibility of a beneficial local mucosal response in preventing replication.

The effect of receiving an active vaccine on *S. Typhi* shedding in the stool was most clearly seen in participants not developing typhoid; fewer positive stool cultures were collected from these participants from 96-hours after challenge. This is in contrast to the previous human challenge studies in Maryland, where no effect of Ty21a vaccination was seen on stool shedding (0-3 days duration, although there was an effect on longer-term shedding of 4-30 days duration),²⁵⁷ and an effect was only seen after Taboral ingestion (an oral acetone-killed Ty2 preparation) when twice the recommended dose was given.¹²³

The time frame immediately prior to diagnosis would be a particular high-risk for transmission in endemic settings, as this would be when patients become increasingly febrile and symptomatic requiring closer care from family members. Notably, during this period M01ZH09 recipients demonstrated less shedding compared to both placebo and Ty21a recipients, thus potentially having more impact on disease transmission.

11.8.7 LIMITATIONS

There are some limitations specific to the data presented in this chapter. A key difference from the initial dose-finding study was the decision to only start collecting blood cultures routinely from Day 5. This was based on data regarding the likely incubation period using the 10^4 CFU dose and, in addition, it was planned to start collecting blood cultures sooner should participants report symptoms or develop abnormal blood parameters. In practice, no participants were overtly symptomatic before Day 5, however as can be seen, bacteraemia was relatively frequently detected in the first sample collected. Whether we could have detected bacteraemia or stool shedding (more likely) on Day 4 when participants were not seen in the clinic is unknown but certainly possible, and should be taken into account in interpreting the data and in future challenge designs.

A further limitation is the lack of corroborating bone marrow or duodenal fluid culture BL data. While both tests would have been relatively invasive to perform and would require additional logistic considerations (personnel, training, consent, compensation etc.) and ethical approvals, they would have provided key data regarding bacterial dynamics at the site of invasion and at a site of replication less affected by the evolving host immune response. Bone marrow BL is less affected by the duration of preceding illness as well as antibiotic exposure and therefore may provide more accurate data regarding the effects of vaccination on bacterial burden.²⁰⁴

In contrast to the data presented in Chapter 7, there was relatively little confirmation of a primary DNAemia, even in samples from unprotected placebo recipients. Reasons for this may include alterations to the sampling schedule (for logistic reasons fewer early collection time points were included) or laboratory processes – while most culture-PCR samples were processed within 24-hours, some cultured pellets were frozen for later extraction, which may have affect the diagnostic yield.

11.8.8 SUMMARY

Challenge studies are a major source of data enabling the pathogenetic mechanisms underlying disease aetiology to be investigated. These findings provide unique insights into the bacterial dynamics occurring after *S. Typhi* exposure and before infection, which is seldom accessible in naturally observed infection and difficult to replicate in the non-naive host. Vaccination with one of two oral live-attenuated *S. Typhi* strains resulted in significant but different effects in altering and reducing the bacterial burden of infection, which could be clearly and accurately documented in this highly-controlled setting. The extent to which these effects may be beneficial in preventing clinical infection or transmission in an endemic setting require investigation.

Chapter 12. ASSESSMENT OF THE PROTECTIVE EFFICACY OF M01ZH09 AND TY21A COMPARED TO PLACEBO USING A HUMAN TYPHOID CHALLENGE MODEL

12.1 INTRODUCTION

A central tenet of this thesis is that a human challenge model using *Salmonella Typhi* may be used to study the efficacy of oral vaccination with M01ZH09 (and Ty21a). Aside from evaluating diagnostic methods, measuring immunogenicity and demonstrating the features of infection after challenge, the question of whether M01ZH09, a novel single-dose oral vaccine, can protect against the development of clinical typhoid infection remains.

An important factor to consider in assessing challenge outcome using a human infection model is the difference in baseline susceptibility/immunity of participants, which may affect both initial vaccine responses and/or subsequent susceptibility to the challenge agent. Evidence of this was seen in the previous Maryland studies, in which many of the volunteers were military veterans (WWII, Korean and Vietnam Wars) and thus either had recent exposure to endemic settings or whole cell vaccination.⁸⁹ The attack rates following challenge with 10^5 bacteria were 48% in those without a military record but 20% in veterans; this attack rate fell to only 6% in vaccinated military veterans.³⁵⁸ Of note, the effect of military service was thought to last for ~12-years after discharge.³⁵⁸ Interestingly, the attack rates found during the course of the Maryland studies gradually increased, which in retrospect may have been a result of less, and a more distant military service record (Myron Levine, personal communication).

Another important determinant of attack rate is the precise study definition of what constitutes 'typhoid fever'. The definitions used here were based on historic data and experience gained during the preliminary dose-escalation study.⁴³⁶ The criteria selected to make a typhoid diagnosis may have been too restrictive, however, particularly given the range of symptoms reported, and the additional infection phenotypes seemingly observed (see: 13.1.8). A major contributory factor is the lack of a suitable gold-standard diagnostic test. Determination of the binary outcome of sick/not sick may therefore be 'improved' or made more robust by the use of composite diagnostic endpoints, as recently suggested.^{537,538}

This chapter will therefore determine the comparative efficacies of M01ZH09 and Ty21a vaccine versus placebo using the *a priori* study definitions for 'typhoid diagnosis', before assessing the attack rates seen using other endpoint classifications. Subsequently, some

assessment of factors contributing to challenge outcome will be explored prior to further attempting to assess the ‘true’ efficacy of the vaccines used in this study.

12.2 METHODS

12.2.1 VACCINE EFFICACY

Vaccine efficacy was calculated according to the methods pre-specified in Chapter 5, using the *per protocol* population i.e., those who were successfully vaccinated and challenged and who were followed-up until at least Day 14. In addition, further outcome measures including time-to-diagnosis, time-to-diagnosis stratified by clinical or microbiological endpoint fulfilment and time to first recording of fever ($\geq 38^{\circ}\text{C}$, any duration) or bacteraemia were summarised using the Kaplan-Meier method.⁵³⁹

12.2.2 STATISTICAL CONSIDERATIONS

Statistical comparisons were made using the methods described in the text, evaluating outcomes for either M01ZH09 or Ty21a in comparison to placebo vaccine recipients. The ‘at-risk’ periods used in time-to-event Kaplan-Meier analyses were 19-days (14-day follow-up plus an additional 5-day blood culture incubation window) for all analyses, except time-to-fever in which data were collected for 21-days after challenge.

12.2.3 SENSITIVITY ANALYSIS

Sensitivity analyses were performed to determine the variations in attack rate using different endpoint criteria. These explored alternative temperature thresholds (both absolute and increases over baseline) and combinations of diagnostic tests to evaluate both attack rates and their effect on vaccine protective efficacy assessment.

12.2.4 ASSESSMENT OF FACTORS CONTRIBUTING TO INFECTION/PROTECTION

To assess the effect of vaccination and, specifically, the humoral response to vaccination in protection of participants after challenge, preliminary univariable analyses were performed. Associations between vaccine responses (anti-LPS, -H and Vi IgG antibody titres), challenge outcome and bacterial endpoints were explored using Pearson's correlation coefficients; skewed data from vaccine responses and QBC bacterial load counts were \log_{10} -transformed. Zero QBC counts were assigned a nominal value of 0.05 (LLOD/2, assumed to be 1CFU/10mL blood) prior to transformation.

Multivariable analyses were performed with the inclusion of further factors contributing to infection outcome, including challenge dose, previous exposure in endemic settings, age and baseline Vi antibody status, in addition to vaccine (M01ZH09 or Ty21a) or placebo receipt. For both logistic and Cox proportional hazard regression models, relevant factors were entered into a model with significance assumed at $p < 0.05$.

12.3 A PRIORI EFFICACY ANALYSIS

12.3.1 PRIMARY ENDPOINT

Using the *a priori* definitions for typhoid diagnosis (Table 12-1), attack rates in the three vaccine groups were 66.7, 58.1 and 43.3% for placebo, M01ZH09 and Ty21a recipients, respectively.

Microbiological definition	
D.	A blood culture positive with <i>Salmonella</i> Typhi was identified from Day 5 onwards, with the presence of one or more objective clinical signs of typhoid infection, or,
E.	A blood culture positive with <i>Salmonella</i> Typhi was identified from Day 7 , with or without objective clinical signs of typhoid infection,
Clinical definition	
F.	The participant developed an oral temperature $\geq 38^{\circ}\text{C}$, persisting continuously for at least 12-hours in the absence of anti-pyretic medication, occurring from 72-hours after challenge .

Table 12-1. Diagnostic definitions for typhoid infection used in the clinical study OVG2012/02.

This resulted in a calculated protective efficacy of 12.9 and 35.1% for the Mo1ZH09 and Ty21a vaccines, respectively (Table 12-2). Therefore neither vaccine resulted in a significant increase in protection in comparison to placebo.

Vaccine group	Challenge Outcome, n (%)		Total	Comparison to placebo	
	nTD	TD		p*	Protective efficacy [95%CI]
Mo1ZH09	13 (41.9)	18 (58.1)	31	0.668	12.9% [-28.9, 41.1]
Placebo	10 (33.3)	20 (66.7)	30	-	-
Ty21a	17 (56.7)	13 (43.3)	30	0.120	35.1% [-5.2, 59.8]
ALL	40	51	91		

Table 12-2. Summary of the protective efficacy against typhoid infection measured following challenge, 28-days after vaccination with either Mo1ZH09 or Ty21a in comparison with placebo.

*Pearson's chi-squared test with continuity correction.

12.3.2 PROTECTION AGAINST CLINICAL/MICROBIOLOGICAL DIAGNOSTIC ENDPOINTS

Analysis of indications for antibiotic initiation demonstrated that, while approximately similar proportions of Mo1ZH09 TD participants met either a microbiological or clinical endpoint (44 and 56% of those diagnosed met each definition, respectively), Ty21a receipt appeared to have a greater effect on the number of clinically-only diagnosed cases (31 and 69%, respectively; Table 12-3.). However both the numbers and differences between the groups were too small to draw any firm differences between outcomes.

Antibiotic indication ^A	Vaccine group, n (%)			ALL
	Mo1ZH09	Placebo	Ty21a	
Microbiological	8 (26)	11 (37)	9 (30)	28 (31)
BC before Day 7	3	7	5	15
BC after Day 7	5	4	4	13
Clinical	10 (32)	9 (30)	4 (13)	23 (25)
Day 14	13 (42)	10 (33)	17 (57)	40 (44)
ALL	31	30	30	91

Table 12-3. Summary of the antibiotic indications for participants completing challenge according to vaccine allocation.

BC, blood culture. A, see Table 12-1 above for category definitions.

12.3.3 TIME-TO-INFECTION

The median [95%CI] time from challenge to diagnosis of typhoid infection by Kaplan Meier analysis was 197 [152-242] and 311 [201-420] hours for the placebo and Mo1ZH09, respectively, and in calculable for Ty21a vaccine recipients (cumulative incidence was less than 50%). Placebo recipients appeared to be diagnosed earlier than those receiving active vaccination, however the delays were not significant when compared using a Log-rank test (Figure 12-1).

Further analysis of time-to-infection was performed using stratification by diagnostic definition fulfilled (Figure 12-2). Firstly, while there was some difference in rates of microbiological diagnoses there were no significant differences in microbiological diagnosis rates between placebo or either active vaccine recipient (Figure 12-2a). Secondly, despite some early clinical diagnoses made in Ty21a recipients and an apparent delay in the clinical diagnosis of Mo1ZH09 recipients, there was no difference in the time to clinical diagnosis for either active vaccine groups recipients compared to placebo (Figure 12-2b).

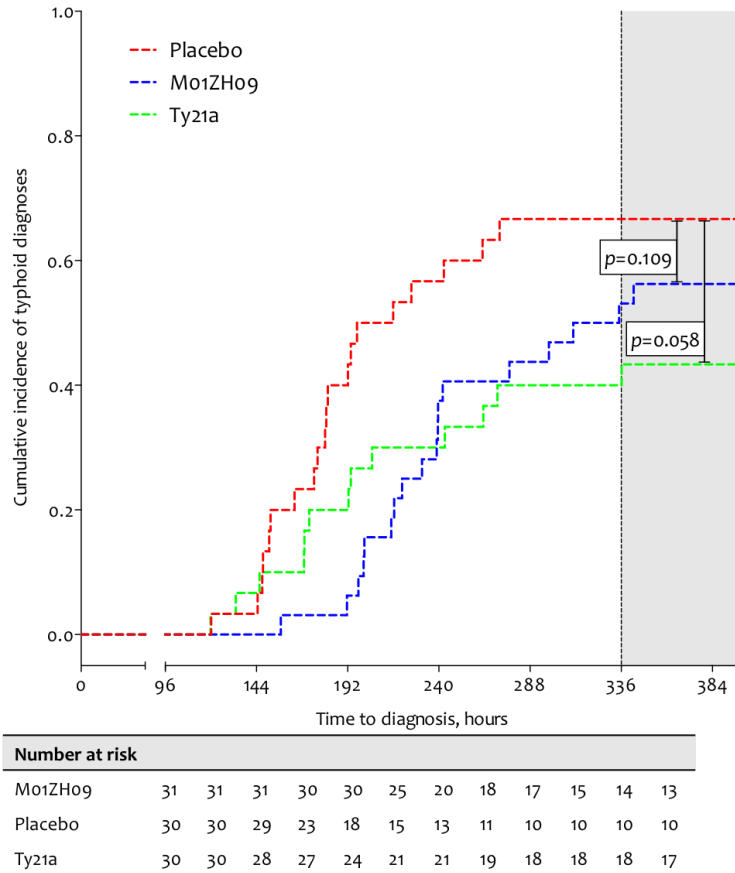


Figure 12-1. Cumulative incidence of typhoid infection (all diagnoses) by vaccine allocation. Grey shading, antibiotics being taken. Non-diagnosed participants were censored in the analysis at t=19 days (456-hours). Log-rank test.

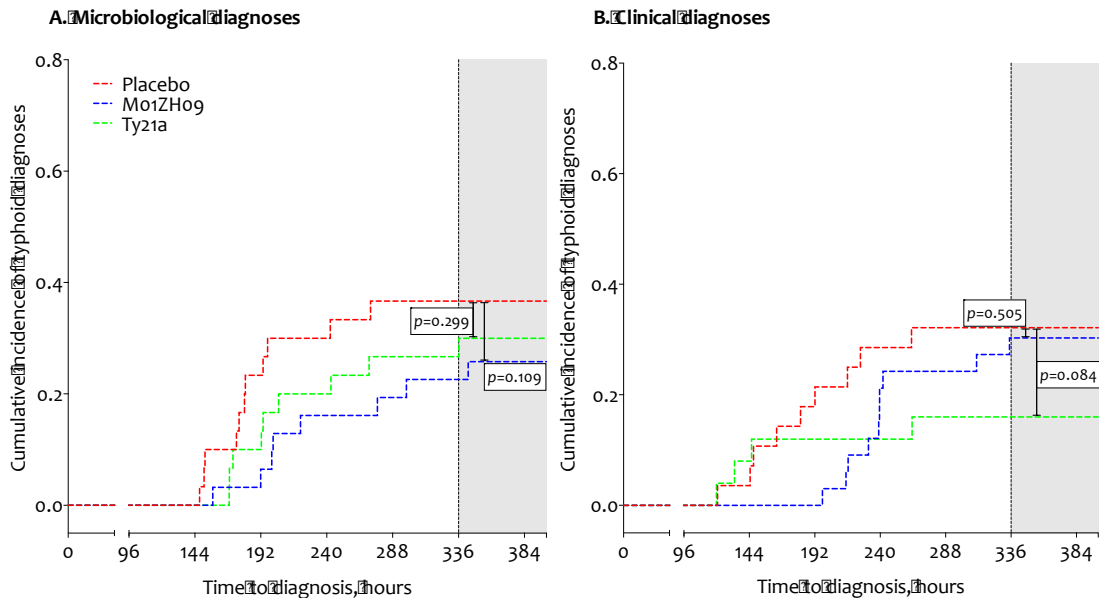


Figure 12-2. Cumulative incidence of A, microbiological, and B, clinical typhoid diagnoses, according vaccine allocation. Numbers at risk are as above. Grey shading, antibiotics being taken. Non-diagnosed participants were censored in the analysis at A: t=19 days (456-hours) and B: t=14 days (336-hours). Participants diagnosed with typhoid using different criteria to those in the plot were censored at the time of diagnosis. Log-rank test.

12.3.4 TIME TO BACTERAEMIA AND FEVER

Analyses of time to first positive blood culture and time to first temperature $\geq 38^{\circ}\text{C}$ supported these findings. Median [95%CI] time to first bacteraemia was 172 [111-233] and 342 [283-401] hours for placebo and vaccine recipients, respectively, and incalculable for the Ty21a group. Cumulative incidence of bacteraemia was significantly lower for both active vaccines compared to placebo (Figure 12-3a).

Median time to first measurement of oral temperature $\geq 38^{\circ}\text{C}$ was 10.5 [6.6-14.4] and 17.5 [14.9-20.1] days in the placebo and M01ZH09 groups, respectively, and was incalculable for Ty21a recipients. Despite some trend the curves were not significantly different by the Log-rank test (Figure 12-3b).

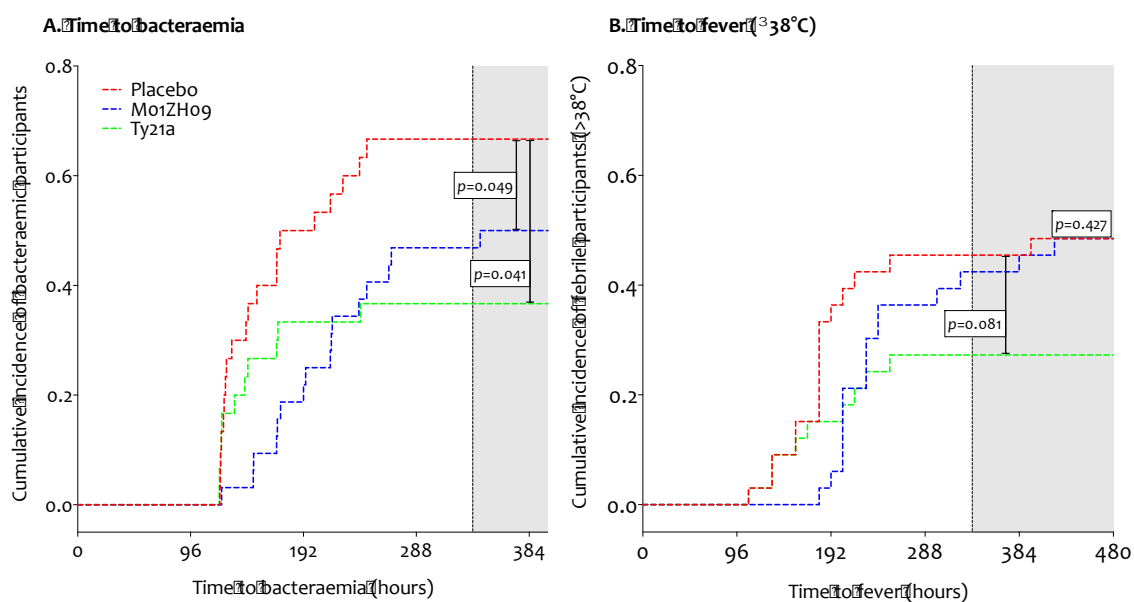


Figure 12-3. Cumulative incidence of A, any bacteraemia, and B, any fever ($\geq 38^{\circ}\text{C}$) irrespective of typhoid diagnosis, according to vaccine allocation.

Grey shading, antibiotics being taken. Non-diagnosed participants were censored in the analysis at A: $t=19$ days (456-hours) and B: $t=21$ days (504-hours). Log-rank test.

12.4 SENSITIVITY ANALYSIS

12.4.1 DIAGNOSTIC DEFINITIONS USED

The diagnostic temperature threshold used ($\geq 38^{\circ}\text{C}$ for ≥ 12 hours) with or without bacteraemia or bacteraemia alone were originally chosen based on data from the previous Maryland challenge studies and for safety reasons. A retrospective review of the data from Maryland by Glynn and colleagues demonstrated the range of attack rates that could be measured using alternative endpoint criteria in order to explore whether a dose-response relationship existed.⁹¹ The effect of endpoint definition was briefly explored during the preliminary dose escalation study, which similarly demonstrated a wide range of attack rates when different criteria were used.⁴³⁶

Exploratory sensitivity analysis around the definitions used for diagnosis demonstrated that small variations in definition resulted in a wide range of efficacy measurements (Figure 12-4). While efficacies ranged from 12.9-51.6% for M01ZH09 and 35.0-80.0% for Ty21a, levels of protection for M01ZH09 remained non-significant regardless of the criteria employed.

12.4.2 TEMPERATURE THRESHOLDS

Exploratory analyses of the temperature thresholds used similarly demonstrated a wide range of effects when used to compare the efficacy of each vaccine against placebo (Figure 12-5). At all temperature cut-offs, Ty21a demonstrated greater efficacy vs. placebo than M01ZH09 vaccination, although confidence intervals overlapped and became increasingly broad due to the rapid reduction in participants meeting fever thresholds.

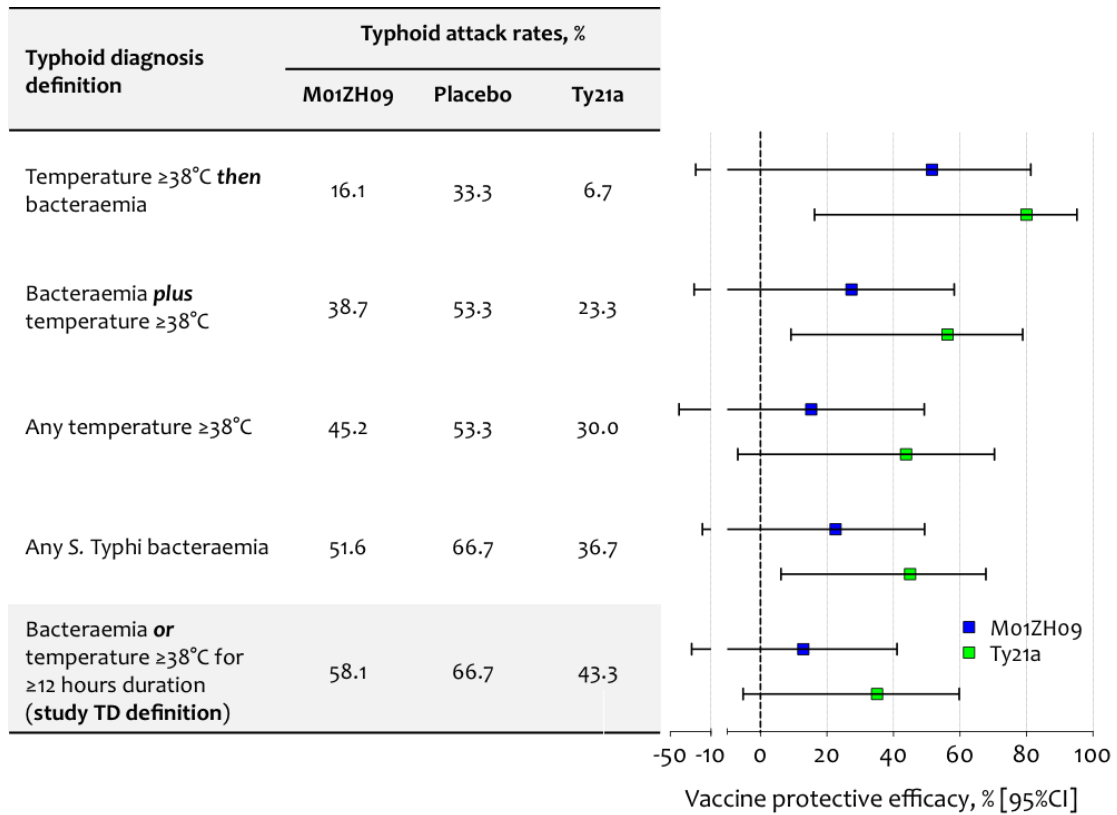


Figure 12-4. Sensitivity analysis demonstrating the variability of observed attack rates and calculated vaccine efficacies according to variations in the diagnostic definitions used.

Note, 'Bacteraemia or temperature $\geq 38^{\circ}\text{C}$ for ≥ 12 hours' is the definition used for the vaccine-challenge study. TD, typhoid-diagnosed.

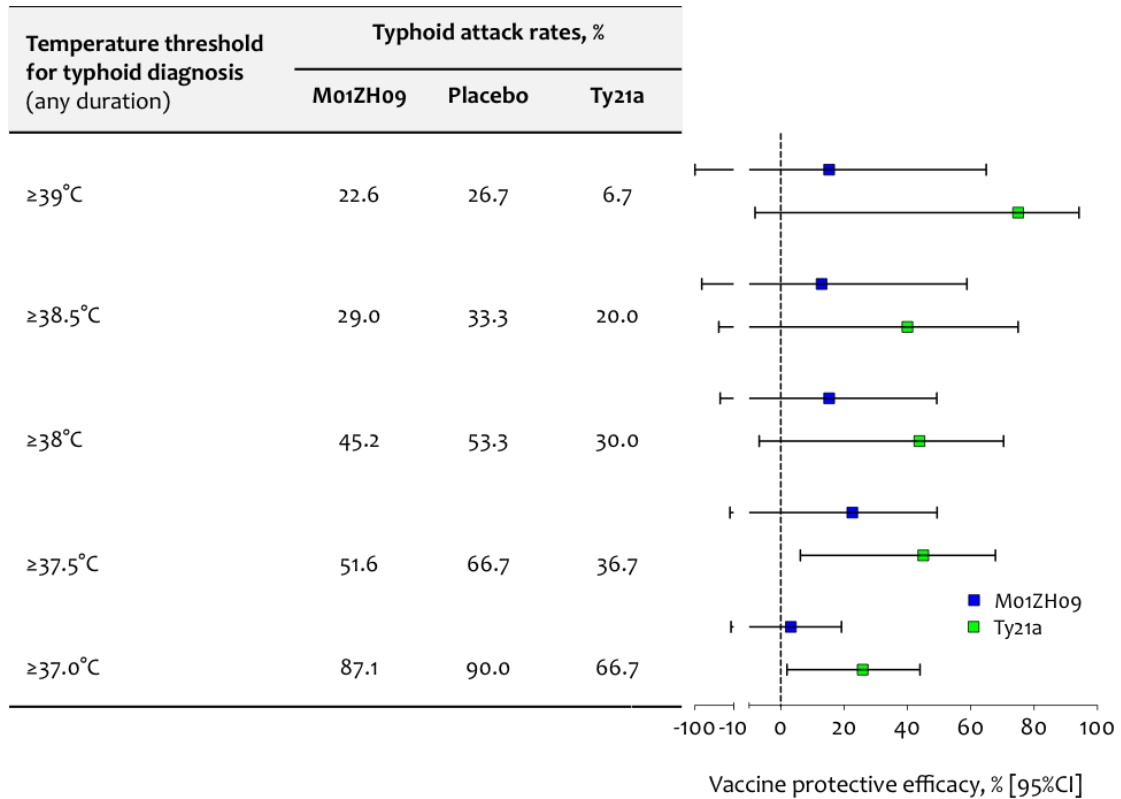


Figure 12-5. Sensitivity analysis demonstrating the variability of observed attack rates and calculated vaccine efficacies according to variations in the temperature thresholds used.

In order to take account of inter-individual variations in temperature patterns and lower values recorded due to the use of oral measurement, further analyses were performed using change in temperature over baseline readings (Figure 12-6). These data demonstrated no clear advantages over using absolute temperature in isolation, however given the greater proportion of placebo recipients who were diagnosed by blood culture (and therefore potentially treated earlier, before the opportunity for fever to develop; Table 12-3), comparative M01ZH09 efficacy was greatly reduced.

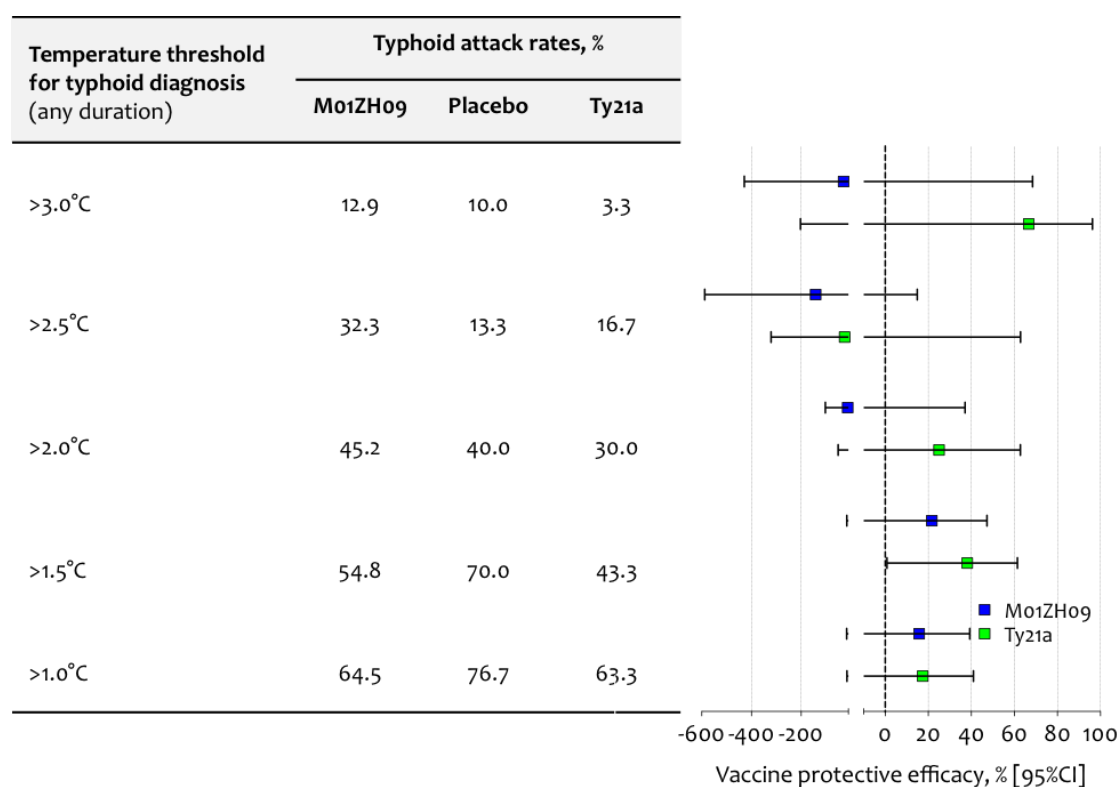


Figure 12-6. Sensitivity analysis demonstrating the variability of observed attack rates and calculated vaccine efficacies according to variations in temperature increments over baseline.

12.4.3 DIAGNOSTIC ASSAYS

Given the apparent insensitivity of blood culture for confirming typhoid diagnosis, further exploratory analyses were performed to assess the effect of alternative diagnostic assays and combinations thereof on calculated vaccine efficacy. Interestingly, while the apparent 'diagnostic sensitivity ranged widely giving resulting attack rates between 66.7-

90.0% in the placebo group, this resulted in little effect on calculated Mo1ZH09 or Ty21a vaccine efficacy.

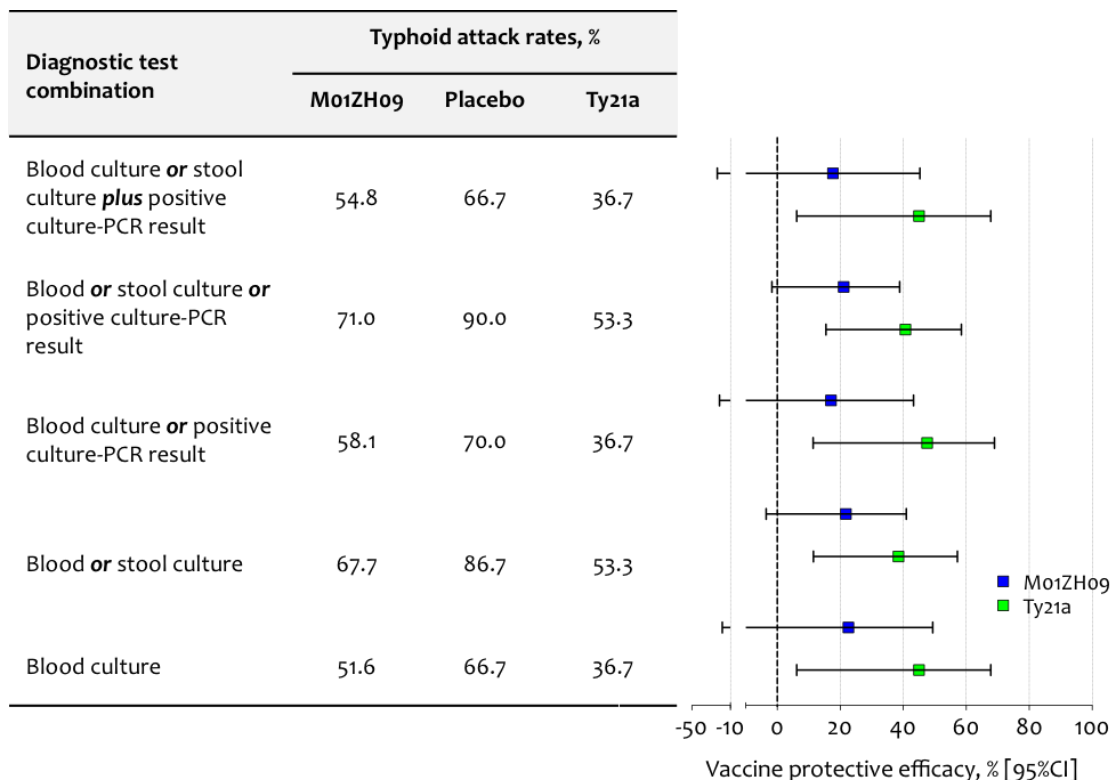


Figure 12-7. Sensitivity analysis demonstrating the variability of observed attack rates and calculated vaccine efficacies with different combinations of diagnostic tests.

12.5 EFFECT OF ANTIBODY TITRES ON CHALLENGE OUTCOME

To assess whether pre-existing immune status or the immune responses after vaccination affected outcome after challenge, exploratory analyses were performed looking for interactions between humoral antibody titres measured and the development of typhoid, time to bacteraemia and other markers of infection severity.

12.5.1 DEVELOPMENT OF TYPHOID AFTER CHALLENGE

Comparison of antibody titres measured before vaccination with those measured prior to challenge demonstrated significant increases in both anti-LPS and anti-H antibody titres as described in Chapter 9. No association between antibody titre and outcome after

challenge was found (Figure 12-9A&B), nor was any association between fold increase following vaccination and outcome (*data not shown*). Post vaccination increases in anti-LPS and anti-H antibody titres were significantly correlated with time-to-diagnosis however (Figure 12-8A&B). These effects were such that a 2x fold change in anti-LPS IgG after vaccination resulted in a 140-hour delay to diagnosis compared to having no vaccine response ($\text{Log}_{10}\text{fold change in anti-LPS titre} = 0.002141 * \text{Time}(\text{hrs}) - 0.2352$; 2-fold: 250 hours, 1-fold, 110 hours).

Anti-Vi IgG antibody titres were not affected by vaccination, however elevated baseline titres were associated with significant protection against development of typhoid after challenge (Figure 12-9C). A pre-vaccination titre $\geq 10 \text{ EU/mL}$ ($= 10 \mu\text{g/mL}$) was detected in 6/51 (12%) of those diagnosed and 14/40 (35%) of those without diagnosis. A high pre-vaccination titre was associated with a significant reduction in the relative risk of being subsequently diagnosed with infection (RR [95%CI]: 0.46 [0.23-0.92], $p=0.005$, Chi-squared).

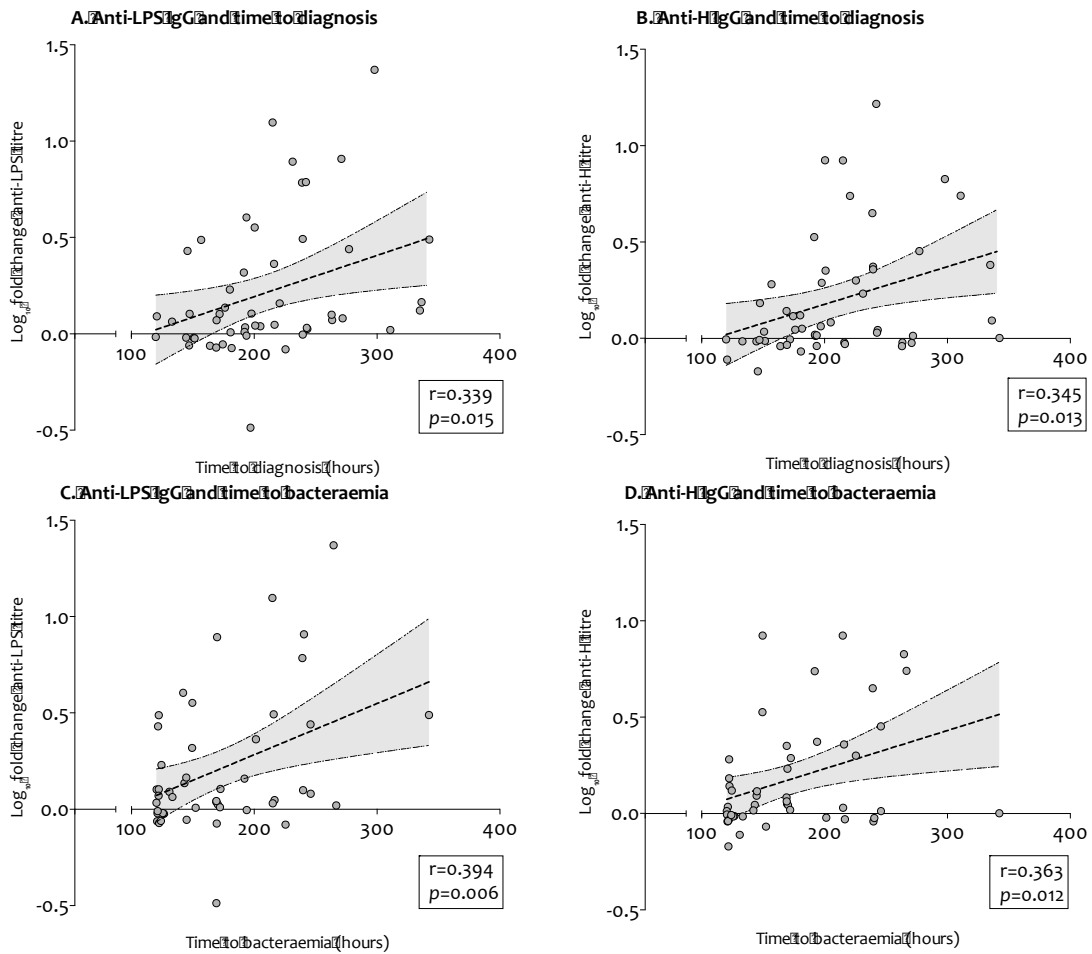


Figure 12-8. Effect of change in antibody titres after vaccination on A & B, time to diagnosis and, C & D, time to bacteraemia, after challenge with *S. Typhi*, Quail's strain. Pearson's correlation coefficient. Non-diagnosed participants excluded.

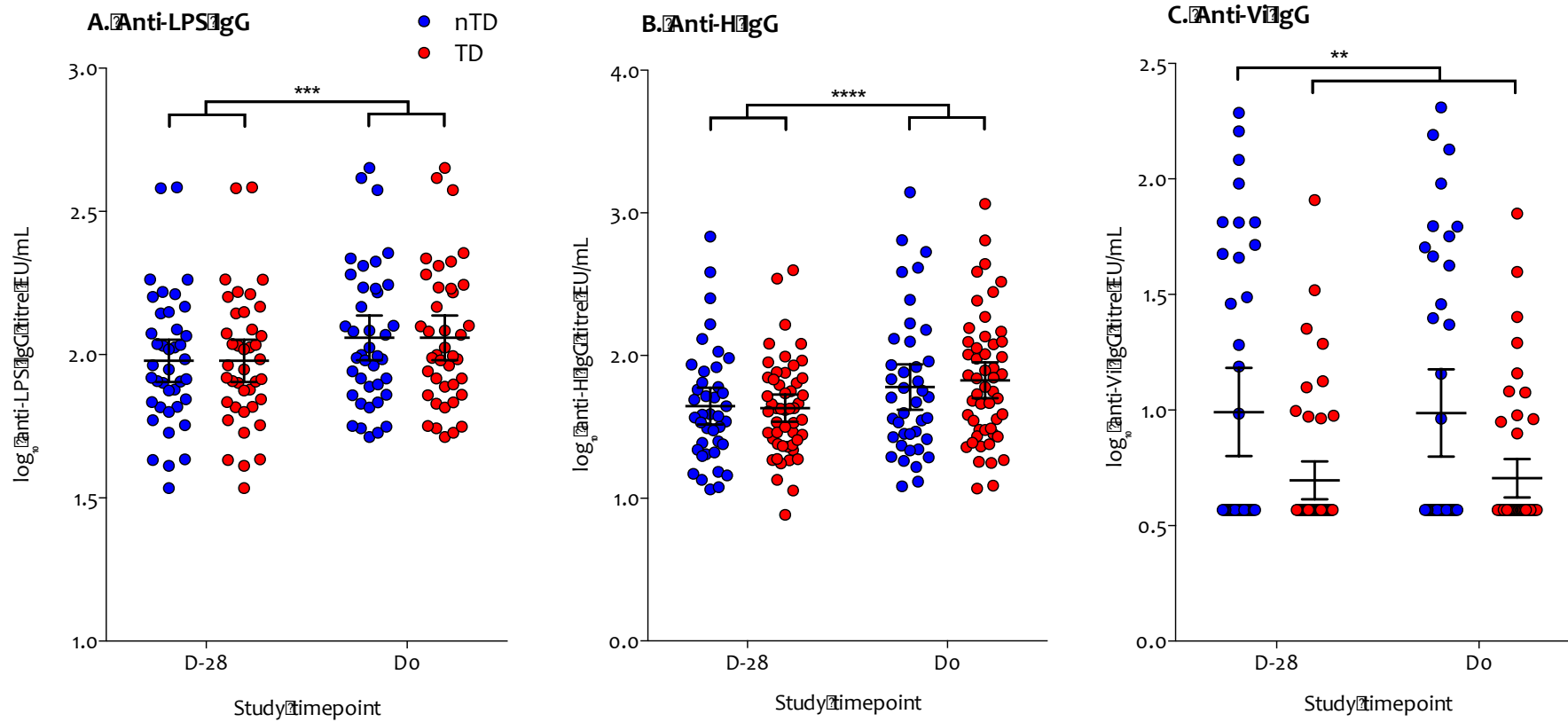


Figure 12-9. Pre-vaccination and pre-challenge IgG antibody according to subsequent challenge outcome.

Significant differences between time points and outcomes and their interactions were assessed by two-way repeated measure ANOVA. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

12.5.2 EFFECT ON DEVELOPMENT OF BACTERAEMIA

To further assess the protective role of antibodies in the development of infection, antibody titres prior to challenge were correlated with time-to-bacteraemia. While there was no correlation with antibody titres measured at Day 0 alone (*data not shown*), anti-LPS and anti-H IgG antibody responses to vaccination, measured as fold-change in titre between Day-28 and Do, were significantly positively correlated to time-to-bacteraemia (first positive blood culture taken; Figure 12-8C&D).

In most participants, even a modest increase in either anti-LPS or anti-H antibody titres seemed to result in low or non-detectable numbers of bacteria being found at diagnosis. Fold change anti-LPS IgG antibody response was significantly correlated with QBC at TD (Figure 12-10C, anti-H IgG increase was non-significant, $p=0.158$). Of note, this relationship became non-significant when zero QBC counts were omitted ($p=0.167$).

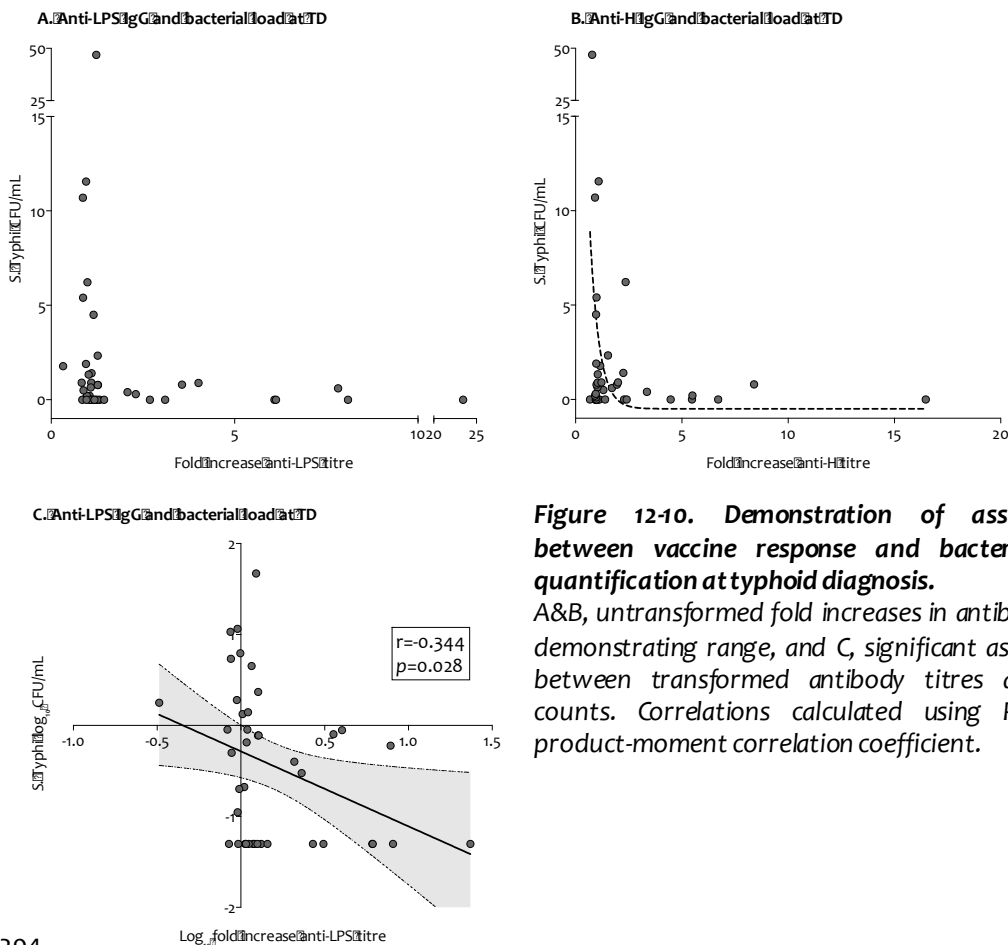


Figure 12-10. Demonstration of associations between vaccine response and bacterial load quantification at typhoid diagnosis. A&B, untransformed fold increases in antibody titre demonstrating range, and C, significant association between transformed antibody titres and QBC counts. Correlations calculated using Pearson’s product-moment correlation coefficient.

12.6 OTHER FACTORS AFFECTING SUSCEPTIBILITY TO/SEVERITY OF INFECTION

12.6.1 AGE AND SEX

Overall, outcome after challenge did not appear to be affected by participant age (median age, years [IQR]: nTD, 27 [22-43]; TD, 24 [21-39], $p=0.247$, MW). Raised pre-existing anti-Vi antibodies were significantly more common in younger study participants however ($V_i < 10$ EU/mL, 27 [21-44], $n=71$; $V_i \geq 10$ EU/mL, 22 [20-27], $n=20$; $p=0.005$, MW). Therefore in Vi-negative participants only, there was a significant difference in age between those who were not ($n=26$, median years [IQR], 39 [24-48]) or were ($n=45$, 25 [21-41]) diagnosed with infection ($p=0.041$, MW test).

There was no association found between gender and challenge outcome, either overall or when stratified by baseline anti-Vi antibody positivity (*data not shown*). No associations were found between age or sex and time to infection or bacteraemia or quantitative bacterial load at typhoid diagnosis (*data not shown*).

12.6.2 PREVIOUS TRAVEL

There were no significant associations found between history of previous travel to an endemic area (<6 months duration) and typhoid challenge outcome. Of note, there was also no significant difference in baseline anti-Vi antibody positivity between those who had travelled previously and those who had not.

12.6.3 CHALLENGE DATE AND DOSE

There was no significant association between date of challenge or challenge dose and typhoid challenge outcome either overall (Figure 12-11), or stratified by baseline Vi-positivity.

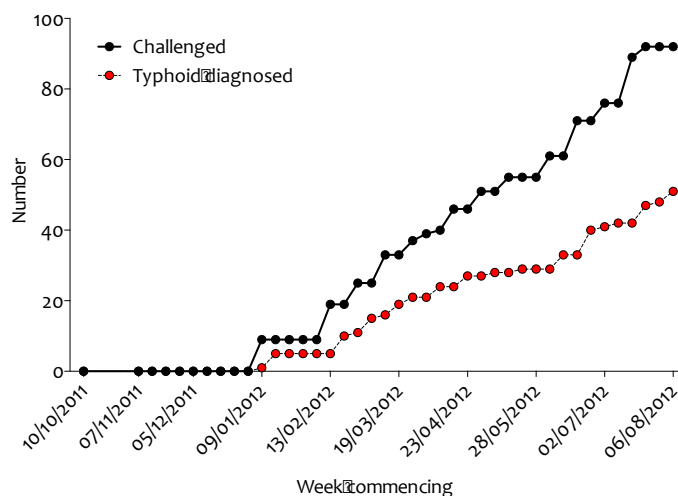


Figure 12-11. Summary of the rate of challenges and subsequent typhoid diagnoses made during the course of the study.

As noted previously there was a significant correlation between challenge dose and bacterial load at diagnosis overall. However, associations between bacterial load and time to infection or time to bacteraemia were found to be significant for only placebo recipients and not for those receiving active vaccination (see: 11.4.2).

12.7 SUMMARY OF FACTORS AFFECTING DEVELOPMENT OF TYPHOID AFTER CHALLENGE

12.7.1 REGRESSION ANALYSIS: TYPHOID DIAGNOSIS

Logistic regression analyses confirmed the protection conveyed by coincidental detection of anti-Vi antibodies at baseline against the subsequent development of typhoid after challenge (Table 12-4). Adjusting for this effect, increasing age was associated with a reduction in risk such that a person aged 40-years would have a 0.46-fold risk of developing typhoid infection compared to a 20-year old (OR 0.21 [i.e. 0.962^{40}]/OR 0.46). Vaccination with Ty21a conveyed a significant protective effect against infection after challenge compared to the placebo recipient reference group, while M01ZH09 did not.

Variable ¹	Odds ratio ²	95% CI	p
Baseline anti-Vi titre <10EU/mL	6.75	1.99-22.88	0.002
Age (per year)	0.96	0.92 - 1.00	0.042
Vaccine group			
Placebo ³			
M01ZH09	0.53	0.17 - 1.67	0.278
Ty21a	0.30	0.10 - 0.96	0.042

Table 12-4. Adjusted odds ratios for the effects of variables including vaccination on diagnosis of typhoid after challenge.

¹, Initial variables entered included gender and challenge dose; ², adjusted for other variables; ³, reference category.

Further analysis of the age effect by quartile, demonstrated an increasing protective effect with advancing participant age, such that the odds (95%CI) of being diagnosed with typhoid in the >39 year old age group was approximately quarter that of a participant <21 years old (OR [95%CI], 0.22 [0.06-0.83], $p=0.026$, adjusted for vaccine group and Vi antibody status).

12.7.2 COX PROPORTIONAL HAZARD ANALYSIS: TIME-TO-INFECTION

Exploratory analysis of factors affecting time-to-diagnosis was performed using Cox proportional hazard regression. This demonstrated that significant factors affecting the hazard ratio included age category, baseline Vi antibody status and vaccine group allocation (Table 12-5), but not fold-change in antibody response or challenge dose ingested.

Risk factor		N	Hazard ratio (95%CI)	p
Baseline anti-Vi titre	<10EU/mL	71	ref	
	≥10EU/mL	20	0.25 (0.10-0.61)	0.002
Age	<21	27	ref	
	21-26	18	0.78 (0.36-1.66)	0.511
	26-39	20	0.58 (0.26-1.29)	0.181
	>39	26	0.45 (0.22-0.93)	0.032
Vaccine group	Placebo	30	ref	
	M01ZH09	31	0.49 (0.25-0.93)	0.029
	Ty21a	30	0.43 (0.21-0.88)	0.022

Table 12-5. Variables and hazard ratios for risk factors of developing typhoid after challenge with *S. Typhi*, Quailles strain.

This confirmed the effect of increasing age on reduction in typhoid risk, and demonstrated that the risk of typhoid in active vaccine recipients (M01ZH09 or Ty21a) was approximately half that of placebo recipients when age and baseline anti-Vi status was adjusted for. This model also demonstrated that there was no evidence for a difference in effect between M01ZH09 and Ty21a vaccine receipt (Figure 12-12), although the sample size was smaller than may have been optimal (assuming 3 predictors and 0.44 negative case proportion, $n=132$ would have been optimal).

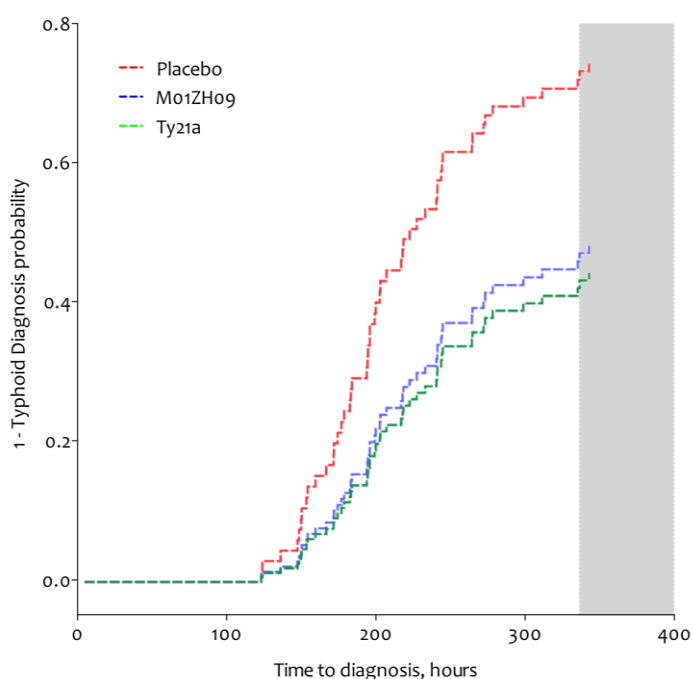


Figure 12-12. Cumulative hazard risk (of typhoid diagnosis) according to vaccine allocation, for the mean of all other covariates (age group and baseline Vi antibody status) in the model.

12.7.3 EFFECT OF SUSCEPTIBILITY FACTORS ON ATTACK RATES AND VACCINE EFFICACY MEASUREMENT

The contribution of factors found to significantly influence the diagnosis of typhoid after challenge was assessed in relation to attack rate and calculated vaccine efficacy. While the attack rates were higher in the population with a baseline anti-Vi IgG titre of <10 EU/mL, increasing to 77.2% in the placebo group, there was a clear distinction between older and younger study participants (Figure 12-13). Using the Vi-negative subgroup

median of 27-years, those <27 and receiving M01ZH09 had a very high risk of infection, whereas those >27 were relatively well protected. Both of these observations were in contrast to the Ty21a group, in whom vaccination appeared to be more effective in the younger age group.

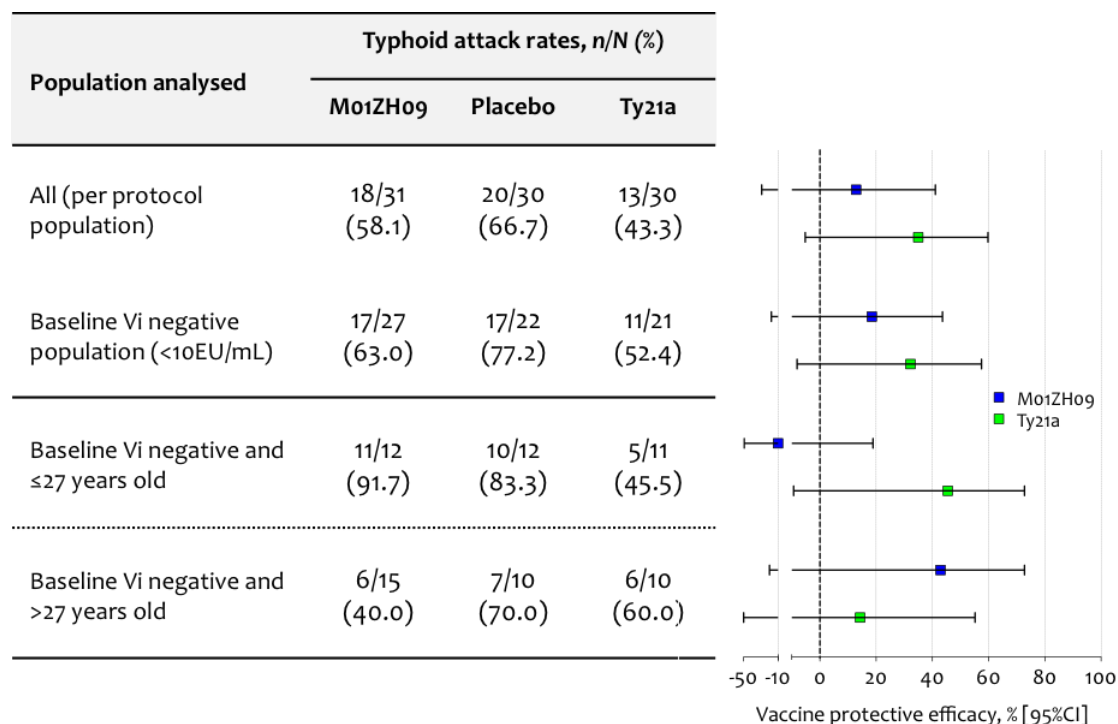


Figure 12-13. Effect of susceptibility/protection criteria on measured attack rates after challenge and calculated vaccine efficacy.

12.8 DISCUSSION

Human challenge studies present a unique opportunity to investigate immunobiological host-pathogen interactions and the subsequent evolution of clinical infection. These studies are particularly vital for human-restricted infections in the absence of convincing animal models. Using such challenge models for the assessment of vaccine efficacy has proven a powerful and cost-effective means to measure protective efficacy in a highly-controlled setting, prior to progressing to large-scale field trials. The intricacy in performing such studies cannot be overlooked, however, and, as demonstrated in the data presented in this chapter, minor alterations to population susceptibility, immune

characteristics, challenge dose and disease definition may produce widely differing estimates.

12.8.1 VALIDITY OF THE CHALLENGE MODEL

The underlying rationale for including Ty21a as a licensed comparator vaccine was to allow comparison with the previous Maryland challenge studies in addition to the broader literature regarding its efficacy. In Maryland, the efficacy of Ty21a (given in 5-8 doses in milk after ingestion of 2g/60mL NaHCO₃ at intervals of 3-4 days) against challenge with 10⁵CFU *S. Typhi* Quail's strain (given in 45mL milk and administered 5-9 weeks after vaccination) was found to be 87% compared to unvaccinated controls.²⁵⁷ Efficacy of Ty21a in the large field-trials subsequently performed demonstrated superior protection from an enteric coated (EC) formulation, for example 71.2% efficacy against natural infection in the Area Occidente region of Santiago, Chile in the first year of follow-up.²⁶⁹

Despite these apparently high efficacy results, however, the performance of Ty21a in preventing infection has varied and appears to depend on the formulation and dosing schedule in addition to the background force of infection and age group under investigation. In the Indonesian field trial, for example,²⁷¹ significant efficacies were only found in younger age groups and protection was higher for the liquid formulation than for the enteric-coated capsule (the latter being used in this current study). Vaccine efficacy ranged from 52.7% (95%CI: 23.9-58.6) in 3-19 year-olds given 3-doses of liquid Ty21a to 23.6% (-78.8-67.3) in 20-44 year-olds given 3-doses of EC capsules during 30 months of follow-up. Reasons given for this included lower attack rates in the older placebo group and smaller sample sizes, which nevertheless numbered 3965 individuals (18 cases) in the control group and 2018 (7 cases) in the EC vaccine group.²⁷¹ In general a much higher attack rate was seen in Indonesia compared to the previous studies (Indonesia:

1206/100,000; Egypt: 49/100,000; Chile: 103/100,000) which may have overcome any possible protective immune advantage conferred by vaccination, although differences either in circulating *S. Typhi* strains or in vaccine strain/production may also have been important.²⁷¹ The close phylogenetic relationship of the Quailles strain to those from Indonesia (Figure 5-2) may indicate higher underlying virulence of these particular strains.

As may be seen from the sample size calculations (see: 5.5.2) choice of a higher challenge dose and thus higher attack rate in the placebo vaccine recipients is a careful balancing act. Lower attack rates and thus larger sample sizes become logistically and ethically progressively more difficult to rationalise (for example in exposing unnecessary numbers of participants to a pathogen) while the higher attack rates and smaller sample sizes risk overwhelming any potentially protective vaccine-derived immune responses. This was seen during evaluations of the early whole cell K and L vaccines in the Maryland studies (*discussed further in Chapter 12*).^{89,359}

A further key feature to take into account is the timing of challenge after vaccination. Although this has not been systematically assessed, there is, for example some field trial evidence that the protective efficacy of oral vaccination is durable and even increases over time, possibly due to on-going boosting by natural exposure or persistence of antigen at reticuloendothelial sites such as the regional lymphnodes.²⁷² Conversely, challenging too soon after vaccination may not allow adequate time for the cell-mediated and humoral responses to mature and may not reflect a fair assessment of vaccine benefit.²⁸⁰

Overall therefore, a protective efficacy of 35.1% found for 3-doses of EC Ty21a given to naïve adult volunteers in this study, suggests that while the exposure dose is likely to be equivalent to more highly endemic settings, the model does represent a valid, if stringent, model of natural typhoid infection.

12.8.2 EFFICACY OF M01ZH09

A single dose of M01ZH09 given as an oral suspension failed to demonstrate any protective efficacy against the predefined typhoid diagnosis endpoint. This is perhaps not unexpected given the task of generating significant levels of protection after ingestion of a single dose. Poor protective responses to oral vaccination are compounded in endemic settings where, for unknown reasons, immunogenicity to enteric vaccine is diminished.¹²⁰ Notably, when given as a single dose during a further study in Santiago, Chile, EC Ty21a only provided 25% efficacy in the first year and 35% in the second – both were non-significant.

There may be multiple reasons for the overall inefficacy of M01ZH09 in preventing typhoid in this challenge model, however, the delay in onset of clinical illness and reduction in bacteraemia and peripheral blood bacterial load suggest that, even after one dose, some significant anti-bacterial protective effects were seen. Alteration or at least delay in disease presentation is often used as a surrogate marker of vaccine efficacy. For example in the controlled human malaria infection (CHMI) setting, delay in time to patency of malarial sporozoites is often used for comparative assessments vaccine candidates.^{392,393}

Although not formally compared, the difference in disease profile (onset, duration, bacteraemia etc.) between M01ZH09 and Ty21a recipients is noteworthy. Those Ty21a participants who developed typhoid became ill early during the challenge period and in a manner more consistent with placebo than M01ZH09 recipients. This suggests a possible underlying difference in mechanism of immune response in these participants. Given that both active vaccines were able to limit bacteraemia possibly through the antibody-mediated responses measured or alternative protective pathways (cytotoxic T-lymphocytes, for example), this may indicate a difference in the ability to control

intracellular bacterial replication during the early stages after invasion, i.e. a difference in mucosal, innate or cell-mediated immunity.

12.8.3 EFFECT OF DIAGNOSTIC DEFINITION ON ATTACK RATE MEASUREMENT

One possible reason for the apparently high protective efficacy demonstrated by Ty21a in the previous Maryland studies, might be the stringency of the diagnostic criteria used in the current study. Using higher temperature thresholds (>39.4°C for ≥24-36hours was used in the Maryland studies),^{90,257} for example, would only include the most severe disease phenotypes, and thus effectively overestimate vaccine efficacy. Previous work performed by Glynn and colleagues retrospectively recalculated attack rates of the Maryland typhoid challenge studies, demonstrating the effect of slight variations in measurable attack rates.⁹⁰ Using an apparently robust definition such as the one chosen *a priori* for this study, leaves room for potential missed diagnoses particularly of more mild infection. Conversely more flexible definitions potentially over ascribe participant symptoms to being related to challenge, which was a significant concern when re-establishment of the Maryland model was being considered using ambulant outpatients.^{96,425} The potential for challenged participants to develop symptoms and fever due to, for example, circulating upper-respiratory tract infections rather than typhoid and thus erroneously get included in calculating the attack rate has led to the more cautious approach taken in development of the Oxford model.⁴³⁶

Data presented in this chapter demonstrate that, were more restrictive definitions for typhoid diagnosis to be used, such as the requirement for a temperature prior to bacteraemia, a lower attack rate would be measured particularly among the vaccine recipients. This would result in a higher measured protective efficacy – this is relevant, as these definitions would be similar to those used for passive surveillance following vaccine introduction in a field trial setting.¹⁹³ Which definition is used is best guided by the

purpose of the study. While a more stringent passive surveillance definition may be useful to calculate future efficacy in a field setting, the development of novel sensitive antibiotic assays relies on much less stringent criteria and the ability to detect all possible cases. Reporting a range of outcomes is likely to be most useful in the context of performing future studies, and uniformity across studies would allow more accurate comparisons (or meta-analyses) to be made between vaccine efficacies in the context of human challenge models.

12.8.4 TEMPERATURE THRESHOLDS

Symptom reporting has not been included in any of the typhoid diagnosis definitions used in challenge studies performed to-date, although symptoms requiring antibiotic initiation was one of the endpoints studied by Glynn and colleagues.⁹⁰ Given the highly subjective nature and inter-personal variability in reporting this would be difficult to quantify, as reflected by the frequency of symptoms reported in this study following placebo vaccine ingestion (Chapter 7). Oral temperature measurement is more objective and easily measurable although still subject to diurnal and interpersonal variation. Improved accuracy of modern oral thermometers should assist in the detection of more subtle increases and therefore detection of potential cases during the earlier stages of disease. These data presented support that a single measurement of a temperature $>37.5^{\circ}\text{C}$ may allow discrimination between those infected/non-infected while retaining adequate specificity for detecting true cases. Limitations to using single measurements of lower temperatures thresholds include the possibility of one-off erroneous readings and earlier abrogation of the clinical course; however, such measurements may be useful as an early predictor of infection onset. Evening measurements may be particularly informative – the link between circadian rhythm and typhoid infection onset requires further investigation, and this model may be a useful setting to investigate the circadian

alteration in gene expression and the apparent link between circadian dynamics, melatonin and endotoxin responses.⁵⁴⁰⁻⁵⁴² Interestingly, loss of the diurnal temperature cycle may be an early indicator of infection developing and also merits further investigation for use as an early marker of impending typhoid onset.

12.8.5 EFFECT OF COMPOSITE DIAGNOSTICS ON ATTACK RATE MEASUREMENT

As has been described in detail (see: 2.5), diagnosis of a patient with typhoid fever is complicated by the non-specific presentation and inaccurate diagnostic tests available. Despite the near optimal conditions for performing blood cultures in this study setting (no prior antibiotic exposure, automated culture machine, high blood volume etc.), we still failed to demonstrate bacteraemia in several participants who were symptomatic and febrile. Use of composite diagnostic endpoints using combinations of diagnostic tests, given the absence of a gold-standard, has been proposed and used with success in several endemic settings and may also usefully be applied to the challenge study setting.^{537,538} Here, sensitivity analysis demonstrated the beneficial effect of culture-PCR either in addition to routine blood culture or as a confirmatory test to a positive stool culture result in increasing the apparent sensitivity of detection by identification of cases at the 'mild' end of the disease spectrum referred to above. Choice of which set of tests might produce optimal case detection at least in the challenge study setting may be improved using methods such as latent class analysis.

12.8.6 ANTIBODIES AND BACTERIA

M01ZH09 is highly immunogenic and resulted in significant increases in anti-LPS and anti-flagellin antibody-secreting cells and antibody responses in the post-vaccination period of this study (Chapter 7). These data presented in this chapter demonstrate that even small (~1.5x fold) increases in antibody titres significantly reduce the bacterial load detectable in

the blood stream. While the role of anti-LPS response as a correlate of protection against infection remains unproven, the evidence for correlation in fold-change antibody titre (thereby presumably increase in *S. Typhi*-specific LPS only) and prolongation of the incubation period and reduction in measurable *S. Typhi* bacterial load demonstrates its disease-modifying potential. This is supported by several observations including the superior efficacy of *S. Typhi*-derived Vi polysaccharide for vaccination compared to that obtained from *Citrobacter freundii* 5396/38, which is thought to be due to inadvertent LPS contamination of the *S. Typhi* vaccine.^{144,286} The previously established relationship between anti-LPS antibody titres and protection in the field setting may therefore either reflect evidence of other, true underlying host-immune protective responses or be a function of the bacterial-lowering effect, thereby reducing the sensitivity of confirmatory blood cultures in field-settings and ability to detect ‘mild’ disease phenotypes.¹⁴²

The protective effect of anti-Vi antibody in preventing typhoid fever has been inadvertently and uniquely demonstrated by the findings of this vaccine study. Early attempts to develop a Vi vaccine and subsequent chimpanzee and human challenge studies to assess its efficacy were unremarkable,^{284,359,543} demonstrating only 25% protection in the Maryland challenge studies.⁸⁹ Reasons for this were obscure at the time, but thoroughly investigated subsequently by Robbins and colleagues; the acetic acid reflux process resulted in denaturation of the Vi-capsular polysaccharide thus reducing the immunogenicity of the vaccine in mice, chimpanzees and humans (see: 2.7.4).²⁸⁶ Despite the screening procedures used in this study and careful selection of infection and vaccine-naïve participants, 21/91 (23%) were found to have detectable antibodies $\geq 10\mu\text{g/mL}$, a level consistent with a recently suggested threshold for protection.³⁰² Participants with pre-challenge titres over this threshold were significantly protected against getting infection in the context of this challenge study (*further discussed in Chapter 12*).

12.8.7 OTHER FACTORS

A further major finding from the analyses presented here is the contribution of other factors in determining the outcome after typhoid challenge. Age, for example, is known to underpin much of the variation in disease burden between different settings, presumed to be due to alteration in behaviour and acquisition of exposure or occasionally vaccine-derived immunity. The findings from this study suggest that additional features may underlie this observation including the apparent decline in febrile response with increasing age (see: 10.7.1). The findings in this chapter also suggest that even in non-immune individuals age may play a role in determining the outcome after exposure. That this effect remains significant after adjusting for vaccine receipt, takes into account underlying immunologic mechanisms such as boosting of vaccine response due to previous antigen exposure – this has been proposed in field trials of Ty21a to support the apparent increased efficacy of Ty21a (and whole cell vaccine) in older age-groups.²⁵⁵ The apparent protective effect of age may therefore be due to detection bias (lack of fever response masking more mild disease phenotypes), non-vaccine related changes to host response (such as diet or activity, or innate immune effects) or bias due to small sample size. Interestingly, a single dose of M01ZH09 was possibly more effective in protecting older participants. This may support an additive role for the humoral immune responses generated in addition to some other underlying immune or behavioural factors.

12.8.8 SUMMARY

Vaccination with a single-dose of oral M01ZH09 vaccine, while immunogenic, resulted in no protection against challenge with 1.5×10^4 CFU *S. Typhi*, Quail's strain. Comparative evaluation with licensed Ty21a suggested that additional factors including unrecognised baseline immunity and age in addition to the challenge dose used might have resulted in lower than expected protection against challenge. M01ZH09 did demonstrate a

significant disease altering affect however, and, with all other factors being equal, vaccinated recipients had similar risk profile after challenge. Whether additional doses, alternative formulations, assessment with a lower challenge dose or selection of a different participant demographic would result in measurable protective efficacy remains tantalisingly unknown.

Chapter 13. DISCUSSION

During their relatively brief, 50,000 year co-existence, *S. Typhi* has been a scourge of humankind. Before knowledge of hygiene and the advent of sanitation practices, typhoid affected rich and poor alike. In the 21st century it is either those who are sufficiently affluent and can afford to travel or the very poorest sections of endemic communities who are most affected. As a human-restricted pathogen with no environmental reservoir, *S. Typhi* has the potential to be much better controlled, if not eradicated, from both settings.

For progress to be made in controlling typhoid infection, an improved understanding of disease pathogenesis, human immunobiological responses and typhoid epidemiology are urgently required. Improving both our understanding of typhoid fever and methods for its control are hindered by inadequate diagnostics and delays in vaccine development, not least due to the lack of a convincing animal model of infection. The rapid emergence and intercontinental spread of antibiotic-resistance and suspicion of a large and under-recognised burden of infection in Africa mandate decisive action.

In this thesis I have described the application of a human typhoid challenge model to specifically evaluating the protective efficacy of oral typhoid vaccines, including a novel, single-dose vaccine candidate M01ZH09, and their associated effects on bacterial dynamics and the host's response to infection. In this process numerous further findings have been made regarding methodological issues relating to challenge design, participant selection and choice of study endpoints. These data should contribute towards improved understanding of typhoid fever and translate into better mechanisms for its detection, control and eventual eradication.

13.1 SUMMARY OF MAIN FINDINGS

13.1.1 CULTURE-PCR DIAGNOSTICS

Currently available diagnostic tests for typhoid infection are limited in sensitivity and specificity. PCR-based approaches have had some success in laboratory studies, but have frequently been unsuccessful when applied to patient samples, likely due to the low bacterial load present at symptom onset.

The development and evaluation of a novel culture-PCR assay described in Chapter 7 demonstrated sensitivity and specificity, at least in this highly-controlled study, and may be a useful adjunct to performing routine blood culture alone in clinical settings. PCR-based diagnostics have to-date performed poorly in detecting bacterial DNA in patient specimens, and therefore these findings are promising in developing a suitable assay for use as a clinical diagnostic tool. In addition to confirming the diagnosis in non-bacteraemic clinically-diagnosed cases, performing this sensitive culture-PCR assay in a challenge study has demonstrated the first objective evidence of the hitherto hypothesized primary bacteraemia that occurs soon after *S. Typhi* ingestion. While transferability and utility of the culture-PCR assay in an endemic-setting laboratory may

be less certain, this assay has provided new insights into bacterial dynamics and the delineation of clinical disease phenotypes, illustrating the useful application of human challenge models to the discovery and development of novel diagnostic approaches.

13.1.2 VACCINE-CHALLENGE STUDY CONSIDERATIONS

Human challenge studies are often perceived as high-risk and offer seemingly little incentive to volunteers. Recruiting participants is therefore often highly logistically intensive to ensure that a healthy, well-informed cohort is screened and selected for study inclusion. There were several specific additional considerations for this vaccine-challenge study: it was the first human challenge study using a CL3 pathogen to apply for regulator (MHRA) approval in the UK; it required additional approvals for deliberate release of a genetically-modified agent (M01ZH09 vaccine); and, to-date it is the largest single challenge study ever performed. The availability of a challenge agent (the Quail strain) that had a long and documented history of use in such studies, and its production to GMP-standard undoubtedly assisted regulators with the approvals process.

Likely due to the inclusion/exclusion criteria, the majority of study participants were young, white and male and, while not fulfilling criteria for exclusion, many had travelled to typhoid-endemic regions previously. Participants from a wide-range of ages, occupations and backgrounds were included in the study, however, with many volunteers commenting that they had not previously been approached to take part in clinical research trials. The enrolment of older adults coincidentally allowed me to demonstrate an apparently age-related protective effect against the development of infection, which was unexpected in this setting but is an opportunity for further detailed investigation. Due to the nature of the study and study participants (most being students), long-term retention to the study was low, with many travelling out of the area (and overseas). This should be considered in future vaccine-challenge study designs. By

their nature these studies are intensive for participants also but require time to allow responses to vaccines to develop and to measure the durability of those responses. Weighting compensation to the latter visits and keeping studies to ≤ 1 -year in duration may increase participant recall.

13.1.3 VACCINE IMMUNOGENICITY

Assessment of vaccine efficacy by challenge provides a direct measure of the degree of protection afforded by vaccination. A major additional benefit is the opportunity to identify and validate potential biomarkers or responses correlating with subsequent protection. The majority of putative correlates reflecting oral vaccine efficacy in field-settings relate to measurement of the humoral response to the major surface-expressed antigens, not least because these measures are more easy to standardise and utilise in new vaccine design and development pathways.¹⁴¹

Both Mo1ZH09 and Ty21a vaccines were well tolerated and generated significant B-cell (ASC) and humoral (antibody) responses. It is interesting to note that in the previous field-trials comparing enteric-coated and NaHCO₃/gelatin formulations of T21a, that rates of anti-LPS seroconversion to the former resulted in a dose-response with efficacy but not the latter.¹⁴² Whether administering vaccine with NaHCO₃ may therefore result in higher humoral immunogenicity but at the expense of the development of protective mucosal/cell-mediated responses, which may do more to protect from subsequent infection may be worth further investigation.

In comparison with previous studies of Mo1ZH09 immunogenicity, the proportion of responders to vaccination was slightly lower than that found in other populations. Reasons for this are speculative, but may include differences in the ages, background exposures or genetic differences in the populations studied (USA, Vietnam, UK), or

vaccine strain viability/formulation differences. Whether this finding had an impact on vaccine efficacy is not known; however, even moderate increases in anti-LPS IgG antibody titre resulted in significant reductions in bacterial load.

The high proportion of participants with measurable levels of anti-Vi antibody at baseline was unexpected. This finding was in contrast to the preliminary dose-escalation study, which, while differences in participant selection are possible, is likely to be due to a change in the assay used. Previously, an in-house ELISA was developed while a standardised commercial assay (developed in collaboration with the NIH/NIBSC) was used in the vaccine-challenge study.^{436,451} While responses were unaffected by the vaccines received as part of the study, participants with measurable Vi titres were significantly protected against challenge (*see below*). There are two important implications of these findings for future vaccine-challenge studies; firstly, that design of selection procedures should consider including anti-Vi IgG measurement (as either an inclusion or exclusion criteria) or that the findings should be stratified according to baseline anti-Vi antibody status; and secondly, that studies of anti-Vi antibody-generating vaccines (either live oral, ViPS or ViPS-conjugates) are likely to demonstrate a significant protective advantage even with the relatively high challenge dose used here (*see below*).

13.1.4 CHALLENGE DOSE AND ATTACK RATES

Key elements in designing a vaccine-challenge study include participant and challenge strain characteristics, the study endpoints chosen and the route and dose of challenge administration.⁹⁶ Each of these may be controlled or varied; to establish the optimal *S. Typhi*, Quail's strain, challenge dose used in this study a preliminary dose-escalation study was performed.⁴³⁶ The target attack rate chosen for both studies (placebo recipients in the vaccine-challenge study) was 60-75% according to the diagnostic criteria chosen; this had to be balanced against the sample size selected (making the study more

logistically complex, more expensive and potentially unnecessarily exposing more individuals to a pathogen) and possibility of overriding any beneficial vaccine-derived protective immune responses by using too high a challenge dose.

Using the same 1.5×10^4 CFU-target challenge dose (actual mean dose given, 1.85×10^4 CFU) in the vaccine-challenge study reproduced the 65% attack rate found in the preliminary dose-escalation study. This, together with the measured incubation periods (IPs) supports the assertion that the model is reproducible. The IPs, bacterial loads measured at diagnosis and clinical symptomatology (and culture-PCR positivity rate) also confirm that the model is consistent with high-level exposure in an endemic setting or the host-bacterial interactions seen in paediatric populations. Repeating the attack rate found in the dose-escalation study also provides confirmation of the sample size calculation used there, which may be important for the development of further challenge models (see *below*).

13.1.5 CHALLENGE MODEL VALIDITY

Three doses of Ty21a were given as a positive control to assess the validity of the challenge model in assessing vaccine efficacy. The attack rate in Ty21a recipients (43.3%) resulted in a calculated vaccine efficacy of 35.1%, which was lower than anticipated and suggested that this model is probably quite stringent. There are multiple examples from the studies performed in Maryland that demonstrate vaccine efficacy but when only using the ID₂₅ dose (1×10^5 CFU); in most cases the ID₅₀ dose negated any benefit from vaccination, and infection onset was even noted to occur earlier than in some unvaccinated challenged participants.^{123,257,265,282,284,358,359,415} Of note, in Maryland the ID₅₀ dose resulted in infection in 65% of participants while the ID₂₅ dose resulted in a 40% attack rate.³⁵⁸ There was no evidence of dose-related worsening of symptoms at typhoid diagnosis in the Maryland studies, or in the preliminary dose-escalation study performed

in Oxford.⁴³⁶ Once participants became ill there was little to separate them. These findings mirror those from the murine-*S. Typhimurium* model, in which, once bacteraemia develops, histological changes and mortality rates are the same.³⁵⁹

Interestingly, analysis of two symptom sets (number and duration) from the non-typhoid diagnosed population in this study independently provides some evidence that the EC₅₀ (~ID₅₀) dose was approximately 1.9×10^4 CFU. This was verified by investigating the dose-response relationship between bacterial load and challenge dose, which similarly (and independently) gave an EC₅₀ dose of $\sim 1.9 \times 10^4$ CFU. These data suggest that, had a challenge dose nearer to 1×10^4 CFU been used, i.e. almost half that of what was given, that increased and more significant protection by Ty21a vaccination compared to placebo may have been seen. A target challenge dose of 1×10^4 CFU would indicate that the effect of NaHCO₃ pre-ingestion and its effect on gastric acidity is to lower the ID₅₀ challenge dose 1- \log_{10} compared to the historical Maryland studies (which used a milk buffer).

13.1.6 HUMAN RESPONSES TO INFECTION

The human host's symptomatic and physiological responses to *S. Typhi* infection have been relatively poorly defined, due in part to late presentation and difficulties in accurately diagnosing true cases of infection. This vaccine-challenge study provided an ideal opportunity to characterise the clinical syndrome in detail and investigate whether oral vaccination had any effect on illness presentation.

Participants developed typhoid infection from Day 5 onwards with Mo1ZH09 recipients having a delayed onset of clinical infection. The symptoms/signs elicited from typhoid-diagnosed participants were similar regardless of vaccine receipt, and responded quickly to treatment initiation, albeit in a biphasic response with a secondary peak of temperature and heart rate increase 6-12-hours following antibiotics commencing. While

non-diagnosed individuals remained relatively asymptomatic, the placebo recipient group demonstrated two peaks of increased symptom reporting. These were commensurate with their typhoid-diagnosed counterparts possibly indicating underlying protective host mechanisms, or responses to release of bacteria/bacterial products from the RES.

13.1.7 BACTERIAL DYNAMICS AFTER CHALLENGE

While much has been learnt from the previous studies performed in animal models and the Maryland human challenge model, much still remains unknown regarding the natural history of *S. Typhi* infection. The development of more accurate and automated culture systems in addition to newer PCR techniques allows a more accurate assessment to be made of bacterial invasion, replication and transition between separate anatomical compartments within the human host. This vaccine-challenge presents the first data available on bacterial quantification in blood and stool and how these levels of bacteria are affected by prior oral live-attenuated vaccination.

Early bacterial shedding was frequently observed, more common in those who subsequently developed infection and also more common in those who had received active vaccination. Increased detection at this stage presumably reflects successful navigation of the gastric acid environment and either active replication or mucosal repulsion of bacteria. Stool shedding was next seen in the days prior to the development of fever and diagnosis, potentially representing a period of high-risk transmission to caregivers (*see below*), although shedding appeared to be reduced in M01ZH09 recipients. Typhoid-diagnosed participants who had received active vaccination had significantly lower bacterial loads measurable at diagnosis. Reasons for this may include the generation of humoral responses to vaccination or a delayed presentation of illness, which is known to reduce measurable bacterial burden in peripheral blood.

13.1.8 PUTATIVE INFECTION PHENOTYPES

One of the benefits of studying infection in a controlled setting and with a carefully selected host range is combining host clinical data with microbiological bacteraemia and shedding profiles to identify distinct infection phenotypes.⁴³⁶ These data and the apparent disconnect between clinical symptomatology and blood bacterial load/stool shedding suggest that a range of infection phenotypes may be apparent, at least in the challenge study setting. While these include those without and with clear (clinical plus microbiological) evidence of infection, additional phenotypes have been noted. These include:

Classical phenotypes

- ‘Infected’, participants with symptomatic bacteraemic infection, who would be identified as typhoid patients in passive surveillance programs.
- ‘non-infected’, participants exposed but with no clinical evidence (fever or unmanageable symptoms) or bacteraemia.

Additional phenotypes

- ‘shedders’, those participants excreting typhoid in stool after challenge who are asymptomatic (at least for the duration of the challenge period);
- ‘asymptomatic bacteraemias’, either blood culture or culture-PCR evidence of bacteria or bacterial products in the bloodstream in the absence of symptoms (or a host inflammatory response)- coincidentally, data from the dose-escalation study suggests the Widal test performed in these participants is often also ‘negative’, suggesting a lack of controlling antibody;
- ‘symptomatic abacteraemics’, profoundly symptomatic participants who fail to demonstrate microbiological evidence of infection either in blood and often in stool.

While these additional phenotypes may represent artefact from a challenge study setting, for example due to the limited follow-up, the 'poor' performance of diagnostic tests, or due to supra-physiological levels of exposure (challenge doses) being used, they may also signify an important range of subclinical infection not detected in endemic field-settings. The identification of asymptomatic individuals shedding *S. Typhi* is of major public health importance, and supports the UK public health approach in contact tracing around confirmed cases of infection.⁵⁴⁴ The frequency of culture/'test' negative symptomatic individuals increases the risk of inappropriate treatment or case management particularly in endemic settings where other causes of febrile infection may prevail.

13.1.9 VACCINE EFFICACY

Human challenge studies present a unique opportunity to directly assess the protective efficacy of novel vaccine candidates in a highly controlled setting. In addition to providing efficacy estimates, challenge studies also allow more detailed interrogation of the mechanisms and the precise nature of the protection afforded. These types of studies are of particular use for infections that have no small animal model or for those that are human-restricted pathogens, such as *S. Typhi*.

Vaccination with a single oral dose of M01ZH09, while immunogenic, resulted in no protection against challenge with 1.5×10^4 CFU *S. Typhi*, Quail's strain. Comparative evaluation with licensed Ty21a suggested that additional factors including unknown baseline immunity (presence of anti-Vi IgG antibodies) and age in addition to the challenge dose used might have resulted in lower than expected protection against challenge. M01ZH09 did demonstrate a significant disease altering effect, however, and, with all other factors being equal, vaccinated recipients had similar risk profiles during the challenge period. Whether additional doses, alternative formulations, assessment

with a lower challenge dose or selection of a different participant demographic would result in measurable protective efficacy remains to be answered.

13.1.10 STERILISING VS. NON-STERILISING IMMUNITY

The ideal characteristics of a typhoid vaccine are hotly debated, and the effect of vaccination in preventing clinical illness versus bacteraemia, which may on its own result in some boosting of the host immune response, are key to the arguments for and against pursuing an oral vaccine development strategy. These data I have presented in this thesis suggest that low levels of bacteraemia (some of which may only be detected by enhanced sensitivity diagnostics, such as the culture-PCR method) may be relatively commonly seen after use of these vaccines or following natural, infection-derived immunity, and don't, *per se*, indicate that the participant would go on to develop typhoid. The arguments for not treating bacteraemic participants in the context of a challenge study are difficult to make, given the priority for participant safety; there is some concern that a delay in treatment may result in more severe illness or sequelae ensuing. Assessment of vaccine efficacy in the context of a challenge study must therefore take this into account; of note the longest incubation period found in the Maryland challenge studies was 56-days.²⁸⁵

13.1.11 APPLICATION TO TRANSMISSION MODELLING

Despite the limitations, these data presented in this thesis may be applicable to a number of different areas. In addition to the characterisation of clinical disease, opportunities to discover, develop and evaluate new diagnostics and vaccines, these data can usefully inform more global policies on typhoid transmission and prevention by vaccination. Existing transmission models make certain assumptions based on endemic-setting field data, including the incidence of disease, age groups affected and the degree to which

immunity may be generated by natural exposure.^{121,122} While not able to supplement these particular parameters, these data presented here do demonstrate some important and possibly unrecognised features of typhoid infection. Firstly, that asymptomatic infection may be common, even following oral vaccination, and may serve to boost both infection-induced clinical or sterile immunity.¹²¹ Secondly, that patients may be shedding *S. Typhi* for several days prior to the onset of clinical symptoms, thereby representing a wider public health risk than has previously been recognised. Thirdly, that exposed individuals receiving oral vaccines may shed more bacteria following subsequent exposure, and that some individuals who are exposed but remain undiagnosed become ‘super-shedders’ for a period of time but do not progress to become carriers (as far as these available data suggest). These and other data extracted from the experience of typhoid challenge models performed so far, will hopefully inform transmission modelling and public health control measures to produce a population level impact.

13.2 LIMITATIONS TO THESE DATA

In addition to the specific limitations to the methods used in each chapter already discussed, there are some additional limitations to the overall study design and approach used.

13.2.1 CHALLENGE FOLLOW-UP

In the context of current ethical and research standards, study participants could only be followed-up without treatment for a relatively short period of time (2-weeks). Reasons for this include the increased risk of severe or complicated infection in the 3rd-4th week of illness, which, while unlikely to have gone unnoticed, was a safety concern. In addition the logistic requirements for staffing, daily visits and blood sampling, and reimbursing participants for additional time off work and travel would have been prohibitive. While

the effect of vaccination in prolonging the incubation period was not entirely unexpected, whether more participants in the M01ZH09 vaccine group would have developed infection after this arbitrary cut-off is a possibility.

13.2.2 EARLY TREATMENT INITIATION

Following the onset of illness, participants were diagnosed at a relatively early stage of infection, at least compared to patients presenting in clinical settings. Reasons for early treatment included reducing the discomfort of diagnosed participants, who, as these data shows, were highly symptomatic by the time of diagnosis. Early treatment undoubtedly limits the extrapolation of data to clinical settings; the rapid normalisation of the white cell count with ciprofloxacin treatment or the relatively low maximal CRP level, as examples, are less likely to be seen in a clinical setting.

13.2.3 SAMPLE COLLECTION

These clinical, microbiological and laboratory data collected are all limited by the relative frequency with which participant visits can be scheduled and performed. Although the intensity of the study schedule was overwhelming at times, these data presented in this thesis open up numerous further areas of enquiry that could only be addressed by more frequent or targeted data collection. Some of these include the capturing of more evidence relating to the primary DNA-emia/bacteraemia by more frequent sampling at an earlier stage after challenge, and the fascinating alterations to diurnal temperature pattern seen in the days prior to infection onset, which may be detected by 24-hour temperature monitoring. As has been mentioned, more invasive sampling to detect bacterial presence in the bone marrow or duodenal fluid or to assess the mucosal immune responses in the GI tract would also enlighten many of the points discussed.

13.2.4 MRS QUAILES' LEGACY

A further general limitation to these data relates to the challenge strain selected. Manufacture of a challenge agent to GMP standard is very expensive and time-consuming, however, as was seen from the Maryland studies, not all challenge strains resulted in the clinical syndrome of typhoid fever. Reasons for this are beyond the scope of this thesis, but it may be interesting to conjecture from the sink hypothesis that virulent strains causing acute infection revert to a more passive, lower virulence phenotype after shedding in the stool or storage in a laboratory. The Quailes strain was selected for its stable Vi-expression, which may make it less representative of circulating wild-type strains, however characterisation and now challenge study experience has demonstrated that it is representative and able to reproduce a characteristic typhoid syndrome after ingestion. Its close relationship to several Indonesian strains is interesting, given the high attack rates and severity of infection seen in that country. Ethical and moral concerns required a fully antibiotic sensitive strain to be used; while this is less representative of the majority of clinical cases currently occurring in the field, resistance may also influence other bacterial virulence factors altering the dynamics of infection and the clinical presentation of cases.

13.2.5 GENERALISATION OF THE RESULTS

Experience in performing the vaccine-challenge trial has demonstrated that, although expensive and labour intensive, data pertaining to vaccine efficacy can be relatively quickly obtained in a safe manner. That the study population is specifically chosen for being antigen-naïve makes comparison with what would occur in an endemic area more difficult. Similarly, the exposure dose leading to 'natural' typhoid fever is not known although likely to be less than that used in this challenge study. Both of these factors are

more likely to have resulted in an under estimate of both Mo1ZH09 and Ty21a efficacy compared to a field trial setting.

13.3 FURTHER WORK

These data presented in this thesis has given rise to multiple further enquiries regarding the host-pathogen interactions and their modulation by previous oral live-attenuated vaccination. Beyond the scope of this work, relevant on-going work to elucidate some of the issues raised includes:

- Investigations of non-humoral immune protection mechanisms (mucosal, CMI, innate), which may mediate protection derived after vaccination with oral vaccines in particular and indicate potential correlates of protection.
- More in-depth exploration of the antibodies generated following vaccination and as a result of infection using protein-microarray, from which interference mechanisms, novel vaccine antigens and enhanced serological diagnostics may be developed.
- Investigation of the effects of iron-related pathway manipulation by *Salmonella* Typhi during the course of infection, and determination of factors affecting severity and duration of bacteraemia.
- Analysis of genetic and faecal microbiome factors predisposing to susceptibility or severity of infection.
- Investigation of alternative diagnostic approaches, including antibody-in-lymphocyte supernatant assays, and metabolomic, proteomic and functional genomic signatures of exposure/infection.

Several further studies are currently underway or in the approvals process to investigate diagnostic approaches (in Kathmandu, Nepal), evaluate Vi and Vi-conjugate vaccines using the challenge model, develop a *Salmonella* Paratyphi challenge model, and to explore the effects of challenge and rechallenge.

13.4 SUMMARY

In endemic settings, typhoid is a debilitating, recurrent problem that disproportionately affects the most impoverished sections of society. Development of accurate diagnostic tests, efficacious vaccines and alternative treatment strategies is restricted by the lack of a convincing small animal model and understanding of host-bacterial interactions. In this thesis I demonstrated how using a human challenge model can directly assess the protective efficacy of novel typhoid vaccine candidates in addition to furthering our understanding of disease processes. This will lead to the accelerated development of new measures to detect, control and, ultimately, eradicate *Salmonella* Typhi.

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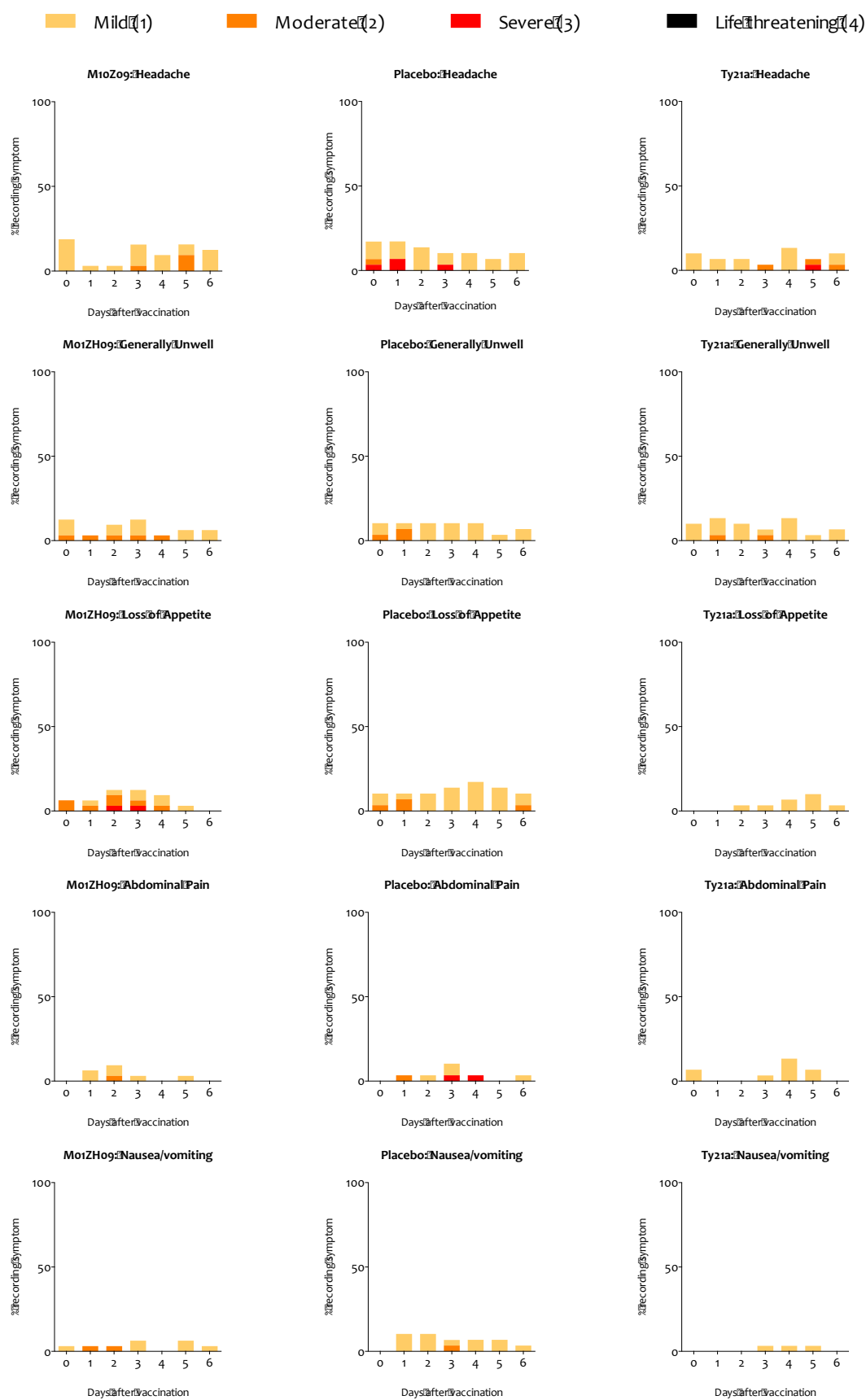
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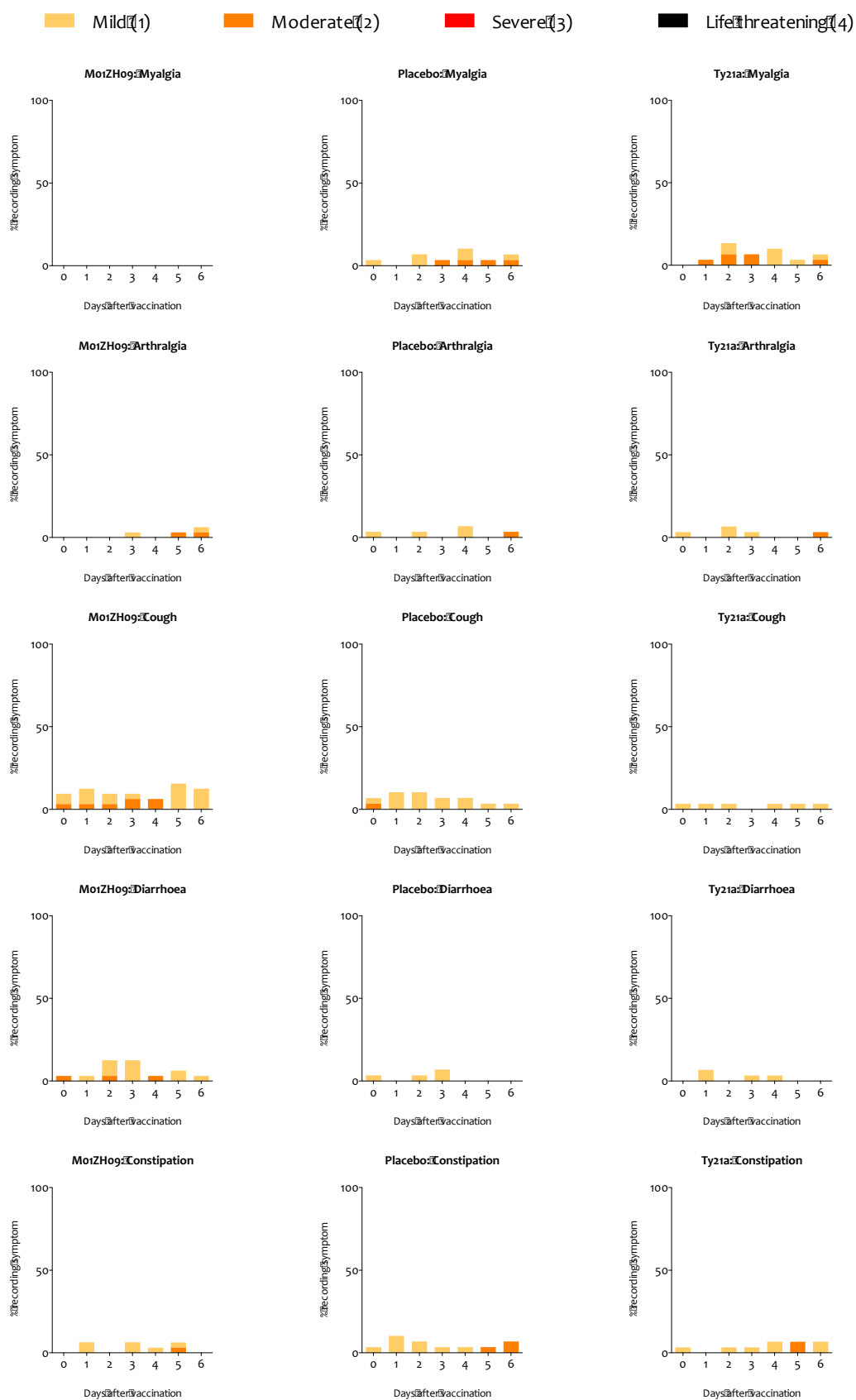
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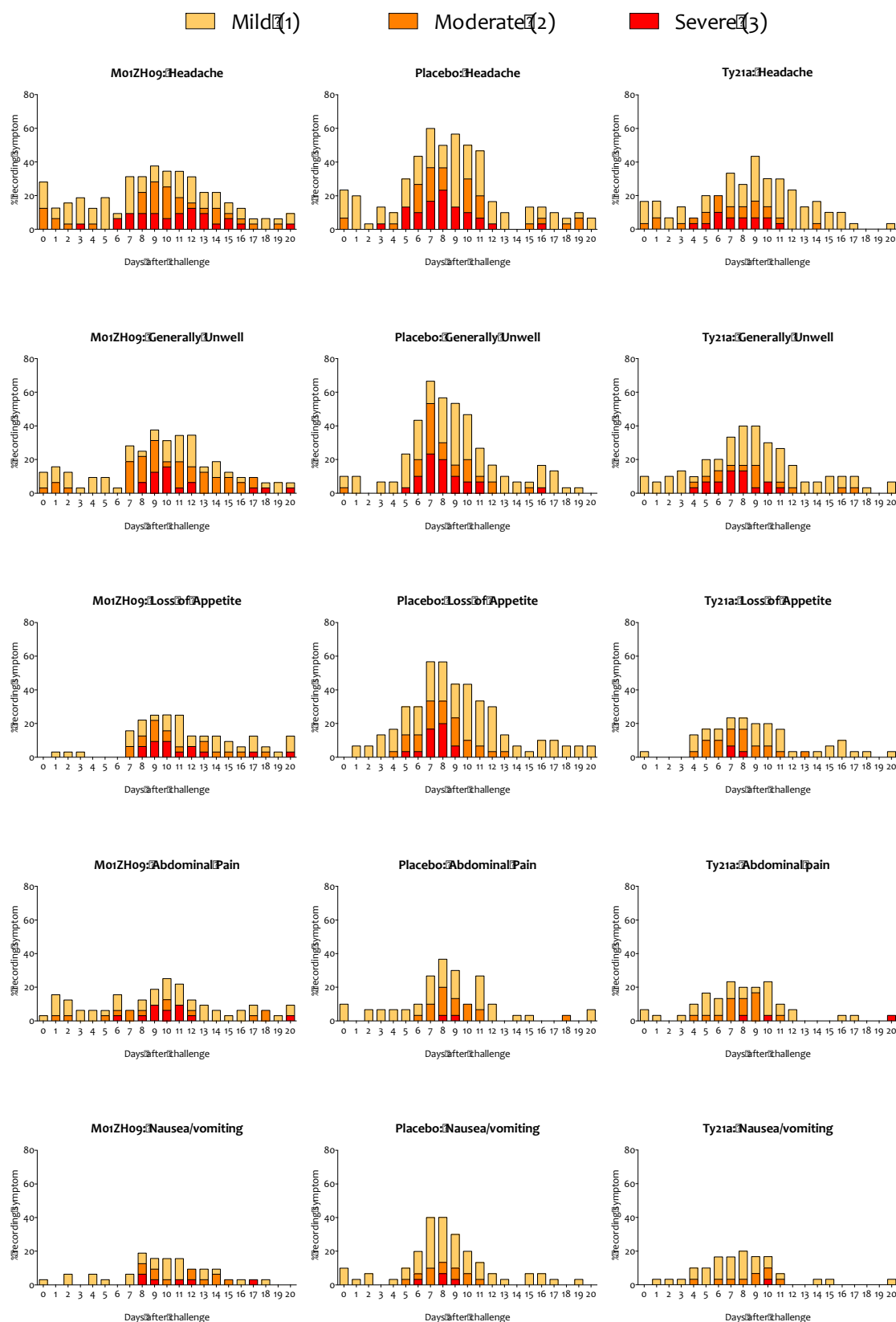
APPENDIX A. SUPPLEMENTARY FIGURES

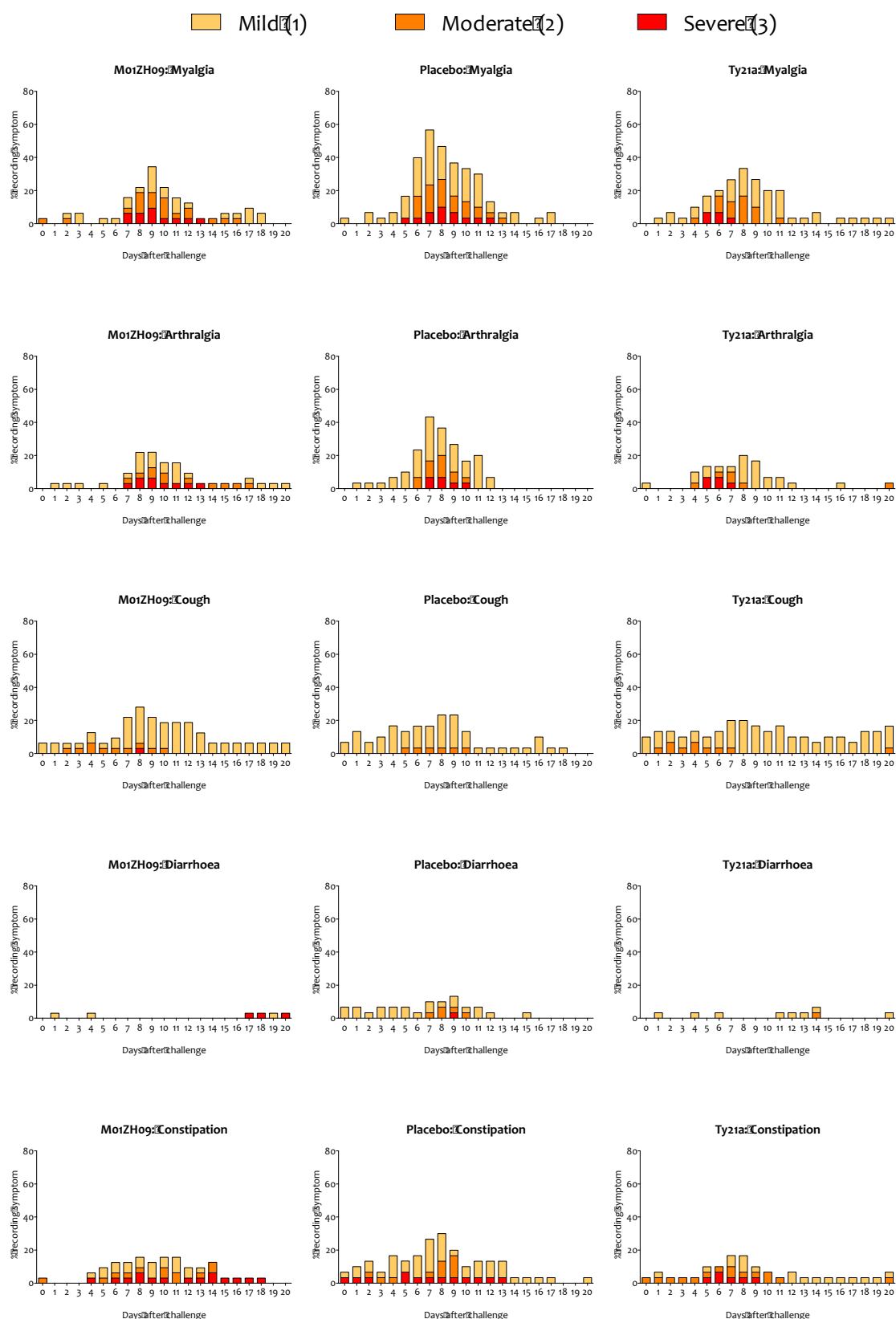
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Study No.	Year pub'd	First author	[ref]	Setting	Study period	Inclusion criteria	n	Mean age, years (med)	Mean time to presentation, days (med)	Main travel destination	% Quinolone resistance
1	2013	Barrett FC	⁵⁴⁵	Copenhagen, Denmark – travellers	02/2006-02/2010	+BC	19	(26)	(16 after return)	84% Indian subcont.	79 (CIP res)
2	2013	Hassing RJ	⁵⁴⁶	Rotterdam, Netherlands – travellers	01/2002-08/2008	+BC	26	30.9		73% India, 27% SE Asia	34.6 (fully sus)
3	2012	Kumar A	⁵⁴⁷	S. Karnatka, India – endemic	01/2005-12/2008	+BC	106	28.8			29.2 (FQ res)
4	2012	Thriemer K	¹⁹¹	Pemba, Tanzania – endemic	03/2009-12/2010	+BC	46	17.7	5.3 days fever		n.s.
5	2013	Farmakiotis D	¹⁹⁴	New York, USA - travellers	01/2006-12/2010	+BC	17	12		71% Bangladesh	76 (NAL res)
6	2012	Khan MI	⁵⁴⁸	Karachi, Pakistan – endemic	08/2002-07/2004	+BC	189	7	6.9 days fever		28 (fully sus)
7	2011	Ahmad KA	⁵⁴⁹	Karachi, Pakistan – endemic	1986-2000	+BC	1650	6.2	9.6		58 (fully sus)
8	2011	Parry CM	⁵⁵⁰	S. Vietnam - endemic	1991-2001	+BC & known ofloxacin MIC	152		8		100 (oflox sus)
9	2010	Mtove G	⁵⁵¹	Muheza, N.E. Tanzania - endemic	03/2008-02/2009	Fever & +BC	14	7.5	10.1 days fever		n.s.
10	2010	Clark TW	⁵¹⁵	Leicester, UK – travellers	01/1999-04/2009	+BC or +StC & clinical	61 ^A	38	(20 days after return)	98% Indian subcont.	45 (NAL res)
11	2008	Yu HR	⁵⁵²	Kaohsiung, Taiwan – mixed (30% travel hx)	01/1987-12/2006	+culture (any site) & clinical	33	58% >17years	8.6 days fever	S.E. Asia, China	n.s.
12	2007	Dimitrov T	⁵¹⁶	Kuwait – travellers	01/2002-12/2005	+BC	135	26.3	6.6	88% Indian subcont.	69.6 (NAL res)
13	2006	Hosoglu S	⁵⁵³	Diyarbakir, S.E. Turkey – endemic	1997-2001	Fever, symptoms & +BC	130 ^B	23.7	10.8		n.s.
14	2005*	Lefebvre N	⁵⁵⁴	Dakar, Senegal – endemic	01/1995-06/2002	+BC	70	16.7			1 (NAL res)
15	2005	Mathura KC	⁵⁵⁵	Kathmandu, Nepal – endemic	07/2004-06/2005	Clinical & +BC		26.17	5.9		4.3 (CIP res)
16	2003	Mathura KC	⁵⁵⁶	Kathmandu, Nepal - endemic	06/2002-06/2003	Clinical & +BC	30	80% 15-30 years			n.s.

Study No.	Year pub'd	First author	[ref]	Setting	Study period	Inclusion criteria	n	Mean age, years (med)	Mean time to presentation, days (med)	Main travel destination	% Quinolone resistance
17	2005	Chowta MN	⁵⁵⁷	Karnatka, India – endemic	1999-2001	+BC, +Widal or Clinical	44	23.9			18.1 (CIP res)
18	2004	Pohan HT	¹⁵⁶	Jakarta, Indonesia - endemic	01/1997-12/1999	+BC	119 ^c	25.0	6.53		n.s.
19	2004	Su CP	⁵⁵⁸	Taipei, N. Taiwan – mixed (50% travel)	01/1993-12/1999	Fever, symptoms & +BC	24	24.7	14.5	S.E. Asia	100 (CIP sus)
20	2001	Abucejo PE	²⁰⁷	Bohol, Philippines – endemic	04/1994-12/1997	+BC	422	26% 15-29 years			100 (CIP sus)
21	2001	Caumes E	⁵⁵⁹	Paris, France – travellers (93%)	01/1998-10/1998	Clinical & culture (any site)	22 ^D	30.5	10.5 days after return	29% Indian subcont., 23% SS Africa etc.	5 (NAL resis)
22	2005	Meltzer E	¹⁵³	Tel Hashomer, Israel – travellers	1995-2003	+BC or +StC & clinical	78 ^E	27.7		74.4% Indian subcont.	n.s.
23	2005	Kahiravan T	⁵⁶⁰	New Delhi, India – endemic	11/2001-10/2003	Fever & +BC	60	15			78 (NAL resis)
24	2007	Malla T	⁵⁶¹	Pokhara, Nepal – endemic	01/2000-12/2005	Clinical & culture (any site) or + Widal	82	6.5			91 (CIP sus)
25	2006	Neopane A	⁵⁶²	Kathmandu, Nepal - endemic	04/2004-08/2005	Presumptive admission diagnosis	53	26.9	10 days		n.s.

Supplementary Table 1. Inclusion criteria and results of the 25 case series (prospective and retrospective) reporting typhoid clinical symptom data since the year 2000. A PubMed search for “typhoid fever clinical symptoms” and 01/01/2000 to 31/12/2014 resulted in 1192 articles from which 52 were selected based on title and available abstract. From these 52, relevant clinical data for 3 or more symptoms was available in 25 manuscripts, which are summarised here.

* Manuscript in French. ^A mixed: 48% *S. Typhi*; ^B data from derivation cohort only; ^C mixed: 95.8% *S. Typhi*; ^D *S. Typhi* only; ^E mixed: 80% *S. Typhi*. BC, blood culture; StC, stool culture; subcont., subcontinent; SS, sub-Saharan; FQ, Fluoroquinolone; CIP, ciprofloxacin; Oflox, ofloxacin; NAL, nalidixic acid; sus, susceptible; res, resistant; n.s. not stated.

	Event severity (grade)				
	Mild (1)	Moderate (2)	Severe (3)	Potentially life-threatening (4)	
	Nausea/ vomiting	No interference with activity or 1–2 episodes/24 hours	Some interference with activity or >2 episodes/24 hours	Prevents daily activity, requires outpatient IV hydration	Emergency department visit or hospitalization for hypotensive shock
	Anorexia	Eats less than normal for 1-2 meals	Misses 1-2 meals completely	Does not eat all meals	Emergency department visit or Hospitalisation
	Abdominal pain	No interference with activity	Some interference with activity	Significant; prevents daily activity	Emergency department visit or Hospitalisation
	Headache	No interference with activity	Some interference with activity	Significant; prevents daily activity	Emergency department visit or Hospitalisation
Adverse event	Malaise	No interference with activity	Some interference with activity	Significant; prevents daily activity	Emergency department visit or Hospitalisation
	Myalgia	No interference with activity	Some interference with activity	Significant; prevents daily activity	Emergency department visit or Hospitalisation
	Arthralgia	No interference with activity	Some interference with activity	Significant; prevents daily activity	Emergency department visit or Hospitalisation
	Cough	No interference with activity	Some interference with activity	Significant; prevents daily activity	Emergency department visit or Hospitalisation
	Diarrhoea	2–3 loose stools/24 hours	4–5 stools/24 hours	6 or more watery stools/24 hours or requires outpatient IV hydration	Emergency department visit or Hospitalisation
	Constipation	No interference with activity	Some interference with activity	Significant; prevents daily activity	Emergency department visit or Hospitalisation
	Flatulence	No increase from normal	Some increase from normal	Significant increase; interferes with daily activity	N/A

Supplementary Table 2. Grading of solicited adverse event severity.

Rash will be classified as present or absent and further described in the case report form by a study investigator.

		Event severity			
		Grade 1	Grade 2	Grade 3	Grade 4*
	Haemoglobin (female): decrease from baseline value (gm/dl)	any decrease-1.5	1.6-2.0	2.1-5.0	>5
	Haemoglobin (male): decrease baseline value (gm/dl)	any decrease-1.5	1.6-2.0	2.1-5.0	>5
	White cell count: increase cell/mm ³	10,800–15,000	15,001–20,000	20,001–25,000	>25,000
	White cell count: decrease (cells/mm ³)	2500-3500	1500-2499	1000-1499	<1000
	Neutrophil count (cells/mm ³)	1500-2000	1000-1499	500-999	<500
	Platelets (cells/mm ³)	125,000-140,000	100,000-124,000	25,000-99,000	<25,000
Laboratory test (units)	Sodium: hyponatraemia (mEq/L)	132–134	130–131	125–129	<125
	Sodium: hypernatraemia (mEq/L)	144–145	146–147	148–150	>150
	Potassium: hyperkalaemia (mEq/L)	5.1–5.2	5.3–5.4	5.5–5.6	>5.6
	Potassium: hypokalaemia mEq/L	3.5–3.6	3.3–3.4	3.1–3.2	<3.1
	Urea (mmol/L)	8.2–8.9	9.0–11	>11	RRT
	Creatinine (µmol/L)	132-150	151-176	177-221	>221 or RRT
	ALT, AST	1.1–2.5 x ULN	>2.6–5.0 x ULN	5.1-10 x ULN	>10 x ULN
	Bilirubin (with increase in LFTs)	1.1–1.25 x ULN	1.26–1.5 x ULN	1.51–1.75 x ULN	>1.75 x ULN
	Bilirubin (with normal LFTs)	1.1–1.5 x ULN	1.6–2.0 x ULN	2.0–3.0 x ULN	>3.0 x ULN
	Alkaline	1.1–2.0 x ULN	2.1–3.0 x ULN	3.1–10 x ULN	>10 x ULN
	Amylase	1.1–1.5 x ULN	1.6–2.0 x ULN	2.1–5.0 x ULN	>5.0 x ULN
	Albumin: hypoalbuminaemia (g/L)	28–31	25–27	<25	N/A
	C-reactive protein	>10-30	31-100	100-200	>200

Supplementary Table 3. Severity grading for laboratory solicited adverse events.

* Potentially life-threatening; RRT: requires Renal Replacement Therapy.

	Finding severity				
	Grade 1	Grade 2	Grade 3	Grade 4*	
Fever (°C) [‡]	38.0-38.4	38.5-38.9	39.0-40.0	>40	
Tachycardia (beats per minute)	101-115	116-130	>130	Emergency department visit or hospitalisation for arrhythmia	
Bradycardia (beats per minute) [*]	50-54	45-49	<45	Emergency department visit or hospitalisation for arrhythmia	
Finding [†]	Hypertension: systolic blood pressure (mmHg), with repeat testing at the same visit	141-150	151-155	>155	Emergency department visit or hospitalisation for malignant hypertension
	Hypertension: diastolic blood pressure (mmHg)	91-95	96-100	>100	Emergency department visit or hospitalisation for malignant hypertension
	Hypotension: systolic blood pressure (mmHg), with repeat testing at the same visit	85-89	80-84	<80	Emergency department visit or hospitalisation for hypotensive shock
	Respiratory rate (breaths per minute)	17-20	21-25	>25	Intubation

Supplementary Table 4. Severity grading of clinical examination findings.

* Potentially life-threatening; [†] Participants should be at rest for measurement of vital signs; [‡] Oral temperature; no recent hot or cold beverages or smoking; ^{*} When resting heart rate is between 60-100 beats per minute - use clinical judgement when characterising bradycardia among some healthy participant populations, for example, conditioned athletes.

Scale	Description	Definition
1	Mild	Transient or mild discomfort (<48 hours); no medical intervention/therapy required
2	Moderate	Some interference with activity not requiring medical intervention
3	Severe	Prevents daily activity and requires medical intervention
4	Serious	Life threatening

Supplementary Table 5. Grading of unsolicited adverse events.

		MoiZH09 (n=32)				Placebo (n=30)				Ty21a (n=29)			
		GMT, EU/mL (95% CI)	x-rise (95% CI)	PIR, % (95% CI)	≥4x-rise, % (95% CI)	GMT, EU/mL (95% CI)	x-rise (95% CI)	PIR, % (95% CI)	≥4x-rise, % (95% CI)	GMT, EU/mL (95% CI)	x-rise (95% CI)	PIR, % (95% CI)	≥4x-rise, % (95% CI)
LPS IgG													
	Pre-vaccine	83.0 (62.0-111)	ref	-		86.3 (63.3-118)	ref	-		118 (86.3-161)	ref		
	Pre-challenge	222 (146-339)	4.64 (2.58-6.70)	50.0 (32.7-67.3)	25.0 (10.0-40.0)	84.1 (62.3-113)	1.04 (0.90-1.18)	6.7 (-2.3-15.6)	0 (0-0)	166 (107-258)	1.89 (1.03-2.75)	24.1 (8.6-39.7)	13.8 (1.2-26.3)
LPS IgA													
	Pre-vaccine	48.0 (36.3-63.4)	ref	-		51.2 (37.8-69.4)	ref	-		65.0 (46.1-91.7)	ref		
	Pre-challenge	85.8 (62.3-118)	2.00 (1.66-2.33)	59.4 (42.4-76.4)	3.1 (-2.9-9.2)	51.23 (38.7-67.9)	1.03 (0.93-1.12)	3.3 (-3.1-9.8)	0 (0-0)	78.1 (52.5-116)	1.43 (1.10-1.76)	24.1 (8.6-39.7)	3.4 (-3.2-10.1)
LPS IgM													
	Pre-vaccine	93.8 (75.7-116)	ref			116 (89.8-149)	ref			105 (85.4-130)	ref		
	Pre-challenge	156 (126-192)	2.02 (1.37-2.68)	40.6 (23.6-57.6)	6.3 (-2.1-14.6)	112 (86.9-145)	0.99 (0.92-1.05)	0 (0-0)	0 (0-0)	115 (94.7-140)	1.16 (1.04-1.28)	3.4 (-3.2-10.1)	0 (0-0)
H IgG													
	Pre-vaccine	40.7 (31.1-53.3)				42.3 (31.2-57.4)	ref			47.8 (32.9-69.4)			
	Pre-challenge	112.8 (73.02-174.2)	4.85 (2.37-7.32)	68.8 (52.7-84.8)	31.3 (15.2-47.3)	44.0 (32.7-59.3)	1.06 (0.97-1.15)	3.3 (-3.1-9.8)	0 (0-0)	50.3 (35.3-71.7)	1.11 (1.01-1.21)	0 (0-0)	0 (0-0)
H IgA													
	Pre-vaccine	11.8 (9.3-14.9)	ref			13.9 (10.8-17.9)	ref			24.6 (18.2-33.1)	ref		
	Pre-challenge	31.2 (22.8-42.6)	4.09 (2.43-5.76)	68.8 (52.7-84.8)	25.0 (10-40)	13.4 (10.5-17.1)	0.99 (0.91-1.07)	0 (0-0)	0 (0-0)	23.6 (17.3-32.2)	1.02 (0.93-1.11)	3.4 (-3.2-10.1)	0 (0-0)
H IgM													
	Pre-vaccine	12.1 (9.4-15.5)	ref			22.2 (15.5-31.8)	ref			17.3 (12.2-24.4)			
	Pre-challenge	348 (181-668)	149.84 (17.1-283)	87.5 (76.0-99.0)	84.4 (71.8-97.0)	18.3 (12.8-26.0)	0.99 (0.78-1.20)	6.7 (-2.3-15.6)	0 (0-0)	22.5 (15.8-32.1)	1.51 (1.17-1.84)	20.7 (5.9-35.4)	3.4 (-3.2-10.1)

Supplementary Table 6. Summary of anti-LPS and anti-H antibody titres before and 28-days after vaccination according to vaccine allocation.

GMT, geometric mean titre; x-rise, mean fold rise; Positive immune response (PIR): IgG and IgM ≥1.7 fold rise; IgA ≥1.5 fold rise; ≥4x-rise.

	M01ZH09		Placebo		Ty21a	
	GMC, ASC/10 ⁶ PBMC (95% CI)	No. (%) Positive ASC response	GMC, ASC/10 ⁶ PBMC (95% CI)	No. (%) Positive ASC response	GMC, ASC/10 ⁶ PBMC (95% CI)	No. (%) Positive ASC response
LPS IgG	n=27		n=22		N=22	
Day -28	0.4 (0.3-0.6)		0.6 (0.4-0.9)		0.4 (0.3-0.6)	
Day -21 (Va)	10.6 (4.7-23.8)	19 (70)	1.0 (0.5-2.0)	5 (23)	1.5 (0.8-2.7)	5 (23)
LPS IgA	n=21		n=15		n=17	
Day -28	0.6 (0.4-1.0)		0.6 (0.4-1.2)		0.6 (0.4-1.0)	
Day -21 (Va)	84.7 (43.4-165.2)	20 (95)	1.5 (0.6-3.8)	5 (33)	9.3 (5.2-16.8)	13 (76)
LPS IgM	n=27		n=21		n=20	
Day -28	0.6 (0.4-0.8)		0.7 (0.4-1.0)		0.5 (0.3-0.9)	
Day -21 (Va)	103.8 (60.7-177.4)	26 (96)	1.5 (0.7-2.8)	6 (29)	5.5 (3.1-10.0)	15 (75)
H IgG	n=27		n=21		n=20	
Day -28	0.6 (0.4-0.9)		0.5 (0.3-0.7)		0.6 (0.4-0.8)	
Day -21 (Va)	20.7 (10.7-40.3)	27 (70)	0.6 (0.4-0.9)	3 (10)	2.1 (1.3-3.5)	10 (33)
H IgA	n=27		n=21		n=20	
Day -28	0.9 (0.6-1.5)		0.8 (0.5-1.2)		1.2 (0.7-2.0)	
Day -21 (Va)	90.0 (57.9-166.0)	32 (97)	1.5 (0.9-2.4)	9 (30)	12.6 (7.6-21.1)	24 (80)
H IgM	n=27		n=21		n=20	
Day -28	0.9 (0.6-1.3)		0.5 (0.3-0.8)		0.9 (0.6-1.4)	
Day -21 (Va)	131.9 (89-195.6)	32 (97)	1.1 (0.7-1.6)	5 (17)	8.4 (4.8-14.6)	21 (70)

Supplementary Table 7. Summary of plasma antibody-secreting cell responses pre-vaccination and 7-days after vaccination with M01ZH09, placebo or Ty21a.

GMC, geometric mean concentration; PBMC, peripheral blood mononuclear cells. Note that high background responders (≥ 4 SFC/10⁶ PBMC) are excluded from the anti-LPS isotypes.

		M01ZH09			Placebo			Ty21a		
		ALL (n=32)	nTD (n=14)	TD (n=18)	ALL (n=30)	nTD (n=10)	TD (n=20)	ALL (n=30)	nTD (n=17)	TD (n=13)
Headache	% (95% CI)	69.7 (54.0-85.4)	57.1 (31.2-83.1)	83.3 (66.1-100.5)	87.1 (75.3-98.9)	75.0 (45.0-105.0)	95.0 (85.4-104.6)	63.3 (45.8-80.9)	35.3 (12.6-58.0)	100.0 (NA)
	Mean severity	1.61	1.07	2.11	2.10	1.38	2.50	1.23	0.41	2.31
Generally Unwell	% (95% CI)	66.7 (50.6-82.8)	50.0 (29.0-96.0)	83.3 (66.1-100.5)	83.9 (70.9-96.8)	62.5 (29.0-96.0)	95.0 (85.4-104.6)	66.7 (49.5-83.8)	41.2 (17.8-64.6)	100.0 (NA)
	Mean severity	1.52	1.00	2.00	2.06	1.13	2.60	1.27	0.47	2.31
Loss of Appetite	% (95% CI)	54.5 (37.6-71.5)	28.6 (4.9-52.2)	77.8 (58.6-97.0)	77.4 (62.7-92.1)	62.5 (29.0-96.0)	85.0 (69.4-100.6)	43.3 (25.3-61.4)	23.5 (3.4-43.7)	69.2 (39.1-99.4)
	Mean severity	1.21	0.57	1.78	1.58	0.63	2.10	0.77	0.24	1.46
Abdominal Pain	% (95% CI)	54.5 (37.6-71.5)	42.9 (16.9-68.8)	66.7 (44.9-88.4)	58.1 (40.7-75.4)	50.0 (15.4-84.6)	65.0 (44.1-85.9)	46.7 (28.5-64.8)	23.5 (3.4-43.7)	76.9 (50.8-103.0)
	Mean severity	1.00	0.64	1.33	0.97	0.75	1.15	0.80	0.35	1.38
Nausea/ vomiting	% (95% CI)	42.4 (25.6-59.3)	28.7 (4.9-52.2)	55.6 (32.6-78.5)	58.1 (40.7-75.4)	25.0 (-5.0-28.6)	80.0 (62.5-97.5)	33.3 (16.2-50.5)	17.6 (-0.5-35.8)	53.8 (16.9-90.8)
	Mean severity	0.82	0.5	1.11	1.03	0.38	1.45	0.60	0.24	1.08
Myalgia	% (95% CI)	42.4 (25.6-59.3)	14.3 (-4.0-32.6)	66.7 (44.9-88.4)	64.5 (47.7-82.4)	25.0 (-0.5-55.0)	85.0 (69.4-100.6)	53.3 (35.2-71.5)	17.6 (-0.5-35.8)	100.0 (NA)
	Mean severity	0.94	0.14	1.61	1.35	0.25	1.95	0.87	0.18	1.77
Arthralgia	% (95% CI)	36.4 (20.0-52.8)	14.3 (-4.0-32.6)	55.6 (32.6-78.5)	54.8 (37.3-72.4)	12.5 (-10.4-35.4)	75.0 (56.0-94.0)	36.7 (19.1-54.2)	23.5 (3.4-43.7)	53.8 (16.9-90.8)
	Mean severity	0.70	0.14	1.17	0.90	0.13	1.30	0.60	0.29	1.00

Cont'd

		MoiZHog			Placebo			Ty21a		
		ALL (n=33)	nTD (n=14)	TD (n=18)	ALL (n=31)	nTD (n=8)	TD (n=20)	ALL (n=30)	nTD (n=17)	TD (n=13)
Cough	% (95% CI)	42.4 (25.6-59.3)	14.3 (-4.0-32.6)	61.1 (38.6-83.6)	54.8 (37.3-72.4)	37.5 (4.0-71.0)	40.0 (18.5-61.5)	46.7 (28.5-64.8)	29.4 (7.8-51.1)	69.2 (39.1-99.4)
	Mean severity	0.52	0.14	0.83	0.45	0.38	0.45	0.57	0.41	0.77
Diarrhoea	% (95% CI)	12.1 (1.0-23.3)	28.6 (4.9-52.2)	0.0 (NA)	38.7 (21.6-55.9)	12.5 (-10.4-28.6)	50.0 (28.1-71.9)	20.0 (5.4-34.6)	17.6 (-0.5-35.8)	23.1 (-24.6-70.8)
	Mean severity	0.24	0.57	N/A	0.58	0.13	0.80	0.23	0.18	0.31
Constipation	% (95% CI)	42.4 (25.6-59.3)	35.7 (10.6-60.8)	50.0 (26.9-73.1)	54.8 (37.3-72.4)	37.5 (4.0-71.0)	65.0 (44.1-85.9)	33.3 (16.2-50.5)	23.5 (3.4-43.7)	46.2 (6.3-86.0)
	Mean severity	0.85	0.64	1.06	0.94	0.50	1.20	0.67	0.47	0.92

Supplementary Table 8. Summary symptom table describing the per cent (95%CI) and severity of symptoms reported according to vaccine group allocation in the 21-day period following challenge.

ALL, all participants allocated to that vaccine; TD, typhoid diagnosed; nTD, non-typhoid diagnosed.

Clinical measurement	Time point	Group	M101ZH09	Placebo	Ty21a	All groups
Oral temperature (°C), mean (95% CI)	Day 0	ALL	36.3 (36.1-36.4)	36.2 (36.1-36.4)	36.0 (35.8-36.2)	36.2 (36.1-36.3)
	Day 7	nTD	36.3 (36.1-36.6)	36.6 (36.1-36.7)	36.4 (36.2-36.6)	36.4 (36.3-35.5)
	Typhoid diagnosis	TD	37.8 (37.5-38.1)	37.8 (37.5-38.1)	37.3 (36.9-37.7)	37.7 (37.5-37.9)
	Day 14	nTD	36.5 (36.4-36.7)	36.7 (36.5-36.9)	36.3 (36.1-36.5)	36.5 (36.3-36.6)
	Day 14	TD	36.6 (36.4-36.7)	36.4 (36.2-36.6)	36.5 (36.3-36.7)	36.5 (36.3-36.6)
Heart rate (b.p.m.), mean (95% CI)	Day 0	ALL	76.0 (71.1-80.1)	76.0 (70.9-81.0)	72.7 (67.4-77.9)	74.9 (72.1-77.8)
	Day 7	nTD	74.2 (69.8-78.5)	82.6 (74.8-90.5)	74.5 (67.8-81.3)	76.4 (72.8-80.0)
	Typhoid diagnosis	TD	94.2 (87.8-100.6)	91.1 (84.5-97.7)	85.3 (76.6-94.0)	90.7 (86.8-94.6)
	Day 14	nTD	75.3 (67.1-83.4)	80.2 (71.2-89.2)	73.7 (69.3-78.1)	75.7 (72.2-79.3)
	Day 14	TD	83.6 (67.1-83.4)	77.2 (71.6-82.8)	77.2 (68.2-86.2)	78.8 (74.5-83.1)
Systolic blood pressure (mmHg), mean (95% CI)	Day 0	ALL	130.1 (124.4-135.8)	129.0 (124.7-133.3)	126.9 (122.5-131.3)	128.7 (126.0-131.4)
	Day 7	nTD	124.5 (114.2-134.8)	126.5 (118.4-134.6)	127.6 (119.2-136.1)	126.4 (121.5-131.2)
	Typhoid diagnosis	TD	129.9 (121.6-138.2)	121.6 (115.2-127.9)	128.1 (122.7-133.4)	126.2 (122.2-130.1)
	Day 14	nTD	123.8 (115.2-132.4)	126.9 (117.5-136.2)	127.1 (122.7-131.4)	127.8 (124.5-131.1)
	Day 14	TD	126.9 (122.5-131.3)	124.8 (118.8-130.7)	120.1 (110.0-130.2)	123.2 (119.1-127.3)
Diastolic blood pressure (mmHg), mean (95% CI)	Day 0	ALL	78.2 (74.7-81.6)	80.8 (77.5-84.2)	80.6 (77.58-83.7)	78.2 (76.6-79.9)
	Day 7	nTD	77.5 (72.2-82.8)	78.6 (73.6-83.6)	76.9 (72.1-81.8)	77.6 (74.9-80.3)
	Typhoid diagnosis	TD	76.6 (72.2-81.0)	82.7 (77.1-88.2)	83.5 (79.2-87.9)	80.5 (77.7-80.3)
	Day 14	nTD	77.0 (69.1-85.0)	78.8 (75.2-82.4)	80.4 (75.2-85.5)	79.1 (76.2-82.0)
	Day 14	TD	75.6 (71.6-79.6)	77.2 (68.9-85.6)	74.9 (69.5-80.3)	75.8 (73.0-78.7)

Supplementary Table 9. Clinical examination findings according to vaccine group allocation, challenge time point and challenge outcome.

ALL, all participants; nTD, non-typhoid diagnosed participants; TD, typhoid diagnosed; B.P.M., beats per minute.

Clinical measurement	Time point	Group	M101ZH09	Placebo	Ty21a	All groups
Haemoglobin, g/dL, mean (95% CI)	Day 0	ALL	14.4 (14.0-14.8)	14.1 (13.5-14.7)	14.7 (14.4-15.0)	14.4 (14.1-14.7)
	Day 8	nTD	13.9 (13.8-14.6)	13.2 (12.2-14.2)	14.2 (13.5-14.9)	13.8 (13.4-14.2)
	Typhoid diagnosis	TD	13.7 (13.2-14.2)	13.8 (13.0-14.7)	14.1 (13.5-14.9)	13.9 (13.5-14.2)
	Day 14	nTD	13.4 (12.3-14.5)	12.6 (6.53-18.7)	14.3 (13.2-15.5)	13.6 (12.7-14.4)
	Day 14	TD	12.8 (12.1-13.5)	12.4 (11.6-13.3)	13.1 (11.8-14.5)	12.7 (12.3-13.2)
Platelets, $\times 10^9/L$, mean (95% CI)	Day 0	ALL	253.7 (232.7-274.7)	249.0 (223.9-274.1)	231.8 (210.2-253.3)	245.0 (232.3-257.5)
	Day 8	nTD	282.5 (250.4-314.7)	248.0 (200.1-295.9)	218.6 (191.3-245.9)	248.8 (228.3-269.3)
	Typhoid diagnosis	TD	185.9 (161.1-210.7)	198.2 (172.8-223.5)	204.4 (179.3-229.4)	195.6 (181.8-209.3)
	Day 14	nTD	252.7 (178.1-327.3)	247.9 (179.7-336.1)	208.1 (164.1-252.1)	238.1 (205.6-270.6)
	Day 14	TD	215.7 (190.6-240.7)	334.3 (269.2-399.4)	305.0 (203.4-406.6)	278.4 (243.7-313.2)
White cells, $\times 10^9/L$, mean (95% CI)	Day 0	ALL	6.7 (5.9-7.5)	6.1 (5.4-6.8)	6.6 (6.0-7.2)	6.5 (6.1-6.9)
	Day 8	nTD	5.9 (4.4-7.5)	5.6 (4.8-6.3)	6.0 (5.0-7.1)	5.9 (5.3-6.5)
	Typhoid diagnosis	TD	6.4 (5.5-7.3)	6.7 (5.9-7.4)	6.6 (5.8-7.5)	6.6 (6.1-7.0)
	Day 14	nTD	5.7 (4.2-7.2)	6.0 (4.3-7.8)	6.1 (5.0-7.2)	5.9 (5.3-6.6)
	Day 14	TD	4.6 (3.8-5.4)	6.0 (4.8-7.2)	5.4 (3.5-7.4)	5.3 (4.7-5.9)
Neutrophils, $\times 10^9/L$, mean (95% CI)	Day 0	ALL	3.8 (3.2-4.5)	3.4 (2.8-3.9)	3.3 (2.9-3.8)	3.5 (3.2-3.8)
	Day 8	nTD	3.6 (2.4-4.8)	3.4 (2.6-4.1)	3.6 (2.8-4.3)	3.5 (3.0-4.0)
	Typhoid diagnosis	TD	4.7 (3.9-5.5)	5.1 (4.3-5.8)	4.4 (3.4-5.5)	4.8 (4.3-5.2)
	Day 14	nTD	3.4 (2.4-4.5)	3.6 (2.3-5.0)	3.4 (2.6-4.1)	3.5 (3.0-4.0)
	Day 14	TD	2.3 (1.8-2.9)	3.0 (2.2-3.7)	2.4 (1.5-3.3)	2.6 (2.2-3.0)

Cont'd

Clinical measurement	Time point	Group	M101ZH09	Placebo	Ty21a	All groups
Lymphocytes, $\times 10^9/L$, mean (95% CI)	Day 0	ALL	2.1 (1.9-2.3)	2.0 (1.7-2.2)	2.4 (2.2-2.7)	2.2 (2.0-2.3)
	Day 8	nTD	1.7 (1.4-2.0)	1.5 (1.3-1.7)	1.7 (1.5-2.0)	1.7 (1.5-1.8)
	Typhoid diagnosis	TD	1.0 (0.9-1.2)	0.9 (0.8-1.0)	1.2 (1.0-1.4)	1.0 (1.0-1.1)
	Day 14	nTD	1.6 (1.1-2.1)	1.6 (1.3-1.9)	1.9 (1.5-2.3)	1.7 (1.5-1.9)
	Day 14	TD	1.6 (1.3-2.0)	2.3 (1.8-2.8)	2.2 (1.2-3.2)	2.0 (1.7-2.3)
Eosinophils, $\times 10^9/L$, mean (95% CI)	Day 0	ALL	0.22 (0.15-0.29)	0.20 (0.16-0.24)	0.24 (0.19-0.29)	0.22 (0.19-0.25)
	Day 8	nTD	0.16 (0.10-0.21)	0.19 (0.07-0.30)	0.18 (0.12-0.23)	0.17 (0.13-0.21)
	Typhoid diagnosis	TD	0.03 (0.00-0.06)	0.02 (0.00-0.03)	0.07 (-0.02-0.16)	0.03 (0.01-0.06)
	Day 14	nTD	0.08 (0.02-0.15)	0.15 (0.07-0.23)	0.21 (0.11-0.31)	0.15 (0.10-0.20)
	Day 14	TD	0.11 (0.03-0.19)	0.15 (0.12-0.19)	0.24 (0.03-0.44)	0.15 (0.10-0.19)

Supplementary Table 10. Haematology blood results according to vaccine group allocation, challenge time point and challenge outcome.

Clinical measurement	Time point	Group	M101ZH09	Placebo	Ty21a	All groups
Sodium, mmol/L, mean (95% CI)	Day 0	ALL	140 (139-141)	140 (140-141)	141 (140-141)	140 (140-141)
	Day 7	nTD	140 (139-142)	141 (140-142)	141 (139-143)	141 (140-141)
	Typhoid diagnosis	TD	137 (135-138)	137 (136-138)	139 (137-141)	137 (137-138)
	Day 14	nTD	139 (139-140)	140 (138-142)	141 (138-142)	140 (139-141)
	Day 14	TD	140 (138-142)	140 (138-142)	140 (139-141)	140 (139-141)
Potassium, mmol/L, mean (95% CI)	Day 0	ALL	3.9 (3.7-4.1)	3.7 (3.6-3.8)	3.7 (3.6-3.8)	3.8 (3.7-3.8)
	Day 7	nTD	4.1 (3.7-4.5)	3.8 (3.6-3.9)	3.8 (3.5-4.1)	3.9 (3.7-4.0)
	Typhoid diagnosis	TD	4.0 (3.5-4.5)	3.5 (3.4-3.7)	3.7 (3.5-3.8)	3.7 (3.5-3.8)
	Day 14	nTD	4.2 (3.2-5.3)	3.8 (3.4-4.2)	3.9 (3.6-4.1)	3.9 (3.6-4.3)
	Day 14	TD	3.9 (3.7-4.0)	3.7 (3.5-3.9)	4.0 (3.8-4.2)	3.8 (3.7-3.9)
Creatinine, µmol/L, mean (95% CI)	Day 0	ALL	75.2 (70.2-79.7)	78.0 (72.5-83.5)	79.1 (75.1-83.1)	77.4 (74.8-80.0)
	Day 7	nTD	77.8 (70.2-85.5)	78.3 (68.8-87.8)	76.8 (69.3-92.3)	77.6 (73.5-81.8)
	Typhoid diagnosis	TD	86.1 (79.8-92.4)	84.1 (76.5-91.6)	85.1 (77.0-93.2)	85.0 (81.0-82.2)
	Day 14	nTD	78.8 (69.1-88.5)	74.4 (63.0-85.7)	78.3 (67.7-88.8)	77.0 (71.8-82.2)
	Day 14	TD	76.0 (70.0-82.0)	75.5 (69.2-81.9)	81.2 (64.4-98.0)	76.6 (72.8-80.5)
Urea, mmol/L, mean (95% CI)	Day 0	ALL	4.8 (4.2-5.5)	4.8 (4.4-5.3)	5.1 (4.7-5.6)	4.9 (4.6-5.2)
	Day 7	nTD	4.8 (4.1-5.5)	4.6 (3.6-5.6)	5.0 (4.2-5.7)	4.8 (4.4-5.2)
	Typhoid diagnosis	TD	3.9 (3.4-4.3)	3.7 (3.2-4.3)	4.0 (3.6-4.4)	3.8 (3.6-4.1)
	Day 14	nTD	5.0 (3.8-6.2)	4.8 (3.9-5.7)	4.6 (3.8-5.4)	4.8 (4.3-5.2)
	Day 14	TD	4.1 (3.5-4.7)	4.7 (4.0-5.4)	4.3 (3.6-5.0)	4.4 (4.0-4.7)

Cont'd

Clinical measurement	Time point	Group	M101ZH09	Placebo	Ty21a	All groups
Amylase, IU/L, mean (95% CI)	Day 0	ALL	59 (52-66)	58 (52-65)	67 (57-76)	61 (57-66)
	Day 7	nTD	60 (48-71)	51 (44-58)	62 (44-80)	58 (51-65)
	Typhoid diagnosis	TD	51 (42-60)	58 (47-69)	60 (50-70)	56 (51-62)
	Day 14	nTD	60 (39-81)	56 (44-68)	62 (41-84)	60 (51-69)
	Day 14	TD	56 (44-68)	68 (58-79)	52 (41-64)	60 (53-67)
Albumin, g/L, mean (95% CI)	Day 0	ALL	47.0 (46.2-47.9)	46.1 (45.2-47.0)	46.0 (45.4-46.6)	46.4 (46.0-46.8)
	Day 7	nTD	45.4 (43.6-47.1)	45.0 (43.1-46.9)	46.7 (45.3-48.1)	45.7 (44.8-46.6)
	Typhoid diagnosis	TD	46.3 (45.0-47.6)	46.2 (44.7-47.6)	47.5 (45.8-49.1)	46.5 (45.7-47.3)
	Day 14	nTD	45.9 (43.5-48.2)	45.6 (43.8-47.4)	46.0 (43.9-48.1)	45.8 (44.8-46.9)
	Day 14	TD	45.2 (43.7-46.7)	45.2 (43.6-46.9)	44.8 (41.8-47.8)	45.1 (44.2-46.1)
Alkaline Phosphatase, IU/L, mean (95% CI)	Day 0	ALL	152 (137-166)	154 (138-171)	153 (133-172)	153 (144-162)
	Day 7	nTD	146 (120-173)	140 (111-168)	148 (122-174)	145 (131-159)
	Typhoid diagnosis	TD	160 (148-173)	160 (143-178)	162 (134-189)	161 (151-171)
	Day 14	nTD	144 (110-179)	152 (111-194)	166 (128-203)	155 (136-174)
	Day 14	TD	170 (131-210)	184 (143-227)	142 (110-175)	172 (148-195)
Alanine aminotransferase, IU/L, mean (95% CI)	Day 0	ALL	22.6 (19.2-26.0)	19.9 (16.7-23.0)	20.7 (17.3-24.1)	21.1 (19.2-23.0)
	Day 7	nTD	19.9 (14.2-25.6)	21.3 (16.0-26.6)	23.2 (17.7-28.7)	21.5 (81.6-24.4)
	Typhoid diagnosis	TD	20.3 (16.2-24.4)	21.2 (16.1-26.2)	22.3 (15.0-29.6)	21.2 (18.3-24.0)
	Day 14	nTD	22.0 (13.3-30.7)	26.6 (16.0-37.1)	26.8 (15.1-38.4)	25.3 (20.0-30.6)
	Day 14	TD	57.4 (27.9-86.8)	146 (-1.30-294)	173 (12.5-334)	108 (50.5-166)

Cont'd

Clinical measurement	Time point	Group	M101ZH09	Placebo	Ty21a	All groups
Bilirubin, $\mu\text{mol/L}$, mean (95% CI)	Day 0	ALL	11.8 (9.6-13.9)	10.9 (8.7-13.0)	10.4 (8.4-12.5)	11.0 (9.9-12.2)
	Day 7	nTD	8.6 (5.9-11.2)	9.3 (7.2-11.4)	11.6 (9.0-12.5)	9.9 (8.5-11.2)
	Typhoid diagnosis	TD	10.7 (8.1-13.3)	10.7 (7.8-13.6)	9.7 (8.4-11.0)	10.5 (9.1-11.9)
	Day 14	nTD	8.8 (5.6-12.0)	7.4 (4.7-10.2)	9.1 (5.2-13.1)	8.5 (6.9-10.1)
	Day 14	TD	7.8 (5.8-9.8)	7.4 (4.2-10.5)	9.2 (2.6-15.9)	7.0 (6.3-9.4)
C-reactive protein, mg/L, mean (95% CI)	Day 0	ALL	3.0 (0.2-5.9)	2.0 (1.0-3.1)	0.7 (0.2-1.2)	2.0 (0.9-3.0)
	Day 7	nTD	0.9 (-0.3-2.1)	1.5 (-0.3-3.3)	2.7 (0.1-5.2)	1.7 (0.7-2.7)
	Typhoid diagnosis	TD	38.0 (24.3-51.7)	43.5 (25.5-61.5)	27.3 (11.8-42.8)	37.4 (28.4-46.4)
	Day 14	nTD	9.7 (-5.7-25.1)	7.2 (-2.9-17.4)	3.0 (-0.5-6.5)	6.4 (1.5-11.2)
	Day 14	TD	32.0 (19.7-44.3)	17.8 (8.5-27.0)	8.5 (1.0-16.0)	22.8 (15.8-29.9)

Supplementary Table 11. Biochemistry blood results according to vaccine group allocation, challenge time point and challenge outcome.