Investigating Enteric Fever – Application of Controlled Human Infection Models to Understand Enteric Fever Pathogenesis and Immunity.



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Abstract : Investigating Enteric Fever – Application of Controlled Human Infection Models to Understand Enteric Fever Pathogenesis and Immunity

The development of novel vaccines for typhoidal *Salmonella* is hampered, in part, by limited understanding of protective host immune responses to infection, the absence of reliable immunological correlates of protection, and uncertainties relating to disease pathogenesis. Controlled human infection models (CHIM) for *Salmonella* Typhi and Paratyphi have recently been re-established. In this thesis I describe the application of two controlled human infection studies (CHIM), designed to offer insights into enteric fever pathogenesis and immunity.

In the first study, I investigate a recently identified virulence factor encoded by *S*. Typhi and Paratyphi, termed the typhoid toxin. Following generation, manufacture and characterisation of challenge strains, I compared the response to challenge with either wild type *S*. Typhi or a typhoid toxin-deficient isogenic mutant strain in 40 healthy volunteers using a novel challenge protocol. There was no significant difference in the rate of infection after challenge with wild type or typhoid toxin deficient strains, although there was a trend toward a more severe clinical syndrome in participants challenged with the typhoid toxin deficient strain. Collectively these data suggest that expression of the typhoid toxin is not an absolute requirement for the establishment of acute typhoid fever.

In a second study, I recruited naïve volunteers with no prior exposure to *Salmonella* Typhi or Paratyphi and compared the response to challenge with a cohort of previously-exposed volunteers. Homologous re-challenge with *S*. Typhi or Paratyphi A was associated with a 37% and 56% relative reduction in attack rates, respectively and a prolonged time to disease. These are the first data indicating prior *Salmonella* Paratyphi exposure confers protection against re-challenge. In contrast, heterologous re-challenge was not associated with significant reduction in attack rates. Protection from disease on re-challenge was most pronounced in older participants and in individuals who did develop disease on primary exposure. Baseline serum antibody and memory B-cell measurements did not correlate with protection after re-challenge, and previous exposure was not associated with a demonstrable boosting of humoral immune responses. Further studies identified that previous exposure and Vi-vaccination were significantly associated with reduced shedding of *S.* Typhi after challenge.

Collectively, these studies demonstrate the expanding application and potential of controlled human infection models to understand of enteric fever pathogenesis and immunity. It is hoped that the data presented herein will ultimately aid efforts aimed at disease control, by identifying correlates of protection and to provide a platform for further vaccine studies.

Publications

The following is a list of publications relating to the typhoid and paratyphoid challenge studies conducted as part of my DPhil, as well as those arising from series of concomitant projects.

Gibani MM, Britto C, Pollard AJ 'Typhoid and Paratyphoid Fever – A Call to Action'
Current Opinion in Infectious Diseases 31, 5, 440-448(2018)

Gibani MM, Voysey M, Jin C et al 'The Impact of Vaccination and Prior exposure on stool Shedding of *Salmonella* Typhi and *Salmonella* Paratyphi A in six controlled human infection studies'
Clin Infect Dis (2018 – Accepted for publication. In Press)

Napolitani G, Kurupati P, Teng K, Gibani MM et al 'Clonal analysis of Salmonella-specific effector T cells reveals serovar-specific and cross-reactive T cell responses'
Nature Immunology 19, 742–754 (2018)

Howson LJ, Napolitani G(…)Gibani MM et al 'MAIT cell clonal expansion and TCR repertoire shaping in human volunteers challenged with Salmonella Paratyphi A'
Nature Communications 253 PMID 29343684 (2018);

Jin C, Gibani MM, Moore M et al. 'Efficacy and immunogenicity of a Vi-tetanus toxoid conjugate vaccine in the prevention of typhoid fever using a controlled human infection model of Salmonella Typhi: a randomised controlled, phase 2b trial'
Lancet 390(10111):2472-2480 (2017)

Dobinson HC, Gibani MM, Jones C, et al. 'Evaluation of the clinical and microbiological response to Salmonella Paratyphi A infection in the first paratyphoid human challenge model.'
Clin Infect Dis. 64(8):1066-1073, (2017)

Meiring J, Gibani MM 'The Typhoid Vaccine Acceleration Consortium (TyVAC): Vaccine effectiveness study designs: Accelerating the introduction of typhoid conjugate vaccines and reducing the global burden of enteric fever'
Vaccine 35(38) 5081-5088 (2017)

Gordon S, Rylance J (…)Gibani MM et al, 'A framework for Controlled Human Infection Model (CHIM) studies in Malawi: Report of a Wellcome Trust workshop on CHIM in Low Income Countries held in Blantyre, Malawi.'
Wellcome Open Research 2:70 (2017) PMID: 29018841

Darton TC, Jones C (…) Gibani MM et al 'Assessment and Translation of the Antibody-in-Lymphocyte Supernatant (ALS) Assay to Improve the Diagnosis of Enteric Fever in Two Controlled Human Infection Models and an Endemic Area of Nepal'
Frontiers in Microbiology (2017) 2031; PMID 29109704

Gibani MM, Jin C, Darton TC, Pollard AJ. 'Control of invasive salmonella disease in Africa: Is there a role for human challenge models?'
Clin Infect Dis 61: S266–71. (2015)

Zhou L, Jones C, Gibani MM, et al. Development and Evaluation of a Blood Culture PCR Assay for Rapid Detection of Salmonella Paratyphi A in Clinical Samples.
PLoS One; 11: e0150576.(2016)

Other Projects

Milligan ID, Gibani MM, Sewell R, et al. 'Safety and Immunogenicity of Novel Adenovirus Type 26– and Modified Vaccinia Ankara–Vectored Ebola Vaccines.'
JAMA 315: 1610. (2016);

Ewer K, Rampling T, Venkatraman N(…)Gibani MM et al. 'A Monovalent Chimpanzee Adenovirus Ebola Vaccine Boosted with MVA.'
New England Journal of Medicine 374: 1635–46 (2016)

Giuntini S, Lujan E, Gibani MM, Dold C, Rollier CS, Pollard AJ, et al. 'Serum Bactericidal Antibody Responses of Adults Immunized with the MenB-4C Vaccine against Genetically Diverse Serogroup B Meningococci.'
Clin Vaccine Immunol . 5;24(1). pii: e00430-16 (2017)

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Declaration and Contribution

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The typhoid toxin deficient strain of *S*. Typhi was generated conducted the Galán laboratory at the Department of Microbial Pathogenesis, Yale University School of Medicine (New Haven, CT, USA). Manufacture of challenge strains was conducted at the Walter Reed Army Institute of Research (MD,USA), co-ordinated by Richard Millward. Environmental persistence testing was performed in collaboration with the Food, Water and Environmental Laboratory, Public Health England, Porton Down and conducted by Frieda Jorgensen. Whole genome sequencing of wild-type and typhoid toxin deficient strain of *S*. Typhi was performed at the Wellcome Trust Sanger Institute (Hinxton, UK) under the supervision of Dr Derek Pickard and Professor Gordan Dougan. Analysis of the sequence data was performed by Dr Eva Heinz (Senior Staff Scientist, Wellcome Trust Sanger Institute).

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The data and analyses presented herein have not been submitted for any degree elsewhere and, unless otherwise stated, all work presented in this thesis is my own.

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Chapter 1 – Introduction: The Diagnosis, Treatment and Prevention of Enteric Fever

1. Introduction

1.1. The Organism

The *Salmonella* genus comprises two species, termed *Salmonella bongori* and *Salmonella enterica.* The latter is divided into six recognised subspecies - *Salmonella enterica* subspecies *arizonae*, *Salmonella enterica* subspecies *diarizonae*, *Salmonella enterica* subspecies *enterica*, *Salmonella enterica* subspecies *houtenae*, *Salmonella enterica* subspecies *indica* and *Salmonella enterica* subspecies *salamae*.[1,2]

Typhoid fever is a disease caused by infection with *Salmonella enterica* subspecies *enterica* serovar Typhi (*S*. Typhi), a Gram-negative facultative anaerobic bacillus. Paratyphoid fever results from infection with the related organism *Salmonella enterica* subspecies *enterica* serovar *Paratyphi* (*S*. Paratyphi), which is divided into three subtypes - *Salmonella Paratyphi* A, B and C. *Salmonella* Typhi and Paratyphi[1] are collectively referred to as typhoidal *Salmonella* serovars and infection with either can result in the clinical syndrome of enteric fever.

1.2. Burden of Disease

Enteric fever is a major global health problem predominantly affecting low- and middle-income countries. The disease has been essentially eliminated as a public-health problem in high-income countries over the past century, owing to improvements in water quality, sanitation and hygiene[3].

[1] In this thesis, *S*. Paratyphi refers to *Salmonella enterica* subspecies *enterica* serovar Paratyphi Type A (*S.* Paratyphi A), unless otherwise stated.

Precise estimates of the burden of disease in low- and middle-income countries are difficult to ascertain for several reasons, such as the non-specific presentation of disease; the lack of reliable diagnostics and laboratory facilities in endemic countries; the insensitivity and poor specificity of commonly used diagnostic tests; regional and sub-national geographic heterogeneity and age-specific variation. Consequently, estimates of disease burden vary widely between studies.

In 2004, typhoid fever was estimated to be responsible for 21.6 million cases and 216,510 deaths [4]. On the assumption that one-fifth of all enteric fever cases were caused by *S.* Paratyphi, paratyphoid fever was estimated to be responsible for an additional 5.4 million cases of enteric fever. These estimates were derived using data from seven studies conducted in Asia and one study conducted in Sub-Saharan Africa, and adjusted for the sensitivity of blood culture[5]. Using United Nations socioeconomic indicators and geographical proximity, the authors extrapolated the incidence of typhoid fever to countries lacking data, classifying regions into low, middle and high incidence areas. These data were updated in 2010 with the addition of one further data set from Kenya, to produce an adjusted estimated global incidence of 26.9 million episodes of disease[6]. The 2016 Global Burden of Disease survey estimates the annual burden of typhoid fever at 11,773,878 (10,227,304 – 13,621,609) cases and paratyphoid fever at 3,758,2000 (3,328,201 – 4,257,4470)[7] cases.

Over recent years, several groups have published studies refining estimates of the global burden of enteric fever, incorporating additional surveillance data from Sub-Saharan Africa and by modelling for specific risk-factors[8–10]. A recent study by Mogasale and colleagues, estimated the burden of typhoid fever cases in low- and middle-income countries in 2010 at 11.9 million cases/year with 129,000 deaths, after adjusting for access to improved water quality[9,11]. Without adjusting for water related-risk, the unadjusted estimate was 20.6 million (17.15-24.2) cases/year with 223 000 (131-344 000 deaths). Antillón and colleagues used a meta-regression approach to

provide an estimate the burden of typhoid fever in low-and-middle income countries. After adjusting for a diverse range of demographic, environmental and economic risk factors, they provide an estimate of 17.8 million annual cases, with a wide confidence interval ranging from 6.9 to 48.8 million[9,10].

A consistent finding of burden-of-disease studies performed to date is the high incidence of typhoid fever in South and South-East Asia. Within these regions, the epidemiology of typhoid is complicated by marked inter- and intra-country variation. For example, data from the Diseases of Most Impoverished (DOMI) programme have described incidence rates varying from 24.2/100 000 in Vietnam up to 493.5/100 000 in parts of India[12]. Surveillance throughout Asia has mostly been undertaken in urban centres with consistently high rates of disease. Less is known about the rates of disease in rural areas, although recent studies have demonstrated high rates of typhoid fever in rural areas of Cambodia and West Africa, suggesting that the disease is not restricted to urban settings with poor sanitation systems[8,13].

The heterogeneity of typhoid epidemiology may be more pronounced in Africa [14]. Surveillance performed in two sites in Kenya between 2006 and 2009 found that the incidence of blood-culture proven typhoid fever in rural and urban sites varied from 29- up to 247-cases/100,000 person-years [15]. Recent data from the Typhoid Fever Surveillance in Africa Program (TSAP) highlighted marked differences in incidence rates between sites in Africa with adjusted rates ranging from 0 in Sudan to 383/100,000 person years in Burkina Faso. This study also demonstrated marked intra-country variation, with higher rates in rural Ghana compared with urban settings [8].

The incidence of enteric fever in children has historically been an area of debate, with conflicting data on the burden of disease in infants and preschool children published from the 1970s to 1990s. More recently, data from surveillance studies, as well as a recent meta-analysis, have

emerged underlining the large burden of disease in pre-school children[16,17]. Recent data derived from ongoing surveillance studies suggest that an estimated 27% of typhoid fever cases requiring medical attention occur in children aged 0-4 years, of which a substantial proportion (~30%) occur at ages below 2 years[18]. Age-specific incidence may vary by country, risk factors and force of infection.

Ongoing surveillance studies (including the Surveillance of Enteric Fever in Asia Project [SEAP], Severe Typhoid in Africa Program [SETA] and the Strategic Typhoid Alliance across Africa [STRATAA programmes] aim to better characterise the burden of severe typhoid disease, refine our understanding of age distribution and to better characterise the role of chronic carriers in transmission dynamics. It is hoped that data generated from these studies will help to inform future prevention strategies[19,20].

The proportion of disease caused by *S.* Paratyphi, as compared with S. Typhi, is highly variable depending on the geographic context. It is typically assumed that *Salmonella* Paratyphi is responsible for approximately one-fifth of all enteric fever cases[4]. However increasing incidence of disease caused by *S.* Paratyphi A has been reported over the past two decades such that this serovar is responsible for an increasing proportion of enteric fever cases in parts of Asia, including in Nepal [21], Cambodia [22,23] and China[24,25]. For example, in a recent retrospective study *S.* Paratyphi A was responsible for 86% of enteric fever cases in Phnom Penh, Cambodia between 2013-2015, increasing from 26% in the period 2008-2012[23]. The highest burden of paratyphoid fever is estimated to occur China, with an estimated annual incidence of 150 cases/100,000 person-years, albeit with marked regional differences[25]. The available data for Africa, whilst limited, indicate that *S.* Paratyphi is responsible for a much lower proportion of enteric fever case (<2%), with the possible exception of Nigeria[15,25–27]. The apparent increasing burden of paratyphoid fever in endemic countries is reflected by an increasing prevalence of paratyphoid fever in returning

travellers. Between 2007 and 2016, S. Paratyphi A was responsible for between 41-51% of enteric fever cases in returning travellers in the UK[28]. It is unclear if the apparent increasing incidence of paratyphoid fever is due to an absolute increase in *S.* Paratyphi A, or a relative increase as a consequence of the decline in enteric fever caused by *S.* Typhi [29].

1.3. Transmission

Transmission of *Salmonella* Typhi and Paratyphi occurs primarily through consumption of contaminated food or water, via short-cycle or long-cycle transmission. Short-cycle transmission is defined as the contamination of food and water in the immediate environment, whereas long-cycle transmission is defined as contamination of the broader environment, such as pollution of water supplies by sewage or inadequate treatment of piped water[30]. The relative contribution of each transmission mode may vary depending on the epidemiological context and may differ between *S.* Typhi and Paratyphi[29]. Direct person-to-person transmission of typhoid is rare, however sexual transmission has rarely been described[31].

1.3.1. Water-borne transmission

The contribution of unsafe drinking water has been recognised as central to the spread of typhoid for over 150 years[32,33]. During the early decades of the 20th century, the introduction of chlorination and filtration of drinking water led to a significant reduction in typhoid fever incidence and mortality in major US cities[3]. For example, the incidence of typhoid fever in the United States was 100/100,000 persons in 1900. Chlorination of municipal drinking water in the United States began in 1908 and became widespread over the subsequent decades resulting in a marked decline in the incidence of typhoid fever from 33.8/100 000 in 1920 to 0.1/100,000 persons in 2006[34]. Whilst provision of safe drinking water will ultimately be central in efforts to eradicate enteric fever, achieving disease control through improved water quality alone is fraught with challenges.

Drinking water can be contaminated with *S*. Typhi and Paratyphi at several points from its source, through to consumption. Flooding and seasonal monsoons are temporally associated with peaks in the incidence of typhoid fever in endemic countries situated in the tropics[35]. Seasonal peaks of typhoid fever in association with rainfall are thought to be due to overflow of drinking water mixing with untreated sewage containing faecal sludge. Consistent with this hypothesis, the concentration of *S*. Typhi and Paratyphi DNA in communal drinking water supplies sourced from stone spouts was found to peak shortly after the rainy season, in Kathmandu, Nepal[36]. Inadequate central treatment of municipal piped water supplies can also result in outbreaks, such as a large outbreak of MDR typhoid fever that occurred in Tajikistan in 1997, associated with inadequate treatment of municipal water supplies. Interventions such as the re-introduction of chlorination of water supplies led to a marked decline in the incidence of typhoid[37].

Piped water is supplied on an intermittent basis in many cities worldwide[38]. Intermittent piped water supplies, as opposed to continuous 24-hour provision, increases the risk of waterborne disease transmission, through a variety of mechanisms including backflow of water and external microbial contamination of unpressurised pipes. Furthermore, when water supplies are intermittent, there is a need for household storage of water in containers, increasing the risk of secondary contamination[39]. In a matched cohort study performed in Hubli-Dharwad, India, residents who received piped 24-hour uninterrupted water supply had a lower incidence of typhoid fever compared with households that received an intermittent piped water supply, despite central water treatment (42% fewer households affected)[40].

Household storage of water is common in many low-income countries as many household's lack 24-hour access to water or an individual piped supply to the household. Water stored in household containers are at risk of secondary contamination through contact with hands and

fomites, even if the stored water is sourced from a continuous piped supply[40,41]. In the absence of high-quality, centralized water treatment systems, point-of-use decontamination of water can significantly reduce the burden of diarrhoeal disease in low-income settings[42–44] although some studies have highlighted limited effectiveness of household water purification in typhoid endemic areas, presumably resulting from heavy contamination of municipal water and recontamination of previously purified water[45]. This emphasises the need to combine water purification strategies with improvements to sanitation and education programmes.

Access to clean, safe drinking water will ultimately be key to reducing the global burden of typhoid fever. Despite improvements in access to clean water globally, achieving universal clean water access requires substantial and sustained investment, and several barriers must be overcome to ensure equity of coverage[46–48].

1.3.2. Food-borne transmission

Several risk factors have been identified in the foodborne transmission of typhoid fever. The consumption of food from street vendors has been associated with increased risk of enteric fever in some epidemiological studies, which most likely results from limited availability of facilities for refrigeration of food and limited availability of hand washing facilities[49,50]. Irrigation of crops using untreated wastewater has been implicated in a number of typhoid fever outbreaks[51,52]. In the early 20th century, shellfish exposed to sewage have also been implicated in large scale outbreaks in the Unites States[53]. The use of contaminated water to manufacture ice products has also increase the risk of typhoid fever[54]. Improperly washed utensils and containers used to store and transport food can result in secondary contamination of food and contribute to the spread of disease[55].

Inadequate hand hygiene amongst individuals involved in food handling and preparation can contribute to the spread of typhoid fever[56–58]. Public health guidelines in the UK define food handlers as "people whose work involves preparing or serving unwrapped food to be served raw or not subjected to further heating"[59]. Studies in South India demonstrated that up to 16% of food handlers in this setting were carriers of typhoid[60]. Similar studies conducted in Pakistan have indicated high carrier rates amongst food handlers, which appears to contribute to sustained transmission of typhoid in this area[61]. Targeted vaccination of food handlers against typhoid is advocated in some countries, such as Malaysia and Sri Lanka, although the efficacy of such strategies have not been formally evaluated[62,63].

1.3.3. Transmission to Laboratory and Healthcare Workers

Infection of laboratory staff handling *Salmonella* Typhi is known to occur, presumably through unsafe laboratory practices[64–67]. In the United Kingdom, *Salmonella* Typhi and *Salmonella* Paratyphi are schedule 5 pathogens and require handling in a containment level 3 laboratory[68]. Routine vaccination of laboratory staff potentially coming into contact with these pathogens, coupled with appropriate biosafety control measures, is typically advocated in this at-risk group[69]

1.4. The disease

1.4.1. Clinical features of uncomplicated disease

The clinical presentation of enteric fever is highly variable, ranging from a mild-illness characterised by low-grade fever and malaise to a severe life-threatening systemic illness with multiple potential complications[70]. Although *S.* Paratyphi has historically been thought to cause a milder disease than *S.* Typhi, data from the largest case series to-date, comprising 609 enteric fever patients in Nepal, indicate that both serovars cause an indistinguishable clinical syndrome[71,72].

Ingestion of the bacteria and systemic invasion is followed by a short-lived period of asymptomatic primary bacteraemia[73]. The incubation period, prior to secondary bacteraemia, typically lasts 7 to 14 days, but can range from 3 to 60 days. The duration of the incubation period is dependent, in part, on the size of the inoculum[74].

Symptoms are usually non-specific and include fever, malaise, anorexia, headache, arthralgia, myalgia, nausea, abdominal discomfort and dry cough[75]. Sporadic, asymptomatic, shedding of the bacteria in the stool can be present prior to the development of symptomatic disease. Altered bowel habit, including constipation in adult patients or diarrhoea in children and HIV positive adults, frequently occur[76]. Clinical signs may include high fever, relative bradycardia, abdominal tenderness, hepatomegaly or splenomegaly. Rose spots are erythematous, maculopapular lesions 2-4mm in diameter, which are typically distributed across the abdomen, chest, arms or back and may occur in 5 to 30% of patients. Altered mental status and reduced coma score may be identified in the context of severe infection. A range of haematological abnormalities have been described, including anaemia, lymphopenia and neutropenia. Levels of the liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) may be elevated to two or three times the upper limit of normal[70].

The presentation of typhoid fever in children under the age of 5 may be non-specific, with features including sepsis-like syndrome, seizures, diarrhoea, pneumonia or a viral-like illness, which may lead to an under recognition of disease in this age group[17,77–79]. Hypothermia may be observed in infants and children who are severely anaemic, malnourished or immunosuppressed may also fail to mount a typical febrile response[16,17].

1.4.2. Complications of severe enteric fever

Enteric fever is associated with a range of systemic complications. The most commonly observed include intestinal haemorrhage, intestinal perforation and encephalopathy. However, complications involving most organ systems have been described, including hepatitis, cholecystitis, myocarditis, pneumonia, acute kidney injury, disseminated intravascular coagulation, thrombocytopenia and psychiatric disturbance[70]. Complications are estimated to occur in 10-15% of hospitalised patients and are more common amongst untreated patients whose illness has persisted for ≥2 weeks[80]. In one case series of 581 patients with culture confirmed typhoid fever in Vietnam, severe typhoid fever occurred in 15.5% of hospitalised patients and was associated with reduced fluoroquinolone sensitivity in the causative isolates – a finding that has also been described amongst enteric fever patients in India[80,81]. The development of severe typhoid fever in this group may result from poor response to antimicrobial therapy or may be associated with strain variation.

1.4.3. Intestinal Haemorrhage

Gastrointestinal haemorrhage in severe typhoid fever arises from ulceration and erosion of necrotic Peyer's patches through to mesenteric vessels. Haemorrhage is estimated to occur in 10% of patients and is considered the most common complication of enteric fever. Bleeding is frequently self-limiting, however severe bleeding occurs in approximately 2% of cases. The majority of cases of typhoid haemorrhage involve small vessels and are self-limiting, although life threatening haemorrhage can occur if large vessels are involved and may require additional intervention to control bleeding, including colonoscopy, surgery or administration of vasopressors[82–85].

1.4.4. Intestinal Perforation

A review of published literature between 1960 and 1990 estimated the frequency of intestinal perforation to be 3% of hospitalised cases, with a mortality rate approaching 40%[86]. However, a review of more recent literature between 1991 and 2011 suggests a decline in case fatality rates to approximately 15%, possibly reflecting improvements in the management of intestinal perforation[87]. Recent reports of typhoid epidemics in Uganda and the Democratic Republic of Congo have described high rates of intestinal perforation (>40%) associated with high mortality (18% – 43%)[88,89].

Delayed or inadequate antimicrobial therapy has been identified as a risk factor for intestinal perforation. Increased mortality is associated with late presentation, the presence of multiple perforations and the development of post-operative complications[90–98]. The pathophysiology of intestinal perforation is thought to result from a hyper-active immune response within Peyer's patches, characterised by infiltration of macrophages, B- and T-lymphocytes[99]. Prior sensitisation may result in a more robust intestinal immune response on re-exposure,[100] which may explain the observation of increased incidence of intestinal preformation in adolescents and young adults, as compared with infants[95,101]. Management involves immediate resuscitation with fluids and blood transfusion as required, followed by prompt surgery. Early surgery appears to reduce mortality compared with conservative management[86,102,103]. Surgical treatment may include primary closure or by-pass of the affected segment. Additional antibiotics, such as metronidazole with or without an aminoglycoside, can be added to cover contaminating enteric bacteria, including anaerobes.[104]

1.4.5. Typhoid Encephalopathy

Neurological complications are frequently observed in severe cases and are associated with a high mortality. The clinical presentation may include an acute confusion, reduced consciousness,

meningitis, myelitis or focal neurological infection. A recent outbreak of typhoid fever in 2009 along the Malawi-Mozambique border was notable for the high rate of observed neurological manifestations, including altered mental status, dysarthria, upper motor neurone syndromes, ataxia, tremors and Parkinsonism[105,106]. Dexamethasone has been shown to be of benefit in severe typhoid fever associated with altered consciousness. In one study conducted in Indonesia, administration of chloramphenicol and dexamethasone (at a dose of 3mg/kg by slow IV infusion, followed by 1mg/kg every six hours) reduced mortality from severe typhoid fever to 10% from 55% in the placebo group[107]. One study conducted in patients with severe typhoid fever in Papua New Guinea, compared the addition of intravenous hydrocortisone (100mg or 400mg IV QDS) combined with a 14-day course of chloramphenicol, to a group of historical controls. In this study, the addition of hydrocortisone failed to confer a survival benefit (mortality rate 44.8% vs. 43.9%) although the unusually high mortality rate observed in this study may have masked any potential benefit[108].

1.4.6. Carriage and Shedding

Chronic carriers are defined as individuals shedding *S.* Typhi or Paratyphi in stool or urine more than 12 months following acute infection. In the absence of effective antimicrobial therapy, approximately 1-5% of patients with acute typhoid infection are thought to become chronic carriers[109]. Chronic carriers may be responsible for maintaining low level transmission of disease, and thus, could complicate disease eradication efforts through sanitation and vaccination programmes[110]. Accurate identification and treatment of chronic carriers will likely form an important component of future disease control efforts. Risk factors for chronic carriage include the presence of gallstones, female sex, older age and inadequate treatment courses[70,109,111,112]. Genetic sequencing of *S.* Typhi obtained from the gallbladder carriers compared with acute infection showed different prevalent strains, whereby carriers had phylogenetically older strains than strains responsible for acute infection, with higher rates of antibiotic susceptibility[113].

Gallstones are present in more than 90% of chronic carriers, and act as a nidus for biofilm formation, thereby allowing bacteria to persist within the gallbladder[114,115]. Although chronic carriers may be asymptomatic, they are at an increased risk of developing gallbladder carcinoma[116–118]. Chronic urinary carriage has also been described and is associated with nephrolithiasis, prostatic hypertrophy or concurrent infection with *Schistosoma haematobium*[119–122].

1.5. Risk Factors

Salmonella Typhi and Paratyphi are acid susceptible and must pass through the gastric acid barrier to establish infection. Reduced gastric acid secretion appears to increase the risk - and possibly severity - of typhoid fever[123–125]. In healthy volunteer studies, administration of sodium bicarbonate prior to challenge with *Salmonella* Typhi, reduces the infectious dose required to cause symptomatic infection, compared with historical controls[74,126]. Prior infection with *Helicobacter pylori* is associated with an increased risk of developing enteric fever[127,128]. The association with *H. pylori* may reflect common environmental risk factors, such as poor hygiene and inadequate sanitation, rather than a direct causal relationship.

In contrast with invasive non-typhoidal *Salmonella* infection, malaria and HIV co-infection do not appear to be major risk factors for enteric fever[129]. Some studies report a reduced incidence of *S.* Typhi bacteraemia in HIV positive patients. The clinical presentation of typhoid fever however may be atypical in the context of HIV infection[76].

1.6. Diagnosis

There remains a pressing need to improve upon current enteric fever diagnostics and to develop a new generation of tests that are accessible, cost-effective, sensitive and specific[130].

1.6.1. Culture Specimens

Bone marrow culture is considered the 'gold-standard' diagnostic test for enteric fever, but is frequently impractical to perform in many endemic settings[131]. Compared with blood culture, the concentration of viable organisms in the bone marrow is ten-fold higher and bone marrow cultures may remain positive despite the initiation of antibiotic therapy[131–133].

Blood culture is therefore the mainstay of typhoid and paratyphoid diagnosis. A recent systematic review estimated the average diagnostic sensitivity of blood culture to be 61.1% (95%CI 51.9 – 70.3%)[9,134]. The sensitivity of blood culture can be affected by a range of factors including a relatively narrow window for detecting circulating organisms, prior antibiotic use, suboptimal volume of blood taken, inadequate dilution of blood in broth and the length of the incubation period in the laboratory[135]. In the absence of blood culture bottles or media, direct plating of the buffy coat (lymphocytes) from 5-10 ml of blood will allow the growth of isolated colonies within 24 hours of specimen collection for around 50% of blood culture positive patients[136].

The isolation of *Salmonella* Typhi or Paratyphi from stool culture in the context of a compatible clinical syndrome is supportive of the diagnosis of enteric fever[135]. There is an 18% increase in isolation rates when stool is cultured at three time points as opposed to a single time point. Rectal swabs may be useful when a stool sample cannot be obtained. Other sites and sources to isolate the organism include rose spots, duodenal string culture, and urine[131,137,138]. A study conducted in Indonesia compared blood culture, rectal swabs, bone marrow and duodenal strings, and gave positivity rates of 54%, 36%, 86% and 58%, respectively[139]. The study also demonstrated a sensitivity of 98% when all four tests were combined.

1.6.2. Colony characteristics

The characteristics of *S.* Typhi and Paratyphi grown on different types of agar are outlines in Table 1-1.

Table 1-1 : Colony characteristics.

Adapted from WHO background document: The diagnosis, treatment and prevention of typhoid fever[140].

Agar	Colony Characteristics
Blood agar	Non-haemolytic smooth, moist white colonies 2-3mm in diameter
MacConkey agar	Lactose non-fermenting smooth colonies
Salmonella Shigella agar	Lactose non-fermenting colonies with black centers (S. Paratyphi A colonies may not have black centers).
Desoxycholate agar	Lactose non-fermenting colonies with black centers (S. Paratyphi A colonies may not have black centers).
Xylose-lysine-desoxycholate agar	Transparent red colonies with black centers (S. Paratyphi A colonies may not have black centers).
Hektoen enteric agar	Transparent green colonies with black centers (S. Paratyphi A colonies may not have black centers).
Bismuth sulphite agar	Black colonies

1.6.3. Serological identification

Salmonella species are classified into serotypes as per the Kauffman-White classification.[141] The serotypes that cause enteric fever have the following antigenic compositions (Table 1-2 & Table 1-3).

Table 1-2 : Antigenic composition.

Antigens in parenthesis are weakly expressed. Adapted from WHO background document: The diagnosis, treatment and prevention of typhoid fever[140].

	O antigen	H antigen	Serogroup Phase 1:2
Salmonella Typhi	9,12,(Vi)	d :	Group D1
Salmonella Paratyphi A	1,2,12	a : (1,5)	Group A
Salmonella Paratyphi B	1,4, (5), 12	b : (1,2)	Group B
Salmonella Paratyphi C	6, 7, (Vi)	c : (1,5)	Group C1

Table 1-3 : Specific O antigens and Phase 1 H antigens.

Adapted from WHO background document: The diagnosis, treatment and prevention of typhoid fever[140].

Organism	Specific O antigen factor	Phase 1 H antigen
Salmonella Typhi	9	d
Salmonella Paratyphi A	2	a
Salmonella Paratyphi B	4	b
Salmonella Paratyphi C	6/7	c

The O-antigen is typically determined by the slide agglutination test, using polyvalent O- or group-specific antiserum, followed by specific O-factor antiserum. Serotypes that express the Vi-antigen, such as *Salmonella* Typhi and *Salmonella* Paratyphi C, may prevent agglutination with O antiserum. Boiling of the culture for 10 minutes will result in removal of the Vi-antigen and allow agglutination with O antiserum[142].

Whereas most *Salmonella* species possess two phases of the H antigen, *Salmonella* Typhi are typically monophasic, expressing the Phase 1 H-d antigen. Some *Salmonella* Typhi isolates from the Indonesian archipelago express an alternative Phase 1 H antigen, known as H-j, and/or a Phase 2 antigen termed Hz66[143–145].

The Vi-capsule is preferentially expressed under conditions of low and medium osmolarlity[146 147]. The mechanisms controlling regulation of Vi-capsule expression are reviewed elsewhere[148].

1.6.4. Rapid Diagnostic Tests

Rapid diagnostic tests (RDTs) for typhoid and paratyphoid fever could theoretically be combined with clinical algorithms to differentiate febrile patients to guide management, particularly in areas lacking well equipped laboratory facilities[135]. Several RDTs for enter fever diagnosis have been developed, the most commonly of which are the Typhidot/Typhidot-M® test, the TUBEX™ test and an anti-O9 IgM based test developed by the Royal Tropical Institute of The Netherlands commercially available as Test-It Typhoid™. These are serological tests based on detecting antibody responses to *S.* Typhi O9 (Tubex & Test-It Typhoid) or a 50kDa outer membrane protein (Typhidot) - reviewed elsewhere[149]. The performance of currently available typhoid RDTs varies between settings and the interpretation of trial data is complicated by the absence of a reliable control population and the lack of a highly sensitive gold standard comparator[150]. A recent Cochrane review compared the performance of RDTs in patients with suspected enteric fever[149]. The meta-analysis showed an average sensitivity of 78% (95%CI 65-87%) and specificity 77% (95%CI 66-86%) for the Typhidot test (excluding indeterminate results); a sensitivity of 78% (95%CI 71-85%) and specificity 87% (95%CI 82-91%) for the TUBEX test, and a sensitivity of 69% (95%CI 59-78%) and specificity 90% (95%CI 78-93%) for Test-It Typhoid. The current generation of typhoid RDTs have only modest sensitivity and specificity determined in meta-analyses and there is insufficient evidence to support their exclusive use for the diagnosis and management of enteric fever[149,150]. An RDT with comparable performance and cost-effectiveness to malaria RDTs would represent a significant advance and, if deployed in the right settings, could prevent indiscriminate antibiotic treatment. Notwithstanding the challenges associated with developing a sensitive and specific RDT, such tests would still require refinements to empirical treatment

guidelines combined with a robust central microbiology reference laboratory to track local patterns in antimicrobial resistance.

1.6.5. Novel Diagnostics

Other diagnostics in development include antibody-in-lymphocyte-supernatant (ALS), which has demonstrated good sensitivity and specificity in endemic settings[151–154].

Several PCR-based methods have also been developed and demonstrate promising results in some small-scale studies, but there are currently no widely used and validated assays in general use and remain poorly sensitive. The sensitivity of PCR based assays can be improved by incorporating a pre-enrichment step[155 156]. Limited laboratory infrastructure, cost and the length of time required to obtain results currently serve as deterrents to scalability and expansive deployment for both molecular diagnostics and ALS.

Future directions for diagnostic biomarker discovery include the application of high-throughput technologies on clinical specimens, including mass spectrometry,[157] next-generation sequencing and antigen arrays[158,159]. Decreasing costs and improvements in analysis of large data sets, provide an opportunity to specifically generate host response profile as diagnostic indications. Using mass spectrometry on serum samples from enteric fever patients, Näsström and colleagues have identified a set of metabolites that was able to distinguish typhoid from paratyphoid fever and enteric fever from febrile, typhoid negative controls and chronic carriers[160–162]. Transcriptional data from individuals with acute typhoid fever could also be used to identify signatures reliably identifying enteric fever cases[163,164]. The feasibility has recently been demonstrated using transcriptional data to identify host signatures (as little as 2 genes) that reliably distinguish bacterial compared with viral infections in a cohort of children in Europe and North America[165,166].

1.6.6. The Need for New Diagnostics

Investment in diagnostics for typhoid and paratyphoid fever will arguably be central in efforts to achieve disease control – allowing for improved patient care, reduced prescriptions of inappropriate antibiotics and improved measures of disease burden. Precise data on disease epidemiology and vaccine effectiveness will help to inform country level decisions on the deployment of vaccines, and the generation of these data are ultimately reliant on reliable diagnostics. Several novel diagnostic modalities are in the pipeline and ideally should be able to rapidly distinguish enteric fever from other causes of undifferentiated fever. The development of novel diagnostics could be accelerated and supported by more formally defining a target product profile, and recognising that different diagnostic modalities will need to be deployed in the range of settings where patients with enteric fever encounter healthcare professionals (inpatient vs. outpatient vs. pharmacy)[130]. Although novel diagnostics are certainly desirable, it should be acknowledged that basic clinical microbiology facilities are often inadequate in low-resource settings where the burden of enteric fever is highest. Investment in basic laboratory infrastructure, equipment (e.g. automated blood culture) and personnel has the potential to achieve an impact that extends beyond improved diagnosis of enteric fever[167].

1.7. Treatment

1.7.1. Multidrug resistant S. Typhi

The mortality rate of enteric fever in the pre-antibiotic era was estimated to be between 10-30%. The availability of traditional first-line antimicrobials over the past nearly 70 years (chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole) has reduced the overall mortality rate to <1%[75,168]. Unfortunately, their use has been limited by the emergence of so-called multidrug resistant (MDR) strains, defined as resistance to all three of the 'traditional' first line antimicrobials. Analysis of a global collection of *S*. Typhi isolates using whole genome sequencing

has demonstrated how a single *S*. Typhi haplotype , termed H58 (or genotype 4.3.1.), has emerged and spread globally[169]. Strains of *S*. Typhi of H58 are associated with multidrug resistance and reduced fluoroquinolone susceptibility, and isolates belonging to this genotype isolates are widely prevalent is South and South-East Asia, as well as in Central/Southern Africa[169–172]. Recent analysis suggests that this haplotype is displacing antibiotic sensitive strains and may possess a fitness advantage compared with other isolates[169,173]. In addition, several MDR typhoid epidemics have recently been described in Africa, evolving independently of the H58 haplotype, suggesting that *S*. Typhi is constantly adapting to new antibiotics and distinct ecological niches[174]. Resistance in MDR strains are typically conferred via IncHI1 plasmids, harbouring resistance genes such as *catA*, *sul1*, *sul2*, *dfrA*, bla_{TEM-1}, *strA*, *strB*, *tetA*, *tetB*, *tetC* and *tetD* on composite transposons. These MDR-associated genes have also been known to integrate within the chromosome of MDR associated H58 *S*. Typhi in isolates from countries including India, Nepal and Bangladesh[169].

MDR strains were responsible for several outbreaks of enteric fever in the 1980/1990s and led to the widespread use of fluoroquinolones as first-line therapy[70]. Despite considerable success in treatment of MDR typhoid, the extensive use of fluoroquinolones has since led to the emergence of intermediate and fully fluoroquinolone resistant strains. Fluoroquinolone resistance occurs mainly via chromosomal mutations in the *gyrA*, *gyrB*, *parC* and *parE* genes[169]. Cumulative mutations correspond to the degree of fluoroquinolone resistance – for example, a single SNP in codon S83F of *gyrA* will produce a low level resistance (ciprofloxacin minimal inhibitory concentration [MIC] of 0.125–0.25 mg/l) whereas additional SNPs in *gyrA* (D87N) and *parC* (S80I) confer a higher level of ciprofloxacin resistance (MIC 8–64 mg/l)[175]. In 2017, the World Health Organisation designated fluoroquinolone resistant *Salmonella* spp. as a high priority pathogen, identified as one of 12 families of bacteria thought to pose the greatest risk to human health through rising antimicrobial resistance[176].

Third generation cephalosporins are commonly used in the empirical treatment of enteric fever and are a valuable empirical treatment option in the setting of MDR and of fluoroquinolone resistant isolates[177]. However, a recent typhoid outbreak in Sindh, Pakistan was attributable to so-called extensively drug resistant (XDR) *S.* Typhi H58 defined as an MDR resistance pattern, combined with fluoroquinolone and cephalosporin resistance[178]. In this outbreak strain, cephalosporin resistance was mediated by the horizontal acquisition of a plasmid encoding the $bla_{\text{CTX-M-15}}$ extended-spectrum β-lactamase (ESBL), in addition to quinolone resistance containing genes such as *qnrB2*, *qnrB4*. The emergence of cephalosporin resistance calls for an urgent reappraisal in the use of cephalosporins for treating enteric fever in South and South-East Asia severely limits potential treatment options and emphasises the importance of disease prevention.

Azithromycin is increasingly used for the empirical treatment of enteric fever, benefitting from once daily oral dosing and excellent tissue penetration. Although currently rare, isolates with increased azithromycin MICs and treatment failure have been reported[179–183]. Azithromycin resistance is known to be mediated via the *ereA*, *msrD* and *msrA* genes[169].

The monobactam aztreonam may be a treatment option for treatment of fluoroquinolone resistant *S.* Typhi, particularly in individuals allergic to penicillin[184]. Alternative treatments include tigecycline or carbapenems, but there are relatively limited studies describing their use for the treatment of typhoid fever and widespread use may be limited by the cost of treatment[185]. In the face of ESBL producing *S.* Typhi, the use of carbapenems for treatment of typhoid fever may be poised to increase.

Combination antibiotic therapy is sometimes used in the treatment of enteric fever, particularly when response to treatment is slow, susceptibilities are unknown and when the diagnosis is uncertain[186]. Combination therapy may have synergistic effects and reduce the rate of emergence

of antibiotic resistant strains. There is currently limited evidence from randomised trials to guide this approach[187,188]. Combination therapy for suspected enteric fever should be actively assessed in future randomised controlled trials, particularly given the absence a well-developed antibiotic pipeline for typhoid fever, rising rates of resistance and limitations of the current generation of diagnostics.

Chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole were seldom used after widespread MDR strains emerged in the 1990's, but recent data suggest that sensitivity to these agents is re-emerging following their declining use. One study spanning a 9-year period in Nepal suggesting that over 95% of isolates were sensitive to all three 'traditional' first-line antimicrobials[189]. A separate case-report described successful treatment of typhoid fever cause by an H58 strain of *S.* Typhi using trimethoprim-sulfamethoxazole monotherapy[190]. There have been similar reports from other endemic settings in South and South-East Asia,[191] although the wider relevance of these findings awaits the conduct of randomised trials[192]. It is almost inevitable that MDR strains will re-emerge over time with the build-up of sufficient antimicrobial pressure via uncoordinated and widespread use of these drugs, particularly if coupled with poor surveillance systems. Nevertheless, these drugs could be used in settings with high fluoroquinolone resistance and evolving cephalosporin resistance – possibly when used in combination or with antibiotic cycling. Robust national and regional surveillance systems to monitor trends in antibiotic resistance will be required to ensure that treatment guidelines provide relevant recommendations in the light of developing resistance or the re-emergence of sensitive strains.

1.7.2. Antimicrobial treatment options

Empirical treatment guidelines for typhoid fever, where available, are frequently outdated and require updating to reflect global trends in antimicrobial resistance. The majority of randomized

trials comparing treatments for enteric fever have a small sample size and lack statistical power to detect meaningful differences between interventions, and the optimal choice of antibiotics and duration of therapy are often uncertain[193]. Differences in study end-points, and failure to report outcomes in culture-negative suspected enteric fever, also complicate the interpretation of trial data[177].

Recommended treatments for enteric fever may vary between regions. In low-resource settings, the diagnosis of enteric fever is often unconfirmed and empirical treatment is frequently commenced in the setting of a compatible clinical syndrome. In culture-confirmed cases, antibiotic treatment should be guided by local sensitivity patterns and direct sensitivity testing where available. Recent studies suggest that fluoroquinolones should not be recommended for empirical treatment of enteric fever in South Asia, due to high risk of treatment failure[177]. Current AMR profiles suggest that ceftriaxone or azithromycin represent appropriate empirical treatment options in South and South-East Asia, whereas fluoroquinolones may represent an appropriate treatment in parts of Africa, where high-level fluoroquinolone resistance is currently less common. Oral azithromycin may be more convenient and cost effective than parenteral ceftriaxone for outpatient management, but widespread use of this drug in many parts of South Asia today may rapidly lead to development of resistance, highlighting the importance of good microbiological surveillance. Some authors have advocated the addition of doxycycline for suspected enteric fever cases in regions of South Asia with high incidence of *Rickettsia* spp.[177,194].

1.7.3. Drivers of Antimicrobial Resistance in *S.* Typhi

The drivers of antimicrobial resistance in enteric fever are multifactorial and are beyond the scope of this chapter[195]. Nevertheless, a key factor for enteric fever is the absence of reliable diagnostics combined with a non-specific clinical syndrome, leading to widespread empirical antibiotic treatment of undifferentiated febrile illness. The purchase of antibiotics from untrained vendors

or unregulated pharmacies is commonplace in several countries with a high burden of enteric fever and likely contributes to the development of resistance[196,197]. In the healthcare setting, the over prescription of antibiotics may stem from limited diagnostics combined with complex cultural drivers and/or patient expectations[198]. Strategies aimed at reducing antibiotic consumption in some settings must be balanced with the need to increase access in parts of the developing world where access to appropriate antibiotics is limited – "balancing access and excess"[199]. The recent emergence of cephalosporin resistant XDR *S.* Typhi in Pakistan has been attributed to the horizontal acquisition of a plasmid encoding ESBL and quinolone resistance genes, which found in a range of widely distributed enteric bacteria including *E. coli*[178]. Antibiotic resistance within *S*. Typhi is a microcosm of the wider AMR challenge, requiring a co-ordinated and global implementation of an AMR action plan, including deployment of vaccines. Notably, of the twelve bacteria identified by the WHO as being priority pathogens "that pose the greatest threat to human health", *Salmonella* Typhi is one of a limited number of bacteria for which vaccines are licensed, pre-qualified and ready to be deployed[176].

1.8. Disease Prevention

Despite improvements in access to clean water globally, achieving universal clean water access requires substantial and sustained investment, and several barriers must be overcome to ensure equity of coverage in the short-term. The sustained high burden of disease, coupled with the emergence of drug resistant strains of *S*. Typhi, makes prevention via vaccination a priority in the short-to-medium term.

At the time of writing, the most commonly used vaccines for typhoid fever are the parenteral Vi-polysaccharide vaccine and the oral live-attenuated vaccine Ty21a. Killed whole cell vaccines (for typhoid and paratyphoid fever[200]) have previously been developed but are no longer recommended owing to high rates of reactogenicity. The Vi-polysaccharide vaccine mediates

protection exclusively through the production of anti-Vi antibodies, although the precise antibody effector mechanism is currently the subject of further study[201]. The Ty21a vaccine is thought to mediate protection through a combination of cellular and mucosal immune effector mechanisms[201]. The Ty21a and Vi-polysaccharide vaccines (Vi-PS) have demonstrated efficacy at 2 years of 58% (95%CI 40-71%) and 59% (95%CI 45-69%) respectively, but have limited use in the youngest age-group of children due to inconvenience in vaccine administration and poor immunogenicity respectively[202,203]. School based campaigns as well as delivery strategies of these vaccines using the available health care structure have previously been effective in terms of coverage and cost-effectiveness in Asia[63].

In 2008, WHO Strategic Advisory Group of Experts (SAGE) on immunisation committee issued a position paper supporting the use of Vi-PS and Ty21a vaccines has been limited – resulting in part from knowledge gaps in key areas required to inform decision making (e.g. disease burden estimates, implementation strategies) and uncertain funding streams[18,204].

1.8.1. Typhoid Conjugate Vaccines

Typhoid conjugate vaccines (TCVs), in which Vi-polysaccharide is covalently linked to carrier proteins, offer several potential advantages over earlier generation typhoid vaccines. The appeal of Vi-conjugate vaccines, relates to their capacity to induce immune responses in infants, enhanced immunogenicity in terms of antibody magnitude, quality and duration, and the potential for boosting of immune responses with re-vaccination. Proof-in-principle of TCV efficacy is derived, primarily, from trials of a prototype Vi-rEPA vaccine, which demonstrated efficacy of up to 91% [95% CI 77-97%] at two years, when given as a two dose schedule in 2-5 year old children[205]. The Vi-rEPA vaccine was efficacious up to at least 5 years and was compatible with co-administered EPI vaccines, but has yet to be commercialised[206,207].

The most advanced TCV is a Vi-tetanus toxoid conjugate vaccine manufactured by Bharat Biotech (Hyderabad, India) - TypbarTCV®[208]. This vaccine is immunogenic and safe in children from as young as six-months of age, as well as demonstrating superior immunogenicity to a Vi-Polysaccharide vaccine. Importantly, TypbarTCV® has demonstrated efficacy of between 54.6 to 87.1% in a stringent controlled human infection model, depending on the efficacy endpoint[209]. Modelling studies have estimated a vaccine efficacy for TypbarTCV® of 85% based on serological data[210]. Cost-effectiveness models indicate that routine infant TCV vaccination is likely to be cost-effective in medium- or high-incidence settings, depending on the intervention strategy used and at a modest vaccine cost (~$2/dose)[211,212]. Additional safety and immunogenicity data will be generated in an upcoming introduction of TCV in Navi Mumbai, India and from three phase IV effectiveness studies conducted as part of the TyVAC consortium [20]. Several TCVs are currently in development, including VI-DT, Vi-rEPA, Vi-CRM_{197} and Vi-tetanus toxoid conjugates, many of which have completed Phase 1 and 2/3 trials[208,213,214].

In October 2017, the WHO SAGE, recommended programmatic use of TCVs in children over six months of age in typhoid endemic countries. A position paper has since been published updating recommendations for on typhoid vaccination, superseding a position paper from 2008[215]. The WHO has recommended the use of TCV in typhoid endemic countries from the age of 6 months onwards, administered as a single dose and programmatic administration in combination with other childhood vaccines at 9 months of age or in the second year of life[215]. Where feasible and supported by epidemiologic data, catch-up vaccination up to 15 years of age was also recommended. The position paper highlights that the roll of TCVs should be prioritised in countries with a high burden of typhoid fever or high rates of antimicrobial resistance. Several areas for further research were highlighted, in particular better understanding of the duration of protection afforded by a single dose, the need for re-vaccination and the need to identify immunological correlates of protection. TYPBAR-TCV® was prequalified by the WHO in January

2018[216] and Gavi has committed an $85 million funding window to support the roll out of these vaccines in eligible countries between 2019-2020.

1.8.2. The Value of Vaccination in Tackling AMR

Immunisation forms a central pillar of the global action plan on AMR and can reduce the development of resistance by multiple mechanisms[195,217]. Programmatic use of TCV is anticipated to reduce overall antibiotic consumption in highly endemic areas, by preventing both confirmed typhoid cases requiring antibiotic treatment, and by reducing the incidence of undifferentiated fever treated with undirected therapy. Conjugate vaccines have reduced the burden of AMR for other invasive bacterial pathogens, including *Streptococcus pneumoniae*[218,219], and effective deployment of TCVs would be anticipated to have a similar effect for *S.* Typhi. TCVs have also be deployed in response to typhoid outbreaks associated AMR, as illustrated by the donation of more than 50,000 doses of a Vi-TT in response to the outbreak of XDR *S.* Typhi in Pakistan[216]. Continuing surveillance studies will be required to assess the impact of TCVs on antimicrobial resistance patterns, but if successful could be used as another example of the impact of vaccines to address AMR.

1.9. Summary

Typhoid and paratyphoid fever are diseases of poverty. Whilst they have been virtually eradicated in the developed world, enteric fever remains a major public-health problem in resource-limited settings. As human restricted diseases, typhoid and paratyphoid fever are potentially amenable to eradication. The development, and impending roll-out, of cost-effective, scalable TCVs represents a major advance in typhoid control and could have a substantial impact on the global burden of disease. TCVs are potentially a valuable tool that overcome some of the limitations of existing typhoid vaccines and – for the first time – offers us a vaccine that is suitable for routine use in childhood immunization programmes. Momentum to achieve control of typhoid and

paratyphoid is building, driven by the availability of effective tools and support from key stakeholders.

Whilst a sustained reduction in disease burden is potentially achievable in the coming decades, limited evidence in several key areas relating to disease transmission, immunity and pathogenesis represent potential pitfalls in efforts to achieve disease control by vaccination. The impending deployment of TCV may apply an evolutionary pressure driving the emergence of Vi-negative strains of *S.* Typhi[220,221], necessitating alternative vaccine approaches. In addition, some studies have described an increase in cases of paratyphoid fever following successful programmatic use of Vi-polysaccharide vaccines, which could be attributed to serovar replacement in an as-yet-unrecognised ecological niche[24]. The apparent increasing burden of enteric fever attributable to *S.* Paratyphi A is of particular concern in the absence of licensed vaccines [222] – leading some experts to label *S.* Paratyphi "*the Achilles heel in the global battle against enteric fever*" [223].

Collectively, novel vaccination strategies are likely to be required to achieve comprehensive control of enteric fever in the coming decades. The development and deployment of new vaccines for typhoidal *Salmonella* is fraught with difficulties, ranging from challenges in access, funding, advocacy and policy more generally, through to uncertainties of disease pathogenesis and protective immunity more specifically. In the next chapter, and in the remainder of this thesis, I will discuss the current understanding of the pathogenesis and immunity to enteric fever, and argue that an improved understanding of these factors could play an important role in contributing towards the control of enteric fever.

Chapter 2 – Understanding Enteric Fever Pathogenesis and Immunity: The Typhoid Toxin and Infection Derived Immunity

2. Introduction

In the previous chapter I have outlined contemporary challenges to the global control of enteric fever and how vaccination is anticipated to play a central role in future disease control efforts. Comprehensive control of enteric fever is likely to require multiple vaccine options, ideally capable of targeting a range of *Salmonella* serovars and conferring herd immunity. In particular, the availability of vaccines for *S*. Paratyphi A is a major unmet need.

In this chapter I will begin by summarising the current understanding of typhoid pathogenesis and review the published literature on a specific virulence factor, which will be the focus of part of my thesis. This is a recently described exotoxin, termed the typhoid toxin. I will describe the current understanding of toxin structure, mechanisms of action and the evidence for its postulated role in the pathogenesis of typhoid fever, before highlighting key knowledge gaps that I intend to address in the first study described in this thesis. I will then review the current understanding of infection derived immunity to enteric fever and highlighting key knowledge gaps that will be addressed in the second study described in this thesis.

2.1. The Pathogenesis of Enteric Fever

Salmonella Typhi and Paratyphi are transmitted faeco-orally. Following ingestion of contaminated food or water, the bacteria must traverse the gastric acid barrier. Both *S*. Typhi and Paratyphi are highly susceptible to stomach acid – studies in human volunteers indicate that neutralization of the stomach acid is associated with a drop in the infectious dose by several orders of

magnitude[74,126]. Once in the intestine, the bacilli invade across the small intestinal mucosa, in part through M cells that overlie gastrointestinal associated lymphoid tissues in the terminal ileum [224 225]. Adhesion to human epithelial cells can occur through multiple mechanisms[226–228]. Once in the lamina propria, the bacteria are thought to be ingested by tissue resident macrophages, within which they reside in a specialized compartment termed the *Salmonella* containing vacuole (SCV) where they avoid killing[229]. Subsequent dissemination of bacteria contained within macrophages through bloodstream dispersal is thought to cause a 'silent' primary bacteraemia[73]. This results in dissemination of *Salmonella* bacteria to the reticuloendothelial system, including the liver, spleen, lymph and bone marrow. The onset of symptoms corresponds with secondary bacteraemia. The majority of bacteria (~60%) are thought to reside in the intracellular compartment during acute illness[230].

In contrast to infection with non-typhoidal *Salmonella* serovars in humans, early intestinal inflammation is not a prominent feature of infection with *Salmonella* Typhi, evidenced by a lack of neutrophil infiltration in the mucosal epithelium[225]. Biopsy specimens taken from volunteers challenged with *Salmonella* Typhi show evidence of granulomatous inflammation in the small intestine in the majority of cases, even in the absence of pronounced gastrointestinal symptoms[231]. Dampening of intestinal inflammation could be mediated through a variety of mechanisms, such as down-regulation of pathogen associated molecular patterns, including flagellin, when the bacteria interact with human cells, resulting in a diminished mucosal inflammatory response[225,232].

2.1.1. Pathogen Genomics

Salmonella Typhi is monophyletic and exhibits limited genetic variation between individual isolates[169]. Classification schemes based on phage-typing or pulsed-field gel electrophoresis have previously been developed but have relatively limited capacity to infer the phylogeny of *S*. Typhi

and Paratyphi. Advances in whole genome sequencing have offered valuable insights into the global population structure of *S*. Typhi. A classification scheme proposed by Roumagnac *et al* divided the *S.* Typhi population into 85 distinct haplotypes, based on 88 single-nucleotide polymorphisms (SNPs)[233]. More recently, Wong and colleagues have proposed a genotyping framework based on 68 SNPs, derived from whole genome sequencing of >1800 geographically distinct *S*. Typhi isolates[234]. In this classification system, global isolates of *S*. Typhi are divided into four broad clusters, within which are contained 16 clades and 49 subclades. Individual isolates are labelled using a structured hierarchical nomenclature system defined sequentially by the cluster, clade and subclade[234]. For example, the globally disseminated multidrug resistant clade labelled H58 according to the Roumagnac *et al*[233] system, is re-labelled as genotype 4.3.1[234]. *Salmonella* Paratyphi A is closely related to *Salmonella* Typhi, but appears to be of more recent evolutionary origin[235,236].

In addition to characterising the phylogeny, genomic studies have also offered several insights into the pathogenic mechanisms of *S.* Typhi and Paratyphi. The genomes of *S.* Typhi and Paratyphi are notable for the accumulation of multiple pseudogenes, several of which correspond to genes known to contribute to the virulence of non-typhoidal *Salmonella* serovars[225]. *S*. Paratyphi appears to have undergone a process of convergent evolution, as evidenced by a similar accumulation of pseudogenes, several of which are shared between the two serovars[235,236].This process of so-called genomic-degradation is thought to reflect the host-restriction properties of *S.* Typhi and *S*. Paratyphi, as a similar process has been observed in other host restricted pathogens including *Mycobacterium leprae*, *Bordetella pertussis* and *Yersinia pestis*[237].

Most strains of *S.* Typhi produce a polysaccharide capsule, termed the Vi antigen. This is encoded by the *viaB* locus, which comprises several genes required for biosynthesis and export of the capsule. The *viaB* locus is unstable and can potentially be excised from the chromosome following

serial passage or long term storage[238,239]. Vi-capsule negative strains of *Salmonella* Typhi have been described, but are currently relatively rare[142,240]. In the absence of the Vi capsule, *Salmonella* Typhi is inherently more sensitive to killing in serum compared with other serovars, such as *Salmonella* Typhimurium[241]. The Vi-capsule possesses immunomodulatory properties that are thought to contribute to disease pathogenesis, including limiting complement deposition, reducing immune activation, assisting with phagocytosis evasion and inhibiting serum bactericidal activity[241–244]. The Vi capsule forms the basis of one of the commercially available typhoid vaccines and new conjugate vaccines. Vi antigen is expressed by other bacteria including *Citrobacter freundii*, *Salmonella* Paratyphi C and *Salmonella* Dublin, but not *Salmonella* Paratyphi A. Notably, *Salmonella* Paratyphi A causes an indistinguishable syndrome to *Salmonella* Typhi, despite lacking the Vi-capsule, suggesting that multiple factors are involved in immune evasion and the pathogenesis of enteric fever[71].

Salmonella Typhi possess approximately 300-400 specific genes not found in other *Salmonella* serovars. Many of these gene products are encoded on *Salmonella* pathogenicity islands (SPIs) relatively unique to *S*. Typhi (e.g. SPI-5, SPI-15, SPI-17 and SPI-18)[225]. One virulence factor shared by *Salmonella* Typhi and Paratyphi A is a recently described exotoxin termed the typhoid-toxin, which will be the focus of the next section.

2.2. The Typhoid Toxin

2.2.1. Historical Context

Exotoxins represent attractive vaccine and therapeutic targets, in part stemming from a long history of targeting exotoxins with active and passive immunisation strategies. In 1901, the first Nobel Prize in Physiology or Medicine was awarded to Emil von Behring for his work on serum therapy targeting diphtheria toxin[245]. Currently licensed vaccines that target bacterial exotoxins – at least in part – include those against tetanus, diphtheria, whooping cough and cholera, and in

cases where exotoxin-based vaccines have proven effective, they provide antibody mediated protection (neutralisation) of exotoxins that have a central role in disease pathogenesis.

Early bacteriological studies of the typhoid bacillus in the late 19th and early 20th centuries attempted to identify a soluble toxin responsible for the symptoms of typhoid fever that could be targeted by passive immunization[246]. In 1889, Ludwig Brieger (1849-1919) first postulated the existence of toxic substance produced by the typhoid bacillus, which he termed 'typhotoxin'. He hypothesized that *"the typhoid bacillus produces symptoms and lesions…which are attributable to toxins rather than to its simple multiplication and parasitic existence in the body"*[247]. The French bacteriologist Andre Chantemesse (1851-1919) later described the production of an anti-serum targeting an exotoxin produced by the bacteria, in which work he states to *"have proved conclusively that exotoxins are produced by the typhoid bacillus if suitable conditions of medium and bacterial strain are chosen"*[248] [249]. Historical studies on the toxins of *S.* Typhi tend to focus primarily on the relative contribution of endotoxins and exotoxins – the existence of a specific exotoxin remained controversial. In a review of 1918, Frederick Gay concludes by stating what is the prevailing wisdom of typhoid pathogenesis, specifically that "*although the typhoid bacillus may produce a type of soluble toxin, its harmful effects must in large part be attributable to the so-called endotoxins*" [250].

2.2.2. 'Re-Discovery' of the Typhoid Toxin

The genes encoding the components of the typhoid toxin were first conclusively identified following the publication of the first complete genome sequence of MDR *Salmonella* Typhi CT18, which described *"a short insert carrying homologues of the Campylobacter toxin cdtB (STY1886) and the Bordetella pertussis toxin genes ptxA and ptxB (STY1890 and STY1891)"* [237].

The components of the typhoid toxin are encoded on a pathogenicity island comprising five genes termed *cdtB*, *pltA*, *pltB*, *ttsA* and *sty1887*[237,251]. Initial studies suggested that typhoid toxin pathogenicity island, was uniquely encoded by the typhoidal *Salmonella* serovars, *S.* Typhi and *S.* Paratyphi A[237]. The apparent restriction of this genomic region to the typhoidal *Salmonella* serovars suggested that this region may encode a virulence factor responsible for the distinct clinical presentation of enteric fever as compared with non-typhoidal *Salmonella* (NTS). However, comparative genomic studies by den Bakker and colleagues have since described the presence of the typhoid toxin pathogenicity island in a range of non-typhoidal *Salmonella* serovars (Figure 2-1 :)[252,253]. Epidemiological data from 46,639 *Salmonella* cases reported in the US between 1996 and 2006, indicated that *Salmonella* isolates associated with invasive diseases possessed the typhoid toxin pathogenicity island[253]. Notably the typhoid toxin pathogenicity island is not found in *Salmonella* Enteritidis or *Salmonella* Typhimurium, which are responsible for largest burden of invasive NTS disease in Africa [253,254].

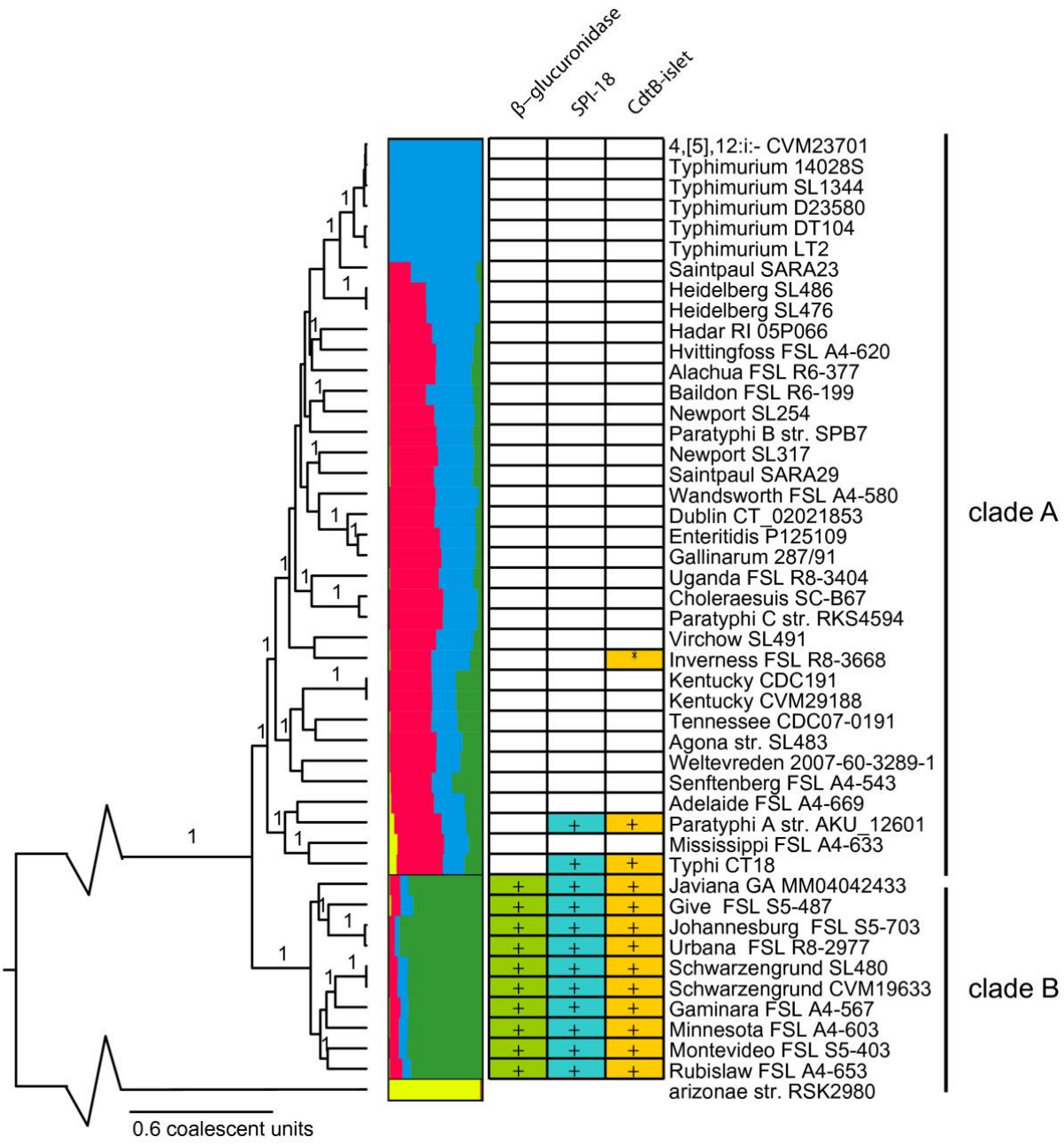


Figure 2-1 : Identification of the typhoid toxin pathogenicity island (CdtB-islet) in typhoidal and non-typhoidal *Salmonella* serovars.

Displayed are the phylogenetic relationships of 46 *Salmonella enterica* subsp. *enterica* strains inferred by multilocus sequence typing. Blocks adjacent to the phylogeny correspond to the presence or absence of specific genes, including the typhoid toxin pathogenicity island (=CdtB-islet [gold]) in the genomes. The typhoid toxin pathogenicity island in S. Inverness is marked with an asterisk because of its position on a putative mobile element. Adapted from den Bakker et al 2011[252]. Reproduced under license under CC BY 4.0 [255].

2.2.3. Composition of the Typhoid Toxin

The typhoid toxin is comprised of three polypeptide sub-units, termed Cytolethal distending toxin B (CdtB), Pertussis like toxin A (PltA) and Pertussis like toxin B (PltB), all of which are encoded within the typhoid toxin pathogenicity island (Figure 2-2). Each of the typhoid-toxin sub-units are homologues of toxin sub-units found in other bacterial species.

CdtB is homologous to the enzymatic component of an exotoxin family known as cytolethal distending toxins (CDT). Cytolethal distending toxins are a family of toxins with DNAse activity, present in a range of bacterial pathogens including *Campylobacter* spp., *E. Coli*, *Shigella* spp, *Haemophilus* spp., and *Yersinia* spp. amongst others[256]. Cells intoxicated with CDT undergo cell-cycle arrest at the G2/M phase of the cell cycle, acquiring a distinct morphology with distension of the cytoplasm and nucleus, and eventually die by apoptosis (Figure 2-2). In most bacterial species, CDT typically exists in a heterotrimeric AB_2 form, comprising three subunits termed CdtA, CdtB and CdtC – toxin-binding and intracellular trafficking is mediated by CdtA/C, whereas DNAse activity is mediated by CdtB[257].

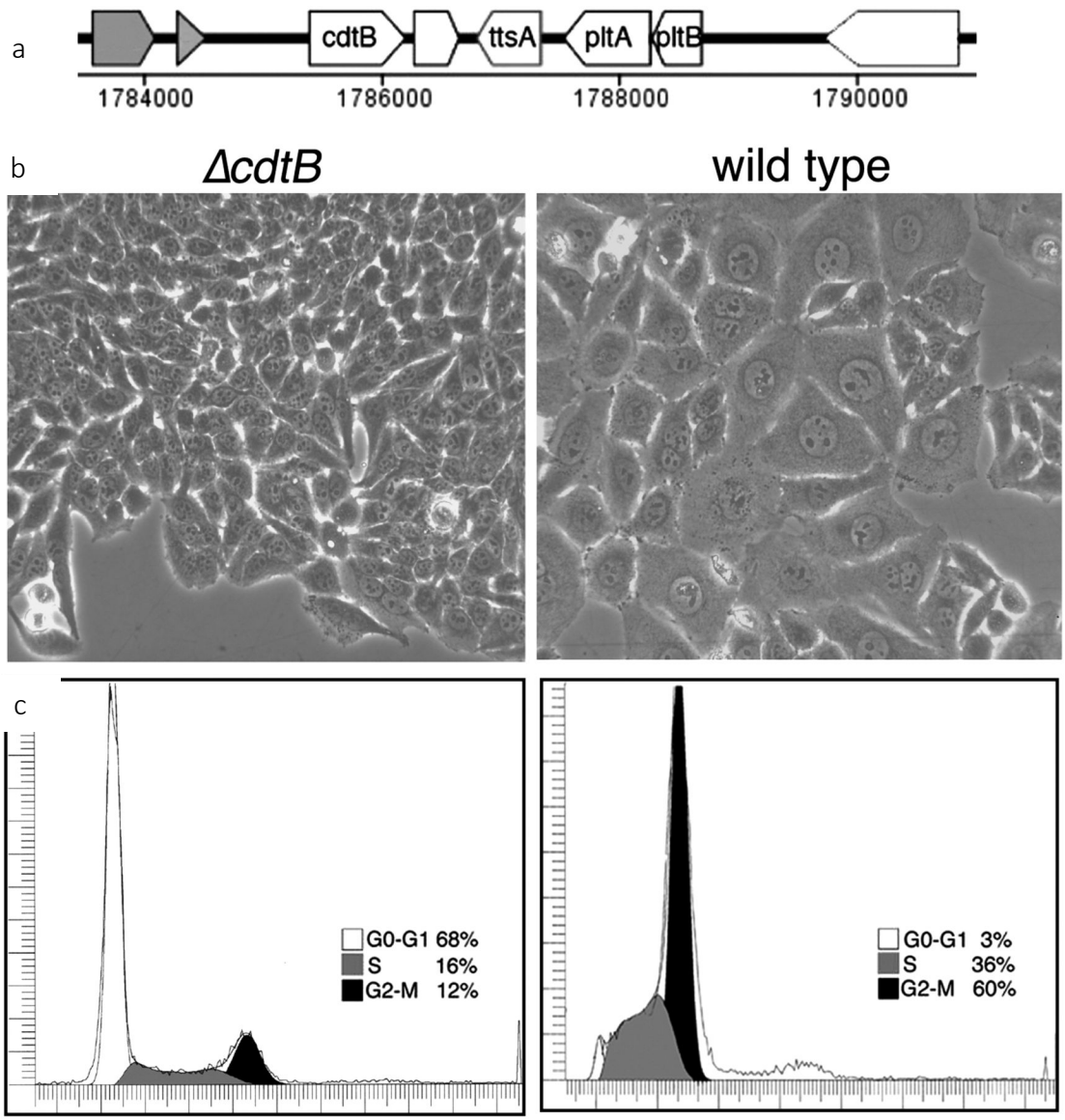


Figure 2-2 : Effect of typhoid toxin on Henle-407 cells in vitro.

(a) Typhoid toxin pathogenicity island. (b) Morphological appearance of cultured epithelial cells by phase contrast microscopy following infection with wild-type *S.* Typhi or cdtB knock-out *S.* Typhi. Cells infected with the wild-type, but not the CdtB knock-out acquire a distinct morphological appearance with a distended nucleus and cytoplasm. (c) Toxicity assay measuring DNA content by flow cytometry, showing the proportion of cells in the G0-G1, S or G2 phase of the cell cycle. Cells undergoing cell cycle arrest show a higher proportion of cells in the G2 phase of the cell cycle (right). Reproduced and modified, with permission, from Galan J (2016) PNAS [251].

The *Salmonella* Typhi and Paratyphi genome encode a homologue of CdtB, but lack homologues of CdtA and CdtC [237]. In typhoidal *Salmonella*, the functions of CdtC and CdtA are instead taken up by homologues of the pertussis toxin, usually expressed by *Bordetella pertussis*. PltA has ADP-ribosylase activity and is homologous to the enzymatic S1 subunit of the pertussis toxin. PltB is homologous to the binding component of other bacterial toxins, including the S2 subunit of the pertussis toxin and SubB subunit of the subtilase cytotoxin expressed by E. Coli [258]. Similar to PtxS2[259] and SubB, the PltB unit possesses a sugar binding pocket that mediates interactions with glycoproteins on the surface of host cells[260,261].

2.2.4. Structure of the Typhoid Toxin

The typhoid toxin has a unique A_2B_5 structure not previously described in other bacterial toxins (**Figure 2-3**). In contrast, 'classical' cytolethal distending toxin is arranged in an AB_2 format[257], whereas the Pertussis toxin is arranged in an AB_5 format[259]. The typhoid-toxin has a height of ~90Å and width of ~60Å, arranged in a pyramid shape[260]. The base is formed by a homo-pentameter of PltB subunits, arranged in a circular fashion with a central pore. The PltA subunit sits atop the homo-pentameric base, with an alpha-helix at the carboxy terminus projecting into the central hydrophobic pore of the PltB channel. CdtB forms the apex of the pyramid, sitting on top of PltA. PltA and CdtB are covalently linked by a disulphide bond between Cys_{214} of PltA and Cys_{269} of CdtB. These two cysteine residues are critical for assembly and export of the toxin, and notably are absent from homologues of PltA (ArtA) and CdtB in other bacterial species – suggesting that these two residues have evolved specifically to mediate interaction between these diverse toxins[260].

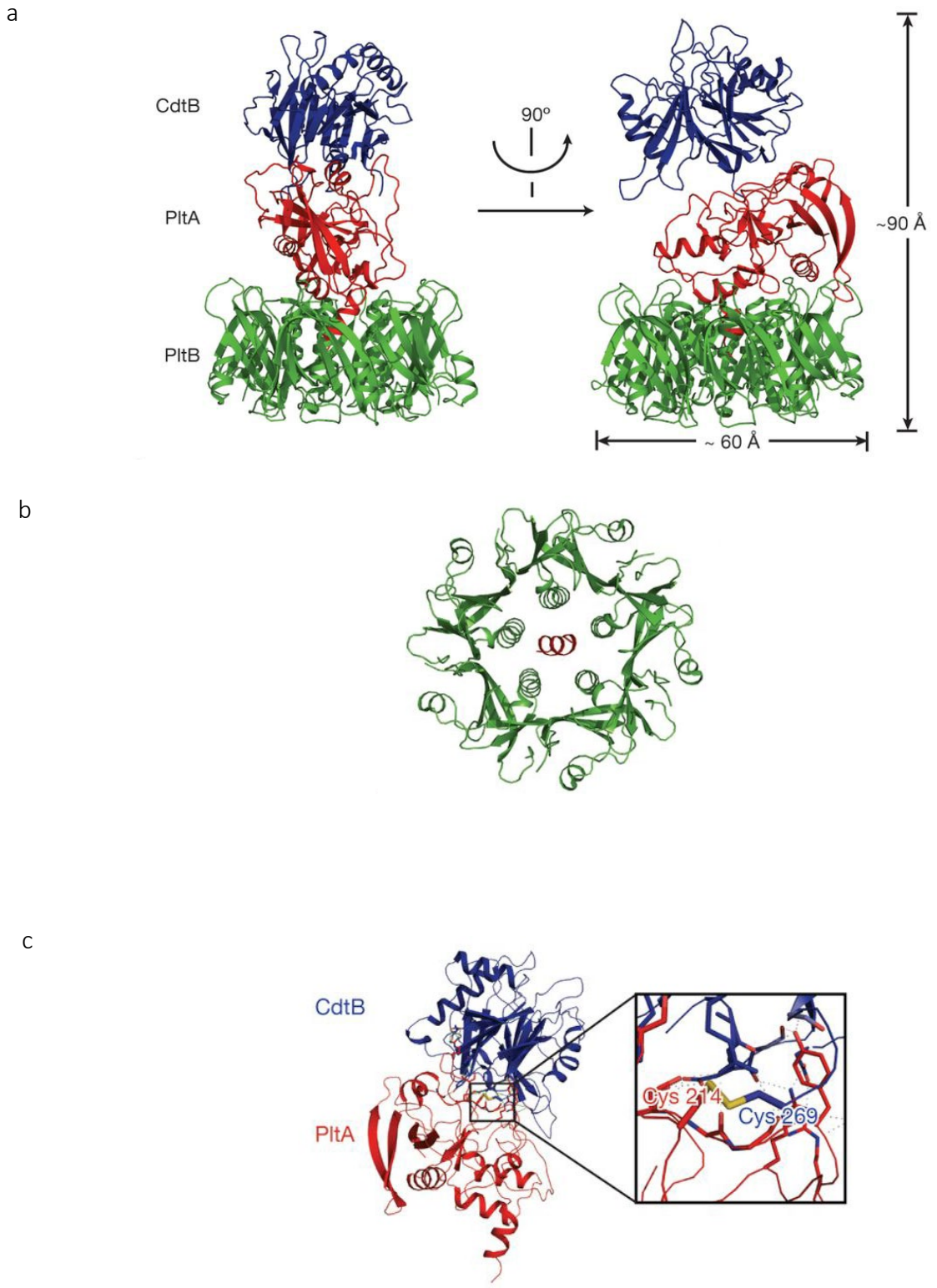


Figure 2-3: Atomic structure of the typhoid toxin.
(a) Two views of the overall structure of the typhoid holotoxin complex shown as a ribbon cartoon and following a 90° rotation about a vertical axis. The CdtB, PltA and PltB subunits are coloured blue, red and green respectively. (b) Bottom view of the channel formed by the PltB pentamer (in green), depicting the PltA C-terminal α-helix projecting into the central pore of the PltB homopentamer. (c) Interface between CdtB and PltA, including the critical disulphide bond between Cys_{214}-PltA and Cys_{269}-CdtB. Adapted, with permission, from Song, Gao and Galán (2013)[260].

2.2.5. Regulation and Mechanisms of Typhoid Toxin Expression

The typhoid toxin is expressed by *S*. Typhi from within the SCV[262]. Transcription of the *cdtB* gene occurs only when *S*. Typhi is grown within intracellular compartment of eukaryotic cells and not when cultured in liquid media, as assessed using a luciferase reporter system[256 262]. Transcriptional regulation of the typhoid toxin involves multiple regulatory components. Triggers for expression of the typhoid toxin pathogenicity island appear to occur only when the bacteria are resident in an intracellular niche. The IgeR protein is able to bind to the *cdtB* promotor and represses expression in an extracellular environment[263]. In addition, other studies have demonstrated that toxin expression occurs in PhoP-dependent manner[264]. Unique expression of the typhoid toxin within the intracellular niche differs from the expression of 'classical' cytolethal distending toxin in other Gram negative bacteria, where the toxin can routinely be detected in the supernatants of bacteria grown in liquid culture[265].

2.2.6. Secretion of the Typhoid Toxin

Expression of an exotoxin from an intracellular bacterium poses several challenges, as the toxin has to cross multiple bacterial and host cellular membranes in order to affect target cells[266]. The typhoid-toxin subunits CdtB, PltA and PltB possess an N-terminal Sec-signal peptide (SecSP), which allows for transport of the toxin-subunits from the bacterial cytoplasm into the periplasm via the SecYEG protein transport complex. Assembly into the A_2B_5 holotoxin likely occurs within the bacterial periplasm[251] (Figure 2-4).

Secretion of the typhoid toxin from the periplasm is mediated by a putative N-acetyl-β-d-muramidase, termed TtsA (Typhoid Toxin Secretion A)[267]. The *ttsA* gene is encoded with the typhoid toxin subunits in the same pathogenicity island. Secretion of typhoid toxin in a TtsA-dependent manner is thought to reflect an adaption of the Holin-Endolysin system, which

typically mediates phage release from bacteria and cell lysis. Holins are a family of proteins that form pores in the inner membrane of bacteria and facilitate transport of cognate endolysins into the periplasm and trigger bacterial cell lysis. The TtsA protein demonstrates close homology to a family of N-acetyl-β-d-muramidases (a sub-class of endolysin) and is presumably of phage origin. The typhoid-toxin pathogenicity island does not encode a cognate Holin protein, as is the case with other Holin-Endolysin pairs. Therefore, transport of the TtsA protein is likely mediated by an as yet uncharacterised Holin protein, expressed elsewhere in the *S*. Typhi genome, and mediates secretion of the typhoid toxin into the extracellular space, dependent on a peptidoglycan binding domain at its C-terminus[267]. The secretion of typhoid toxin across the bacterial outer membrane is not accompanied by cell lysis and suggests that a system designed for phage release has been adapted for secretion of toxins by bacteria(Figure 2-4)[251].

After the typhoid toxin crosses the bacterial outer membrane, it enters the lumen of the SCV, from where it is packaged into vesicle carrier intermediates and transported to the extracellular environment. Different models have been proposed to explain the trafficking of the typhoid-toxin from the SCV to the extracellular environment where it can affect target cells. Using a model of *S.* Typhimurium engineered to express the typhoid-toxin, Guidi and colleagues propose that the typhoid-toxin containing outer-membrane vesicles (OMVs) bud from the membrane and are trafficked from trafficked from the SCV to the extracellular space along microtubule and actin tracks[266]. Spano and colleagues propose a subtly different model, whereby trafficking of the typhoid-toxin to the extracellular environment is dependent on the Rab GTPase, Rab29[268]. Rab29 is recruited to vesicles containing *S.* Typhi and *S*. Paratyphi after bacterial internalisation in epithelial (Henle-407, COS-1) and macrophage (THP-1) cell lines. Recruitment of Rab29 occurred ~90-120 minutes after internalisation and was maximal after ~3 hours. In contrast, Rab29 was not recruited to vesicles containing non-typhoidal *Salmonella* serovars Typhimurium, Enteritidis and Dublin that typically possess a broad host range. These non-typhoidal *Salmonella* serovars

are shown to possess an effector protein termed GtgE, which is secreted by the Type III secretion system and is absent from S. Typhi. GtgE cleaves Rab29 and prevents export of the typhoid toxin from the SCV to the extracellular space[268]. Sorting of the typhoid-toxin into vesicle carrier intermediates requires an interaction between the PltB sub-unit and sialylated glycans on the inner membrane of the SCV[269]. In the model proposed by Spano and colleagues, the typhoid toxin is released into the extracellular environment as a soluble toxin, as opposed to being packaged within OMVs[251] (Figure 2-4).

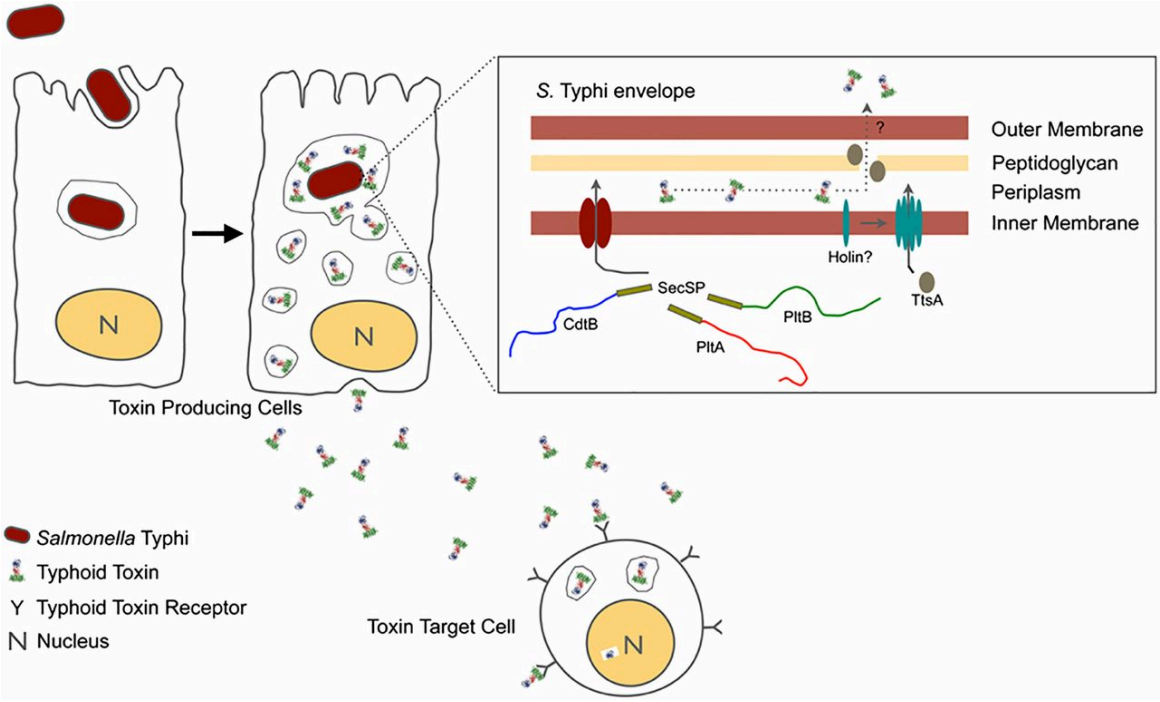


Figure 2-4 : Proposed mechanisms for expression of the typhoid toxin.

S. Typhi uptake into host cell and formation of the *Salmonella* containing vacuole (SCV). Transcription of the typhoid toxin with de-repression of IgeR [263] and under control of PhoP[264]. Proteins translated within the cytoplasm cross the inner membrane under control of the SecSP signal sequence. Formation of the holo-toxin within the periplasmic space. Secretion of the holo-toxin mediated by TtsA with the aid of as as-yet-uncharacterised protein[267]. Packaging of toxin into vesicle carrier intermediates and transport outside of the host cell[268]. Targeting host-cells in a paracrine and autocrine fashion. Binding to host-cells surface glycoproteins terminating in N-Acetylneuraminic Acid [261]. Entry into host cells via endocytosis. Retrograde trafficking to Golgi complex and endoplasmic reticulum, from where it crosses the nuclear membrane to cause single strand- and double-strand breaks in host DNA. Host cell undergoes apoptosis as part of DNA damage response. Adapted, with permission, from Galan J (2016)[251].

2.2.7. Cellular Targets

After release from the infected cell, the typhoid toxin is postulated to target surrounding cells in an autocrine and paracrine fashion (Figure 2-4). Addition of a blocking antibody prevents *S*. Typhi infected cells from intoxicating surrounding cells[262,269]. The typhoid toxin interacts with target cells by binding of the PltB sub-unit to a number of cell surface receptors, including podocalyxin-like protein 1 (PODXL) on epithelial and endothelial cells, and CD45 on haematopoietic cells[260]. Typhoid toxin can target a broad range of host-cells – including epithelial cells, endothelial cells, granulocytes, T-cell, B-cells and macrophages – suggesting that the typhoid toxin can interact with multiple distinct cellular receptors. The common feature of receptors capable of binding typhoid toxin is the presence of a specific terminal carbohydrate moiety, N-Acetylneuraminic Acid (Neu5AC)[260,261]. A specific residue (Ser_{35} on PltB) has been shown to mediate the interaction between PltB and Neu5AC[260].

Entry of typhoid toxin into the target cell occurs via endocytosis. Once inside target cells, the typhoid toxin is predicted to undergo retrograde trafficking through the Golgi complex and endoplasmic reticulum, before crossing the nuclear membrane to cause single-strand breaks (SSB) and double-strand breaks (DSB) in host DNA[265,266]. The location and mechanisms of typhoid-toxin sub-unit dissociation, and the mechanisms of transport across the nuclear membrane are currently uncharacterised[265]. Damage to host DNA activates the cells' DNA damage response, resulting in cell cycle arrest at G2/M phase and cellular distension, which – ultimately – is presumed to be mechanism by which the typhoid toxin is harmful to the host.

2.2.8. In Vivo Evidence for Typhoid Toxin as a Key Virulence Factor

A number of *in vivo* studies in small mammals suggest that the typhoid-toxin may represent an important virulence factor in acute typhoid disease. Administration of purified typhoid toxin to

C57BL/6 mice by intravenous injection results in an acute illness, characterised lethargy, stupor weight loss, leukopaenia and neutropaenia[260]. Toxin administration is also associated with a severe impairment of motor function and seizures in a mouse model[270]. As a comparator, oral administration of *S*. Typhimurium in the same mouse model resulted in a clinical phenotype of stupor, hunched posture and neutrophilia, but not the neurological dysfunction or neutropaenia associated with typhoid toxin administration[270]. Notably, typhoid toxin administration does not induce fever in the mouse model [260 270]. Administration of typhoid-toxin variants with mutations in the catalytic site of CdtB ($CdtB^{H160Q}$) – or at the binding site of PltB ($PltB^{S35A}$) – does not result in the acute illness phenotype associated with administration of the wild-type typhoid toxin. Toxin variants with a mutation in the putative catalytic site of PltA ($PltA^{E133A}$) retain the capacity to induce toxicity *in vitro* and *in vivo* [260,270].

Other studies have suggested that the typhoid-toxin contributes to the establishment of chronic infection. CDT belongs to a family of genotoxins which have been associated with chronic infection and colonisation in a range of bacterial infections, along with the development of cancers. Notably, chronic carriage of *S*. Typhi is associated with an increased risk of gallbladder carcinoma[271]. Using a model of *S.* Typhimurium (MC1 strain) engineered to express the typhoid toxin, Del Bel Belluz and colleagues demonstrated that oral challenge of 129S6/SvEvTac mice with a typhoid-toxin expressing *S*. Typhimurium was associated with enhanced survival, reduced intestinal inflammation and the establishment of a chronic infection state, compared with an isogenic *S*. Typhimurium strain lacking expression of the typhoid toxin[272].

2.2.9. Contribution of the Typhoid Toxin to Human Host Specificity of *Salmonella* Typhi

Recent evidence suggests that the unique binding properties of typhoid toxin may, in part, account for the host restriction properties of typhoidal *Salmonella*. The typhoid toxin binds to a broad range of human cells via the PltB sub-unit. Binding is mediated by an interaction with a

range of cell-surface glycoproteins, which terminate in specific sialoglycans known as N-Acetylneuraminic Acid (Neu5AC). In contrast to humans, sialoglycans on the surface of cells from other old-world primates and most mammals possess a different configuration, terminating in N-Glycolylneuraminic Acid (Neu5GC). This is because humans lack a specific enzyme (CMP-N-acetylneuraminic acid hydroxylase (CMAH)) required to convert Neu5AC to Neu5GC. Typhoid toxin preferentially binds, and is toxic to, cells expressing the human signature Neu5AC, as in comparison to cells expressing Neu5GC, as found in most other mammals(Figure 2-5)[261].

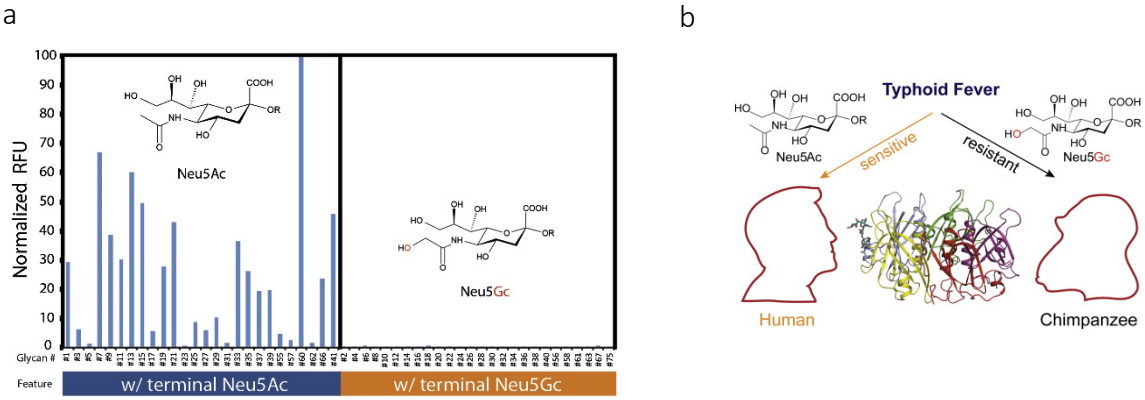


Figure 2-5 : Binding of typhoid toxin PltB to sialoglycans on cell surface glycoproteins
(a)Comparison of Typhoid Toxin Binding to Paired Neu5Ac- and Neu5Gc-Terminated Glycans by a Customized Microarray (b) Differential binding to human and chimpanzee tissues. Binding is mediated by an interaction with a range of cell-surface glycoproteins, which terminate in specific sialoglycans - N-Acetylneuraminic Acid (Neu5AC). In contrast to humans, sialoglycans on the surface of cells from other old-world primates and most mammals possess a different configuration, terminating in N-Glycolylneuraminic Acid (Neu5GC). This is because humans lack a specific enzyme (CMP-N-acetylneuraminic acid hydroxylase [CMAH]) required to convert Neu5AC to Neu5GC. Typhoid toxin preferentially binds, and is toxic to, cells expressing the human signature Neu5AC, as in comparison to cells expressing Neu5GC, as most other mammals. Reproduced, with permission, from Deng, Song, Gao et al (2014) [261].

2.2.10. The Role of Typhoid Toxin in Host Inflammatory Responses

The role of typhoid toxin in the induction of inflammatory responses is poorly understood. Administration of purified typhoid toxin to mice fails to induce fever,[260] suggesting that that the typhoid toxin itself does not directly stimulate the production of classical pyrogens such as IL-1, IL-6 or TNF-α. Following infection with a *S*. Typhimurium strain engineered to express the typhoid toxin, mouse colonic cells had reduced transcription of genes encoding pro-inflammatory cytokines, including *Tnfa*, *Ifng*, *Cxcl5*, *Cxcr5* and *Il22*, when compared with mice infected with a *S*. Typhimurium strain lacking the typhoid toxin[272]. Cytolethal distending toxins from other bacterial species have been shown to reduce secretion of pro-inflammatory cytokines from $CD4^+$ T-cells[273]. These data suggest that the typhoid toxin may play an anti-inflammatory and immunomodulatory role in typhoid infection, helping to establish chronic infection. Strains of *S.* Typhi lacking the typhoid toxin remain capable of activating the NF-κB and mitogen activated protein kinases Erk, Jnk, and p38 in a similar fashion to wild-type *S*. Typhi, although the effects on downstream cellular responses or cytokine production has not been studied[274].

2.2.11. Adaptive Immune Responses to the Typhoid Toxin

C57BL/6 mice immunised with a typhoid-toxoid in a prime-boost regimen (2µg subcutaneous injection, two weeks apart) induced strong IgG responses against typhoid toxin. Transfer of antibodies from immunised mice were able to prevent induction of cell cycle arrest in vitro and clinical illness in vivo following administration of typhoid toxin, suggesting that humoral responses against the typhoid toxin can prevent clinical illness[270].

Several studies indicate that typhoid toxin is highly immunogenic in humans. IgG antibodies against CdtB have been identified in the serum of typhoid fever patients from Vietnam, the presence of which was used to discriminate healthy controls and from patients infected with NTS[158]. Similar findings have been found using ALS samples from typhoid fever patients in

Bangladesh[275] and other studies have also demonstrated strong IgM and IgG responses against components of the typhoid toxin [159,276,277]. A recent study using samples derived from a controlled human infection model, illustrates that challenge with *S*. Typhi and Paratyphi are associated with the development of T-cell responses to CdtB measured by IFNγ ELISPOT [278]. These data indicate that the typhoid toxin is expressed by the bacterium during natural infection – although whether anti-typhoid toxin immune responses are correlated with protection against re-infection in humans is currently unknown.

2.2.12. Knowledge Gaps

Converging lines of evidence suggest that typhoid toxin may have an important role of in the pathogenesis of typhoid fever. Based on the wealth of pre-clinical evidence, some authors have postulated that the typhoid toxin could be a promising antigen to target in novel vaccines or therapeutics for typhoidal Salmonella[251].

Although the evidence gathered to date is strongly suggestive that typhoid toxin is an important virulence factor, definitive evidence in human infection is lacking and a number of important knowledge gaps remain. There are currently no robust *in vivo* data indicating at what stage in the life-cycle of *S*. Typhi the typhoid toxin is expressed, both temporally and anatomically. In vitro data show that typhoid-toxin expression occurs only within the SCV; that expression of typhoid toxin genes begins from ~2-6 hours after cellular invasion and that export from inside cells occurs ~24 hours after invasion[262]. Typhoid toxin can be detected in the liver of infected mice 10 days after oral infection – although this was the first time point at which tissue was sampled[272]. It is unclear how this relates to typhoid-toxin expression within the human host – specifically whether the toxin is expressed in vivo at the intestinal stage of infection (12-24hrs), during the incubation period in the reticuloendothelial system (~days 2-7), during acute bacteraemia or in the establishment of chronic carriage in the gall bladder.

Variations in mouse strains, route of administration, challenge dose and challenge method (toxin injection vs. mutant *S.* Typhimurium strains) complicate the interpretation of in vivo data on typhoid toxin, with regards to human infection. Additionally, the endpoints used to measure disease in mice – including weight loss, lethargy, motor dysfunction – are not directly comparable to clinically relevant endpoints for enteric fever in field studies.

Collectively, a large volume of knowledge has been gathered on the composition, structure, mechanisms of action and potential functions of typhoid toxin. However, the lack of biologically relevant challenge model has hampered the ability to study the importance of typhoid toxin in the pathogenesis of typhoid fever in a biologically relevant system. The advent of controlled human infection models could potentially provide a platform to address this question and will be discussed in the next chapter.

2.3. Immunity to Enteric Fever

In addition to understanding the pathogenic mechanisms underlying disease, the development of new vaccines against enteric fever could be aided by studying the human immune response during infection. The extent, characteristics and duration of immunity conferred by natural infection with *S.* Typhi and Paratyphi are currently incompletely understood and there remains no readily reproducible immunological correlate of protection. Limitations in understanding of mechanisms and determinants of immunity stem in part from the lack of a relevant small-animal model in which the disease can be studied, as both *S.* Typhi and *S.* Paratyphi are human-restricted pathogens.

The life cycle of *Salmonella* Typhi and Paratyphi suggests that host immune responses to the typhoidal *Salmonella* serovars are complex. From the point of ingestion, through to bacteraemia and shedding, the bacilli will be resident in a range of different anatomical compartments, at

various points transitioning between an intracellular and an extracellular state[225]. It follows that different immune effector mechanisms – ranging from innate, mucosal, humoral and cellular immune responses - will be engaged at different anatomical locations and at different times[279] (Figure 2-6).

The relative contributions of different immune effector mechanisms to protection against typhoidal *Salmonella* are poorly understood. The efficacy of plain Vi-polysaccharide vaccines - which can only induce antibody - attests to role of antibodies in conferring protection against typhoid fever [5,203,280]. However, Vi-antibody levels measured by ELISA are imperfect correlates of protection and the precise effector mechanisms through which Vi-antibodies mediate protection are poorly understood[209]. In addition, the live-attenuated oral vaccine Ty21a has moderate efficacy against typhoid fever, thought to be mediated by cellular and/or mucosal immunity[201]. Notably, Ty21a lacks expression of the Vi-capsule, emphasising that vaccine-derived immunity to typhoid fever can be achieved through Vi-dependent and Vi-independent mechanisms[279]. Improved understanding of protective immune responses induced by natural infection and vaccination could aid the rational design of novel vaccines for typhoidal *Salmonella* or the improvement of existing vaccines.

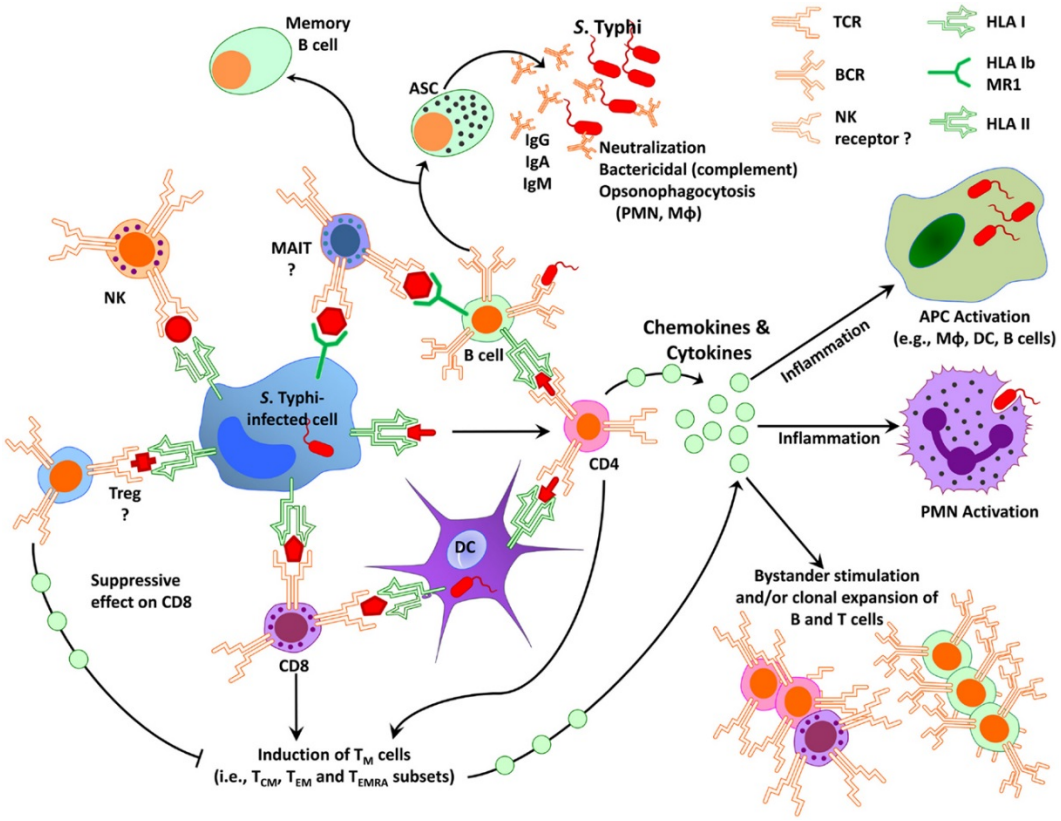


Figure 2-6 : Adaptive immune responses to typhoidal *Salmonella*.

Adapted from Sztein, Salerno-Goncalves and McArthur (2014) Frontiers in Immunology[279]. Licensed under CC BY 4.0 [255]

S. Typhi and Paratyphi controlled human infection studies provide a platform for the detailed study of host immune responses, allowing for longitudinal collection of a diverse range of tissue samples at baseline and at different stages of the bacterial life-cycle – from early after infection, during the incubation period, during acute disease and convalescence. Several studies using samples derived from the typhoid and paratyphoid challenge model have begun to provide insights into the role of different immune effectors during acute infection, including dendritic cells and monocytes[281]; NK cells [282]; plasma cytokines including gamma-interferon[163,283]; B-cells[284]; bactericidal antibodies[159,285]; CD4+ T-cells[278]; CD8+ T-cells[286,287]; regulatory T-cells[281] and MAIT cells[288,289]. Whilst these studies can describe the characteristics of the host immune response that develops during acute disease – presumably contributing to the clearance of infection – their role

in conferring protection against future disease remains unknown. In this section I review the current understanding of naturally acquired immunity to *S.* Typhi and *S.* Paratyphi and discuss how controlled human infection studies can be applied to offer mechanistic insights that can inform vaccine development.

2.3.1. Kinetics of natural, humoral immunity to *S.* Typhi and *S.* Paratyphi

The capacity for humans to acquire immunity to S. Typhi and Paratyphi is supported by the epidemiology of disease in endemic countries, where the peak incidence is observed in infants and school-age children, and declines with age[4,7,12]. The declining incidence with age is frequently assumed to reflect the acquisition of immunity through cumulative sub-clinical environmental exposure and boosting of host immune responses. The immunological mechanisms underlying these observations have historically proven difficult to disentangle in the absence of a reliable disease model and measurable correlate of protection.

As an example, Pulickal and colleagues studied the kinetics of natural humoral immunity to *S.* Typhi using an outpatient surveillance study in Kathmandhu, Nepal.[290] In this study, venous blood samples were collected between June and July 2006, from consecutive individuals presenting to their outpatient facility, excluding those presenting with confirmed enteric ever, febrile illness or known history of typhoid vaccination. Cord blood samples were collected from consecutive deliveries in the maternity unit and samples from children and adults collected as part of healthy volunteer studies in Oxford, UK were used for comparison. Humoral immune responses were compared in different age groups by measuring serum bactericidal titres and anti-Vi IgG concentrations. In the Nepali cohort, bactericidal antibody titres were elevated in cord-blood samples, consistent with maternal antibody transfer. Bactericidal antibody titres fell in the age-groups from 6 months up to 8 years, but subsequently increased in age-groups from 9 years up to 75 years. A similar pattern was observed when anti-Vi IgG concentrations were measured,

which also described elevated anti-Vi IgG in the Nepali cohort compared with matched UK age-groups. The age-related increase in bactericidal antibody titres suggest that humoral immunity could be acquired through cumulative environmental exposure to *S.* Typhi over time. Whilst serum bactericidal activity has not consistently correlated with protection against typhoid fever, the data illustrated in this study broadly mirror the age-distribution of typhoid fever in epidemiological studies.

2.3.2. Observational studies

In his book of 1918, Frederick Gay reviewed the literature relating to acquired immunity following recovery from typhoid fever in the pre-antibiotics era [250]. The majority of the studies reviewed are drawn from case-series and observational studies in Western Europe and North America. In it, he estimates that approximately 1-5% of typhoid cases gave a self-reported history of previous episodes of typhoid. For example, Eichhorst reported on a consecutive series of 666 cases of typhoid fever in Zurich, of whom 4% gave a self-reported history of previous typhoid disease [291]. Some studies report that second attacks of typhoid were of milder severity than the preceding episode, whereas others state that the severity and mortality rates of the first and second episodes were comparable. It is also noted that second episodes frequently occur in the context of explosive outbreaks and epidemics, thought to be associated with a higher infectious dose. He concludes by stating that "*recovery from typhoid is, as is true for most biological phenomena of this sort, relative and not absolute*" [250].

The reliance on self-reported histories, coupled with the lack of accurate diagnostics distinguishing typhoid other febrile illnesses, make it difficult to draw reliable conclusions from the historical literature. Nevertheless, a seminal report of a typhoid outbreak provided by Marmion and colleagues in 1953 provides valuable insights to naturally acquired immunity to typhoid, that continues to inform many our assumptions on immunity to typhoid fever to this

day[292]– and indeed are used to parameterise contemporary modelling for typhoid transmission dynamics [293,294].

In this report, Marmion and colleagues describe two outbreaks of typhoid fever that occurred amongst RAF servicemen stationed in the Suez Canal Zone, Egypt in 1950. The first outbreak occurred between April to May 1950, where a total of 84 of the 657 of the servicemen were infected, confirmed by either culture or serological methods. The outbreak was attributed to a local cook involved in preparing food for the station and the outbreak subsided when he was removed from duty. All affected persons were successfully treated with Chloramphenicol.

In July of the same year, a second explosive outbreak occurred that affected 235 of 699 (34%) servicemen now stationed at the camp. This outbreak could be traced to specific communal meal, where the majority of the camp were assumed to have been 'exposed'. Of the 699 people at risk in the second outbreak, 54 had been diagnosed with typhoid in the first outbreak and of these, 11/54 (20%) also developed typhoid fever for a second time. No comment is made as to relative severity of the second disease. The rate of infection amongst individuals with previous confirmed disease was noted to be ~35% lower than individuals who had not been exposed during the first outbreak (78/250; 31%) as well as being lower than individuals who were exposed in the first outbreak but who did not succumb to disease (146/384; 38%) (Table 2-1).

Table 2-1 : Second attacks of typhoid infection following two outbreaks of typhoid fever.

Outbreak 1 occurred between April to May 1950 and affected 84 of 657 (13%) people at risk. Outbreak 2 occurred between July and August 1950 and affected 235 of 688 (34%). Rates of typhoid infection in individuals without known prior exposure, individuals diagnosed with typhoid infection in Outbreak 1 and individuals exposed but without disease in Outbreak 2. *1 – Relative risk. **Newcomers to station between outbreak 1 and outbreak 2, who were exposed to infection in outbreak 2. Data adapted from Marmion, Naylor & Stewart (1953) [292]

Group	Number at Risk	Number Diagnosed with Typhoid Fever	Attack Rate	Relative Risk (95% Confidence Interval)	Estimated Protective Effect*
No known previous exposure**	250	78	31.2%	-	-
Typhoid Diagnosis in Outbreak 1	54	11	20.4%	0.65 (0.37 – 1.10)	35%
Typhoid Exposure in Outbreak 1, No Disease	384	146	38.2%	1.22 (0.97 – 1.53)	-22%

From this observational study, it can be inferred that second attacks of typhoid fever can occur following recovery from disease, in as little as 4-5 months between episodes. The lower rate of infection in the re-challenged group hints that recovery from disease may be associated with the acquisition of partial immunity. The second outbreak was assumed to be associated with a higher force of infection, as evidenced by the high proportion of servicemen affected, suggesting that immunity can be overcome with a high enough infectious dose. The authors conclude that "*the specific immunity conferred by an attack of typhoid fever is of no more than moderate degree and, in the event of re-exposure to infection, recurrences are not very unusual*" [292].

2.3.3. Challenge Studies

Additional insights into infection-derived immunity are provided by a re-challenge study reported by DuPont and colleagues in 1971[295]. This study was conducted as part of the original programme of typhoid challenge studies conducted in Maryland between 1958 and 1974 (reviewed in Section 3.1). In this study, 22 volunteers who had previously been challenged with the Quailes strain of *S*. Typhi were re-challenged with 10^6 CFU of the same strain, two to twelve months after the primary exposure. All had met the case definition for typhoid fever on primary exposure (defined as a temperature of >39.5°C for >36 hours plus clinical symptoms of typhoid fever). Of the re-challenge cohort, 5/22 (23%) met the case definition for typhoid fever on re-challenge, compared with 11/34 (30%) of a cohort of naïve controls challenged contemporaneously – corresponding to an estimated protective effect of 30% (**Table 2-2**). As a comparator, the protective effect of the Ty21a vaccine in the same challenge model was 87% [296].

Table 2-2 : The impact of previous typhoid disease on rates of typhoid infection in historical typhoid challenge studies.

Comparison of attack rates in re-challenge participants with control participants without prior S. Typhi exposure. Data adapted from DuPont et al.[295] Challenge was performed with S. Typhi Quailes strain at a dose of 10^6 CFU. Individuals were enrolled in the re-challenge group only if they developed typhoid fever following their primary exposure. Re-challenge was performed within 12 months of primary exposure. The criteria for initiation of treatment was a fever of 103°F (39.4°C) for >36 hours and symptoms of typhoid fever, including abdominal pain, constipation and presence of rose spots. *1 – Relative risk.

Group	Number at Risk	Number Diagnosed with Typhoid Fever	Attack Rate	Relative Risk (95% Confidence Interval)	Estimated Protective Effect*
Control Challenge	34	11	32%	-	-
Re-Challenge	22	5	23%	0.70 (0.28 – 1.64)	30%

In an unpublished monograph, William Woodward (the lead investigator of the Maryland challenge programme) describes nineteen challenge volunteers who were re-challenged 2 to 82 months after their primary challenge[297]. It is unclear if any or all of these overlap with the volunteers described in the study published by DuPont et al[295]. Of these, 15 had developed typhoid disease on primary exposure, 5 of whom developed disease on re-challenge. Four participants who did not develop disease during the first challenge were re-challenged, of whom 1 developed disease on re-challenge. One participant who was challenged three times did not develop disease on first exposure, developed disease on second exposure and did not develop disease on third exposure.

Whilst this re-challenge study is broadly consistent with the findings of Marmion and Gay (i.e that previous exposure is associated with partial, but incomplete, protection against subsequent disease) there are a number of limitations to this study. In particular, the interval between primary challenge and re-challenge is not clearly defined, nor is it made clear how participants were selected or recruited for re-challenge. No data is presented on the serological response to re-challenge, the response to re-challenge in individuals who were protected on primary challenge, nor in previously vaccinated individuals.

2.3.4. Modelling Studies

Several groups have published studies modelling typhoid transmission dynamics and the impact of vaccinations programmes on the burden of typhoid disease (reviewed by Watson and Edmunds [298]). Each of the models incorporates infection-derived immunity as a central parameter. Empirical data to fit the models are relatively limited and these studies typically rely on assumptions derived from observational studies[290] and historical human challenge studies[295]. As noted by Watson and Edmunds "*there is a paucity of data from microbiological, immunological or epidemiological studies to parameterise models or to validate assumptions*"[298].

Two recent modelling studies have been published by Saul et al (2013)[294] and Pitzer et al (2014 - Figure 2-7) [293]. Both models differentiate between hypothetical immune states, described as sterile-immunity and clinical-immunity. The former describes a state of complete resistance to infection (including sub-clinical diseases) and the absence of shedding of the organism within the stool. Clinical immunity describes resistance to clinically overt typhoid disease but allows for sub-clinical infection and shedding of *S*. Typhi within the stool. Both Saul et al[294] and Pitzer et al[293] suggest that natural immunity to typhoid is likely to be long-lived. These studies also assume that immunity to typhoid remains partial and 'leaky' – that is, immunity wanes over time and individuals with prior exposure and partial immunity can be overcome given a sufficiently high infectious dose[292]. Using a stochastic agent-based model, Saul suggest that multiple episodes of typhoid exposure - predicted to be approximately three infection episodes - are required to confer functional immunity against subsequent infection.[294] This is in keeping with the long-standing hypothesis of asymptomatic boosting leading to the development of immunity over time. This hypothesis is supported by epidemiological data on the age distribution of typhoid fever and data showing acquisition of bactericidal antibodies with age[290].

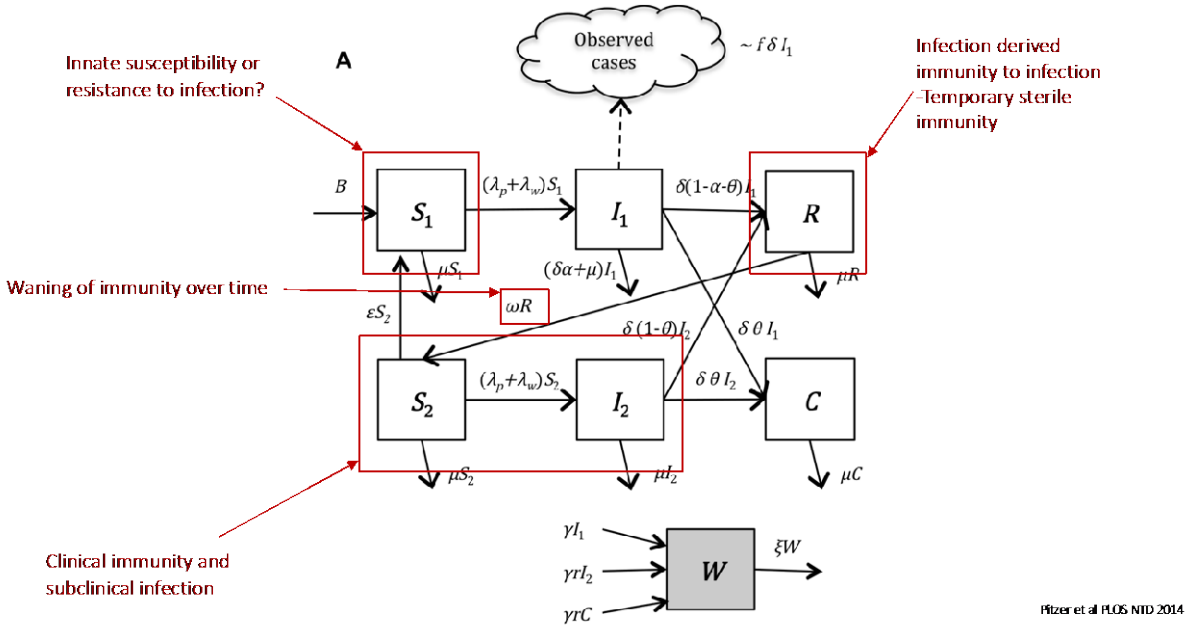


Figure 2-7 : Model of *S.* Typhi transmission dynamics

As proposed by Pitzer et al [293]. The model assumes that individuals are born susceptible to infection and disease caused by Salmonella Typhi (S1). Susceptible individuals are infected through contaminated food and water in the immediate environment (short-cycle transmission) as well as bacterial contamination of water supplies and the broader environment (long-cycle transmission). Individuals experiencing primary infection (I1) remain infectious for a period, some will experience disease-induced mortality, a fraction develop chronic carriage (C), and the remaining individuals recover and are temporarily immune (R). Immunity to reinfection wanes, leaving the individual partially susceptible (S2). If reinfection occurs prior to waning of partial immunity, the model assumes these reinfections (I2) are subclinical and do not require medical attention. Sub-clinically infected individuals can again recover or become chronic carriers. It is assumed that all infectious individuals shed bacteria into the environmental reservoir (W) and the bacteria remain infectious for a period of time. Labels in red highlights areas of uncertainty in relation to immunity. Adapted from Pitzer et al (2014) [293] Licensed under CC BY 4.0 [255]

2.3.5. Knowledge Gaps

Collectively, the available literature suggests that previous infection with typhoid fever is associated with partial – but incomplete – protection against subsequent disease. Modelling studies have proven useful in contextualising the role of infection derived immunity in transmission networks, but a number of important uncertainties and knowledge gaps remain, including the existence of a state of sterile immunity; the rate of waning immunity over time; the occurrence of an altered clinical phenotype on reinfection to name but a few. In addition, the immunological mechanisms underlying this effect are poorly understood which has arguably hampered the identification of correlates of protection. In the absence of reliable small animal models of infection, human infection models could be adapted to address these specific knowledge gaps.

2.4. Summary

The development of novel vaccines for typhoidal *Salmonella* is hampered, in part, by limited understanding of protective host immune responses to infection and uncertainties relating to disease pathogenesis. An improved understanding of infection derived immunity to enteric fever, coupled with improved understanding of disease pathogenesis could aid the rational development of novel vaccines and therapeutics for typhoidal *Salmonella*. Several studies using distinct approaches are currently ongoing to address these knowledge gaps. In the next chapter I will discuss how controlled human infection studies offer one means of addressing these knowledge gaps and review how they can be applied to offer mechanistic insights that can inform vaccine development.

Chapter 3 - Rationale and Aims

3. Rationale

In previous chapters I have described some of the key challenges in efforts to achieve global control of enteric fever. These challenges are many and varied, ranging from uncertainties in burden and distribution of disease; an increasing incidence of *S*. Paratyphi A; limitations of existing diagnostics; the emergence and dissemination of multidrug resistant strains; inadequate vaccine options, together with difficulties to achieving improved water quality and sanitation infrastructure in low-and-middle income countries. Whilst there is increasing optimism that the advent of new preventative tools – including typhoid conjugate vaccines[215] – can achieve a degree of disease control in the short-to-medium term, novel strategies will be needed to address existing and future challenges.

The primary aim of this thesis is to describe how the novel application of controlled human infection models can form part of efforts to achieve control of enteric fever. Specifically, I aim to describe how controlled human infection models can be applied to address fundamental questions relating to disease pathogenesis and immunity, thereby acting as a catalyst for vaccine development.

3.1. Controlled Human Infection Models for *Salmonella* Typhi and Paratyphi

Experimental challenge of human volunteers with *Salmonella* Typhi has a long history. In a report describing the development of prototype vaccines for typhoid fever in 1896, Sir Almroth Wright described inoculating two medical personnel from the Indian Medical Service with a killed typhoid vaccine – two weeks after which one volunteer was "*injected in the flank* (with) *virulent typhoid*

bacilli" without developing symptoms of typhoid fever[299]. Other occurrences of accidental oral challenge with *S*. Typhi[300,301] and *S*. Paratyphi A[302] were subsequently described in the literature.

The first systematic and reproducible programme of typhoid human challenge studies was conducted by the University of Maryland School of Medicine (USA), beginning in the early 1950s. Between 1952 and 1976, close to 2000 volunteers underwent challenge with *S.* Typhi in a series of studies that provided valuable insights into the pathogenesis and immunological control of typhoid fever (reviewed elsewhere [297,303]). These included dose finding studies[304]; descriptions of asymptomatic bacteraemia[305]; descriptions of histopathological changes in gut mucosa during acute infection[231]; the effect of chloramphenicol treatment[304]; the response of challenge with different strains (Vi+ve vs. Vi-ve)[74]; the role of endotoxin in pathogenesis[306] and a number of vaccine studies assessing parenteral acetone-killed/heat-killed whole cell vaccines[307], oral acetone-killed whole cell vaccines [295] and the Ty21a vaccine [296]. The Maryland programme was conducted in volunteers recruited from amongst adult inmates of the Maryland House of Corrections, where a dedicated research facility was established in 1962[308 303]. The Maryland programme eventually concluded, in part due to concerns regarding the use of prisoners and other vulnerable groups in clinical research, despite the comparatively progressive measures instituted to ensure appropriate volunteer consent[297,303,309,310].

In recognition of the ongoing global challenges of enteric fever and to provide a platform to assess new vaccine candidates, a typhoid challenge model was re-established by the University of Oxford in 2011[126], followed in 2014 by the establishment of the first paratyphoid challenge model[311]. The Oxford typhoid and paratyphoid models differs from earlier challenge studies conducted in Maryland in a number of key areas, including challenge setting and population, the duration of follow-up, the infectious dose used [126,304] and endpoint definitions[312]. An important difference in the Oxford model is the use of sodium bicarbonate as a gastric acid neutralising buffer prior to

challenge, allowing a lower challenge dose to be used and resulting in a more consistent pattern of clinical infection between studies[126,313]. Early studies allowed the identification of an optimal challenge doses ($ID_{60\text{-}75}$ 1-5 x 10^4 CFU and 1-5x10^3 CFU for S. Typhi and S. Paratyphi respectively)[126,311]. Subsequent studies have since applied the typhoid challenge model to test novel live-attenuated typhoid vaccines (MO1ZH09)[314] and Vi-conjugate vaccines (Typbar TCV)[209]; to assess novel diagnostics[73,154,159,315] and to study the host response to infection[159,163,278,281–289].

The studies conducted in Oxford since 2011 have established safe and reproducible, protocols for typhoid and paratyphoid challenge in healthy adult volunteers. In part this has been facilitated by a relatively permissive regulatory environment in the UK for conducting controlled human infection studies and detailed consideration of the ethical issues surrounding human infection studies[316,317]. This has also required the development of challenge stocks of *S.* Typhi and *S.* Paratyphi strains, which have undergone detailed characterisation and produced to a Good Manufacturing Practice (GMP) standard. Overall, the experience of conducting typhoid and paratyphoid challenge studies in Oxford since 2011 has established a platform to expand the translational application of controlled human infection studies to address new questions relating to disease pathogenesis and immunity.

3.2. Specific Aims

The work described in this thesis is derived from two *S.* Typhi and *S.* Paratyphi controlled human infection studies conducted in healthy adult volunteers between 2015 and 2018. These studies represent part of a larger project to understand host-pathogen interactions during *S.* Typhi and Paratyphi infection, with the overarching aim of accelerating vaccine development.

The first study described in this thesis is entitled 'Investigating the role of typhoid toxin in the pathogenesis of typhoid fever: A randomised, double blinded outpatient controlled human

infection study'(OVG2016/03 TYGER). This study aims to compare the response to challenge with wild-type *Salmonella* Typhi with an isogenic knock-out strain lacking a putative virulence factor termed the typhoid toxin. The specific aims of this project are:

- To generate and characterise a typhoid toxin deficient challenge strain of *Salmonella* Typhi (Chapter 5).
- To design, establish and obtain approvals for a controlled human infection study incorporating deliberate release of a genetically modified strain of *Salmonella* Typhi (Chapter 5).
- To compare the rate of typhoid infection following challenge with wild-type *Salmonella* Typhi or a typhoid toxin-deficient isogenic mutant *Salmonella* Typhi (Chapter 6).
- To compare the clinical, microbiological, haematological and biochemical features of typhoid infection following challenge with wild-type *Salmonella* Typhi Quailes strain or typhoid toxin-deficient isogenic mutant *Salmonella* Typhi (Chapter 6).
- To compare the host immune response following challenge with wild-type *Salmonella* Typhi Quailes strain or typhoid toxin-deficient isogenic mutant *Salmonella* Typhi (Chapter 6).

The second study described in this thesis is entitled 'Investigating the Mechanisms and Determinants of Immunity to *Salmonella* Typhi and *Salmonella* Paratyphi A in Naïve and Previously Challenged Individuals – A Challenge and Re-Challenge study' (OVG2014/01 PATCH study). This study aims to describe the extent of infection-derived immunity conferred by previous exposure and to identify potential correlates of protection. The specific aims of this project are:

- To design, establish and obtain approvals for a challenge-re-challenge study of *Salmonella* Typhi and *Salmonella* Paratyphi infection (Chapter 7).
- To compare the rate of typhoid and paratyphoid infection in naïve individuals with rates of infection after homologous re-challenge with *Salmonella* Typhi and *Salmonella* Paratyphi (Chapter 7).
- To compare the rate of typhoid and paratyphoid infection in naïve individuals with rates of infection after heterologous re-challenge with *Salmonella* Typhi and *Salmonella* Paratyphi (Chapter 7).
- To describe the human clinical response to *S*. Typhi or *S*. Paratyphi in naïve and previously challenged individuals (Chapter 7).
- To describe the characteristics of bacterial dynamics after challenge in naïve and previously challenged individuals, including onset and duration of bacteraemia, bacterial burden at diagnosis, and stool shedding (Chapter 7).
- To describe the humoral immune response to naïve challenge and re-challenge with *S*. Typhi or *S*. Paratyphi, including B-cell and antibody responses (Chapter 8).

The final component of this thesis will be an integrative analysis of data collected across a series of typhoid and paratyphoid controlled human infection studies. The specific aims of these chapters will be:

- To describe variables associated with susceptibility or resistance to typhoid and paratyphoid fever in naïve and previously exposed individuals (Chapter 7 & Chapter 8).
- To describe variables associated with shedding of *Salmonella* Typhi and *Salmonella* Paratyphi in stool (Chapter 9).

Chapter 4 - Methodology

4. Introduction

Since the re-establishment of *S.* Typhi and *S.* Paratyphi challenge models in Oxford from 2010 onwards, protocols and study procedures have progressed through multiple iterations. Protocols may be refined for several reasons, such as to capture additional data; to minimise risk to study participants or to address study-specific questions. Certain core procedures and endpoints have however remained constant between all studies, including the principal eligibility criteria; challenge strains, doses and stocks used, and the primary definition of typhoid/paratyphoid disease. Consistency in core procedures can allow for comparisons to be made across studies. In this chapter I will detail challenge study procedures and methods that are consistent between the studies described in this thesis. Study specific methods are detailed the relevant chapters.

4.1. Study Site

Studies were undertaken at the Centre for Clinical Vaccinology & Tropical Medicine (CCVTM), Churchill Hospital, Oxford, UK.

4.2. Study Population

Healthy adults aged 18-60 years, without prior residency in an enteric fever endemic country for ≥6 months, were considered eligible for enrolment. Participants with a known and documented history of typhoid vaccination with either Vi-polysaccharide or Ty21a (Vivotif®) were excluded from naïve cohorts. Key exclusion criteria included significant medical, surgical or psychiatric history, gallbladder disease and high-risk occupations as defined by Public Health England guidelines.[59] A full description of inclusion and exclusion criteria is provided in Appendix 1.

4.3. Recruitment

Healthy volunteers were approached for recruited using a variety of methods, including targeted mailouts, healthy volunteer databases, poster advertisements and social media. Volunteers who expressed an interest in the study were assessed for eligibility by telephone screening.

4.4. Screening and Consent

Those who met the basic eligibility criteria were invited for face-to-face screening with a study doctor, where the purpose of the study and study procedures were explained in detail. Written, informed consent was obtained from all participants. Understanding of study procedures was assessed using a multiple-choice consent quiz. Screening of volunteers who consented to participate included a medical history, physical examination, screening blood tests, urine dipstick, an ECG and an ultrasound scan of the liver and biliary tree. Screening blood tests included urea & electrolytes, liver function tests, C-reactive protein, serum amylase, full blood count, erythrocyte sedimentation rate, coeliac serology, anti-Vi IgG serology and serology for HIV, Hepatitis B and Hepatitis C. A volunteers' medical history and suitability for participation was reviewed with their GP prior to enrolment.

Written consent was repeated on the day of enrolment and consent to continue in the studies was repeated at each clinic visits. Participants were free to withdraw from the study at any time and consent to complete a course of antibiotic treatment if they withdrew prior to meeting diagnostic criteria or day 14.

4.5. Challenge strains

In the United Kingdom, microbial challenge strains are not considered investigation medial products and, as such, approvals from the Medicines and Healthcare Products Regulatory agency

(MHRA) are not required prior to their use. Challenge agents must have a defined passage history and be manufactured to a GMP standard.

4.5.1. *Salmonella* Typhi

The Quailes strain *Salmonella* Typhi has been used extensively for human challenge studies. The strain was originally isolated from the gallbladder of a chronic carrier of typhoid in 1958 and was used in a series of human challenge studies performed by the University of Maryland School of Medicine between 1959 and 1974[303]. The Quailes strain was provided by the University of Maryland to establish a GMP master cell bank in Oxford, which was subsequently transferred to Public Health England (PHE,Porton Down, Salisbury, UK) and manufactured to GMP standards. Full antibiotic sensitivity of the strain stored in the master cell bank has been demonstrated and further characterization work including genome-sequencing was completed at the Wellcome Trust Sanger Institute, Hinxton UK prior to use in the original challenge studies (Figure 4-1). Prior to the start of these studies, the Quailes strain has been used to safely challenge 132 participants in Oxford since 2010[126,314]. The PHE GMP batch of *S.* Typhi Quailes strain was used in the OVG2014/01 (PATCH) study (Chapter 7). A new GMP batch of wild-type *S*. Typhi Quailes strain was manufactured in parallel with a batch of typhoid-toxin deficient *S*. Typhi SB6000, for the OVG2016/03 study as detailed in Chapter 5.

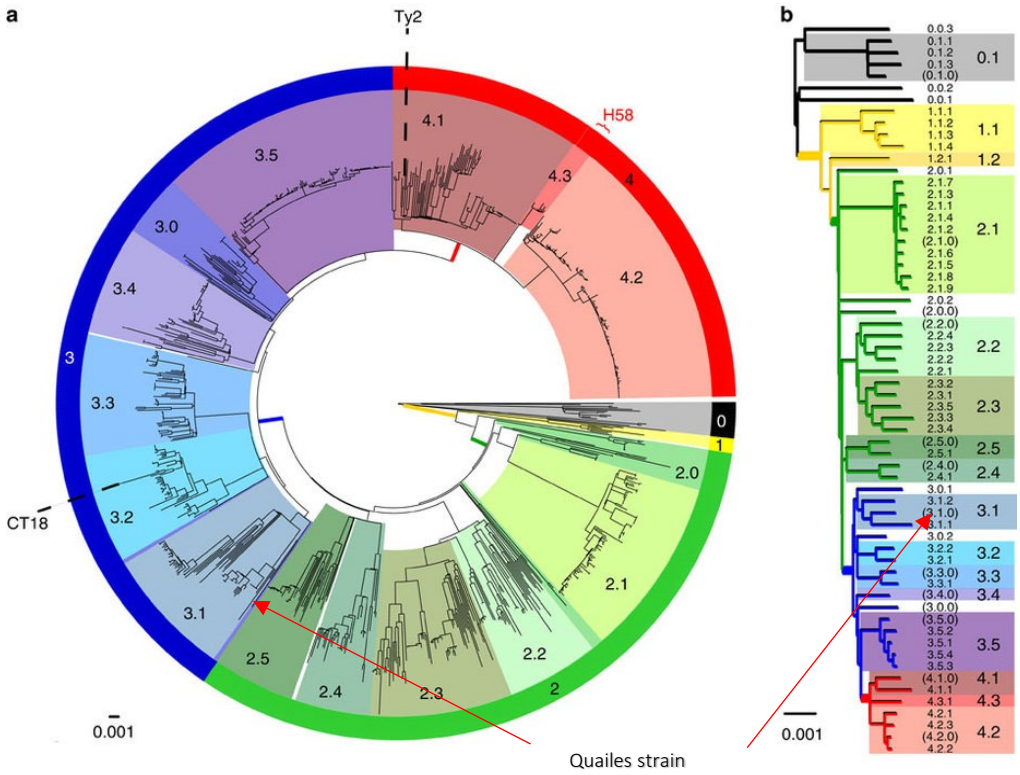


Figure 4-1 : Phylogenetic position of *S.* Typhi Quailes strain

Genotype in relation to a global collection of *S*. Typhi isolates collated by Wong et al(2016) Nature Communications[234]. (a) Whole-genome tree of 1,831 global *S*. Typhi isolates, coloured according to primary clusters. Groups are sub-divided into clades and sub-clades (b). Labelled are The location of *S.* Typhi reference genomes CT18 (accession number AL513382), Ty2 (accession number AE014613) and Subclade 4.3.1 (H58, marked in red). Reproduced and modified under license under CC BY 4.0[255].

4.5.2. *Salmonella* Paratyphi

The *S.* Paratyphi A strain NVGH308 was isolated in 2006 from a patient with paratyphoid fever in Kathmandu, Nepal and is susceptible to most commonly used antibiotics, including ciprofloxacin[318] . Doses used for challenge were manufactured to GMP standards (GenIbet, Portugal) and supplied for study purposes by GSK Vaccines Institute for Global Health, Siena, Italy (formerly Novartis Vaccines Institute for Global Health). Prior to the start of these studies, the NVGH308 strain had been used to safely challenge 40 participants in Oxford since 2014[319].

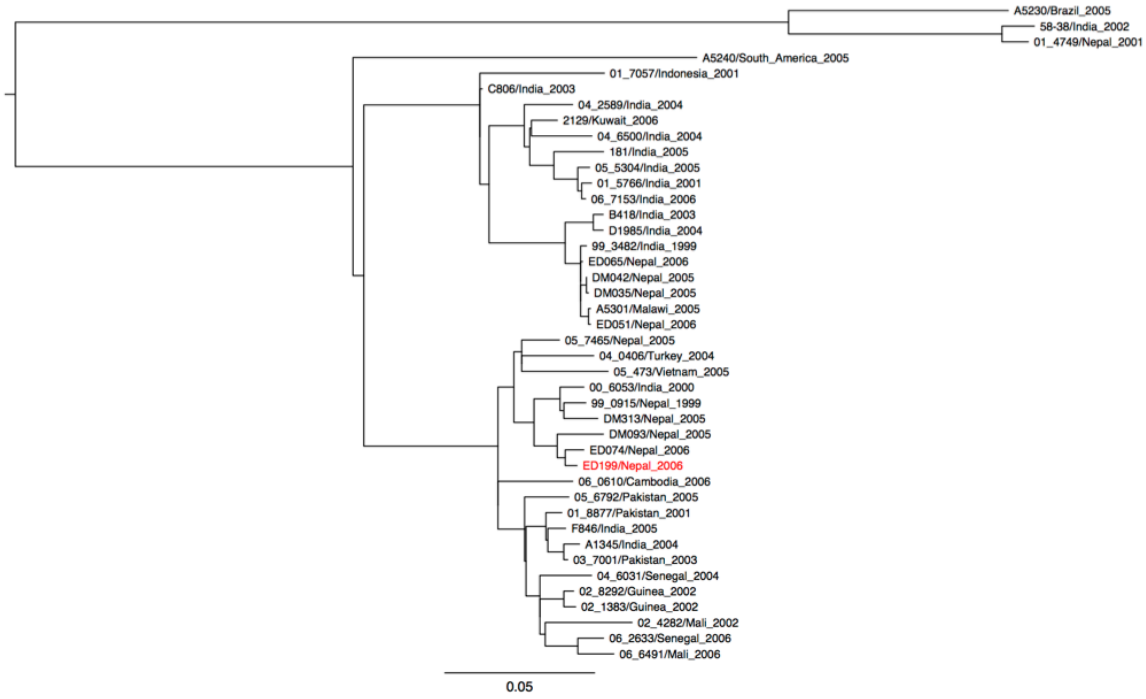


Figure 4-2 : Phylogenetic position of *S.* Paratyphi A NVGH308.

Maximum Likelihood phylogeny generated from 1,539 SNPs (Single Nucleotide Polymorphisms) identified in the whole genome sequences of 42 previously sequenced global isolated of *S*. Paratyphi A isolates [320]. The NVGH308 challenge strain (labelled ED199/Nepal_2006) is highlighted in red; strain names are expanded to show the location and time of isolation. The scale bar indicates the number of substitution per variable site. Genomic DNA from representative *S.* Paratyphi A isolates were extracted using the Wizard genome DNA extraction kit (Promega, USA) and sequenced using the Illumina Hiseq platform to generate 75 paired-end reads. Reads were mapped to the reference sequence of *S*. Paratyphi A strain AKU_12601 (accession no: FM200053) using SMALT (version 0.7.4). Single nucleotide polymorphisms (SNPs) in the core genomes were called and filtered using SAMtools.[320,321] Figure adapted from Dobinson, Gibani, Jones et al (2017) CID. [311]. Reproduced under license under CC BY 4.0[255]

4.6. Challenge agent preparation

The *Salmonella* Typhi (Quailes strain) and *Salmonella* Paratyphi (NVGH308) challenge strains are stored as a frozen suspension in soya tryptone medium containing 10% sucrose. Vials of *S.* Typhi Quailes strain (stock concentration 1-5x10^5 CFU/ml) *or Salmonella* Paratyphi NVGH308 (stock concentration 1-5x10^4 CFU/ml) were thawed and diluted immediately prior to use.

Stock vials of *S.* Typhi Quailes strain were diluted to a target challenge dose of 1-5x10^4 CFU. This challenge dose was selected to achieve a target attack rate of 60-75% in the naïve challenge group, as has been shown in earlier studies[126,314]. Stock vials of *S.* Paratyphi NVGH308 strain were diluted to a target challenge dose of 1-5x10^3 CFU. This challenge dose was selected to achieve a target attack rate of 60-75% in the naïve challenge group, as has been shown in earlier studies[311].

The dose administered ('actual dose') was calculated by direct plating of aliquots on Tryptone soya agar (TSA) plates (Oxoid/PO0163A) in triplicate and colony counting after 24 hours incubation at 37°C with 5%CO_2.

4.7. Challenge Procedure

Participants fasted for 90 minutes prior to challenge. Two minutes prior to challenge, participants drank a $NaHCO_3$ solution (2.1g/120ml) to neutralise stomach acid. The oral challenge inoculum was administered suspended in a $NaHCO_3$ solution (0.53g/30ml) and was kept on ice prior to administration within two hours of preparation. Participants were observed for 90 minutes post challenge.

4.8. Post Challenge Monitoring

After challenge, participants were seen once more on the day of challenge (D0+12h) and then daily for fourteen days. Clinic visits were conducted as outpatients, and included as a minimum continued consent, monitoring of vital signs (heart rate, blood pressure and oral temperature), review of solicited symptoms, sample collection and reinforcement of hygiene precautions. Sample collection timetables are detailed in Appendix 5 and Appendix 6. Grading of abnormalities in vital signs are detailed in Table 4-1.

Table 4-1 : Grading the severity of clinical observations

Observation	Grade 0	Grade 1	Grade 2	Grade 3
Oral temperature (°C)	35.5 – 37.5	37.6 – 38.0	38.1 – 39.0	>39
Tachycardia (beats/min)	55-100	101-115	116-130	>130
Bradycardia (beats/min)	55-100	50-54	45-49	<45
Systolic hypertension (mmHg)	90-140	141-150	151-155	>155
Diastolic hypertension (mmHg)	90-140	91-95	96-100	>100
Systolic hypotension (mmHg)	90-140	85-89	80-84	<80

4.9. Diagnostic Criteria

The primary outcome in all studies is the rate of enteric fever, defined as a composite endpoint of either a positive blood culture for *S*. Typhi/ *S.* Paratyphi A (taken ≥72 hours after challenge to avoid detection of primary bacteraemia) and/or oral temperature ≥38°C persisting for ≥12 hours[318]. Full criteria for diagnosis are detailed in Table 4-2.

Table 4-2 : Composite diagnostic criteria for typhoid and paratyphoid fever OVG2014/01 and OVG2016/03 study.

Enteric fever is diagnosed if ANY of the following apply:
A positive blood culture for *Salmonella* Typhi or Paratyphi from 72 hours post-challenge
A positive blood culture for *Salmonella* Typhi or Paratyphi within 72 hours post-challenge, with one or more signs/symptoms of enteric fever (such as recorded temperature ≥38 ^{o}C)
Persistent positive blood cultures (two or more blood cultures taken at least 4 hours apart) for *Salmonella* Typhi or Paratyphi within 72 hours post-challenge.
Oral temperature ≥38^{o}C persisting for 12 hours

4.10. Treatment

Antibiotic treatment was initiated upon fulfilment of composite diagnostic criteria or at day 14 for those without illness. Both the *S*. Typhi Quailes strain and *S*. Paratyphi NVGH308 strains were fully susceptible to all common first, second and third line treatments for typhoidal *Salmonella*, including the 'traditional' first line agents of chloramphenicol, trimethoprim-sulfamethoxazole and amoxicillin. Participants were treated at the time of typhoid diagnosis or at day 14 for those who did not develop disease.

First line treatment in the OVG2016/03 (TYGER) was ciprofloxacin 500mg PO twice daily for 14 days.

First line treatment in the OVG2014/01 study varied depending on the time of enrolment. From March 2015 to October 2016, the first line treatment was oral azithromycin 500mg once daily for 14 days. After October 2016, first line treatment was changed to ciprofloxacin 500mg twice-daily for 14 days. The change in first line therapy was prompted by a recommendation from the data

safety monitoring committee, following review of safety data pertaining to antibiotic treatment from a parallel typhoid challenge study (OVG2014/08)[209].

Antibiotic treatment was directly observed in the outpatient clinic for the first 96 hours and all patients received daily prompts by SMS or email thereafter. Participants could be switched to second-line (ciprofloxacin or azithromycin), third-line treatment (trimethoprim/sulfamethoxazole 160/800 mg once daily for 14 days), fourth-line (amoxicillin 500mg TDS for 14 days) at the discretion of the attending physician in consultation with the principal investigator, in cases where a switch of antibiotics was deemed necessary owing to confirmed/suspected hypersensitivity, antibiotic intolerance, delayed treatment response or confirmed/suspected antibiotic-related adverse effects.

Concomitant medications were prescribed at the discretion of the attending physician to manage symptoms of enteric fever. Participants were advised to avoid antipyretic medications prior to commencing antibiotics (Table 4-3).

Table 4-3 : Concomitant medications for symptom control.

Drug	Indication	Dose	Route	Frequency
Paracetamol	Fever and/or pain (after antibiotic therapy started)	1 gram	Oral	PRN, max QDS
Codeine	Pain	30-60mg	Oral	PRN max. 240mg/24hours
Senna	Constipation	2-4 tablets	Oral	PRN
Domperidone	Nausea	10-20mg	Oral	PRN (max 80mg in 24hrs)
Chlorpheniramine	Allergy symptoms	4mg	Oral	PRN 6-8 hourly, max 24mg in 24hrs

4.11. Safety monitoring

Regular participant safety monitoring occurred through daily clinical review for a minimum of 14 days. Participants had 24-hour access to a study physician and consented to be contactable 24-hours a day. Emergency contact details from a nominated contact were collected from all participants. Emergency contacts were provided with written study information and agreed to be contacted in cases where the participants were uncontactable.

4.12. Solicited symptoms

Solicited symptoms and twice daily temperature measurements were recorded in an electronic diary for 21 days following challenge. Symptoms were categorized as not present, mild, moderate or severe as outlined in Table 4-4 [318].

Table 4-4 : Grading of solicited symptoms recorded after challenge.

Solicited Symptoms & Expected Adverse Events of Enteric Fever	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Headache	Not present	Present but no interference with activity	Some interference with activity	Significant; any use of codeine phosphate or prevents daily activity	Hospital visit required
Malaise	Not present	Present but no interference with activity	Some interference with activity	Significant; any use of codeine phosphate or prevents daily activity	Hospital visit required
Anorexia	Not present	Eat less than normal for 1-2 meals	Miss 1-2 meals completely	Miss all meals completely	Hospital visit required
Rash	Not present	Present but no interference with activity	Some interference with activity	Significant or prevents daily activity	Hospital visit required

Constipation	Not present	Present but no interference with activity	Some interference with activity	Significant or prevents daily activity	Hospital visit required
Diarrhoea	Not present	3 – 4 loose stools in 24 hours (≥200mls volume)	5 – 6 loose stools in 24 hours (≥200mls volume)	>6 loose stools in 24 hours (≥200mls volume)	Hospital visit required
Abdominal pain/distension	Not present	Present but no interference with activity	Some interference with activity	Significant or prevents daily activity	Hospital visit required
Myalgia	Not present	Present but no interference with activity	Some interference with activity	Significant or prevents daily activity	Hospital visit required
Arthralgia	Not present	Present but no interference with activity	Some interference with activity	Significant or prevents daily activity	Hospital visit required
Cough	Not present	Present but no interference with activity	Some interference with activity	Significant or prevents daily activity	Hospital visit required
Nausea and/or vomiting	Not present	Present but no interference with activity or 1 – 2 episodes in 24 hours	Some interference with activity or more than 2 episodes in 24 hours	Significant or prevents daily activity	Hospital visit required

4.13. Severe Enteric Fever

The criteria for a diagnosis of severe enteric fever are detailed in Table 4-5. Criteria for disease severity were defined to reflect complications of severe typhoid fever (1.4.2) and were consistent with the definitions used in previous enteric fever challenge studies[126,314].

Table 4-5 : Criteria for severe enteric fever.

The grading of laboratory abnormalities are outlined in Table 4-6[309,310].

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Severe enteric fever is diagnosed if ANY of the following apply
Oral temperature ≥ 40ºC

Systolic blood pressure ≤ 85 mmHg
Significant lethargy or confusion
Gastrointestinal bleeding
Gastrointestinal perforation
Any grade 4 laboratory abnormality

Table 4-6 : Grading the severity of laboratory abnormalities.

Parameter				
	Grade 1	Grade 2	Grade 3	Grade 4
Haemoglobin: decrease from baseline value (gm/dl)	< 1.5	1.5-2.0	2.1-5.0	>5
White cell count: elevated (cell/mm3)	10,800–15,000	15,001–20,000	20,001–25,000	>25,000
White cell count: depressed (cells/mm3)	2500-3500	1500-2499	1000-1499	<1000
Neutrophil count (cells/mm3)	1500-2000	1000-1499	500-999	<500
Lymphocyte count (cells/mm3)	750-1000	500-749	250-499	<249
Platelets (cells/mm3)	125,000-140,000	100,000-124,000	25,000-99,000	<25,000
Sodium: hyponatraemia (mmol/L)	132–134	130–131	125–129	<125
Sodium: hypernatraemia (mmol/L)	144–145	146–147	148–150	>150
Potassium: hyperkalaemia (mmol/L)	5.1–5.2	5.3–5.4	5.5–5.6	>5.6
Potassium: hypokalaemia (mmol/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 and/or AST (IU/L)	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 (umol/L)	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 (umol/L)	1.1–1.5 x ULN	1.6–2.0 x ULN	2.0–3.0 x ULN	>3.0 x ULN
Alkaline phosphatase (U/L)	1.1–2.0 x ULN	2.1–3.0 x ULN	3.1–10 x ULN	>10 x ULN
Albumin: hypoalbuminaemia (g/L)	28–31	25–27	<25	Not applicable
C-reactive protein	>10-30	31-100	100-200	>200

4.14. Microbiology Procedures

4.14.1. Automated Blood Culture

Aerobic blood cultures (10ml volume, BACTEC Plus Aerobic vials, BD) for microscopy, culture and sensitivity were collected daily[126]. Sample processing was undertaken by the Microbiology

laboratory, John Radcliffe Hospital, Oxford University Hospitals NHS foundation trust, according to national guidelines[322]. Blood culture bottles were cultured for up to five days at 35-37°C. Samples with no evidence of growth after five days were labelled as negative. On detection of growth, the study team was informed, and a Gram stain was performed. If Gram-negative rods were by microscopy, further subculture and identification was performed according to national guidelines as previously described[309,322]. In the context of the study, participants were commenced on treatment when Gram negative rods were first detected, prior to speciation.

4.14.2. Quantitative Blood Culture

Quantitative blood culture samples were collected immediately prior to antibiotic treatment by inoculation of 10 mL blood into a Wampole Isolator 10 tube (Alere, UK) and processed according to the manufacturer's instructions[126]. Briefly, ten millilitres of whole blood were collected into a 10ml Isolator 10 blood collection tube and inverted the isolator tube 5 times. The sample was centrifuges at 3000G for 30 minutes, the supernatant removed, and pellet re-suspended by vortexing. The contents of the tube were split equally between two Xylose Lysine Deoxycholate (XLD) plates, placed in an incubator overnight at 37°C and 5% CO_2, and colonies counted the following day. The number of colonies counted was summed across two plates and divided by 10, to give the number of S. Typhi/Paratyphi A CFUs/ml. For values below the lower limit of detection (0.1 CFU/ml), an arbitrary value of 0.05 CFU/ml was assigned.

4.15. Stool culture

Daily stool samples were requested from each participant before and daily for 14 days after challenge. Participants were instructed to collect stool samples as close as possible to their scheduled clinic visit and to deliver samples using insulated transport bags containing cool-packs.

Stool samples were submitted for standard microbiological culture at the microbiology laboratory, John Radcliffe Hospital, Oxford University Hospital NHS Foundation Trust. Stool cultures were performed according to local procedures and based on national guidance[323]. Briefly, selenite broth was inoculated with ~1g faeces and mixed by vortexing. An XLD Agar plate was directly inoculated with 10µL of the suspension, and after 18-24 hours incubation at 37°C the selenite was inoculated onto chromogenic agar (BBL CHROMagar *Salmonella* agar, BD Diagnostics) for the detection of *Salmonella* spp.

In order to demonstrate clearance, three convalescent stool samples were collected at least one week after completion of antibiotics, followed by two further stool samples collected at least 48 hours apart.

4.16. Study outcomes and endpoints

4.16.1. Primary endpoint

The primary outcome was the attack rate after challenge, defined as the proportion of participants developing clinical or microbiologically proven enteric fever as defined in Table 4-2 following oral challenge with 1-5 x 10^4 CFU *S.* Typhi or 1-5 x 10^3CFU S. Paratyphi NVGH308 strain.

4.16.2. Secondary clinical and microbiological endpoints

Secondary clinical and microbiological outcome variables are listed by below and described in more detail in the relevant subsections. Immunogenicity endpoints are described separately in the relevant chapters.

- Time to diagnosis - Time from date/time of challenge to date/time of first temperature ≥38^{o}C that subsequently lasted for ≥12hours OR the date/time of first positive blood culture collection (whichever occurs earliest).

- Time to first blood culture positive for *S.* Typhi/Paratyphi - Time from date/time of challenge to date/time of blood culture collection.
- Time to clinical diagnosis (fever ≥38^{o}C lasting ≥ 12 hours) - Time from date/time of challenge to date/time of first recorded temperature ≥38^{o}C which subsequently lasted 12 hours.
- Mode of diagnosis - The proportion of participants diagnosed with typhoid/paratyphoid fever based upon either clinical criteria (persistent fever ≥38^{o}C for ≥ 12 hours) OR microbiological criteria (blood culture positive for S. Typhi/Paratyphi collected ≥ 72 hours from diagnosis)
- Detailed mode of diagnosis - Proportion of participants diagnosed with typhoid/paratyphoid fever based upon either of the following specific diagnostic criteria:
    - Temperature ≥38^{o}C preceding positive blood culture;
    - Temperature ≥38^{o}C without positive blood culture;
    - S. Typhi/Paratyphi bacteraemia preceding temperature ≥38^{o}C;
    - S. Typhi/Paratyphi bacteraemia without temperature ≥38^{o}C.
- Time to first fever - Time from date/time of challenge to date/time of first recorded temperature ≥38^{o}C.
- Fever clearance time - Time from initiation of antibiotics or start of fever (whichever was later) to first recorded temperature <38^{o}C persisting for at least 48hours. Only diagnosed participants with fever were included in the analysis.
- Symptom severity - Severity of symptoms in each challenge group were assessed by:
    - The proportion of participants with maximum symptom severity score graded as mild, moderate or severe following challenge.
    - The proportion of participants meeting the criteria for severe enteric fever (see Table 4-5).

- Individual enteric fever severity scores calculated by summing numerical values assigned to the severity of individual solicited symptoms, clinical observations (heart rate, systolic blood pressure, diastolic blood pressure and temperature) and laboratory measurements between Day 0 to Day 21 (0=not present; 1=mild; 2=moderate; 3=severe; 4 = Hospitalisation – see Table 4-4
- Duration of bacteraemia - Time (Hours/Days) from collection of first positive blood culture until date/time of the last positive blood culture.
- Bacteraemia clearance time - Time (Hours/Days) from collection of first positive blood culture until date/time of the first negative blood culture remaining persistently negative. Participants with missing data (e.g. no negative blood cultures after commencing antibiotics) were censored in the analysis at the time point of the last culture taken.
- Stool shedding - Daily stool culture(s) positive for S. Typhi/Paratyphi for 14 days post-challenge.

- Quantitative blood culture - Concentration of bacteria in 10ml blood taken at the time of diagnosis using the Wampole™ Isostat® Isolator system (Colony forming units/ml). For values below the lower limit of detection (0.1 CFU/ml), a value of 0.05 CFU/ml was assigned.
- Haematological and biochemical end-points - The following haematological parameters were measured from time of challenge to Day 28 and/or Day 90.
    - Total Haemoglobin (g/L)
    - Haemoglobin change from baseline (Hb g/l D0 – Hb g/l D14)
    - Total White Cell Count ($x10^9$/l)
    - Platelet counts ($x10^9$/l)
    - Neutrophil count ($x10^9$/l)
    - Lymphocyte count ($x10^9$/l)

- Monocyte count (x10^9/l)
- Eosinophil count (x10^9/l)
- Monocyte/Lymphocyte ratio
- Urea & Electrolytes (Na, K+, Urea, Creatinine –mmol/l)
- C-reactive protein (mg/l)
- Liver function tests (Bilirubin [umol/l], aspartate transaminase (AST IU/l), alkaline phosphatase (ALP IU/l), alanine transaminase (ALT IU/l), Albumin (g/L)

- Safety outcome measures - Adverse events, adverse events of special interest, SAE's and SUSARs according to each study group.

4.17. Statistics

4.17.1. General Considerations

Normally distributed continuous variables are summarised using mean, standard deviation and range values (after log transformation, if required, for assumption of normality, in which case the original geometric means and confidence intervals on these will also be presented). Variables that are to be analysed non-parametrically (or variables far from normal distribution) are summarised using median, interquartile range and range values. Categorical values are presented as counts, percentages and/or centiles. 95% confidence intervals on means and on geometric means will be calculated, to demonstrate the plausible effects of random variation. Both numbers and proportions are presented for binary data. All statistical tests were 2-sided and P values less than 0.05 were considered significant. Secondary outcomes were assessed using Mann Whitney U tests for non-normally distributed variables and unpaired t-tests on normal or log-normal values. Skewed variables were $\log_{10}$ transformed prior to analysis.

4.18. Characteristics of participants

Patient throughput from referral, through randomisation, follow up and analysis are presented in a CONSORT flow diagram. This contains the numbers of participants randomly assigned to each group, challenged, completed the study and analysed for the primary outcomes. It also includes a breakdown of reasons for withdrawal.

4.19. Definition of population for analysis

The Per Protocol (PP) population was used for evaluation of the primary endpoint. For the OVG2014/01 and OVG2016/03 studies, the PP population was defined as all participants who:

- Have signed the requisite consent forms and successfully completed screening assessments, and
- Have been successfully challenged with the allocated challenge organism (or the actual challenge agent received in case of randomisation error), and
- Have received no bias or interference that may interfere with challenge, either according to the protocol or in the view of the study investigators.

If a participant withdrew from the study, data up until that point was included in the analysis.

4.20. Attack Rate Calculations

When calculating enteric fever diagnosis proportions, the numerator was taken as the number of participants who meet the criteria for diagnosis and the denominator included all participants challenged (excluding those who withdrew or were treated prior to day 14 without prior diagnosis of typhoid). The difference in proportions between the naïve and re-challenge groups was analysed using Pearson's chi-squared test or Fisher's Exact test if expected counts in any group were less than 5. The protective effect of prior typhoid/paratyphoid challenge was calculated as 1 – Relative Risk.

$$PE = 100 \times (1 - AR_{re\text{-}challenge}/AR_{naive}),$$

The 95% confidence intervals for protective effect were calculated as previously described and were derived from the upper and lower limits of relative risk of developing enteric fever in naïve and re-challenge groups[324].

Clinical data, recorded on a web-based database (OpenClinica Enterprise), and e-Diary data was extracted using Microsoft Excel. Data analysis detailed in this transfer report was performed using R version 3.4.4 and GraphPad Prism version 7.0.

4.21. Ethics Statement

All studies were approved by Oxfordshire Research Ethics Committee A (10/H0604/53; 11/SC/0302; 14/SC/0004; 14/SC/1204; 14/SC/1427; 16/SC/0358) and performed according to the provisions of the Declaration of Helsinki and Good Clinical Practice guidelines.

Chapter 5 - Design of a controlled human infection model to characterise the role of typhoid-toxin in the pathogenesis of typhoid fever.

5. Introduction

In Chapter 2 I summarised the evidence supporting the postulated role of the typhoid toxin as a key virulence factor in the pathogenesis of typhoid fever. This evidence is derived primarily from *in vitro* studies of cultured cells[256,260,261]; *in vivo* studies of the response of small animal models to administration of the toxin[260,261], along with serological and host response data from patients with acute typhoid fever[158,276,278]. The structural complexity and limited-distribution of the toxin amongst *Salmonella* serovars suggests that the typhoid-toxin confers a survival advantage to typhoidal *Salmonella*. Whilst there is an abundance of high-quality pre-clinical studies suggesting that the typhoid toxin is an important virulence factor, definitive evidence characterising its role in human disease is currently lacking.

The absence of a reproducible small-animal model of typhoid infection complicates the study of putative virulence factors expressed by *Salmonella* Typhi. Controlled human infection models have previously been applied to study bacterial virulence factors where animal models are not available [325]. For example, definitive evidence of the role of the Vi-capsule in contributing to the virulence of *S*. Typhi was provided in human challenge studies, where challenge with Vi-negative strains was associated with a 50% reduction in the risk of symptomatic typhoid fever (RR 0.50; 0.27-0.88; p=0.03)[326]. Challenge models using targeted knock-out strains have also been used to study putative virulence factors in other bacteria, such as in the study of outer-membrane proteins, cytolethal distending toxin and haemolysin in ulcer formation caused by infection with

Haemophilus ducreyi[327–329]. The availability of a reproducible human model of typhoid-infection offers a platform to assess the role of typhoid toxin in contributing to typhoid infection in a biologically relevant system.

The typhoid toxin has been proposed as a potential target for a new generation of vaccines or therapeutics to tackle the emergence of widespread antimicrobial resistance amongst *S.* Typhi [251]. As the typhoid toxin is also expressed by *S*. Paratyphi A, it may also represent a vaccine target to address the increased incidence of paratyphoid fever [223]. However, traditional approaches to develop typhoid-toxin based vaccines or therapeutics could face several challenges – progression of a product from pre-clinical testing through to phase 3 clinical trials would require sustained investment over several years, with little guarantee of success[330]. The study of the typhoid toxin in a controlled human infection model could – theoretically – help to 'de-risk' the development of a new generation of typhoid vaccines or therapeutics that target this antigen.

In order to investigate the role of typhoid toxin, the response to challenge with a wild type strain of *S.* Typhi can be compared to challenge with a typhoid toxin-deficient isogenic mutant strain in the context of a controlled human infection model. In this chapter, I will detail the construction, manufacture and characterisation of the challenge strains to be used in a human challenge study. In particular, I highlight issues pertinent to the design of studies involving deliberate release of genetically modified challenge strains and regulation of human challenge studies.

5.1. General considerations in the establishment of new challenge studies

5.1.1. Strain selection

The choice of strain is a key variable in the design of controlled human infection models. There are multiple factors underpinning strain selection that vary from model-to-model, but consistently include safety profile; passage history; expression of key virulence factors;

antimicrobial susceptibility and relationship to circulating or clinically significant isolates [325]. To minimize the theoretical risk of transmissible spongiform encephalopathy, bacteria strains used in challenge studies should not have been propagated on media containing animal products.

Early typhoid challenge studies have demonstrated variability in attack rate and clinical response between different strains of *S.* Typhi. For example, early studies in the Maryland challenge programme failed to induce typhoid fever after challenge with $>10^6$ CFU of the Ty2 strain of *S.* Typhi[307]. Subsequently, most typhoid challenge studies have used the Quailes strain of *S.* Typhi, a Vi-expressing strain isolated from a chronic carrier, which successfully induces clinical typhoid infection in healthy volunteers (section 4.5.1), albeit with varying attack rates between studies[295,331]. Strain-to-strain and study-to-study variation in attack rates in historical studies are more likely attributable to the mode of administration than the challenge strain *per-se*[303]. Modifications to the challenge procedure – specifically pre-treatment with sodium bicarbonate – has led to highly reproducible attack rates between studies [126,209,314].

The Quailes strain of *S.* Typhi benefits from a long-history of use, full antibiotic susceptibility, a defined passage history and a safety profile in >1000 volunteers. Quailes is classified as *S.* Typhi genotype 3.0.1. according to the most recent classification scheme [234]. Whilst Quailes is relatively distantly related to strains of S. Typhi causing the majority of disease in South Asia (genotype 4.3.1 [H58]), *S.* Typhi is a highly clonal, monophyletic pathogen with relatively limited strain-to-strain variation [169 234]. In order to facilitate comparisons with earlier challenge studies and to minimize risk to study participants, the Quailes strain was used as the parent strain from which the typhoid toxin-deficient knock-out strain was generated.

5.1.2. Genetic modification of challenge strain

As outlined in 2, the toxicity of the typhoid-toxin *in vitro* and *in vivo* models is thought to be mediated primarily by the CdtB component, which can be abrogated by a single point mutation in the catalytic site ($CdtB^{H160Q}$) [260,261]. Additionally a single point mutation in the binding PltB subunit ($PltB^{S35A}$) also results in a loss of cellular intoxication[260,261]. While it is possible to inactivate the toxin by introducing point mutations in the sub-units of the typhoid toxin, this approach would lower the barrier to reversion to virulence and would not account for potential unknown activities of other components of the typhoid toxin, including PltA. To avoid these caveats a *S.* Typhi mutant lacking all three components of typhoid toxin – CdtB, PltB and PltA – was created.

5.1.3. Legislation for deliberate release of genetically modified organisms

The deliberate release of a genetically modified organism (GMO) is defined as "*any intentional introduction into the environment of a GMO or a combination of GMOs for which no specific containment measures are used to limit their contact with, and to provide a high level of safety for, the general population and the environment*"[332]. After challenge, participants will shed GM *S.* Typhi in stool that will enter the broader environment via the sewage system. Contained release of the organism is not possible without substantial modification to challenge procedures and a dedicated inpatient facility. Previous typhoid challenge studies involving attenuated oral typhoid vaccines have required specific approvals for deliberate release of GMOs[314], although such approvals have not previously been sought for challenge strains of *Salmonella* Typhi.

In the UK, legislation governing the deliberate release of genetically modified organisms is provided by the Environmental Protection Act 1990 section 111 and 112[333], and the Genetically Modified Organisms (Deliberate Release) Regulations 2002[334]. The Department for the Environment, Food and Rural affairs co-ordinates applications for deliberate release of GMOs. An application for deliberate release comprises two components: Part A provides details relating to

organism; methods of genetic modification; an environmental and human health risk assessment; information relating to conditions of release, and containment and disposal of waste. Part B details information about the release to be included on a public register. The precise dates, location and GPS co-ordinates of the planned release are included in the application. Following submission of an application, a public notification must be made in the form of an advertisement in a national newspaper. This is followed by a minimum 60-day public consultation period, during which time additional information can be requested. Details relating the planned deliberate release of the genetically modified challenge strain are available publicly online [335]. Applications are reviewed by the Advisory Committee on Releases to the Environment (ACRE), who provide recommendations to the Secretary of State, who ultimately responsible for granting consent for release.

5.2. Methods

5.2.1. Construction of a typhoid-toxin deficient strain of *Salmonella* Typhi

The typhoid toxin deficient strain of *Salmonella* Typhi was generated by the Galán laboratory at the Department of Microbial Pathogenesis, Yale University School of Medicine (New Haven, CT, USA) as outlined in Appendix 2.

5.2.2. GMP Manufacture

Manufacture of challenge strains was performed by the Walter Reed Army Institute of Research (WRAIR - MD, USA). Challenge batches were required to pass a series of release tests (Table 5-1) prior to administration to challenge participants. The GMP products was released by the manufacturer's GMP Qualified person (QP) against the agreed specification.

Table 5-1 : Release testing required for *S.* Typhi challenge strains.

MCB = Master cell bank. MIC = Minimum inhibitory concentration.

Product release	Identity			Purity		Content
	API-20E (+ Gram stain)	Antibiotic sensitivity (MIC)	Slide Agglutination	Microbial limits	Gross contamination	Viable Cell Count
GMP-MCB (Wild Type and Toxin Negative)	x	x	x	x	x	x
GMP Challenge material (Wild Type and Toxin Negative)	x	x	x	x	x	x

5.2.3. Antimicrobial Sensitivity

To ensure safety of study participants and to allow for unanticipated antibiotic intolerance/allergy, the strains of *S*. Typhi used in the model must be sensitive to first, second and third line treatment options for typhoid fever. Contemporary treatment guidelines for typhoid fever are outdated and require updating to reflect trends in antimicrobial resistance [140]. Nevertheless, ciprofloxacin or azithromycin represent appropriate oral treatment options for fully sensitive strains of *S*. Typhi. Antimicrobial sensitivity of challenge strains was determined against ampicillin (10 µg), ceftazidime (30 µg), imipenem (10 µg), tazobactam (10 µg), amoxicillin with clavulanic acid (20/10 µg), cephalothin (30 µg), ceftriaxone (30 µg), gentamicin (10 µg), meropenem (10 µg), trimethoprim (5 µg), nalidixic acid (30 µg), cefuroxime (30 µg), azithromycin (15 µg). The minimum inhibitory concentration (MIC) for ciprofloxacin and azithromycin was also determined.

5.2.4. Microbial limits testing

Microbial limits testing was performed to confirm absence of other specified pathogenic bacteria, namely *Escherichia coli*, *Pseudomonas spp.*, *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella spp*. (other than *S.* Typhi), yeasts and moulds and <100 non-pathogenic bacteria and <20 fungi/

ml of the strain material. The culture media and selective media used are outlined in *Table 5-2*. Microbial limits testing was performed by BioScreen ® Testing services Inc (CA, USA) on behalf of WRAIR. Testing was performed based upon a protocol provided by Emergent BioSolutions® (Ref MP: E/0083).

Table 5-2 : Microbial limits testing for challenge stocks of *S.* Typhi.

Enrichment broth and selective agar used to detect each specified contaminant. Protocols supplied by Emergent BioSolutions (Ref MP E/0083).

Specified Contaminant	Enrichment Broth	Selective Agar
Escherichia coli	Fluid Lactose Medium	MacConkey Agar Medium
Salmonella typhimurium	Fluid Lactose Medium (Primary enrichment) Fluid Tetrathionate Medium (Secondary enrichment broth) Fluid Selenite-Cystine medium (Secondary enrichment broth)	Xylose-Lysine-Deoxycholate (XLD) Agar Medium Bismuth Sulphite Agar Medium
Pseudomonas aeruginosa	Fluid Lactose Medium Fluid Soybean-Casein Digest Medium	Cetrimide Agar Medium
Bacillus cereus	Fluid Soybean-Casein Digest Medium	Bacillus cereus Agar
Staphylococcus aureus	Fluid Soybean-Casein Digest Medium	Baird-Parker Agar Medium
Aspergillus niger	N/A	Acidified Potato Dextrose Agar Medium
Candida albicans	N/A	Sabouraud Dextrose Agar Medium + Kanamycin

5.2.5. DNA extraction and Sequencing.

Methods for DNA extraction and sequencing are detailed in Appendix 3 – Whole genome sequencing of typhoid toxin deficient S. Typhi SB6000 strain.

5.2.6. Slide agglutination assays

Slide agglutination was performed in accordance with local SOPs (OVGL040) and Public Health England guidelines[336]. Testing was performed in duplicate on glycerol stocks from the following isolates:

1. *Salmonella* Typhi Quails strain BN ASS/VPU/2010/0002; Vial number 660;
2. Master cell bank *Salmonella* Typhi Quails strain wild type; BPR –1201 –00; Vial number 60; Lot 1962; Date of Manufacture 01-Dec-2015
3. *Salmonella* Typhi Quails strain wild type; BPR-1202-00; Vial number 190; Lot 964; Date of manufacture 03-Dec-2015
4. Master cell bank *Salmonella* Typhi Toxin deficient Quails strain SB6000 (Pre-GMP); BPR-1217-00; Vial number 17; Lot 1976; Date of Manufacture 02-Feb-2016
5. *Salmonella* Typhi Toxin deficient Quails strain SB6000 (GMP batch); BPR-1218-00; Vial number 24; Lot 1977; Date of Manufacture 05-Feb-2016

Isolates from glycerol stocks were inoculated and spread on tryptone soya agar using a sterile 10μl loop and cultured overnight. Simultaneously, isolates were inoculated into 10ml of Lysogeny broth and cultured overnight in a shaking incubator at 200-220rpm, 37°C. Isolates grown in liquid culture were spun down at 3,000 rpm for 10 min and the supernatant discarded, giving a suspension of bacteria used to assess agglutination.

The following anti-sera were used: *Salmonella* agglutinating sera O9 (Remel Europe Ltd, R30957301 [ZC17]); *Salmonella* agglutinating sera Hd (Pro-lab diagnostics; R30957301 (ZC17)); *Salmonella* agglutinating sera Vi (Remel Europe Ltd, R30957301 [ZC18]) and *Salmonella* agglutinating sera Ha (Remel Europe Ltd, R30160201 [ZD05]). Antisera were removed from storage at 4°C and allowed to reach room temperature prior to use. Twenty microliters of each antiserum were pipetted onto a glass slide (2 per slide). A colony of *Salmonella* Typhi was selected from an agar plate using a 10μl loop and mixed into the antisera to form a smooth milky

suspension. The slide was gently rocked for up to 1 minute to observe clumping of the suspension with a clearing of the background.

5.2.7. Growth characteristics in liquid culture

Growth curves of wild-type and toxin-deficient strains of *S.* Typhi were performed in LB using isolates listed in section 5.2.6. Isolates were inoculated into 10ml of LB and grown overnight in a shaking incubator at 200-220rpm and 37°C. The following day, cultures were vortexed and diluted 1:10 in fresh LB (100 µl culture + 900 µl LB). The OD600 was read in a cuvette using a mini photospectrometer against a LB-only blank, multiplying by the dilution factor (x10) to give the actual OD of the overnight culture. The dilution required to reduce the culture OD to 0.05 in a 30 ml volume of LB was calculated using:

$$OD1*V1 = OD2*V2$$

Where, OD1 = OD of the overnight culture; V1 = volume of the overnight culture to be added to the new mix; OD2 = OD of the new inoculum (0.05) and V2 = volume of new inoculums (30 ml). The calculated volume of overnight cultures (V1) was added to 30ml of fresh LB, the OD600 read to ensure the OD of the new culture was 0.05 and subsequently returned to the shaking incubator. Samples of culture were removed from incubator at regular intervals and the OD600 measured in a 50:50 mix of the culture and fresh LB, against a LB blank. OD600 readings were multiplied by the dilution factor (x2) to give values for the undiluted culture and plotted against time to give the growth curve.

5.2.8. Cellular Invasion assay

Henle-407 intestinal epithelial cells were infected with wild type *S*. Typhi Quailes or the toxin-deficient SB6000 derivative for 2 hours at three MOI levels (50,100 & 500). Cells were washed, and gentamicin (50 µg/ml) was added to the culture medium. After two hours, cells were washed again, lysed and colony counts of both strains was determined by plating dilutions of the cell

lysates. The invasive ability was expressed as the percentage of the bacterial inoculum that survived gentamicin treatment. These assays were performed by the Galán laboratory at the Department of Microbial Pathogenesis, Yale University School of Medicine.

5.2.9. Typhoid toxin activity assay

Activity of the typhoid toxin was assessed using previously published methods[256,260]. Briefly, Henle-407 intestinal epithelial cells were infected with wild-type Quailes or typhoid-toxin deficient SB6000 *S.* Typhi 1 hour. Cells were washed and culture medium containing gentamicin (50 µg/ml) was added, then incubated for 2 hours. Cell were then washed and medium containing 5 µg/ml gentamicin was added and infection continued for 48 hours. Cells were collected from dishes by trypsinization (subsequently neutralized with serum containing media). The cell suspensions were centrifuged for 5 min at 1500 rpm, the supernatant discarded, and cell pellets resuspended in 0.5 ml of phosphate-buffered saline (PBS) at room temperature. Cell suspensions were slowly added to tubes containing 4 ml of cold 90% ethanol solution with continuous mixing. Cells were kept in fixative for 2 hours on ice. The fixed cells were collected by centrifugation, and the fixative decanted thoroughly. The pellets were washed once with 5 ml of PBS, and the cell pellet were resuspended in 1 ml of a solution containing 0.1% Triton X-100, DNase-free ribonuclease A (20 mg/ml), and propidium iodide (20 µg/ml; Molecular Probes) in PBS. The stained cells were analyzed by flow cytometry with a FACStar Plus flow cytometer (Beckton Dickinson). Intoxicated cells show a larger proportion of cells in the G2/M phase of the cell cycle and thus exhibited a larger amount of DNA content[337]. These assays were performed by the Galán laboratory at the Department of Microbial Pathogenesis, Yale University School of Medicine.

5.2.10. Establishment of challenge dose

Challenge agents were prepared in batches for a maximum of 6 participants at any one time. All work was performed in the containment level 3 facility at the Centre for Clinical Vaccinology & Tropical medicine (Oxford, UK) in a class II biological safety hood dedicated for challenge agent preparation.

Two GMP master stock vials of typhoid-toxin negative *Salmonella* Typhi SB6000 strain (BPR—1218-00, Lot 1977, Cell concentration 1 x 10^6) or wild type *Salmonella* Typhi Quailes strain (BPR—1218-00, Lot 1977, Cell concentration 9.8 x 10^5) were selected at random from stocks stored in a -80°C freezer. Vials were thawed at room temperature for ~10 minutes and mixed by vortexing. The contents of two GMP master stock vials were transferred to a fresh the tube labelled 'Master stock' and mixed for 6-10 seconds by vortexing. A 1:10 dilution in sodium bicarbonate was performed by transferring 1600ul from the tube labelled 'master stock' to the 50ml falcon labelled as 'master stock 1:10'. To create the challenge inoculum of the toxin negative strain, 1.74ml from the 'master Stock 1:10 dilution' was transferred to a sterile culture flask containing 4.2g sodium bicarbonate dissolved in 240ml bottled mineral water ('Challenge Flask'). The challenge inoculum for the wild type strain was generated by transferring 1.85ml from the 1:10 dilution into an equivalent challenge flask. The challenge agents were then prepared by transferring 30ml from the challenge flask to a prelabelled 50ml falcon tubes, sealed and stored on ice.

The challenge dose was confirmed by pipetting 200μl from challenge dose onto six Tryptone Soya Agar (TSA) plates (Oxoid/PO0163A). The bacterial suspension was spread over the source of the agar using an L-shaped spreader and cultured in an incubator overnight at 37°C, 5% CO2. On the following day, colony counts were manually counted and checked by a second operator. The CFU of the challenge inoculum was calculated by multiplying the mean of the CFU counts for the plates

by the dilution factor of the volume plated (x150 = total challenge inoculum of 30ml, plating 0.2ml = [30ml/0.2 = 150x]).

Sodium bicarbonate for administration prior to challenge agent administration was prepared by dissolving 2.1g sodium bicarbonate (Waitrose Cooks' Ingredients) in 120ml bottled mineral water.

5.3. Results

5.3.1. Development of Wild-Type and Typhoid Toxin deficient *S.* Typhi challenge strain.

The steps involved in the generation of new challenge strain batches are outlined in Figure 5-1. Two vials of GMP-grade *S*. Typhi Quailes strain were supplied to the Galán laboratory (Department of Microbial Pathogenesis, Yale School of Medicine, New Haven, CT, USA), who undertook a targeted knock-out of the typhoid-toxin pathogenicity island. To allow for non-specific changes that may occur in the processes of genetic modification and passage, wild-type *S*. Typhi Quailes strain underwent parallel handling and culture, with the exception of targeted knock-out of typhoid toxin genes. In all cases, strains were propagated in animal-product-free media.

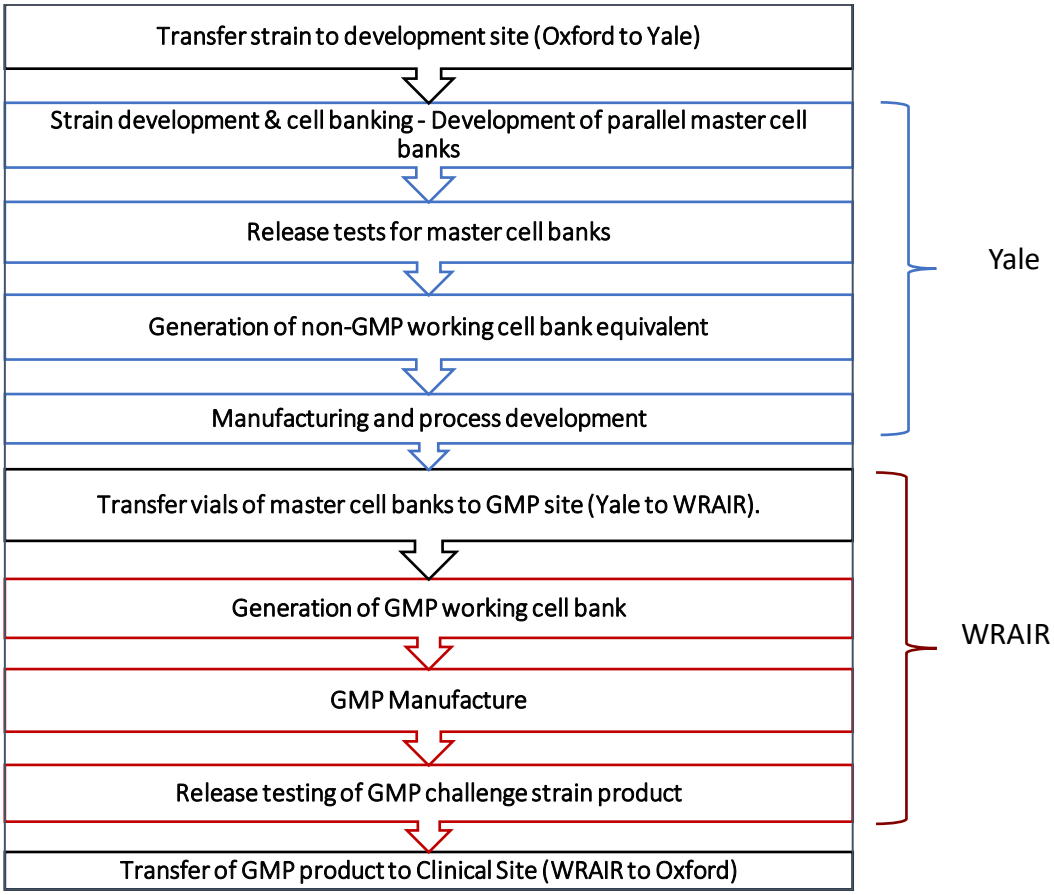


Figure 5-1 : Development of *S.* Typhi challenge agents.

WRAIR = Walter Reed Army Institute of Research (MD, USA).

5.3.2. Generation of typhoid toxin deficient *Salmonella* Typhi

Construction of the knock-out strain is illustrated in Figure 5-2. Due to the genomic organization of the typhoid toxin pathogenicity island, the *cdtB* gene was deleted first and followed by simultaneous deletion of the *pltA* and *pltB* genes (encoded immediately adjacent to one another) in the *ΔcdtB* strain. Full details of strain construction are provided in Appendix 2 – Construction of typhoid toxin deficient Salmonella Typhi strain.

Deletion of typhoid toxin sub-unit genes was carried out using the R6K-derived, suicide vector pSB890. The pSB890 plasmid is unable to replicate in *S.* Typhi as it requires the bacteriophage *λpir* protein for replication. The plasmid vector also encodes a counter selectable marker *sacB*,

encoding an enzyme that is lethal to bacteria when grown in the presence of sucrose. The pSB890 plasmid vector is maintained in a specially constructed strain of *Escherichia coli*, which encodes the bacteriophage *λpir* protein. This *E. coli* strain also carries a deletion mutation in the *asd* gene(*Δasd)*, which encodes for the aspartate semialdehyde dehydrogenase required for peptidoglycan synthesis – growth of this strain will only occur in media supplemented with Lysine diaminoheptanedioate (L-DAP).

Chromosomal DNA fragments encoding sequences upstream and downstream of the target genes were expanded by PCR and cloned into the pSB890 plasmid, maintained in the *E.* coli *Δasd λpir* strain. The plasmid vector encoding the cloned sequences was then transferred to *S.* Typhi by conjugation, counter-selecting the donor *E* coli strain by plating the trans-conjugants in media lacking L-DAP (counter-selecting for the donor *E.* Coli). Trans-conjugants of *S.* Typhi possessing deletions of the toxin integrated into the chromosome were identified by plating in sucrose that counter-selects for the plasmid vector. Colonies were screened by PCR to identified mutants carrying the specific deletions.

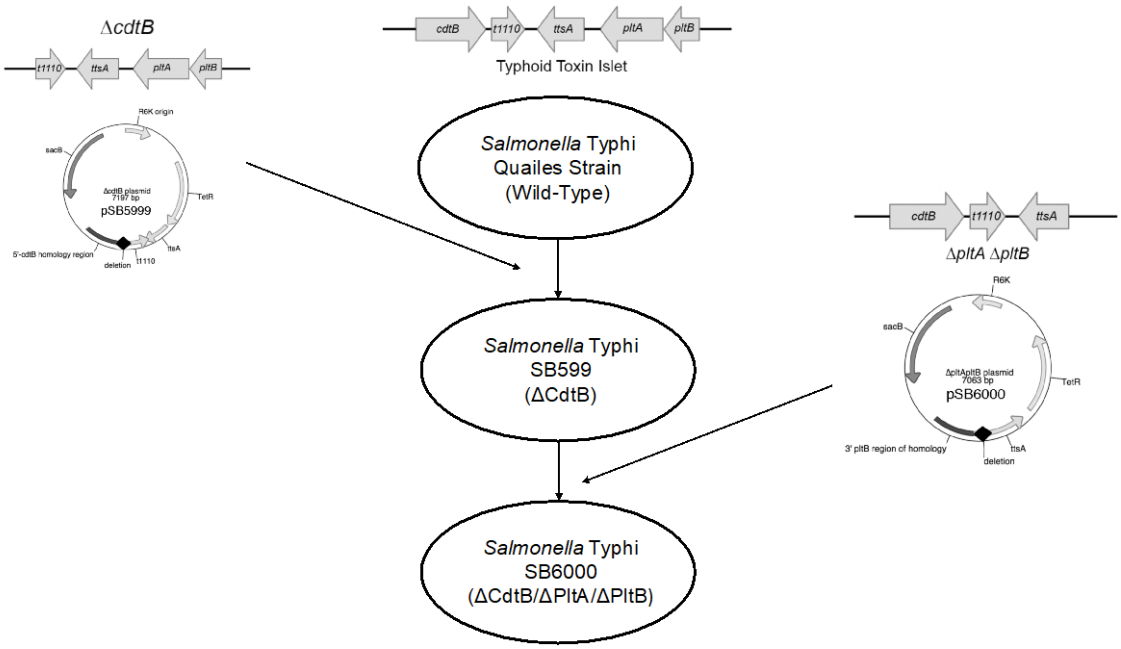


Figure 5-2 : Construction of *Salmonella* Typhi ΔCdtB, ΔPltA, ΔPltB knock-out strain (SB6000)

5.3.3. Manufacture of challenge strains

Manufacture of challenge strains was undertaken at the Pilot Production Facility of the Walter Reed Army Institute of Research (WRAIR – MD, USA). Both challenge stocks were fully susceptible to all antibiotics tested as outlined in section 5.2.3. Challenge stocks were certified as being free of *E. Coli*, *P. aeruginosa*, *B. cereus*, *S. aureus*, *A. niger* and *C. albicans*. Two hundred vials each of wild-type S. Typhi Quailes strain and typhoid toxin deficient *S.* Typhi SB6000 strain were manufactured (Figure 5-3). Fifty vials of each challenge strain were retained by the manufacturer for stability testing.

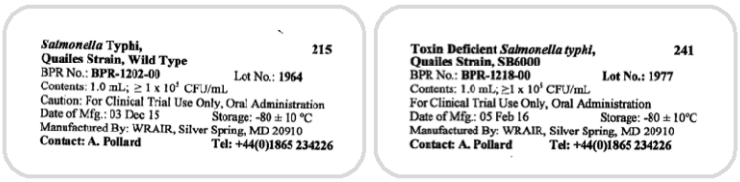


Figure 5-3 : Labelling of GMP challenge batches.

5.3.4. Sequencing of challenge strains.

Sequencing of the challenge strains was performed at the Wellcome Trust Sanger Institute as detailed in Appendix 3 – Whole genome sequencing of typhoid toxin deficient S. Typhi SB6000 strain.

Whole genome sequencing of challenge strains confirmed the expected absence of the typhoid-toxin pathogenicity islet in the SB6000 GMO strain. We observed no differences between the wild-type and SB6000 GMO strains in relation to key virulence factors. In particular, no differences between strains were observed in SPI-7, a region encoding genes required for expression of the Vi-capsule. Differences between strains were confined to highly-variable regions encoding phage proteins, which were not predicted to impact on bacterial survival in the environment or persistence in the human host (Appendix 3 – Whole genome sequencing of typhoid toxin deficient S. Typhi SB6000 strain.

5.3.5. Phenotypic Characterisation of Challenge Strains

5.3.5.1. Growth curves

The growth characteristics in liquid culture of wild type and toxin-negative *S.* Typhi were compared as outlined in section 5.2.7 and growth curves are illustrated in Figure 5-4. As

illustrated in the figure, we observed comparable growth characteristics in liquid culture between wild-type and toxin-negative *S*. Typhi.

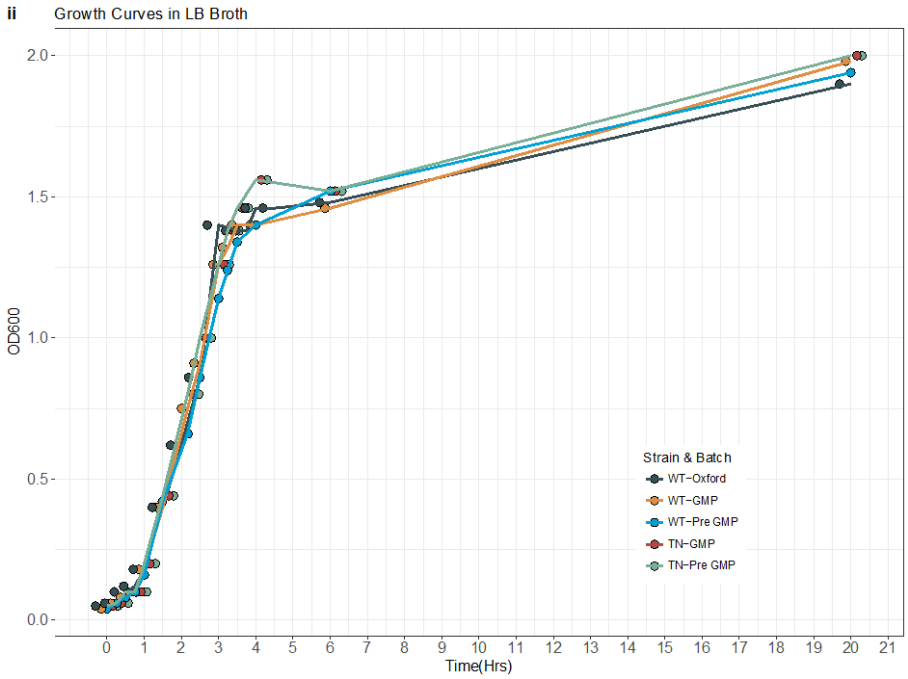


Figure 5-4 : Growth curves of wild-type (WT) and typhoid-toxin deficient (TN) S. Typhi

Growth in Lysogeny broth. Data points and lines are coloured according to challenge strain and batch. Pre-GMP and GMP batches were compared to a GMP batch of *S*. Typhi Quailes strain used in previous challenge studies.

5.3.5.2. Agglutination assays

Expression of the Vi-capsule is thought to have an important role in the pathogenesis of typhoid fever. The genes required for expression of the Vi capsule are encoded in a mobile and potentially unstable region of the *Salmonella* Typhi genome termed the *viaB* locus within SPI-7 [225]. Spontaneous loss of Vi expression and SPI-7 has been described following serial passage or long term storage [142,338]. Detection of Vi antigen by slide agglutination can be affected by a number of factors including osmolality and growth conditions used [148,339].

In order to determine if steps involved in the manufacture of the challenge strain were associated with altered Vi-capsule expression, I performed slide agglutination assays on the GMP batches of wild-type and toxin-negative challenge strains.

Reactions to *Salmonella* agglutinating sera are recorded in Table 5-3. Both wild-type and toxin-negative strains expressed the Vi-capsule. Agglutination using O9 anti-serum was initially negative. Cultures were heated to 95-100^{o}C in a heat block for 10 minutes and testing repeated, after which satisfactory agglutination was observed.

Table 5-3 : Results of slide agglutination assays.

Antigen	Strain Variant				
	1 *Salmonella* Typhi Quailes strain (Oxford)	2 Master cell bank *Salmonella* Typhi Quailes strain wild type	3 *Salmonella* Typhi Quailes strain wild type (WRAIR)	4 Master cell bank *Salmonella* Typhi Toxin deficient Quailes strain SB6000	5 *Salmonella* Typhi Toxin deficient Quailes strain SB6000 (GMP batch)
Cultures on Solid Media (TSA Plates)					
Vi	+	+	+	+	+
H-a (negative control)	-	-	-	-	-
Liquid cultures in LB broth					
Vi	+	+	+	+	+
O-9	+	+	+	+	+
H-d	+/-	Weak	Weak	Weak	Weak
H-a (negative control)	-	-	-	-	-

5.3.6. Cellular Invasion Assay

As outlined in section 2.2.6, expression of the typhoid toxin occurs when the bacteria are resident within the intracellular compartment[262]. To determine whether knock-out of the typhoid-toxin pathogenicity island was associated with altered capacity to invade host cells, cellular invasion of wild-type and toxin-deficient S. Typhi were compared using a gentamicin protection assay. No differences between the invasive ability of the two strains were detected (Figure 5-5)[337].

5.3.7. Typhoid toxin activity assay

Comparison of typhoid toxin activity between wild-type and toxin-deficient strains was performed at the by the Galán laboratory at the Department of Microbial Pathogenesis, Yale University School of Medicine. Compared with the typhoid toxin-deficient strain SB6000, cells infected with wild type *S.* Typhi Quailes showed a higher proportion of cells in the G2-M phase of the cell cycle, indicative of cell-cycle arrest caused by intoxication with the typhoid toxin(Figure 5-5)[256 260].

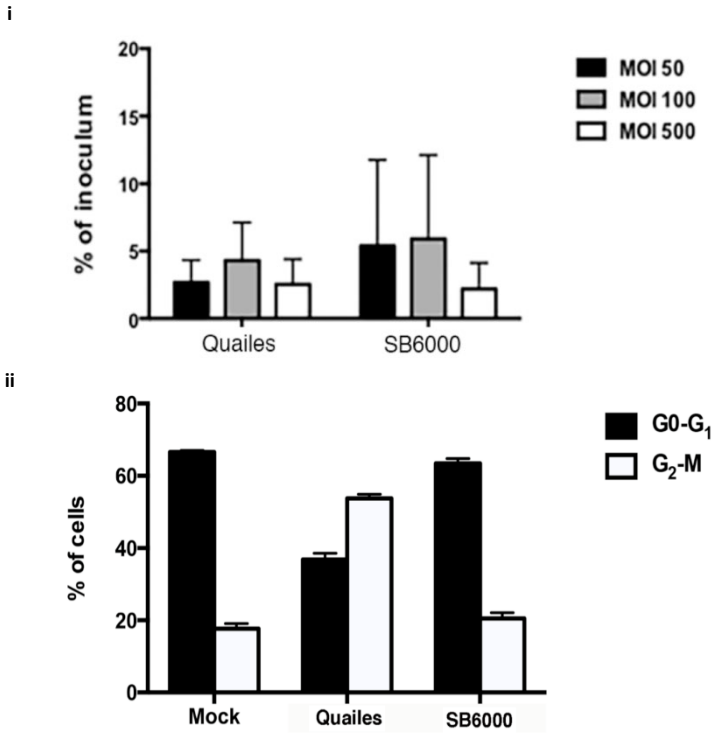


Figure 5-5 : Characterisation *S* of. Typhi SB6000 typhoid toxin deficient strain.

(i) Cellular invasion; (ii) Typhoid toxin activity assay. Assays performed by Maria Lara-Tejero, Xuyaou Jiao and Shu-Jung Chan (Department of Microbial Pathogenesis, Yale School of Medicine, New Haven, CT, USA). Figures courtesy of Jorge Galan.

5.3.8. Establishment of Challenge Dose

Previous challenge *S.* Typhi challenge studies have been performed using frozen vials of GMP challenge stocks of the Quailes strain, diluted to a target concentration of 1-5 x 10^4 CFU. With sodium bicarbonate pre-treatment, challenge at this dose level aims to achieve an attack rate of 60-75% in unvaccinated, typhoid naïve volunteers - across all typhoid studies performed to date, the average attack rate in naïve controls is 69% (95% CI 60-77%; n=121) [126,209,314]. To ensure comparable protocols and dose-administration with previous typhoid challenge studies, I re-validated standard operating procedures for challenge agent preparation for the newly generated challenge stocks.

Vials of wild-type *S*. Typhi were GMP released at a concentration of 9.8 x 10^5 CFU/ml. Vials of toxin-negative *S*. Typhi SB6000 were GMP released at a slightly higher concentration of 1 x 10^6 CFU/ml.

The results of challenge agent preparation testing are illustrated in Figure 5-6. All test runs achieved the target dose range of 1-5 x 10^4 CFU. Early test runs (batches 1 & 2 Figure 5-6) were associated with differences of ~5000 - 10 000 CFU between wild-type and toxin-negative challenge strains, likely reflecting differences in the concentration of bacteria in the challenge vials. In subsequent test runs, the protocol was modified to ensure that a more comparable dose was obtained for both challenge strains (see methods 5.2.10).

To ensure consistency of dose between participants, the challenge agents were prepared fresh on the morning of challenge, maintained on ice after preparation and administered to within 2-3 hours of preparation. To check that challenge dose did not alter significantly over this time period, I performed serial colony counts at multiple timepoints from three batches of challenge agents stored at ice and at room temperature (see methods 5.2.10). Within the period of challenge dose administration, the challenge doses remained within the target dose range for both wild-type or toxin negative strains (Figure 5-7).

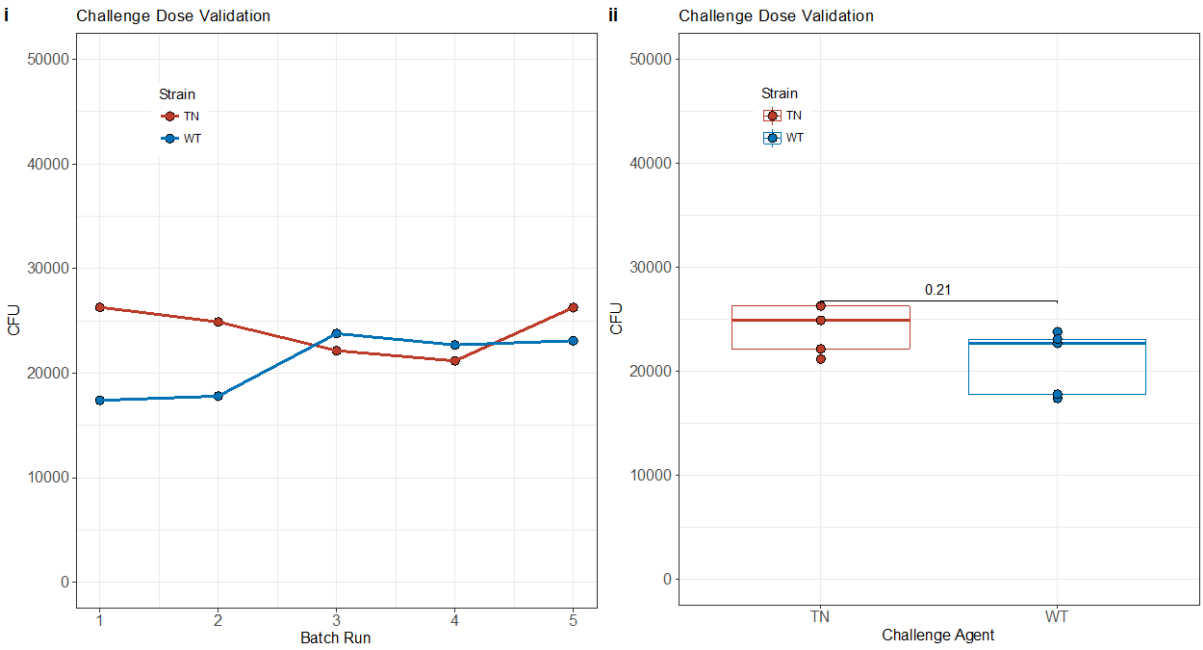


Figure 5-6 : Establishment of challenge dose

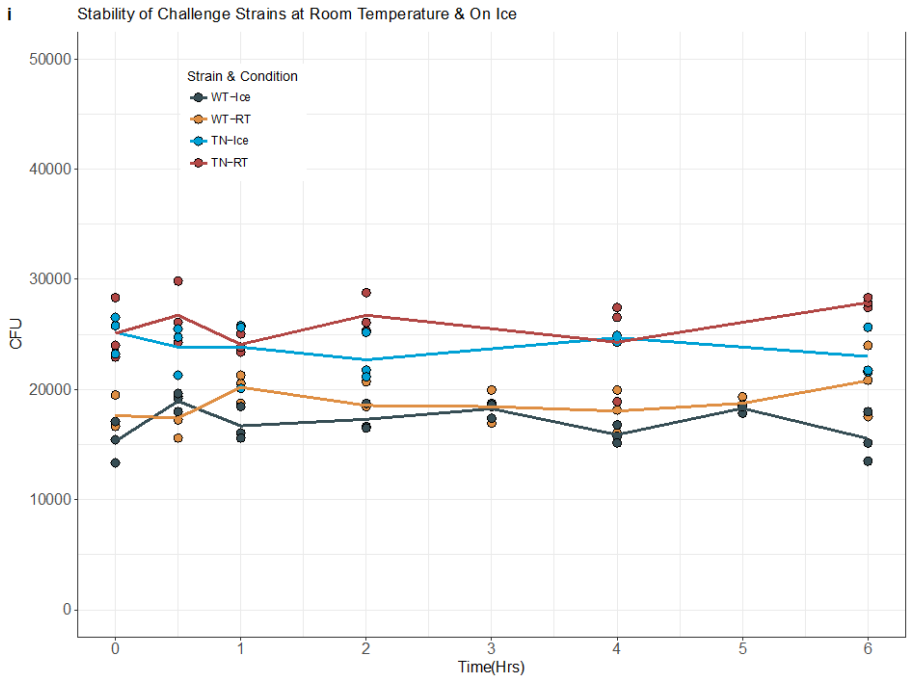
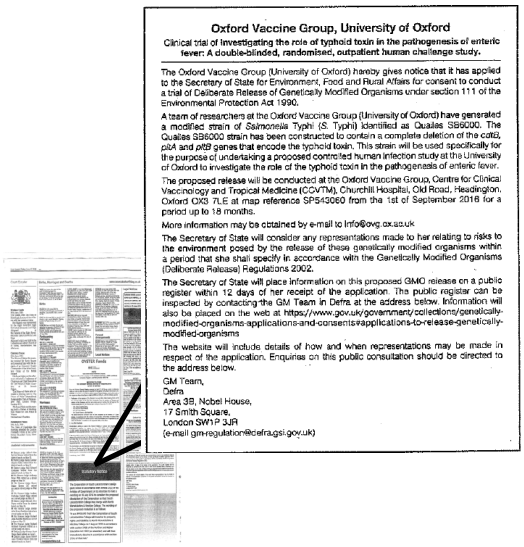


Figure 5-7 : Stability of challenge strains on ice and room temperature (RT).

Data points are coloured according to challenge strain and condition. WT = Wild type S. Typhi Quailes strain. TN = Typhoid toxin deficient S. Typhi SB6000. Each strain and condition were tested in triplicate. Coloured lines connect mean colony counts at different time points.

5.3.9. Deliberate release of a genetically modified challenge strain

An application for deliberate release of the typhoid toxin deficient strain of Salmonella Typhi was submitted on the 8th June 2016. The full application can be reviewed online [335]. On the 8th July 2016, the ACRE committee responded requesting additional information to be included in the application. These included data relating to whole genome sequencing of the challenge strain (detailed in 5.3.4) and additional environmental persistence testing to determine "*that the GMO has the same survival and persistence characteristics as the parent strain*" (detailed in 5.3.10). Following completion of testing and provision of this information, final approvals were granted on the 22nd February 2017.



Oxford Vaccine Group, University of Oxford

Clinical trial of investigating the role of typhoid toxin in the pathogenesis of enteric fever: A double-blinded, randomised, outpatient human challenge study.

The Oxford Vaccine Group (University of Oxford) hereby gives notice that it has applied to the Secretary of State for Environment, Food and Rural Affairs for consent to conduct a trial of Deliberate Release of Genetically Modified Organisms under section 111 of the Environmental Protection Act 1990.

A team of researchers at the Oxford Vaccine Group (University of Oxford) have generated a modified strain of *Salmonella* Typhi (*S.* Typhi) identified as Quailes SB6000. The Quailes SB6000 strain has been constructed to contain a complete deletion of the *cdtB*, *pltA* and *pltB* genes that encode the typhoid toxin. This strain will be used specifically for the purpose of undertaking a proposed controlled human infection study at the University of Oxford to investigate the role of the typhoid toxin in the pathogenesis of enteric fever.

The proposed release will be conducted at the Oxford Vaccine Group, Centre for Clinical Vaccinology and Tropical Medicine (CCVTM), Churchill Hospital, Old Road, Headington, Oxford OX3 7LE at map reference SP543060 from the 1st of September 2016 for a period up to 18 months.

More information may be obtained by e-mail to info@ovg.ox.ac.uk

The Secretary of State will consider any representations made to her relating to risks to the environment posed by the release of these genetically modified organisms within a period that she shall specify in accordance with the Genetically Modified Organisms (Deliberate Release) Regulations 2002.

The Secretary of State will place information on this proposed GMO release on a public register within 12 days of her receipt of the application. The public register can be inspected by contacting the GM Team in Defra at the address below. Information will also be placed on the web at https://www.gov.uk/government/collections/genetically-modified-organisms-applications-and-consents#applications-to-release-genetically-modified-organisms

The website will include details of how and when representations may be made in respect of the application. Enquiries on this public consultation should be directed to the address below.

GM Team,
Defra
Area 3B, Nobel House,
17 Smith Square,
London SW1P 3JR
(e-mail gm-regulation@defra.gsi.gov.uk)

Figure 5-8 Public notification of deliberate release of genetically modified challenge strain.

Advertisement in The Times newspaper 16th June 2016 fulfilling requirements under Genetically Modified Organisms (deliberate release) Regulations 2002 [334].

5.3.10. Environmental persistence testing

To demonstrate that toxin-negative *S*. Typhi SB600 had the same survival and persistence characteristics as the parent strain, both strains were cultured in a variety of environmental

media as outlined in Appendix 4 - Environmental persistence testing of challenge strains. Overall, both strains survived in environmental samples for longer than expected, although we observed no significant difference in the survival characteristics of wild-type and toxin-negative *S*. Typhi.

When both strains were cultured in river water, from a high starting inoculum (10^7 CFU/ml) and incubation at 21°C, there was only a single $\log_{10}$ decline over first 40 days. Beyond, 40 days the there was a more rapid decline in colony counts, such that by day 68, both wild-type and knock-out strains were below limit of detection (Figure 5-9). Consistent with previous reports, survival was prolonged when cultured at 4°C, with only a one $\log_{10}$ of wild-type *S*. Typhi by Day 68 [340]. When the strains were grown in sea-water and incubation at 21°C, there was a one $\log_{10}$ decline over first 20 days, after which the colony counts declined more rapidly. By day 51, both wild-type and knock-out strains were below limit of detection (Figure 5-10). Similar to growth in river water, culture at 4°C in sea-water was associated with prolonged survival compared with incubation at 21°C. When the strains were grown in soil, there was a three $\log_{10}$ decline over first 20 days and both strains were below limit of detection by day 44 (Figure 5-11).

Data on environmental persistence were provided to DEFRA as part of a re-submission package to obtain approvals for deliberate release of the GM strain of *S*. Typhi.

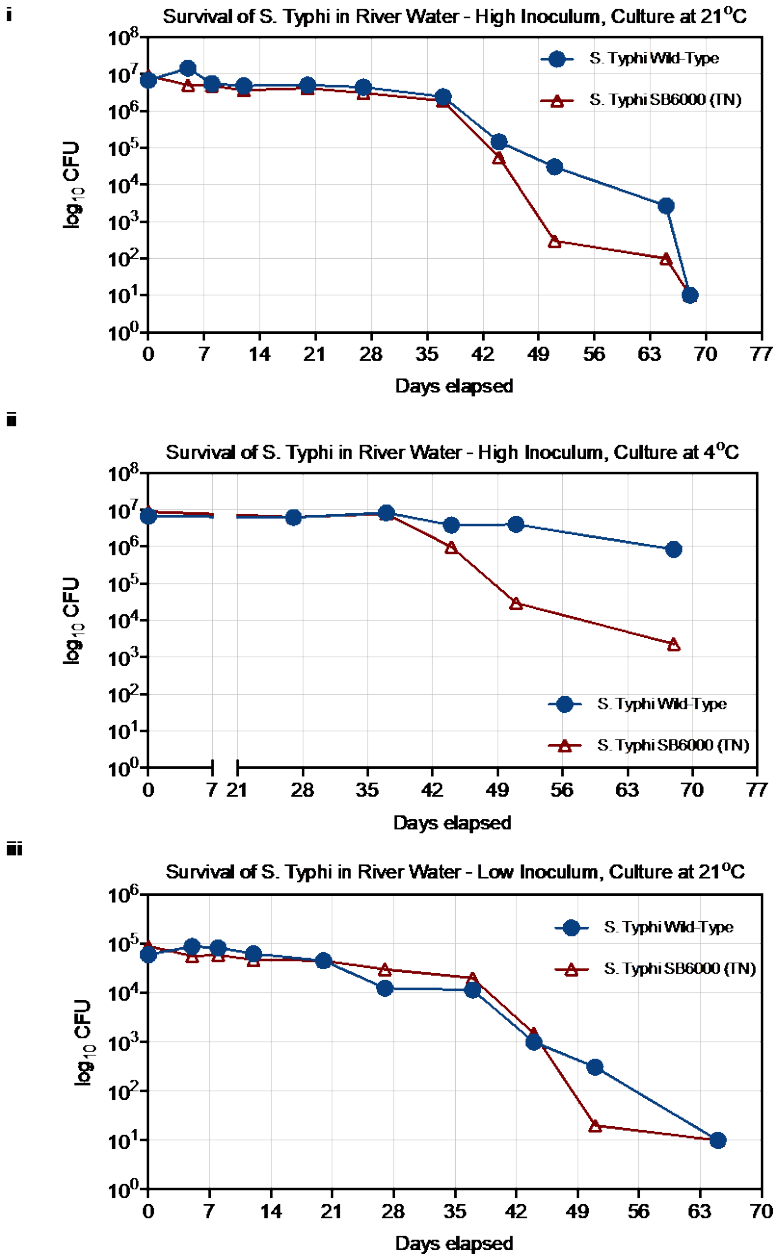


Figure 5-9 : Survival of wild-type and toxin-negative S. Typhi in river water.

(i) Staring inoculum 10^7 CFU, culture at 21°C; (ii) Staring inoculum 10^7 CFU, culture at 4°C; (iii) Staring inoculum 10^5 CFU, culture at 21°C. Data generated and provided courtesy of Frieda Jorgensen, Public Health England.

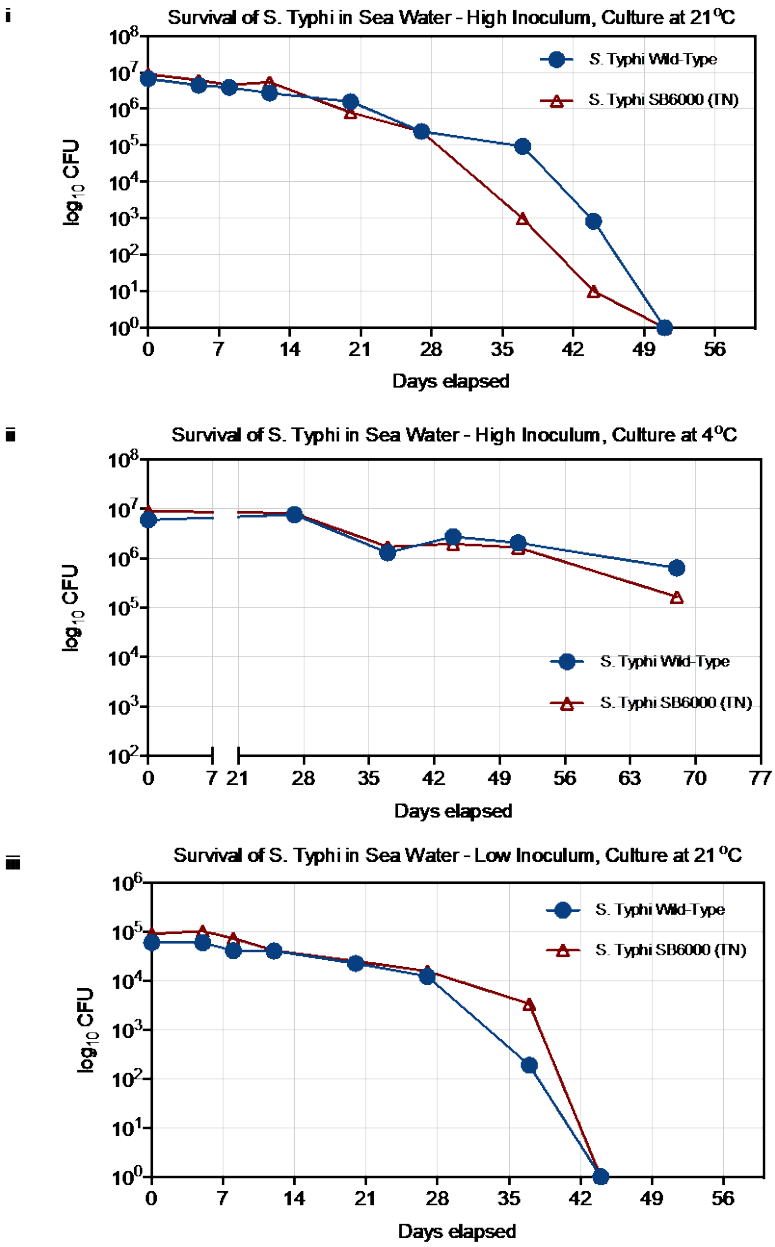


Figure 5-10 : Survival of wild-type and toxin-negative S. Typhi in sea-water.

(i) Staring inoculum 10^7 CFU, culture at 21°C; (ii) Staring inoculum 10^7 CFU, culture at 4°C; (iii) Staring inoculum 10^5 CFU, culture at 21°C. Data generated and provided courtesy of Frieda Jorgensen, Public Health England.

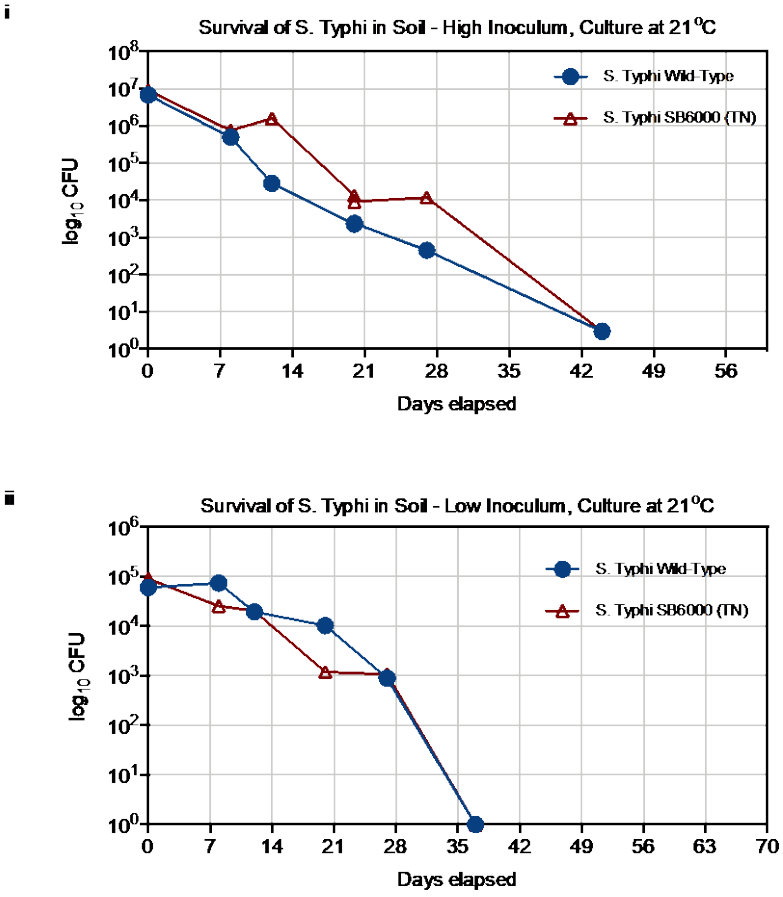


Figure 5-11 : Survival of wild-type and toxin-negative S. Typhi in soil.

(i) Staring inoculum 10^7 CFU, culture at 21°C; (ii) Staring inoculum 10^5 CFU, culture at 21°C. Data generated and provided courtesy of Frieda Jorgensen, Public Health England.

5.3.11. Discussion

In this chapter I have described the design, manufacture and characterisation of wild-type and typhoid toxin deficient strains of *S.* Typhi for use in a controlled human infection study. The primary aim of this work was to ensure (at the pathogen level) that any differences observed after challenge with the wild-type and mutant strains could be attributed to the presence or absence of the typhoid toxin. This work represents, to my knowledge, the first occasion in which a genetically modified strain of *Salmonella* Typhi has been manufactured specifically for use as a challenge agent in a controlled human infection model. A detailed programme of work was

undertaken to characterise the challenge strains genotypically and phenotypically prior to administration in healthy volunteers. These studies demonstrated that both challenge strain variants were comparable across multiple measures, including antibiotic susceptibility, growth characteristics, Vi-capsule expression and cellular invasion. In addition, I have validated a standard operating procedure for preparation of challenge doses, to ensure comparable and reproducible doses between studies and challenge batches.

The regulation of human challenge studies varies substantially between countries. Under current UK guidance, microbial challenge agents are not considered as investigational medical products. As such, formal approvals by the Medicines and Healthcare products Regulatory Agency (MHRA) are not currently required for challenge studies not involving investigational products. In contrast, other European countries and the US Food and Drug Administration (FDA) consider microbial challenge agents as Investigational New Drugs (INDs) and investigators must submit specific a IND application prior to their use[341]. Challenge agents in the US – and protocols for challenge studies – are therefore subject to safety, quality and manufacturing review by the FDA, which may even extend to reviewing third-party risks[342,343].

The differing regulatory requirements for challenge studies between countries, raises important safety and ethical questions. Approval by a research ethics committee does not guarantee the safety – or the scientific quality – of a challenge study. The UK is viewed as a relatively 'permissive' environment to conduct challenge studies and the introduction of additional regulatory requirements could represent an additional administrative and financial burden - potentially hindering research and development, such as occurred in efforts to develop a human challenge model of Zika virus infection[342,344]. It can be argued that a permissive regulatory environment allows challenge studies to "fulfil their potential" in accelerating development and de-risking products for emerging global health challenges. In contrast, the relatively limited oversight by

government agencies could – theoretically – expose volunteers to risks of unsafe challenge agents or poorly designed study protocols. Investigators and sponsors may also expose themselves to additional reputational, legal or financial risks by conducting challenge studies in the absence of a strong regulatory framework. The regulatory framework of human challenge studies – including those relevant to low-and middle-income countries - are currently being considered by consortia established by major funding bodies[345,346].

In this study, we manufactured two new master cell banks and challenge agent batches in a GMP facility familiar with production of challenge agents and live attenuated vaccines. As challenge agents are not considered IMPs in the UK, there is no specific requirement for GMP manufacture. However, the MHRA recommends that microbial challenge agents are manufactured 'in the spirit of GMP' [310]. Manufacture to GMP offers a degree of quality control and confidence in the agents administered to volunteers, facilitating batch-to-batch consistency between challenge doses and allowing for comparisons between studies.

The process of GMP manufacture is costly, time consuming and requires engagement with multiple partners – the process described in this chapter spanned a period of twenty months from shipment of the challenge strain for manufacture, to administration of the first challenge dose. Production of large batches of challenge stocks removes the need to repeat the manufacture for each study but introduces the need for regular stability testing. The production of infectious material for use in controlled human infection studies is a problem shared across distinct types model and is arguably an issues central to the future development of controlled human infection models[347].

In the set-up of this study, we obtained approvals for deliberate release of a genetically modified strain of *S*. Typhi. Additional approvals were obtained from local bodies, including university and

NHS trust committees overseeing GM organism use. The study site also underwent a routine inspection by the Health and Safety executive during the study to ensure compliance with control measures. We implemented measures to ensure appropriate disposal of waste, including autoclaving of all study samples, prior to incineration. In contrast with the regulatory environment for challenge studies and challenge agent manufacture, detailed legislation and regulations exist governing the deliberate release of genetically modified organisms. As stated by the UK government, these "*strict legislations*" exist "*to protect human health and the environment*"[348]. One might ask why the same does not apply to virulent strains of *S.* Typhi without genetic modifications.

The process of obtaining approvals for deliberate release of GM strains of *S*. Typhi spanned a period of 9 months and was delayed primarily by the need to generate additional data to support the application (5.3.4 & 5.3.10). The experience of obtaining approvals for deliberate release of GM pathogens – and knowledge of the data required to obtain approvals – could help to inform the set-up of future challenge studies involving GM challenge strains.

Studies on the survival of challenge strains in different water samples yielded unexpected insights into the environmental persistence of *S.* Typhi. Whilst both wild-type and GM challenge strains had similar survival patterns, the persistent survival of *S.* Typhi in environmental media was unexpected. Survival of the bacteria was greatest in river water, compared with sea water, which in turn was greater than that in top-soil. In river water and sea-water, the bacteria survived at high levels for up to 40 days and 20 days respectively before significant decline in viable cell counts. In all conditions tested, the incubation temperature had an important impact on survival, such that the organisms survived longer when cultured at 4^{o}C compared with 21^{o}C. Direct culture of S. Typhi from environmental water samples is difficult [36], in part as the bacteria can enter a viable-non-culturable state[349]. Nevertheless, the organism must be able to survive for long

periods in the environment, as supported by these data and detailed surveillance of water supplies using molecular methods [36]. The conditions in which survival were tested (inoculation of high concentration of bacteria into a small volume of water) may not be representative of how *S.* Typhi contaminates water supplies in field settings and additional work on the chemical composition of water samples were not undertaken. Future studies could include comparative studies of *S*. Paratyphi A survival in the environment and detailed studies of survival in treated and untreated sewage. The limitations notwithstanding, these data could be used to inform future environmental surveillance strategies for *S*. Typhi, following the impending implementation of typhoid conjugate vaccines.

5.3.12. Summary

In summary, I have described the successful generation, manufacture and testing of a typhoid-toxin deficient strain of *S*. Typhi. The data presented in this chapter highlight the economic, ethical and regulatory hurdles associated with the production of genetically modified challenge agents. The absence of a reliable small-animal model of typhoid infection complicates the study of typhoid pathogenesis and screening of virulence factors. To partially overcome this challenge, controlled human infection models could represent a valuable means of assessing specific virulence factors for which there is substantial pre-clinical evidence supporting their role in disease pathogenesis – such as is the case with the typhoid toxin. In the following chapter I will describe the application of this challenge strain in a controlled human infection model.

Chapter 6 - Comparison of the response to challenge with wild-type and typhoid toxin deficient *Salmonella* Typhi

6. Introduction

The availability of a controlled human infection model offers a platform to assess the role of typhoid toxin acute typhoid fever. In the previous chapter I described the manufacture and testing of wild-type and typhoid-toxin deficient strain of *S*. Typhi for used in a controlled human infection study. If the typhoid toxin acts a virulence factor central to the pathogenesis of acute typhoid disease, the absence of the typhoid toxin should be associated with an altered disease phenotype in the challenge model. Extrapolating from animal and *in vitro* data, we hypothesized that the attack rate and/or the severity of disease in healthy participants challenged with a typhoid toxin deficient strain (TN) would be reduced compared to challenge with a wild type (WT) strain of *S*. Typhi.

In this chapter, I describe the design and set-up of a controlled human infection study that aims to test this hypothesis. I begin by discussing central aspect of study design, objectives, study power and statistical considerations. I then present the results of the controlled human infection study comparing challenge with WT and TN strains, focussing on primary endpoints along with clinical and microbiological secondary endpoints. Finally, I will discuss the implications of these data with relation to typhoid fever pathogenesis and global disease control efforts.

6.1. Methods

6.1.1. Study design

The OVG2016/03 (TYGER) study was a randomised, double-blinded, controlled human infection study comparing the response to challenge with wild-type *S*. Typhi with a typhoid-toxin deficient isogenic mutant strain *S*. Typhi SB6000. The study was designed as an outpatient challenge study, conducted in a cohort of healthy community adult volunteers in a setting non-endemic for typhoid fever.

6.1.2. Primary Objective

The primary objective of this study was to compare the proportion of participants developing clinical or microbiologically proven typhoid infection (attack rate, AR) following oral challenge with 1-5 x 10^4 CFU wild type *S.* Typhi Quailes strain (WT), in comparison to challenge with 1-5 x 10^4 CFU of a typhoid toxin-deficient isogenic mutant of *S.* Typhi Quailes strain SB6000 (TN). The null and alternate hypotheses were:

$$H_0 = AR_{WT} = AR_{TN}$$

$$H_1 = AR_{WT} \neq AR_{TN}$$

Where AR_{WT} and AR_{TN} were the proportion of participants meeting the diagnostic criteria for typhoid infection, who had been challenged with WT or TN strains respectively.

6.1.3. Secondary Objectives

The secondary objectives of the study were to compare disease severity using clinical and laboratory endpoints following challenge with either wild-type *S.* Typhi Quailes strain (WT) or typhoid toxin-deficient isogenic mutant *S.* Typhi Quailes strain SB6000 (TN), with specific reference to:

- Diagnostic endpoints and mode of diagnosis
- time to diagnosis

- time to onset of clinical symptoms and signs
- duration of illness including fever clearance time
- symptom severity and symptom profiles
- time to onset of bacteraemia
- duration of bacteraemia
- quantification of bacteraemia at time of diagnosis
- time to onset and pattern of stool shedding
- inflammatory response (e.g. C-reactive protein, leukocyte counts etc.)
- haematological and biochemical parameters (as outlined in Chapter 4)

6.1.4. Study Population

Healthy, adult volunteers who consented to challenge were eligible for enrolment as outlined in Chapter 4 and Appendix 1 - Inclusion & Exclusion Criteria for the OVG2014/01(PATCH) and OVG2016/03 (TYGER) Studies.

6.1.5. Study Procedures

Study procedures and follow up are outlined Chapter 4. Typhoid fever was diagnosed if participants met the composite diagnostic endpoint of either a positive blood culture for *S.* Typhi and/or oral temperature ≥38°C persisting for ≥12 hours. Treatment was initiated at the time of typhoid diagnosis, or at day 14 post-challenge for individuals who did not meet the diagnostic criteria. The study visit schedule and study procedures are outlined in Table 6-1. The sample collection schedule is detailed in Appendix 5.

6.1.1. Challenge Agent Preparation & Administration

Wild-type and toxin negative challenge strains were prepared, and dose calculated, as outlined in section 5.2.10 and 5.3.8. The challenge agent was administered to participants within three hours of preparation. Both agents had the appearance of a transparent, colourless liquid. Measures to maintain blinding are outlined in section 6.1.5.

6.1.2. Calculation of Challenge Dose

The challenge dose administered was calculated from aliquots of the challenge agent inoculated onto six TSA plates as described in 5.2.10 and 5.3.8.

Table 6-1 : Summary of Study Procedures OVG2016/03 (TYGER) study.

[1] Physical examination was performed at other times in the study if clinically indicated and at the discretion of the study team. [2] Stool samples were collected at least one week after completion of a 14-day course of antibiotics, until 3 successive stool samples are negative for S. Typhi. If persistent stool shedding occurs after completion of antibiotics, participants were referred to the Infectious Diseases Consultant at the Oxford University Hospitals NHS Trust. [3] Public Health England and participant GPs were notified at the time of stool shedding clearance.

	Screening	D-7	D0	D0+12hrs	D1-6	D7	D8-13	D14	D28	TD	TD+12hs	TD+24 – 96	D90 – D180	D365
Informed consent	x													
Consent quiz	x													
Revalidation of consent		x												
Obtain 24 hr contact details		x												
Medical history	x													
Interim medical history		x	x	x	x	x	x	x	x	x	x	x	x	x
Physical examination[1]	x									x				
Vital signs (HR,BP,Temp)	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Urine pregnancy test	x	x	x					x		x				
Urine sample	x		x		x			x	x	x				
Stool sample[2]			x		x	x	x	x	x	x		x	x	x
Blood sample	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Saliva sample	x	x	x					x		x				
Nasal Swab	x	x	x					x		x				
12 lead ECG	x													
Ultrasound scan	x													
Mood assessment	x	x				x		x		x				
Challenge with *S.* Typhi (WT or TN Quailes strain)			x											
Issue study pack		x												
e-Diary entries		x	x	x	x	x	x	x	x	x	x	x		
Randomisation		x												
Commence antibiotics								x		x				
Participant questionnaire										x				
Notification of Public Health England and GP[3]			x						x		x			
Letter informing close contacts			x											
Letter to close contacts offering screening									x		x			

6.1.3. Sample Size

A total of 40 participants were randomised 1:1 to receive challenge with WT or TN strains. The sample size was dictated primarily by the number of participants that could be feasibly enrolled within the time-frame and budget of the study, and therefore represents a convenience sample. Assuming that the typhoid toxin is central to the clinical presentation of acute typhoid fever, it was anticipated that the attack rate following challenge with the TN strain would be reduced compared with the WT strain, although the effect size was unknown. Assuming an attack rate of 65% following WT challenge (as observed in previous studies) and 50% attack rate following TN strain, and accounting for 10% drop out, 20 participants in each group gave 95% confidence intervals for attack rate of 41% to 85% in the WT group and 27% to 73% in TN group. The confidence intervals for observed attack rates of 0 – 70% using a sample size of 20 in each group are displayed in Table 6-2. Twenty participants per arm provided 95% power to detect an absolute reduction in attack rate of 55% (65% with WT strain versus 10% with TN strains - corresponding to an 85% relative risk reduction) and 80% power to detect an absolute reduction in attack rate of 47% (65% with S. Typhi wild type strain versus 18% with S. Typhi toxin-negative strain – corresponding to a 72% relative risk reduction) based on Fisher's Exact test with 5% alpha.

Table 6-2 : Confidence intervals for observed attack rates using sample size 20 in each group

Confidence Level	Sample Size (N)	Proportion diagnosed (P)	Lower Limit	Upper Limit	Actual Width	Width if P = 0.5
0.95	20	0	0	0.168	0.168	0.456
0.95	20	0.35	0.154	0.592	0.438	0.456
0.95	20	0.4	0.191	0.639	0.448	0.456
0.95	20	0.45	0.231	0.685	0.454	0.456
0.95	20	0.5	0.272	0.728	0.456	0.456
0.95	20	0.55	0.315	0.769	0.454	0.456
0.95	20	0.6	0.361	0.809	0.448	0.456
0.95	20	0.65	0.408	0.846	0.438	0.456
0.95	20	0.7	0.457	0.881	0.424	0.456

6.1.4. Randomisation

Participants were randomised 1:1 to challenge with either wild-type strain *Salmonella* Typhi (WT) or toxin-negative strain *Salmonella* Typhi (TN) in varying block sizes. Randomisation was stratified by anti-Vi IgG measured at screening (low [<7.4EU/mL] or high [≥7.4U/mL]). The exception was a sentinel group of two participants who were randomised 1:1 to receive the WT strain or TN knock-out strain, using a block size of two. Randomisation was performed at the pre-challenge visit, one week prior to challenge using the computerised randomisation software 'Sortition' (Department of Primary Care, University of Oxford). The software generated a randomisation number, corresponding to challenge allocation group. A locked, challenge agent randomisation allocation list was maintained by the study statistician and unblinded laboratory team responsible for challenge agent preparation.

6.1.5. Blinding

The study was conducted in a double-blind from the time of randomisation until participant unblinding, such that the participant and clinical staff undertaking follow-up visits were unaware of which challenge agent they had received. Designated laboratory staff prepared the WT and TN challenge agents on the morning of challenge. Prior to preparation of challenge agent, the unblinded laboratory team matched participant ID numbers and a randomisation number with the corresponding challenge agent allocation (WT or TN strain) detailed in a challenge agent randomisation allocation sheet.

Both WT and TN strains were prepared suspended in sodium bicarbonate and had an indistinguishable appearance (transparent, colourless liquid). Challenge strains were delivered to the clinic rooms labelled with participant identification number, participant initials and randomisation number (Figure 6-1). This information was recorded on the eCRF by blinded clinical staff prior to administration of the challenge agents.

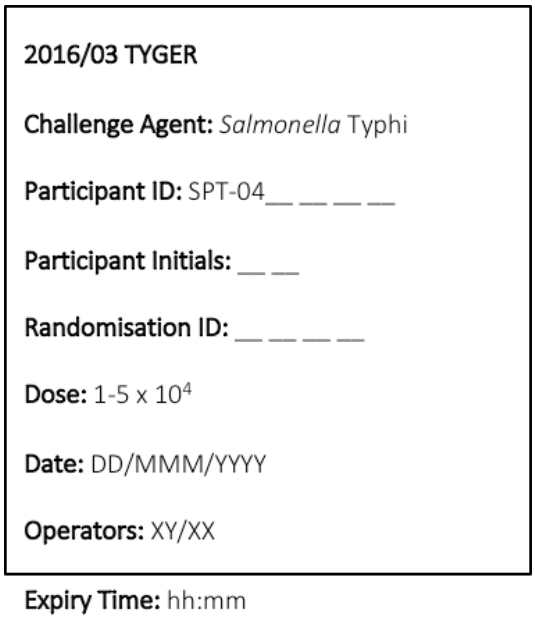


Figure 6-1 : Blinding of challenge agent administration.

Label applied to challenge agent. Unblinded staff recorded participant ID, participant initials and randomisation on label, in addition to date of preparation, time of expiry and initials of operator. Blinded staff recorded corresponding information onto eCRF at time of administration

6.1.6. Unblinding

Participants were unblinded when the final participant reached their Day 28 post-challenge visit. Investigators were blinded for laboratory assays. Participants were informed of the challenge agent they have received at the time of unblinding. A letter was also sent to the participant's GP notifying them of unblinding and challenge agent assignment. Unblinding could occur during the course of the study in the event of participant withdrawal, or the occurrence of SAEs, SARs or SUSARs, conducted under the guidance of the Data Safety and Monitoring Committee.

6.1.7. Anti-Vi IgG ELISA

IgG responses to the Vi-antigen were measured using a commercial ELISA kit (VaccZyme, The Binding Site Ltd, Birmingham, UK) according to the manufacturer's instructions, as detailed in section 8.2.1.[209].

6.1.8. Ex-Vivo ELISPOT

Ex-vivo antibody secreting cell (ASC) responses to *S*. Typhi O9:LPS and H-d were at baseline, 7 days after challenge and at the time of enteric fever diagnosis using an ELISOT assay as previously described [314]. Assay details are provided in section 8.2.2.

6.1.9. Analysis

6.1.9.1. Statistical considerations

The attack rate was defined as the proportion of participants with a diagnosis of typhoid fever, where the numerator was taken as the number of participants who meet the criteria for diagnosis and the denominator included all participants with the exception of those who withdrew or were treated prior to Day 14 without prior diagnosis of typhoid. Results are presented as percentages with accompanying 95% binomial exact (Clopper-Pearson) confidence intervals. Difference in proportions between the wild-type challenge and typhoid-toxin negative challenge control groups were analysed using Fisher's Exact Test and are also presented as a relative risk (95% CI) of typhoid diagnosis.

$$RR = AR_{SB6000}/AR_{Quailes}$$

Time-to-event analyses of individual components of typhoid diagnosis (e.g. time to diagnosis, time to bacteraemia, time to temperature ≥38.0°C, fever clearance time etc.) were conducted using the Kaplan-Meier method and included all participants. Participants were censored in the analysis at the time of diagnosis or at final monitoring for those undiagnosed (i.e. Day 14). The time variable in time-to-diagnosis analyses was taken as the time at which a blood culture positive blood sample was collected/time first temperature ≥38.0°C was recorded rather than the time at which it was recognised to be positive.

6.1.10. Symptom Severity Score

Solicited symptom scores were calculated by scoring symptoms as 0 for none, 1 for mild, 2 for moderate, 3 for severe, and 4 for life-threatening/hospitalisation, for each symptom and for each day of recording (Day 1 – 21). These were summed to provide a cumulative score for all symptoms or a cumulative score for individual symptom components.

6.1.11. Principal component analysis

Solicited symptoms were scored by individual variables according to the scheme outlined in 6.1.10. Laboratory parameters were assigned a numerical score from 0 to 4 corresponding to the severity grade as outlined in chapter 4 and summed for all timepoints measured. Similarly, vital signs were assigned a numerical score from 0 to 3 according to severity grading outlined in chapter 4 and summed for all timepoints measures. Other variables included in the analysis were fever clearance time, time to fever, time to bacteraemia, duration of bacteraemia and quantitative blood culture. Participants not meeting the criteria for diagnosis were assigned a value of 0 for these variables. Variables were normalised by Z-score prior to inclusion in the principal component analysis. The principal component analysis was performed using the FactoMineR package [350] with R version 3.4.4.

6.1.1. Correlation Matrix

The correlation matrix was created using the Hmisc package[351] with R version 3.4.4. Correlation coefficients were calculated using the Spearman correlation method for non-parametric variables.

6.1.2. Funding – OVG2016/03

The OVG2016/03 (TYGER) study was funded by the Bill & Melinda Gates foundation (OPP1126235). The study funders had no role in study design, data collection or analysis.

6.1.3. Study Sponsorship & Approvals - OVG2016/03

The OVG2016/03 (TYGER) study was sponsored by the University of Oxford (Clinical Trials & Research Governance). Ethical approvals for the primary protocol, and any study amendments, were obtained from the South-Central Oxford A research ethics committee (16/SC/0358). Approvals for deliberate release of the genetically modified strain of S. Typhi were obtained from the Minister for State for Agriculture, Food and Fisheries at the UK Department for the Environment, Food & Rural Affairs (16/R48/01).

6.1.4. Study registration

The OVG2016/03 (TYGER) study was registered with clinicaltrials.gov, number NCT03067961[352].

6.1.5. Safety monitoring

An independent data safety monitoring committee (DSMC) provided oversight of the study safety and conduct. The DSMC were provided with planned safety updates after the sentinel cohort of two participants had completed challenge, after the next 10 participants had been challenged and when all participants reached Day 28 post-challenge. Any serious adverse events and adverse events of special interest were reported to the DSMC.

6.2. Results

6.2.1. Recruitment

Participants were enrolled between the 10th April 2017 and 1st August 2017. Consistent with previous typhoid and paratyphoid CHIM, approximately half (54%) of all of participants who

attended a screening visit were enrolled for challenge (Figure 6-2). A total of 41 participants were enrolled into the study and randomized to challenge with either strain variant, of whom 40 completed challenge and were included in the final analysis. One participant randomized to challenge with wild-type *S.* Typhi subsequently declined to participate in the study. As this participant withdrew prior to challenge, they were excluded from all further analyses (Figure 6-2). Study groups were well matched at baseline (Table 6-3). A slightly higher proportion of participants randomized to challenge with the knock-out strain had detectable anti-Vi IgG at baseline (11/19 [58%] vs 10/21 [48%]; p=0.54), with no significant between-group differences in baseline anti-Vi IgG titers (Figure 6-3).

Table 6-3 : Baseline participant characteristics OVG2016/03 Study.

[1] - Participants with prolonged travel or residence in an enteric fever endemic country were ineligible for enrolment. [2-] Detectable anti-Vi IgG defined as any titre >7.4 EU/ml.

	Challenge Group		
	All	*S.* Typhi Quailes (Wild-Type)	*S.* Typhi SB6000 (Toxin Negative)
Number	40	21	19
Male sex, n (%)	24 (59%)	13 (62%)	11 (58%)
Age, Years, median (range)	29.6 (19.4-51.6)	27.8 (20.7 – 51.6)	30.6 (19.4 – 44.2)
Ethnicity, n (%)			
White British	30/40 (75%)	15/21 (71%)	15/19 (79%)
White (Other)	6/40 (15%)	4/21 (19%)	2/19 (11%)
Asian (Chinese)	1/40 (2.5%)	0/21 (0%)	1/19 (5%)
Black (Other)	1/40 (2.5%)	1/21 (5%)	0/19 (0%)
Other ethnic group	1/40 (2.5%)	0/21 (0%)	1/19 (5%)
Mixed	1/40 (2.5%)	1/21 (5%)	0/19 (0%)
Previous travel to enteric fever endemic area <6 months, n (%)[1]	17/40 (43%)	10/21 (48%)	7/19 (37%)
Alcohol consumption, any, n (%)	27/40 (64%)	15/21 (71%)	12/19 (63%)
Tobacco smoker, any, n (%)	7/40 (18%)	4/21 (19%)	3/19 (16%)

Detectable anti-Vi IgG at screening[2]	21/40 (53%)	10/21 (48%)	11/19 (58%)

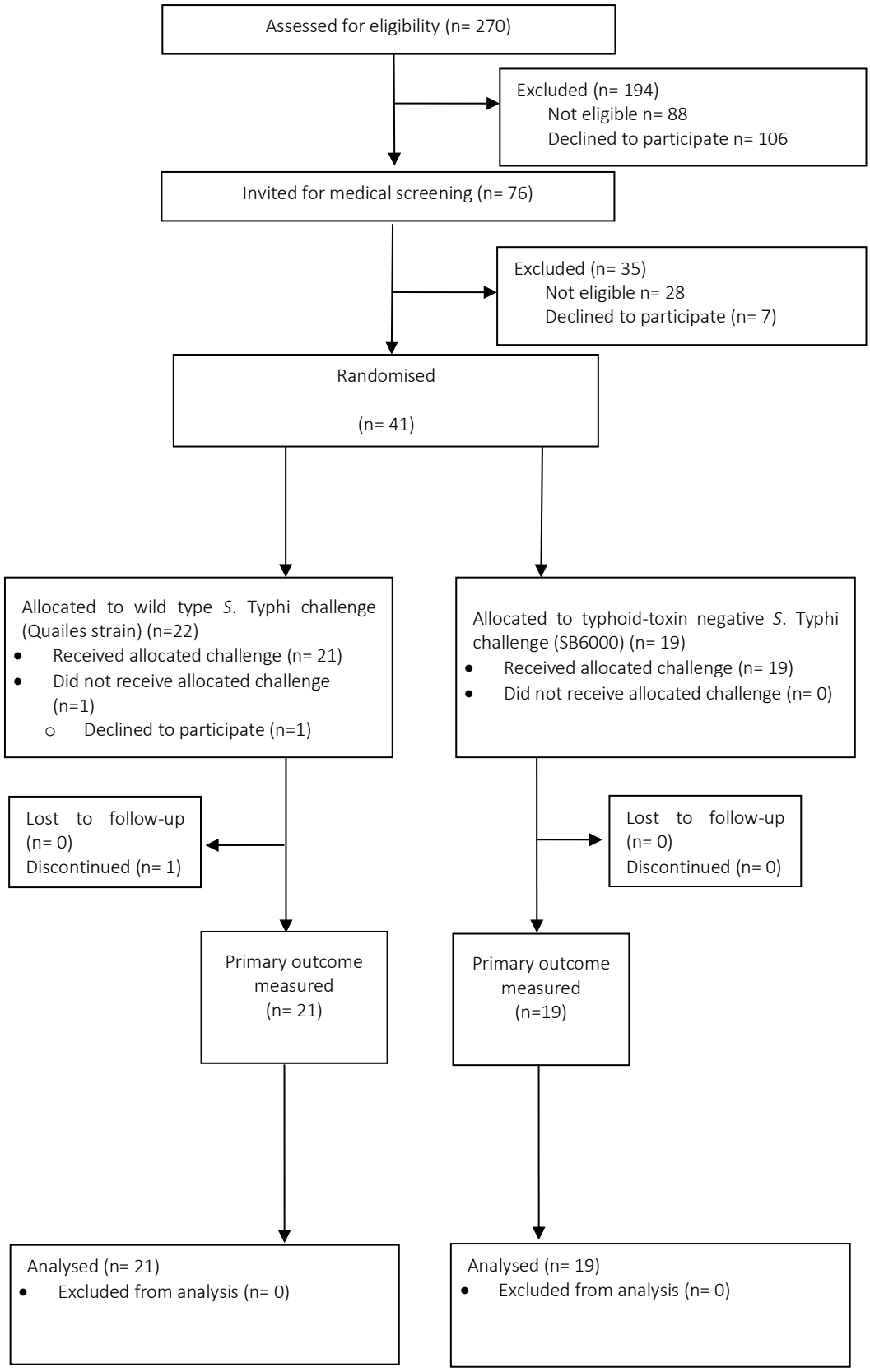


Figure 6-2 : OVG2016/01 Trial profile

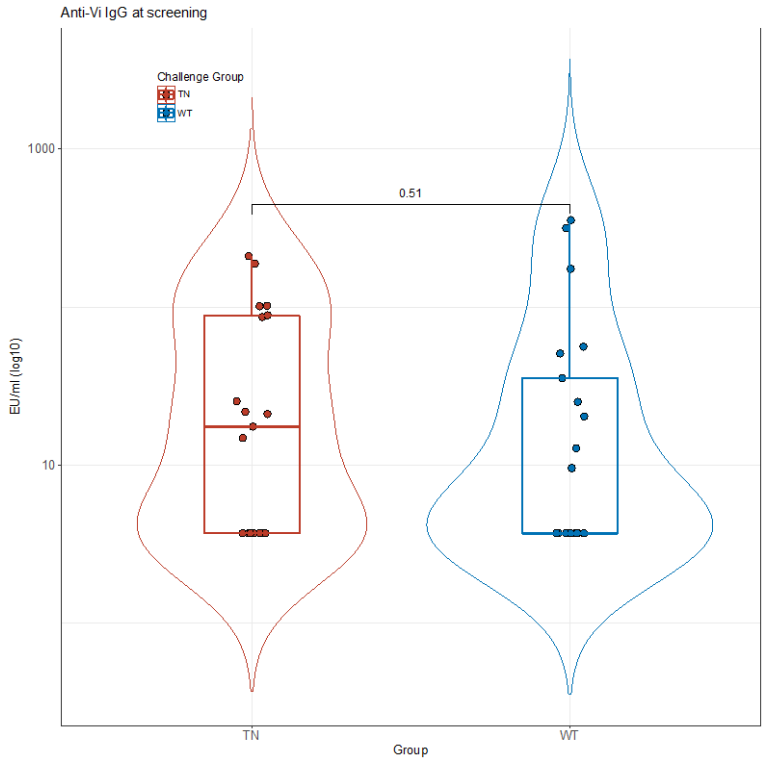


Figure 6-3 : Baseline anti-Vi IgG OVG2016/03 study.

TN = Typhoid toxin deficient S. Typhi SB6000 strain; WT = Wild-type S. Typhi Quailes strain. p = Mann-Whitney U test. Box plots represent median, interquartile range. Overlaid violin plots illustrate distribution of the data points and their probability density. Samples below the lower limit of detection of the ELISA (7.4 EU/ml) were assigned an arbitrary value corresponding to half the lower limit of detection (3.7 EU/ml)[209].

6.2.2. Challenge dose

All participants were challenged with the challenge strain to which they were randomised. No episodes of unplanned or emergency unblinding occurred. All participants were challenged within the target dose range of 1-5 x 10^3 CFU and within 3 hours of challenge agent preparation from frozen stocks. The average dose administered was higher in participants challenged with wild-type *S.* Typhi (Median dose [IQR]; 2.55 x 10^4 CFU [2.36-2.8] vs 2.50 x 10^4 CFU [2.14-2.61); p=0.02), albeit within the target dose range. There was no significant difference in the dose administered and the outcome of challenge Figure 6-4.

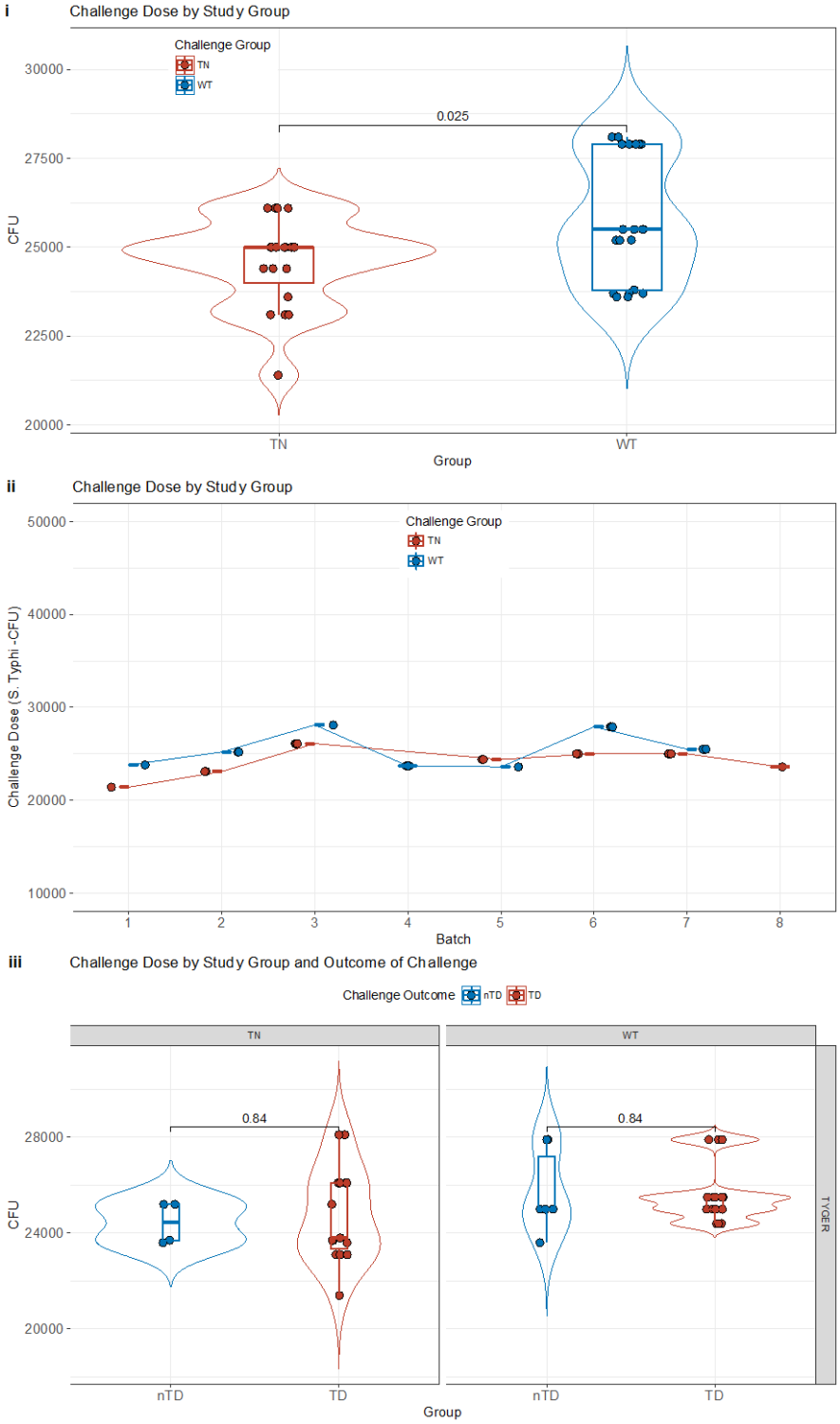


Figure 6-4 : Challenge dose administered.

TN = Typhoid toxin deficient S. Typhi SB6000 strain; WT = Wild-type S. Typhi Quailes strain.(i) Comparison of actual challenge dose administered between wild-type and toxin-negative challenge groups. (ii) Actual challenge dose administered by batch. (iii) Comparison of challenge dose administered according to outcome and challenge strain. TD = Typhoid diagnosis; nTD = No typhoid diagnosis. p = Mann-Whitney U test. Box plots represent median, interquartile range. Overlaid violin plots illustrate distribution of the data points and their probability density.

6.2.3. Primary endpoint – Attack rate according to challenge group

Using the primary composite diagnostic endpoint (*S*. Typhi bacteraemia and/or fever ≥38C° persisting ≥ 12hrs), there was no significant difference in the rate of typhoid disease between participants challenged with wild-type or typhoid-toxin deficient *S*. Typhi (15/21 [71%] vs. 15/19 [79%]; relative risk 1.11 [95%CI 0.8 – 1.6]; p = 0.58 - Table 6-4). Participants challenged with typhoid-toxin deficient *S*. Typhi developed typhoid fever approximately 2 days earlier than participants challenged with wild-type *S*. Typhi (median [IQR] days to diagnosis 5.25 [5.01 – 6.14] vs. 7.05 [5.08 – 8.83]; p =0.23 - Figure 6-5).

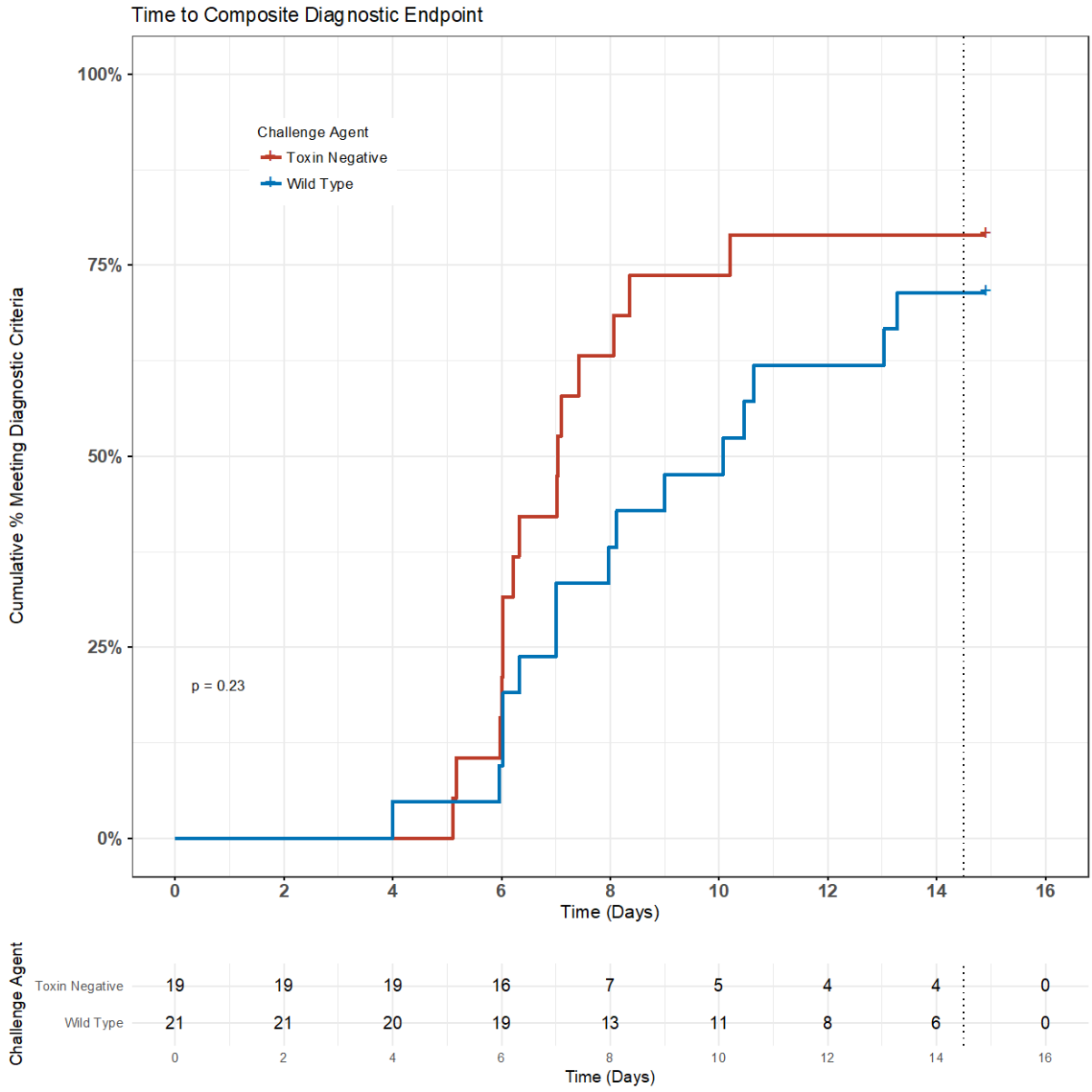


Figure 6-5 : Time to diagnosis after challenge with wild-type and typhoid-toxin deficient *S.* Typhi.

Composite diagnostic endpoint defined as *S.* Typhi bacteraemia and/or fever ≥38C° persisting ≥ 12hrs. Participants who did not meet the diagnostic criteria for typhoid diagnosis were censored at day 14. Shaded areas represent 95% confidence intervals for survival curves. p = log rank test.

Table 6-4 : Primary and secondary outcomes after challenge with wild-type *S.* Typhi Quailes strain and *S.* Typhi SB6000 typhoid-toxin deficient strain.

Composite diagnostic endpoint = *S*. Typhi bacteraemia from blood culture sample collected ≥72 hours from challenge and/or temperature≥38°C lasting ≥12 hours. Time to diagnosis defined as time from date/time of challenge to date/time of first temperature ≥38°C that subsequently lasted for ≥12hours OR the date/time of first positive blood culture collection (whichever occurs earliest). CFU = Colony forming units. *P values were derived using Fishers exact test for categorical variables, the log rank test for time-to-event data and the Mann-Whitney U test for continues variables.

	Challenge Group		RR (95% CI)	P*
	S. Typhi Quailes (Wild-Type) (n= 21)	*S.* Typhi SB6000 (Toxin Negative) (n=19)		
Primary Outcome				
Typhoid Fever Diagnosis, n (% attack rate – Composite endpoint)	15/21 (71%)	15/19 (79%)	1.11 (0.8 - 1.6)	0.58
Attack rate 95% Confidence Interval, %	41-89%	54-94%	-	-
Primary Clinical Diagnosis, n (% diagnosed)	2/15 (13%)	0/15 (0%)	0 (0-1.7)	0.48
Primary Microbiological Diagnosis, n (% diagnosed)	13/15(87%)	15/15 (100%)	0.8 (0.5-1.0)	0.22
Detailed Mode of Diagnosis				
S. Typhi bacteraemia without Fever ≥38°C, n (% diagnosed)	5/15(33%)	6/15(33%)	0.83 (0.3-2.1)	0.9
S. Typhi bacteraemia preceding Fever ≥38°C, n (% diagnosed)	8/15 (53%)	9/15(53%)	0.89 (0.5-1.7)	0.9
Fever ≥38°C for ≥12hrs without *S.* Typhi bacteraemia, n (% diagnosed)	1/15 (7%)	0/15 (0%)	0 (0-3.6)	0.9
Fever ≥38°C preceding *S.* Typhi bacteraemia, n (% diagnosed)	1/15 (7%)	0/15 (0%)	0 (0-3.6)	0.9
Time to diagnosis, Days, median (IQR)	7.05 (5.08 - 8.31)	5.25 (5.01-6.14)	-	0.23
Severe Enteric Fever (Any), n	1/15(7%)	4/15(27%)	0.25 (0.04 - 1.4)	0.32
Systolic blood pressure ≤ 85mmHg	0/15 (0%)	0/15 (0%)	-	-
Oral Temperature ≥40°C	0/15 (0%)	1/15 (7%)	0 (0-3.6)	0.9
Significant lethargy or confusion	0/15 (0%)	0/15 (0%)	-	-
Gastrointestinal bleeding and/or perforation	0/15 (0%)	0/15 (0%)	-	-
Grade 4 laboratory abnormality	1/15 (7%)	3/15 (20%)	0.33 (0.05 - 2.1)	0.33

Clinical Endpoints				
Any temperature ≥38°C n, (% diagnosed)	10/15 (67%)	9/15 (60%)	1.11 (0.6 - 2.0)	0.9
Time to first temperature ≥38°C , Days, Median (IQR)	6.43 (3.47 - 13.6)	6.03 (4.54 - 7.98)	-	0.77
Fever Clearance Time, Hrs, Median (IQR)	53.15 (23.0 - 87.4)	44.92 (12 - 96.6)	-	0.71
Microbiology - Blood culture				
Any *S.* Typhi bacteraemia, n (% diagnosed)	14/15 (93%)	15/15 (100%)	0.93 (0.7 - 1.1)	0.9
Time to *S.* Typhi bacteraemia, Days, Median (Range)	6.59 (4.16 - 12.7)	5.25 (3.93 - 9.10)		0.13
Overall duration of *S.* Typhi bacteraemia, Days, Median (range)	30.3 (3.6 - 49.4)	47.6 (28.9 - 97.0)	-	<0.001*
S. Typhi bacteraemia ≥ 96 hours, n (% diagnosed)	0/15 (0%)	1/15 (7%)	0 (0-3.6)	0.9
Quantitative Blood Culture (CFU/ml), Median (range)	0.20 (0 - 21)	0.55 (0 - 3)	-	0.44
Microbiology - Stool culture				
Any *S.* Typhi stool shedding, n (% total)				
All	13/21 (62%)	11/19 (58%)	1.07 (0.6 - 1.8)	0.9
Typhoid fever diagnosed (TD)	9/15 (60%)	9/15 (60%)	1 (0.5 - 1.8)	0.9
Typhoid fever NOT diagnosed (nTD)	4/6 (67%)	2/4 (50%)	1.33 (0.4 - 4.8)	0.9

6.2.4. Mode of diagnosis

The majority of participants in the wild-type (13/15; 87%) and toxin-negative (15/15; 100%) challenge groups were diagnosed based upon microbiological criteria. A breakdown of detailed mode of diagnosis is shown in Table 6-4. In participants challenged with typhoid-toxin deficient *S*. Typhi, the diagnosis of typhoid fever was confirmed by a positive blood culture for *S*. Typhi in 15/15 (100%) of participants who met the composite diagnostic criteria. One participant challenged with wild-type *S*. Typhi (1/15; 7%) met the clinical criteria for typhoid diagnosis, without culture confirmation (Table 6-4).

6.2.5. Alternative diagnostic criteria

We compared attack rates between wild-type and toxin-negative groups using a series of different disease definitions, including an endpoint that approximates the presentation of typhoid in a field setting (fever ≥38^{o}C preceding bacteraemia). Regardless of the disease definition that was applied, we observed no significant difference in the attack rate of typhoid fever between wild-type and toxin-negative groups (Table 6-5).

Table 6-5 : Attack rates according to alternative diagnostic criteria.

After challenge with wild-type S. Typhi Quailes strain and S. Typhi SB6000 typhoid-toxin deficient strain.

Diagnostic Criteria	*S.* Typhi Quailes (Wild-type) (n=21)	*S.* Typhi SB6000 (Toxin negative) (n=19)	RR (95% CI)	p
Fever ≥37·5˚C (any duration)	14/21 (67%)	13/19 (68%)	0.96 (0.5-1.9)	0.9
Fever ≥38˚C (any duration)	10/21 (48%)	9/19 (48%)	1.01 (0.5-1.8)	0.9
Fever ≥37·5˚C (any duration) + *S.* Typhi bacteraemia	12/21 (63%)	13/19 (57%)	0.8 (0.4-1.5)	0.46
Fever ≥38·0˚C (any duration) + *S.* Typhi bacteraemia	9/21 (45%)	9/19 (43%)	0.92 (0.5-1.7)	0.9
Fever ≥38·0˚C (any duration) with subsequent bacteraemia	7/21 (35%)	7/19 (33%)	0.93 (0.5-1.7)	0.9
S. Typhi bacteraemia or stool shedding	19/21 (90%)	17/19 (89%)	1.05 (0.5 – 3.6)	0.9

6.2.6. Safety endpoints

No participants required hospitalisation during the challenge period and the symptoms experienced were in-keeping with those expected for typhoid fever (see 6.3.2).

Two serious adverse events were reported in the course of the study. One participant allocated to wild-type challenge experienced a self-limiting generalised tonic-clonic seizure seven months after completion of challenge. On further questioning, he reported a history of seizures in childhood that was not disclosed at screening. A second participant (allocated to WT challenge) attended the emergency department following an episode of deliberate self-harm 6 months after completion of challenge, attributed to an acute life stressor. No specific treatment was administered. Given the interval between these episodes and challenge, neither case was considered related to *S.* Typhi challenge or study procedures.

6.3. Clinical Endpoints

6.3.1. Severe enteric fever

Five participants met the pre-specified criteria for severe typhoid fever, of whom one participant was randomised to wild-type challenge (1/15; 7%) and four (4/15; 27%) were randomised to the typhoid toxin knock-out challenge. In the wild-type challenge group, severe typhoid was diagnosed based upon a grade 4 laboratory abnormality (serum K^+ 2.8mmo/l at 48 hours post typhoid diagnosis). In the typhoid toxin negative challenge group, severe typhoid was diagnosed based upon an oral temperature of 40.1°C 24 hours post typhoid diagnosis in one case and grade 4 laboratory abnormalities in three cases (Serum K^+ 2.8mmo/l at 48 hours post typhoid diagnosis, C-reactive protein 214mg/l 48 hours post typhoid diagnosis and C-reactive protein 375mg/l 48 hours post typhoid diagnosis.

6.3.2. Symptom profiles

In participants diagnosed with typhoid fever, the most common solicited symptoms reported by participants were headache (30/30; 100%), malaise (30/30; 100%), anorexia (26/30; 87%) and abdominal pain (23/30; 77%). The profile of symptoms and self-reported severity was comparable between participants challenged with wild-type or toxin-negative *S*. Typhi (Figure 6-6). The severity of disease was also assessed using a severity score of solicited symptoms, as previously described [311]. Using the symptom severity score, there was no significant difference in the clinical severity of typhoid disease between individuals challenged with wild-type or toxin-negative *S.* Typhi (Figure 6-7).

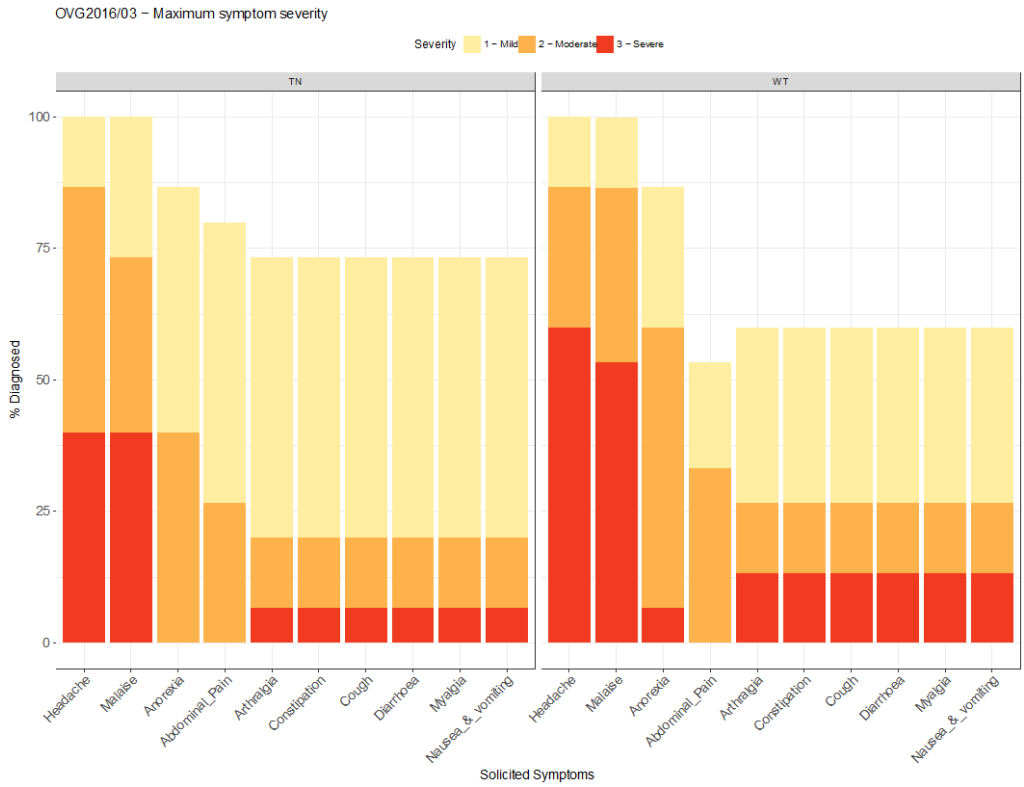


Figure 6-6 : Maximum symptom severity score in participants diagnosed with typhoid fever

Categorised according to study group. TN = Typhoid toxin deficient *S*. Typhi SB6000 strain; WT = Wild-type *S*. Typhi Quailes strain. Percentage of participants reporting one or more events. Symptoms were recorded using an electronic diary from Day 0 up to Day 21 post challenge. Stacked columns display percentage of participants reporting maximum symptom severity graded as mild (present but no interference with daily activity), moderate (some limitation of daily activity) or severe (unable to perform normal daily activity).

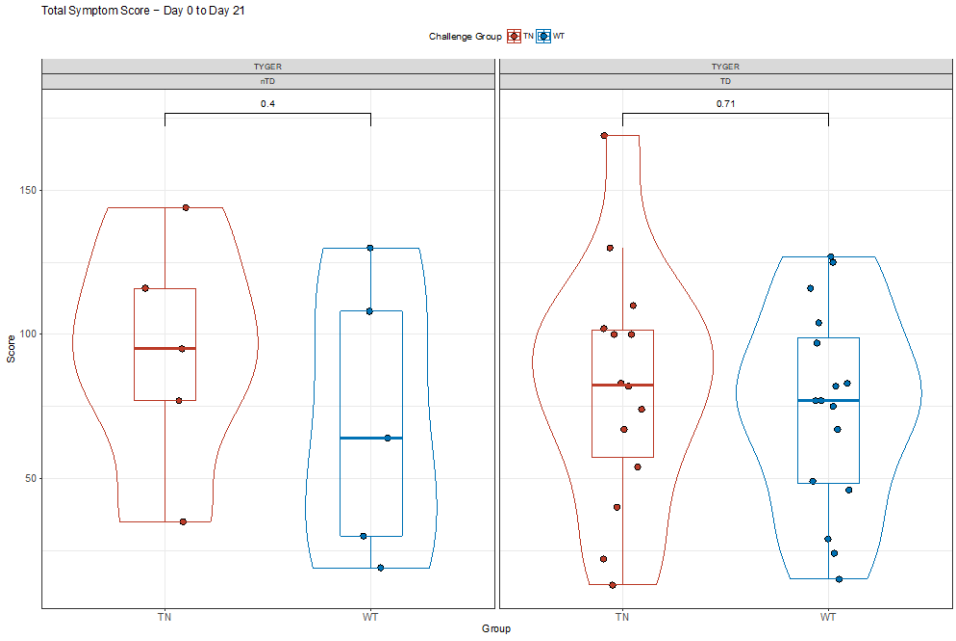


Figure 6-7 : Comparison of cumulative symptom severity scores

Categorised according to challenge outcome and study group. Symptom scores were calculated by summing numerical values assigned to the severity of individual symptoms between day 0 and day 21 (0, not present; 1, mild; 2, moderate; 3, severe).Total symptom score TN = Typhoid toxin deficient S. Typhi SB6000 strain; WT = Wild-type S. Typhi Quailes strain. TD = Typhoid diagnosis; nTD = No typhoid diagnosis. p = Mann-Whitney U test

6.3.3. Fever clearance time

Fever clearance times were compared between participants diagnosed with typhoid fever following challenge with wild-type or toxin-negative strains of *S.* Typhi. Approximately two-thirds of participants reported any fever ≥38°C during the challenge period (Table 6-3) and the time to first recorded ≥38°C fever was comparable between challenge groups (Figure 6-8). All participants diagnosed with typhoid fever were treated with ciprofloxacin 500mg twice daily. The time to fever clearance was comparable between the wild-type and knock-out groups (median FCT 53.15[23.0 - 87.4] vs. 44.92 [12 - 96.6] hours; p = 0.71 - Figure 6-9).

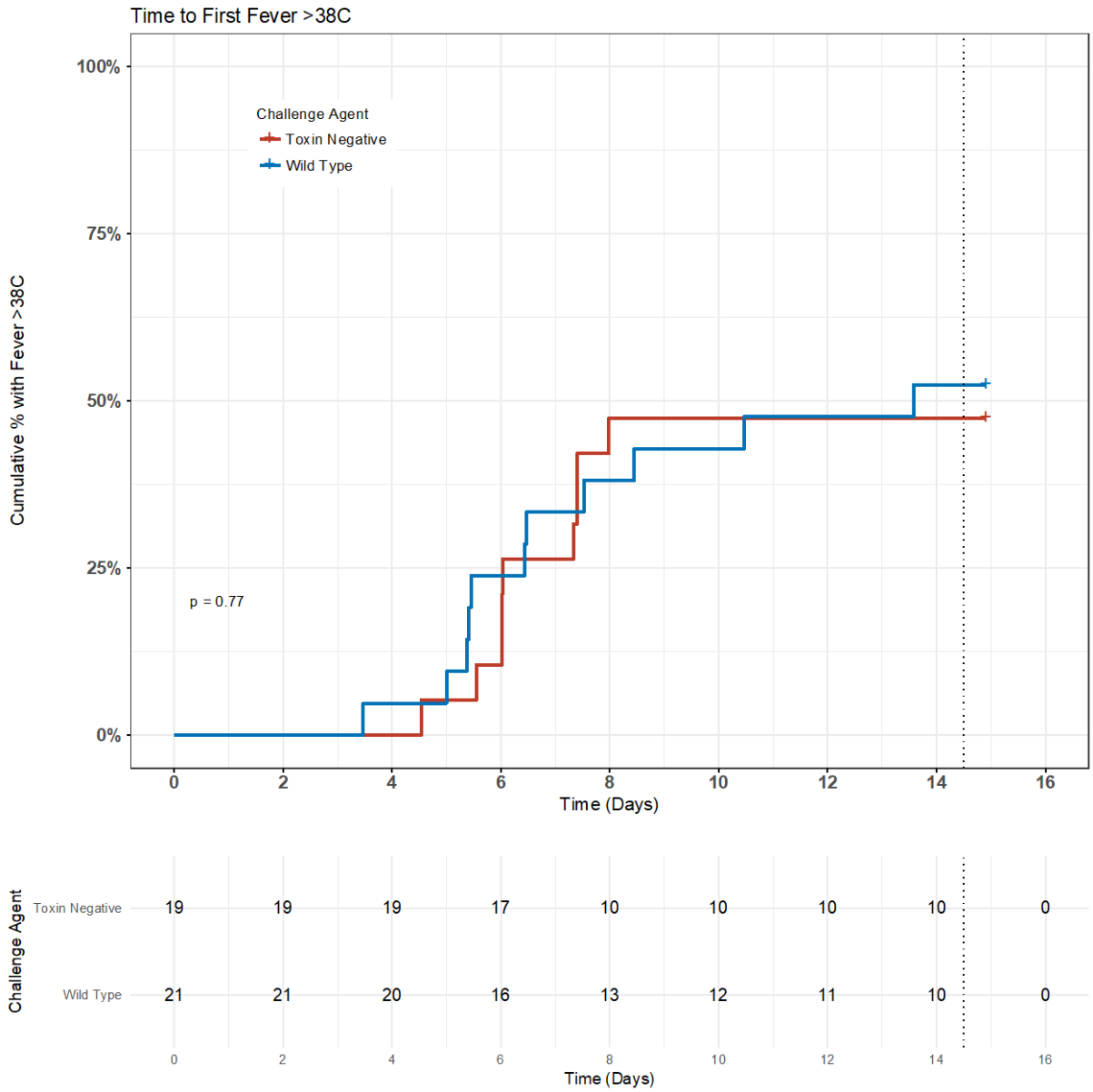


Figure 6-8 : Time to first fever >38°C categorised according to study group.

Kaplan-Meier survival curve showing the cumulative proportion of participants with any fever >38°C by challenge group. Oral temperature measurements were recorded twice daily (morning and evening) Participants with no recorded fever were censored at the day of last monitoring (day 14). Shaded areas represent 95% confidence intervals for individual survival curves. p = log rank test.

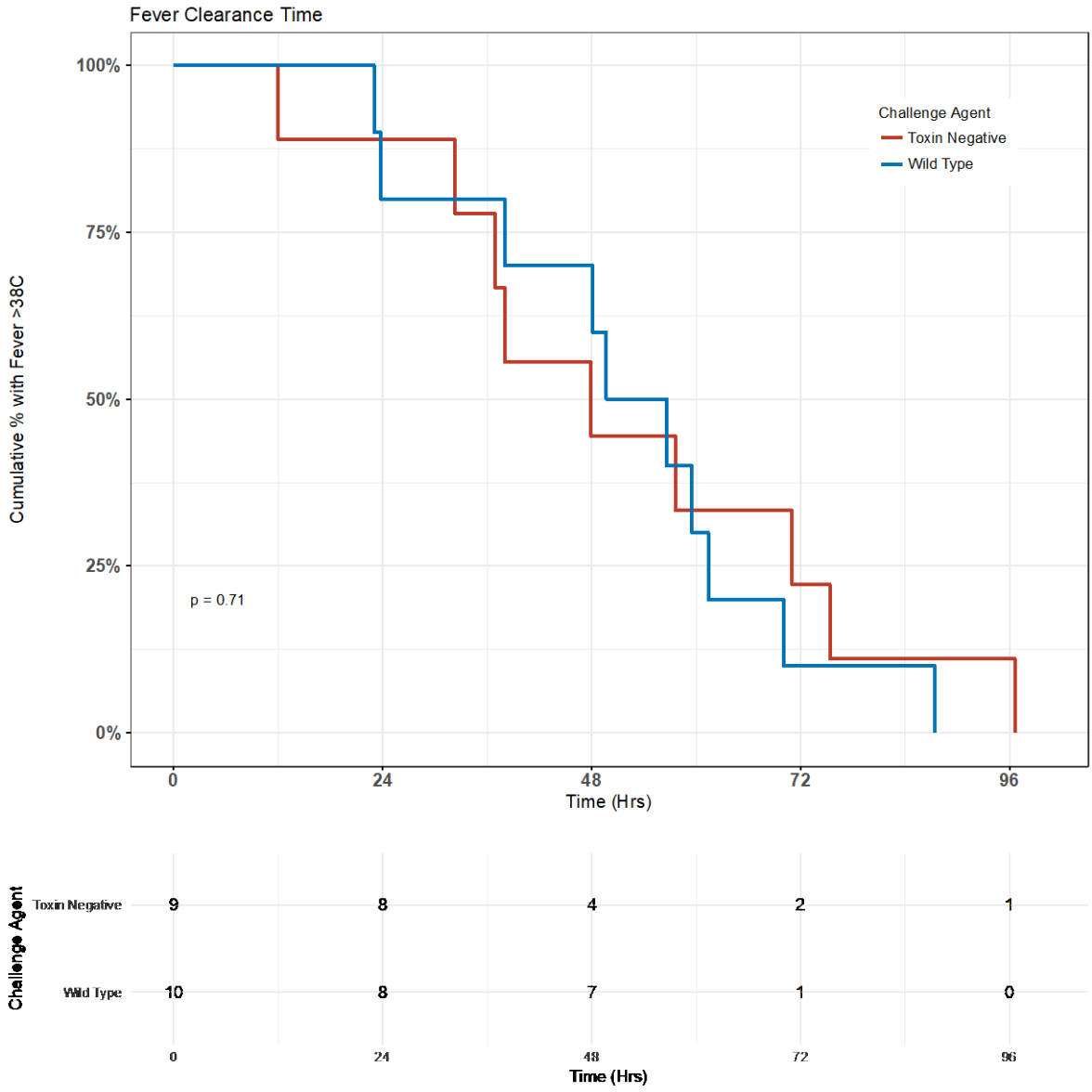


Figure 6-9 : Fever clearance time in participants diagnosed with typhoid fever

Categorised according to study group. Kaplan-Meier survival curve showing the cumulative proportion of participants with fever at the time of treatment initiation remaining febrile by challenge group. Only participants febrile at the time of treatment initiation were included. Shaded areas represent 95% confidence intervals for individual survival curves. p=log rank test.

6.4. Microbiological endpoints

6.4.1. *Salmonella* Typhi Bacteraemia

The pattern of bacteraemia following challenge with wild-type or typhoid-toxin deficient S. Typhi is illustrated in (Figure 6-10). As the majority of participants were diagnosed based upon microbiological criteria, the time to bacteraemia mirrored the time to composite diagnosis and was shorter in participants challenged with the knock-out strain (Figure 6-11). Despite all patients receiving an identical treatment regimen (ciprofloxacin 500mg twice daily), the duration of bacteraemia was significantly longer in participants challenged with the typhoid-toxin deficient strain compared with the wild-type strain (47.6 hrs [28.9 - 97.0] vs. 30.3 [3.6 - 49.4]; p=<0.001 - Figure 6-12).

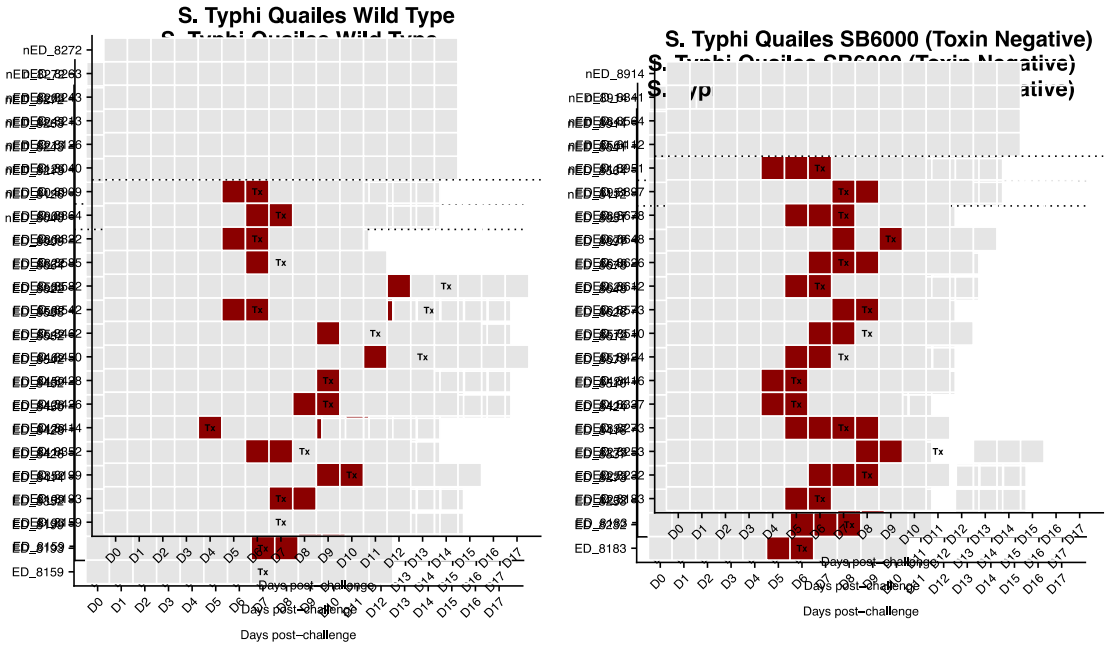


Figure 6-10 - Pattern of bacteraemia after wild type or toxin-negative *S.* Typhi challenge.

Each row corresponds to an individual participant. Grey squares = Negative sample, Red squares = Positive blood culture, White squares = No sample collected. Tx = Day of treatment initiation. Participants above the dotted line did not meet the composite criteria for typhoid diagnosis.

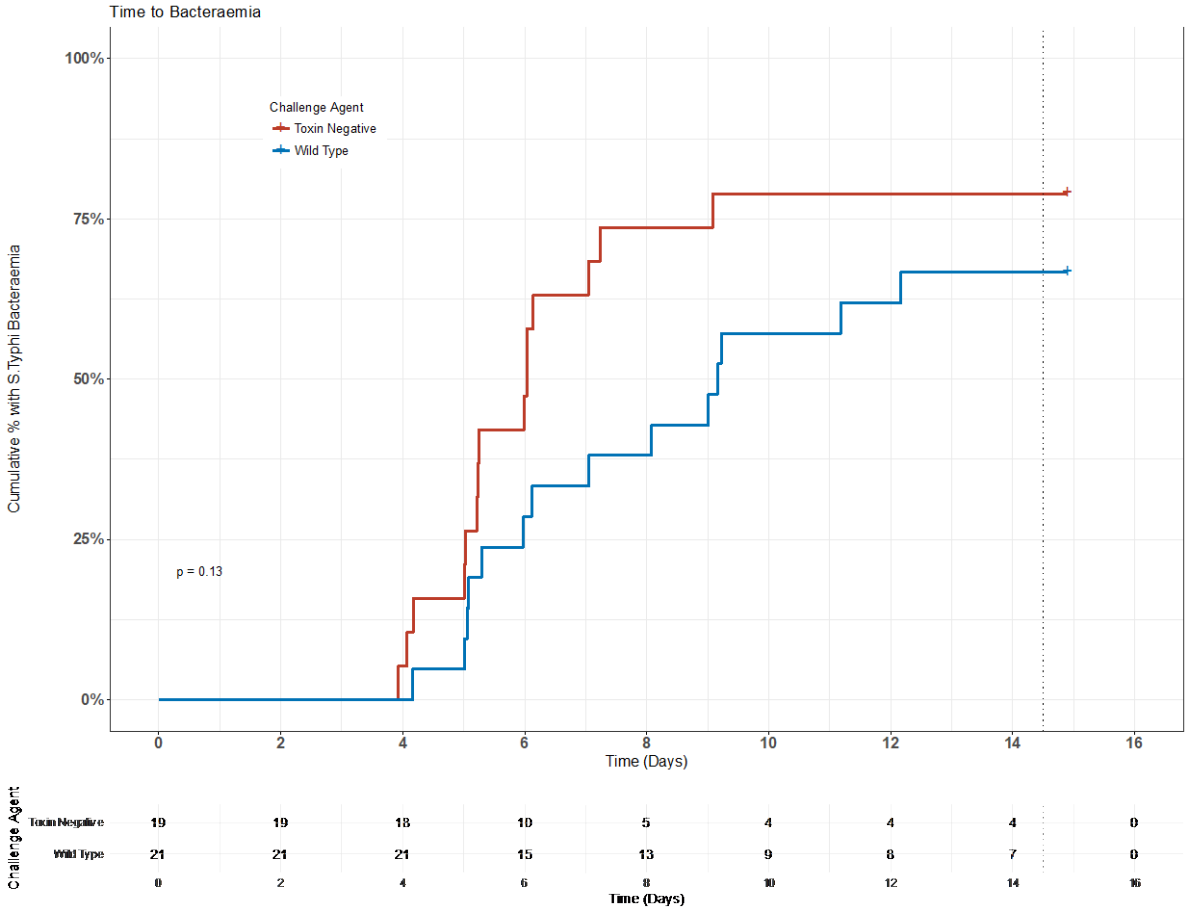


Figure 6-11 : Time to first detected *S.* Typhi bacteraemia after challenge

Categorised according to study group. Kaplan-Meier survival curve showing the cumulative proportion of participants with bacteraemia after challenge. Participants who did not meet the diagnostic criteria for typhoid diagnosis were censored at day 14. p = log rank test.

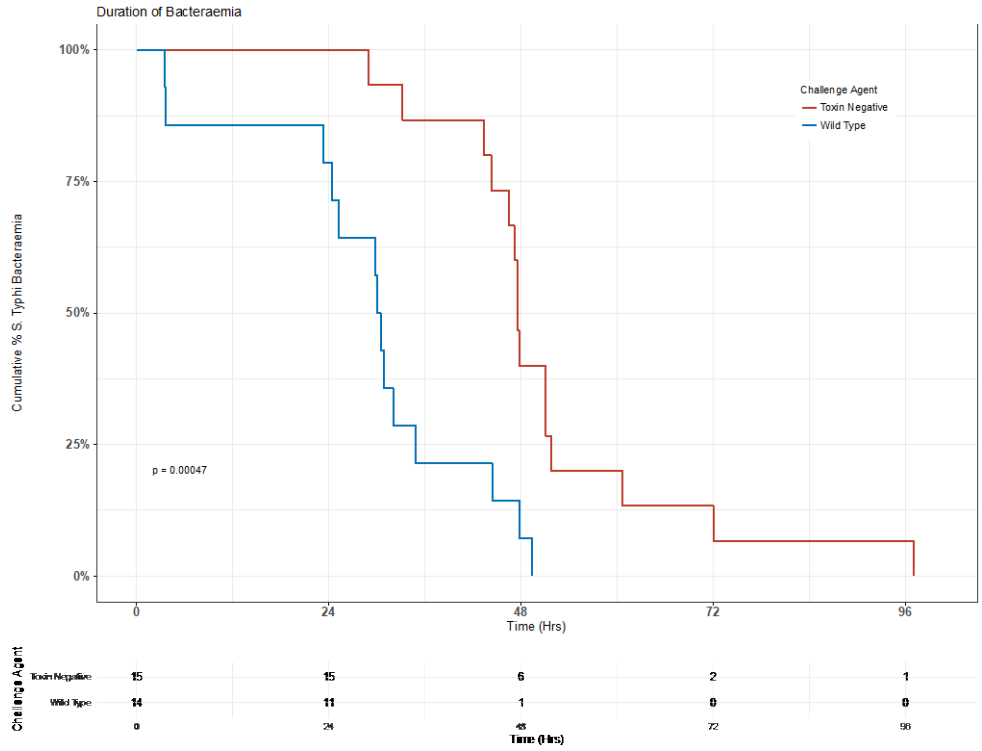


Figure 6-12 : Duration of bacteraemia following challenge with wild-type and typhoid-toxin negative *S.* Typhi.

Kaplan-Meier survival curve showing the cumulative proportion of participants with ongoing bacteraemia measured from time of treatment initiation to first persistently negative blood culture, categorised according to challenge group.P = log rank test.

6.4.2. Quantitative Blood culture

To assess the impact of typhoid toxin on the number of circulating *S.* Typhi bacteria during acute typhoid disease, we compared quantitative colony counts at the time of typhoid diagnosis in individuals challenged with wild-type or the typhoid-toxin knock-out strains, using as quantitative blood culture system as previously described[209,314]. The number of circulating bacteria during acute typhoid disease is reduced by typhoid vaccination and has been used a surrogate for disease severity in previous typhoid controlled human infection studies[209,314]. Consistent with data from field studies and previous typhoid CHIM, quantitative blood culture counts during acute typhoid disease were low (≤ 1CFU/ml)[230]. We observed no significant difference in circulating

colony counts between individuals challenged with wild-type or typhoid-toxin negative *S.* Typhi (0.55 CFU/ml [0-3] vs. 0.2 CFU/ml [0-21]; p = 0.44 - Figure 6-13 Table 6-4).

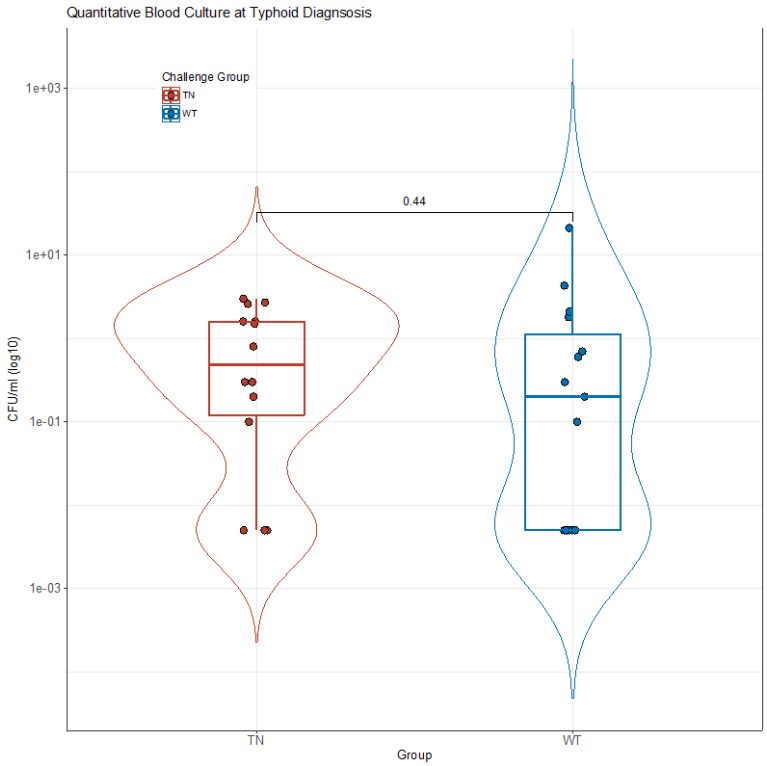


Figure 6-13 : Quantitative blood culture at time of typhoid diagnosis.

TN = Typhoid toxin deficient *S.* Typhi SB6000 strain; WT = Wild-type *S.* Typhi Quailes strain. p = Mann-Whitney U test. Box plots represent median, interquartile range. Overlaid violin plots illustrate distribution of the data points and their probability density. Samples with no colonies were assigned an arbitrary value corresponding to half the lower limit of detection (0.05 CFU/ml).

6.4.3. Stool Shedding

The pattern of stool shedding is illustrated in Figure 6-14. We isolated at least one positive stool culture of S. Typhi from 13/21 (62%) of participants challenged with wild-type S. Typhi and 11/19 (58%) challenged with the knock-out strain. The pattern of stool shedding was comparable between the two challenge strains, characterised by an early peak 24-48 hours after challenge, followed by a second peak in week 2 as participants are diagnosed with typhoid Figure 6-15. There was no difference in the probability of shedding over the entire challenge period following

challenge with in the typhoid toxin negative strain of *S*. Typhi compared with wild-type (Odds Ratio 0.638, 95%CI 0.165 - 2.465; p = 0.51).

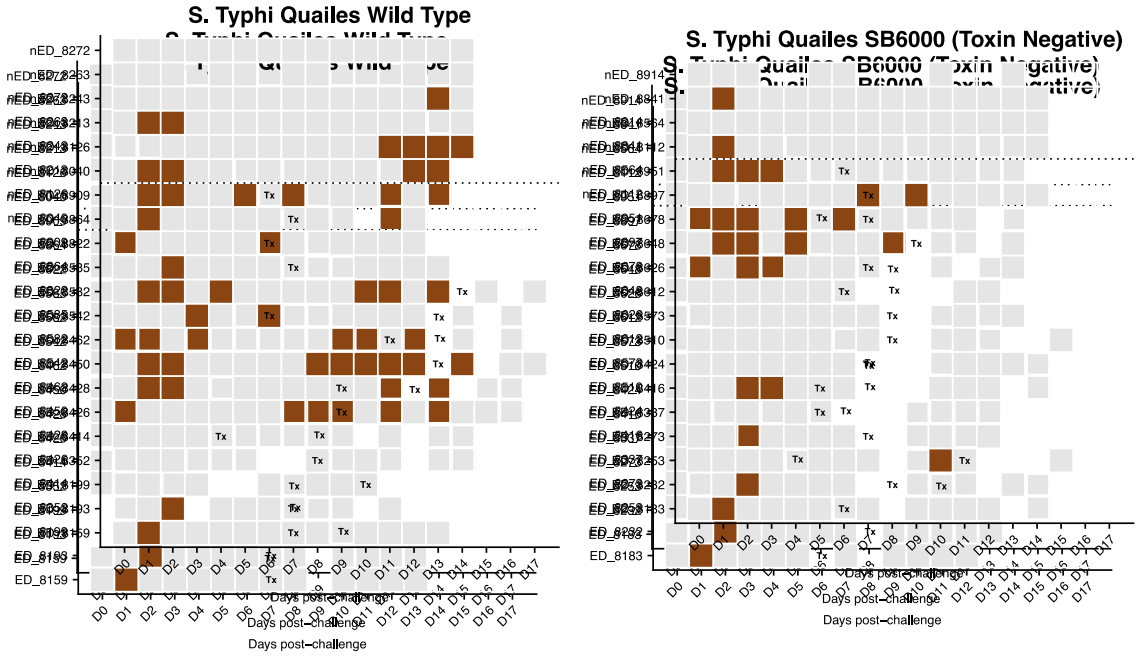


Figure 6-14 : Pattern of stool shedding after *S.* Typhi challenge.

Each row corresponds to an individual participant. Grey squares = Negative sample, Brown squares = Positive stool culture, White squares = No sample collected. Tx = Day of treatment initiation. Participants above the dotted line did not meet the composite criteria for typhoid diagnosis.

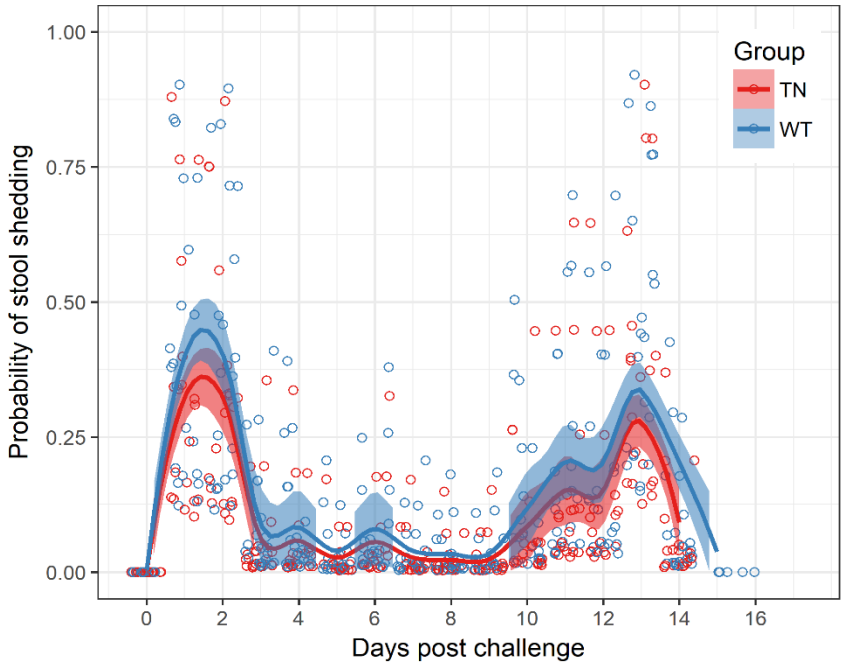


Figure 6-15 : Probability of shedding *S.* Typhi over time after challenge wild-type or typhoid toxin negative *S.* Typhi

6.4.4. Laboratory parameters

Routine haematology and biochemistry samples were collected during the challenge period to monitor participant safety. A summary of haematology results is presented in Figure 6-16. In both challenge groups, the total white cell count and neutrophil count fell during acute typhoid disease, reaching a nadir approximately 72 hours after diagnosis, before recovering. In contrast, the lymphocyte count begins fell 24-48 hours prior to diagnosis, typically reaching a nadir 24 hour after diagnosis. Routine biochemistry results are presented in Figure 6-17. As expected, C-reactive protein measurements peaked 48-72hrs after diagnosis. Median peak C-reactive protein measurements were higher in the toxin-negative group compared with the wild-type group, although did not meet significance (peak CRP, median [IQR] 90mmol/l [30.1-162.2] vs. 60.2mmol/l [18.25 – 120.8]; p = 0.33).

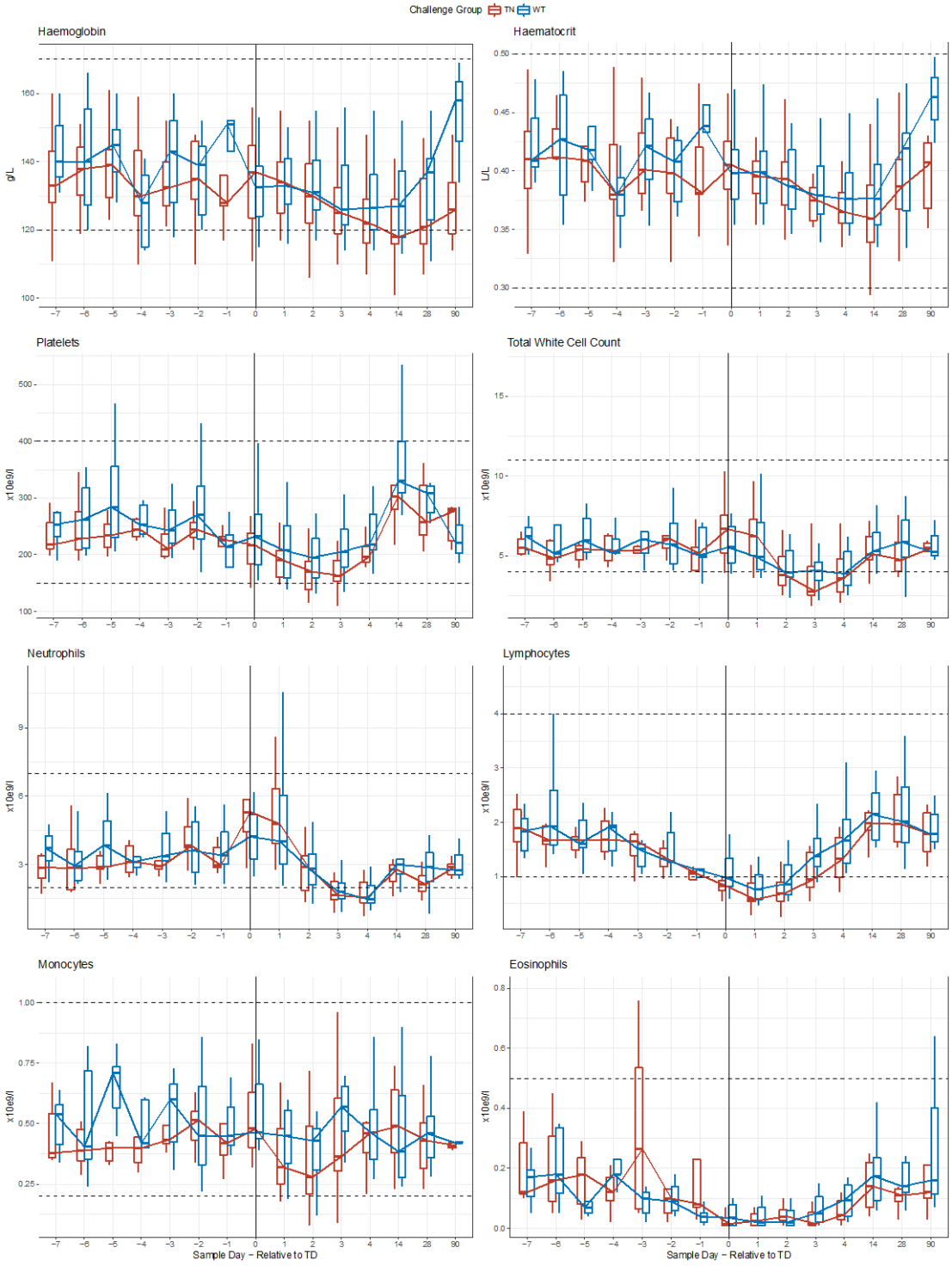


Figure 6-16 : Haematology laboratory parameters (OVG2016/03)

Participants challenged with typhoid-toxin deficient (TN- red) and wild-type (WT - blue) S. Typhi and diagnosed with typhoid fever. Data are presented relative to the time of typhoid diagnosis (Timepoint 0 = Day of diagnosis). Dot plots corresponds to individual values coloured according to challenge group (TN- red, WT – blue). Box-plots illustrate median and IQR, coloured and grouped according to challenge group. Solid, coloured lines connect median values at each timepoint. Dashed lines represent upper and lower reference limits for the individual parameters measured.

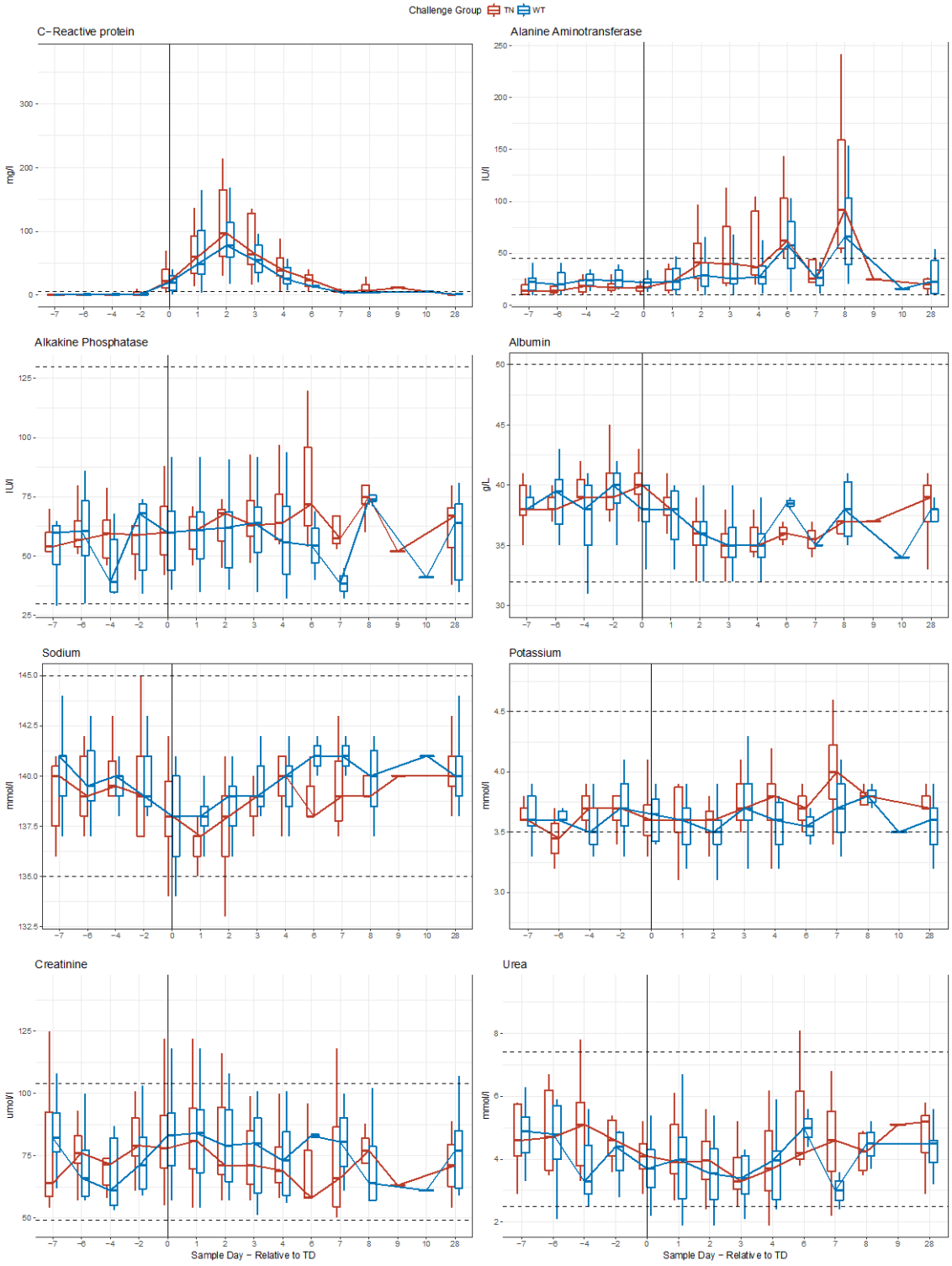


Figure 6-17 Biochemistry laboratory parameters (OVG2016/03).

Participants challenged with typhoid-toxin deficient (TN- red) and wild-type (WT - blue) S. Typhi and diagnosed with typhoid fever. Data are presented relative to the time of typhoid diagnosis (Timepoint 0 = Day of diagnosis). Dot plots corresponds to individual values coloured according to challenge group (TN- red, WT – blue). Box-plots illustrate median and IQR, coloured and grouped according to challenge group. Solid, coloured lines connect median values at each timepoint. Dashed lines represent upper and lower reference limits for the individual parameters measured.

6.4.5. Antibody secreting cell response

Ex-vivo IgG, IgA and IgM producing antibody secreting cell (ASC) responses against O-antigen and H-antigen were measured at baseline, at day 7 and at the time of typhoid diagnosis in those meeting the diagnostic criteria. There was a significant increase in anti-O9:LPS and anti-Hd specific circulating ASCs at the time of typhoid diagnosis in both challenge groups (Figure 6-18 Figure 6-19). The magnitude of the O9:LPS-anitgen and Hd-antigen specific ASC response at typhoid diagnosis was generally greater in participants challenged with toxin-negative *S*. Typhi (Figure 6-20). IgM producing ASC response at the time of typhoid diagnosis was significantly greater in participants challenged with toxin-negative *S*. Typhi compared with the wild-type strain.

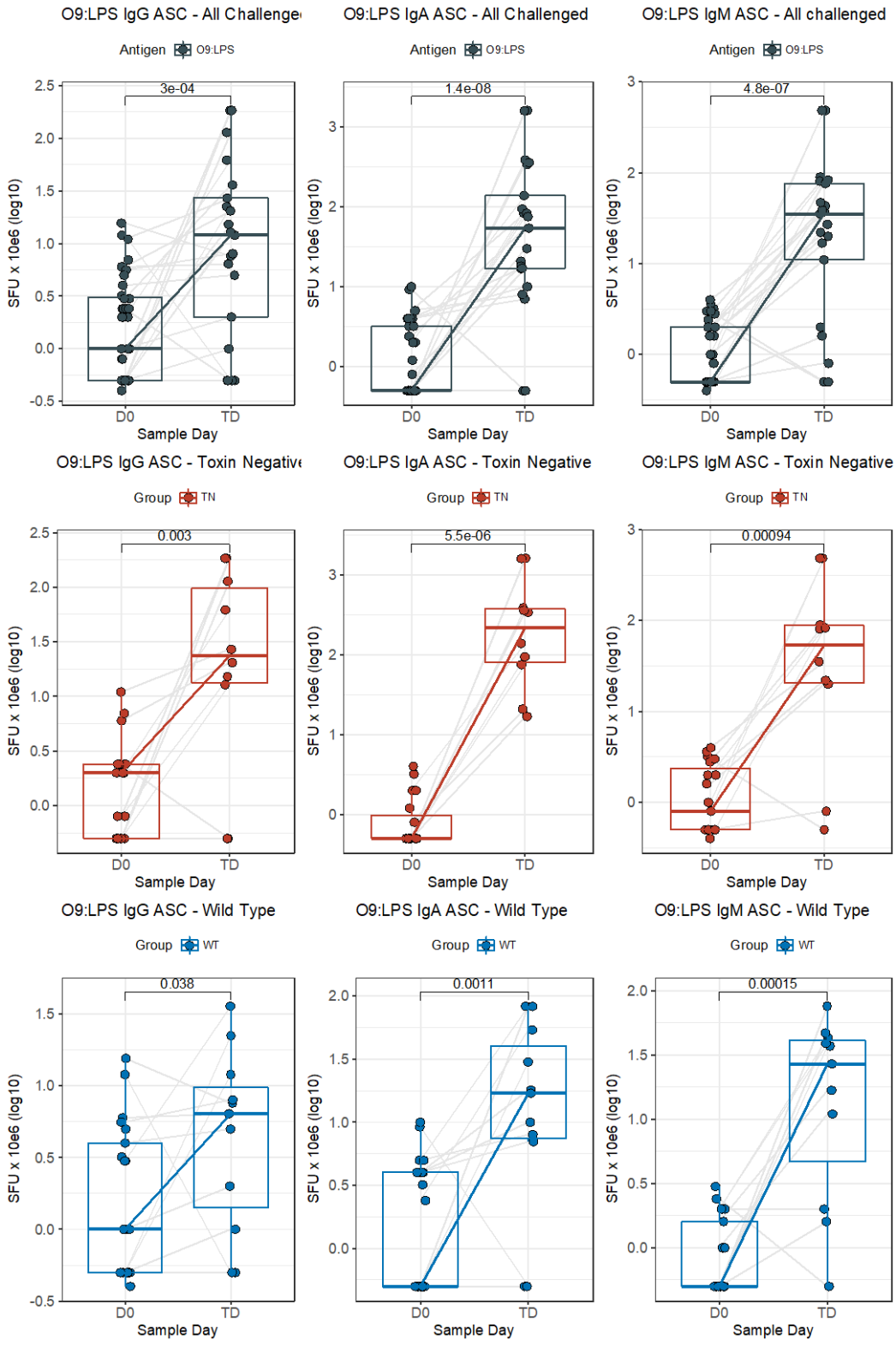


Figure 6-18 : O9:LPS specific ex-vivo IgG, IgA and IgM antibody secreting cell responses.

Participants diagnosed with typhoid, illustrated as $\log_{10}$ spot forming units (SFU) per 10^6 PBMCs. D0 = Baseline. TD=Time of diagnosis (samples processed 24-48hrs after initiation of treatment).(i) All challenged; (ii) Typhoid toxin deficient S. Typhi SB6000 challenge; (iii) Wild-type S. Typhi Quailes challenge. Measurements were taken at baseline (Day 0) and at the time of typhoid diagnosis (TD). p = Wilcoxon matched-pairs signed rank test.

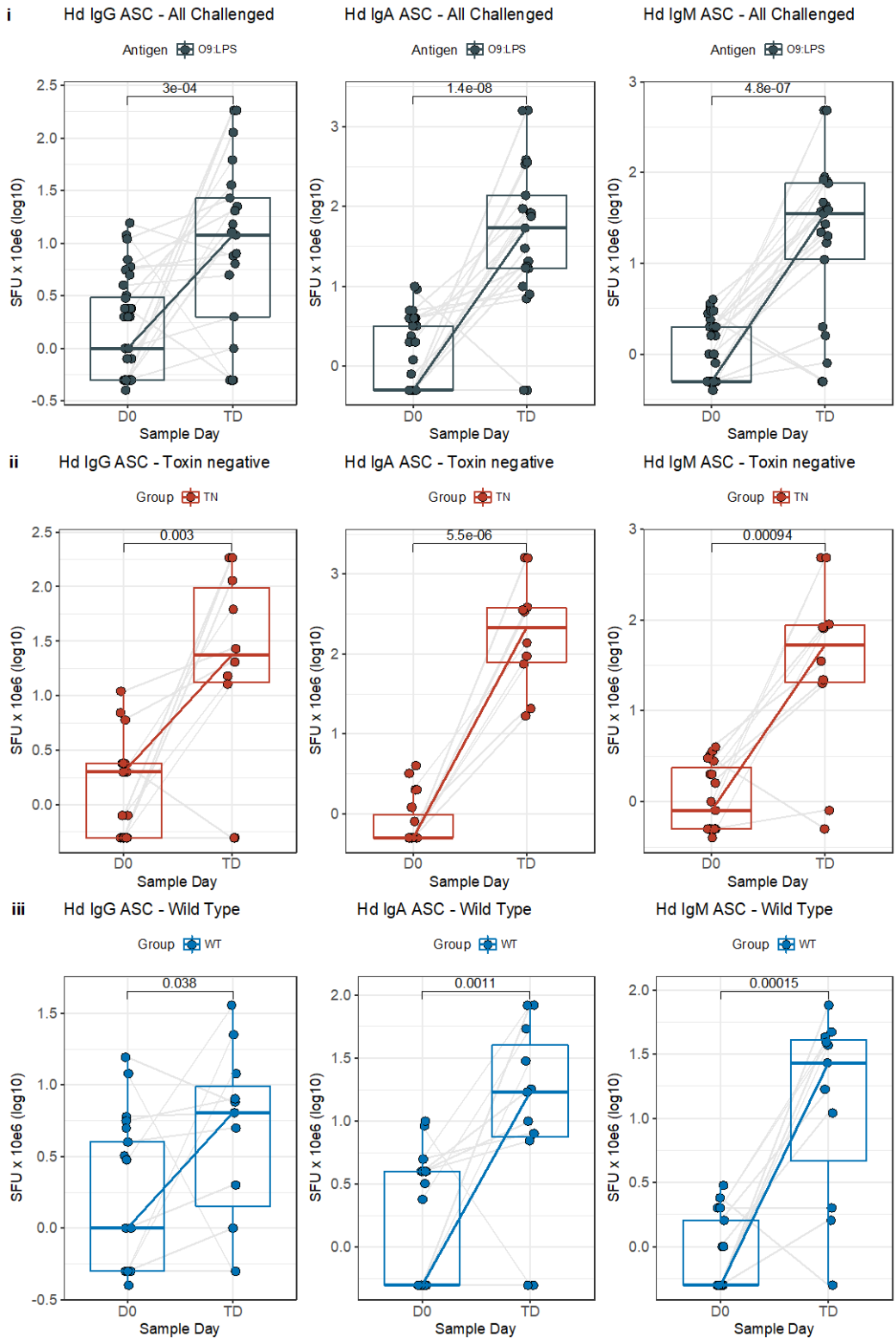


Figure 6-19 : Hd specific ex-vivo IgG, IgA and IgM antibody secreting cell responses.

Participants diagnosed with typhoid, illustrated as $\log_{10}$ spot forming units (SFU) per 10^6 PBMCs. D0 = Baseline. TD=Time of diagnosis (samples processed 24-48hrs after initiation of treatment). (i) All challenged; (ii) Typhoid toxin deficient S. Typhi SB6000 challenge; (iii) Wild-type S. Typhi Quailes challenge. Measurements were taken at baseline (Day 0) and at the time of typhoid diagnosis (TD). p = Wilcoxon matched-pairs signed rank test.

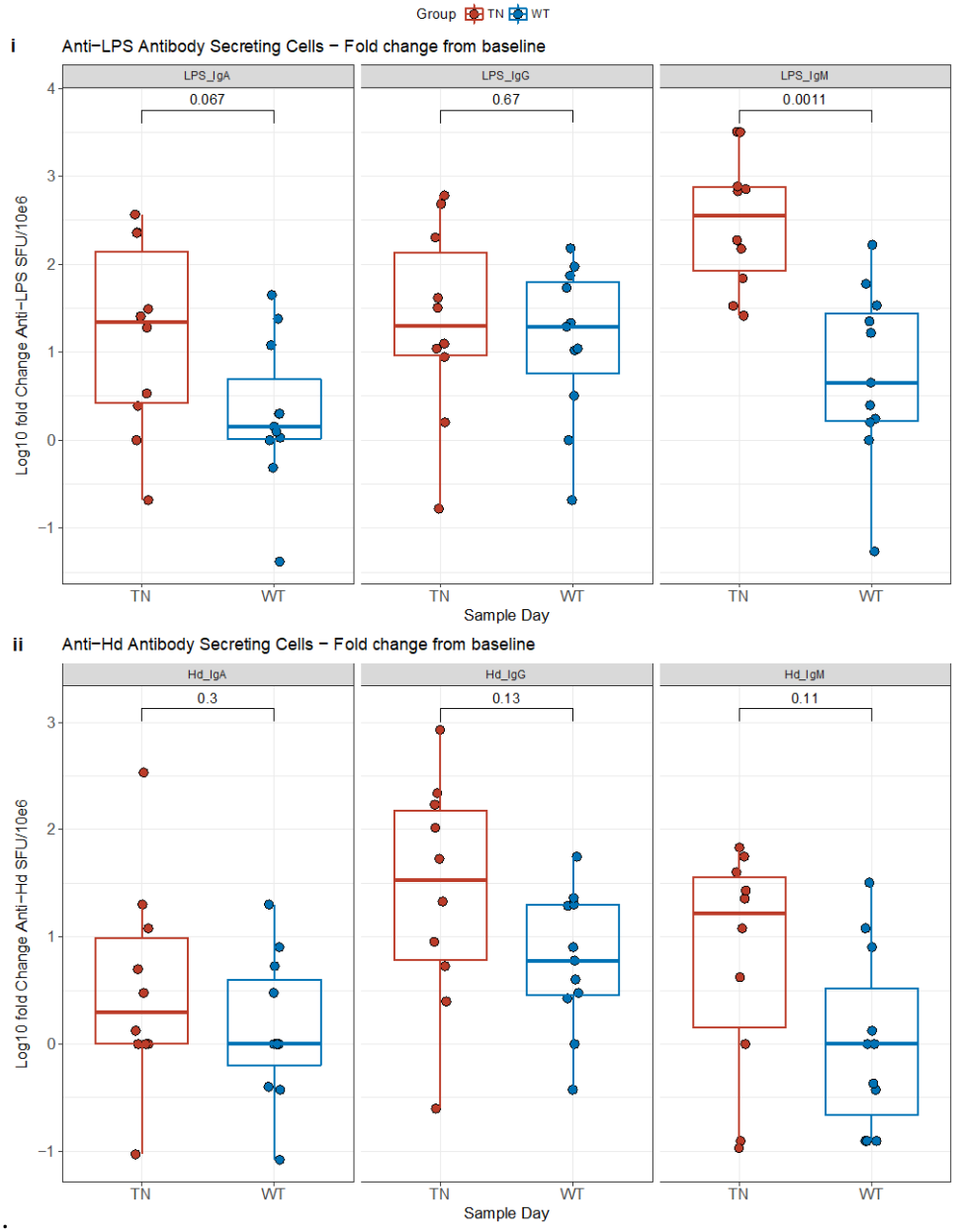


Figure 6-20 : Magnitude of antibody secreting cell response at typhoid diagnosis.

Represented as log_{10} fold change in spot forming units (SFU) per 10^6 PBMCs at time of diagnosis, from baseline. Grouped according to antigen, isotype and challenge agent allocation. TN = Typhoid toxin deficient S. Typhi SB6000 strain; WT = Wild-type S. Typhi Quailes strain. p = Mann-Whitney U test.

6.4.6. Principal component analysis

In order to better characterise differences or similarities between the study groups, I performed a principal component analysis of disease severity, using a range of clinical, microbiological and laboratory measures collected during the course of the challenge study. This approach offers the

theoretical benefit of identifying hidden patterns within the data, that are inapparent when individual measures are analysed independently. Several of the variables characteristic of typhoid fever that were included within the principal component analysis were strongly correlated with one another (Figure 6-21). When all participants were included in the analysis, participants diagnosed with typhoid fever clearly cluster separately from individuals who did not develop disease (Figure 6-22). Notably, there was substantial variation within the group categorised as being diagnosed with typhoid fever, emphasising the heterogeneity in clinical presentation observed within the challenge model.

In order to determine if the absence of the typhoid toxin was associated with a distinct disease phenotype, we performed a separate analysis of individuals diagnosed with typhoid fever. The percentage contribution of each variables to principal components 1 and 2 is illustrated in Figure 6-23. When all variables were included in the analysis, there was no clustering of participants by challenge group, suggesting that challenge with a typhoid-toxin deficient strain of *S*. Typhi was associated with an indistinguishable clinical phenotype to that caused by wild-type *S*. Typhi (Figure 6-24).

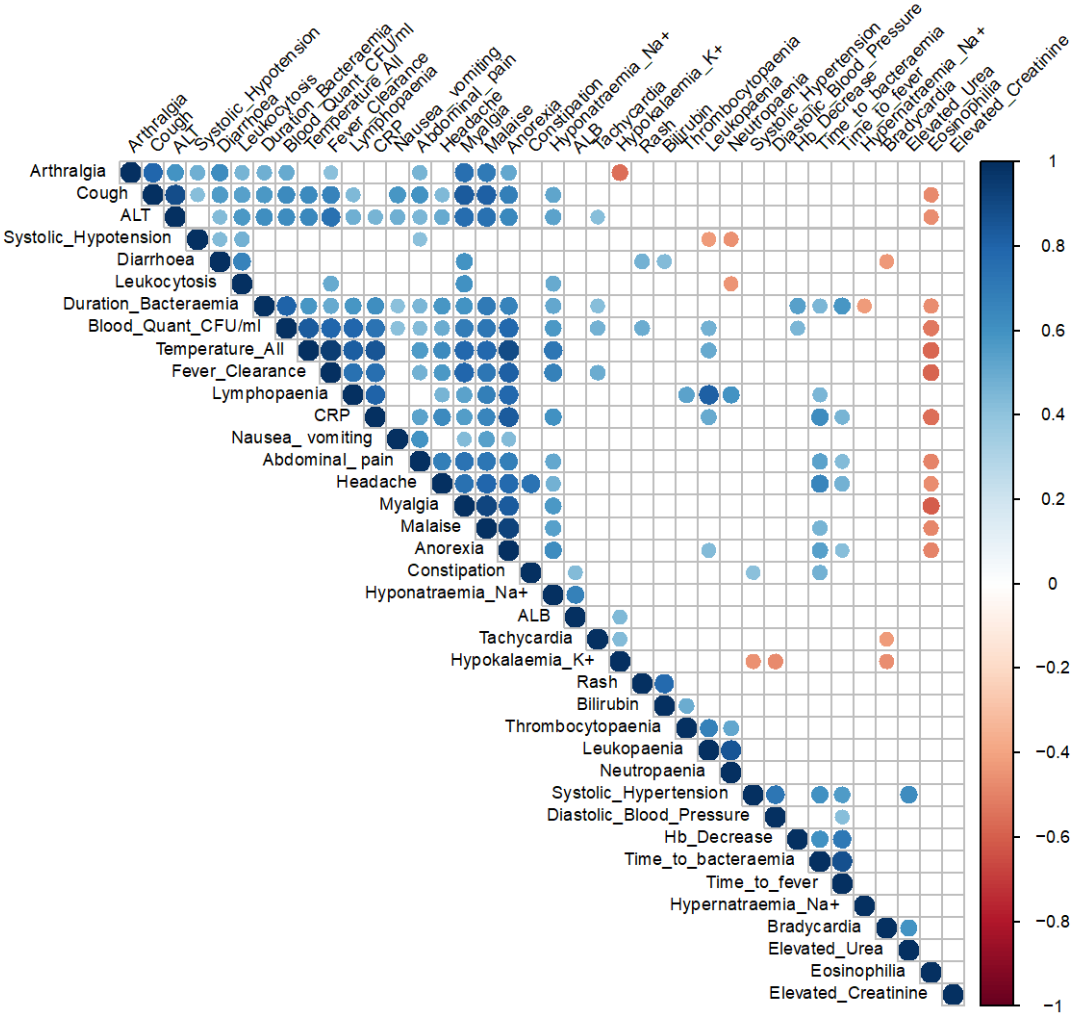


Figure 6-21 : Typhoid Severity Score Correlation matrix

Illustrating Spearman correlation coefficients of multiple variables contributing to symptom severity score (OVG2016/03 TYGER Study). Positive correlations are illustrated in blue, negative correlations are coloured in red. Circle size and colour are scaled according to the size of the correlation coefficients (+1 to -1). Correlations with a p value of ≥0.01 are left blank, whilst all correlations with a p value of <0.01 are plotted on the matrix [353].

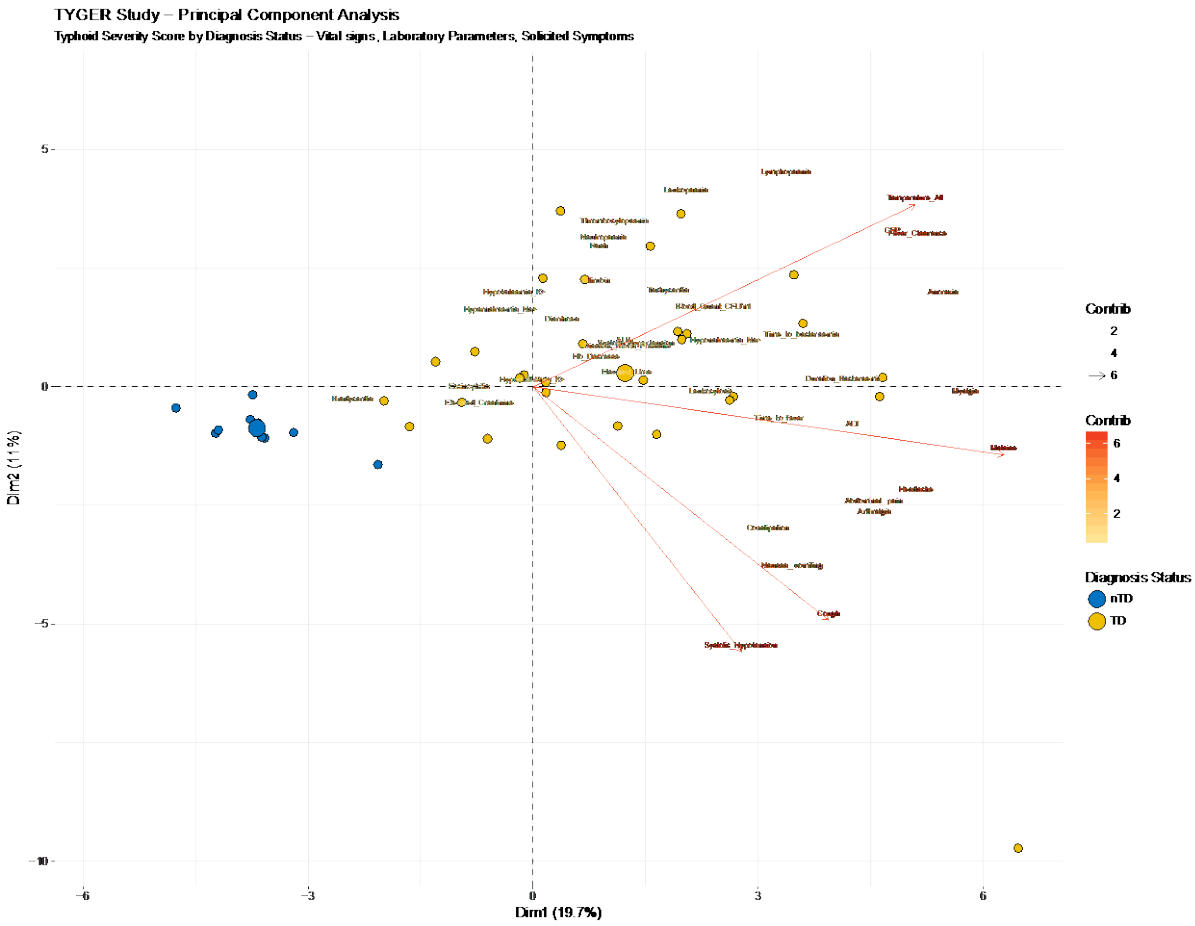


Figure 6-22 : Principal Component Analysis (PCA) biplot of disease severity by outcome of challenge (OVG2016/03).

Participants challenged with typhoid-toxin negative (TN) or wild-type (WT) *S*. Typhi based on the typhoid severity score. Data points are coloured by outcome of challenge (yellow [TD] = Typhoid diagnosis; blue (nTD) = No typhoid diagnosis). Ellipses represent 95% confidence levels for grouping variable (diagnosis status). Biplot arrows represent the contributions of individual variables to a given principal component, colour- and size scaled according to their relative contribution. Positively correlated variables are grouped together, whilst variables negatively correlated with one another are positioned on opposed quadrants. The length of the arrows represents the quality of the variables on the factor map, such that variables located further away from the origin contribute to greater variation along a principal component axis [354].

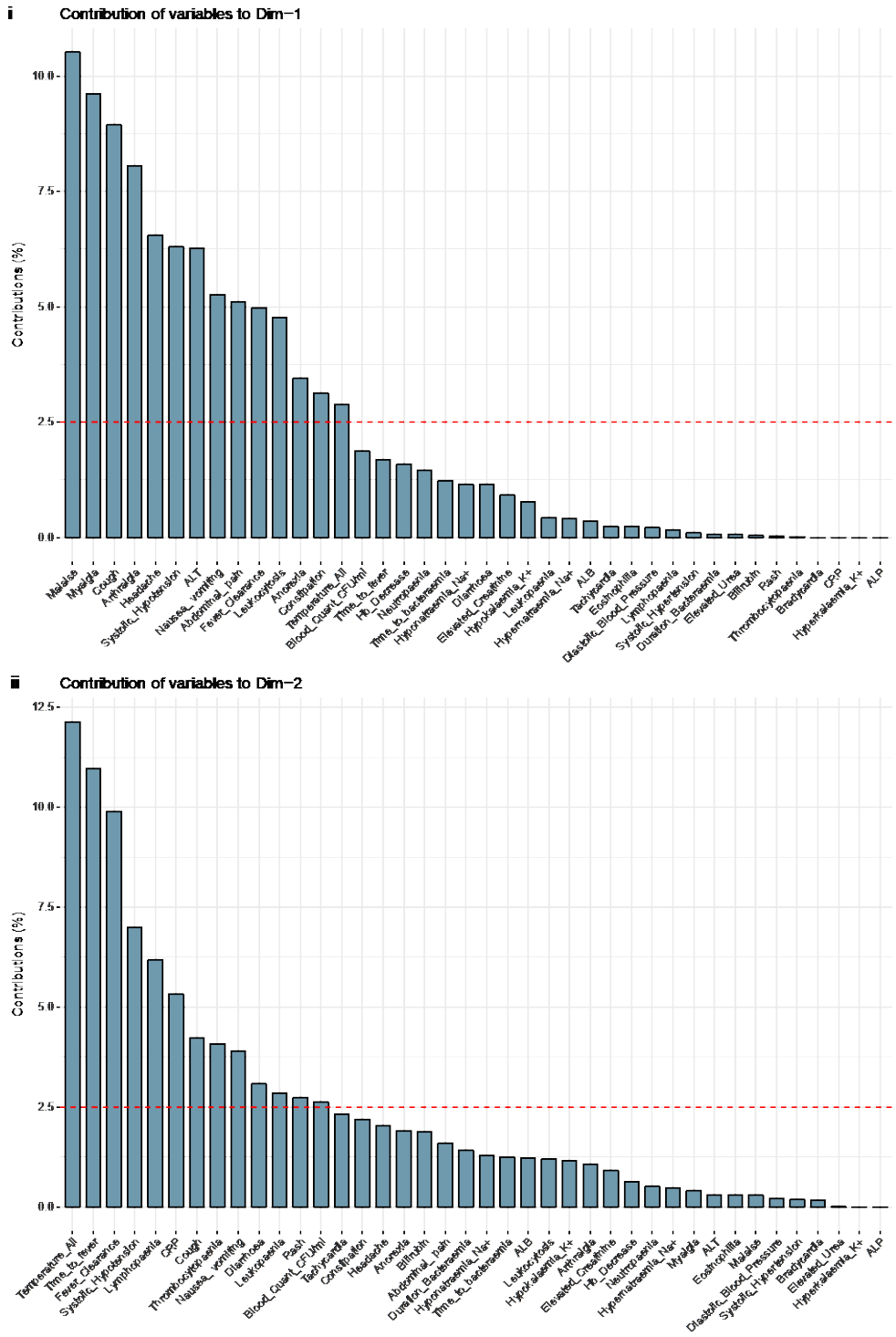


Figure 6-23 : Contributions of individual variables to PCA of disease severity.

(i)Principal component 1 and (ii) Principal component 2 in participants diagnosed with typhoid fever after challenge, expressed as percentage contribution. Variables listed on the X-axis represent variables included in the original principal component analysis. Red dashed line indicates the expected average contribution if the contribution were uniform, corresponding to 1/n(variables). Variables over the cut-off are considered important contributors to that principal component [354].

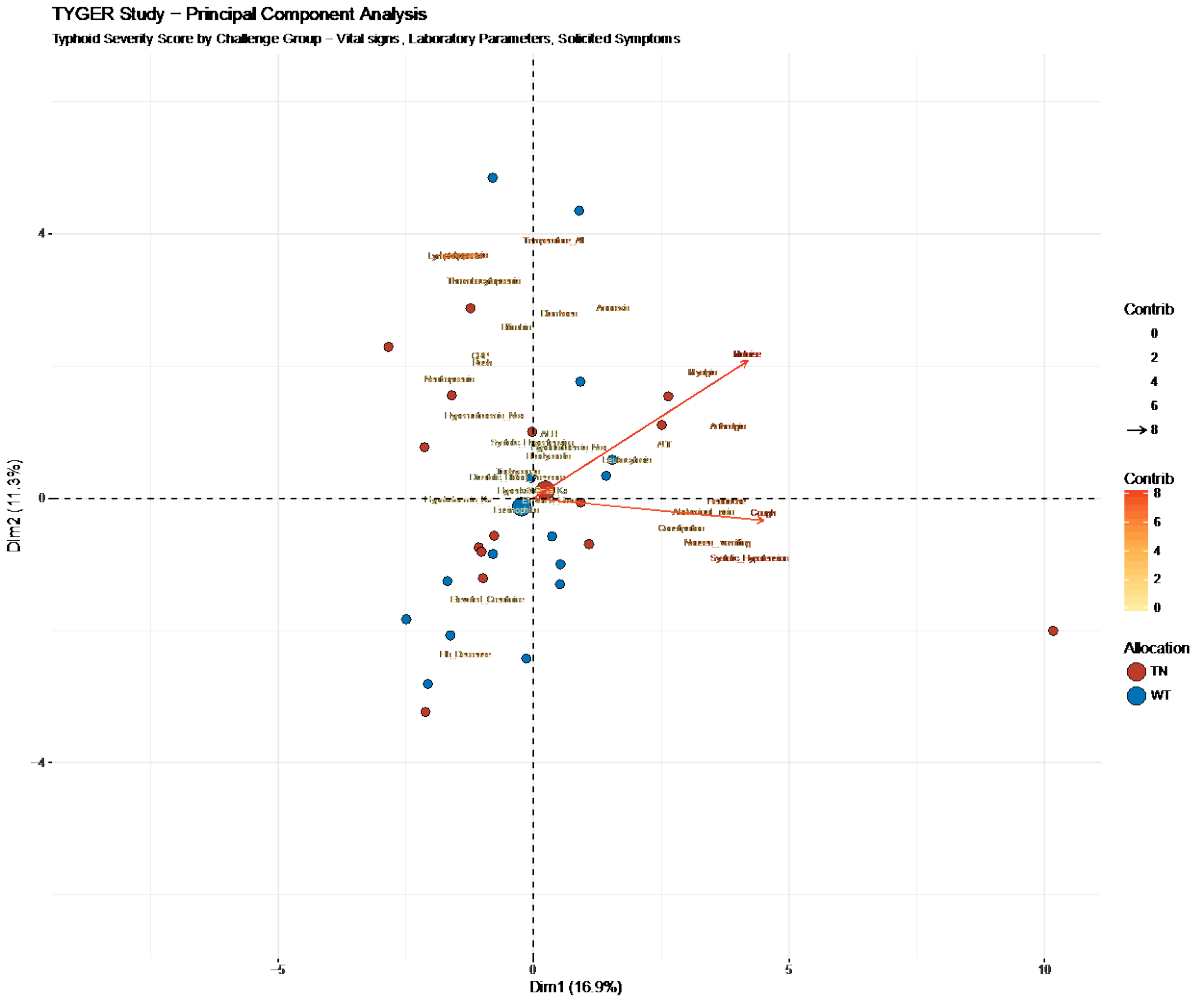


Figure 6-24 : Principal Component Analysis (PCA) biplot of disease severity by challenge agent.

Principal Component Analysis (PCA) biplot of participants diagnosed with typhoid fever following challenge with typhoid-toxin negative (TN) or wild-type (WT) S. Typhi based on the typhoid severity score. Data points are coloured by challenge agent allocation. Ellipses represent 95% confidence levels for grouping variable (challenge agent). Biplot arrows represent the contributions of individual variables to a given principal component, colour- and size scaled according to their relative contribution. Positively correlated variables are grouped together, whilst variables negatively correlated with one another are positioned on opposed quadrants. The length of the arrows represents the quality of the variables on the factor map, such that variables located further away from the origin contribute to greater variation along a principal component axis[354].

6.5. Discussion

In this chapter, I have described the design and results of a controlled human infection study aimed at assessing the contribution of the typhoid toxin to acute *S.* Typhi infection. This study represents the first application of a typhoid controlled human infection model to prospectively study the role of a specific virulence factor in the pathogenies of typhoid fever. In a cohort of 40 healthy volunteers, I observed no significant difference in the rate of typhoid fever between participants challenged with wild-type or typhoid-toxin deficient *S.* Typhi. Aside from a significantly prolonged duration of bacteraemia in participants randomised to typhoid-toxin negative challenge, the clinical presentation and disease severity was indistinguishable between the two study groups when measured using a variety of clinical, microbiological and laboratory endpoints.

Study groups were well matched at baseline and were comparable in demographic profile to earlier typhoid challenge studies. The clinical symptoms (fever, malaise, headache, abdominal pain) and laboratory abnormalities (elevated CRP, lymphopenia, neutropaenia) were all consistent with the expected presentation of typhoid fever in the field [70]. In addition, the attack rate observed in the wild-type *S*. Typhi challenge group (71%) was within the target range of 60-75%. This was consistent with that observed across multiple previous challenge studies [126,209,314] and provides re-assurance that the GMP batch of wild-type *S*. Typhi Quailes manufactured for this study (administered at a dose of 1-5 x 10^4 CFU with sodium bicarbonate) is broadly consistent with earlier GMP batches.

6.5.1. Deletion of the typhoid-toxin is not associated with reduced rate of disease in a human challenge model of infection

In contrast with what was predicted from pre-clinical studies, no differences in the rate, or severity of disease, were observed following challenge with a toxin-negative strain of *S.* Typhi. Typhoid toxin is said to "reproduce many of the pathognomonic symptoms of the acute [typhoid] disease" in mice models [251,260]. It remains unclear how surrogate endpoints of illness in mice – such as lethargy, weight loss, behavioural and motor changes[261,270] – are representative of acute typhoid fever in humans. Notably administration of typhoid toxin does not cause pyrexia in mice[260,270].

The discrepancy between pre-clinical studies and the findings in human volunteers may in part be attributable to differences in outcome measures. The primary diagnostic criteria for typhoid fever (*S.* Typhi bacteraemia and/or fever ≥38°C for ≥12 hours) was applied to minimise risk to study participants by commencing treatment at the earliest time point [126], but could mask differences between groups by treating self-limiting disease that may not have reached clinical attention in a field setting (see section 6.5.4). The disease endpoint is distinct to the WHO definition for typhoid fever (Fever ≥ 38°C for ≥3 days, with laboratory-confirmed culture from blood, bone marrow, bowel fluid)[140] and from the disease definition used in historical challenge studies (Oral temperature ≥39.4°C for 24-36h)[74]. Indeed, estimates of vaccine efficacy within the challenge model can be increased or decreased by applying more strict or liberal disease endpoints [209 314]. In contrast with previous challenge studies, no significant differences in attack rates were observed when alternative diagnostic criteria were applied, but early initiation of treatment may preclude the application of clinical diagnostic criteria.

6.5.2. General Limitations

The typhoid controlled human infection model is designed to study acute typhoid fever within a highly controlled experimental setting. Whilst this offers several advantages, ethical, safety and practical considerations in the conduct of these studies introduce some limitations to the wider applicability of these findings.

The sample size represented the number of participants that could be feasibly enrolled within the financial constraints and timeframe of the study. We enrolled a total of 40 participants into one of two challenge groups. Previous challenge studies studying specific virulence factors have enrolled between 5 to 10 participants per group [327–329,355,356]. The study is likely underpowered to detect subtle differences between challenge strains, with 95% power to detect a relative risk reduction in attack rate of 85% in the absence of the typhoid toxin. As a comparison, Vi-negative strains of *S*. Typhi are associated with a 50% relative risk reduction in the risk of symptomatic typhoid fever[326]. Consequently, differences between challenge groups would only have been observed in this study if the typhoid toxin explains the majority of *S*. Typhi virulence.

Modification of SB6000 strain may have resulted in phenotypic changes to the knock-out strain unrelated to the typhoid toxin. Characterisation of the strain prior to challenge comprised a basic set of assays to compare growth, cellular invasion and expression of some surface antigens. The absence of experiments showing reversion to virulence after complementation of the typhoid toxin genes in vitro is a limitation of this project. Additional studies to characterise gene expression of the typhoid toxin knock-out strain *in vitro*[357], *in vivo* [358 359] or phenotypic behaviour under diverse conditions[360] could help to address whether deletion of the typhoid toxin is associated with an altered phenotype beyond loss of toxicity.

6.5.3. Typhoid Toxin - Enhanced disease severity?

In several of the secondary endpoints measured, there was a trend towards a more severe disease phenotype in the toxin negative group. These measures include a shorter time to diagnosis; higher number of cases meeting the criteria for severe enteric fever; elevated ASC response; higher peak CRP and a prolonged duration of bacteraemia.

The causes for an apparently more severe disease phenotype in the toxin negative arm are unclear. The typhoid toxin is known to target leukocytes, including neutrophils,[260] and it has been suggested that it this is one mechanism by which the typhoid bacillus facilitates immune evasion[262]. The absence of the typhoid toxin could therefore be associated with a more pronounced host immune and inflammatory response, manifesting as enhance disease severity. Alternatively, it could be speculated that the absence of the typhoid toxin is associated with reduced detection of bacteria-encoded virulence factors by the innate immune system. A similar mechanism of so-called anti-virulence immunity has been observed with other bacterial exotoxins[361]. Diminished activation of innate immune system early in infection could result in reduced pathogen killing and more severe infection – although notably there was no difference in the concentration of circulating bacteria during acute typhoid disease.

The absence of an objective and reproducible measure of typhoid disease severity make these data difficult to interpret and with the exception of the duration of bacteraemia, none of these observations met statistical significance. Further studies of gene expression profiles in whole blood and plasma cytokine profiles are ongoing and could better characterise differences in host immune responses in response to challenge with wild-type and toxin-negative strains.

6.5.4. Typhoid toxin – Beyond acute febrile illness?

Based on these data, we cannot exclude a role for the typhoid toxin in features of typhoid disease beyond the stage acute febrile illness. It is conceivable that large differences in attack rate between participants challenged with WT or TN strains were not observed as the illness were halted prior to expression of the typhoid toxin or application of its biological activity *in vivo*. In *vitro* data demonstrate that typhoid-toxin expression occurs within 3-5 hours of intracellular infection and extracellular trafficking occurs within 24 hours[262], making it highly likely that the toxin had the opportunity to be expressed during the course of the challenge period. Additional studies are ongoing to characterise the host immune response to the typhoid-toxin.

Typhoid encephalopathy with a reduced level of consciousness is a characteristic feature of severe typhoid fever[70]. A number of pre-clinical studies have suggested that trafficking of typhoid-toxin to the brain is associated with the CNS depression observed in typhoid fever. Typhoid toxin can be detected bound to CNS arterioles following typhoid toxin administration to C57BL/6 mice [270] and a consistent feature of mouse studies is the occurrence of profound CNS depression following challenge with purified typhoid toxin [261,270]. In a similar vein, Chimpanzees are capable of being infected with *S.* Typhi, but do not demonstrate the "characteristic" CNS depression and reduced level of consciousness observed in severe typhoid fever, which is often cited as evidence that the disease phenotype in Chimpanzees is different to that observed in humans [362] [363]. Notably, the typhoid toxin is incapable of binding to cell surface receptors of other primates and cellular toxicity is not observed *in vitro*[261]. For self-evident safety reasons, typhoid encephalopathy is not an endpoint that can be studied within the context of the controlled human infection model, but could theoretically be addressed by a detailed study of patients with severe typhoid fever in the field[19,20].

Some studies have suggested that the typhoid toxin is associated with the establishment of chronic infection and enhanced host survival using a mouse model of infection [272]. If the typhoid-toxin expresses its action at a later stage in the life cycle of *S*. Typhi within the human host (i.e. in the establishment of chronic infection with the gall-bladder or urinary tract), this would not be detected in the human challenge model. Whilst the data presented here do not support a role for typhoid toxin in acute typhoid fever, they do not exclude a role for the toxin in chronic typhoid infection. Chronic carriers of S. Typhi are predicted to have a central role in the transmission dynamics of typhoid [293] and it could be speculated that strategies to target the typhoid toxin could have a role in preventing progression to the typhoid carrier state. The major challenges in the prospective identification of typhoid carriers presents a major – if not insurmountable – barrier to testing this hypothesis in field studies [364].

6.5.5. Study of virulence factors using controlled human infection models

The data presented in this chapter highlight the challenges and dilemmas associated with studying virulence factors and disease pathogenesis using controlled human infection models. The typhoid toxin is a virulence factor for which there is arguably abundant, high-quality pre-clinical evidence supporting a role in typhoid disease[251]. Despite this, no effect was observed in the challenge model when participants were challenged with a typhoid toxin deficient strain. It is unclear if this is due to limitations in the study design, intrinsic restrictions to the challenge model and endpoints, or a true lack of effect. Overall both the wild-type and toxin-negative strains of *S*. Typhi employed in this study, possess several putative virulence factors – including the Vi-capsule. It is plausible that the typhoid toxin is one of multiple redundant mechanisms involved in disease pathogenesis. The approach employed in this study may, therefore, be overly reductive.

Challenge models may not be well suited to high-throughput screening of pathogen virulence factors and no objective criteria exist to determine which virulence factors to study based upon

pre-clinical data. As an example, *S*. Typhi expresses a pore-forming exotoxin termed Haemolysin E (HlyE), belonging to the haemolysin superfamily of toxins that is the target of host strong cellular[278] and humoral responses[158,159,276], and may have an important role in disease pathogenesis. A HlyE knock-out strain of *S*. Typhi could theoretically be tested in a challenge model similar to that described in this chapter. In order to assess this, a new challenge strain would require manufacture and the processes described in this and earlier chapters repeating with no guarantee of success. It is reasonable to ask if this approach helps – or hinders – product development.

6.6. Summary

The study described in this chapter is the first to specifically investigate the role of typhoid toxin in the definitive host of *S*. Typhi. In summary, these data suggest that the typhoid toxin is not an essential virulence factor required for the development of acute typhoid fever within the context of a controlled human infection model. Conceivably, earlier bacteraemia and higher inflammatory responses might be followed by a more self-limiting disease with less involvement of the reticuloendothelial system. We cannot definitely exclude a role for the typhoid toxin in natural infection in the field; in the occurrence of severe complications of typhoid fever nor in the establishment of chronic infection. The observation of a more severe disease phenotype and more rapid onset of disease in the absence of the typhoid toxin, suggests that it may have an important role in modifying host inflammatory responses. Additional *in vitro* studies and deeper analysis of challenge samples are ongoing to further characterise the immunobiological role of typhoid toxin in the pathogenesis of typhoid fever. Overall, these data highlight some of the benefits – and challenges – of studying bacterial virulence factors using controlled human infection models.

Chapter 7 - Homologous and Heterologous Re-Challenge with *Salmonella* Typhi and *Salmonella* Paratyphi A in a Controlled Human Infection Model

7. Introduction

The development of new vaccines against enteric fever can be aided by studying the human immune response during infection and understanding the pathogenic mechanisms underlying disease. The extent, characteristics and duration of immunity conferred by natural infection with *S.* Typhi and Paratyphi are currently incompletely understood and there remains no readily reproducible immunological correlate of protection.

Three vaccines are currently pre-qualified by the WHO for control of typhoid fever,[215] but no vaccines are currently available for control of paratyphoid fever[222]. The effectiveness of licensed typhoid vaccines is currently the subject on ongoing trials[20], but the impact of these vaccines in different populations and different epidemiologic settings remains unknown. Studies modelling the impact of typhoid vaccination campaigns incorporate infection-derived immunity as a central variable,[293,294] but there are limited empirical data on the extent, mechanisms and duration of immunity conferred by prior typhoid infection. Data from case-studies indicate that repeated typhoid infections can occur, particularly when there is exposure to large infectious doses[292]. Historical challenge studies indicate that previous typhoid fever confers moderate (~30%) protection against subsequent disease[295]. Modelling data suggest that multiple episodes of typhoid exposure are required to confer functional immunity against future typhoid infection[293294].

Much less is known regarding immunity derived from previous paratyphoid infection. Similarly, it is unknown whether infection with *S.* Typhi confers heterologous protection against *S.* Paratyphi and vice-versa. Infection with *S.* Typhi and live attenuated *S.* Typhi vaccines induce cross-reactive cellular and humoral immune responses against *S.* Paratyphi A and B,[365–367] but it is unknown whether these responses correlate with protection. Data from field trials indicate that the Ty21a vaccine can confer moderate protection against *S.* Paratyphi B, but less is known regarding cross-protection against *S.* Paratyphi A [368 369].

Challenge/re-challenge studies have been used extensively to investigate the extent of infection-derived immunity to a range of enteric pathogens, including *Vibrio cholerae*[370,371], enterotoxigenic *E. Coli*[372], *Campylobacter jejuni* [373–375], *Shigella* spp. [313,376,377] and *Giardia lamblia*[378] (Table 7-1). Such studies can offer insights into mechanisms and determinants of immunity to guide vaccination strategies. Controlled human infection models for *S.* Typhi[126] and *S.* Paratyphi[311] can provide a unique opportunity to investigate infection-derived immunity within the context of a strictly monitored experimental setting.

To better characterise protective immunity to *S.* Typhi and *S.* Paratyphi, I conducted a homologous and heterologous re-challenge study of participants previously enrolled in *S.* Typhi and *S.* Paratyphi CHIM studies. The primary objective of the study was to compare the rate of enteric fever in re-challenge groups with naïve controls. Secondary objectives of the study were to compare the clinical features, microbiological profile and humoral immune responses between groups.

Table 7-1 : Controlled human infection studies describing re-challenge with enteric pathogens.

Relative risk calculated from published data. Protective effect calculated as 1-RR. P=Fishers exact test.

Re-Challenge Organism & Strain	Re-Challenge	Re-Challenge Interval	Challenge Dose	Primary Endpoint	Attack Rate Control Group	Attack Rate Re-Challenge Group	Protective Effect	Reference
Shigella flexneri 2a (2457T strain)	Homologous Re-Challenge	2 to 12 months	10^4 CFU	Fever ≥100°F or Diarrhoea ≥4 stools/24 hours	22 /39 (56%)	3/15 (20%)	65% (14-88%) p = 0.03	376
Shigella flexneri 2a	Homologous Re-Challenge	2 months	1.4 x 10^3 CFU Administered with sodium bicarbonate	Any Illness of Fever and/or Diarrhoea and/or Dysentery	11/12 (92%)	3/11 (27%)	70% (35-89%) p = 0.003	313
Shigella sonnei (strain 53G)	Homologous Re-Challenge	2 months	~500 CFU	Diarrhoea	7/12 (58%)	0/6 (0%)	100% (35-100%) p = 0.037	379
Vibrio cholerae O1 Inaba (strain 569B)	Homologous Re-Challenge – Disease on primary exposure	4 – 12 months	10^6 CFU administered with sodium bicarbonate	Any Diarrhoea	47/58 (81%)	0/21 (0%)	100% (81-100%) p<0.0001	370
Vibrio cholerae O1 Inaba (strain 569B)	Homologous Re-Challenge – No disease on primary exposure	4 – 12 months	10^6 CFU administered with sodium bicarbonate	Any Diarrhoea	47/58 (81%)	2/3 (67%)	18% (-21 -75%)	370

Vibrio cholerae O1 Ogawa (strain 395)	Heterologous Re-Challenge (Primary Challenge - V. cholera Inaba strain 569B)	4 – 12 months	10^6 CFU administered with sodium bicarbonate	Any Diarrhoea	47/58 (81%)	4/6 (67%)	18% (-17% - 63%) p = 0.59	[370]
Vibrio cholerae O1 Ogawa	Homologous Re-Challenge	2-3 months	10^6 CFU	Any Diarrhoea	5/5 (100%)	0/4 (0%)	100% (51 – 100%) p = 0.008	[371]
Vibrio cholerae O1 Ogawa	Heterologous Re-Challenge (Primary Challenge - V. cholera Inaba)	2-3 months	10^6 CFU	Any Diarrhoea	9/10 (90%)	0/5 (0%)	100% (51-100%) P = 0.002	[371]
Vibrio cholerae O1 Inaba	Heterologous Re-Challenge (Primary Challenge - V. cholera Ogawa)	2-3 months	10^6 CFU	Any Diarrhoea		0/7 (0%)	100% (57-100%) p = 0.0007	[371]
E. Coli (Strain H010407)	Homologous Re-Challenge	2-3 months	2 x 10^7CFU (Administered with sodium bicarbonate buffer)	Diarrhoea		1/10 (10%)	28/35 (80%) p = 0.0001	[372]
Giardia lamblia GS/M isolate	Homologous Re-Challenge	3 months	5 x 10^4 cysts	Infectivity (Cysts in multiple stool samples, humoral response to Giardia antigen)		2/2 (100%)	-	[378]

Campylobacter jejuni (Strain A3249)	Homologous Re-Challenge	1 month	10^8 CFU	Diarrhoea or Fever		0/2 (0%)	100% (34-100%) p = 0.0476	[373]
Campylobacter jejuni (Strain 81-176)	Homologous Re-Challenge	1 month	10^9 CFU	Diarrhoea or Fever		0/7 (0%)	100% (0 – 21%) p= 0.044	[373].
Campylobacter jejuni (Strain 81-176)	Homologous Re-Challenge	1-2 months	10^9 CFU	Campylobacteriosis		0/8	100% (65-100%) p <0.0001	[374]
Campylobacter jejuni (Strain 81-176)	Homologous Re-Challenge	12 months	10^9 CFU	Campylobacteriosis		4/7 (57%)	38% (6-73%) p = 0.045	[374]
Campylobacter jejuni (Strain CG8421)	Homologous Re-Challenge	3 months	5.5 x 10^5 CFU, administered with sodium bicarbonate	Campylobacteriosis (Clinical illness with documented C. jejuni infection)		8/8 (100%)	-7% (-90% - 29%) P= 0.99	[375]

7.1. Methods

7.1.1. Study Design

The OVG2014/01 (PATCH) study was a single-blinded, randomised, outpatient controlled human infection study using *S.* Typhi or *S.* Paratyphi, undertaken in a cohort of healthy community adult volunteers in a non-endemic setting (Chapter 4).

Study volunteers were recruited into one of three groups, defined *a priori* according to prior challenge with typhoidal *Salmonella* – either (i) no prior challenge (naïve cohort); (ii) previous challenge with *Salmonella* Typhi or (iii) previous challenge with *Salmonella* Paratyphi. Participants within each group were randomised to challenge with either *S*. Typhi or *S*. Paratyphi, giving a total of six study sub-groups (Figure 7-1).

Participants within the re-challenge cohort were randomised to either homologous or heterologous re-challenge. Homologous re-challenge was defined as re-challenge with the same *Salmonella* serovar and strain as occurred on primary exposure (*S*. Typhi Quailes strain or *S*. Paratyphi NVGH308). Heterologous re-challenge was defined as re-challenge with the alternative *Salmonella* serovar (Typhi or Paratyphi) to that which the participant was exposed to on primary challenge.

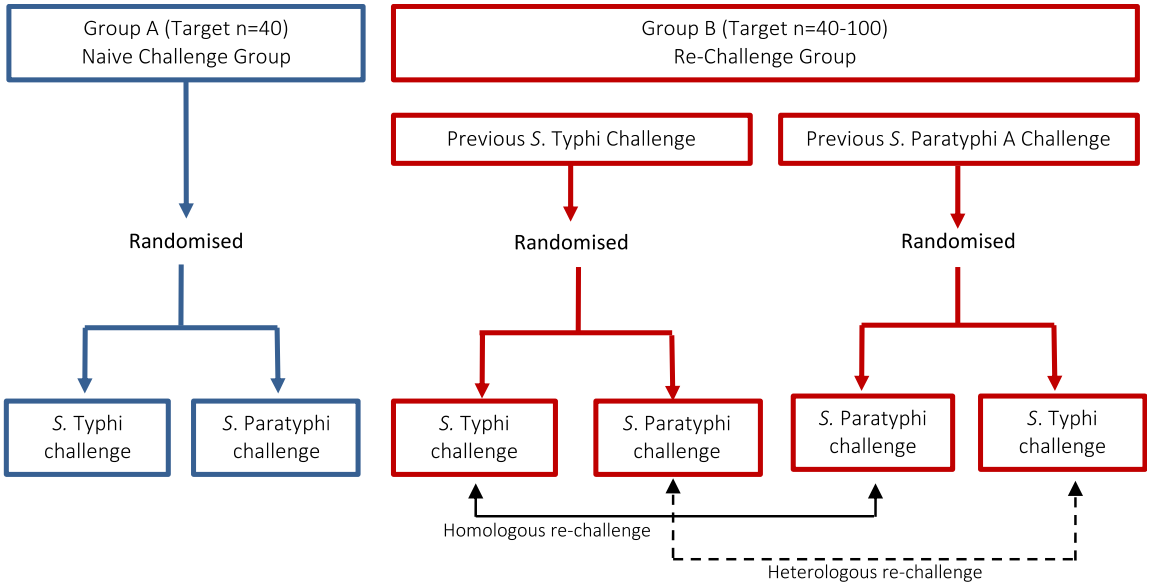


Figure 7-1 : OVG2014/01 Study overview.

7.1.2. Study Visits

The study visit schedule and study procedures are outlined in Table 7-2. Details of study procedures, challenge agent administration, challenge dose calculation and endpoint definitions are outlined in Chapter 4. Inclusion and Exclusion criteria are detailed in Appendix 1 and the sample collection schedule is detailed in Appendix 6.

Table 7-2 : Summary of Study Procedures OVG2014/01 (PATCH) study.

[1] Physical examination was performed at other times in the study if clinically indicated and at the discretion of the study team. [2] Stool samples were collected at least one week after completion of a 14-day course of antibiotics, until 3 successive stool samples are negative for S. Typhi. If persistent stool shedding occurs after completion of antibiotics, participants were referred to the Infectious Diseases Consultant at the Oxford University Hospitals NHS Trust. [3] Public Health England and participant GPs were notified at the time of stool shedding clearance.

	Screening	Endoscopy	D-7	D0	D0+12hrs	D1-6	D7	D8-13	D14	D28	ED	ED+12hs	ED+24 – 96	Endoscopy	D90 – D180	D365
Informed consent	x															
Consent quiz	x															
Revalidation of consent			x													
Obtain 24 hr contact details			x													
Medical history	x															
Interim medical history		x	x	x	x	x	x	x	x	x	x	x	x		x	x
Physical examination[1]	x										x					
Vital signs (HR,BP,Temp)	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Urine pregnancy test	x	x	x	x					x		x			x		
Urine sample	x			x		x			x	x	x					
Upper GI Endoscopy		x												x		
Stool sample[2]				x		x	x	x	x	x	x		x		x	x
Blood sample	x		x	x	x	x	x	x	x	x	x	x	x		x	x
Saliva sample	x		x	x					x		x					
Nasal Swab	x		x	x					x		x					
Wireless Capsule Endoscopy					x								x			
12 lead ECG	x															
Ultrasound scan	x															
Mood assessment	x		x				x		x		x					
Challenge with *S.* Typhi/S. Paratyphi				x												
Issue study pack			x													
e-Diary entries			x	x	x	x	x	x	x	x	x	x	x			
Randomisation			x													
Commence antibiotics									x		x					
Participant questionnaire											x					
Notification of Public Health England and GP[3]				x						x		x				

Letter informing close contacts				x											

7.1.3. Re-Challenge Cohort – Special Considerations

All participants who had previously participated in any *S*. Typhi or *S*. Paratyphi controlled human infection study conducted at the Centre for Clinical Vaccinology & Tropical Medicine, Oxford, UK were considered for enrolment into the re-challenge cohort. At the onset of study, the potential pool of re-challenge volunteers comprised 172 individuals who had participated in three separate studies between March 2011 and November 2014[126,311,314]. Participants originally enrolled into the naïve cohort were also approached for re-enrolment into the re-challenge cohort after completion of the one-year follow up visit. In November 2016 a substantial amendment to the study protocol was submitted and approved, allowing participants from a parallel *S*. Typhi vaccine/challenge study (OVG2014/08) [209] to be considered eligible for re-challenge, following completion of one-year safety follow up in their primary study.

All participants who had been previously challenged with *S*. Typhi or *S*. Paratyphi were eligible to be screened for enrolment into the re-challenge cohort, regardless of the diagnostic outcome of primary challenge (enteric fever diagnosis vs. no enteric fever diagnosis) and including those who had received typhoid vaccines as part of earlier challenge studies[209,314]. A separate post-hoc sub-group analysis was performed according to outcome of primary challenge and previous vaccination status (any vaccine vs. no vaccine and by specific vaccine type). We collected data on the date of primary challenge, date of primary vaccination (if applicable), previous challenge dose administered, the diagnostic outcome of primary challenge, date of initial enteric fever diagnosis (if applicable) and study vaccination status (if applicable).

7.1.4. Antibiotic Treatment

Participants were treated at the time of typhoid diagnosis or at day 14 for those who did not develop disease. From March 2015 to October 2016, the first line treatment was oral azithromycin 500mg once daily for 14 days. After October 2016, first line treatment was changed to ciprofloxacin 500mg twice-daily for 14 days. The change in first line therapy was prompted by a recommendation from the data safety monitoring committee, following review of safety data pertaining to antibiotic treatment from a parallel typhoid challenge study (OVG2014/08)[209].

7.1.5. Sample Size

No formal sample size calculations were made in the design of this study. All participants who had participated in previous challenge studies and who had consented to be contacted for future studies were approached to participate in the re-challenge group. The number of volunteers enrolled into the re-challenge group was dependent on the number of participants who consented to re-challenge and who were eligible after screening. We aimed to enrol 20 volunteers into each of the S. Typhi and S. Paratyphi naïve groups, giving 95% confidence intervals (CIs) for attack rates of between 36%–81% and 46%–88% assuming a measured attack rates of 60%-75%[126].

7.1.6. Inclusion of historical controls

As the main comparisons of interest in this study had low statistical power and did not constitute randomised comparisons, a sensitivity analysis was conducted to incorporate additional non-study data. Specifically, naïve participants from earlier studies were included in an additional post-hoc analysis as follows,

7.1.6.1. Typhoid challenge historical controls – naïve challenge

Data from previously unexposed participants enrolled and challenged with wild-type *S*. Typhi in challenge studies (OVG2009/10, OVG2011/02, OVG2014/08, OVG2016/03)[126 314 352] were included in a separate post-hoc analysis if they have not received a study vaccine and if they were exposed to the same challenge protocol, strain, and the same dose (1-5 x 10^4 CFU) as was used in the OVG2014/01 study (Table 7-3).

7.1.6.2. Paratyphoid challenge historical controls – naïve challenge

Data from previously unexposed participants enrolled and challenged in the OVG2013/07 [311] study were included in a separate post-hoc analysis if they were challenged with same challenge protocol, strain, and the same dose (1-5 x 10^3 CFU) as was used in the OVG2014/01 study (Table 7-3).

Table 7-3 : *Salmonella* Typhi and Paratyphi controlled human infection studies conducted in Oxford 2011-2017.

Study	Challenge agent	Study type	Vaccine	Description	Ref
OVG2009/10 (T1)	*S.* Typhi Quailes strain	Observational	-	Dose finding study Low dose: 1-5 x 10^3 CFU (n=20) High dose: 1-5 x 10^4 CFU (n=20)	126
OVG2011/02 NCT01405521 (T2)	*S.* Typhi Quailes strain	Vaccine RCT	M01ZH09 (n=31) Ty21a (n=30) Placebo (n=30)	Ty21a or M01ZH09 vaccines compared with control. Challenge dose 1-5 x 10^4 CFU	314
OVG2013/07 NCT02100397 (P1)	*S.* Paratyphi A NVGH308 strain	Observational	-	Dose finding study High dose: 1-5 x 10^3 CFU (n=20) Low dose: 0.5-1 x 10^3 CFU (n=20)	311
OVG2014/08 NCT02324751 (VAST)	*S.* Typhi Quailes strain	Vaccine RCT	Vi-Polysaccharide (n=35) Vi-TT conjugate (n=37) Placebo (n=31)	Vi-PS (Typhim Vi ®, Sanofi Pasteur) or Vi-TT (TypbarTCV ®, Bharat Biotech) vaccines compared with control. Challenge dose 1-5 x 10^4 CFU	209
OVG2014/01 NCT02192008 (PATCH)	*S.* Paratyphi A NVGH308 strain *S.* Typhi Quailes strain	RCT	-	Naïve challenge (S. Typhi and S. Paratyphi) vs Re-challenge (homologous and heterologous) S. Typhi challenge dose 1-5 x 10^4 CFU S. Paratyphi challenge dose 1-5 x 10^3 CFU	380
OVG2016/03 NCT03067961 (TYGER)	*S.* Typhi Quailes strain/ *S.* Typhi SB6000 (Typhoid-toxin deficient)	RCT	-	Wild Type S. Typhi Quailes strain (n=20) SB6000 Typhoid toxin negative strain (n=20) Challenge dose 1-5 x 10^4 CFU	352

7.1.7. Randomisation & Blinding

Randomisation was undertaken using the computer randomisation system Sortition (Nuffield Department of Primary Care Health Sciences, University of Oxford). Participants in the naïve cohort were randomised 1:1 to receive either *S.* Typhi or *S.* Paratyphi. Participants in the re-challenge cohort were stratified according to their previous exposure and then randomised 1:1 to receive either *S.* Typhi or *S.* Paratyphi. To elaborate, participants who were previously challenged with *S.* Typhi were randomised 1:1 to receive either *S.* Typhi or *S.* Paratyphi. Participants who were previously challenged with *S.* Paratyphi were randomised 1:1 to receive either *S.* Typhi or *S.* Paratyphi.

Randomisation was performed after screening investigations were completed and when the participant had their first study procedure (upper GI endoscopy) and study samples are taken. Varying block sizes were used. Participants were blinded to the allocation of the challenge strain until Day 28 post challenge. Investigators administering challenge agents were blinded to challenge agent allocation. Processing of blood culture samples for diagnosis was performed in an independent clinical microbiology laboratory, who were blinded to challenge agent allocation.

7.1.8. Statistical Analysis

Data were analysed using R version 3.4.4. Attack rates and 95% confidence intervals were calculated for each challenge group for the per-protocol population (i.e. participants who completed the fourteen-day challenge period) as the primary endpoint. Participants who were challenged but who commenced antibiotics prior to day 14 without meeting the diagnostic criteria were excluded from the primary analysis.

The difference in attack rate between naïve and re-challenge groups was calculated using Fisher's exact test and are also presented as a relative risk (RR with 95% CI) of typhoid/paratyphoid diagnosis.

$$RR = AR_{Re\text{-}Challenge}/AR_{Naive}$$

Protective efficacy was calculated as the 1-RR. To increase power, we performed a post-hoc analysis comparing attack rates in the re-challenge groups with the combined attack rates from vaccine-naïve/control individuals challenged in previous *S*. Typhi and *S*. Paratyphi challenge studies at equivalent challenge doses (Section 7.1.6).

Time to diagnosis, time to first fever and time to bacteraemia were summarised using the Kaplan-Meir method, with participants censored at Day 14. Group comparisons were performed using a log-rank test.

Blood quantification CFU's less than the lower limit of detection (0.1 CFU/ml) were assigned a value of 0.05 CFU/ml. Comparisons between groups were performed using Mann-Whitney U tests.

The principal component analysis was performed using the FactoMineR package[350] with R version 3.4.4. Variables were normalised by Z-score prior to inclusion in the principal component analysis.

7.1.9. Funding – OVG2014/01

The OVG2014/01 (PATCH) study was funded by the Medical Research Council (MR/K021222/1). The study funders had no role in study design, data collection or analysis.

7.1.10. Study Sponsorship & Approvals - OVG2014/01

The OVG2014/01 (PATCH) study was sponsored by the University of Oxford. The study (ClinicalTrials.gov identifier NCT02100397) was approved by Oxfordshire Research Ethics Committee A (14/SC/0004) and performed according to the provisions of the Declaration of Helsinki and Good Clinical Practice guidelines.

7.1.11. Safety monitoring

An independent data safety monitoring committee (DSMC) provided oversight of the study safety and conduct. The DSMC were provided with planned safety updates after the first 10 participants had been challenged and when all participants reached Day 28 post-challenge. Any serious adverse events and adverse events of special interest were reported to the DSMC.

7.2. Results

7.2.1. Recruitment

We enrolled 124 participants between 17th March 2015 and 24th August 2017 (Figure 7-2). Nine participants withdrew or were withdrawn from the study after randomisation but prior to challenge. A total of 115 participants were challenged, of whom two participants commenced antibiotics prior to day 14 without meeting the criteria for enteric fever diagnosis and were excluded from the primary analysis. Baseline characteristics were comparable across groups (Table 7-4).

Of participants eligible for re-challenge at the time of study initiation, 51/172 (30%) were recruited for re-challenge (Table 7-5). The median interval between primary challenge and re-challenge in participants previously challenged with *S.* Typhi was 38 months (range 12-68) compared with 16.9 months (13.9-27.7) in participants previously challenged with *S.* Paratyphi (p=0.09), reflecting the timing of previous challenge studies (Figure 7-3).

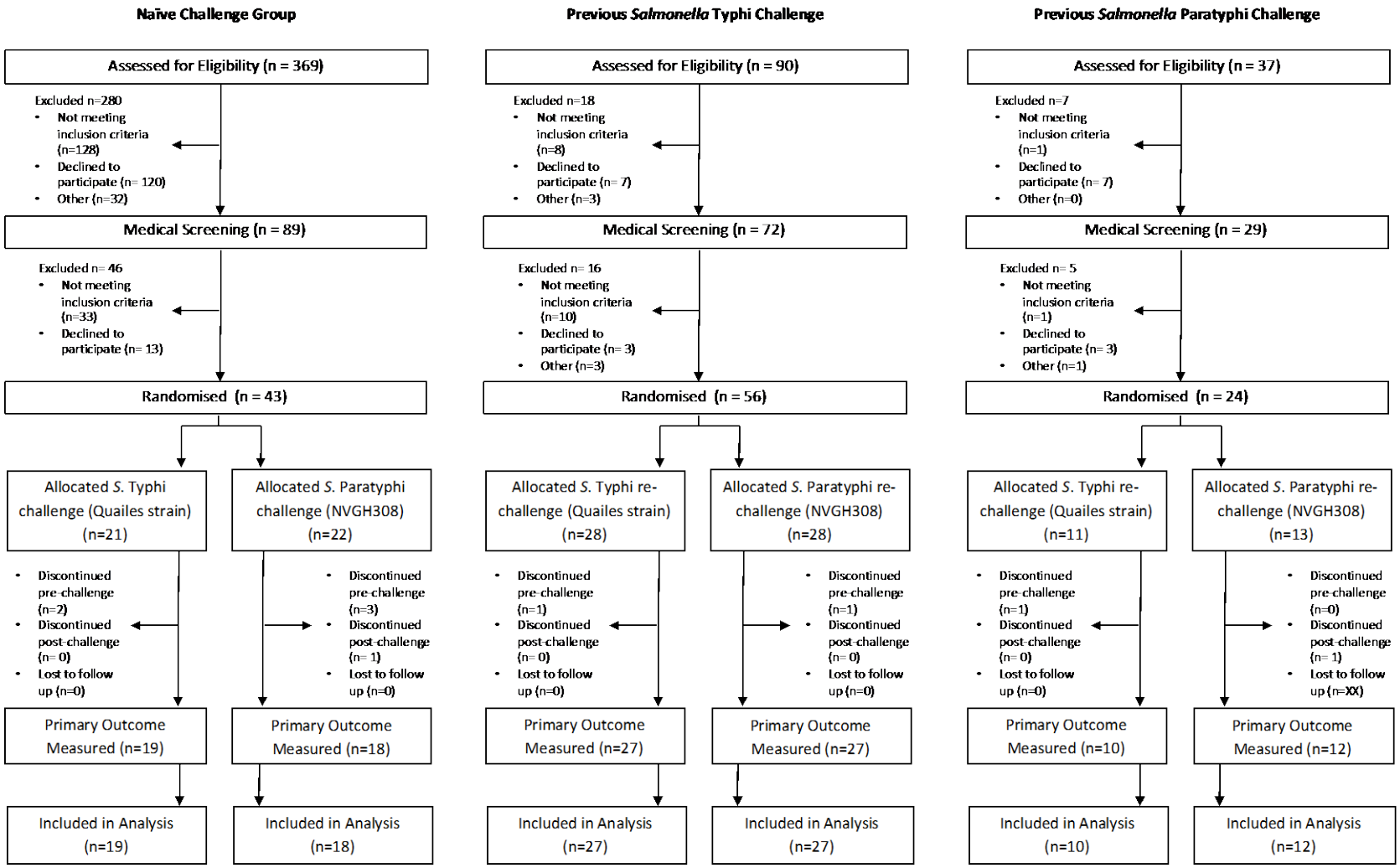


Figure 7-2 : OVG2014/01 Study profile.

Participants were recruited into one of three study groups defined a priori according to prior challenge status.

Table 7-4 : Participant characteristics OVG2014/01(PATCH) study.

ST-ST Re-Challenge = Homologous re-challenge with S. Typhi following previous S. Typhi challenge. SPT-SPT Re-Challenge = Homologous re-challenge with S. Paratyphi following previous S. Paratyphi challenge. ST-SPT Re-Challenge = Heterologous re-challenge with S. Paratyphi following previous S. Typhi challenge. SPT-ST Re-Challenge = Heterologous re-challenge with S. Typhi following previous S. Typhi challenge. Prior known typhoid vaccination of any type was an exclusion criterion for enrolment into the naïve arm of the study. Participants with prior history of Vi-polysaccharide vaccination were eligible for enrolment in previous paratyphoid challenge studies. Participants with a history of Vi-polysaccharide, Vi-tetanus toxoid conjugate, Ty21a, M01ZH09 vaccination received either vaccine as part of previous human challenge studies assessing vaccine efficacy. [314] [209] Minimum interval between primary challenge and re-challenge was 12 months.

	Challenge Group						
	All	*S.* Typhi Challenge			*S.* Paratyphi (A) Challenge		
		ST Naïve	ST-ST Re-Challenge	SPT-ST Re-Challenge	SPT Naïve	SPT-SPT Re-Challenge	ST-SPT Re-Challenge
Number	113	19	27	10	18	12	27
Male sex, n (%)	71/113 (63%)	14/19 (74%)	15/27 (56%)	9/10 (90%)	11/18 (61%)	6/12 (50%)	16/27 (59%)
Age, Years, median (range)	27.8 (18.8-60.8)	27.2 (19.6-59.7)	33.2 (21.3-60.8)	26.7 (22.2-52)	27.1 (18.8-42.6)	23.8 (21-44.1)	33.5 (19.3-55)
Ethnicity, n (%)							
White British	84 /113 (74%)	13 /19 (68%)	19/27 (70%)	9/10 (90%)	12/18 (66%)	9/12 (75%)	22/27 (82%)
White (Other)	23/113 (20%)	6/19 (31%)	6/27 (22%)	1/10 (10%)	4/18 (22%)	2/12 (17%)	4/27 (15%)
Mixed	5/113 (4%)	0/19 (0%)	2/27 (7%)	0/10 (0%)	2/18 (11%)	0/12 (0%)	1/27 (4%)
Asian (Indian)	1/113 (1%)	0/19 (0%)	0/27 (0%)	0/10 (0%)	0/18 (0%)	1/12 (8%)	0/27 (0%)
Previous travel to enteric fever endemic area, n (%)	42/133 (37%)	5/19 (26%)	10/27 (37%)	8/10 (80%)	5/18 (28%)	8/12 (67%)	6/27 (22%)
Alcohol consumption, any, n (%)	93/113 (82%)	14/19 (73%)	22 /27 (82%)	9/10 (90%)	16/18 (89%)	8/12 (67%)	24/27 (89%)
Tobacco smoker, any, n (%)	31/113 (27%)	5/19 (26%)	8 /27 (30%)	3/10 (30%)	4/18 (22%)	5/12 (42%)	7/27 (26%)
Previous *Salmonella* Challenge, n (%)							
S. Typhi	54/113	-	27/27	-	-	-	27/27

	(48%)		(100%)				(100%)
Challenge Dose (CFU): 1-5x10^3	5/113 (4%)	-	3/27 (11%)	-	-	-	2/27 (7%)
Challenge Dose (CFU): 1-5x10^4	49/113 (43%)	-	24/27 (89%)	-	-	-	25/27 (93%)
S. Paratyphi	22/113 (19%)	-	-	10/10 (100%)	-	12/12 (100%)	-
Challenge Dose (CFU):0.5-1 x 10^3	12/113 (11%)	-	-	6/10 (60%)	-	6/12 (50%)	-
Challenge Dose (CFU): 1-5 x 10^3	10/113 (9%)	-	-	4/10 (40%)	-	6/12 (50%)	-
Re-Challenge Interval, months, median (range)[c]	19 (12 – 67.9)	-	38 (12-60.6)	17.3 (13.9-24.9)	-	17 (14.2-27.2)	39 (12.2 – 67.9)
Previous enteric fever diagnosis, n (%)	37/113 (33%)	-	12/27 (44%)	7/10 (70%)	-	5/12 (41%)	13/27 (48%)
Previous typhoid vaccination, n (%)[b] Any Vaccine	28/113 (25%)	0/19 (0%)	11/27 (41%)	1/10 (10%)	0/18 (0%)	0/12 (0%)	16/27 (59%)
Vi-Polysaccharide	8/113 (7%)	0/19 (0%)	4/27 (15%)	1/10 (10%)	0/18 (0%)	0/12 (0%)	3/27 (11%)
Vi-Tetanus toxoid conjugate	11/113 (10%)	0/19 (0%)	3/27 (11%)	0/10 (0%)	0/18 (0%)	0/12 (0%)	8/27 (30%)
Ty21a	4/113 (3.5%)	0/19 (0%)	3/27 (11%)	0/10 (0%)	0/18 (0%)	0/12 (0%)	1/27 (4%)
M01ZH09	5/113 (4%)	0/19 (0%)	1 /27 (4%)	0/10 (0%)	0/18 (0%)	0/12 (0%)	4/27 (15%)

Table 7-5 : Recruitment into re-challenge cohort.

Number (%) of participants enrolled for re-challenge from previous challenge studies. Participants from the OVG2014/01 (n=113) and OVG2014/08 (n=103) studies were eligible for re-challenge after 12 months had elapsed from their primary challenge. As these studies were running contemporaneously, only a proportion of participants from these studies were eligible for re-challenge by the end of recruitment.

Study	Primary Challenge	Number Eligible for Re-Challenge	Number (%) Enrolled for Re-Challenge
OVG2009/10 (T1) [126]	*S.* Typhi Quailes strain	40	14 (35%)
OVG2011/02 (T2) NCT01405521 [314]	*S.* Typhi Quailes strain	94	15 (16%)
OVG2013/07 (P1) NCT02100397 [311]	*S.* Paratyphi A NVGH308 strain	40	22 (55%)
OVG2014/08 (VAST) NCT02324751 [209]	*S.* Typhi Quailes strain	-	23
OVG2014/01 (PATCH) NCT02192008 [380]	*S.* Paratyphi A NVGH308 strain/ *S.* Typhi Quailes strain	-	3

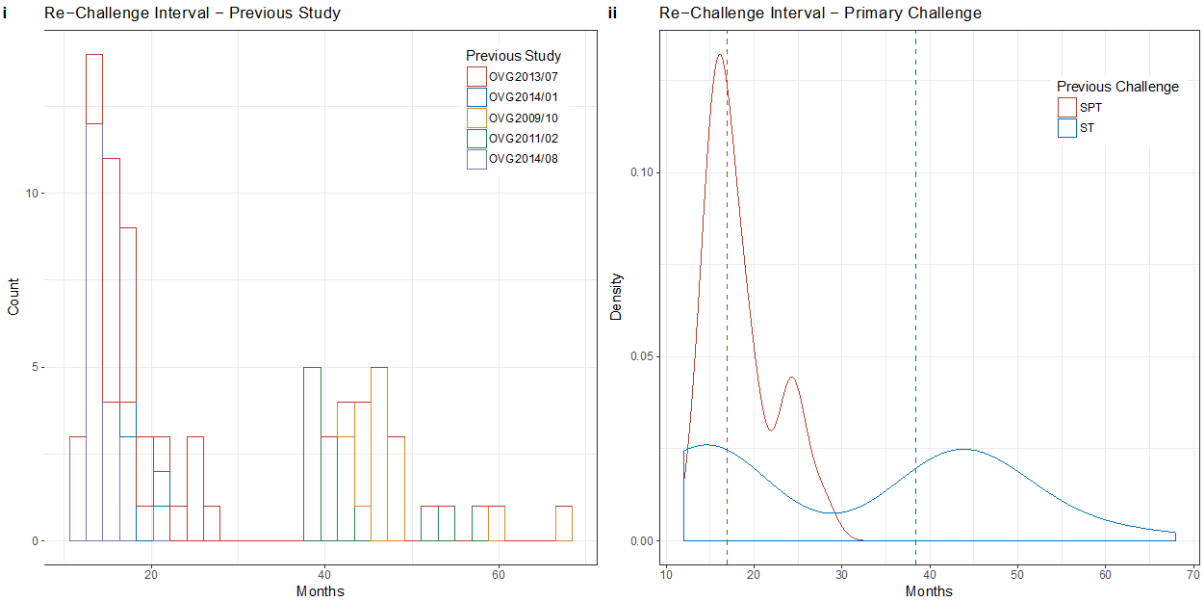


Figure 7-3 : Re-Challenge Interval OVG2014/01 study.

(A) Histogram coloured according to previous challenge study, (B) Density plot according to previous challenge agent allocation. Vertical lines represent median re-challenge interval for participants previously challenged with S. Typhi (blue) and S. Paratyphi (red).

7.2.2. Homologous *Salmonella* Typhi Re-challenge

A total of 56 participants were challenged with *S.* Typhi across three groups. All participants were challenged within the target dose range of 1-5x10^4CFU (Table 7-6).

Of the naïve participants challenged with *S*. Typhi, 12/19 (63%) met the composite primary endpoint for typhoid fever, compared with 12/27 (44%) after homologous re-challenge group, corresponding to a 30% relative risk reduction in the risk of typhoid diagnosis (Relative risk 0.70; 95% CI 0.40 to 1.23; p = 0.24) (Table 7-6). The diagnosis of typhoid fever was confirmed by a positive blood culture in 12/12 (100%) of the naïve cohort and 11/12 (92%) of the re-challenge cohort and the majority of participants in both groups were diagnosed based upon microbiological criteria (Table 7-6). The median time to diagnosis was prolonged by approximately 2 days in the typhoid homologous re-challenge group compared with naïve controls (Median days [range] 8 [4-10.9] vs. 6.2 [5-10.1]; p = 0.099) (Figure 7-4). Homologous re-challenge was also associated with a prolonged time to bacteraemia and time to fever compared with naïve controls (Figure 7-5).

As the main comparisons of between naïve and re-challenge cohorts had low statistical power with confidence intervals, we performed a post-hoc analysis comparing the rate of typhoid infection in the re-challenge cohort with the combined attack rate from all naïve volunteers enrolled in typhoid challenge studies between 2011 and 2017 (Table 7-3). The observed attack rate across all naïve volunteers (n=121) was consistent from study-to-study with an average attack rate of 69% (95%CI 60-77% - Figure 7-6)

When compared with all historical controls, the rate of typhoid infection was significantly reduced in the *S*. Typhi re-challenge group compared with naïve controls, corresponding to a 36% relative

risk reduction (12/27 [44%] vs. 84/121 [69%]; RR 0.64 [0.39-0.93]; p = 0.02 Fisher's exact test). The time to diagnosis was also prolonged in the typhoid re-challenge group when compared with all naïve controls but did meet significance (median days [range] 6.2 [5-10.1] vs. 7[4-14]; p=0.07 - Figure 7-7).

Table 7-6 : Primary and secondary outcome measures OVG2014/01 Study.

ST-ST Re-Challenge = Homologous re-challenge with S. Typhi following previous S. Typhi challenge. SPT-SPT Re-Challenge = Homologous re-challenge with S. Paratyphi following previous S. Paratyphi challenge. ST-SPT Re-Challenge = Heterologous re-challenge with S. Paratyphi following previous S. Typhi challenge. SPT-ST Re-Challenge = Heterologous re-challenge with S. Typhi following previous S. Paratyphi challenge.

	All	Challenge Group					
		S. Typhi Challenge			S. Paratyphi Challenge		
		ST Naïve	ST-ST Re-Challenge	SPT-ST Re-Challenge	SPT Naïve	SPT-SPT Re-Challenge	ST-SPT Re-Challenge
		A1	B1	B3	A2	B4	B2
Included in Primary Outcome Analysis	113	19	27	10	18	12	27
Enteric Fever Diagnosis, n (% attack rate)	58/113 (51%)	12/19 (63%)	12/27 (44%)	7/10 (70%)	10/18 (56%)	3/12 (25%)	14/27 (52%)
Attack rate 95% Confidence Interval, %	42-61%	38-84%	25-65%	35-93%	31-78%	5-57%	25-65%
Primary Clinical Diagnosis, n (% diagnosed)	8/58 (13.8%)	0/12 (0%)	3/12 (25%)	0/7 (0%)	2/10 (20%)	1/3 (33.3%)	2/14 (14.3%)
Primary Microbiological Diagnosis, n (% diagnosed)	50/58 (86.2%)	12/12 (100%)	9/12 (75%)	7/7 (100%)	8/10 (80%)	2/3 (66.7%)	12/14 (85.7%)
Bacteraemia without Fever ≥38°C, n (% diagnosed)	22/58 (37.9%)	3/12 (25%)	4/12 (33.3%)	4/7 (57.1%)	4/10 (40%)	2/3 (66.7%)	5/14 (35.7%)
Bacteraemia preceding Fever ≥38°C, n (% diagnosed)	28/58 (48.3%)	9/12 (75%)	5/12 (41.7%)	3/7 (42.9%)	4/10 (40%)	0/3 (0%)	7/14 (50%)
Fever ≥38°C for ≥12hrs without Bacteraemia, n (% diagnosed)	3/58 (5.2%)	0/12 (0%)	1/12 (8.3%)	0/7 (0%)	1/10 (10%)	0/3 (0%)	1/14 (7.1%)
Fever ≥38°C preceding Bacteraemia, n (% diagnosed)	5/58 (8.6%)	0/12 (0%)	2/12 (16.7%)	0/7 (0%)	1/10 (10%)	1/3 (33.3%)	1/14 (7.1%)
Actual challenge dose administered, CFU x 10^3, median (IQR)	-	24.4 (21.9-29.5)	22.9 (21.3-24.4)	26.2 (21.5-29.3)	2.1 (2.1-2.6)	2.5 (2.2-2.7)	2.6 (2.4 -2.8)
Previous Typhoid Diagnosis, n (% attack rate)	-	-	8/12 (67%)	-	-	-	10/13 (77%)
Previous Paratyphoid Diagnosis, n(% attack rate)	-	-	-	6/7 (86%)	-	1/5 (20%)	-
Previous Typhoid Exposure (No Diagnosis), n (% attack rate)	-	-	4/15 (27%)	-	-	-	4/14 (29%)
Previous Paratyphoid Exposure (No Diagnosis), n(% attack rate)	-	-	-	1/3 (33%)	-	2/7 (29%)	-

Previous Vaccine Diagnosed, (% attack rate)							
None	-	-	7/16 (44%)	6/9 (67%)	-	3/12 (25%)	6/11(55%)
Any	14/28 (50%)	-	5/11 (45%)	1/1 (100%)	-	0/0 (0%)	8/16 (50%)
Vi-PS	4/8 (50%)	-	2/4 (50%)	1/1 (100%)	-	0/0 (0%)	1/3 (33%)
Vi-TT	5/11 (45%)	-	0/3 (0%)	0/0 (0%)	-	0/0 (0%)	5/8 (63%)
Ty21a	2/4 (50%)	-	2/3 (67%)	0/0 (0%)	-	0/0 (0%)	0/1 (0%)
M01ZH09	3/5 (60%)	-	1/1 (100%)	0/0 (0%)	-	0/0 (0%)	2/4 (50%)
Time to diagnosis, Days, median (Range)	7.1 (4.0-12.1)	6.2 (5.0-10.1)	8.0 (4.0-10.9)	7.0 (4.1-8.9)	7.0 (4.3-12.1)	9.4 (6.0-11.0)	8.0 (5.1-11.1)
Time to first fever >38°C, Days, median (Range)	8.51 (5.35-14.4)	7.29 (5.43-10.6)	10.25 (5.47-13)	6.39 (5.99-10.12)	7.97 (5.35-12.2)	9.44 (-)	9.47 (5.76-14.4)
Severe Enteric Fever (Any), n	6/58 (10%)	2/12 (17%)	0/12 (0%)	1/10 (10%)	0/10 (0%)	0/3 (0%)	3/14 (21%)
Systolic blood pressure ≤ 85mmHg	0	0	0	0	0	0	0
Oral Temperature ≥ 40°C	0	0	0	0	0	0	0
Significant lethargy or confusion	0	0	0	0	0	0	0
Gastrointestinal bleeding and/or perforation	0	0	0	0	0	0	0
Grade 4 laboratory abnormality	6	2	0	1	0	0	3
Microbiology - Blood culture							
Time to bacteraemia, Days, Median (Range)	7.1 (4.0-14.2)	6.3 (5.96-10.1)	8 (4.0-10.9)	6.97 (4.1-8.9)	6.95 (4.3-12.1)	9.99 (5.96-10.97)	11.0 (6.97-14.2)
Duration of bacteraemia,Hrs, Median (IQR)	81.4 (60.2-96)	24.5 (4.49-76.3)	24 (15.1-68.4)	92 (88.4-96)	93.7 (67.5-96)	72.8 (49.5-96)	74.8 (45.6)
Positive at ED + 96 hours, n (% diagnosed)	21/58 (36%)	2/12 (17%)	1/12 (8%)	6/7 (86%)	5/10 (50%)	1/3 (33%)	6/14 (43%)
Quantitative blood culture, CFU/ml, Median (IQR)	-	0.1 (0-1.8)	0.1 (0-1.3)	0.2 (0 -1.1)	0.3 (0-0.7)	0 (0-0.2)	1.2 (0.05-3.15)
Any stool shedding, n (% total)							
All	54/113 (47%)	9/19 (47%)	15/27 (56%)	9/10 (90%)	8/18 (44%)	3/12 (25%)	10/27 (37%)
Enteric fever diagnosed (ED)	36/58 (62%)	7/12 (58%)	7/12 (58%)	7/7 (100%)	5/10 (50%)	2/3 (67%)	8/14 (57%)
Enteric fever NOT diagnosed (nED)	18/75 (24%)	2/7 (29%)	8/15 (53%)	2/3 (67%)	3/8 (38%)	1/9(11%)	2/13 (15%)

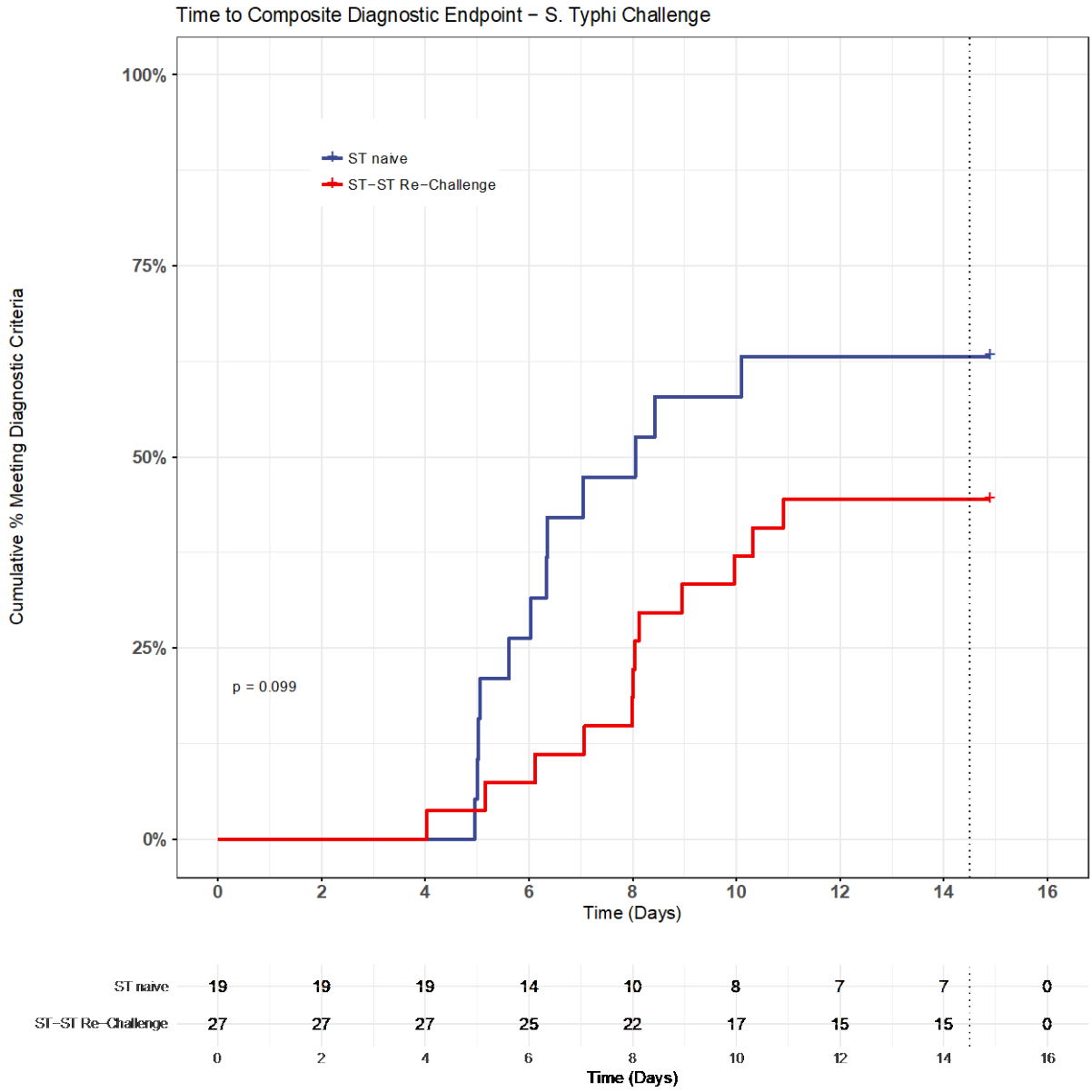


Figure 7-4 : Time to Diagnosis after naïve *S.* Typhi challenge and homologous *S.* Typhi Re-challenge.

Kaplan-Meier survival cumulative incidence of participants meeting the composite diagnostic endpoint, measured from challenge agent ingestion to development of first fever ≥38°C or first positive blood culture sampling. Non-diagnosed participants censored at day 14 hours. P value from log-rank test. ST = S. Typhi naïve challenge. ST-ST = Homologous Re-Challenge with S. Typhi.

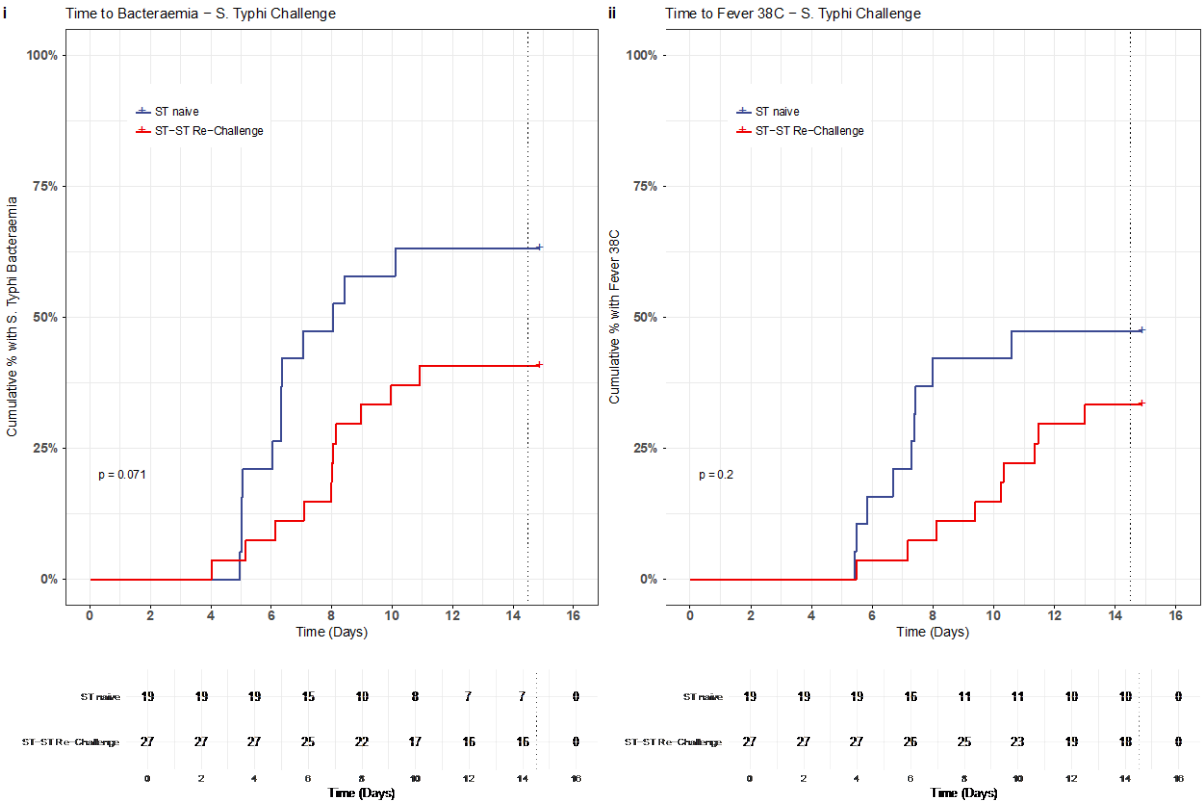


Figure 7-5 : Time to bacteraemia and first fever following *S.* Typhi challenge in naïve and homologous re-challenge participants.

(i) Time to first detected S. Typhi bacteraemia after challenged. Participants who did not meet the diagnostic criteria for typhoid diagnosis were censored at day 14. (ii) Time to first fever >38°C. Kaplan-Meier survival curve showing the cumulative proportion of participants with any fever >38°C by challenge group. Oral temperature measurements were recorded twice daily (morning and evening) Participants with no recorded fever were censored at the day of last monitoring (day 14). Shaded areas represent 95% confidence intervals for individual survival curves. p = log rank test.

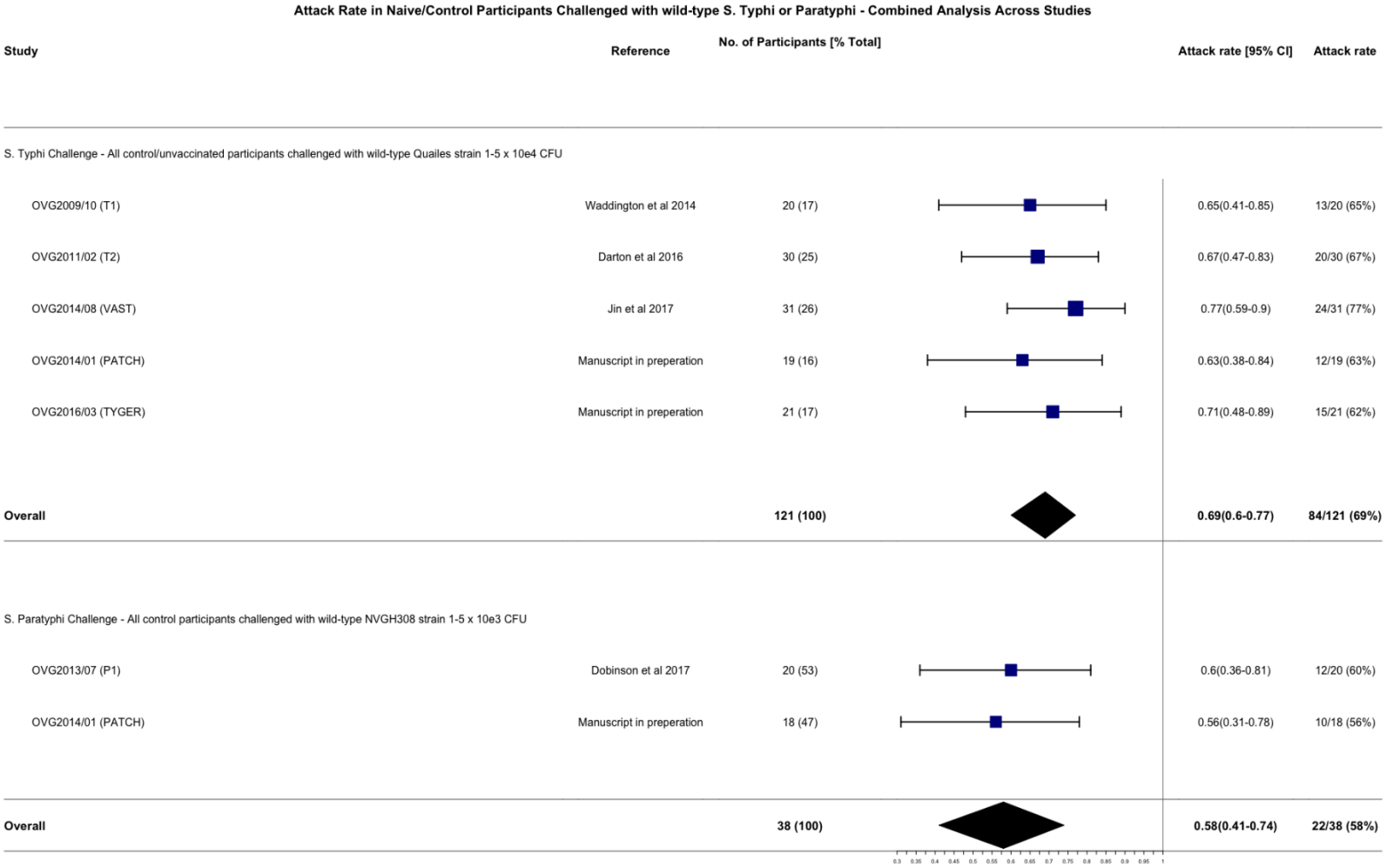


Figure 7-6 : Combined Attack Rates in *S.* Typhi and *S.* Paratyphi challenge studies.

Forest plot illustrating attack rates in naive cohorts of S. typhi (top) and S. Paratyphi (bottom) challenge studies.

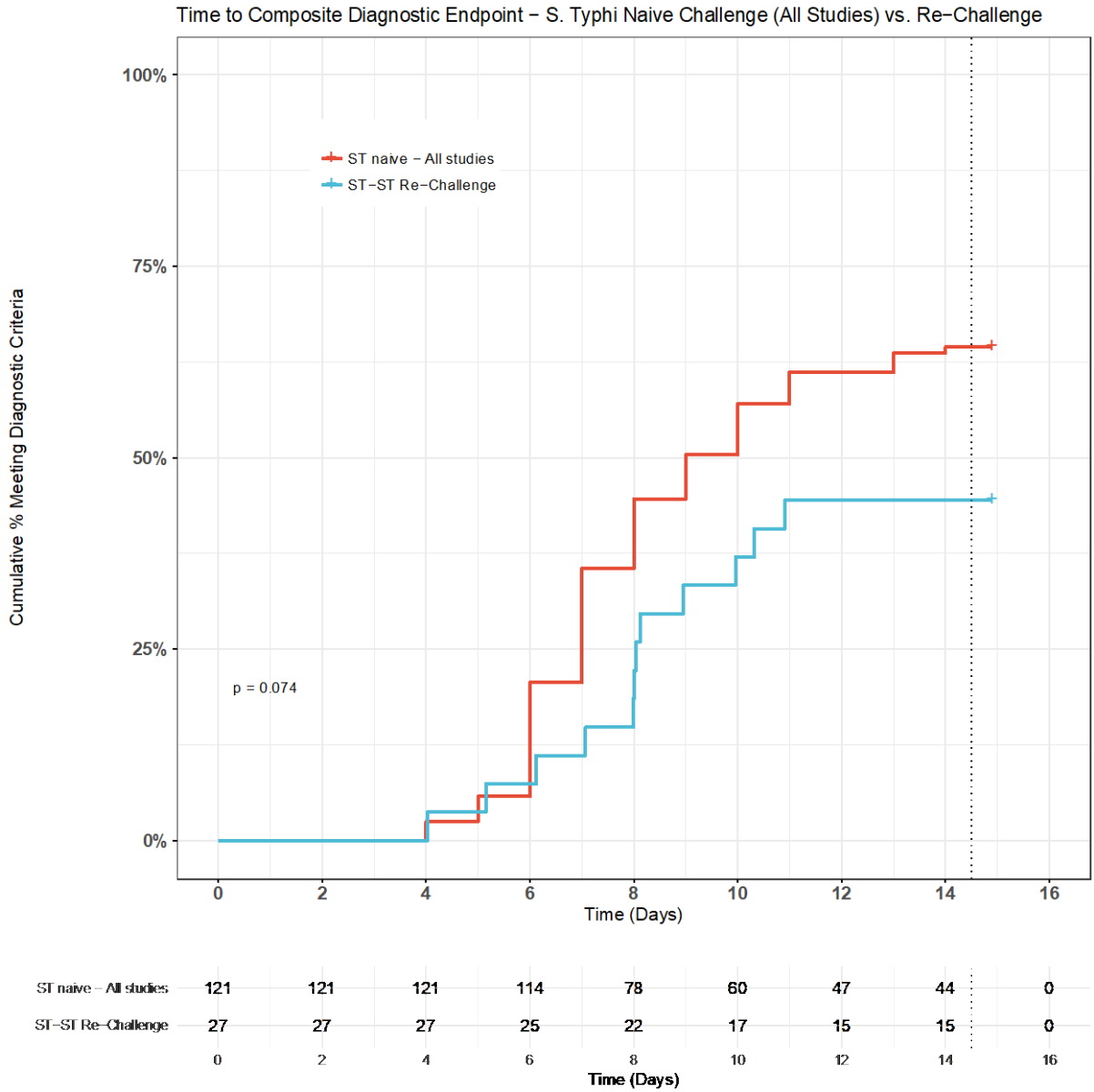


Figure 7-7 : Comparison with combined *S.* Typhi challenge controls. Time to Diagnosis after naïve *S.* Typhi challenge and homologous *S.* Typhi Re-challenge.

Cumulative incidence of typhoid fever after challenge in naïve (ST) and homologous re-challenge (ST-ST) groups. Time to composite diagnostic endpoint, measured from challenge agent ingestion to development of first fever ≥38°C or first positive blood culture sampling. Non-diagnosed participants censored at day 14 hours. P value from log-rank test. ST = Combined *S.* Typhi naïve challenge (**Figure 7-6**). ST-ST = Homologous Re-Challenge with S. Typhi.

7.2.3. Heterologous Re-Challenge

Ten participants with previous exposure to *S.* Paratyphi underwent heterologous re-challenge with *S.* Typhi, after a median interval of 17.3 months (range 13.9-24.9 - Table 7-6). Overall, we observed no significant cross-protection between *S.* Typhi and *S.* Paratyphi. Typhoid fever occurred in 7/10 (70%) of participants challenged with *S.* Typhi following prior *S.* Paratyphi challenge compared with 12/19 (63%) in the naïve cohort (RR 1.11; 95%CI 0.58 to 1.87; p = 0.99), with a comparable time to diagnosis (7.0[4.1-8.9] vs. 6.2 [5-10.1]; p=0.84 - Figure 7-8). Similarly, no significant difference was observed when we compared secondary endpoints of time to bacteraemia and time to fever between naïve and heterologous re-challenge groups (Figure 7-9).

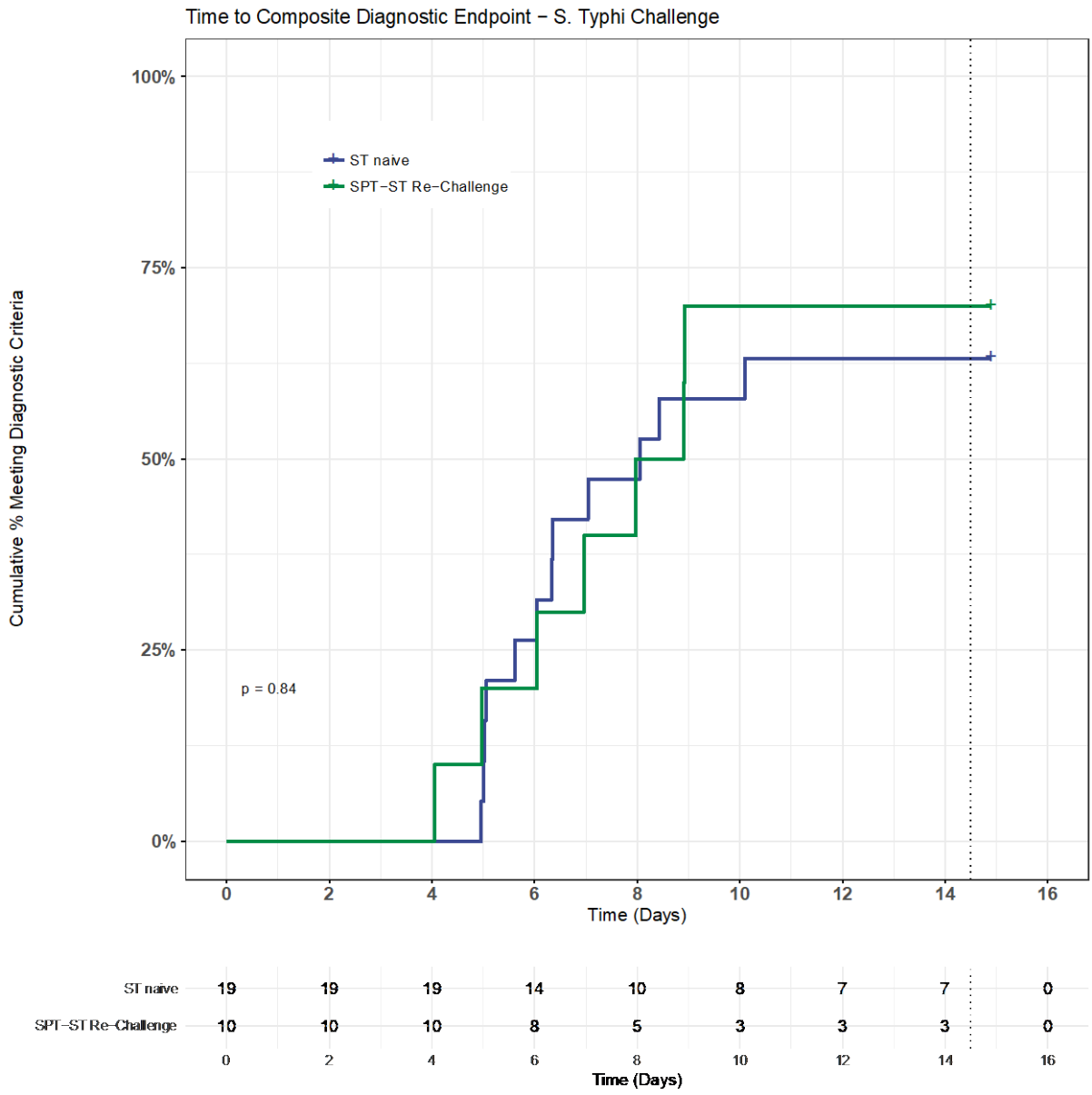


Figure 7-8 : Time to Diagnosis after naïve *S.* Typhi challenge and heterologous *S.* Typhi Re-challenge.

Cumulative incidence of typhoid fever after naïve challenge and heterologous S. Typhi re-challenge. Time to composite diagnostic endpoint, measured from challenge agent ingestion to development of first fever ≥38°C or first positive blood culture sampling. Non-diagnosed participants censored at day 14 hours. P value from log-rank test. ST = S. Typhi naïve challenge. SPT-ST Re-Challenge = Heterologous re-challenge with S. Typhi following previous S. Paratyphi challenge.

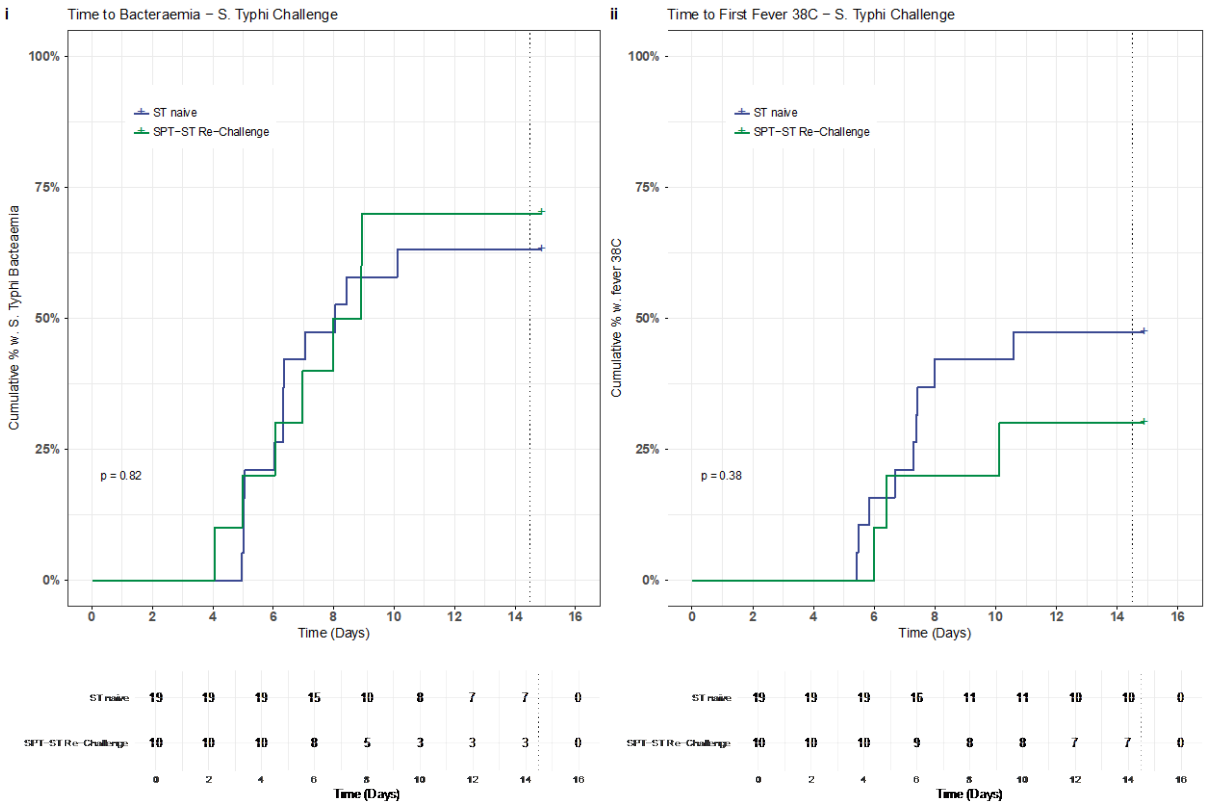


Figure 7-9 : Heterologous Re-Challenge. Time to bacteraemia and first fever following *S.* Typhi challenge in naïve and heterologous re-challenge participants.

(i) Time to first detected S. Typhi bacteraemia after challenged. Participants who did not meet the diagnostic criteria for typhoid diagnosis were censored at day 14. (ii) Time to first fever >38°C. Kaplan-Meier survival curve showing the cumulative proportion of participants with any fever >38°C by challenge group. Oral temperature measurements were recorded twice daily (morning and evening) Participants with no recorded fever were censored at the day of last monitoring (day 14). Shaded areas represent 95% confidence intervals for individual survival curves. p = log rank test.

7.2.4. Homologous *Salmonella* Paratyphi A Re-Challenge

A total of 58 participants were challenged with *S.* Paratyphi A across three groups (Table 7-6). Two participants (one in the naïve cohort and one in the homologous re-challenge cohort) commenced antibiotics prior to meeting the diagnostic endpoint and prior to day 14 and were

excluded from analysis of the primary endpoint[2]. All participants were challenged within the target dose range of 1-5x10^3 CFU.

Of the naïve participants challenged with *S*. Paratyphi, 10/18 (56%) met the composite primary endpoint This attack rate in the naïve cohort (56%) was comparable to the 60% attack rate observed in earlier challenge studies[311] although lower than the target attack rate of 60-75%.

The median interval between primary challenge and re-challenge was 17 months (range 14.2-27.2). Previous challenge with *S*. Paratyphi was associated with a 55% reduced risk of paratyphoid infection compared with naïve controls (3/12[25%] vs. 10/18 [56%]; RR 0.45; 95% CI 0.15 to 1.13; p = 0.14). In addition, the median time to diagnosis was prolonged by approximately 2 days in the paratyphoid re-challenge cohort compared with naïve controls (median days [range] 9.4 [6-11] vs. 7.0 [4.3-12.1]; p = 0.13 - Figure 7-10). Only 1/12 (8%) participants diagnosed with paratyphoid fever in the homologous re-challenge group were febrile at any point, compared with 6/19 (32%) in the naïve group. Homologous re-challenge was also associated with a non-significantly prolonged time to bacteraemia and time to first fever compared with naïve controls (Figure 7-10).

When compared with combined historical naïve controls in a post-hoc analysis, previous challenge with *S.* Paratyphi was also associated with a borderline significant reduction the rate of paratyphoid disease (3/12[25%] vs. 22/38 [58%]; RR 0.43-0.99; p=0.047 Chi-Squared test; p= 0.1 Fishers exact test). Time to diagnosis was also borderline significantly prolonged in the re-challenge group when historical controls were included (p=0.049 - Figure 7-12).

[2] One participant developed diarrhoea at day 3 post challenge and requested to be withdrawn from the study. One participant was commenced on antibiotics in error at day 10 due to a laboratory labelling error.

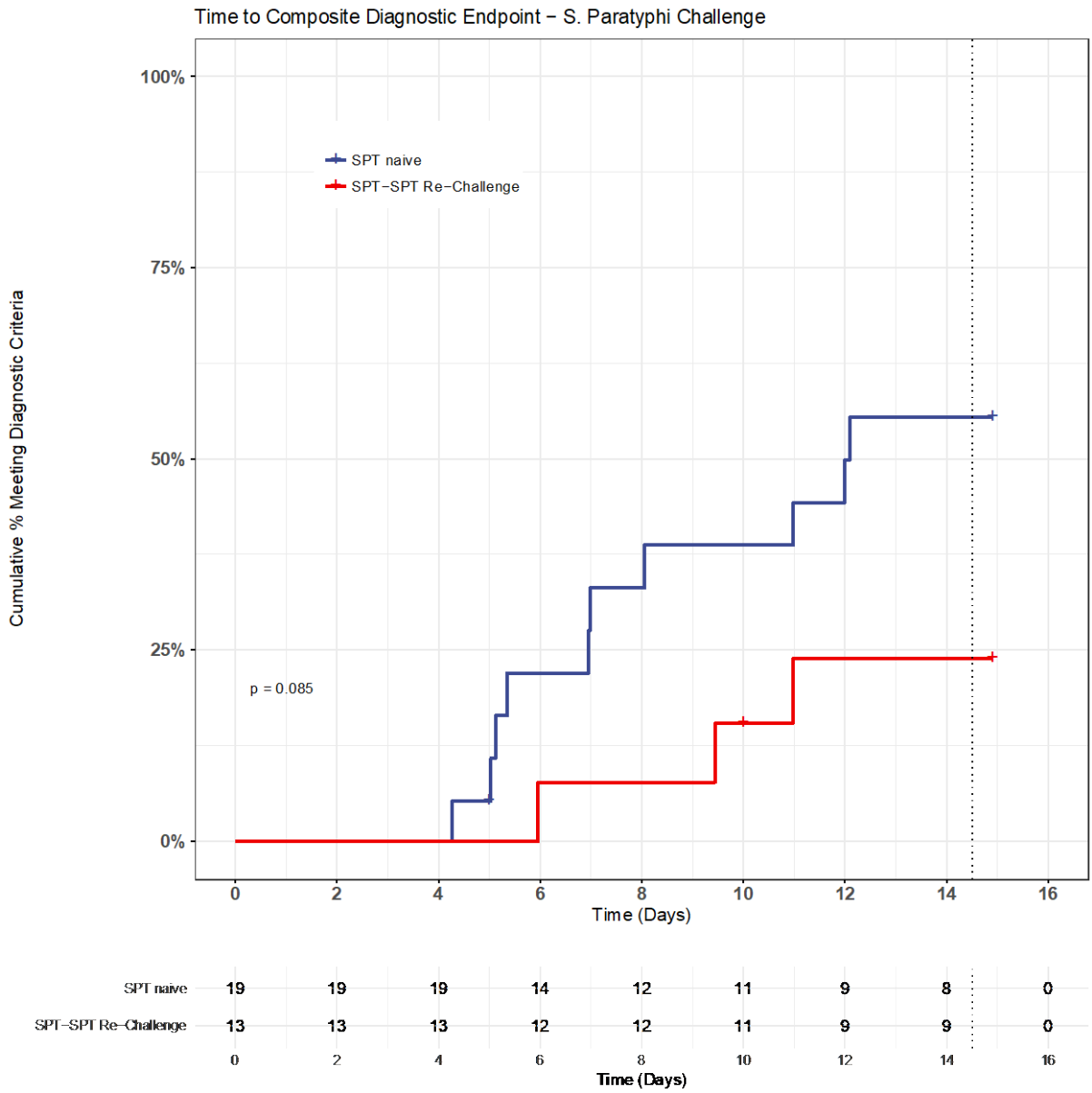


Figure 7-10 : Time to Diagnosis after naïve *S.* Paratyphi challenge and homologous *S.* Paratyphi Re-challenge.

Cumulative incidence of paratyphoid fever after challenge in naïve (SPT) and homologous re-challenge (SPT-SPT) groups. Time to composite diagnostic endpoint, measured from challenge agent ingestion to development of first fever ≥38°C or first positive blood culture sampling. Non-diagnosed participants censored at day 14 hours. P value from log-rank test comparing both groups. SPT = S. Paratyphi naïve challenge. SPT-SPT = Homologous Re-Challenge with S. Paratyphi.

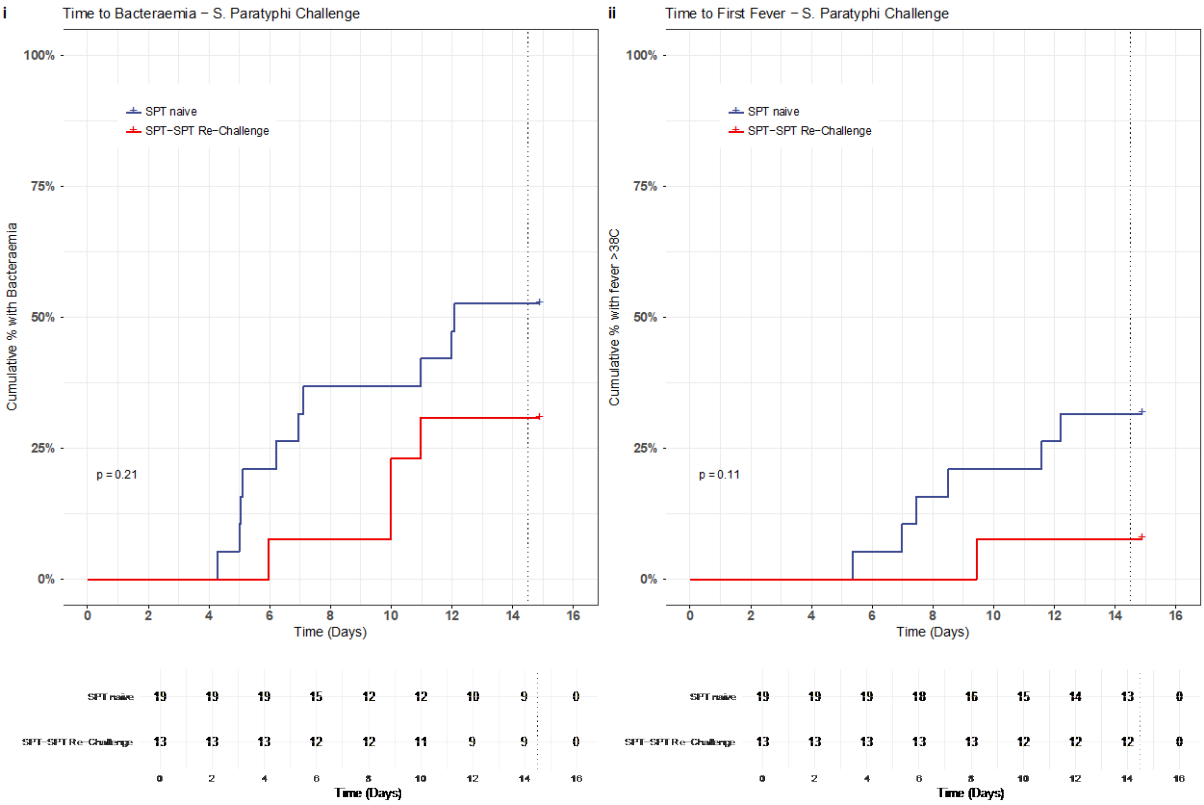


Figure 7-11 : Time to bacteraemia and first fever following *S.* Paratyphi challenge in naïve and homologous *S.* Paratyphi re-challenge participants.

(i) Time to first detected *S.* Paratyphi bacteraemia after challenged. Participants who did not meet the diagnostic criteria for typhoid diagnosis were censored at day 14. (ii) Time to first fever >38°C. Kaplan-Meier survival curve showing the cumulative proportion of participants with any fever >38°C by challenge group. Oral temperature measurements were recorded twice daily (morning and evening) Participants with no recorded fever were censored at the day of last monitoring (day 14). Shaded areas represent 95% confidence intervals for individual survival curves. p = log rank test.

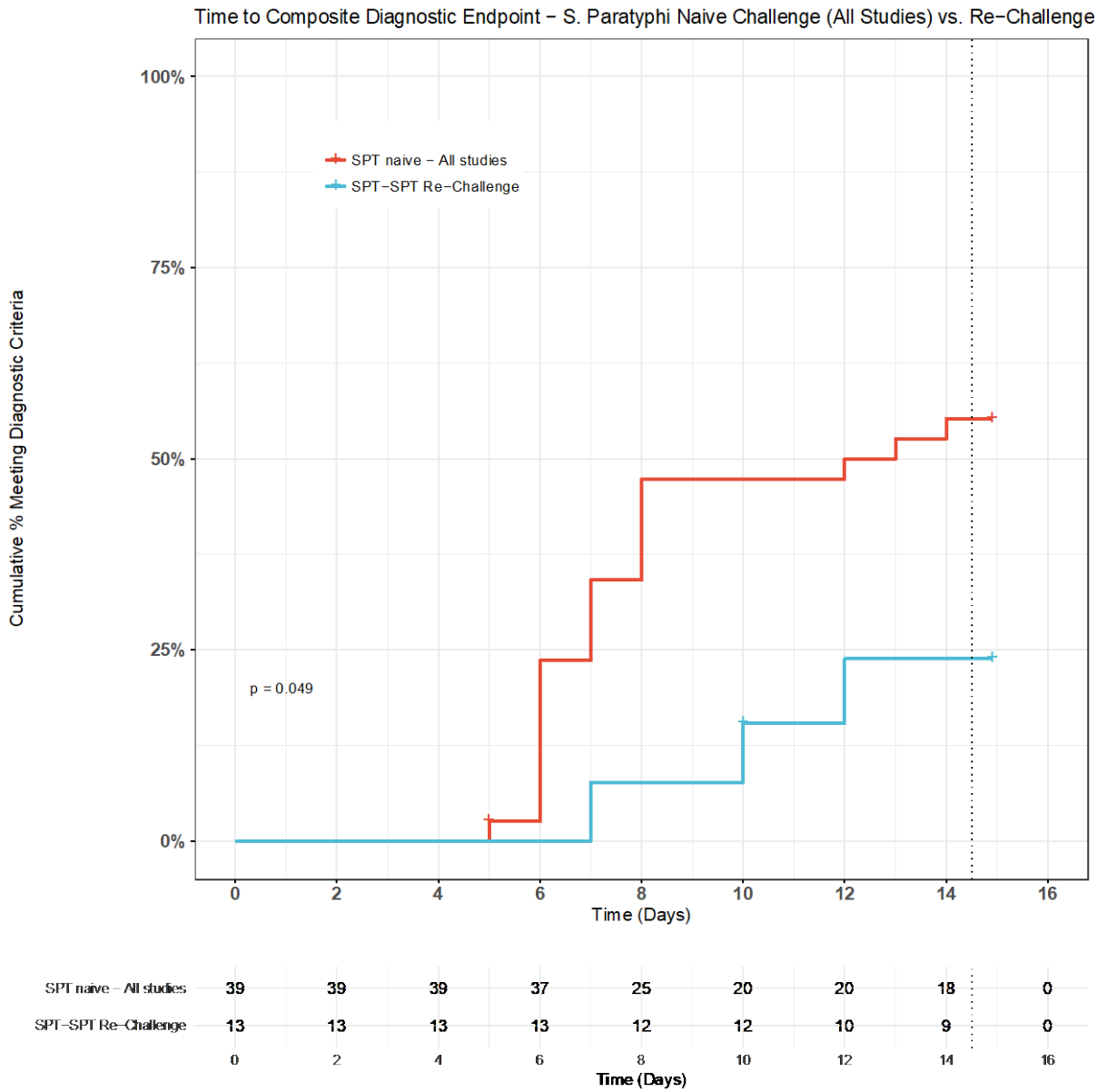


Figure 7-12 : Comparison with combined *S.* Paratyphi challenge controls. Time to Diagnosis after naïve *S.* Paratyphi challenge and homologous *S.* Paratyphi Re-challenge.

Cumulative incidence of paratyphoid fever after challenge in naïve (SPT) and homologous re-challenge (SPT-SPT) groups. Time to composite diagnostic endpoint, measured from challenge agent ingestion to development of first fever ≥38°C or first positive blood culture sampling. Non-diagnosed participants censored at day 14 hours. P value from log-rank test comparing. SPT = *S.* Paratyphi naïve challenge. SPT-SPT = Homologous Re-Challenge with *S.* Paratyphi.

7.2.5. Heterologous re-challenge

A total of 27 participants with previous exposure to *S*. Typhi underwent heterologous re-challenge with *S*. Paratyphi. The median interval between primary challenge and re-challenge was 39 months (range 12.2-67.9). The attack rate in the *S.* Paratyphi heterologous re-challenge group was not reduced compared with naïve controls (14/27 (52%) vs. 10/18 (56%); RR 0.93; 95% CI 0.54 to 1.69; p =0.99 Fishers exact test - Figure 7-13 Table 7-6). In keeping with this, there was no difference in time to bacteraemia and time to first fever following heterologous re-challenge (Figure 7-14).

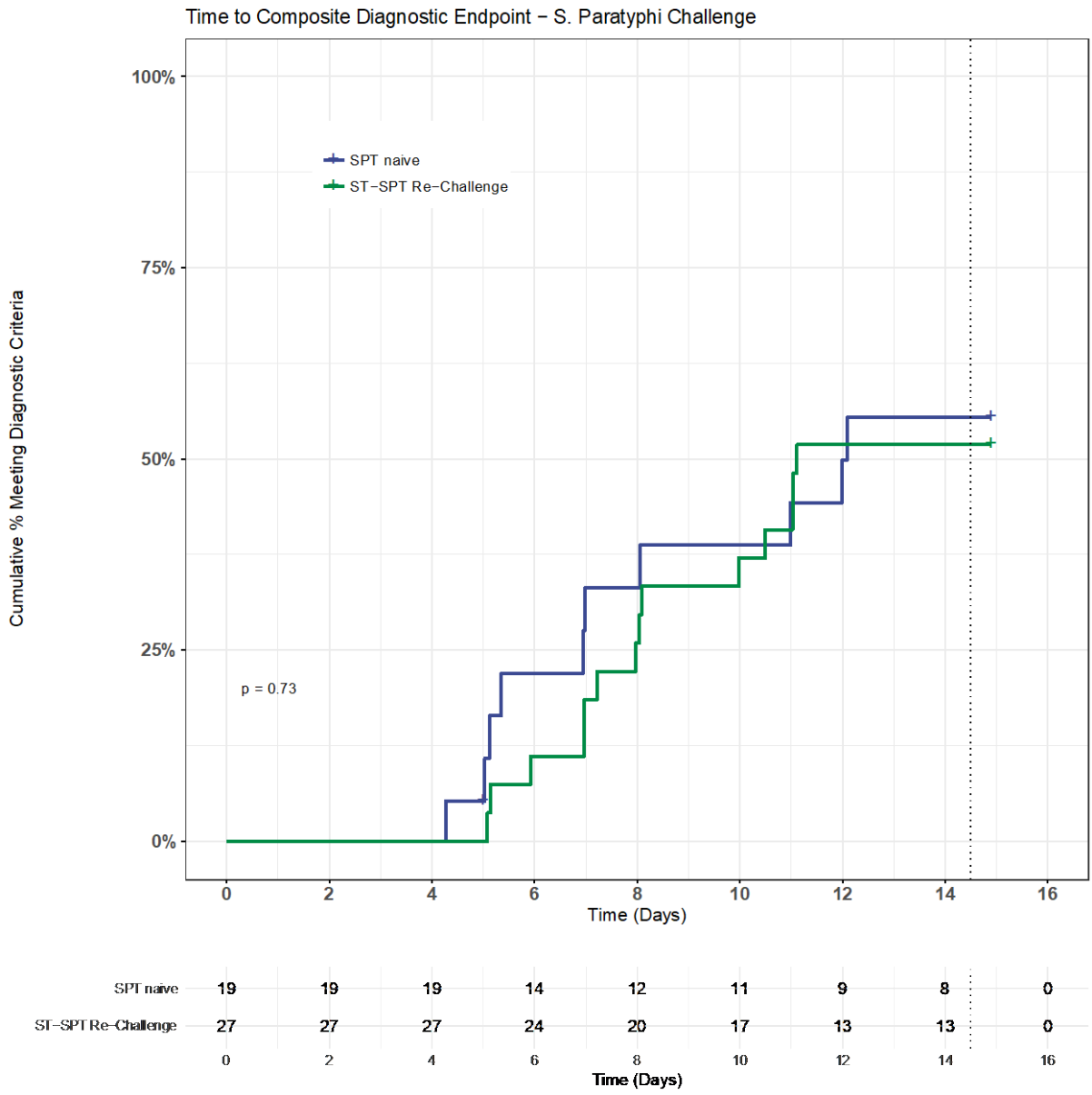


Figure 7-13 : Heterologous S. Paratyphi re-challenge. Time to Diagnosis after naïve *S.* Typhi challenge and heterologous *S.* Paratyphi Re-challenge

Cumulative incidence of paratyphoid fever after naïve challenge and heterologous S. Paratyphi re-challenge. Time to composite diagnostic endpoint, measured from challenge agent ingestion to development of first fever ≥38°C or first positive blood culture sampling. Non-diagnosed participants censored at day 14 hours. P value from log-rank test. SPT = S. Typhi naïve challenge. ST-SPT Re-Challenge = Heterologous re-challenge with S. Paratyphi following previous S. Typhi challenge.

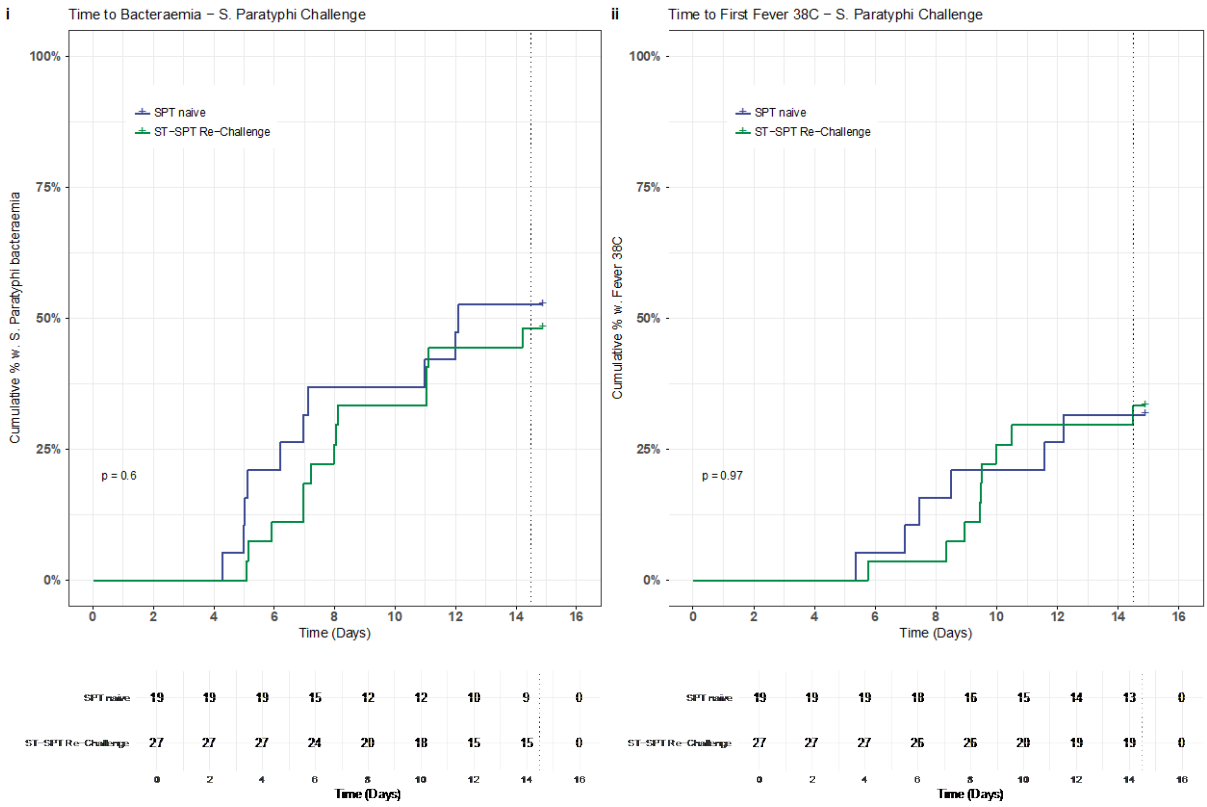


Figure 7-14 : Time to bacteraemia and first fever following *S.* Paratyphi challenge in naïve and heterologous S. Paratyphi re-challenge participants.

(i) Time to first detected *S.* Paratyphi bacteraemia after challenged. Participants who did not meet the diagnostic criteria for typhoid diagnosis were censored at day 14. (ii) Time to first fever >38°C. Kaplan-Meier survival curve showing the cumulative proportion of participants with any fever >38°C by challenge group. Oral temperature measurements were recorded twice daily (morning and evening) Participants with no recorded fever were censored at the day of last monitoring (day 14). Shaded areas represent 95% confidence intervals for individual survival curves. p = log rank test.

7.2.6. Alternative Diagnostic Criteria

I compared attack rates between naïve and re-challenge groups using a series of different disease definitions, including an endpoint that approximates the presentation of typhoid in a field setting (fever ≥38^{o}C preceding bacteraemia). Regardless of the disease definition that was applied, we observed no significant difference in the attack rate of typhoid fever between naïve and re-challenge groups (Table 7-7).

Table 7-7 : Attack rates according to alternative diagnostic criteria.

ST = S. Typhi. SPT = S. Paratyphi A. p = Fishers exact test.

	Challenge Group					
	S. Typhi Challenge			S. Paratyphi Challenge		
	ST Naïve	ST-ST Re-Challenge	SPT-ST Re-Challenge	SPT Naïve	SPT-SPT Re-Challenge	ST-SPT Re-Challenge
	A1	B1	B3	A2	B4	B2
Composite Criteria, n (% attack rate)	12/19 (63%)	12/27 (44%)	7/10 (70%)	10/18 (56%)	3/12 (25%)	14/27 (52%)
RR (95% CI)	-	0.7 (0.40-1.23)	1.10 (0.59-1.87)	-	0.15 (0.15-1.14)	0.93 (0.54-1.68)
p	-	0.24	0.99	-	0.14	0.99
Fever ≥38˚C (any duration)	9/19 (47%)	9/27 (33%)	3/10 (30%)	6/18 (33%)	1/12 (8%)	9/27 (33%)
RR (95% CI)	-	0.70 (0.35-1.45)	0.63 (0.21-1.60)	-	0.25 (0.04 – 1.29)	1 (0.45-2.35)
p	-	0.37	0.44	-	0.19	0.99
Fever ≥38·0˚C (any duration) + *S.* Typhi bacteraemia	9/19 (47%)	7/27 (26%)	3/10 (30%)	5/18 (27%)	1/12 (8%)	8/27 (30%)
RR (95% CI)	-	0.54 (0.25-120)	0.63 (0.21-1.60)	-	0.3 (0.05-1.61)	1.07 (0.44 -2.8)
p	-	0.20	0.44	-	0.35	0.99
Fever ≥38·0˚C (any duration) with subsequent bacteraemia	0/19 (0%)	4/27 (15%)	0/10 (0%)	2/18 (11%)	1/12 (8%)	2/27 (7%)
RR (95% CI)	-	-	-	-	0.75 (0.10 – 5.14)	0.67 (0.12 – 3.6)
p	-	-	-	-	0.99	0.99
S. Typhi bacteraemia OR stool shedding	14/19 (74%)	20/27 (74%)	9/10 (90%)	14/18 (77%)	5/12 (42%)	16/27 (59%)

RR (95% CI)	-	1 (0.71-1.51)	1.22 (0.77 – 1.80)	-	0.75 (0.10 – 5.14)	0.76 (0.50 – 1.17)
p	0	0.99	0.63	-	0.06	0.30
S. Typhi bacteraemia AND stool shedding	7/19 (37%)	6/27 (22%)	7/10 (70%)	4/18 (22%)	2/12 (17%)	7/27 (26%)
RR (95% CI)	-	0.6 (0.24-1.49)	1.9 (0.9 – 3.9)	-	0.75 (0.18-2.96)	1.17 (0.43-3.38)
p	-	0.33	0.12	-	0.99	0.99
Any Shedding	9/19 (47%)	15/27 (56%)	9/10 (90%)	9/18 (50%)	4/12 (33%)	10/27 (37%)
RR (95% CI)	-	1.17 (0.67-2.19)	1.9 (1.10 – 3.37)	-	0.67 (0.25-1.54)	0.74 (0.38 – 1.48)
p	-	0.76	0.043	-	0.47	0.54

7.2.7. Safety endpoints

All study participants were successfully treated, with no episodes of disease relapse or recrudescence. One participant had convalescent shedding of *S.* Paratyphi in the stool following a 14-day course of ciprofloxacin. Following treatment with azithromycin, all subsequent stool samples were negative.

Four serious adverse events were reported during the course of the study (Table 7-8). Six participants met the criteria for severe enteric fever across all challenge groups (Table 7-9). Hypokalaemia was observed in five participants, which was attributed to pseudohypokalaemia resulting from delayed sample transport over periods of high ambient temperature[381].

Table 7-8 : Serious adverse events OVG2014/01 study.

Date reported	Challenge Group	Criteria	Event	Challenge related	Comment
18/05/2016	*S.* Typhi (Naïve)	In-patient hospitalisation or prolongation	Collapse	No	Episode of collapse with loss of consciousness on 12 months after challenge. Reported left arm and left leg weakness 24 hours' duration. Diagnosed as possible generalised seizure following neurology review. Instructed not to drive for 6/12. No medication prescribed, and no subsequent events.
18/11/16	*S.* Paratyphi (Naïve)	In-patient hospitalisation or prolongation	Hospital admission for IV fluids	Yes	Nausea and vomiting, unresponsive to oral anti-emetics. Tachycardia. Admitted overnight for IV fluids and anti-emetics. Treatment switched to IV Ceftriaxone. Discharged after <24 hours and completed course of oral ciprofloxacin.
24/04/17	NA	Medically important event	Renal Colic	No	Right loin pain with evidence of renal calculus on CT KUB. Diagnosed after enrolment but prior to challenge. Withdrawn from study and not challenged.
06/06/2017	*S.* Paratyphi (Heterologous re-challenge ST-SPT)	Medically important event	Raised ALT	Yes	Alanine aminotransferase elevated to 898 IU/L 5 days after diagnosis. Ascribed to paratyphoid fever plus possible adverse drug reaction (azithromycin + paracetamol). Antibiotic switched from azithromycin to ciprofloxacin. Paracetamol withheld. Resolved.

Table 7-9 : Participants meeting pre-specified criteria for severe enteric fever OVG2014/01 study.

Challenge Group	Criteria	Event	Challenge related	Comment
S. Typhi Naïve	Grade 4 laboratory abnormality	Hypokalaemia	No	K+ 2.9 mmol/l At time of typhoid diagnosis
S. Typhi Naïve	Grade 4 laboratory abnormality	Hypokalaemia	No	K+ 2.8 mmol/l 24 hours after typhoid diagnosis
S. Paratyphi (Heterologous re-challenge ST-SPT)	Grade 4 laboratory abnormality	Hypokalaemia	No	K+ 2.9 mmol/l
S. Paratyphi (Heterologous re-challenge ST-SPT)	Grade 4 laboratory abnormality	Hypokalaemia	No	K+ 2.9 mmol/l 48hours after typhoid diagnosis
S. Paratyphi (Heterologous re-challenge ST-SPT)	Grade 4 laboratory abnormality	Hypokalaemia	No	K+ 2.8 mmol/l
S. Typhi (Heterologous re-challenge SPT-ST)	Grade 4 laboratory abnormality	Elevated CRP	Yes	CRP 231mmol/l 48 hours after typhoid diagnosis

7.2.8. Clinical Endpoints

In order to determine if prior exposure was associated with an altered clinical phenotype, we compared solicited symptoms between naïve and re-challenge groups. The most common symptoms reported by all participants diagnosed with enteric fever were headache (97%), malaise (90%), anorexia (78%) and myalgia (78%). Symptom profiles and disease severity are shown in Figure 7-15. Symptom profiles were broadly similar between all study groups, with the exception of participants diagnosed in the *S.* Paratyphi homologous re-challenge group who reported fewer severe symptoms. The severity of disease was also assessed using a symptom severity score as previously described [311]. There was marked heterogeneity in the severity of

symptoms reported, with some participants reporting symptoms characteristic of enteric fever syndrome, whilst others reported few or no symptoms despite bacteraemia (Figure 7-16). Overall, we observed no difference in the severity of disease between all individuals diagnosed with typhoid or paratyphoid fever. Similarly, there was no significant difference in the symptom severity scores between naïve and re-challenge groups when comparing cumulative symptom scores between day 0 and day 21 (Figure 7-16).

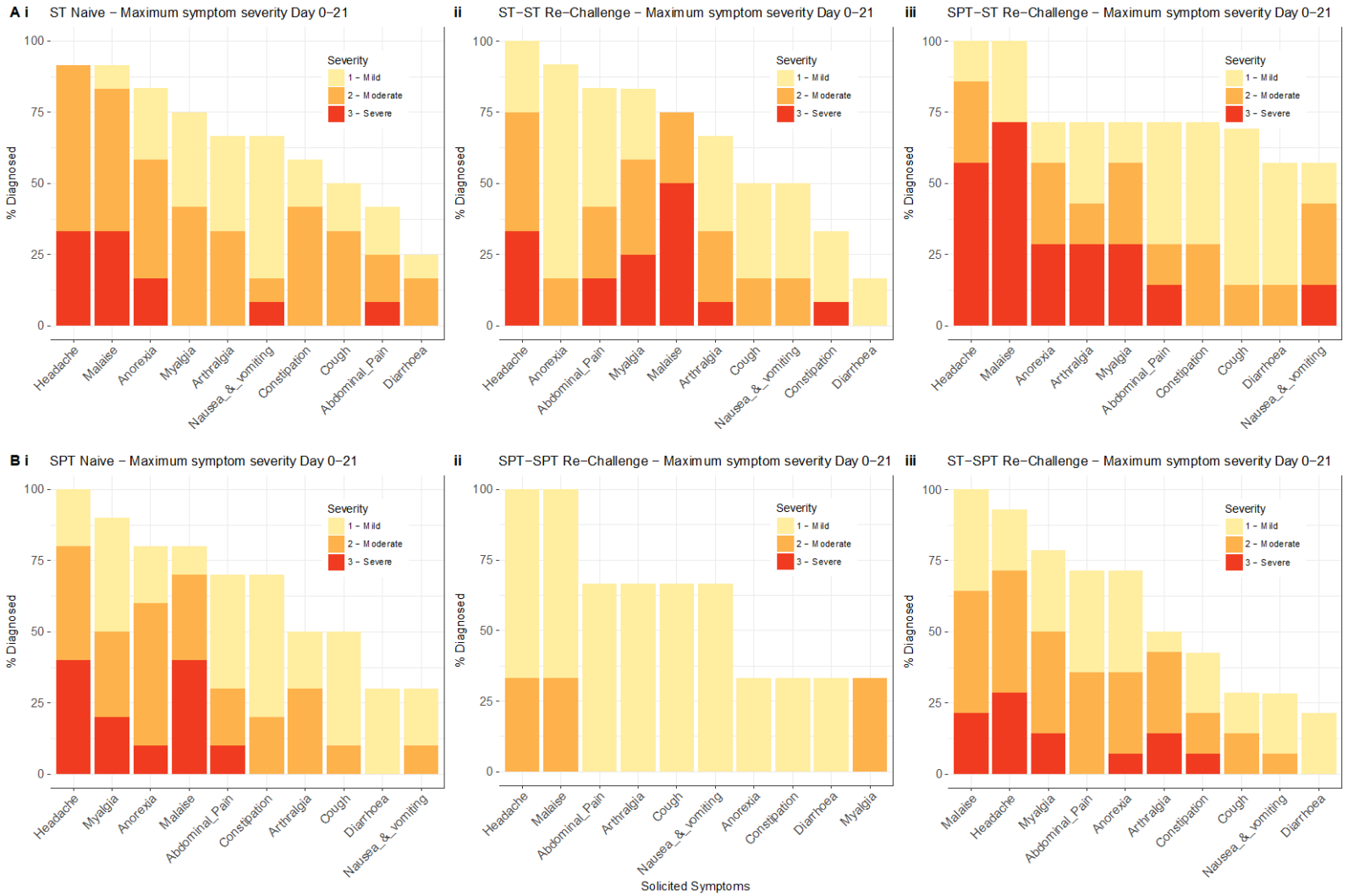


Figure 7-15 : Maximum symptom severity score.

Participants diagnosed with typhoid (A) or paratyphoid (B) fever categorised according to challenge agent and study group. ST = S. Typhi, SPT = S. Paratyphi. Percentage of participants reporting one or more events. Symptoms were recorded using an electronic diary from Day 0 up to Day 21 post challenge. Stacked columns display percentage of participants reporting maximum symptom severity graded as mild (present but no interference with daily activity), moderate (some limitation of daily activity) or severe (unable to perform normal daily activity)

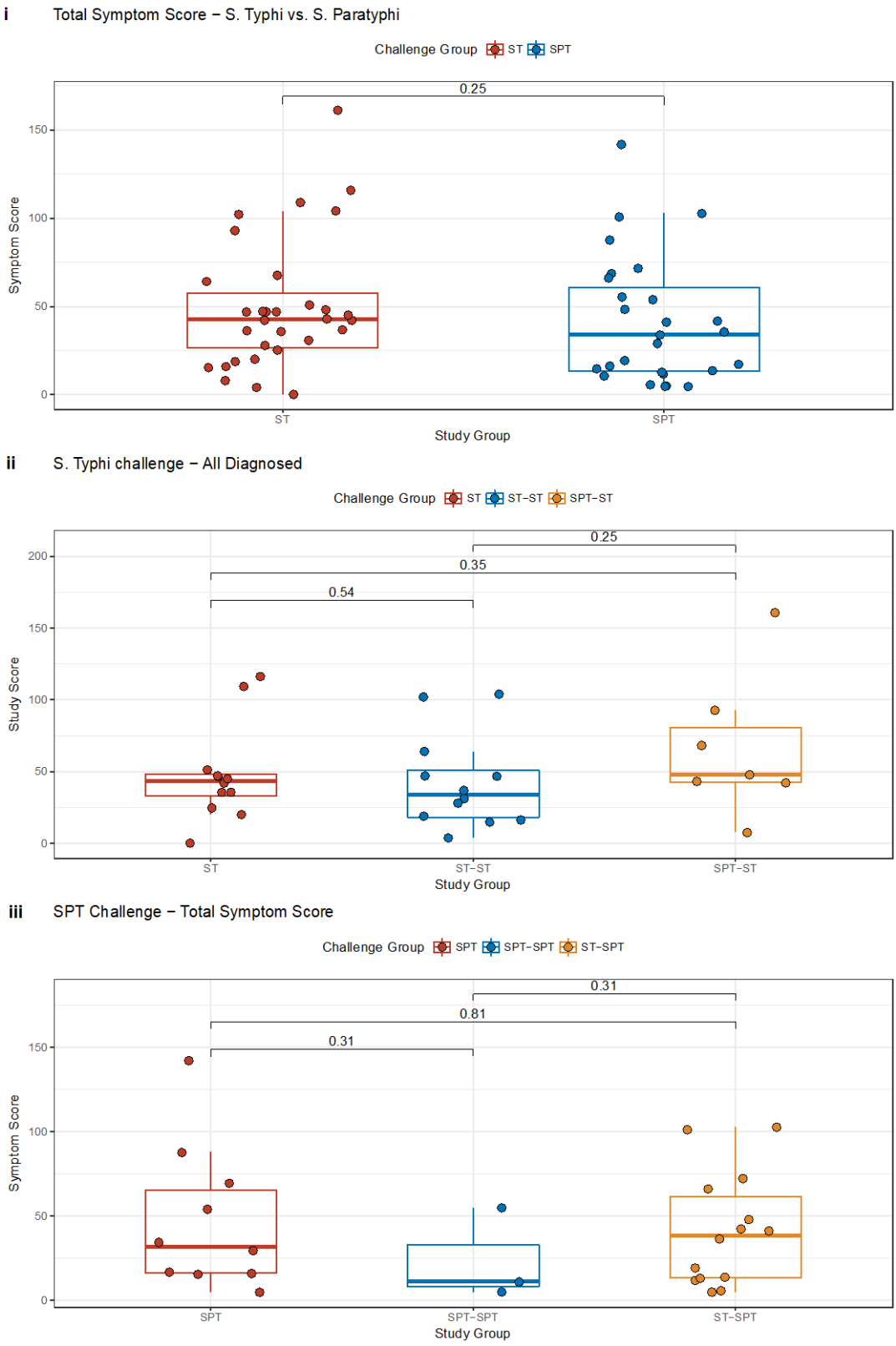


Figure 7-16 : Total symptom score by study group OVG2014/01 study.

Comparison of cumulative symptom severity scores in participants diagnosed with enteric fever categorised according to study group. Symptom scores were calculated by summing numerical values assigned to the severity of individual symptoms between day 0 and day 21 (0, not present; 1, mild; 2, moderate; 3, severe). Total symptom score ST = S. Typhi, SPT = S. Paratyphi. p = Mann-Whitney U test.

Solicited symptom scores are potentially subject to reporting bias and inter-individual variability in severity grading. To assess a more objective measure of clinical disease severity, we compared fever clearance times in participants diagnosed with typhoid or paratyphoid fever. There was no significant difference in the proportion of participants recording any fever between naïve and re-challenge groups (Table 7-7). At the time of diagnosis, 18/31 (58%) and 14/27 (52%) of participants diagnosed with typhoid or paratyphoid were febrile. We observed no difference in fever clearance times between individuals diagnosed with typhoid or paratyphoid fever (Median [IQR] hrs 44.3[40-95.6] vs. 35.8[9.9-69.6]; p=0.86 - Figure 7-17). Fever clearance times were also comparable between naïve and re-challenge sub-groups (Figure 7-18). Overall, this suggests that prior exposure was not associated with an altered rate of fever nor fever clearance time.

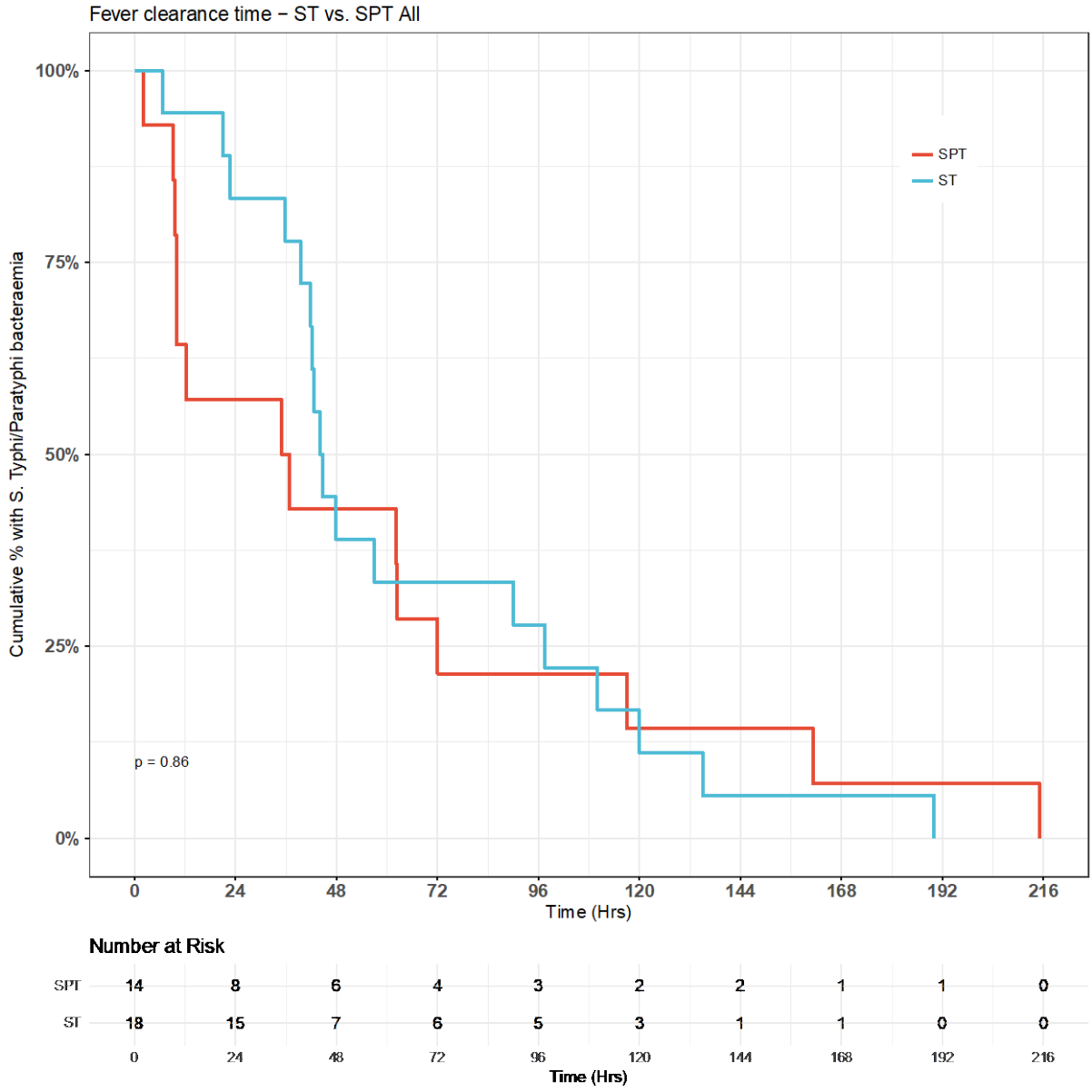


Figure 7-17 : Fever Clearance Time according to challenge agent allocation.

Kaplan-Meier survival curve showing the cumulative proportion of participants with fever at the time of treatment initiation remaining febrile by challenge group.

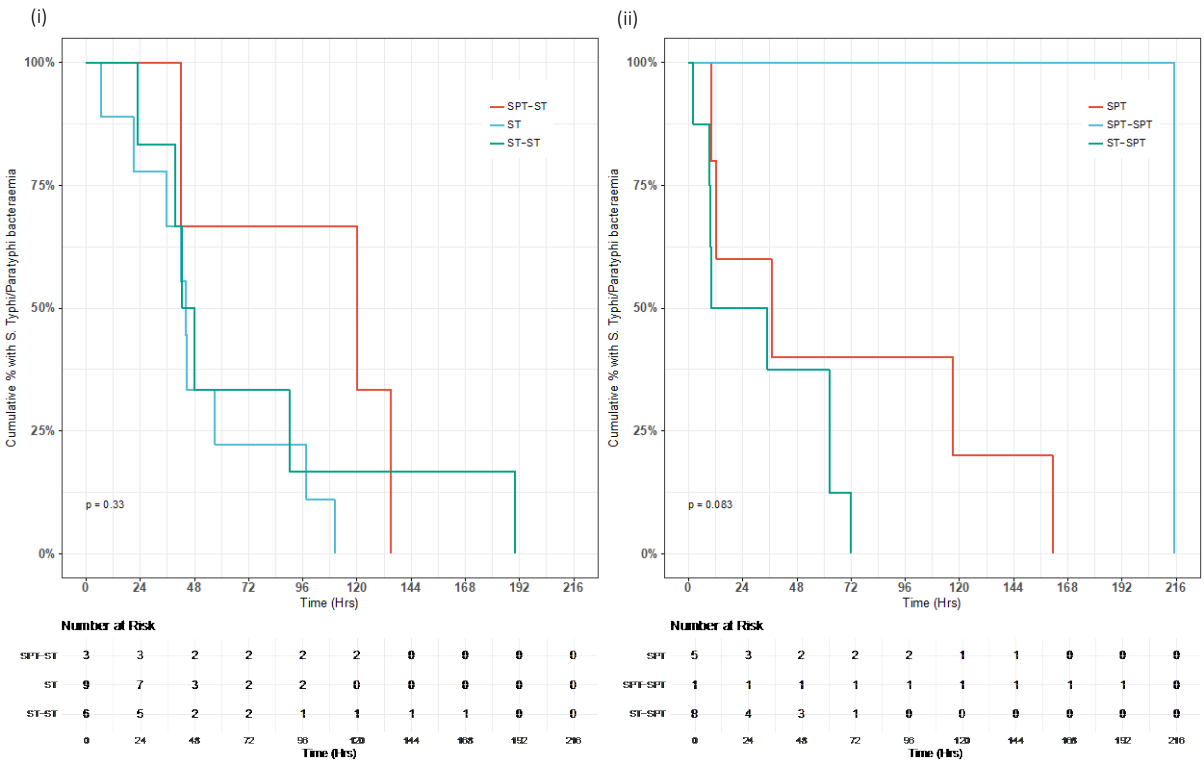


Figure 7-18 : Fever clearance time according to study sub-group.

Kaplan-Meir plots illustrating cumulative proportion with fever at the time of treatment initiation remaining febrile by study group (i) S. Typhi challenge; (ii) S. Paratyphi challenge.

7.2.9. Microbiological Endpoints

7.2.9.1. *S.* Typhi and Paratyphi bacteraemia

The pattern of bacteraemia following challenge with *S.* Typhi and Paratyphi is illustrated in Figure 7-19. There was marked heterogeneity in the pattern of bacteraemia, with some participants having a single positive blood culture, whilst others were bacteraemic for a total of 5-6 days (Figure 7-19). In participants who met the composite criteria for typhoid or paratyphoid fever, the diagnosis was confirmed by positive blood culture in 30/31 (97%) and 25/27 (93%) cases respectively. The duration of *S.* Paratyphi bacteraemia after treatment initiation was generally prolonged compared with *S.* Typhi bacteraemia, although did not meet significance (blood culture clearance time, median [IQR] hours 81.4 [63.8-96] vs. 40.8 [21-92]; p=0.39 - Figure 7-20). There was no significant difference in the duration of bacteraemia between naïve and re-challenge

groups, although the small numbers per group complicate the interpretation of these observations (Figure 7-21 & Table 7-6).

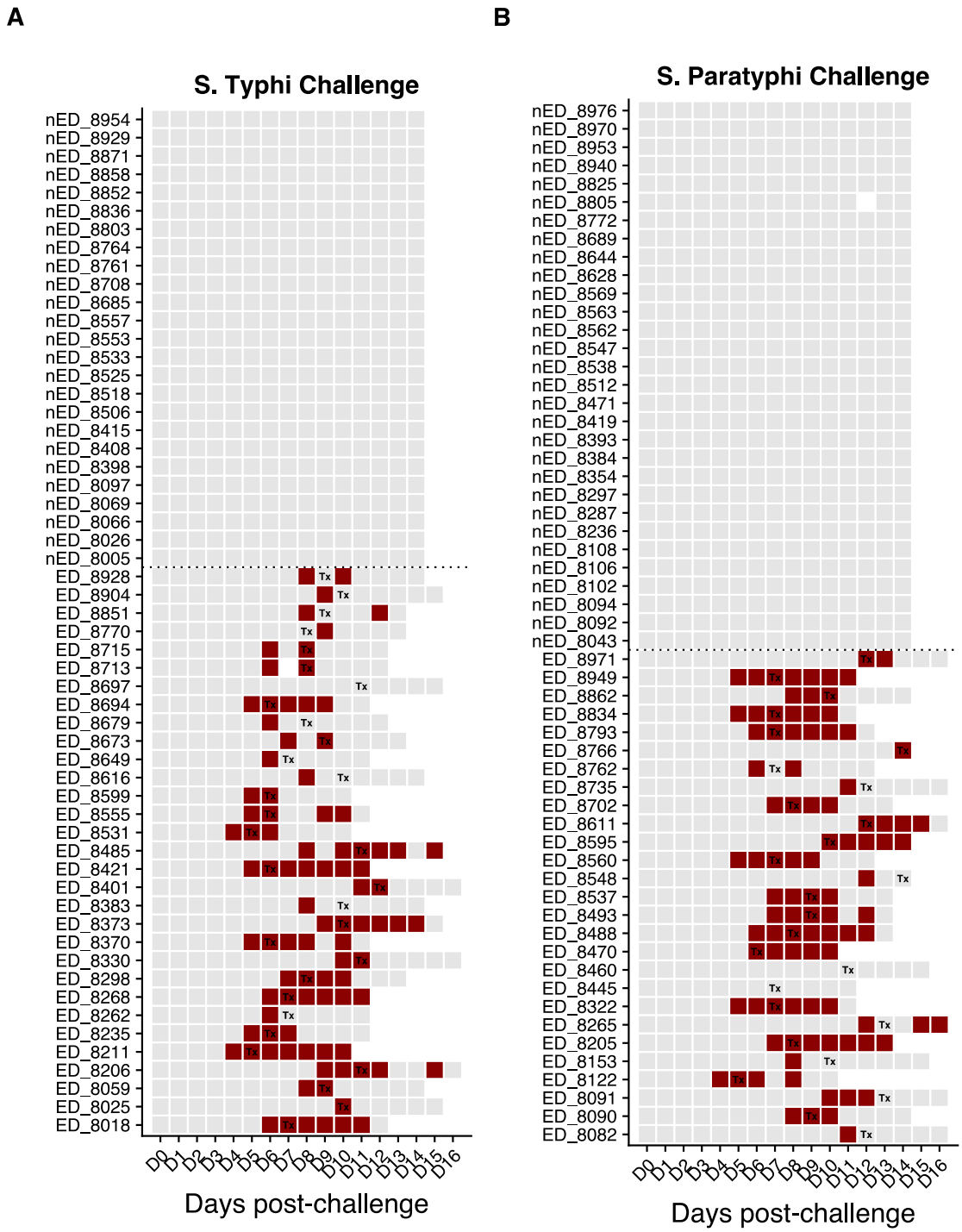


Figure 7-19 : Pattern of bacteraemia following *S.* Typhi and *S.* Paratyphi challenge.

Each row corresponds to an individual participant. Grey squares = Negative sample, Red squares = Positive blood culture, White squares = No sample collected. Tx = Day of treatment initiation. Participants above the dotted line did not meet the composite criteria for typhoid or paratyphoid diagnosis.

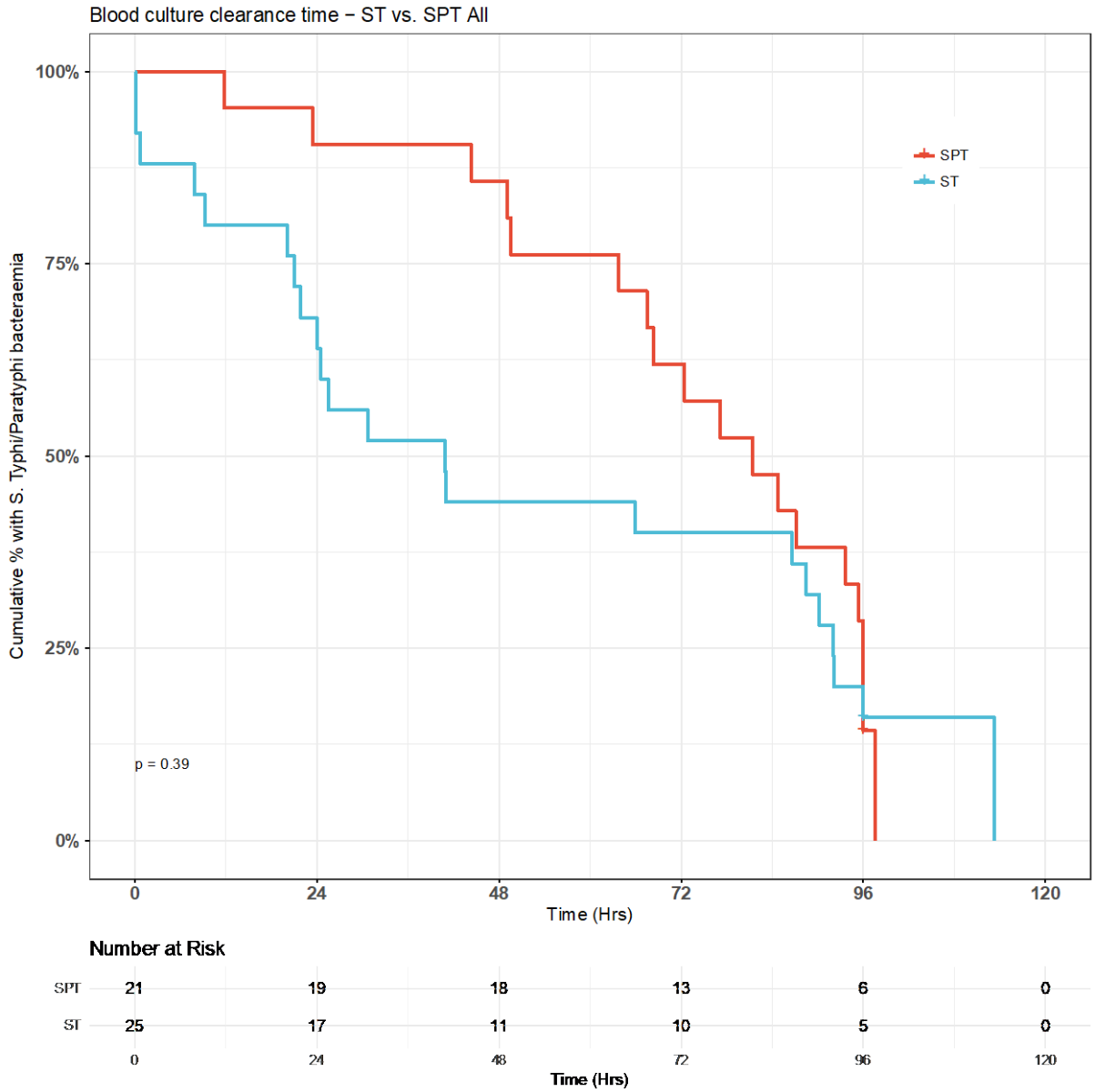


Figure 7-20 : Comparison of blood culture clearance time following S. Typhi and S. Paratyphi challenge.

(A) Blood culture Clearance time. Kaplan-Meier survival curve showing cumulative proportion of participants with Salmonella Typhi or Paratyphi bacteraemia from initiation of treatment to first persistently negative blood culture. Participants without contiguous negative blood culture beyond 96 hours from initiation of antibiotics (last routine follow-up visit) were censored at the time of last positive blood culture sampling.

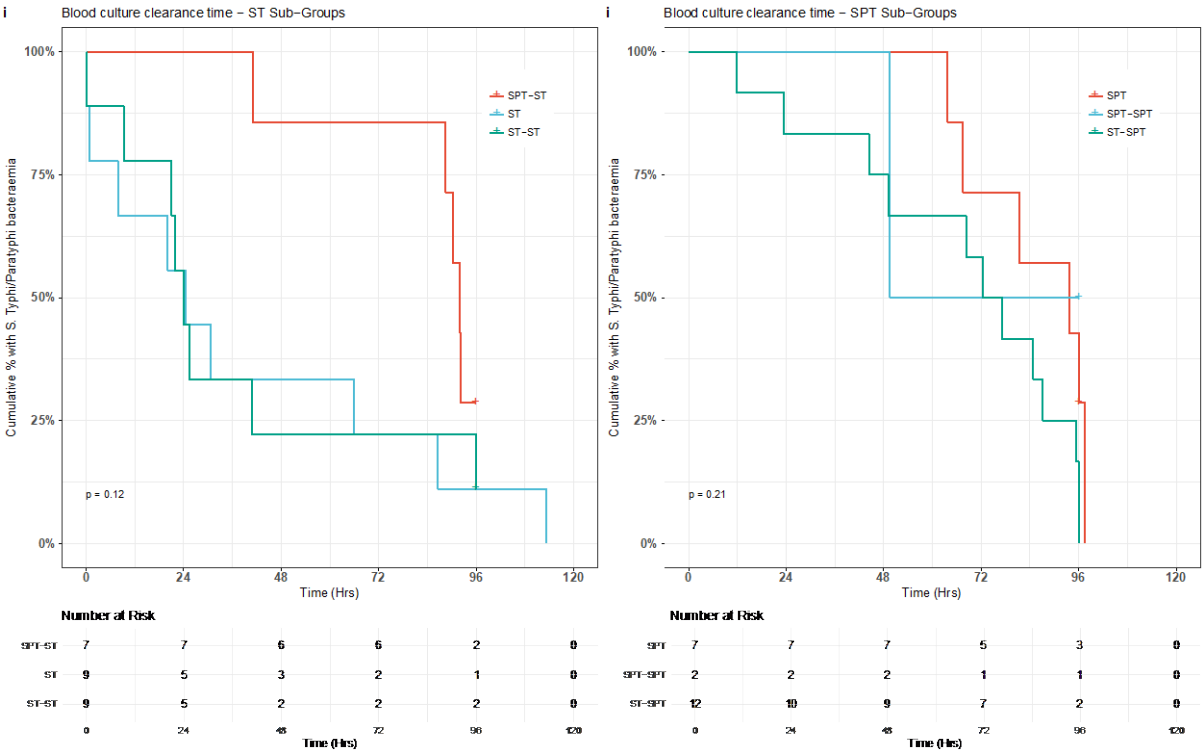


Figure 7-21 : Blood culture clearance times according to study sub-groups.

Kaplan-Meir plots illustrating cumulative proportion with bacteraemia after treatment initiation. (i) S. Typhi challenge sub-groups. (ii) S. Paratyphi challenge sub-groups. Participants without contiguous negative blood culture beyond 96 hours from initiation of antibiotics (last routine follow-up visit) were censored at the time of last positive blood culture sampling.

7.2.10. Quantitative blood culture

Consistent with our previously published data[311], we observed no difference in the number of circulating bacteria at the time of typhoid or paratyphoid diagnosis (Median [IQR] 0.1 CFU/ml [0-1.1.8] vs. 0.2 CFU/ml [0-2.4]; p=0.46) (Figure 7-23). In order to determine if previous challenge was associated with a reduced bacteraemia at the time of diagnosis, we then compared quantitative blood culture between all challenge groups. In all study groups compared, we observed no significant difference in the number of circulating organisms at the time of typhoid or paratyphoid diagnosis (p=0.74 and 0.16 respectively, Kruskal-Wallis test - Figure 7-23 & Table 7-6).

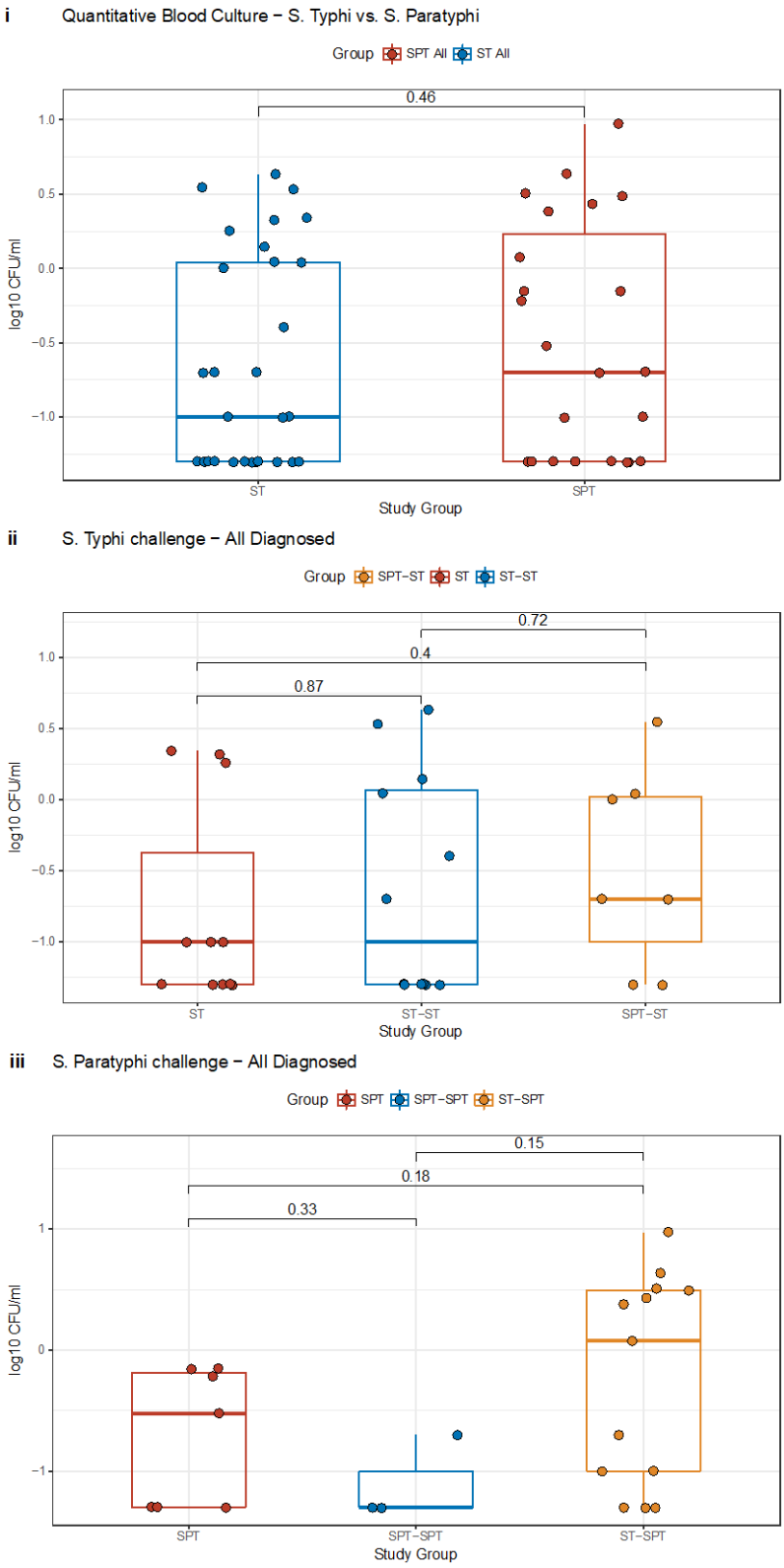


Figure 7-22 : Quantitative blood culture at time of enteric fever diagnosis.

Values are presented as log_{10} CFU/ml. (i) Comparison of all participants diagnosed with typhoid (ST) vs. paratyphoid (SPT) fever; (ii) Comparison quantitative blood culture values in S. Typhi challenge sub-groups. ST = ST naïve, ST-ST = Homologous S. Typhi re-challenge, SPT-ST = Heterologous S. Typhi re-challenge; (iii) Comparison quantitative blood culture values in S. Paratyphi challenge sub-groups. SPT = S. Paratyphi naïve, SPT-SPT = Homologous S. Paratyphi re-challenge, ST-SPT = Heterologous S. Paratyphi re-challenge. P = Mann Whitney U test.

7.2.11. Stool shedding

The pattern of stool shedding after *S.* Typhi and *S.* Paratyphi challenge is illustrated in Figure 7-23. There was marked heterogeneity in the pattern of shedding both between and within groups diagnosed and not diagnosed with enteric fever. Participants who met the diagnostic criteria for enteric fever were significantly more likely to have at least one positive stool culture than participants who did not develop disease (36/58 [62%] vs. 18/75 [24%]; RR 2.59 [95%CI 1.67-4.10]; p<0.0001 -Table 7-6). The same pattern was observed when typhoid and paratyphoid challenge groups were analysed separately, although only met significance in the paratyphoid challenge group (TD vs. nTD 21/31 [68%] vs. 12/24 [50%]; RR 1.36 (0.87-2.260; p=0.26, and PD vs. nPD 15/27 [56%] vs. 6/30 [20%]; RR 2.78 [1.32-6.20]; p=0.007).

Notably, almost a quarter of participants 18/75 (24%) who did not develop disease shed *S.* Typhi/Paratyphi in stool, demonstrating that a proportion of individuals exposed to typhoidal *Salmonella* will act as an 'intermediate host' and transiently shed into the environment in the absence of overt clinical disease. Factors impacting stool shedding across all challenge studies are discussed in detail in Chapter 9.

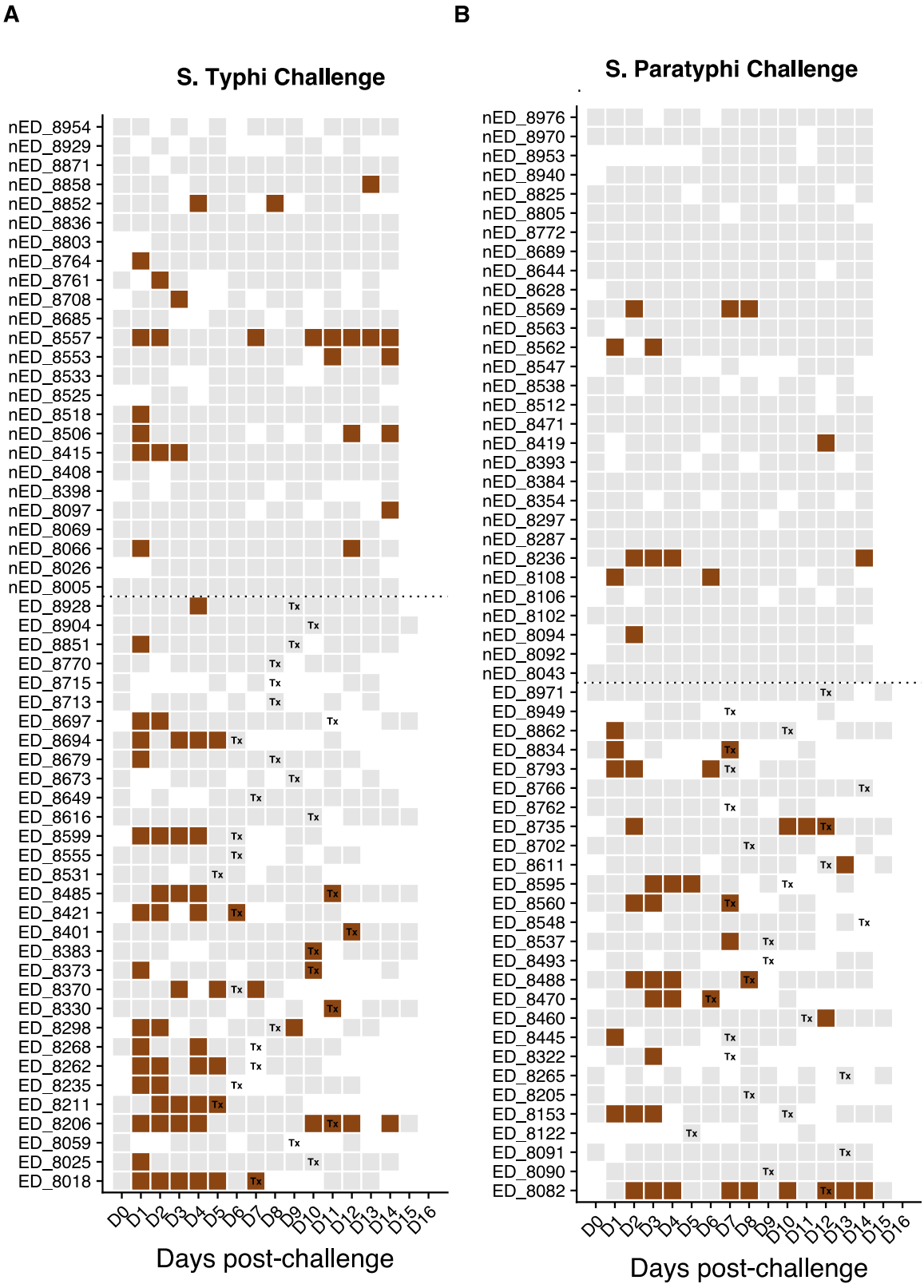


Figure 7-23 : Pattern of stool shedding after *S.* Typhi and *S.* Paratyphi challenge.

Each row corresponds to an individual participant. Grey squares = Negative sample, Brown squares = Positive stool culture, White squares = No sample collected. Tx = Day of treatment initiation. Participants above the dotted line did not meet the composite criteria for typhoid diagnosis.

7.2.12. Impact of Antibiotic Choice on Treatment Response

Mid-way through study recruitment, data from a contemporaneous typhoid CHI comparing treatment response to azithromycin and ciprofloxacin became available. These data suggested that participants randomised to azithromycin had a significantly delayed treatment response compared with volunteers randomised to ciprofloxacin (OVG2014/08 – C Jin personal communication). Based upon these data, the data safety monitoring committee recommended changing first line antibiotic treatment from azithromycin to ciprofloxacin [209]. Between March 2015 to October 2016, participants were treated with was oral azithromycin 500mg once daily for 14 days as first line treatment. From October 2016 to August 2018, first line treatment was changed to ciprofloxacin 500mg twice-daily for 14 days.

Treatment responses to azithromycin and ciprofloxacin are detailed in (Table 7-10). Thirty-one participants met the criteria for typhoid diagnosis, of whom 20/31 (65%) were treated with azithromycin and 11/31 (35%) were treated with ciprofloxacin. In an intention to treat analysis, the duration of *S*. Typhi bacteraemia was significantly prolonged in individuals treated with azithromycin compared with those treated with ciprofloxacin - median 86.6 hours (range 0.17-113) vs. 22.5 (0.1-88.4), p = 0.005 log rank test (Figure 7-24). The fever clearance time was also significantly prolonged in typhoid participants treated with azithromycin compared with those treated with ciprofloxacin (median 90 hours (range 21-190) vs. 44 (6.6-135)]; p=0.24), without meet the significance threshold.

Twenty-seven participants met the criteria for paratyphoid diagnosis across all groups, of whom 15/27 (56%) were treated with azithromycin and 12/27 (44%) were treated with ciprofloxacin. The duration of *S*. Paratyphi bacteraemia was significantly prolonged in individuals treated with azithromycin compared with those treated with ciprofloxacin - median 94.8 hours [range 11.8-97.6] vs. 72.4 hours [23.4 – 95.3], p = 0.028 (Figure 7-24). Fever clearance time was also prolonged in those treated with azithromycin, without meeting significance (62.3 [9.3-215] vs. 34.8 [2-72];

p = 0.17), likely due to the small sample size in this group. These data imply that the choice of treatment has a greater impact on clinical and microbiological outcome measures than prior immune exposure.

Table 7-10 : Comparison of response to treatment with azithromycin and ciprofloxacin

Participants diagnosed with typhoid and paratyphoid fever OVG2014/01 study. Prolonged treatment response was defined as persistent S. Typhi or S. Paratyphi positive blood culture and/or persistent fever ≥38°C for ≥72 hours after commencing antibiotics and/or change in antibiotic treatment due to adverse reactions or clinical concerns. P=Fisher's exact test for categorical variables and log-rank test for time-to-event data. ns = p>0.05.

	OVG2014/01 Study: *S.* Typhi Challenge			OVG2014/01 Study: *S.* Paratyphi Challenge		
	Azithromycin	Ciprofloxacin	p	Azithromycin	Ciprofloxacin	p
Any *S.* Typhi/Paratyphi bacteraemia	20/20 (100%)	10/11 (91%)	ns	14/15 (93%)	11/12 (92%)	ns
S. Typhi/Paratyphi bacteraemia after commencing antibiotics	17/20 (85%)	8/11 (73%)	ns	12/15 (80%)	9/12 (75%)	ns
Time to first negative blood culture (Hours, Median [Range])	86.6 (0.17-113)	22.5 (0.1-88.4)	0.005	94.8 (11.8-97.5)	72.4 (23.4-95.3)	0.03
Clinical Endpoints						
Any fever ≥38°C	10/20 (50%)	10/11 (91%)	0.0472	8/15 (53%)	8/12 (66%)	ns
Fever ≥38°C after commencing antibiotics	9/20 (45%)	9/11 (82%)	ns	7/15 (47%)	7/12 (58%)	ns
Fever Clearance Time <38°C (Hours, Median [Range])	90 (21-190)	44 (6.6-135)	0.24	62.3 (9.25-215)	34.8 (2.08-72)	0.17
Any symptoms of typhoid/paratyphoid fever	20/20 (100%)	11/11 (100%)	ns	15/15 (100%)	11/12 (92%)	ns
Time to complete symptom resolution (Days, Median [Range])	8.5 (0-15)	4 (2-9)	0.004	4 (2-13)	4.5 (0-11)	ns
Prolonged Treatment Response						
All	9/20 (45%)	1/11 (9%)	0.055	9/15 (60%)	5/12 (42%)	ns
Positive blood culture with *S.* Typhi for ≥72 hours	8/20 (40%)	1/11 (9%)	0.106	8/15 (53%)	5/12 (42%)	ns
Persistent fever ≥38°C for ≥72 hours	5/20 (25%)	1/11 (9%)	ns	3/15 (20%)	0/12 (0%)	ns

Both positive blood culture and persistent fever ≥38°C for ≥72 hours	4/20 (20%)	1/11 (9%)	ns	3/15 (20%)	0/12 (0%)	ns
Antibiotic change due to adverse reaction or clinical concern (not meeting above definitions)	3/20 (15%)	0/11 (0%)	ns	4/15 (27%)	2/12 (16%)	ns
Treatment Change						
Antibiotic treatment change (all participants)	3/34 (9%)	0/22 (0%)	ns	4/15 (27%)	2/12 (16%)	ns
Antibiotic treatment change in diagnosed participants	3/20 (15%)	0/11 (0%)	ns	4/15 (27%)	2/12 (16%)	ns

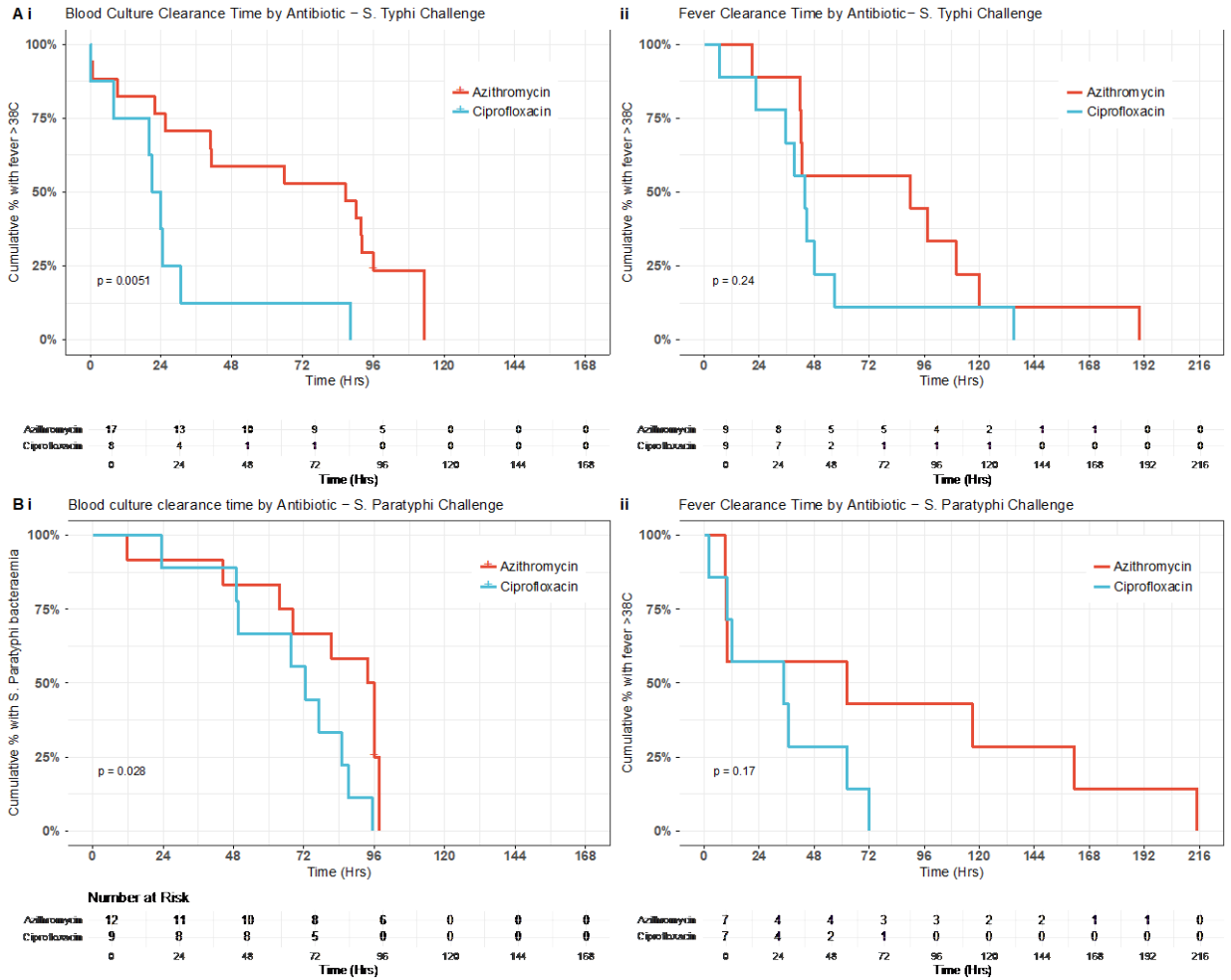


Figure 7-24 : Treatment response by antibiotic treatment in all participants with typhoid and paratyphoid fever.

(A) Blood culture Clearance time. Kaplan-Meier survival curve showing proportion of participants with Salmonella Typhi (A-i) and Salmonella Paratyphi (B-i) bacteraemia from initiation of treatment to first persistently negative blood culture. Participants without contiguous negative blood culture beyond 96 hours from initiation of antibiotics (last routine follow-up visit) were censored at the time of last positive blood culture sampling; (B) Kaplan-Meier survival curve showing the proportion of participants with fever at the time of treatment initiation remaining febrile by treatment group, Salmonella Typhi (A-ii) and Salmonella Paratyphi (B-ii). P = Log rank test.

7.2.13. Principal Component Analysis

In order to determine if previous exposure to *S.* Typhi or Paratyphi was associated with an altered disease presentation on re-challenge, I performed a principal component using a range of clinical, microbiological and laboratory measures collected during the course of the challenge study. This approach offers the theoretical benefit of identifying hidden patterns within the data, that are inapparent when individual measures are analysed independently. When all participants were included in the analysis, the variables with the greatest degree of variation in principle components 1 & 2 were symptoms of malaise, anorexia and headache, combined with temperature and C-reactive protein measurements (Figure 7-25)– likely reflecting differences between participants who did, or did not, develop enteric fever after challenge.

Consistent with this, participants who did meet the criteria for enteric fever formed a distinct cluster in the PCA plot when participants were grouped according to diagnostic outcome (Figure 7-26). The overall clinical presentation and disease severity for participants diagnosed with enteric fever was highly heterogeneous. Notably, some participants who met the criteria for enteric fever clustered with other participants who did not develop disease. These individuals likely had a milder disease phenotype at the time of their diagnosis. It could be speculated that these individuals had a mild disease phenotype that may not have reached clinical attention within a field setting. Alternatively, these individuals may have commenced treatment early in the course of disease preventing the full development of symptoms. In addition, two participants who commenced antibiotics prior to meeting the composite endpoint and day 14, clustered with the non-diagnosed group. In contrast with the distinct clustering that is observed when participants were grouped according to outcome of challenge, no clustering was observed when participants were grouped according to challenge agents (*S.* Typhi vs. Paratyphi - Figure 7-27) or when grouped according to challenge group (naïve vs. re-challenge - Figure 7-28). This analysis suggests that, within the context of the challenge model, participants with prior exposure to *S.*

Typhi or *S.* Paratyphi had a similar clinical phenotype to naïve individuals on exposure to the bacteria

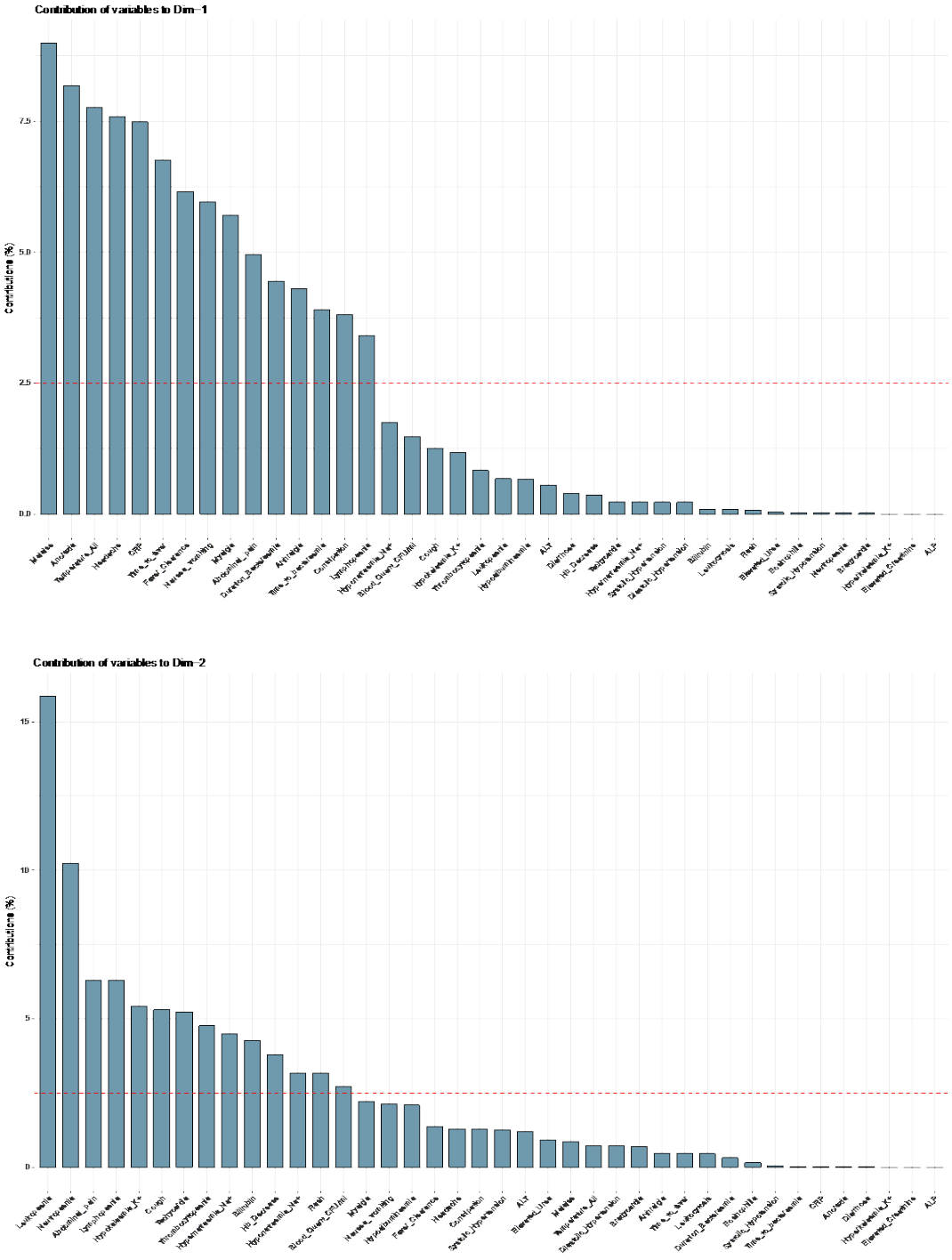


Figure 7-25 : Contributions of individual variables to Principal Component Analysis (OVG2014/01 PATCH study)

(i)Principal component 1 and (ii) Principal component 2 in participants challenged with *S.* Typhi and *S.* Paratyphi, expressed as percentage contribution. Variables listed on the X-axis represent variables included in the original principal component analysis. Red dashed line indicates the expected average contribution if the contribution were uniform, corresponding to 1/n(variables). Variables over the cut-off are considered important contributors to that principal component. [354]

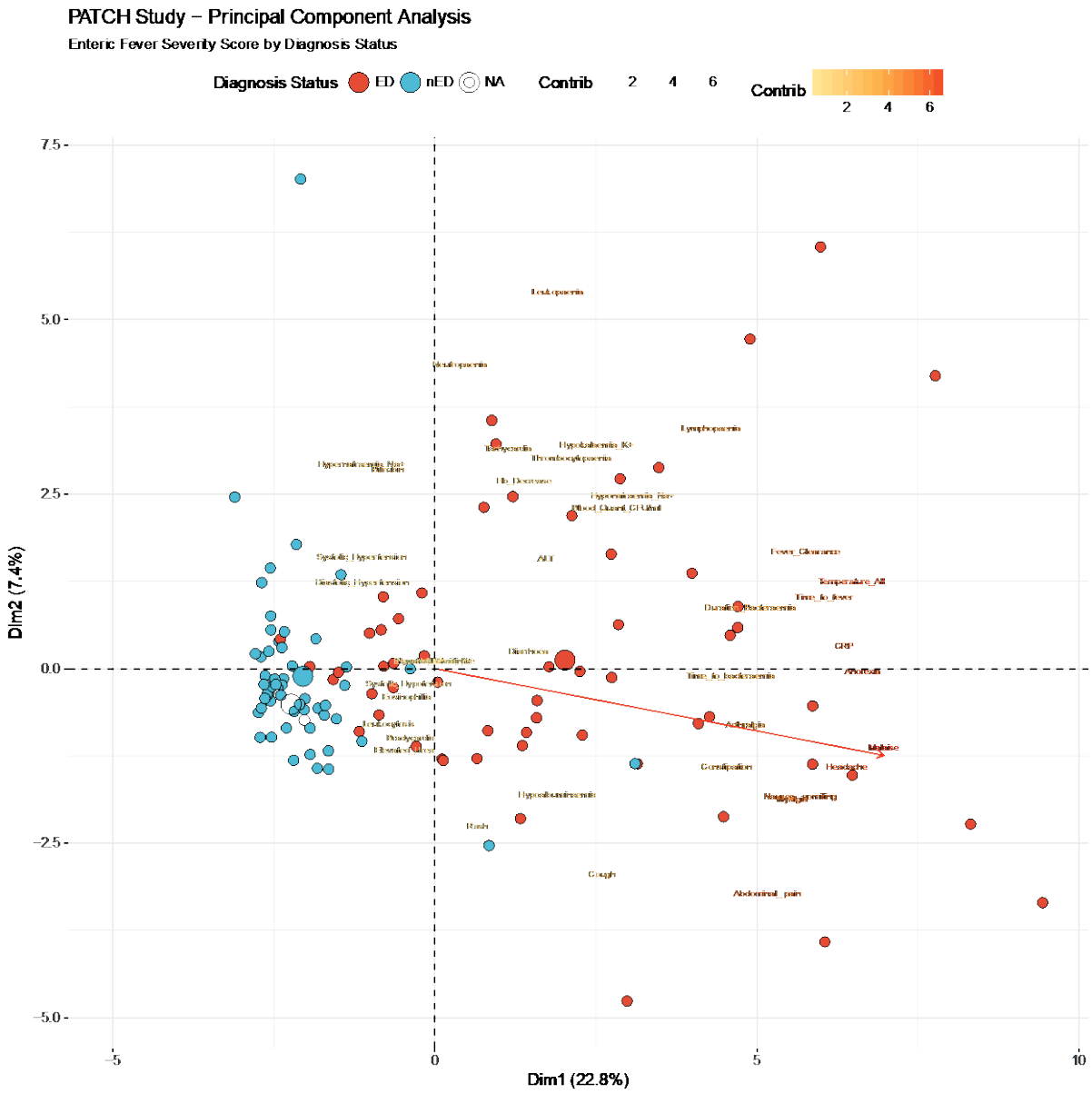


Figure 7-26 : Principal Component Analysis (PCA) biplot of disease severity by outcome of challenge (OVG2014/01 PATCH Study).

Variation in clinical presentation according to diagnosis status (OVG2014/01 study). Principal Component Analysis (PCA) biplot of naïve and re-challenge participants following S. Typhi and or S. Paratyphi challenge derived from disease severity scores. Data points are coloured by challenge outcome (ED = Enteric Fever Diagnosis; nED = No enteric fever diagnosis; NA = Early withdrawal). Ellipses represent 95% confidence levels for grouping variable (challenge group). Large circles represent mean position for grouping variable. Biplot arrows represent the contributions of individual variables to a given principal component, colour- and size scaled according to their relative contribution. Positively correlated variables are grouped together, whilst variables negatively correlated with one another are positioned on opposed quadrants. The length of the arrows represents the quality of the variables on the factor map, such that variables located further away from the origin contribute to greater variation along a principal component axis.[354]

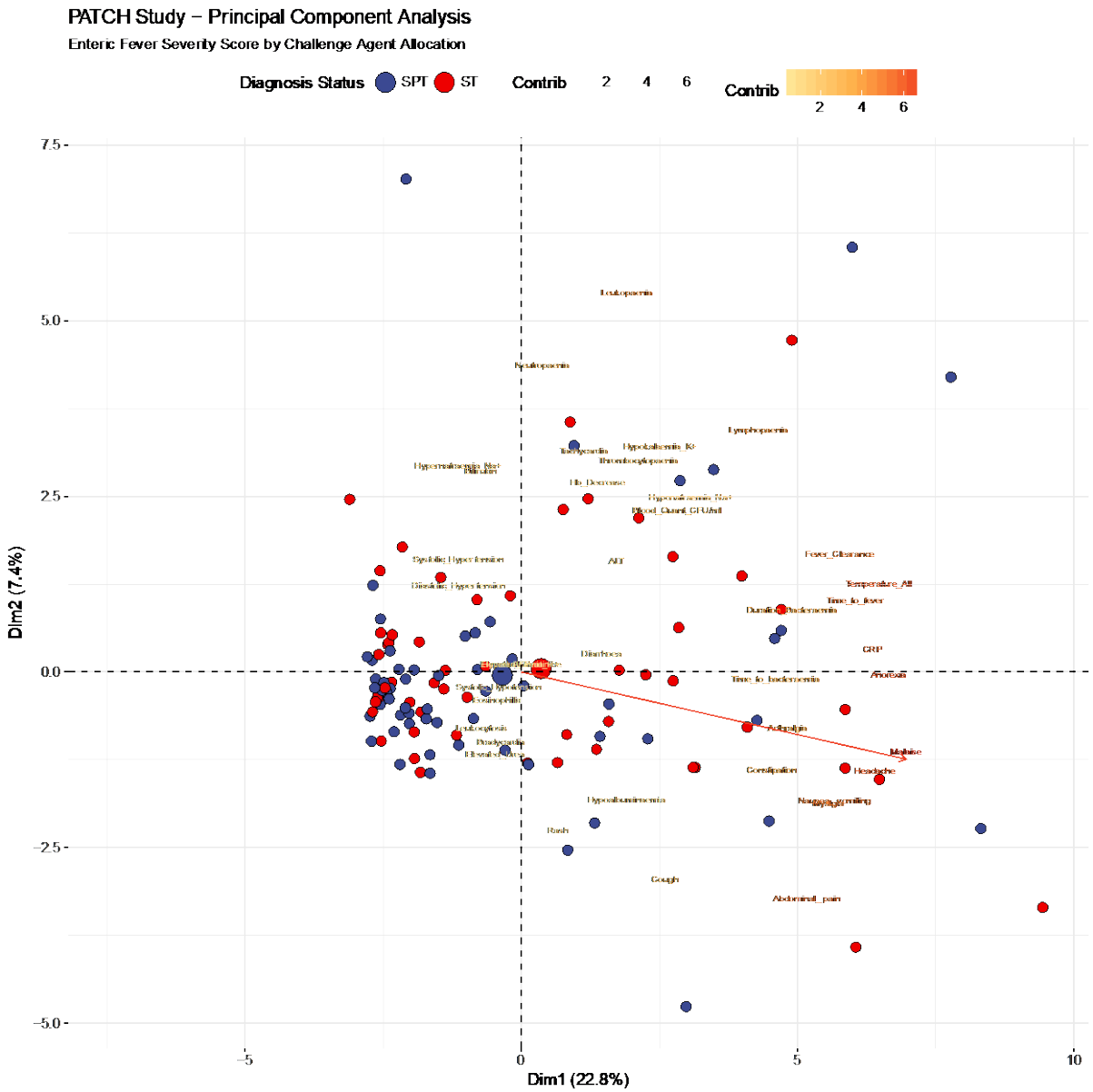


Figure 7-27 : Principal Component Analysis (PCA) biplot of disease severity by challenge agent (OVG2014/01 PATCH Study).

Variation in clinical presentation according to challenge agent (OVG2014/01 study). Principal Component Analysis (PCA) biplot of naïve and re-challenge participants following S. Typhi and or S. Paratyphi challenge derived from disease severity scores. Data points are coloured by challenge serovar (ST = S. Typhi; SPT = S. Paratyphi). Ellipses represent 95% confidence levels for grouping variable (challenge group). Large circles represent mean position for grouping variable. Biplot arrows represent the contributions of individual variables to a given principal component, colour- and size scaled according to their relative contribution. Positively correlated variables are grouped together, whilst variables negatively correlated with one another are positioned on opposed quadrants. The length of the arrows represents the quality of the variables on the factor map, such that variables located further away from the origin contribute to greater variation along a principal component axis.[354]

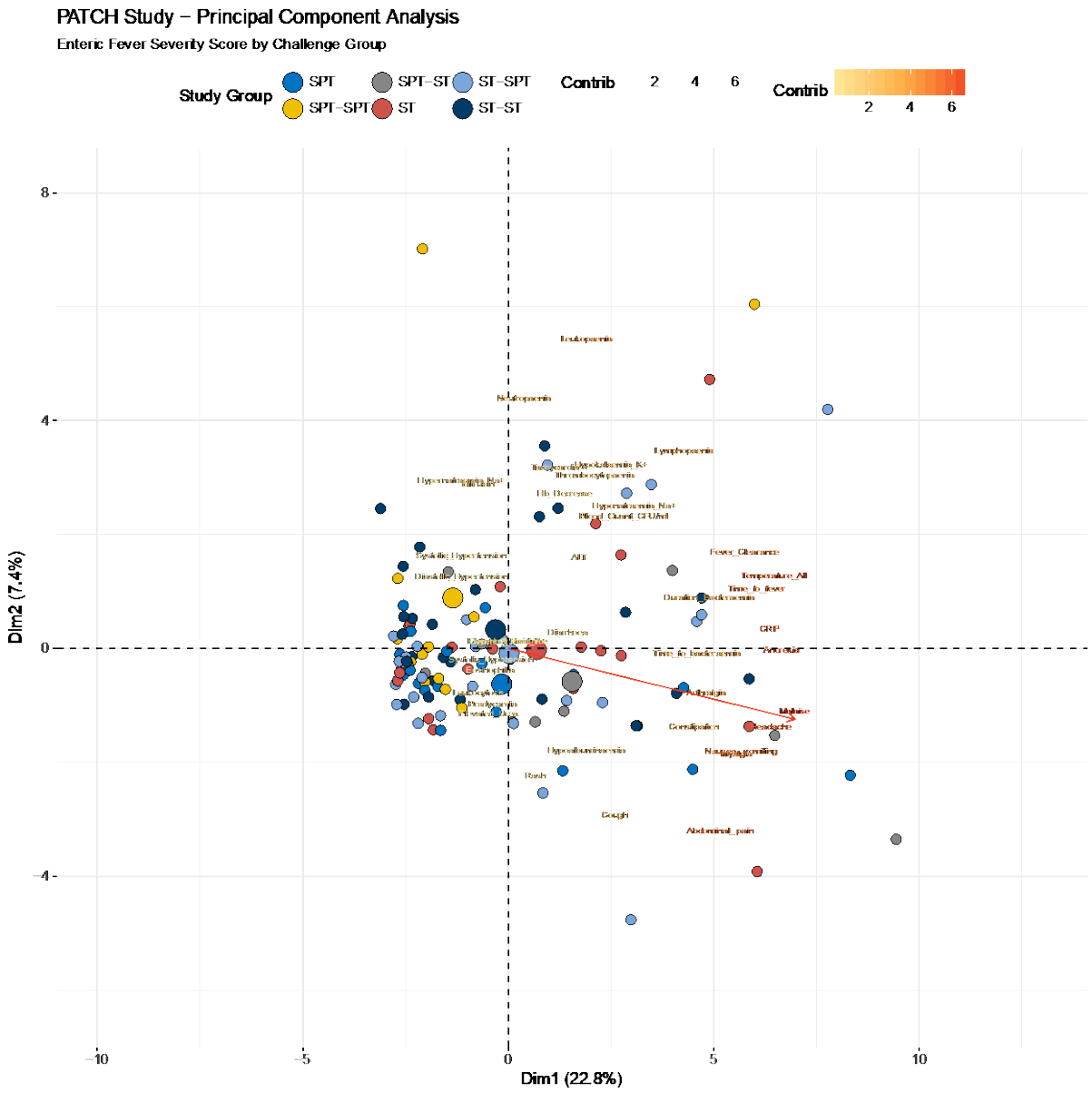


Figure 7-28 : Principal Component Analysis (PCA) biplot of disease severity by study group (OVG2014/01 PATCH Study).

Variation in clinical presentation according to study group (OVG2014/01 study). Principal Component Analysis (PCA) biplot of naïve and re-challenge participants following S. Typhi and or S. Paratyphi challenge derived from disease severity scores. Data points are coloured by challenge agent allocation and group (naïve, homologous re-challenge, heterologous re-challenge). Ellipses represent 95% confidence levels for grouping variable (challenge group). Large circles represent mean position for grouping variable. Biplot arrows represent the contributions of individual variables to a given principal component, colour- and size scaled according to their relative contribution. Positively correlated variables are grouped together, whilst variables negatively correlated with one another are positioned on opposed quadrants. The length of the arrows represents the quality of the variables on the factor map, such that variables located further away from the origin contribute to greater variation along a principal component axis.[354]

7.2.14. Factors affecting response to re-challenge

Participants enrolled into the re-challenge cohorts were heterogeneous with respect to several variables that might impact the outcome of re-challenge. These included vaccination status, outcome of primary challenge (disease vs. no disease) and interval between primary and secondary challenge. We analysed sub-groups within the re-challenge cohort to determine which factors might impact the response to re-challenge.

Participants with a history of typhoid vaccination had an equivalent rate of infection on re-challenge, compared with those without a history of prior typhoid vaccination (AR 5/11 [45%] vs. 7/16 [43%]; p= 0.70). No participants (0/3) who had previously received a Vi-tetanus toxoid conjugate vaccine [209] developed typhoid fever on re-challenge.

The rate of infection on re-challenge of individuals who did not develop enteric fever on primary exposure was significantly lower than participants who had been diagnosed with typhoid after their first exposure (11/39 [28%] vs. 25/37 [68%]; RR 0.42, 95%CI 0.24 – 0.70; p=0.001). Of the 76 participants enrolled in all re-challenge groups, 37 (49%) were diagnosed with enteric fever on primary exposure. With the exception of the *S.* Paratyphi homologous re-challenge group, where only three volunteers were diagnosed, the attack rate was consistently higher in participants who had developed disease on primary exposure compared with those who were protected on primary exposure (Table 7-11).

Table 7-11 : Comparison of attack rates in re-challenge cohorts according to outcome of primary challenge.

	Previous Disease	No Previous Disease	RR (95% CI)	p
All	25/37(68%)	11/39 (28%)	0.42 (0.24-0.70)	<0.0001
ST-ST	8/12(67%)	4/15 (27%)	0.46 (0.18-1.07)	0.12
SPT-ST	6/7(86%)	1/3 (33%)	0.39 (0.07-1.15)	0.18
SPT-SPT	1/5(20%)	2/7(29%)	1.43 (0.24-9.68)	0.99
ST-SPT	10/13(77%)	4/14(29%)	0.37 (0.15-0.81)	0.02

A comparison of attack rates in re-challenge sub-groups compared with naïve control challenge contemporaneously is shown in Figure 7-29. In order to increase power, we also compared the re-challenge cohort with combined naïve controls from previous challenge studies (Figure 7-30). Both analyses gave comparable results. In particular, participants in the *S*. Typhi homologous re-challenge group who did not develop typhoid on primary exposure were less likely to develop typhoid on re-challenge than naïve controls (4/15 vs. 84/121; RR 0.38 (0.16-0.76); p=0.002).

In order to further characterise factors associated with protection in the re-challenge study, we performed a univariate and multivariate logistic regression analysis of a range of variables recorded at baseline (Appendix 7). The dependent variable in the model was diagnosis status (disease =1, no disease = 0). The independent variables included within the model were outcome of primary challenge (disease vs. no disease), previous vaccination, interval from primary challenge and study. The unadjusted and adjusted odds ratios are presented in Appendix 7 Supplementary Table 5 .

After adjusting for vaccine status and re-challenge interval, participants who did not develop disease on primary exposure were significantly less likely to develop disease on re-challenge (OR 0.17 [95% CI 0.02-0.82], p=0.043 - Appendix 7 Supplementary Table 5). The number of previously-vaccinated participants who underwent re-challenge was too small to draw any

meaningful conclusions. The interval between primary challenge and re-challenge was not significantly associated with risk of enteric fever diagnosis when analysed as a categorical (Appendix 7 Supplementary Table 5) or as a continuous variable (OR 1.01 [0.97-1.05]; p=0.58 – Appendix 7 Supplementary Figure 1). When all re-challenge participants were analysed together there was a trend towards increased risk of enteric fever diagnosis with prolonged challenge interval (Appendix 7 Supplementary Figure 2).

S. Typhi & Paratyphi Re-Challenge - Relative Risk of Composite Diagnositic Endpoint by Subgroup

Subgroup	No. of Participants [%]	Re-Challenge Group % AR	Naive Group % AR	RR[95%CI]	P Value
Overall S. Typhi-S. Typhi Re-challenge	**27 (100)**	**12/27(44%)**	**12/19(63%)**	**0.7(0.41-1.23)**	**0.24**
Outcome of Primary Challenge					
Typhoid Challenge - Typhoid Diagnosis	12 (44.4)	8/12(67%)	12/19(63%)	1.06(0.58-1.79)	0.99
Typhoid Challenge - No previous Diagnosis	15 (55.6)	4/15(27%)	12/19(63%)	0.42(0.16-0.94)	0.045
Previous Vaccine & Typhoid Challenge					
Any Vaccine	11 (41)	5/11(45%)	12/19(63%)	0.72(0.32-1.39)	0.45
Previous Vi-PS	4 (15)	2/4(50%)	12/19(63%)	0.95(0.35-1.29)	0.99
Previous Vi-TT	3 (11)	0/3(0%)	12/19(63%)	0(0-0.96)	0.08
Previous Ty21a	3 (11)	2/3(67%)	12/19(63%)	1.06(0.32-1.94)	0.99
Previous M01ZH09	1 (4)	1/1(100%)	12/19(63%)	1.58(0.32-9.93)	0.99
No Previous Vaccine	16 (59)	7/16(44%)	12/19(63%)	0.69(0.34-1.29)	0.31
Overall S. Paratyphi-S. Typhi Re-challenge	**10 (100)**	**7/10(70%)**	**12/19(63%)**	**1.11(0.59-1.87)**	**0.99**
Paratyphoid Challenge - Paratyphoid Diagnosis	7 (70)	6/7(86%)	12/19(63%)	1.36(0.73-2.18)	0.37
Paratyphoid Challenge - No previous Diagnosis	3 (30)	1/3(33%)	12/19(63%)	0.53(0.1-1.47)	0.54
Overall S. Paratyphi-S.Paratyphi Re-Challenge	**12 (100)**	**3/12(25%)**	**10/18 (56%)**	**0.45(0.15-1.13)**	**0.14**
Outcome of Primary Challenge					
Paratyphoid Challenge - Paratyphoid Diagnosis	5 (42)	1/5(20%)	10/18 (56%)	0.36(0.06-1.31)	0.32
Paratyphoid Challenge - No previous Diagnosis	7 (58)	2/7 (29%)	10/18 (56%)	0.51(0.14-1.38)	0.38
Overall S. Typhi-S.Paratyphi Re-Challenge	**27 (100)**	**14/27(52%)**	**10/18 (56%)**	**0.93(0.54-1.68)**	**0.99**
Outcome of Primary Challenge					
Typhoid Challenge - Typhoid Diagnosis	13 (48)	10/13 (77%)	10/18 (56%)	1.39(0.8-2.4)	0.28
Typhoid Challenge - No previous Diagnosis	14 (52)	4/14 (29%)	10/18 (56%)	0.51(0.2-1.19)	0.16
Previous Vaccine & Typhoid Challenge					
Any Vaccine	16 (59)	8/16 (50%)	10/18 (56%)	0.9(0.46-1.71)	0.99
Previous Vi-PS	3 (11)	1/3 (33%)	10/18 (56%)	0.6(0.11-1.76)	0.59
Previous Vi-TT	8 (29)	5/8 (63%)	10/18 (56%)	1.13(0.51-2.12)	0.99
Previous Ty21a	1 (4)	0/1 (0%)	10/18 (56%)	0(0-1.65)	0.47
Previous M01ZH09	4 (15)	2/4 (50%)	10/18 (56%)	0.9(0.26-1.99)	0.99
No Previous Vaccine	11 (4)	6/11(55%)	10/18 (56%)	0.98(0.46-1.88)	0.99

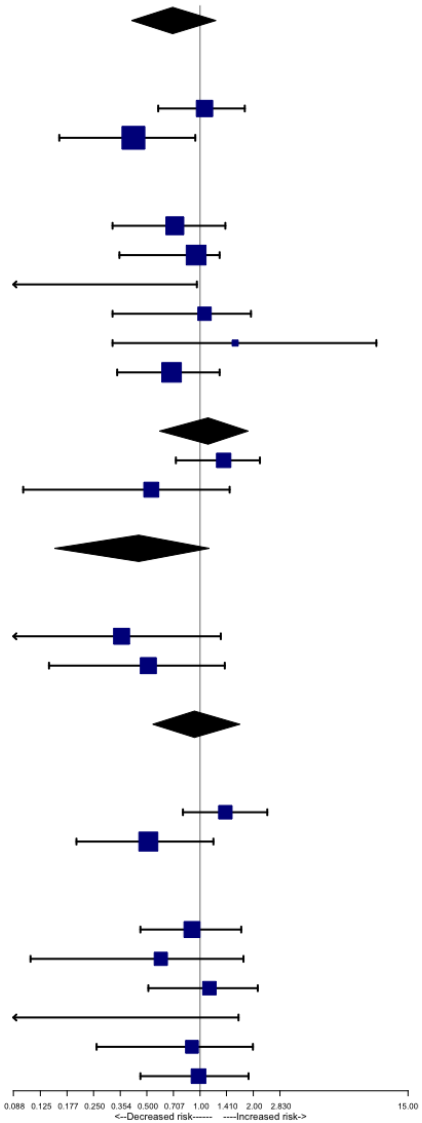


Figure 7-29 : Sub-Group analysis OVG2014/01 study.

Forest plot comparing relative risk of typhoid or paratyphoid diagnosis following re-challenge compared with naïve controls from OVG2014/01 study. Box-plots represent relative risk and 95% confidence intervals scaled according to size of sub-group. Diamonds represent combined relative risk of diagnosis in each of the re-challenge cohorts. P = Fishers exact test.

S. Typhi & Paratyphi Re-Challenge - Relative Risk of Composite Diagnositic Endpoint by Subgroup (vs. Combined Naive Controls)

Subgroup	No. of Participants [%]	Re-Challenge Group % AR	Naive Group % AR	RR[95%CI]	P Value
Overall S. Typhi-S. Typhi Re-challenge	**27 (100)**	**12/27(44%)**	**84/121(69%)**	**0.64(0.39-0.93)**	**0.02**
Outcome of Primary Challenge					
Typhoid Challenge - Typhoid Diagnosis	12 (44.4)	8/12(67%)	84/121(69%)	0.96(0.56-1.29)	0.99
Typhoid Challenge - No previous Diagnosis	15 (55.6)	4/15(27%)	84/121(69%)	0.38(0.16-0.76)	0.002
Previous Vaccine & Typhoid Challenge					
Any Vaccine	11 (41)	5/11(45%)	84/121(69%)	0.65(0.3-1.06)	0.18
Previous Vi-PS	4 (15)	2/4(50%)	84/121(69%)	0.86(0.33-1.31)	0.64
Previous Vi-TT	3 (11)	0/3(0%)	84/121(69%)	0(0-0.82)	0.03
Previous Ty21a	3 (11)	2/3(67%)	84/121(69%)	0.96(0.3-1.41)	0.99
Previous M01ZH09	1 (4)	1/1(100%)	84/121(69%)	1.44(0.3-3.24)	0.99
No Previous Vaccine	16 (59)	7/16(44%)	84/121(69%)	0.63(0.33-0.99)	0.05
Overall S. Paratyphi-S. Typhi Re-challenge	**10 (100)**	**7/10(70%)**	**84/121(69%)**	**1.01(0.57-1.34)**	**0.99**
Outcome of Primary Challenge					
Paratyphoid Challenge - Paratyphoid Diagnosis	7 (70)	6/7(86%)	84/121(69%)	1.24(0.7-1.51)	0.67
Paratyphoid Challenge - No previous Diagnosis	3 (30)	1/3(33%)	84/121(69%)	0.48(0.09-1.16)	0.23
Overall S. Paratyphi-S.Paratyphi Re-Challenge	**12 (100)**	**3/12(25%)**	**22/38(58%)**	**0.43(0.15-0.99)**	**0.1**
Outcome of Primary Challenge					
Paratyphoid Challenge - Paratyphoid Diagnosis	5 (42)	1/5(20%)	22/38(58%)	0.36(0.06-1.31)	0.32
Paratyphoid Challenge - No previous Diagnosis	7 (58)	2/7 (29%)	22/38(58%)	0.51(0.14-1.38)	0.38
Overall S. Typhi-S.Paratyphi Re-Challenge	**27 (100)**	**14/27(52%)**	**22/38(58%)**	**0.9(0.55-1.39)**	**0.8**
Outcome of Primary Challenge					
Typhoid Challenge - Typhoid Diagnosis	13 (48)	10/13 (77%)	22/38(58%)	1.33(0.81-1.95)	0.32
Typhoid Challenge - No previous Diagnosis	14 (52)	4/14 (29%)	22/38(58%)	0.49(0.2-1.03)	0.12
Previous Vaccine & Typhoid Challenge					
Any Vaccine	16 (59)	8/16 (50%)	22/38(58%)	0.86(0.46-1.42)	0.77
Previous Vi-PS	3 (11)	1/3 (33%)	22/38(58%)	0.58(0.1-1.5)	0.57
Previous Vi-TT	8 (29)	5/8 (63%)	22/38(58%)	1.08(0.51-1.75)	0.99
Previous Ty21a	1 (4)	0/1 (0%)	22/38(58%)	0(0-1.46)	0.44
Previous M01ZH09	4 (15)	2/4 (50%)	22/38(58%)	0.86(0.25-1.67)	0.99
No Previous Vaccine	11 (4)	6/11(55%)	22/38(58%)	0.94(0.46-1.56)	0.99

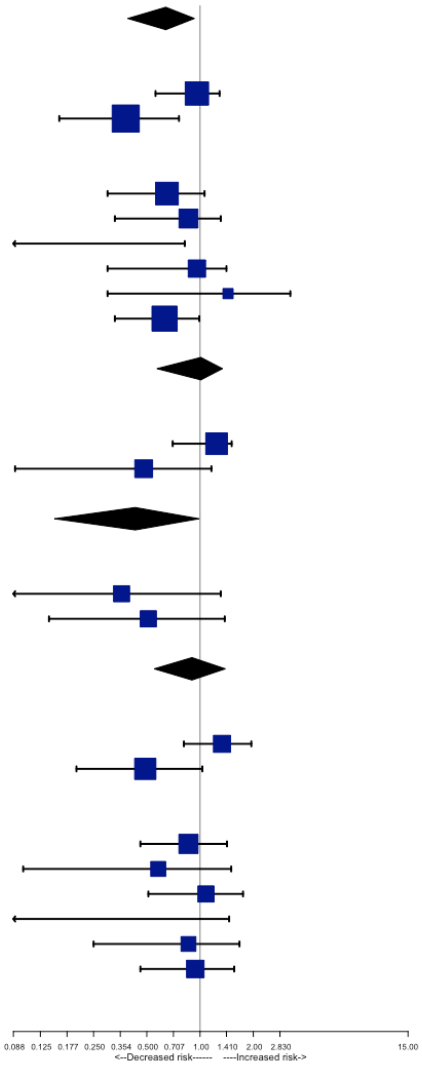


Figure 7-30 : Sub-Group analysis OVG2014/01 study compared with historical controls.

Forest plot comparing relative risk of typhoid or paratyphoid diagnosis following re-challenge compared with combined naïve controls. Box-plots represent relative risk and 95% confidence intervals scaled according to size of sub-group. Diamonds represent combined relative risk of diagnosis in each of the re-challenge cohorts. P = Fishers exact test

7.3. Discussion

In this study we have undertaken the first *S.* Typhi re-challenge study in over 40 years, as well as the first description of *S.* Paratyphi re-challenge in a controlled human infection model. These data demonstrate that a single previous exposure to *S.* Typhi was associated with an approximately 36% reduced risk of typhoid disease on re-challenge compared with naïve controls. Previous exposure to *S.* Paratyphi was associated with an estimated 57% reduced risk of paratyphoid disease compared with naïve controls, although the protective effect was non-significant, with a wide confidence interval, due to the small sample size in the re-challenge arm. Heterologous re-challenge with *S.* Typhi or Paratyphi was not associated with a reduced rate of infection. In those participants who did develop disease, the clinical and microbiological features of naïve and re-challenge participants was indistinguishable.

The protective effect of prior *S.* Typhi challenge was remarkably similar to that observed in previous studies describing infection derived immunity to typhoid fever. As previously discussed, Marmion and colleagues describe a 35% relative risk reduction in previously infected patients in the context of two consecutive typhoid outbreaks[292], and Dupont and colleagues report a 30% relative risk reduction in the context of a human challenge study[295]. Comparisons between historical and contemporary typhoid challenge studies are complicated by key methodological differences, particularly relating to study population, challenge dose and mode of administration. New insights from this study include a substantially longer interval between challenge and re-challenge compared with previous studies (median 38 months vs. ≤12months), and the inclusion of participants who did not develop disease on primary exposure[292,295]. Overall, these data support the view that a single episode of typhoid infection induces moderate, but incomplete, protection against subsequent disease.

Prior *S.* Paratyphi exposure was associated with a slightly larger protective effect within the context of the challenge model (57%) but did not meet the significance threshold due to the small sample size within the homologous re-challenge group (n=12). In those participants who developed disease, homologous re-challenge with *S*. Paratyphi was associated with a significant delay in time to disease onset. The estimate of protective effect from prior paratyphoid exposure is comparable to that conferred by Vi-conjugate vaccines following typhoid challenge [209] and is likely to be an underestimate owing to the strict diagnostic endpoint applied[209]. These observations raise the intriguing possibility that prior paratyphoid infection could confers at least partial immunity against re-infection and would be supportive of an approach to test live-attenuated oral paratyphoid vaccines this model (e.g. CVD-1902[222]). Differences in the magnitude of protection associated with prior *S.* Typhi or *S.* Paratyphi challenge could be explained by differences between the serovars (e.g. lack of Vi-capsule expression by *S*. Paratyphi), challenge dose (10^3 vs 10^4CFU) or a longer interval between challenge episodes (38 vs. 17 months) associated with waning immunity. The precision of these observations is limited by the small sample size in each study sub-group and will require further validation in field studies or future challenge studies.

This study also represents the first description of heterologous re-challenge with *S*. Typhi and *S*. Paratyphi with the aim of studying cross-reactive immune responses between these closely related serovars. When individuals previously challenged with *S*. Typhi were re-challenged with *S*. Paratyphi, the attack rate was comparable to that in naïve controls (52% vs. 56%; RR0.93 [0.54-1.68]; p=0.99). Patients with typhoid fever and individuals vaccinated with oral typhoid-vaccines have detectable cross-reactive humoral immune responses against Paratyphi A in vitro.[365–367] However, field studies in highly endemic areas have shown that Ty21a does not appear to protect against S. Paratyphi A,[368] but there is evidence for protection against *S*. Paratyphi B from a retrospective analysis (49% efficacy; 95%CI 8-73%)[369]. Whilst these comparisons are also

underpowered, these data suggest that prior exposure to *S.* Typhi is not associated with a significant protection against *S.* Paratyphi A infection within the context of the challenge model.

The effect of previous *S.* Paratyphi infection on protection against *S.* Typhi disease has been less extensively studied. The attack rate in the *S.* Typhi heterologous re-challenge group was similar to naïve controls challenged with S. Typhi (63% vs. 70%; 1.10 (0.59-1.87); p=0.99), albeit in a small sample of volunteers (n=10). As these serovars are co-endemic and share a primary of transmission, an improved understanding of cross-reactive immune responses between these two serovars will be important in future disease control efforts – particularly in the context of the impending deployment of Vi-conjugate vaccines that offer no protection against *S.* Paratyphi A.

Whilst previous exposure to S. Typhi and Paratyphi is associated with a moderate protection against disease on re-challenge, the clinical presentation in re-challenged participants was comparable to those who were immunologically naïve. Modelling studies postulate a spectrum of hypothetical immune states following prior typhoid infection, ranging from sterile immunity through to clinical immunity to complete susceptibility [293,294]. In particular, a state of clinical immunity is proposed to be characterised by milder infection, with or without shedding, that is less likely to require clinical attention. There are no validated metrics of typhoid/paratyphoid severity. When measured using a range of clinical and microbiological endpoints, we observed no difference in the observable clinical syndrome between naïve and re-challenge groups, although early treatment initiation limits the conclusion that can be drawn from these endpoints. When analysed by a principal component analysis incorporating a range of diverse variables, no clustering was observed by challenge groups Figure 7-28. Data from this study suggest that prior exposure is not demonstrably associated with an attenuated clinical phenotype and clinical immunity compared with naïve exposure, although the significance of this to field settings is as yet unclear.

7.3.1. Prior disease vs. Prior exposure

In the re-challenge study, we employed an unbiased recruitment approach by contacting all consenting participants of previous typhoid and paratyphoid studies in the UK, regardless of the outcome of their primary exposure. Re-challenge studies of other enteric pathogens typically re-challenge only those participants who had developed disease on their first exposure (Table 7-1) – beyond this, the selection criteria of participants for re-challenge is rarely clear from study reports[313,370–376,378,379]. Participants in the re-challenge study had a documented episode of a S. Typhi/S. Paratyphi exposure, at a recorded date and time, at a set dose and known mode of delivery. The data derived from this study should best be viewed as a study of re-challenge following previous *S.* Typhi/Paratyphi exposure – as distinct from a study of re-challenge following previous disease.

Intriguingly, we observed that individuals who did not develop enteric fever on primary exposure were more likely to be protected on re-challenge, compared with individuals diagnosed on primary exposure (Table 7-11 & Figure 7-30). Host factors might explain why apparent susceptibility to typhoid infection differs between individuals. For example, genome wide association studies in Vietnam and Nepal have identified variation at HLA-DRB1 to be strongly associated with resistance to enteric fever[382]. Additional risk factors associated with susceptibility to enteric fever include variations in the *cftr* locus [226,227] and carriage of *Helicobacter pylori*[127,128]. The effect of the host microbiome on susceptibility to enteric fever is currently the subject of further study[383]. It is unclear if the development of immunity following S. Typhi/Paratyphi challenge requires the development of clinical disease/bacteraemia, or whether immunity can develop following asymptomatic exposure. Previous studies by our group, have shown that the majority of participants challenged with S. Typhi produce a transient peak in cytokine production as early as 12 hours after challenge, which is independent of the subsequent development of

typhoid disease, suggesting that early innate interactions occur in most participants challenged.[163] However, in the challenge model effective priming of the adaptive immune system appears to require the development of symptomatic infection and/or bacteraemia, as individuals who did not develop typhoid/paratyphoid disease after challenge failed to mount a humoral and cellular response to *S.* Typhi/Paratyphi antigens[126,278,287–289,311,384].

These observations raise the possibility that the protection observed following homologous re-challenge is not mediated by a 'classical' adaptive memory immune response following primary exposure. Differences in infection rates after re-challenge may instead be accounted for by inter-host variation in genetic or epigenetic factors impacting innate immune responses and the development of trained innate immunity after primary infection[385]. Alternatively, protection may be mediated by adaptive immune responses that have yet to be measured – such as those mediated by T- and B-cells at the gastrointestinal mucosa[386]. Further studies are currently underway to analyse patterns in whole blood gene expression, DNA methylation and in flow cytometric analysis of gut resident T-cells that could be associated with protection from disease.

7.3.2. General Limitations

Ethical and safety considerations in the design of this study, including early initiation of rescue therapy, limit the extent that our findings can be extrapolated to endemic settings[110]. It is plausible that some individuals who did not develop enteric fever during the 14-day observation period may have progressed to symptomatic disease/bacteraemia had antibiotic treatment not been initiated in all participants. Conversely, some participants who were diagnosed based on bacteraemia in the absence of clinical signs of disease may not be representative of typhoid fever in the field – indeed, spontaneous clearance of asymptomatic *S*. Typhi bacteraemia in the absence of treatment has been described in historical challenge studies [305]. As only a single strain was used in this study we cannot conclusively rule out a strain-specific effect for our observations.

However, the NVGH308 strain is a recent clinical isolate from a symptomatic case with bacteraemia, and is closely related to currently circulating strains [311]. The Quailes strain of *S*. Typhi is also related to other known disease causing isolates [234 126]. Both *S.* Typhi and *S.* Paratyphi are clonally monomorphic pathogens containing limited genomic variation[17], suggesting that the pathogenicity and immune response to both the Quails and NVGH308 strains should translate to other wild-type strains.

It could be speculated that a larger protective effect would have been observed had we employed a shorter interval between challenge episodes. Other re-challenge studies of enteric pathogens have typically used an interval of 1-≤12months (Table 7-1). The minimum interval between primary challenge and re-challenge in this study was set at 12 months, which was defined primarily to ensure the safety and comfort of study participants in mind. In particular, this interval would allow recovery of haemoglobin levels and haematocrit after intensive venepuncture during the course of the challenge study[126 311], and to allow for recovery of microbial diversity within the intestinal microbiome after antibiotic treatment[389]. Whilst a shorter challenge interval may have enhanced the protective effect observed, it can be argued that the wide range of challenge intervals in this study closely mirrors the stochastic nature of exposure to *S*. Typhi in a field setting [35].

7.3.3. Limitation in sample size

The study was broadly underpowered to detect anything other than large differences in attack rates between naïve and re-challenge groups. No formal sample size calculations were made in the design of this study as the size of the re-challenge cohort was dependent on the number of participants who were willing and consented to challenge a second challenge. The likely retention rate between re-challenge studies was unknown at the onset of the start of the study. Thirty percent of all participants eligible for re-challenge were enrolled into this study and the number of volunteers who were willing to undergo re-challenge rises to 41% (71/172) when volunteers

excluded after screening are considered (Table 7-5). The retention rate for re-challenge across challenge studies is respectable given the study requirements and transitory nature of many study participants (e.g. student volunteers). This illustrates that experience of previous typhoid/paratyphoid challenge was not necessarily an impediment to recruitment. However, only 49% of those eventually challenged had developed disease on primary exposure, suggesting a slight recruitment bias towards those who were protected on primary exposure (Table 7-4). Additional participants could be re-challenged to increase the power and validate the preliminary findings of this study but is unlikely to be cost-effective.

7.3.4. Impact of antibiotic treatment

Controlled human infection (CHI) models offer a distinctive platform to study and compare treatment responses to different antibiotics, benefitting from close monitoring of trial participants, daily sample collection and the capacity to integrate treatment in the context of other measures of host immunity. Early typhoid challenge models were used to study the use of chloramphenicol for the treatment of typhoid fever[304]. The switch in first line antibiotic treatment mid-way through the study provided an opportunity to undertake a non-randomised, observational comparison of azithromycin and ciprofloxacin treatment in the context of controlled typhoid and paratyphoid infection.

Whilst all patients were successfully treated, we observed a delayed response to treatment of *S.* Typhi and *S.* Paratyphi infection in those receiving azithromycin compared with those receiving ciprofloxacin. These findings were consistent across multiple studies, including a randomised trial comparing azithromycin and ciprofloxacin (C Jin personal communication – CJ, MMG manuscript in preparation).

While our results may not be directly applicable to treatment options in field settings, there are several relevant observations from this study that should be considered when managing uncomplicated enteric fever. The emergence and dissemination of multidrug resistant, fluoroquinolone resistant and extremely drug resistant (XDR) strains of *S.* Typhi[178] represents a threat to the global control of enteric fever. At the time of writing, azithromycin is one of the few oral treatment options to which the majority of global *S.* Typhi and Paratyphi isolates remain sensitive. Azithromycin is known to be a successful treatment option for enteric fever, but has been associated with fever clearance times of ≥ 96hrs[187,390–393] and prolonged bacteraemia[392,393] in multiple field trials. Several recent studies suggest that fluoroquinolones should not be recommended for empirical treatment of enteric fever in South/South East Asia, due to high risk of treatment failure.[177] Further studies are required to determine the optimal dosing regimen in the context of typhoid and paratyphoid infection, as well as to assess novel treatment strategies, including combination therapies and antibiotic cycling[187,188,394].

7.3.5. Future work

This approach adopted in this study offers the advantage of studying immunity to typhoid and paratyphoid fever in a highly controlled, experimental setting. Multiple samples were collected from participants, including DNA, RNA, PBMCs, serum, plasma, gastrointestinal biopsies, saliva and stool. Deep analysis of samples collected from naïve and re-challenge participants could help to identify host immune responses that correlate with the protective effect observed after typhoid and paratyphoid re-challenge, which in turn could help to inform the development of future vaccines. Samples generated from the challenge study have begun to offer insights into the role of MAIT cells [289] and effector CD4+ T-cells [278] in the response to typhoid fever, and additional work is ongoing to analyse other aspects of host immunity following challenge.

Previous studies have demonstrated that the typhoid challenge model can be successfully applied to test candidate typhoid vaccines [209]. To date, vaccines for *S.* Paratyphi have yet to be tested in the model to determine if comparable protection can be conferred by vaccination as is observed following prior infection. Protocols could be readily adapted to assesses candidate vaccines, including O-specific polysaccharide conjugate vaccines and live-oral vaccines [222].

7.4. Summary

In this chapter I have described the application of a controlled human infection model to study infection derived immunity to *S.* Typhi and Paratyphi infection. By comparing groups of individuals who have been previously exposed to these pathogens with immunologically-naïve individuals, this approach aims to assess the contribution of infection-derived immunity in the prevention (or otherwise) of subsequent disease. This approach has proven valuable in vaccine development for other enteric pathogens, such as *Shigella* spp[379] and *Vibrio Cholerae*[370,371]. Improved understanding of infection-induced immunity could provide valuable data to refine modelling of transmission dynamics and vaccine impact measures[293], in addition to aiding the identification of correlates of protection to expedite vaccine development for *S.* Typhi and Paratyphi.

Chapter 8 - Humoral Responses to *Salmonella* Typhi and *Salmonella* Paratyphi Challenge and Re-Challenge

8. Introduction – Humoral Immunity to typhoidal *Salmonella*

The efficacy of plain Vi-polysaccharide vaccines - which can only induce antibody - attests to role of antibodies in conferring protection against typhoid fever [5,203,280]. Humoral responses to surface antigens of *S.* Typhi and *S.* Paratyphi - including Vi, Hd, Ha, O:9 and O:2 – can typically be detected in the serum of exposed individuals. Within the context of the Oxford challenge model, the development of serum antibodies to O and H antigen after a single exposure occurs primarily in individuals who developed typhoid/paratyphoid infection, whereas individuals challenged who do not go on to develop illness typically did not mount an antibody response to these antigens[126,311,314]. It is unknown whether antibodies against these surface components correlate with protection on re-exposure.

The protective role of anti-O and anti-H humoral responses has been studied primarily in the context of live-oral typhoid vaccination[201,395]. There is weak evidence associating humoral responses to these antigens with protection against typhoid fever. For example, there is some evidence that the magnitude of anti-O IgA ASC response after immunisation correlates with efficacy of different schedules and formulations of the Ty21a vaccine[309,396,397]. Additionally, the efficacy of Ty21a in some field studies correlated with the proportion of individuals mounting antibody responses to O-antigen after vaccination[398]. Antibodies targeted against the O-antigen are capable of mediating complement mediated killing of *S.* Typhi[241,285], although this is partly dependent on the level of Vi-capsule expression.

Infection with *S*. Typhi can still occur despite robust anti-O and anti-H humoral responses. In general, antibody responses to O-antigen after Ty21a vaccination are modest, occurring in ~60% of vaccinees receiving a three-dose schedule[397–399]. Whilst serum O antibody broadly correlates with protection after Ty21a vaccination, it is not believed to be the main operative mechanism through which Ty21a acts [398,400]. The oral vaccine MO1ZH09 is associated with a strong ASC response and seroconversion to O- or H-antigens,[401,402]but does not protect against typhoid fever within the context of the challenge model[314]. Anti-O:LPS antibodies appear to mediate bactericidal activity after live-oral typhoid vaccination and challenge, but do not appear to confer sterile immunity[285]. Collectively, these studies support the hypothesis that multiple immune effector mechanisms could be involved in conferring protection against enteric fever[201].

The potential for emergence of Vi-negative strains of *S*. Typhi has led to the development of O9-specific-polysaccharide conjugate vaccines for typhoid fever [220 221]. Anti-09:LPS responses offer the theoretical advantage of targetting Vi-negative strains of *S.* Typhi, as well as other *Salmonella* serovars responsible for invasive disease (e.g. *Salmonella* Enteritidis)[309]. Additionally, anti-O responses are associated with protection against *S.* Typhimurium in mice models[403], but there is currently limited evidence to support the role of anti-O responses as correlates of protection against typhoid fever in humans.

An improved understanding of the role of humoral responses to the O-antigen is particularly pertinent in the development of vaccines for *S*. Paratyphi A. The O-antigen is predicted to be a a key target antigen in the development of future paratyphoid vaccines[404 222]. This assumption is based, in part, on animal models showing that O-specific polysaccharide (OSP) conjugate vaccines were capable of inducing high levels of bactericidal antibodies in mice,[405] as well as extrapolating from the postulated role of anti-O specific antibodies in the protection against onther enteric pathogens (e.g. *Shigella spp*[406]). O-specific polysaccharide conjugate vaccines are some of the

leading paratyphoid conjugate vaccines in development [222] [407]. An OSP-tetanus toxoid conjugate vaccine has completed phase 1 and 2 trials in Vietnam, and was shown to be safe and immunogenic in adults, adolescents and children as young as 2 years[408]. The immunogenicity of another leading paratyphoid vaccine candidate – the live-attenuated oral-vaccine CVD-1902 – has also been assessed by measuring serum antibody and peripheral blood ASC responses against the *S.* Paratyphi O-antigen (Myron M Levine – personal communication). However, there is currently limited evidence that humoral responses against O-specific-polysaccharide antigens correlate with protection against paratyphoid infection.

Memory B-cells (Bm) are postulated to have an important role in protection against typhoid and paratyphoid fever[279]. Vaccination with the experimental live-attenuate oral typhoid vaccine CVD-909 can induce anti-flagellar IgA/IgG and anti-Vi IgA producing Bm cells in 73% and 64% of vaccinated volunteers respectively, when measured by Enzyme linked Immunospot (ELISPOT)[409]. Similarly, vaccination with Ty21a vaccine can induce LPS and flagellar specific Bm cells in 30-50% of vaccinated volunteers [409], as well as inducing memory B-cells targeted against outer membrane proteins of *S.* Typhi and Paratyphi[366]. In most studies of oral typhoid vaccination, IgA producing Bm cells predominate over IgG producing cells[366,409]. The frequency of Bm cells post vaccination also correlated strongly with levels of serum antibody[409]. When measured using flow cytometry, data from the challenge studies indicate that a population of LPS-specific circulating memory B-cell populations express activation markers and decrease in frequency during acute typhoid disease (unswitched [CD27+IgD+] and switched [CD27+IgD-] Bm cells)[284]. Whilst these studies indicate that Bm cells are induced and activated by typhoid infection[284] or vaccination[409], it is unknown if the frequency or activation status of Bm cells correlates with protection against disease.

8.1.1. Aims

In this chapter I will describe humoral immune responses following controlled challenge with *Salmonella* Typhi and *Salmonella* Paratyphi A, focussing on serum antibody, antibody secreting cell, and memory B-cell responses[279.] I will describe how humoral immune responses are modulated by prior exposure to *Salmonella* Typhi and Paratyphi by comparing responses in the homologous and heterologous re-challenge groups, as compared with naïve individuals with no prior exposure to typhoidal *Salmonella*. Similar measures have been recorded in earlier challenge and vaccine studies performed in Oxford, including in the assessment of naïve challenge and live-oral typhoid vaccines Ty21a and MO1ZH09[314]. This will allow for comparisons of infection-derived humoral immunity with vaccine derived humoral immunity. Furthermore, these measures will provide direct information as to the value of these immunological measures as putative correlates of infection-derived immunity.

8.2. Methods

8.2.1. Serum Antibody ELISA

Antibody responses were measured by ELISA at 28 and 90 days after challenge and compared with those at baseline.

8.2.1.1. O-antigen and H-antigen ELISA

Antibody responses to *S*. Typhi O9:LPS (*S*. Typhosa LPS, L2387; Sigma-Aldrich, Dorset, UK), *S*. Typhi H-d antigen (University of Maryland CV0150622) and *S*. Paratyphi O:2 antigen (GSK Vaccines for global health) were measured using an in-house ELISA[126,311,314]. Ninety-six well flat-bottom ELISA plates (Nunc 442404, Thermo Fisher) were coated 100µl/well of the target antigens suspended in 50mM carbonate-bicarbonate buffer (final concentration 15µg/ml O9:LPS; 1µg/ml H-d antigen and 1µg/ml O2 antigen), sealed and stored overnight at 4°C (Figure 8-1). Plates were washed five

times with 300µl/well PBS + 0.05% Tween®20, before being blocked with 200µl/well PBS + 1 % bovine serum albumin (BSA) at room temperature for 90 minutes. After blocking, plates were washed five times with PBS + 0.05% Tween®20 and subsequently incubated at ambient temperature for 90 mins with 100µl/well of test serum samples diluted to 1:200, 1:600, 1:1800, 1:5400. All samples were run in duplicate. After incubation with the primary antibody, plates were again washed five times with PBS + 0.05% Tween®20 and subsequently incubated with secondary antibody at room temperature for 60 minutes (goat anti-human IgG:HRP at 1:20,000 [Bio Rad 204005] or goat anti-human IgA:HRP [Bio Rad STR141] at 1:6,000). After washing, plates were developed by adding 100µl/well of 3,3', 5, 5'-Tetramethylbenzidine (TMB T0440, Sigma Aldrich, UK) and incubating out of direct light at room temperature for ~20-30 minutes, until absorbance at 630nm reading for the top concentration of standard curve reached 0.5-0.55. The reaction was stopped by adding 50µl/well of H_2SO_4. The final absorbances were read at 450 nm and 630nm using an automated microplate reader (Biotek ELx808 and associated Gen software v5.2).

The standard curve was generated by preparing a serial dilution of standard serum per plate. The standard serum was supplied by Emergent BioSolutions® from volunteers vaccinated with the live oral typhoid vaccine MO1ZH09. A standard serum of nominal concentration of 30,000 units/ml was made by adding 200µl of positive sera with 170µl of LPS negative sera, also supplied by Emergent BioSolutions®. From this, an intermediate positive control serum (IPCS), with a nominal concentration of 300units/ml, was prepared by mixing 300µl of standard sera with 29.7ml PBS/BSA, stored in 1ml aliquots at -20°C. We added 200µL/well of standard serum in duplicate at a starting concentration of 1/100, which was two-fold serial diluted to 1/12800 with PBS/BSA. High, intermediate and low-quality control (QC) sera were also prepared using IPCS standard sera to defined concentrations varying according to test antigen, respectively, and 100ul/well was added to the plate. PBS/BSA alone was used in the blank control wells and plates were incubated at RT for 1-hour.

An R^2 value for the standard curve of each plate was calculated and the plate rejected if the $R^2 <$ 0.98. Additionally, the plate was rejected if the average blank OD value was > 0.1. The results of each ELISA plate were accepted only if the OD values of the high, intermediate and low-quality control wells were within the range of their known values ± two standard deviations of the blank wells. Plates were rejected if the within-dilution percentage coefficient of variance (%CV) of the three QC dilutions was > 10% or, if the mean values for the three QC dilutions were outside the predetermined ranges.

For test sera sample to be accepted, at least 2 consecutive serial dilutions with a within-dilution %CV of < 20% for the duplicates of each dilution needed to be present. If samples fell into the bottom range of the standard curve close to the detection limit (absorbance LLQ) a within-dilution %CV of < 35% was accepted if no further dilution was available.

	Standard Serum		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	1/200	1/200	1/200	1/200	1/200	1/200	1/200	1/200
B	1/200	1/200	Med QC	Med QC	1/600	1/600	1/600	1/600	1/600	1/600	1/600	1/600
C	1/400	1/400	Low QC	Low QC	1/1800	1/1800	1/1800	1/1800	1/1800	1/1800	1/1800	1/1800
D	1/800	1/800	Blank	Blank	1/5400	1/5400	1/5400	1/5400	1/5400	1/5400	1/5400	1/5400
					Test Sera 5		Test Sera 6		Test Sera 7		Test Sera 8	
E	1/1600	1/1600	Blank	Blank	1/200	1/200	1/200	1/200	1/200	1/200	1/200	1/200
F	1/3200	1/3200	Blank	Blank	1/600	1/600	1/600	1/600	1/600	1/600	1/600	1/600
G	1/6400	1/6400	Blank	Blank	1/1800	1/1800	1/1800	1/1800	1/1800	1/1800	1/1800	1/1800
H	1/12800	1/12800	Blank	Blank	1/5400	1/5400	1/5400	1/5400	1/5400	1/5400	1/5400	1/5400

Figure 8-1 : Plate layout for O9:LPS, Hd and 02:LPS ELISA.

Eight test samples per 96 well plate. Numbers in boxes represent dilutions of test sera. Blank = PBS/BSA.

8.2.1.2. Vi-antigen ELISA

IgG responses to the Vi-antigen were measured using a commercial ELISA kit (VaccZyme, The Binding Site Ltd, Birmingham, UK) according to the manufacturer's instructions [209]. 100µl of each test sample at a dilution of 1/100 were added in duplicate to the plate and incubated at room temperature for 30 minutes. The plate was washed 3 times with 350µl of wash buffer, after which the conjugate antibody was added and incubated at room temperature for a further 30 minutes, then the wash step repeated. TMB was added to each well and incubated for 20 minutes. The final absorbances were read at two wavelengths (405 nm and 490 nm) using an automated microplate reader (Biotek ELx808 and associated Gen software v5.2). Plates passed if the standard curve had an $R^2 > 0.95$, and the high and low controls were within the range provided by the manufacturer. Individual samples were accepted if the %CV was < 15% for the duplicates of each sample. Samples which fell below the lower limit of detection for the assay kit (7.4U/mL), were assigned an arbitrary value of half the lower limit of detection (3.7U/mL) [209].

8.2.2. Ex-Vivo Antibody secreting cell ELISPOT assay

Ex-vivo antibody secreting cell (ASC) responses to *S*. Typhi O9:LPS and H-d, and S. Paratyphi O:2 and H-a were measured at baseline, 7 days after challenge and at the time of enteric fever diagnosis using an ELISOT assay as previously described[314].

Multiscreen filtration ELISPOT plates (Millipore/MAHAS4510) were coated as outlined in Figure 8-2 with *S*. Typhi O9:LPS, *S*. Typhi H-d antigen, *S*. Paratyphi O:2 antigen (as above), *S*. Paratyphi Ha (University of Maryland CVD1902D lot CVD141113-01) and Pan goat anti-human immunoglobulin (Invitrogen H170000) each at a final concentration of 10µg/ml in carbonate-bicarbonate buffer and incubated overnight at 4°C. Plates were blocked with 200µl/well of R10 medium for 1 hour prior to use at 37°C/5% CO_2. Peripheral blood mononuclear cells were

separated using Accuspin™ tubes (Sigma-Aldrich), counted and re-suspended in R10 media. Peripheral blood mononuclear cells (PBMCs) at a concentration of $2.5x10^5$ were added in duplicate to the ELISPOT plate (100µl/well) and incubated overnight at 37°C/5% CO_2. Plates were washed four times with PBS-0.25% Tween, once with PBS and soaked with PBS for 5 minutes. Goat anti-human IgG, IgM and IgA secondary antibodies conjugated to alkaline phosphatase (Calbiochem #401442, #401132, #401902) were diluted to 1:5000 in PBS/FBS and incubated for four hours at room temperature. After incubation, the plates were washed five times with PBS-0.25% Tween and four times with d.s.H_2O. Alkaline phosphatase substrate (Bio-Rad #170-6432) was added at 50µl/well, allowed to develop over ~10minutes and stopped with d.s.H_2O as spots begin to develop.

	Sample 1: Cells→ 2.5x10⁵/well						Sample 2: Cells→ 2.5x10⁵/well					
	1	2	3	4	5	6	7	8	9	10	11	12
A	LPS(T)	LPS(T)	LPS(T)	H(P)	H(P)	H(P)	LPS(T)	LPS(T)	LPS(T)	H(P)	H(P)	H(P)
B	LPS(T)	LPS(T)	LPS(T)	H(P)	H(P)	H(P)	LPS(T)	LPS(T)	LPS(T)	H(P)	H(P)	H(P)
C	LPS(T)	LPS(T)	LPS(T)	H(T)	H(T)	H(T)	LPS(T)	LPS(T)	LPS(T)	H(T)	H(T)	H(T)
D	LPS(T)	LPS(T)	LPS(T)	H(T)	H(T)	H(T)	LPS(T)	LPS(T)	LPS(T)	H(T)	H(T)	H(T)
E	LPS(P)	LPS(P)	LPS(P)	Ig	Ig	Ig	LPS(P)	LPS(P)	LPS(P)	Ig	Ig	Ig
F	LPS(P)	LPS(P)	LPS(P)	Ig	Ig	Ig	LPS(P)	LPS(P)	LPS(P)	Ig	Ig	Ig
G	LPS(P)	LPS(P)	LPS(P)	CB	CB	CB	LPS(P)	LPS(P)	LPS(P)	CB	CB	CB
H	LPS(P)	LPS(P)	LPS(P)	CB	CB	CB	LPS(P)	LPS(P)	LPS(P)	CB	CB	CB

Figure 8-2 : Ex-vivo antibody secreting cell ELISPOT plate layout and coating antigen.

LPS(T) = S. Typhi LPS. LPS(P) = S. Paratyphi O:2 antigen. H(T) = Purified Salmonella Typhi Hd antigen. H(P) = Purified S. Paratyphi A Ha antigen. Ig = Positive control immunoglobulin wells. CB = Negative control blank wells.

ELISPOT plates were read using an automated ELISPOT reader (AID ELR03/ELR030408215) and AID ELISPOT software V5.0. Study and antigen specific count settings for spot intensity, size and gradient were applied to plate counts and manually verified to remove artefacts. Raw counts (spots/$2.5x10^5$ PBMCs) were averaged across duplicate well and multiplied by four to give the

number of spot forming units (SFUs) per 10^6 PBMCs. The number of background spots detected in blank wells were subtracted from the test samples to give the final cell count per sample.

8.2.3. Memory B-cell ELISPOT

Antigen specific memory B-cells (Bm) were analysed by ELISPOT, using the method originally described by Crotty and colleagues[410], which has previously been applied to study Bm responses after oral typhoid vaccination[366,409].

Bm ELISPOT assays were performed on frozen PBMCs collected at baseline, 28 days after challenge and 90 days after challenge. Frozen vials of PBMCs were defrosted inside a class II safety cabinet by gentle swirling in warmed water and transferred to complete media (RPMI plus 50ml heat inactivated fetal bovine serum, 5ml of penicillin/streptomycin, 5ml L-Glutamine, 5ml NEAA, 5ml Sodium Pyruvate and 500µl 2- Mercaptoethanol) warmed to 37°C. Thawed cells were spun at 250G for ten minutes, washed with PBS-EDTA+0.5% BSA, counted and resuspended in complete media to a concentration of $2x10^6$ PBMC/ml. Cells were added to round bottom 96-well plates (100µl/well, $2x10^5$ cells), to which 100µl/well of a polyclonal antigen stimulation mix was added. The antigen stimulation mix comprised CpG (200µg/ml, 1:58 dilution BioScience UK #15230162), pokeweed mitogen (1mg/ml, 1/6000 dilution, Sigma L-9379) and *Staphylococcus aureus* Cowan's strain suspension (VWR international #507861-50) made up in complete media. Cells suspended in antigen stimulation mix were cultured for 5 days at 37°C/5% CO_2, before harvesting and washing twice with wash buffer at 250G/10 minutes. Cultures cells were counted, re-suspended in complete media to a concentration of $2x10^6$ PBMC/ml, added to blocked ELISPOT plates and subsequently processed as outlined above for ex-vivo ELISPOTS.

8.2.4. Statistical Considerations

Baseline comparisons across groups were performed by Kruskal-Wallis test for non-parametric measures. Paired samples across timepoints were measured using Wilcoxon matched-pairs signed rank test. Between group comparisons were performed using the Mann-Whitney U test. Serum ELISA data was $\log_{10}$-transformed to approximate a normal distribution. A nominal value of 3.7EU/mL was assigned to all unmeasurable anti-Vi IgG titres, corresponding to half lower-limit of detection.

Antibody-secreting cell counts were $\log_{10}$-transformed prior to analysis. Zero counts were assigned a nominal value of 0.5.

8.3. Results

8.3.1. Baseline antibody status

Baseline IgG antibody levels against O9:LPS, Hd and Vi antigens were compared between three groups randomised to challenge with *S*. Typhi.

To determine if previous exposure was associated with persistence of *S*. Typhi/Paratyphi specific antibody in serum, I began by comparing antibody levels at baseline. Overall, previous challenge was not associated with elevated levels of anti-O9:LPS and anti-Hd IgG in serum (Figure 8-3). Similarly, in participants randomised to challenge with *S.* Paratyphi, there were no significant differences in baseline serum anti-O2 IgG and IgA (Figure 8-4). Surprisingly, baseline anti-Vi IgG was significantly higher in the *S*. Typhi naïve group compared with the homologous re-challenge group (p=0.006 - Figure 8-3). Elevated anti-Vi IgG in individuals ostensibly naïve to *S*. Typhi exposure may be the result of undisclosed Vi-vaccination not detected at screening or cross-

reactivity to Vi-antigen from other sources (e.g. *Citrobacter freundii*, *Salmonella enterica.* serovar *Dublin*).

Previous studies have demonstrated that anti-O and anti-H antibody levels returned to baseline 3-6 months after challenge[74,297]. To determine if baseline serum antibody concentrations had declined since primary challenge, we examined the relationship between re-challenge interval and antibody concentration. There was a weak negative correlation between re-challenge interval and baseline anti-O9:LPS IgG (r=-0.39, p= 0.047). For all other antigens and antibody isotypes tested, there was no association between baseline antibody levels and re-challenge interval (Figure 8-5).

In previous challenge studies, seroconversion to O-antigens was observed primarily in those participants who develop disease after challenge[126 311]. To determine if antibody levels at baseline differed depending on the outcome of primary challenge, we compared baseline levels in the re-challenge cohort categorised according to the outcome of primary challenge. For all antigens and antibody isotypes tested, the outcome of primary challenge was not significantly associated with baseline serum antibody levels (Figure 8-6). Finally, there was no association between baseline antibody titres and age at time of challenge (Figure 8-7).

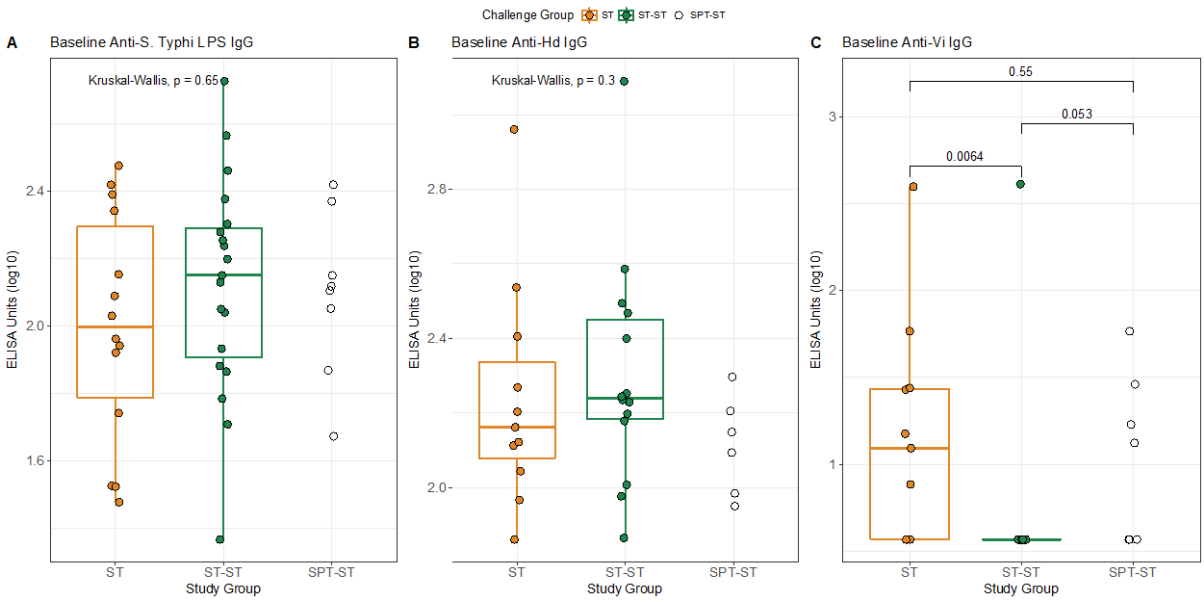


Figure 8-3 : Baseline anti-O9:LPS,anti-Vi and anti-Hd IgG between challenge groups randomised to *S.* Typhi challenge.

Boxplots illustrate median and interquartile range. (A) *S.* Typhi LPS; (B) *S.* Typhi Hd (C) Vi-antigen. Comparisons across all groups were performed using the Kruskal-Wallis test. Between group comparisons in (C) were performed using the Mann-Whitney U-test. ST = *S.* Typhi naïve; ST-ST = Homologous *S.* Typhi re-challenge. SPT-ST = Heterologous *S.* Typhi re-challenge

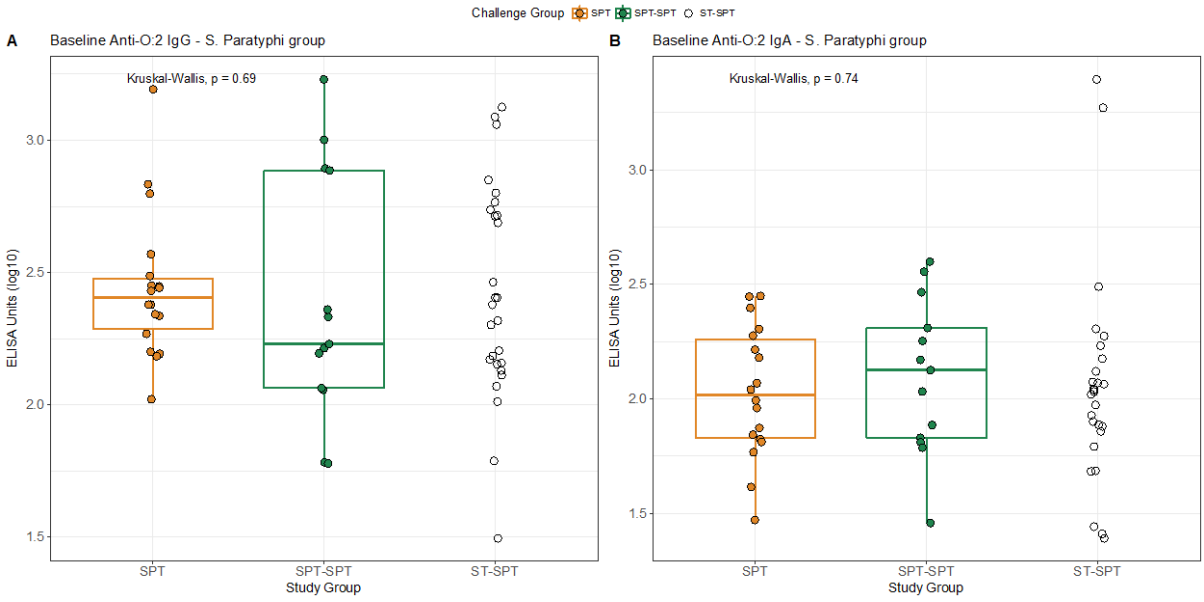


Figure 8-4 : Baseline anti-O:2 IgG and IgA between challenge groups randomised to S. Paratyphi challenge.

Boxplots illustrate median and interquartile range. SPT = *S.* Paratyphi naïve; SPT-SPT = Homologous *S.* Paratyphi re-challenge. ST-SPT = Heterologous *S.* Paratyphi re-challenge.

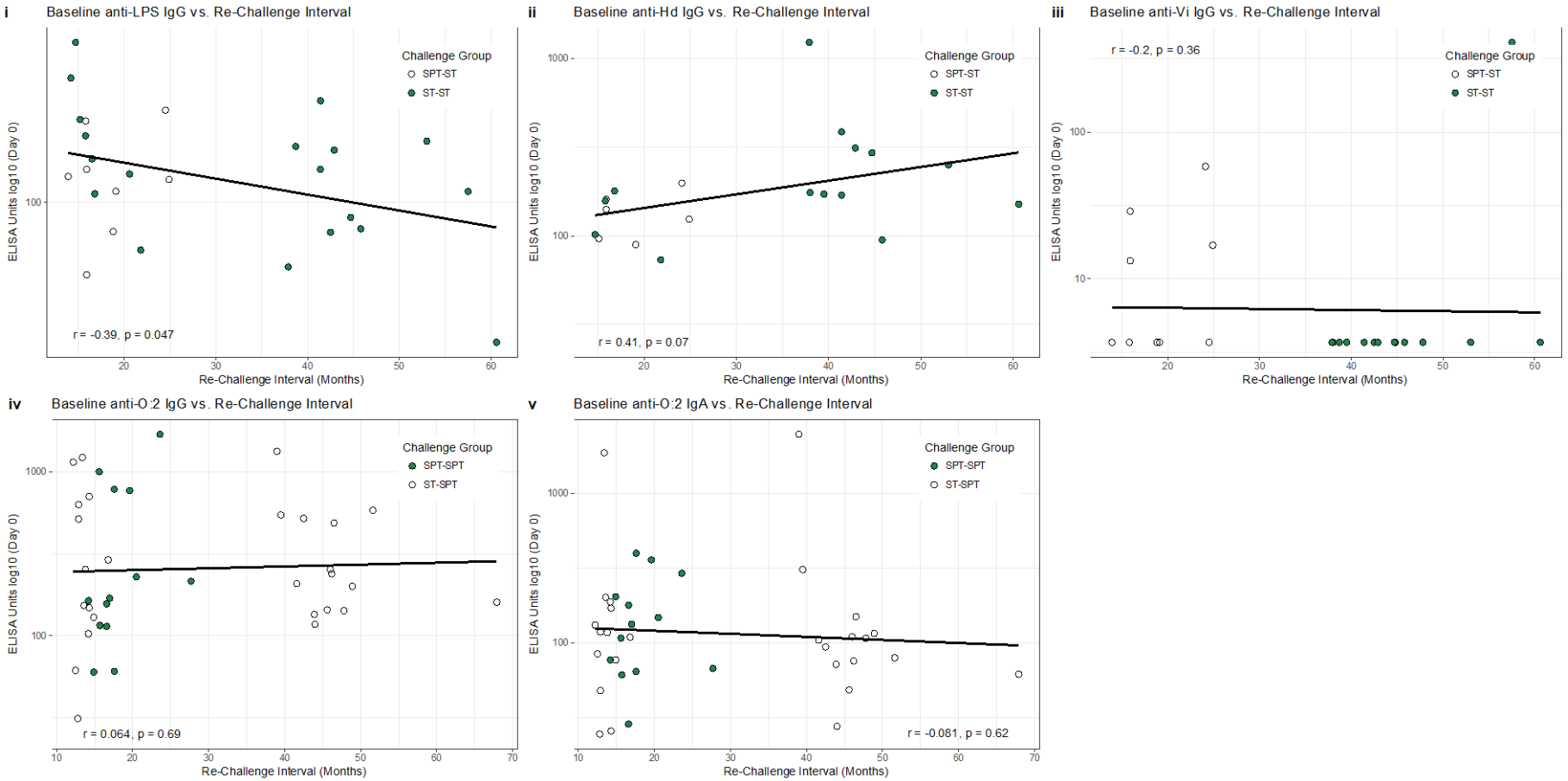


Figure 8-5 : Relationship of baseline antibody titre with re-challenge interval.

(i-iii) *S*. Typhi homologous re-challenge groups; (iv-v) *S*. Paratyphi homologous re-challenge groups only. r = Spearman's correlation coefficient.

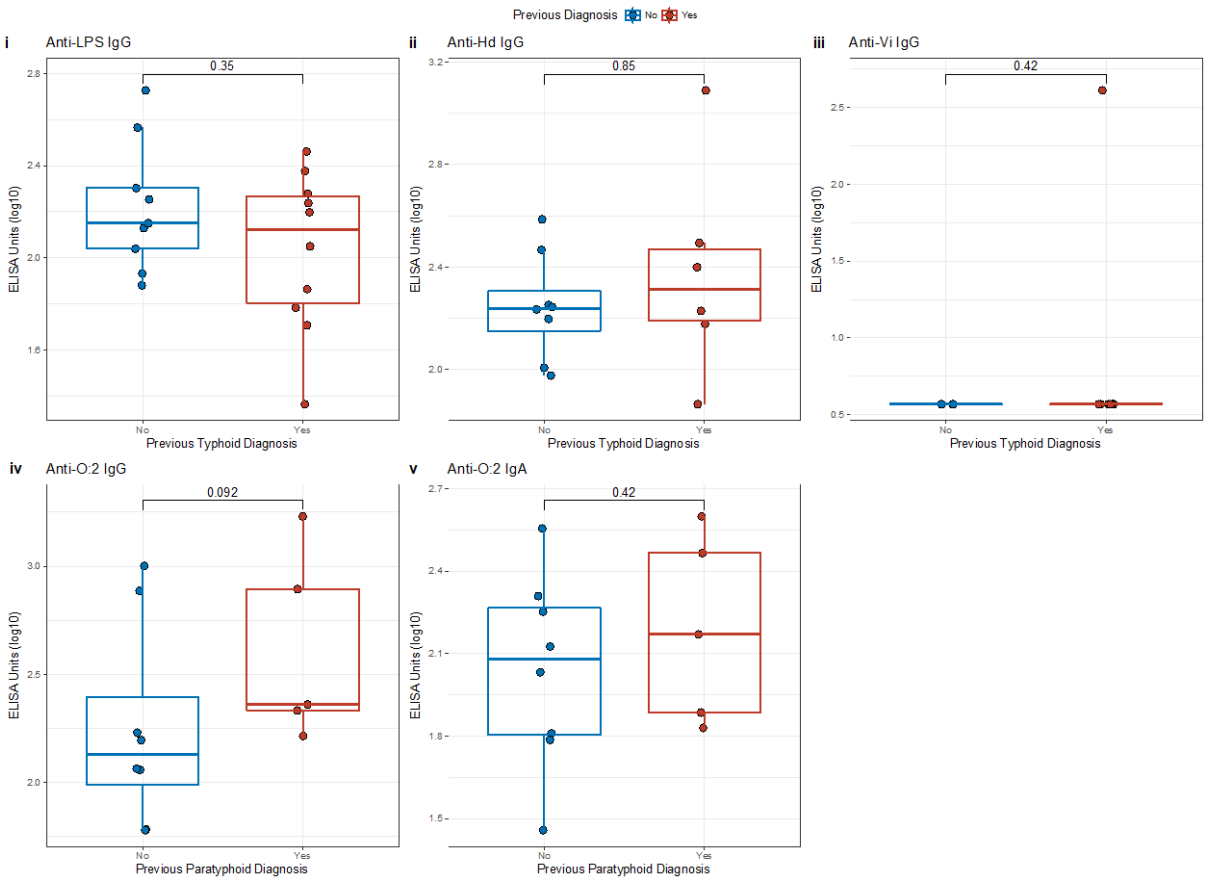


Figure 8-6 : Baseline (Day 0) antibody status by outcome of primary challenge.

(i-iii) *S.* Typhi homologous re-challenge groups; (iv-v) *S.* Paratyphi homologous re-challenge groups only. p = Mann-Whitney U test.

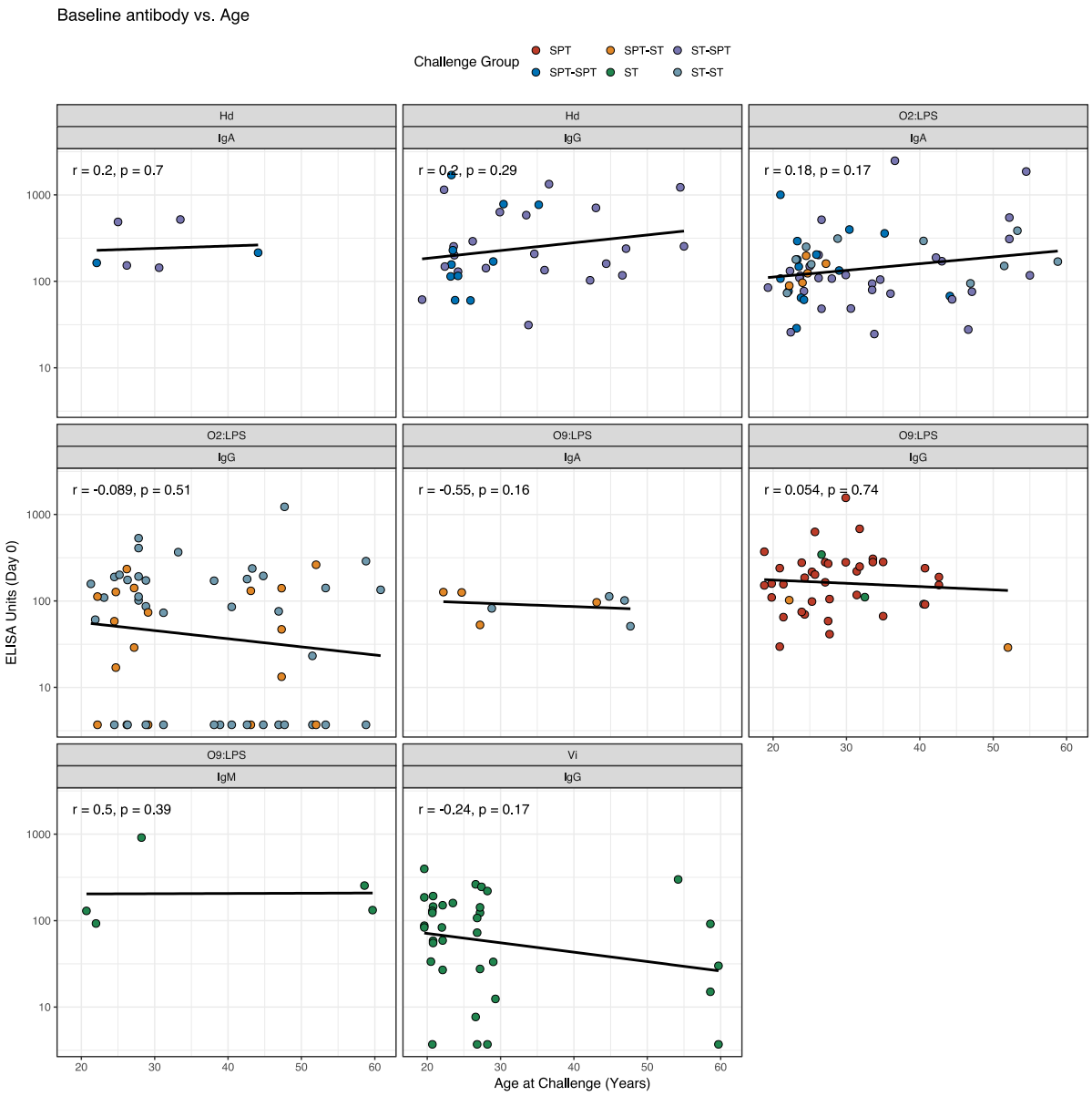


Figure 8-7 : Association between baseline antibody levels and age at time of challenge.

Grouped according to antigen and antibody isotype. Individuals coloured according to study challenge group. r = Spearman's correlation coefficient.

8.3.2.S. Typhi challenge - Relationship of serum antibody with challenge outcome

To determine if pre-existing levels of circulating antibody were related to protection against typhoid fever after *S.* Typhi challenge, we began by comparing baseline serum anti-O9, anti-Vi and anti-Hd IgG in all participants challenged. When all groups were combined, there was no significant difference in baseline anti-O9 and anti-Hd IgG concentration between individuals who

progressed to typhoid disease compared with those who did not develop disease after challenge (Figure 8-8). Baseline anti-Vi IgG in participants were non-significantly elevated in participants who developed disease after challenge (p=0.07 - Figure 8-8). The same pattern was observed when naïve (ST) and re-challenge groups (ST-ST and SPT-ST) were analysed separately, although the analysis was limited by small sample size within each group (Figure 8-9). This analysis suggests that baseline anti-O9:LPS, anti-Hd IgG and anti-Vi IgG levels were not directly correlated with protection against typhoid disease after challenge.

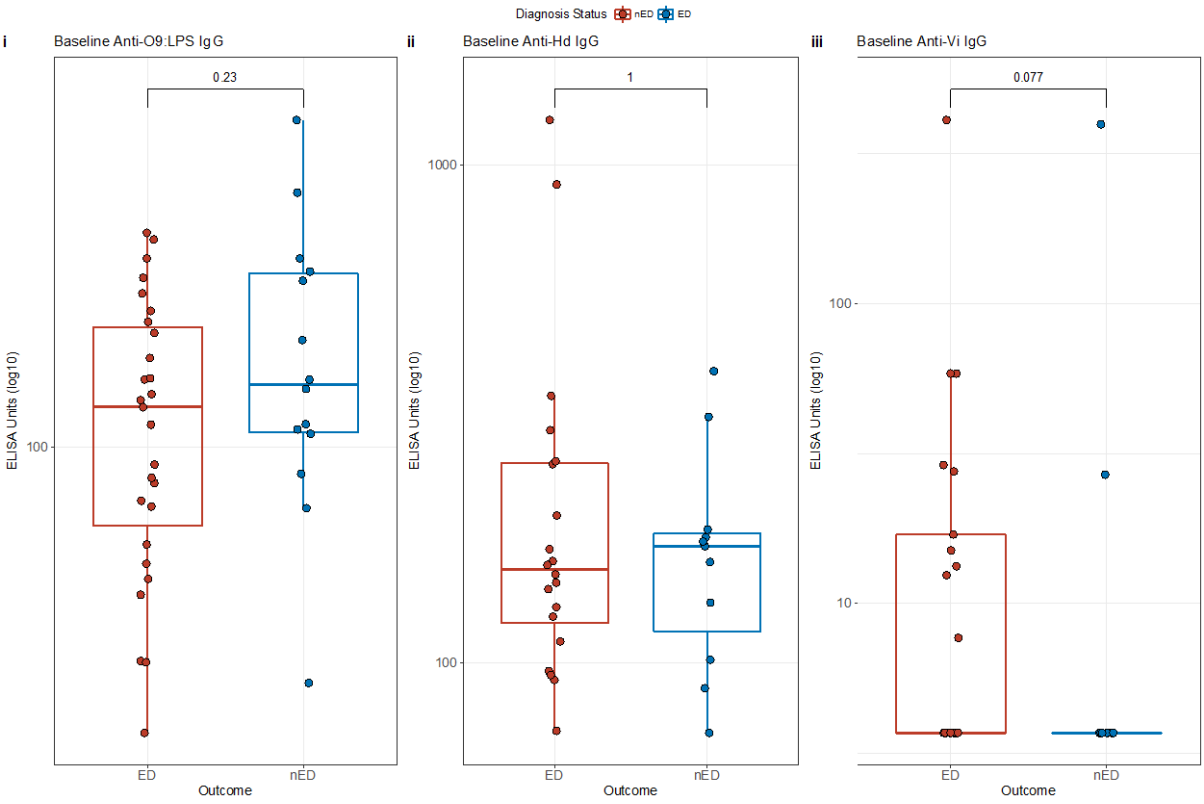


Figure 8-8 : Relationship of baseline antibody with challenge outcome (S. Typhi challenge).

Baseline anti-O9:LPS IgG (i), anti-Hd IgG (ii) and anti-Vi IgG (iii), in participants challenged with Salmonella Typhi according to challenge outcome. Mann-Whitney U test. ED = Met criteria for enteric fever diagnosis. nED = Did not meet criteria for enteric fever diagnosis.

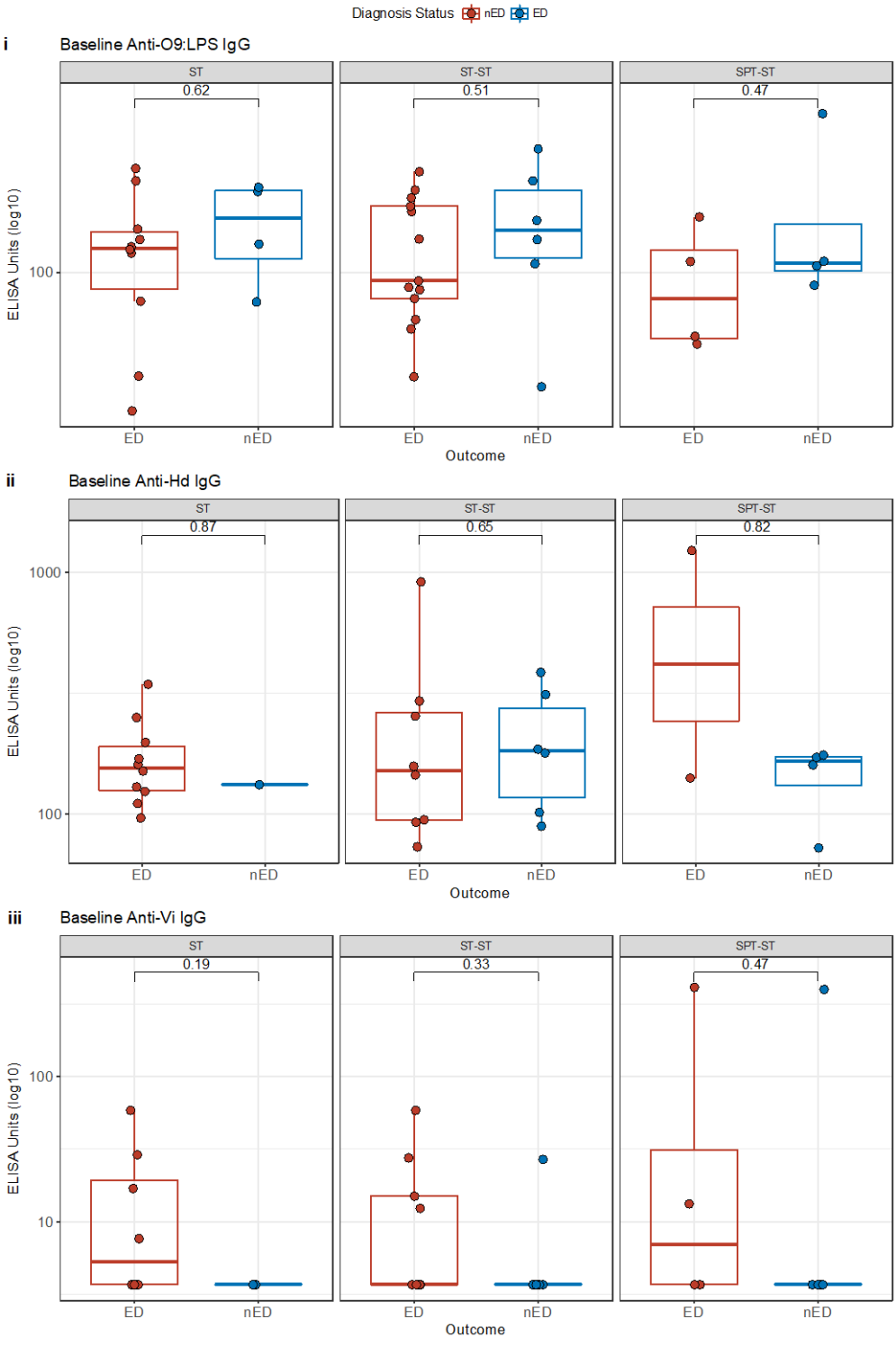


Figure 8-9 : Relationship of baseline antibody with challenge outcome (*S.* Typhi challenge) by challenge group.

Baseline anti-O9:LPS IgG (i), anti-Hd IgG (ii) and anti-Vi IgG (iii), in participants challenged with *Salmonella* Typhi according to challenge outcome. Mann-Whitney U test. ED = Met criteria for enteric fever diagnosis. nED = Did not meet criteria for enteric fever diagnosis. ST = *S.* Typhi naïve; ST-ST = Homologous *S.* Typhi re-challenge. SPT-ST = Heterologous *S.* Typhi re-challenge.

We next compared serum anti-O:9, anti-Hd IgG and anti-Vi IgG responses following *S.* Typhi challenge. Participants challenged with *S.* Typhi typically mounted a serum antibody response against O9:LPS, but not against Hd- or Vi-antigen (Figure 8-10). Responses peaked at day 28 and remained elevated from baseline when measured at day 90. Consistent with previous challenge studies, the anti-O9:LPS IgG response was driven by participants who developed typhoid fever, as compared with those who did not develop typhoid fever after challenge (Figure 8-10), suggesting that seroconversion was more pronounced in the context of clinical symptoms and/or bacteraemia.

To determine if previous exposure to typhoidal *Salmonella* was associated with a boosting of antibody response on re-challenge, we compared the level of post-challenge antibody in naïve and re-challenge participants who developed typhoid disease. Overall, there was no significant difference in the magnitude of the anti-O9:LPS IgG response at day 28 and day 90 when naïve and homologous-re-challenge groups were compared (Figure 8-11). Notably, previous exposure to *S.* Paratyphi was associated with the greatest increase in anti-O9:LPS IgG at day 28 and day 90 following *S.* Typhi, which was significantly greater than the naïve and homologous re-challenge groups (Figure 8-11).

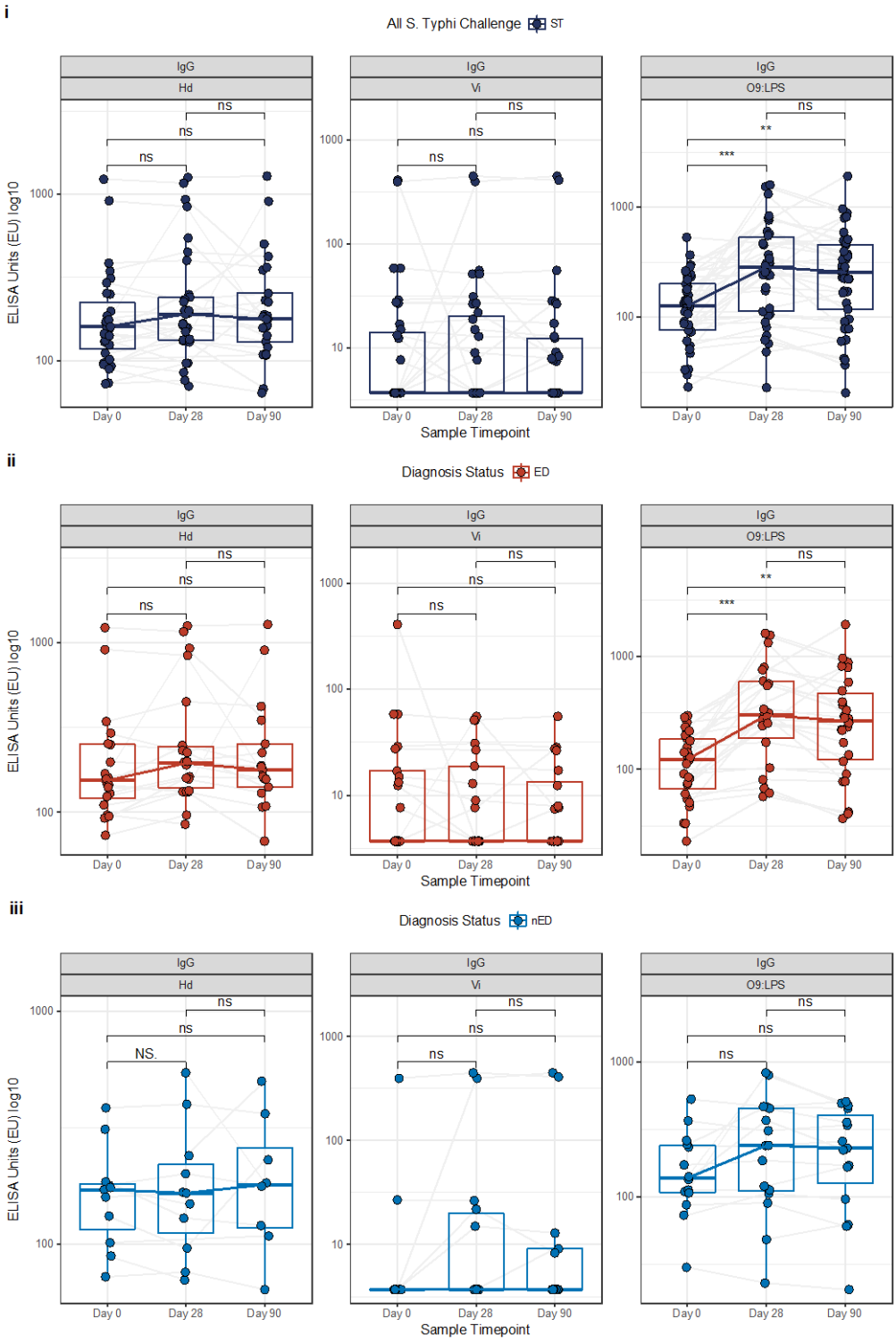


Figure 8-10 : Serum antibody responses following Salmonella Typhi challenge across all challenge groups.

(i) All *S.* Typhi Challenge regardless of diagnostic outcome; (ii) Participants meeting diagnostic criteria for enteric fever; (iii) Participants not meeting criteria for enteric fever. Coloured lines connect median values for each timepoint. Grey lines connect paired samples across timepoints. Wilcoxon matched-pairs signed rank test. *** p <0.001, **p<0.01, ns = p >0.05.

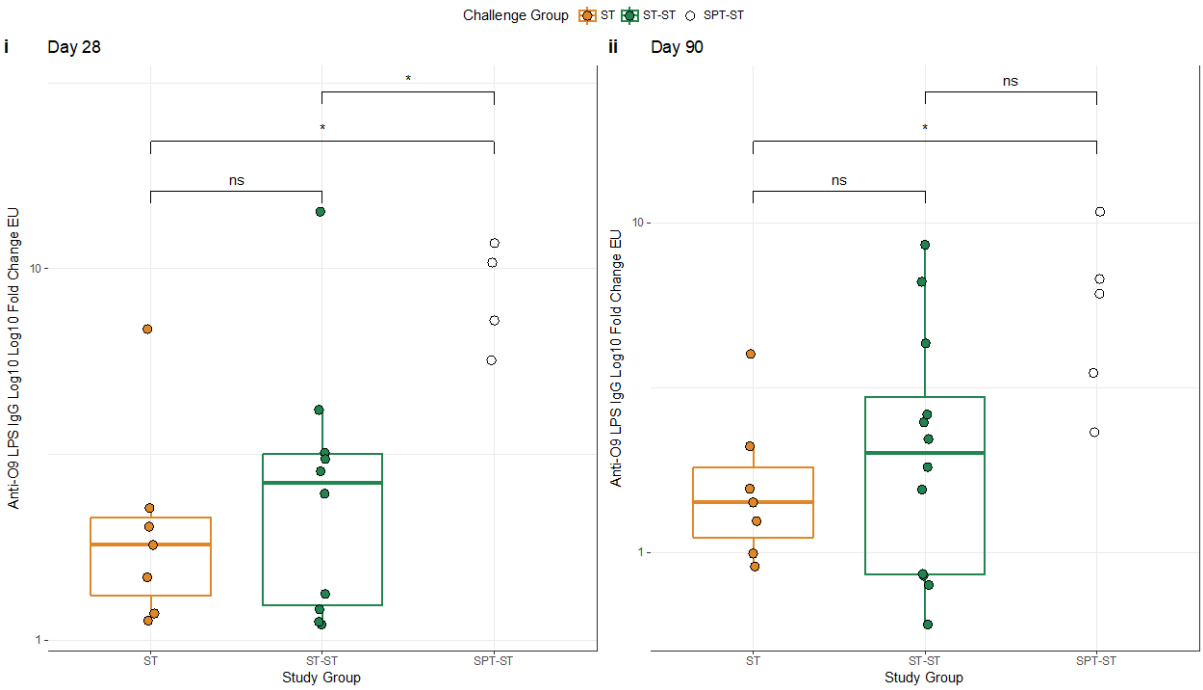


Figure 8-11 : Magnitude of Anti-O9:LPS IgG antibody responses in participants diagnosed with typhoid fever

Stratified by challenge group, represented as log_{10} fold change from baseline. (i) Day 28 post challenge. (ii) Day 90 post challenge. Mann-Whitney U test.*p<0.05. ST = *S.* Typhi naïve; ST-ST = Homologous *S.* Typhi re-challenge. SPT-ST = Heterologous *S.* Typhi re-challenge

8.3.3.S. Paratyphi challenge - Relationship of serum antibody with challenge outcome

To determine if pre-existing levels of anti-O2 IgG and IgA were related to protection after *S.* Paratyphi challenge, we began by comparing baseline serum anti-O2 in all participants challenged. When all groups were combined, there was no significant difference in baseline anti-O2 IgG and IgA between individuals who progressed to paratyphoid disease compared with those who did not develop disease after challenge (Figure 8-12). The same pattern was observed when naïve (SPT) and re-challenge groups (SPT-SPT and ST-SPT) were analysed separately (Figure 8-13). This analysis suggests that baseline anti-O2 IgG and IgA levels were not correlated with protection against paratyphoid within the context of the challenge model.

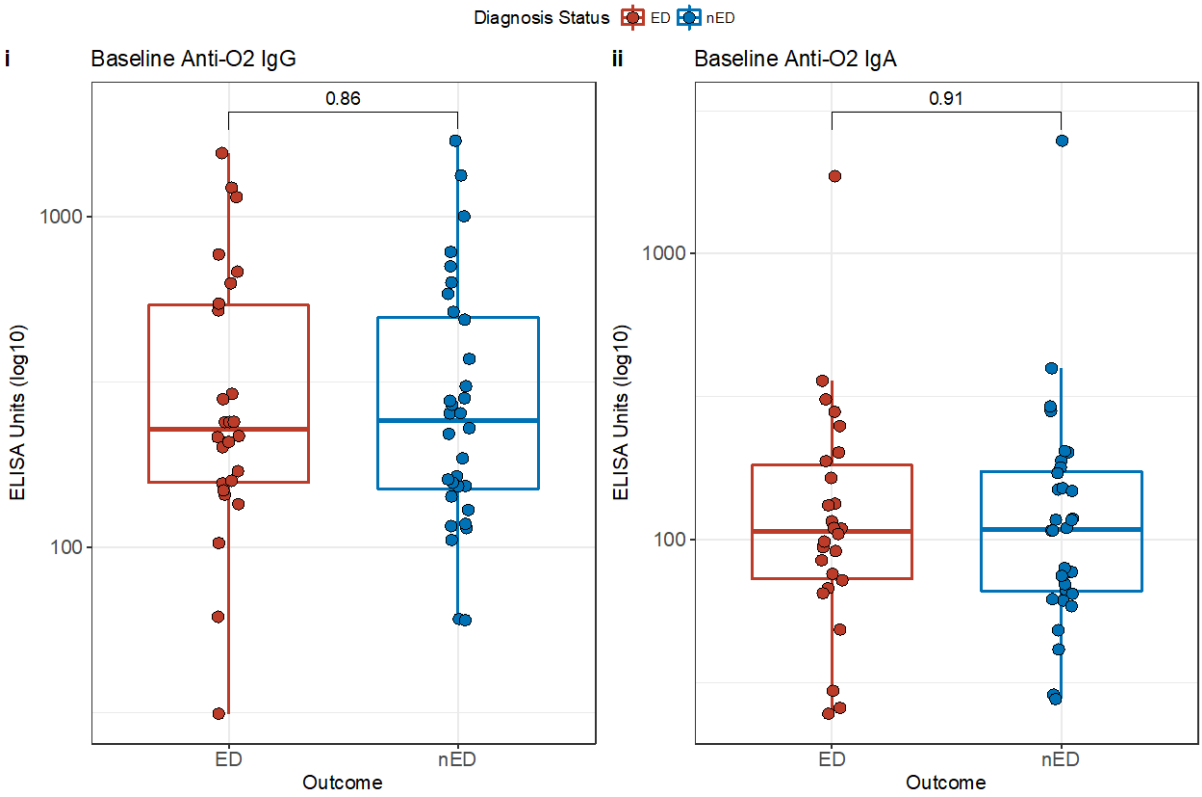


Figure 8-12 : Relationship of baseline antibody with challenge outcome (*S*. Paratyphi challenge).

Baseline anti-O2 IgA (left) and IgG (right) across all groups in participants challenged with *Salmonella* Paratyphi according to challenge outcome. ED = Meet criteria for enteric fever diagnosis. nED = Did not meet criteria for enteric fever diagnosis. P = Mann-Whitney U test.

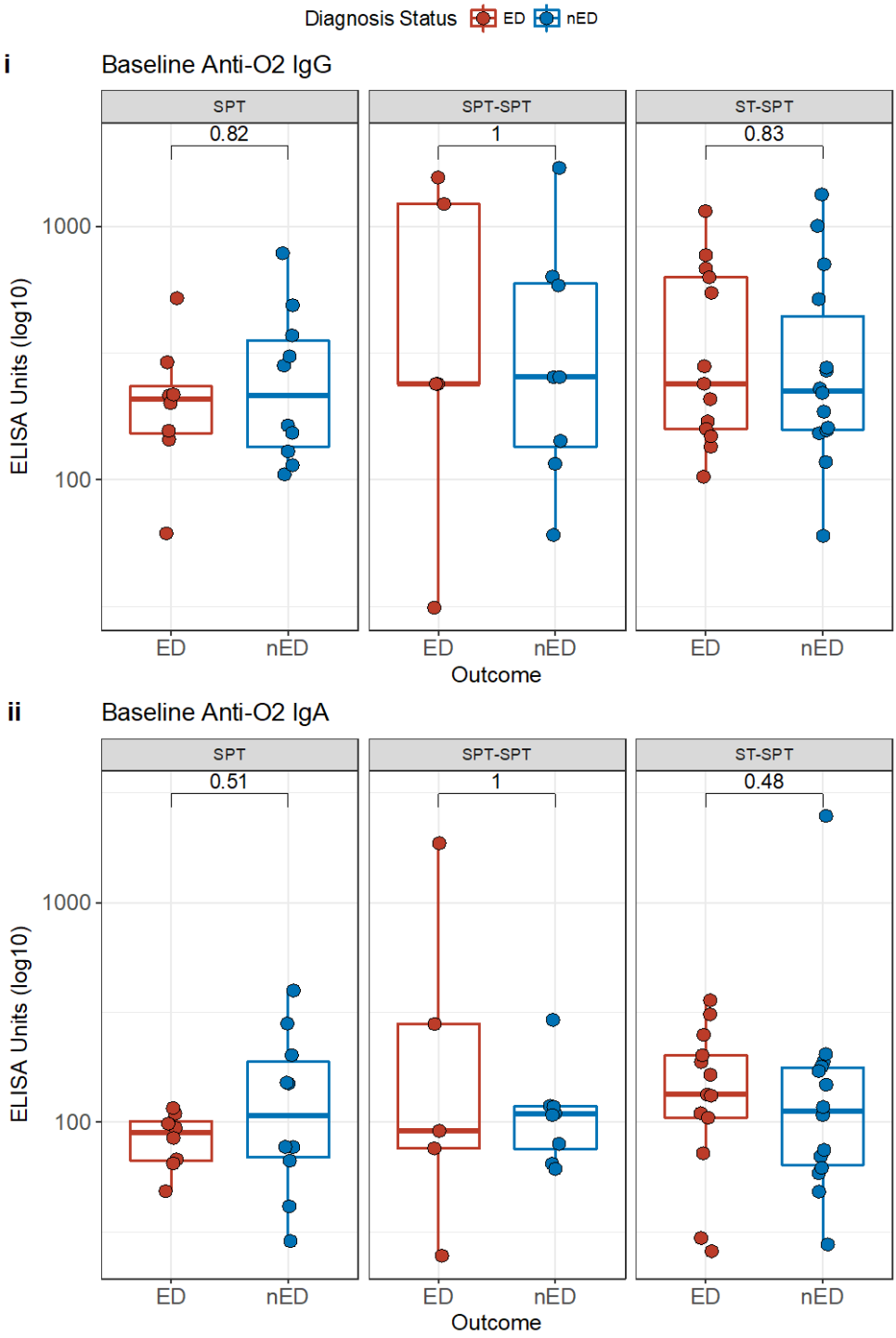


Figure 8-13 : Relationship of baseline antibody with challenge outcome (*S.* Paratyphi challenge) by challenge group.

Baseline anti-O2:LPS IgG (i) and anti-Vi IgA (ii), in participants challenged with *Salmonella* Paratyphi according to challenge outcome. Mann-Whitney U test. ED = Met criteria for enteric fever diagnosis. nED = Did not meet criteria for enteric fever diagnosis.

We next compared serum anti-O2 IgG and IgA responses following *S.* Paratyphi challenge. Consistent with earlier studies, participants who developed paratyphoid disease displayed a significant increase in anti-O2 IgG and IgA, which peaked 28 days after challenge and remained elevated at day 90 (Figure 8-14). When analysed as a group, anti-O2 IgG and IgA responses in the non-diagnosed group did not significantly increase at day 28 and day 90 compared with baseline (Figure 8-14– iii). However, when individual paired samples are analysed, a small proportion of individuals had an increase in anti-O2 IgG and IgA titres from day 0 to day 28, which may be indicative of sub-clinical disease (Figure 8-14– iii).

The magnitude of the anti-O2 IgG and IgA responses in participants who developed paratyphoid disease did not significantly differ significant between naïve and homologous re-challenge groups (Figure 8-15), suggesting that prior exposure to S. Paratyphi was not associated with a boosting of antibody levels on-challenge. Previous exposure to *S.* Typhi was not associated with an enhanced anti-O2 IgG and IgA response following *S.* Paratyphi re-challenge (Figure 8-15).

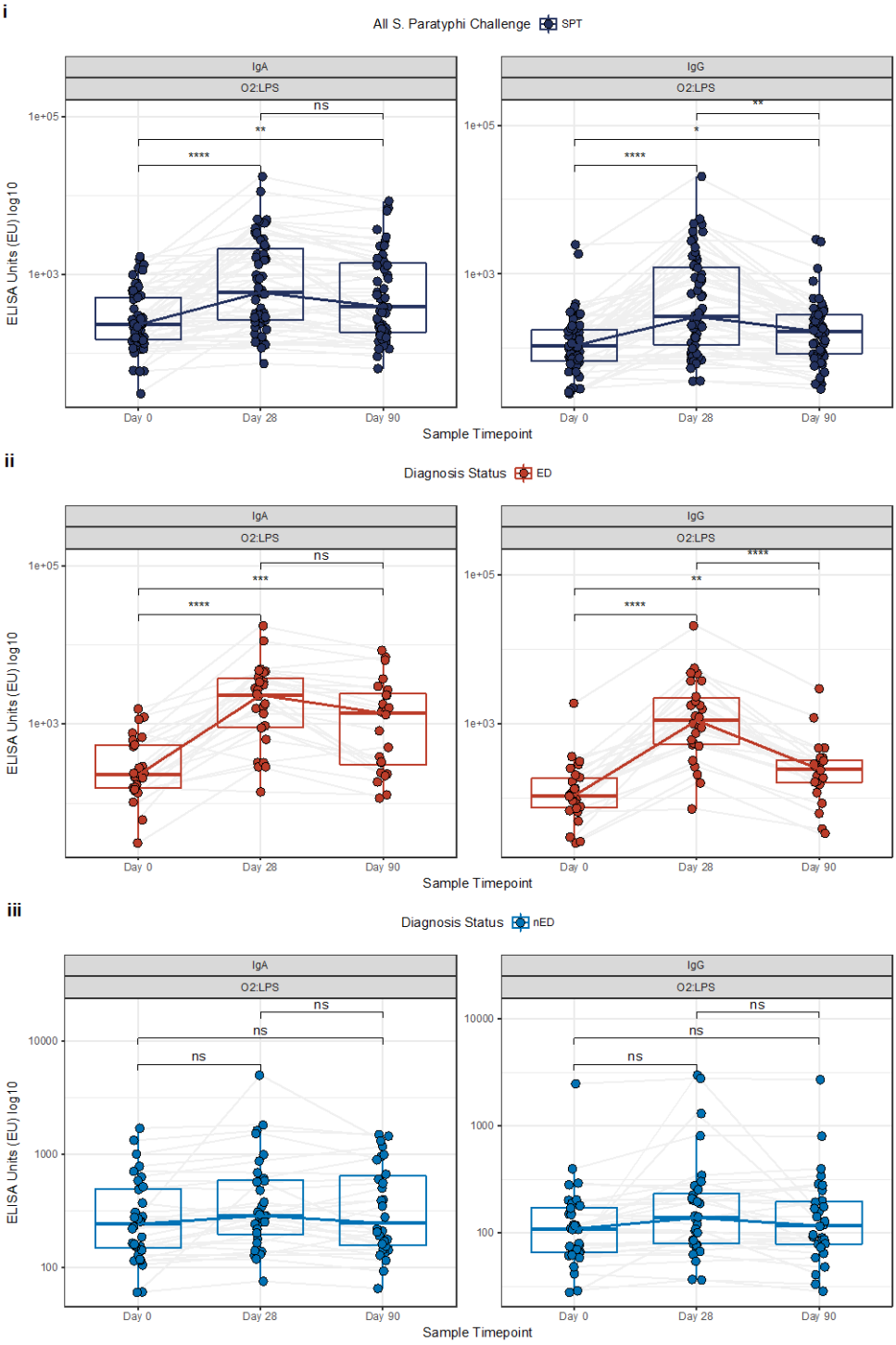


Figure 8-14 : Serum antibody responses following *Salmonella* Paratyphi challenge across all challenge groups.

(i) All *S.* Paratyphi Challenge regardless of diagnostic outcome; (ii) Participants meeting diagnostic criteria for enteric fever; (iii) Participants not meeting criteria for enteric fever. Coloured lines connect median values for each timepoint. Grey lines connect paired samples across timepoints. Wilcoxon matched-pairs signed rank test.**** p<0.0001, *** p <0.001, **p<0.01, ns = p >0.05.

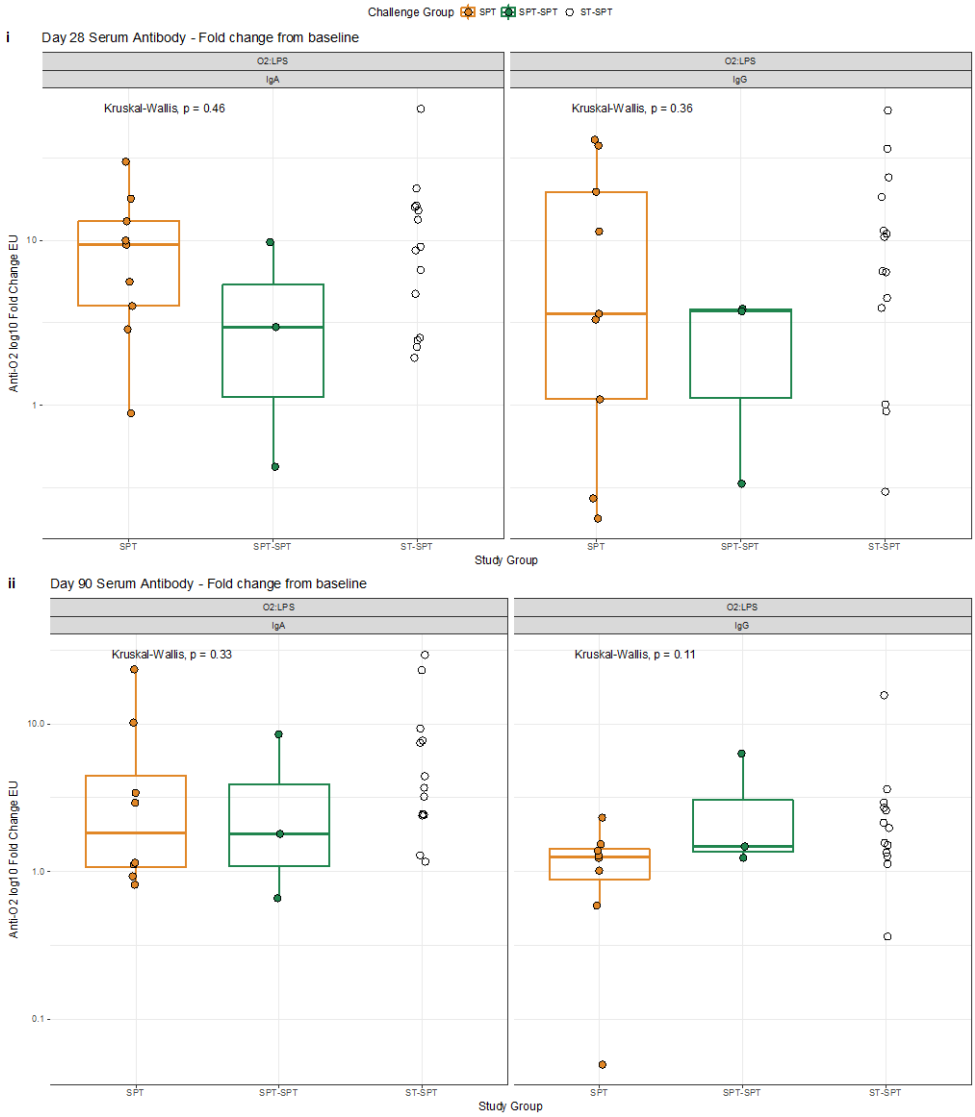


Figure 8-15 : Magnitude of anti-O2 LPS IgG and IgA antibody responses in participants diagnosed with paratyphoid fever

Stratified by challenge group, represented as $\log_{10}$ fold change from baseline. (i) Day 28 post challenge. (ii) Day 90 post challenge. SPT = *S.* Paratyphi naïve; SPT-SPT = Homologous *S.* Paratyphi re-challenge. ST-SPT = Heterologous *S*. Paratyphi re-challenge.

8.3.4. Kinetics of Serum Antibody Response after S. Typhi and S. Paratyphi challenge and re-challenge

In order to characterise persistence and temporal dynamics of serum antibody, anti-O9:LPS IgG and anti-O2:LPS IgA/IgG levels from individuals undergoing homologous re-challenge with *S.* Typhi and *S.* Paratyphi were plotted longitudinally. Matched serum samples were available for 14/27 (52%) individuals undergoing homologous *S.* Typhi re-challenge and 13/13 (100%) individuals undergoing homologous *S.* Paratyphi re-challenge, of whom 9/14 (64%) and 5/13 (38%) were diagnosed with enteric fever at primary challenge. The median interval between primary challenge and re-challenge was 1179 days (range 512 – 1842) and 510 days (426-831) respectively.

8.3.4.1. Paired S. Typhi Challenge & Re-Challenge

The kinetics of serum antibody responses to *S.* Typhi challenge and re-challenge are illustrated in Figure 8-16. Participants who developed typhoid fever after primary challenge mounted a strong anti-O9:LPS response, which peaked at day 28 after challenge. Antibody levels began to wane by day 60-90 after primary challenge and had returned to baseline immediately prior to re-challenge. There was no significant difference in paired baseline antibody levels prior to primary challenge and those measured immediately prior to re-challenge (p=0.78 - Figure 8-17). Two individuals who did not meet the diagnostic criteria for typhoid fever at primary challenge had a >4-fold rise in anti-O9:LPS, possibly indicative of subclinical infection at primary exposure (Figure 8-16 -iii).

In order to assess if re-challenge was associated with boosting of antibody responses, we compared peak antibody responses after primary challenge with those measured after re-challenge. The magnitude of the anti-O9:LPS IgG response was significantly higher after primary

challenge than after re-challenge (p=0.017 - Figure 8-18). This observation is in keeping with the apparent lack of a booster response on homologous re-challenge (Figure 8-11).

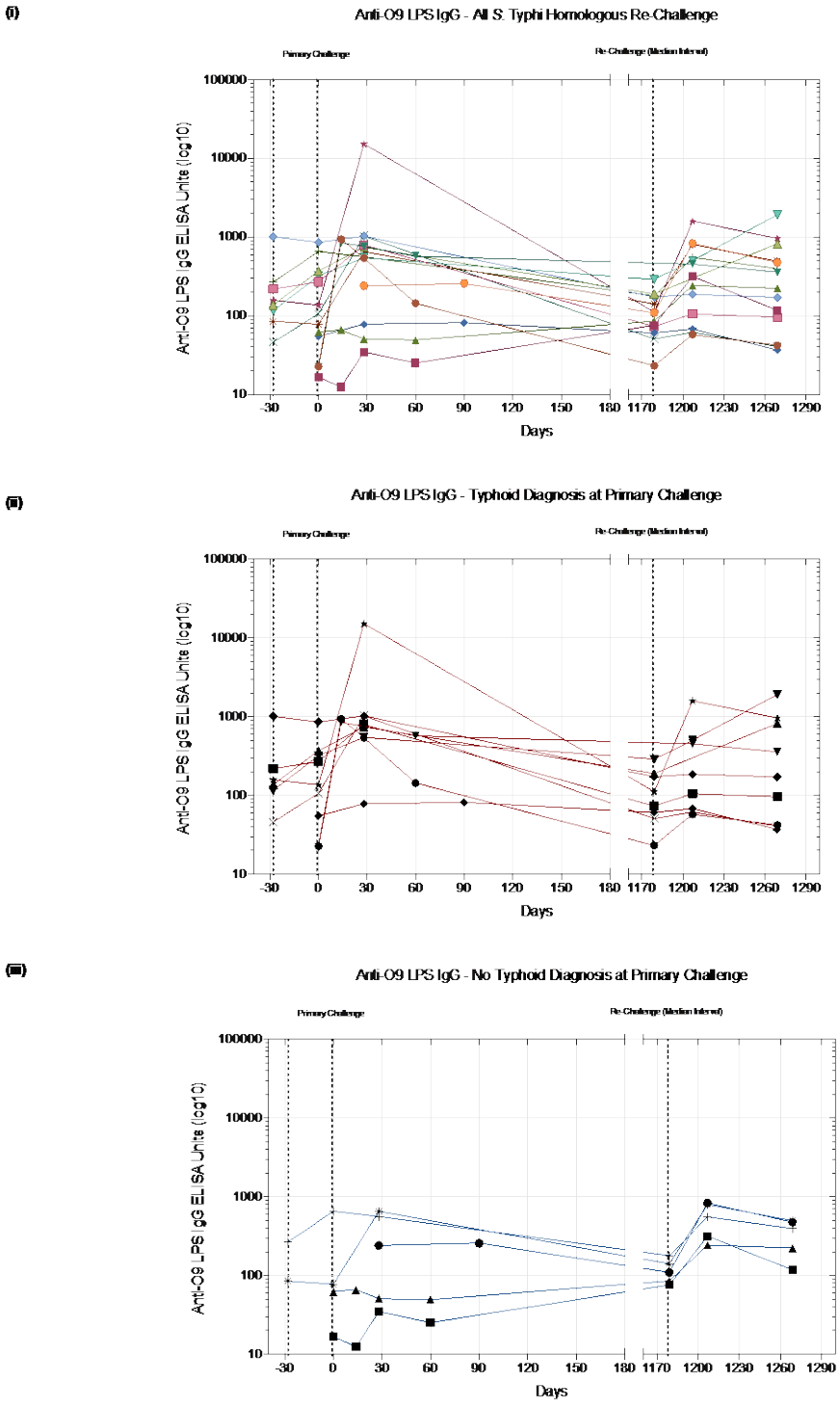


Figure 8-16 : Temporal dynamics of serum anti-O9 LPS IgG in S. Typhi-S. Typhi homologous re-challenge group.

(i) All challenged participants. (ii) Participants meeting composite criteria for typhoid fever at primary challenge. (iii) Participants not meeting composite criteria for typhoid fever at primary challenge. Time point of re-challenge normalised to median re-challenge interval between primary challenge and re-challenge (median 1179 days, range 512 – 1842 days).

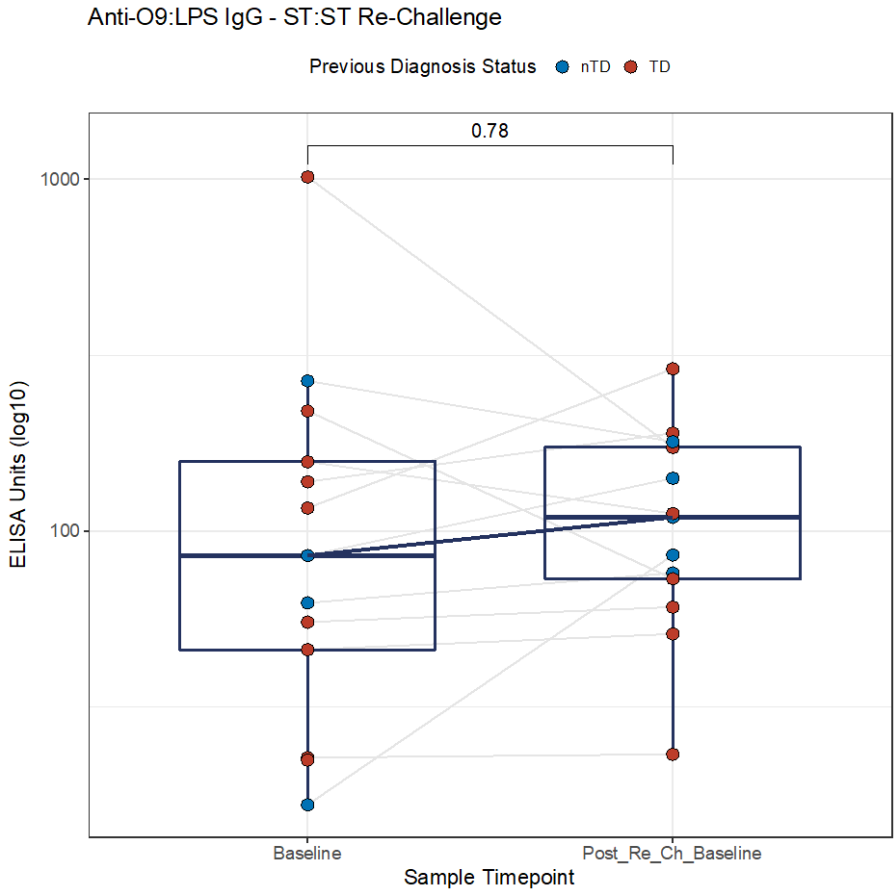


Figure 8-17 : Persistence of serum anti-O9 IgG

Comparison of paired serum antibody responses within individuals prior to first challenge (Baseline) and immediately prior to re-challenge (Post_Re_Ch_Baseline), coloured according to outcome of primary challenge. (i)Anti-O9:LPS IgG in individuals enrolled in the S. Typhi-S. Typhi re-challenge group. Data points are coloured according to outcome of primary challenge (nTD = No typhoid disease, TD = Typhoid Disease). Coloured line connects median at the two timepoints. Grey lines connect paired samples. Median interval from primary challenge to re-challenge 1179 days (range 512 – 1842). P = Wilcoxon matched-pairs signed rank test.

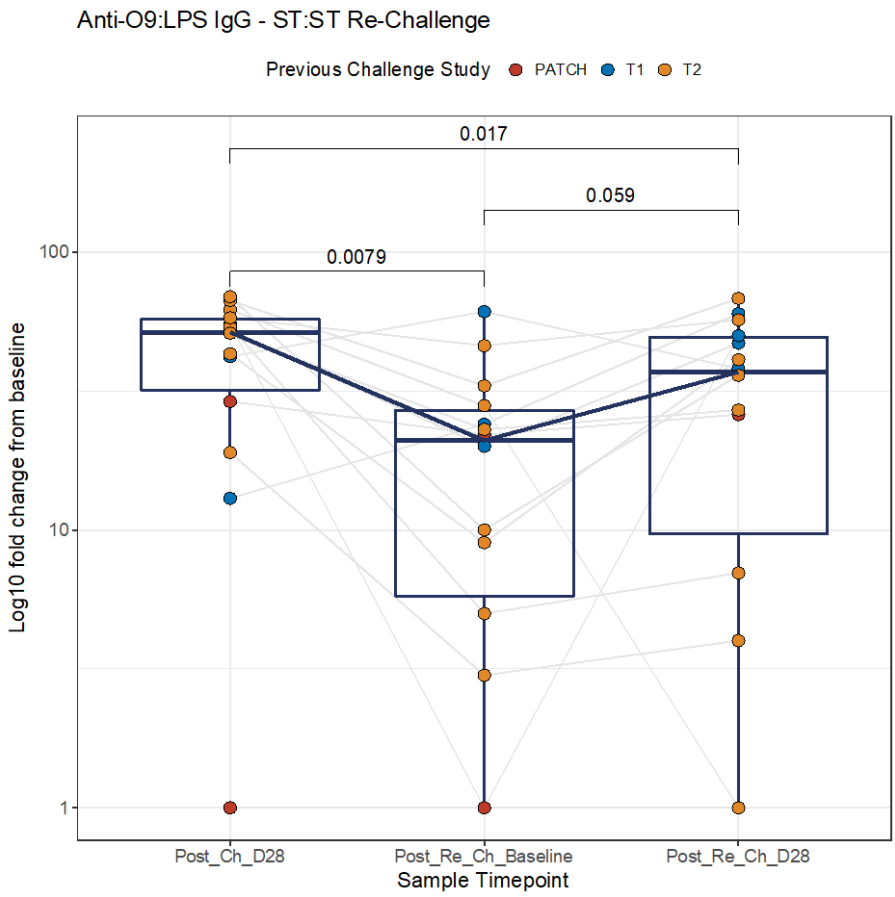


Figure 8-18 : Comparison of the magnitude of serum antibody response after primary challenge and re-challenge.

(i) Anti-O9 LPS IgG in S. Typhi homologous re-challenge group. Time points: Post_Ch_D28 = Serum IgG at 28 days after primary challenge; Post_Re_Ch_Baseline = Serum IgG measured immediately prior to re-challenge; Post_Re_Ch_D28 = Serum IgG measured 28 days after re-challenge. Data points are coloured according to primary study. Coloured line connects median at the two timepoints. Grey lines connect paired samples. P = Wilcoxon matched-pairs signed rank test.

8.3.4.2. Paired *S.* Paratyphi Challenge & Re-Challenge

We performed a similar analysis of the kinetics of anti-O2:LPS response in individuals enrolled in the *S.* Paratyphi-*S.* Paratyphi homologous re-challenge group (Figure 8-19 & Figure 8-20). Similar to *S.* Typhi challenge, individuals who developed paratyphoid fever after challenge mounted a strong antibody response that peaked at day 28 and began to wane by day 90. As was observed in the S. Typhi re-challenge group, the majority of individuals who did not develop paratyphoid fever at primary challenge failed to mount an antibody response and levels remained stable prior to re-challenge. Two participants mounted anti-O2 IgG and IgA responses, suggestive of subclinical infection.

In contrast with S. Typhi re-challenge, paired anti-O2 IgG/IgA levels measured immediately prior to re-challenge were significantly higher than those measured prior to primary challenge ($p = 0.001$ and 0.03 for IgG and IgA respectively - Figure 8-21), suggesting that antibody levels induced after primary infection had not completely returned to their pre-challenge baseline. This observation is possibly attributable to the shorter re-challenge interval for *S.* Paratyphi than for *S.* Typhi (Figure 7-3).

In participants re-challenged with *S.* Paratyphi, the magnitude of the anti-O2 IgG and IgA response was not significantly increased after re-challenge compared with primary challenge, suggesting that re-challenge was not associated with a boosting of antibody levels within the context of the challenge model (Figure 8-22).

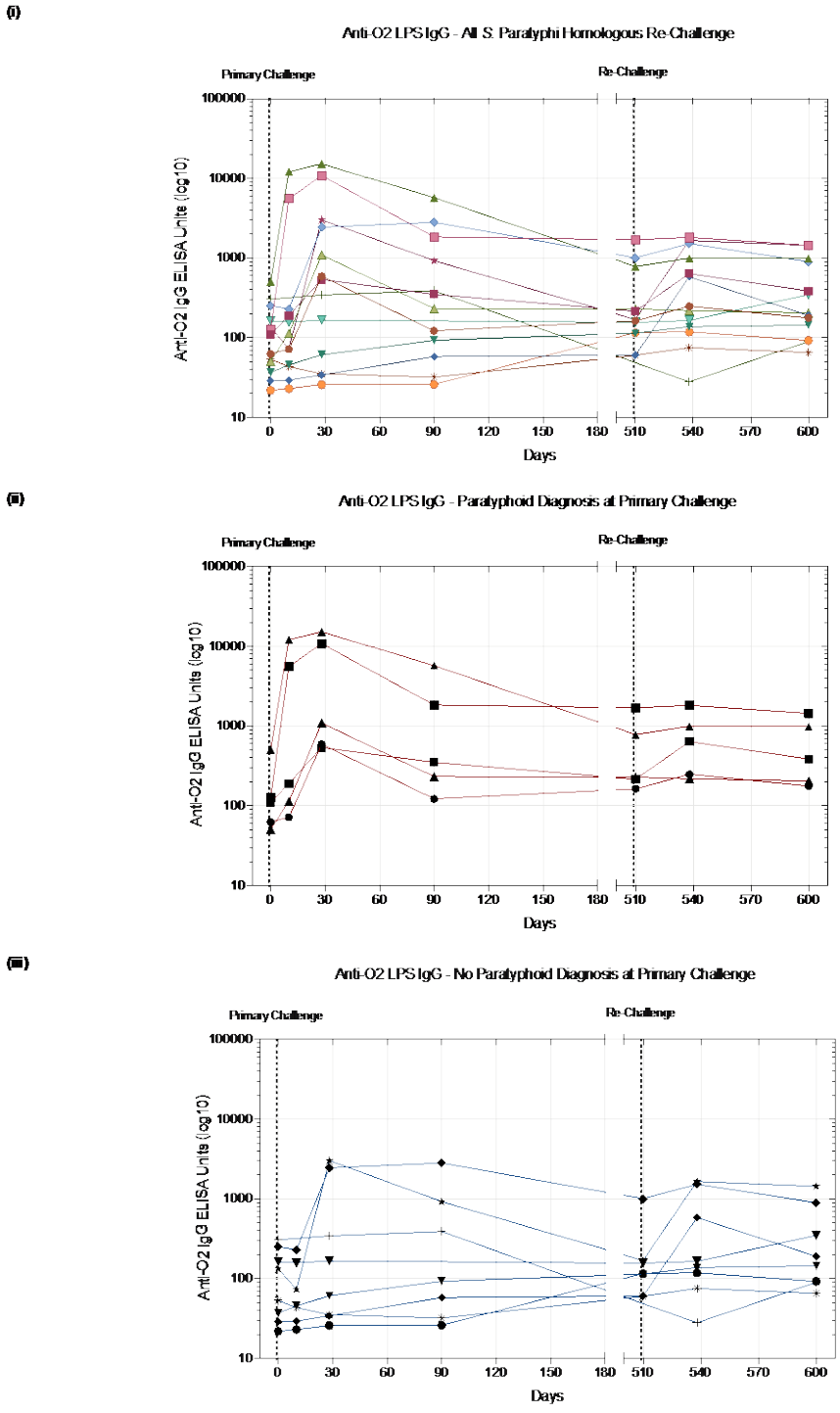
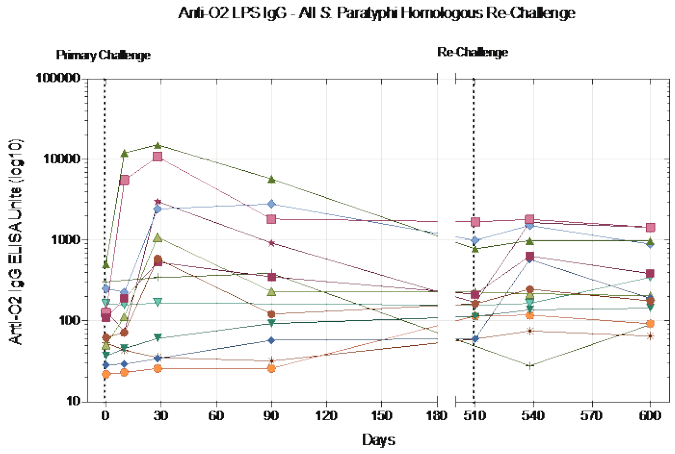


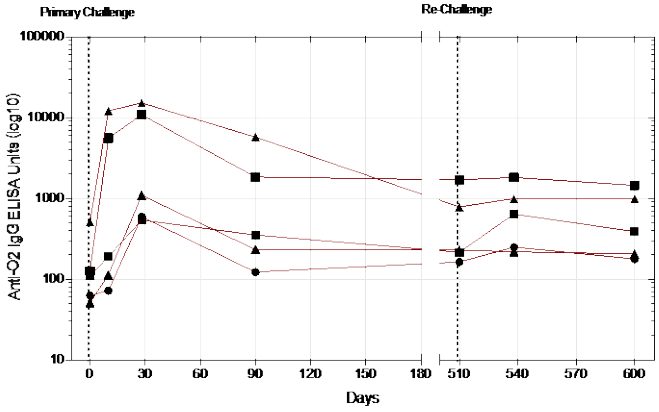
Figure 8-19 : Temporal dynamics of serum anti-O2 LPS IgG in S. Paratyphi-S. Paratyphi homologous re-challenge group.

(i) All challenged participants. (ii) Participants meeting composite criteria for paratyphoid fever at primary challenge. (iii) Participants not meeting composite criteria for paratyphoid fever at primary challenge. Time point of re-challenge normalised to median re-challenge interval between primary challenge and re-challenge (median 510 days, range 426-831 days).

(i)



(ii)



(iii)

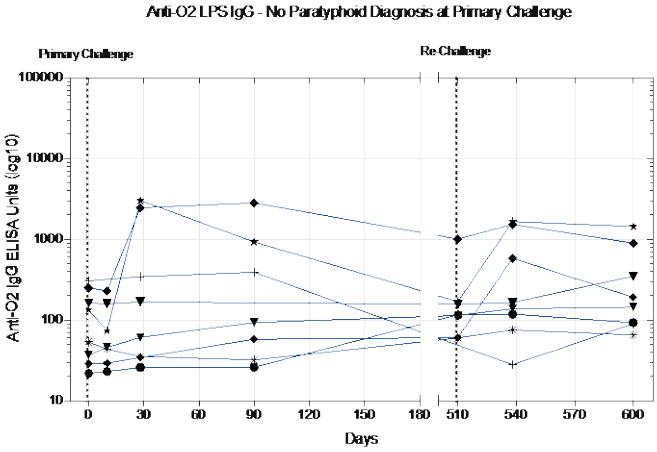


Figure 8-20 : Temporal dynamics of serum anti-O2 LPS IgA in S. Paratyphi-S. Paratyphi homologous re-challenge group.

(i) All challenged participants. (ii) Participants meeting composite criteria for paratyphoid fever at primary challenge. (iii) Participants not meeting composite criteria for paratyphoid fever at primary challenge. Time point of re-challenge normalised to median re-challenge interval between primary challenge and re-challenge (median 510 days, range 426-831 days).

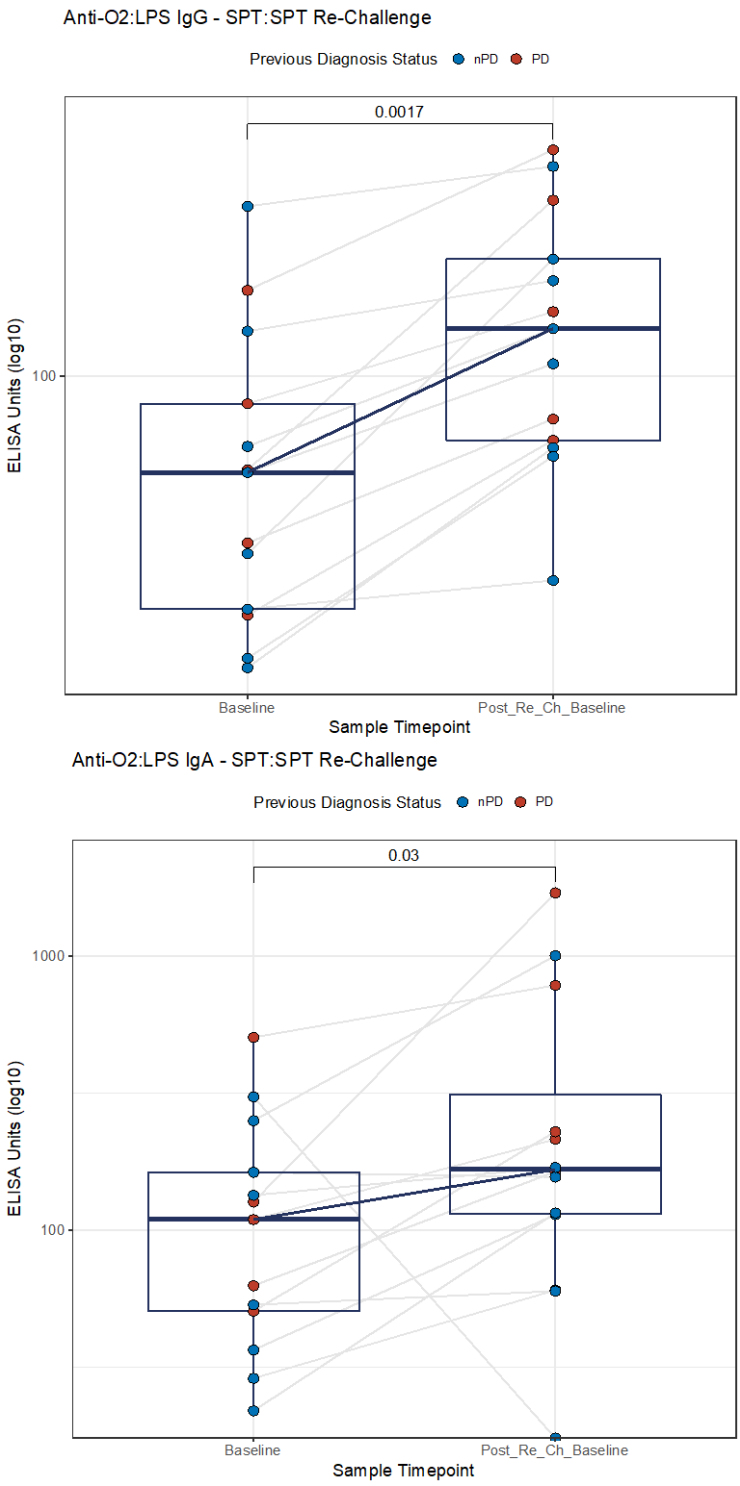


Figure 8-21 : Persistence of serum anti-O2 IgG and IgA.

Comparison of paired serum antibody responses within individuals prior to first challenge (Baseline) and immediately prior to re-challenge (Post_Re_Ch_Baseline). Anti-O2:LPS IgG (top) and IgA (bottom) in individuals enrolled in the S. Paratyphi-S. Paratyphi re-challenge group. Data points are coloured according to outcome of primary challenge (nPD = No Paratyphoid disease, PD = Paratyphoid Disease). Coloured line connects median at the two timepoints. Grey lines connect paired samples. Median interval from primary challenge to re-challenge 510 days (426-831). P = Wilcoxon matched-pairs signed rank test.

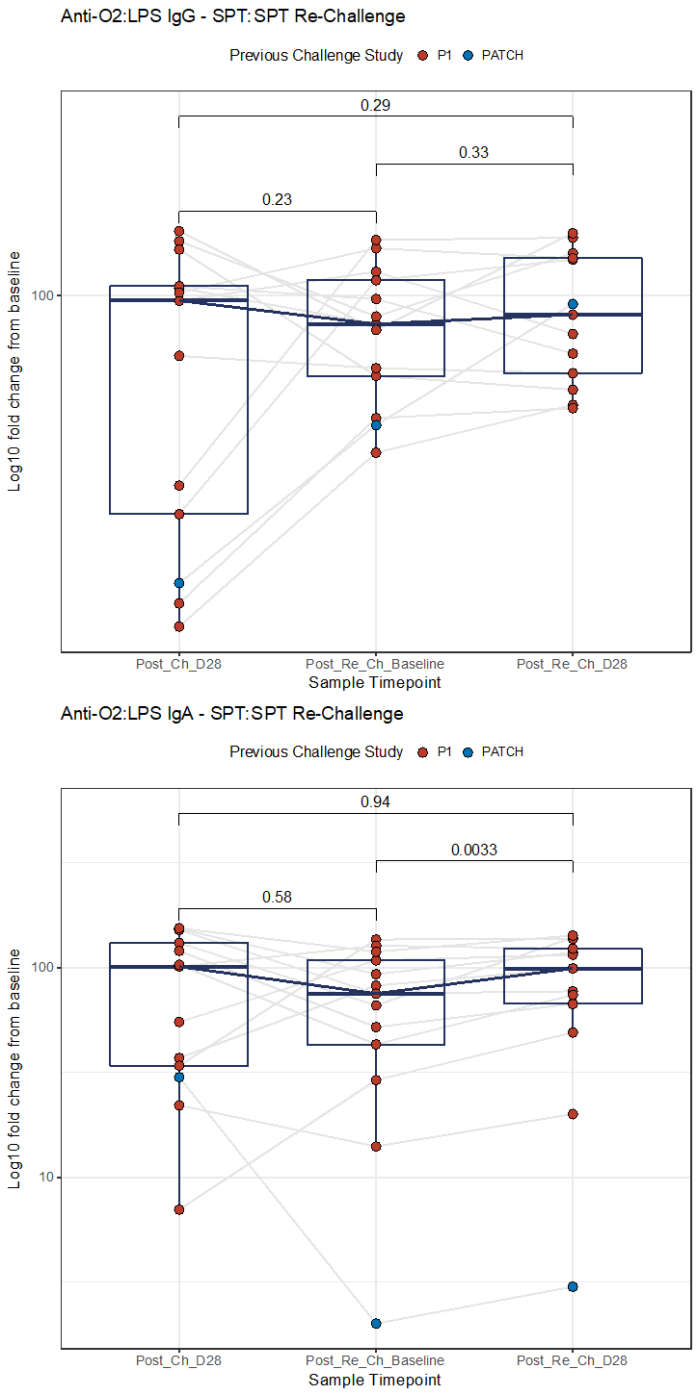


Figure 8-22 : Comparison of the magnitude of serum antibody response after primary *S.* Paratyphi challenge and re-challenge.

Anti-O2 LPS IgG (top) and IgA (bottom) in *S.* Paratyphi homologous re-challenge group. Time points: Post_Ch_D28 = Serum IgG at 28 days after primary challenge; Post_Re_Ch_Baseline = Serum IgG measured immediately prior to re-challenge; Post_Re_Ch_D28 = Serum IgG measured 28 days after re-challenge. P = Wilcoxon matched-pairs signed rank test.

8.3.5. Antibody secreting cells

In order to further characterise the humoral immune response after primary infection and re-challenge, we compared the antibody secreting cell (ASC) response to *S.* Typhi and *S.* Paratyphi surface antigens. Ex-vivo ASC ELISPOT assays were performed as outlined in section 8.2.2 and were measured at baseline, at 24-48 hours after enteric fever diagnosis and at day 7 in individuals not meeting the criteria for enteric fever diagnosis.

8.3.5.1. Antibody Secreting Cell responses after S. Typhi challenge

The number of anti-O9:LPS IgG, IgA and IgM producing ASCs was significantly increased at the time of enteric fever diagnosis when compared with baseline (Figure 8-23). In contrast, we observed no significant increase in anti-Hd IgG, IgA and IgM producing ASCs at the time of typhoid diagnosis (Figure 8-24), consistent with the lack of a strong anti-Hd antibody response after challenge.

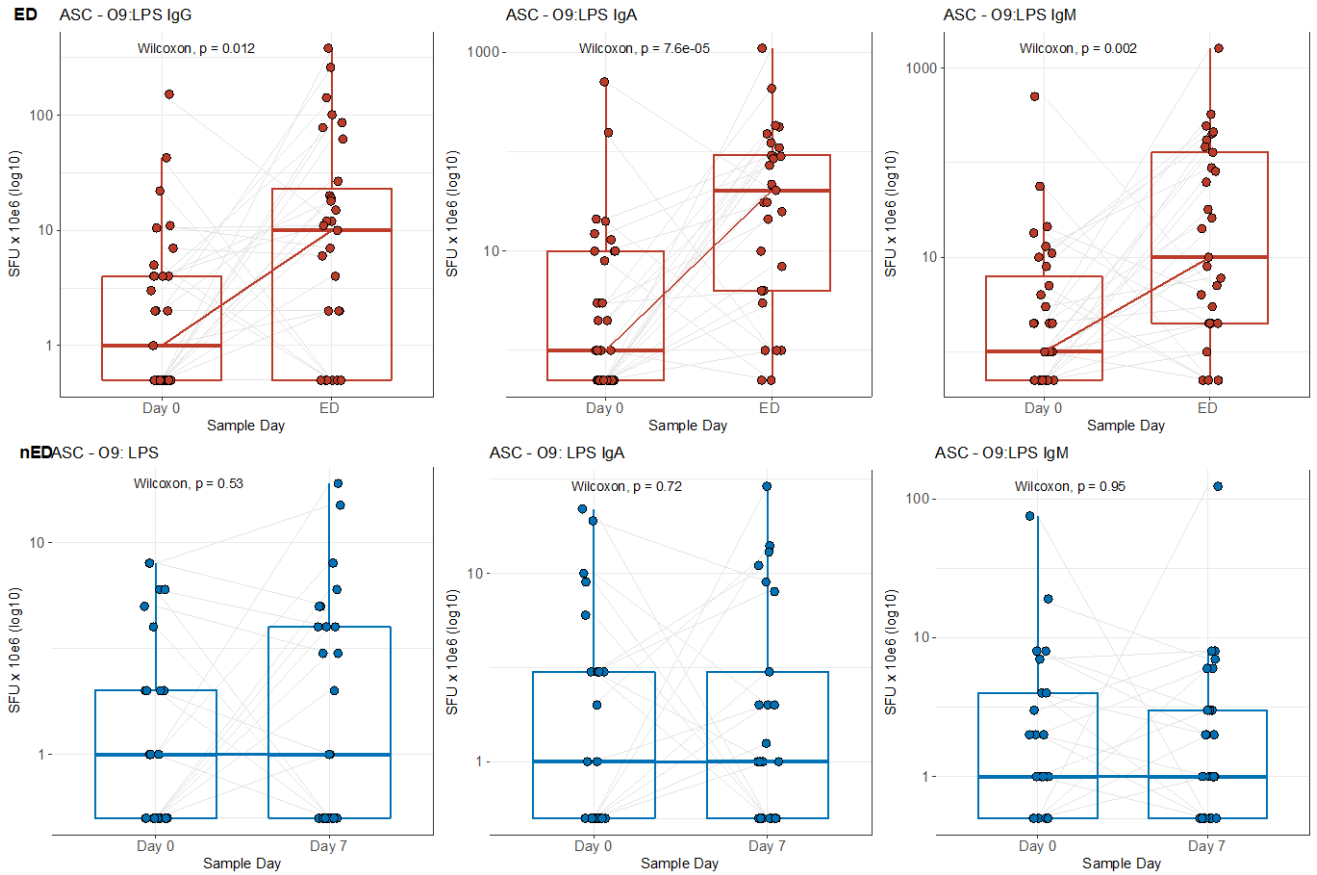


Figure 8-23 : O9:LPS-Specific antibody secreting cell (ASC) response to *S.* Typhi challenge

Measured by ex-vivo ELISPOT in all participants challenged with *S.* Typhi. Grouped according to challenge outcome (ED = Top panel, nED = Bottom panel), ASC isotype and antigen specificity. Measurement were taken at baseline (Day 0) and at the time of paratyphoid diagnosis (ED), or at day 7 in participants not meeting the diagnostic criteria for paratyphoid fever. Box-plots represent median and IQR. Coloured lines connect median values for each time point. Grey lines connect paired data points from different timepoints. p = Wilcoxon matched-pairs signed rank test.

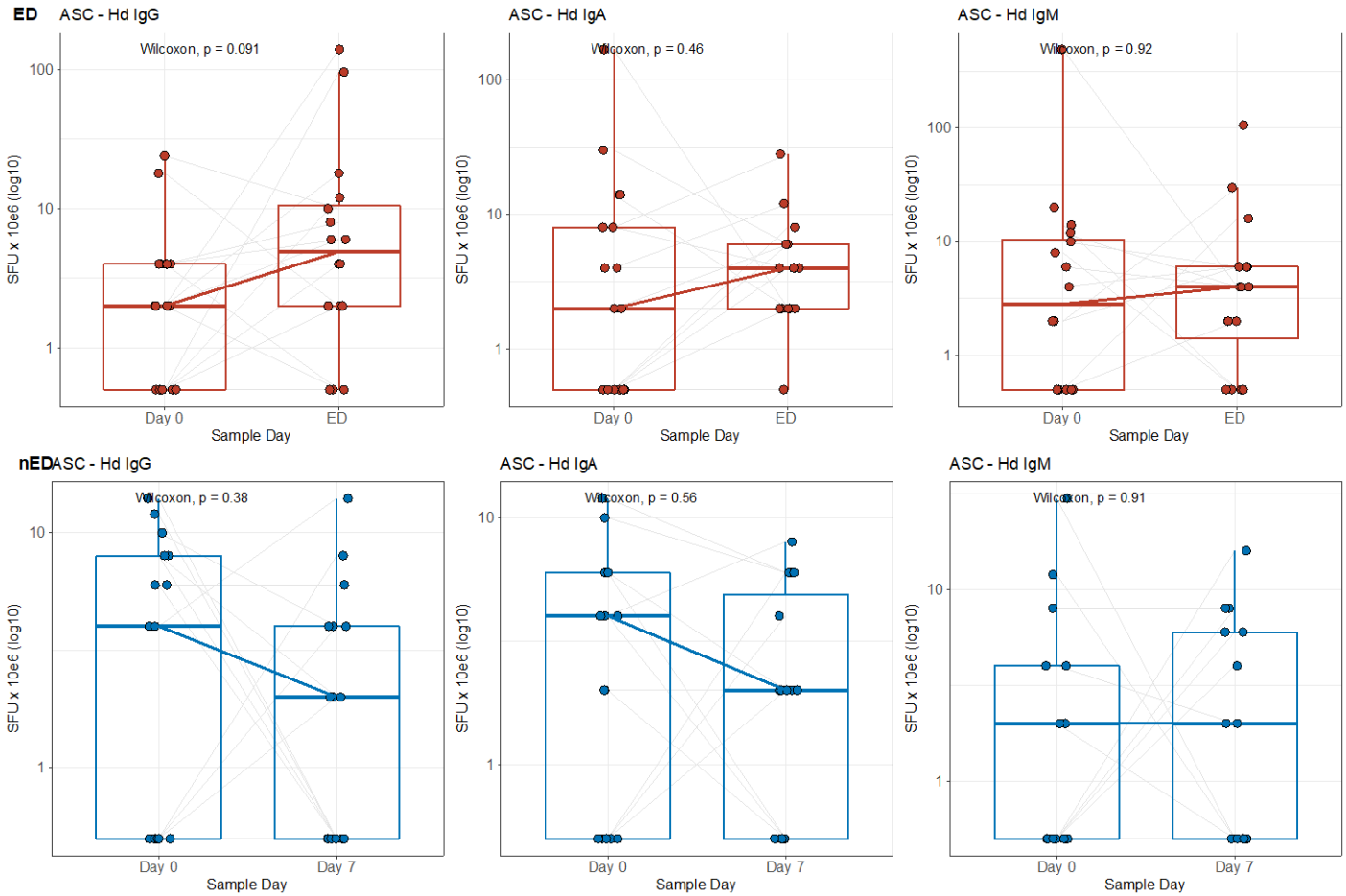


Figure 8-24 : Hd-Specific antibody secreting cell (ASC) response to *S.* Typhi challenge

Measured by ex-vivo ELISPOT in all participants challenged with *S.* Typhi. Grouped according to challenge outcome (ED = Top panel, nED = Bottom panel), ASC isotype and antigen specificity. Measurement were taken at baseline (Day 0) and at the time of paratyphoid diagnosis (ED), or at day 7 in participants not meeting the diagnostic criteria for paratyphoid fever. Box-plots represent median and IQR. Coloured lines connect median values for each time point. Grey lines connect paired data points from different timepoints. p = Wilcoxon matched-pairs signed rank test

To assess if previous challenge was associated with enhanced ASC responses, we compared the magnitude of the increase in ASC in diagnosed volunteers from naïve and re-challenge groups. Overall, there was no significant difference in the magnitude of the anti-O9:LPS IgG, IgA and IgM ASC response between naïve and homologous re-challenge groups, expressed as fold change from baseline (Figure 8-25). Noticeably, the magnitude of IgG, IgA and IgM ASC responses to anti-O9:LPS was significantly higher in the heterologous re-challenge group (previous *S*. Paratyphi challenge, *S*. Typhi re-challenge) compared with naïve or homologous re-challenge groups (Figure 8-25). Participants challenged with *S.* Typhi also mounted a significant IgG, IgA and IgM ASC responses to the *S.* Paratyphi antigen O2:LPS at the time of diagnosis. Comparable to the response to O9:LPS, the ASC response to O2:LPS was greatest in the heterologous re-challenge group (Figure 8-26). Collectively, these data suggest that O9:LPS specific ASCs are activated during acute typhoid disease, but that previous exposure is not associated with an enhanced response on re-challenge.

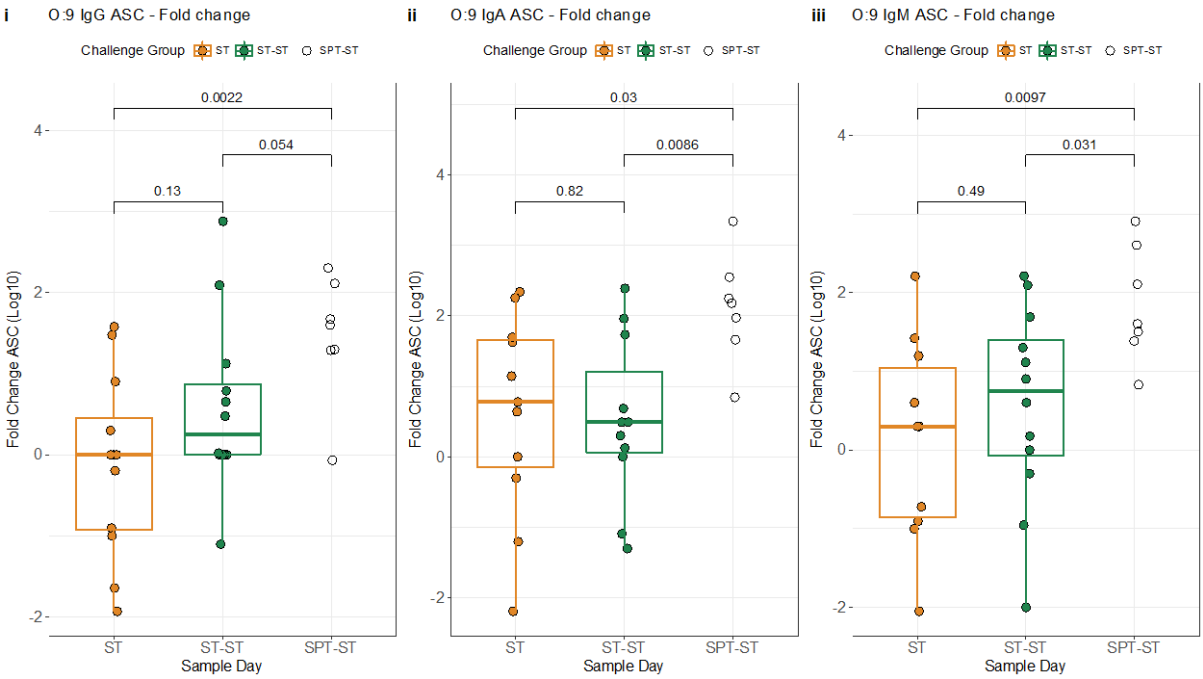


Figure 8-25 : *S.* Typhi challenge. Magnitude of ASC responses in naïve and re-challenge participants

Comparison of antibody secreting cell response to *S.* Typhi challenge between naïve and re-challenge groups. (i) O9:LPS IgG ASC; (ii) O9:LPS IgA ASC; (iii) O9:LPS IgM ASC. Presented as $\log_{10}$ fold change from baseline. Between group comparisons performed using Mann-Whitney U test.

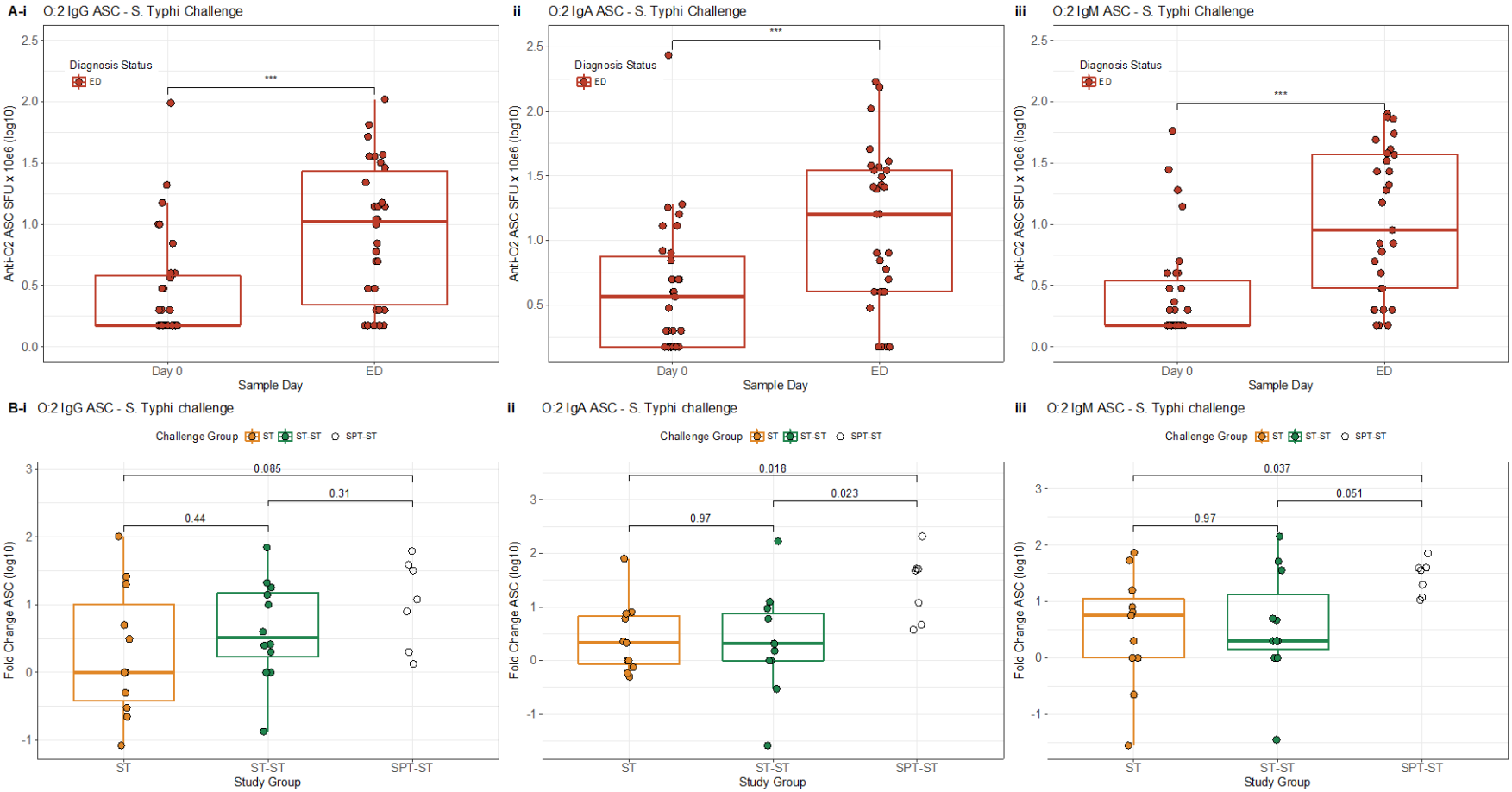


Figure 8-26 : Antibody secreting cell (ASC)response to heterologous S. Paratyphi O2 antigen in participants challenged with S. Typhi.

(A) All participants meeting criteria for typhoid diagnosis. Day 0 = Baseline, ED = Time of diagnosis. Paired comparisons performed by Wilcoxon matched-pairs signed rank test. *** p <0.001, **p<0.01, * p<0.05, ns = p >0.05. (B) ASC response at time of diagnosis grouped according to study group. Between group comparisons performed using Mann-Whitney U test.

8.3.5.2. Antibody Secreting Cell responses after S. Paratyphi challenge

O:2 and Ha specific antibody secreting cells were measured at baseline and at paratyphoid diagnosis following S. Paratyphi challenge. Across all groups, a large proportion of participants had background responses at baseline. The number of anti-O2:LPS IgG, IgA and IgM producing ASCs typically increased from baseline at the time of paratyphoid diagnosis, although was not significant due to the high background (Figure 8-27). Consistent with the limited antibody responses to Ha, anti-Ha specific IgG, IgA and IgM producing ASCs did not significantly increase at the time of paratyphoid diagnosis, although inter-individual responses differed widely (Figure 8-28). When naïve and re-challenge groups were compared, we observed an increased O:2 specific IgG and IgM ASC response in participants previously challenged with *S.* Typhi compared with naïve participants (Figure 8-29).

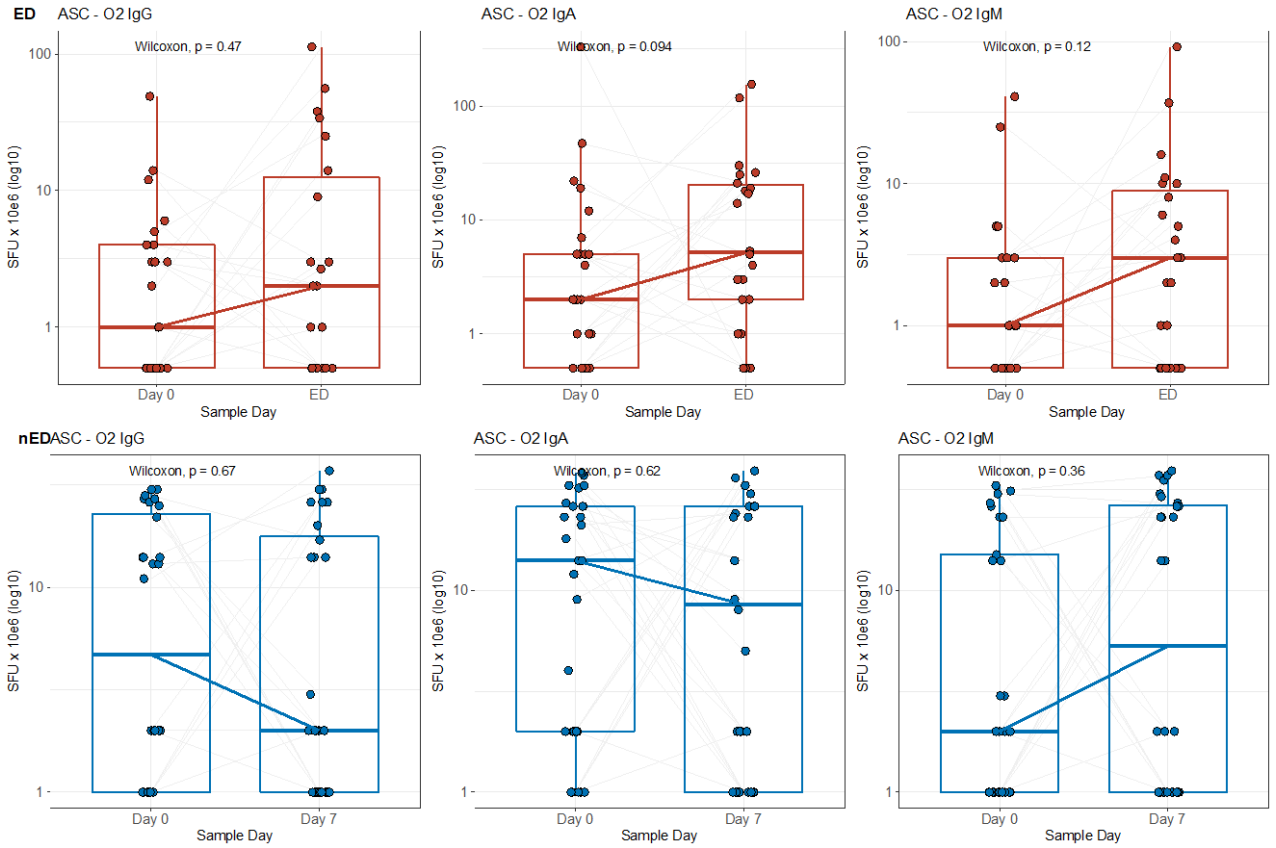


Figure 8-27 : O2-Specific antibody secreting cell (ASC) response to *S.* Paratyphi challenge

Measured by ex-vivo ELISPOT in all participants challenged with *S*. Paratyphi. Grouped according to challenge outcome (ED = Top panel, nED = Bottom panel), ASC isotype and antigen specificity. Measurement were taken at baseline (Day 0) and at the time of paratyphoid diagnosis (ED), or at day 7 in participants not meeting the diagnostic criteria for paratyphoid fever. Box-plots represent median and IQR. Coloured lines connect median values for each time point. Grey lines connect paired data points from different timepoints. p = Wilcoxon matched-pairs signed rank test.

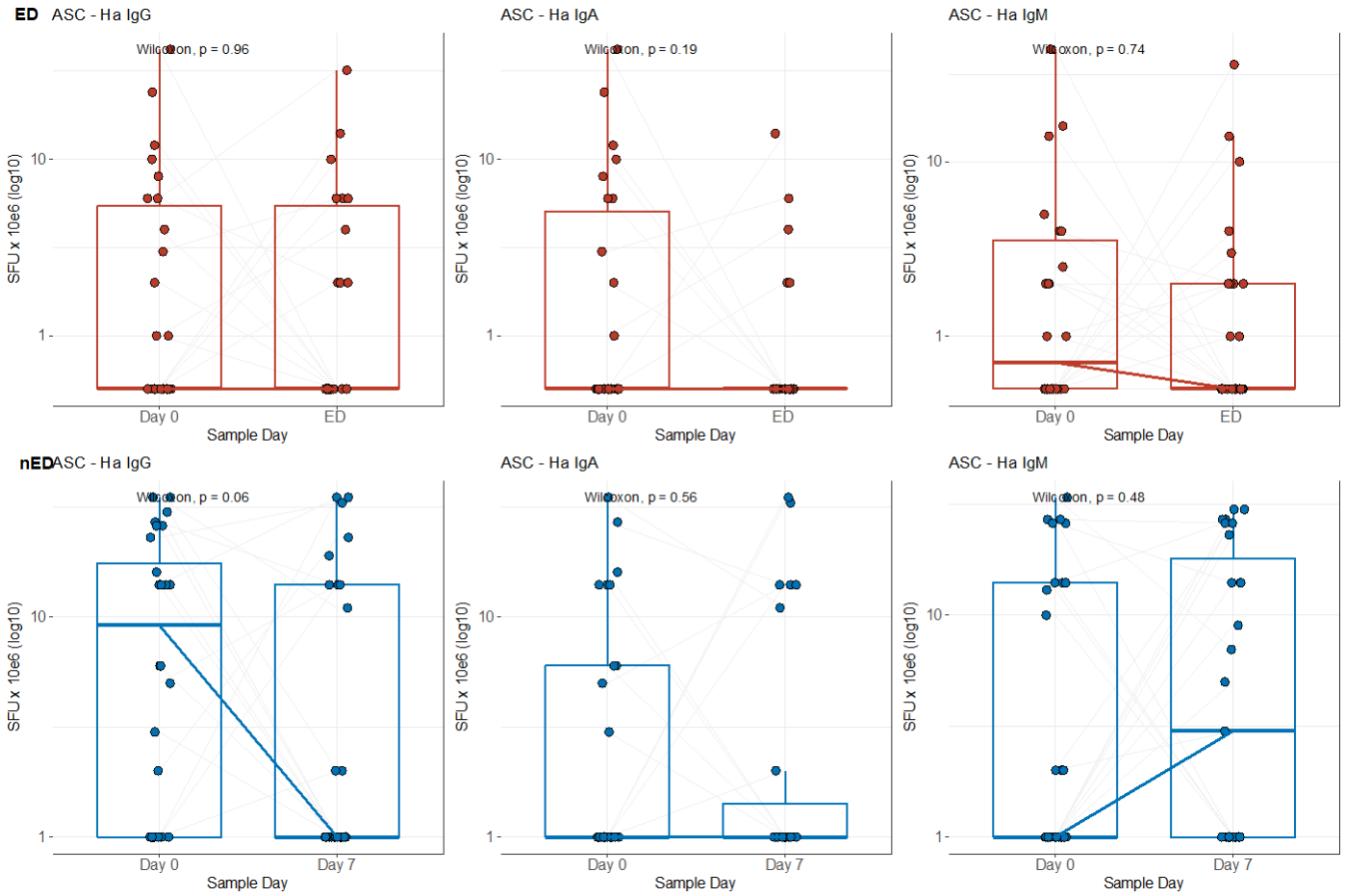


Figure 8-28 : Ha-Specific antibody secreting cell (ASC) response to *S.* Paratyphi challenge

Measured by ex-vivo ELISPOT in all participants challenged with *S*. Paratyphi. Grouped according to challenge outcome (ED = Top panel, nED = Bottom panel), ASC isotype and antigen specificity. Measurement were taken at baseline (Day 0) and at the time of paratyphoid diagnosis (ED), or at day 7 in participants not meeting the diagnostic criteria for paratyphoid fever

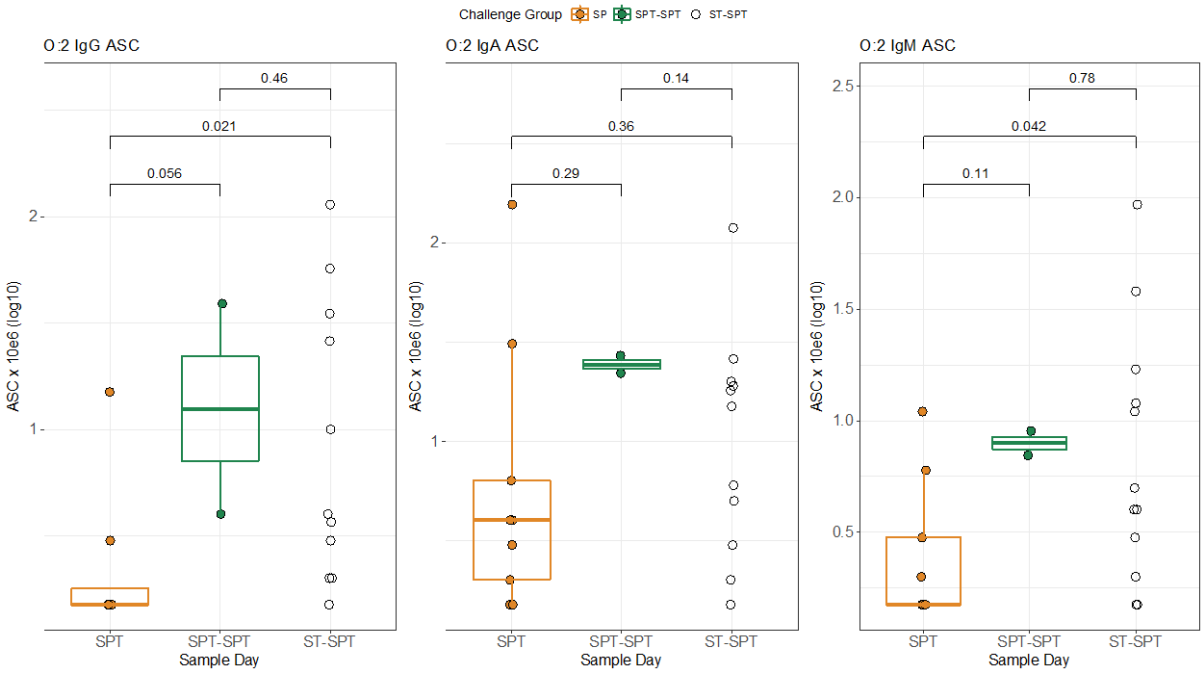


Figure 8-29 : Magnitude of antibody secreting cell response to S. Paratyphi challenge

Comparison of naïve and re-challenge groups. (i) O2:LPS IgG ASC; (ii) O2:LPS IgA ASC; (iii) O2:LPS IgM ASC. Presented as $\log_{10}$ fold change from baseline. Between group comparisons performed using Mann-Whitney U test. SPT = *S.* Paratyphi naïve; SPT-SPT = Homologous *S.* Paratyphi re-challenge. ST-SPT = Heterologous *S.* Paratyphi re-challenge.

8.3.6. Memory B-cell ELISPOT

8.3.6.1. Memory B-cell response after S. Typhi Challenge

O:9:LPS and Hd specific memory B-cells were quantified at baseline, and at day 28 and day 90 post challenge. Levels of anti-09:LPS and anti-Hd memory B-cells at baseline were generally higher in participants with previous exposure to *S.* Typhi than naïve participants, although only anti-Hd IgG producing cells were significantly higher in the re-challenge group (Figure 8-30). There was no significant difference in the level of baseline *S.* Typhi specific memory B-cells between participants who did, or did not, go on to develop typhoid fever (Figure 8-31).

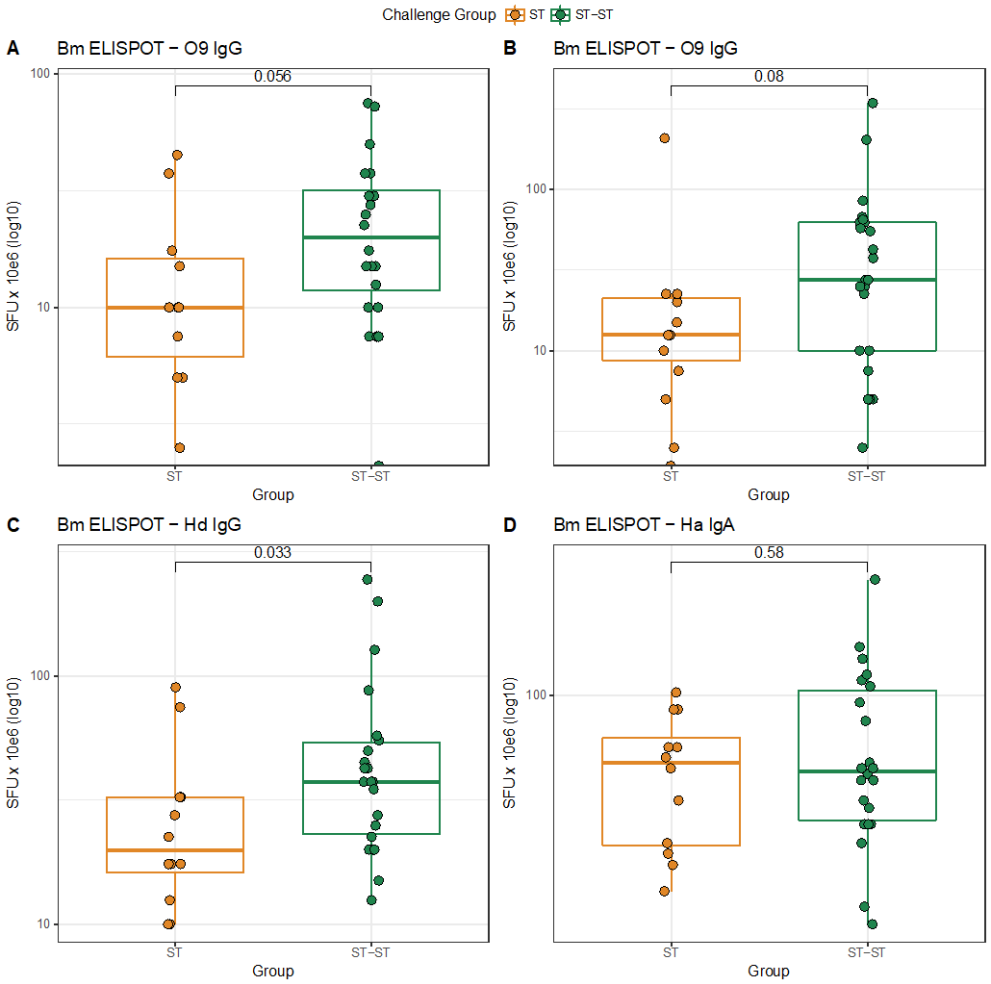


Figure 8-30 : Anti-O9:LPS and anti-Hd memory B-cells at baseline

Participants challenged with *S*. Typhi. Grouped according to challenge group allocation, measured by Bm ELISPOT. P = Mann-Whitney U test.

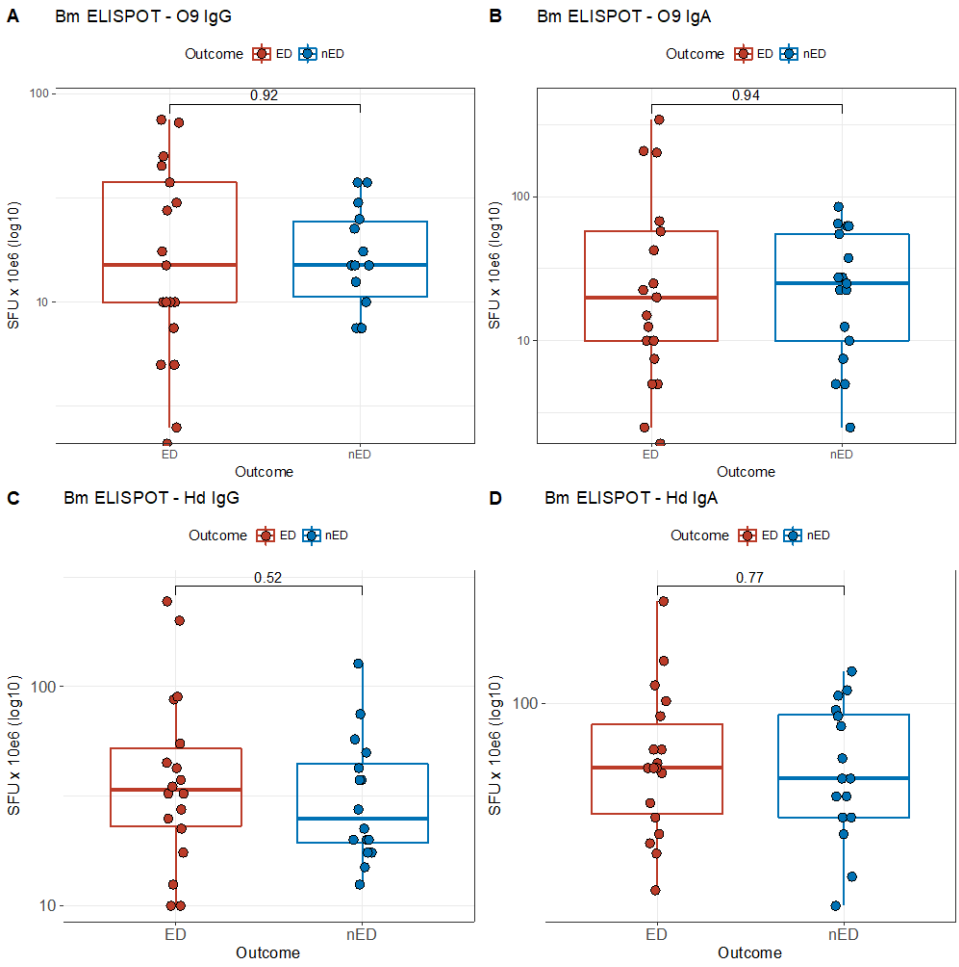


Figure 8-31 : Anti-O9:LPS and anti-Hd memory B-cells at baseline according to outcome of challenge.

Participants challenged with S. Typhi. Measured by Bm ELISPOT. P = Mann-Whitney U test.

Following challenge, participants who developed typhoid had a significant increase in circulating anti-O9:LPS IgG producing cells at day 28, which began to fall by day 90. Notably, anti-O9:LPS IgG producing cells were also increased at day 28 in those participants who did not develop disease (Figure 8-32). Previous exposure to *S.* Typhi was not associated with an increased in the magnitude of the memory B-cell response at day 28 (Figure 8-33).

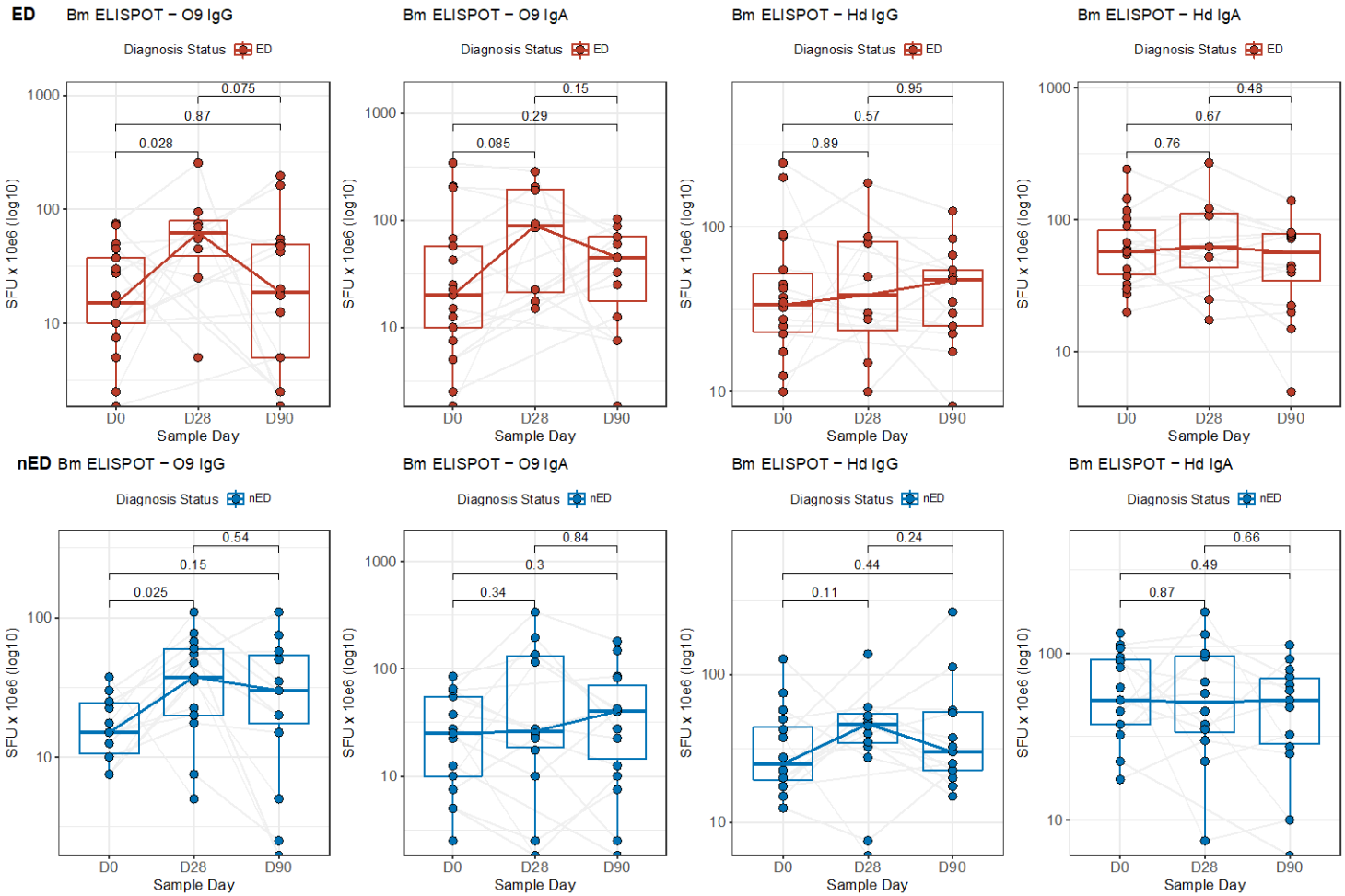


Figure 8-32 : Anti-O9:LPS and anti-Hd specific memory B-cell kinetics after *S.* Typhi challenge.

Anti-O9:LPS and Hd specific IgG and IgA producing memory B-cells measured by ELISPOT, presented as log10 spot forming units (SFU) per 10^6 PBMCs. ED (top) = Enteric fever diagnosis. nED (bottom) = No enteric fever diagnosis. Box-plots represent median and IQR. Coloured lines connect median values for each time point. Grey lines connect paired data points from different timepoints. P = Wilcoxon matched-pairs signed rank test.

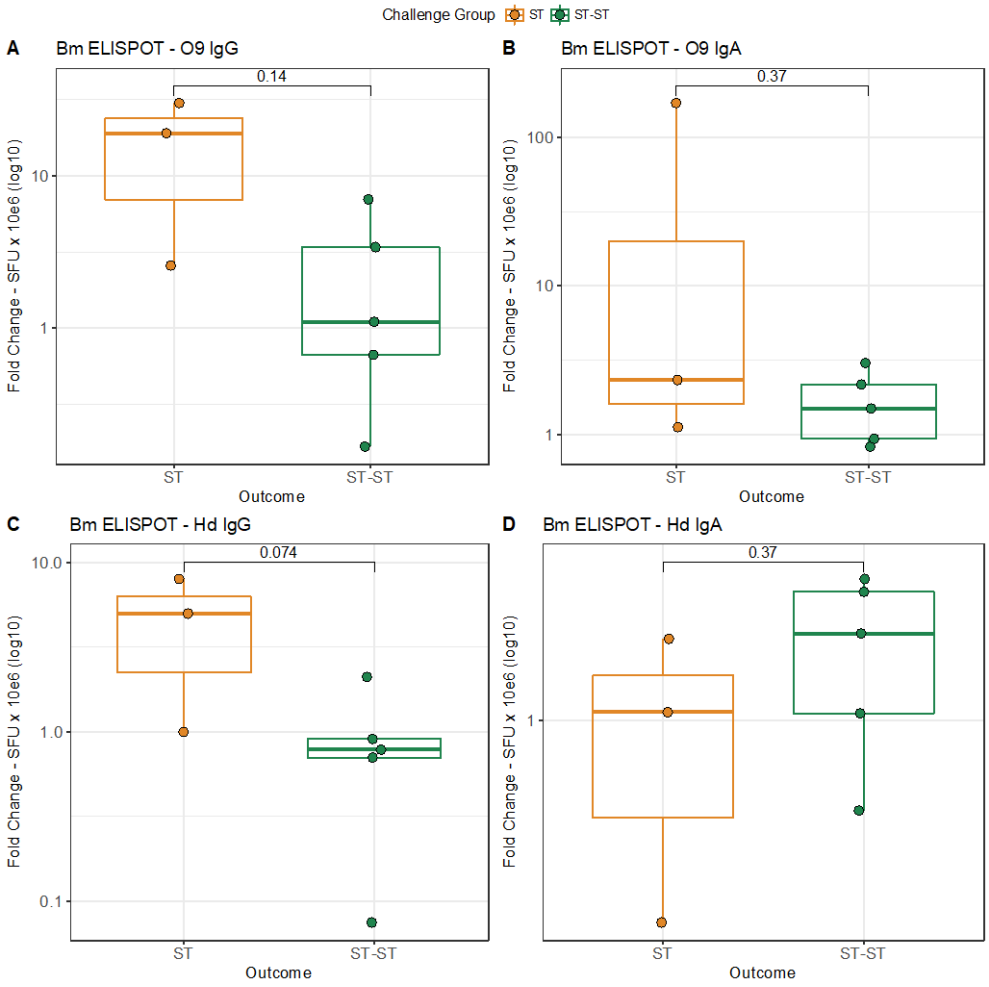


Figure 8-33 : Magnitude of anti-09:LPS and anti-Hd memory B-cell response in naïve and re-challenge participants

Measured at day 28 post challenge in participants diagnosed with typhoid, expressed as log fold change from baseline. P = Mann Whitney U test.

8.3.6.2. Memory B-cell response after S. Paratyphi Challenge

O:2 and Ha specific memory B-cells were quantified at baseline, and at day 28 and day 90 post challenge. There was no difference in baseline O2 and Ha specific memory B-cells between naïve and re-challenge groups (Figure 8-34), nor by subsequent outcome of challenge (Figure 8-35). In participants who were diagnosed with paratyphoid infection, the number of circulating memory B-cells was increased at day 28 compared with baseline, before declining by day 90. The increase at day 28 was significant for all antigen-isotype combinations tested, with the exception of anti-

Ha IgG producing memory B-cells (p=0.19 - Figure 8-36). Notably, we observed a significant increase in anti-O2 IgA producing memory B-cells at day 28 in participants not diagnosed with paratyphoid. No significant changes were observed in all other antigen-isotype combinations in the non-diagnosed group (Figure 8-36). In participants diagnosed with paratyphoid, the magnitude of the memory B-cell response was comparable between naïve and re-challenge groups (Figure 8-37).

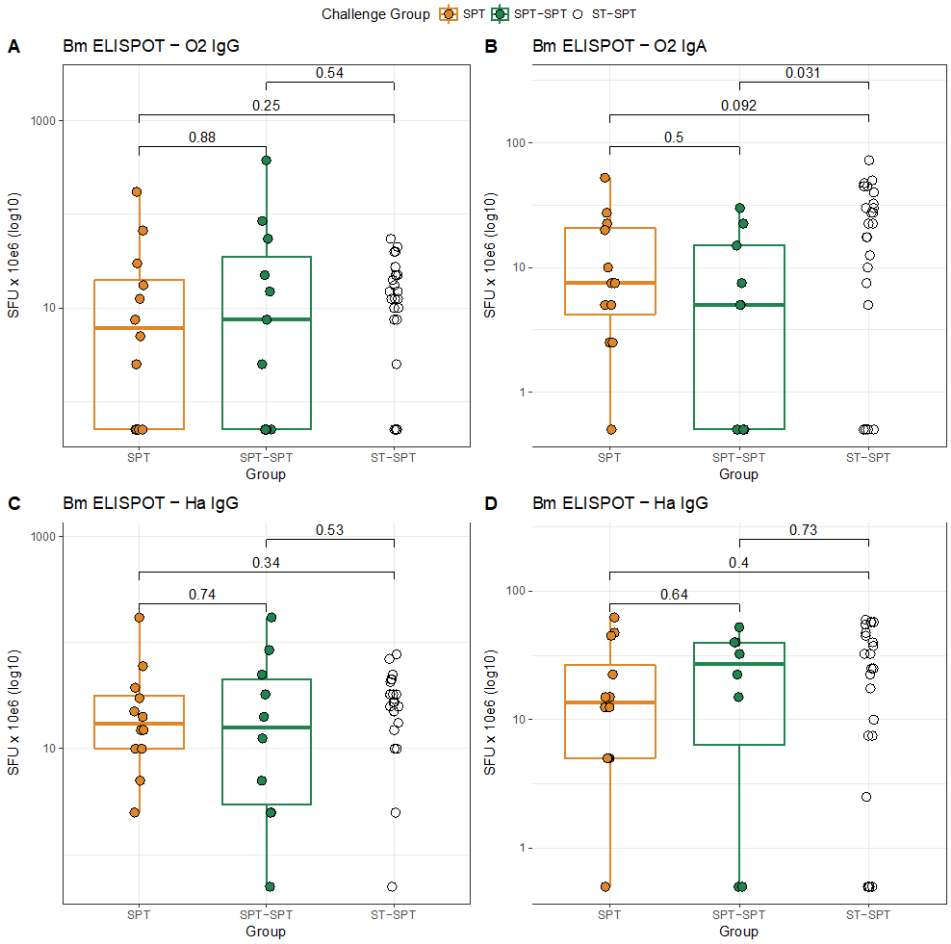


Figure 8-34 : Anti-O2 and anti-Ha memory B-cells at baseline according to challenge group allocation.

Participants challenged with S. Paratyphi. p = Mann-Whitney U test. SPT = *S.* Paratyphi naïve; SPT-SPT = Homologous *S.* Paratyphi re-challenge. ST-SPT = Heterologous *S.* Paratyphi re-challenge.

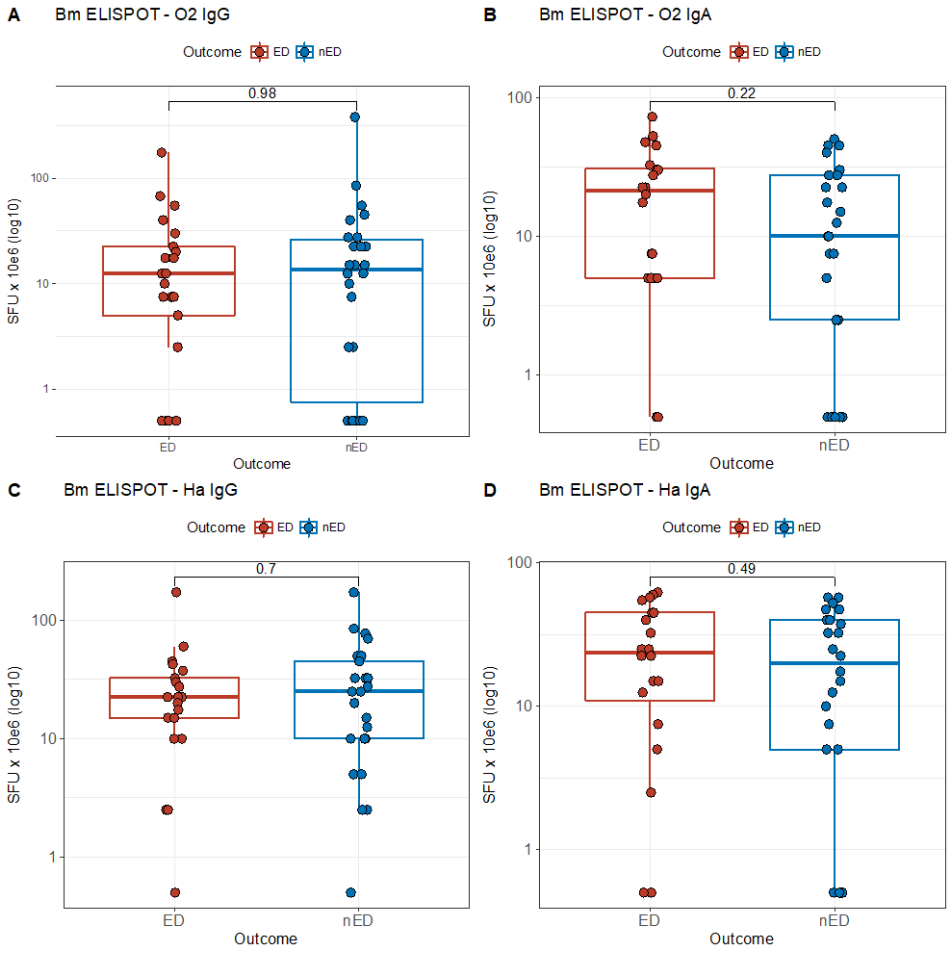


Figure 8-35 : Anti-O2 and anti-Ha memory B-cells at baseline according to outcome of challenge.

Participants challenged with *S*. Paratyphi. P = Mann-Whitney U test.

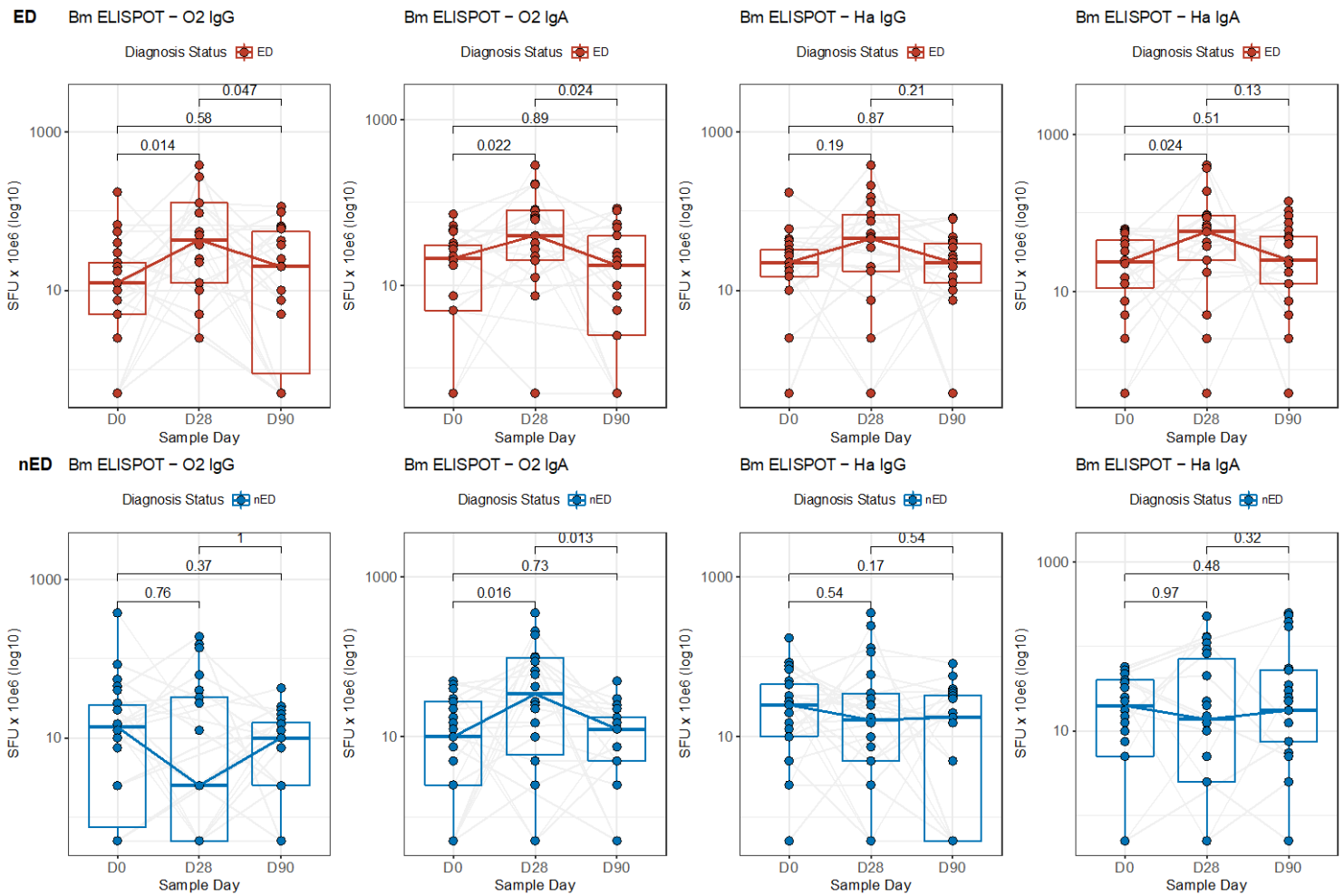


Figure 8-36 : Anti-O2 and anti-Ha specific memory B-cell kinetics after *S.* Paratyphi challenge.

Memory B-cell ELISPOT measurements after S. Paratyphi challenge. Anti-O2 and Ha specific IgG and IgA producing memory B-cells measured by ELISPOT, presented as log10 spot forming units (SFU) per 10^6 PBMCs. ED (top) = Enteric fever diagnosis. nED (bottom) = No enteric fever diagnosis. Box-plots represent median and IQR. Coloured lines connect median values for each time point. Grey lines connect paired data points from different timepoints. p = Wilcoxon matched-pairs signed rank test.

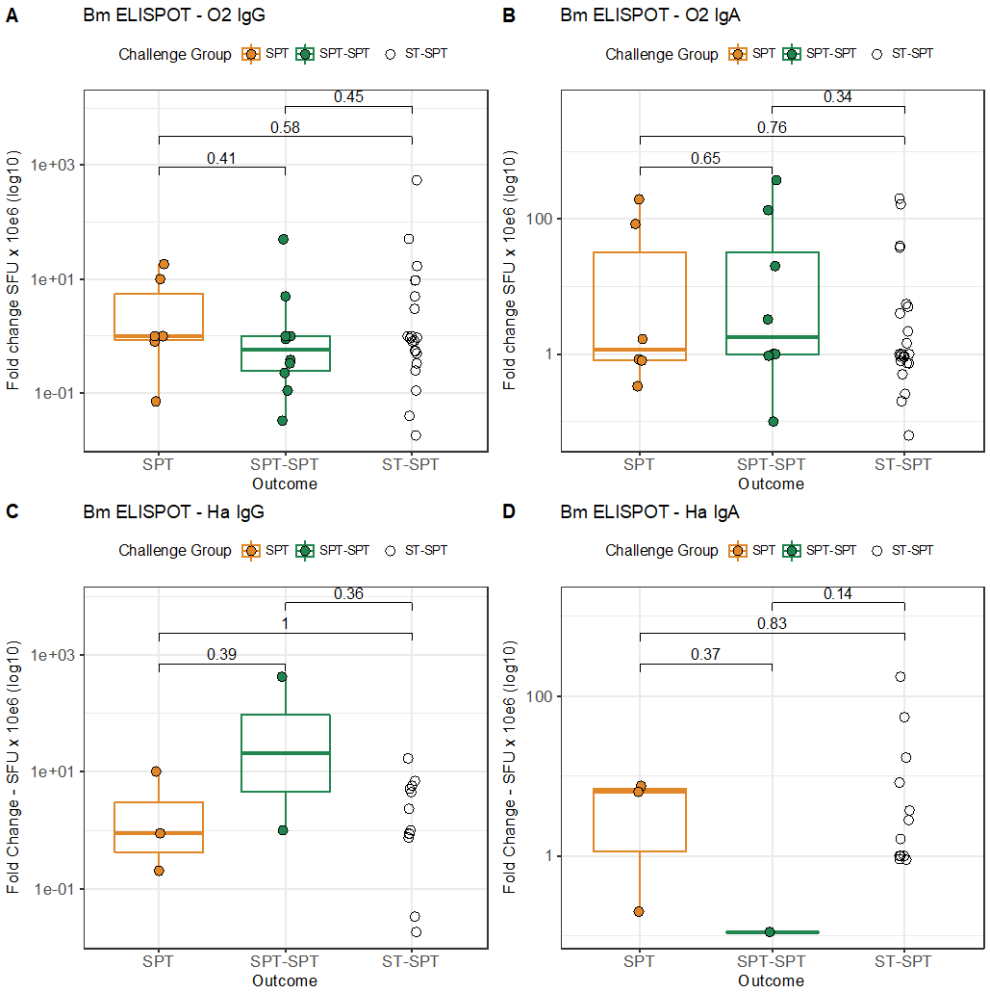


Figure 8-37 : Magnitude of anti-O2 and Ha specific memory B-cell response in naïve and re-challenge participants.

Participants challenged with S. Paratyphi. Measured day 28 post challenge in participants diagnosed with paratyphoid disease, expressed as log fold change from baseline. SPT = *S.* Paratyphi naïve; SPT-SPT = Homologous *S.* Paratyphi re-challenge. ST-SPT = Heterologous *S.* Paratyphi re-challenge.

8.4. Discussion

The data presented in this chapter represents the first description of humoral immune responses to common surface antigens of *S.* Typhi and Paratyphi in a cohort of naïve and re-challenged volunteers. The challenge-re-challenge study provided an opportunity to relate humoral immune responses with protection against typhoid and paratyphoid fever. Consistent with previous studies, humoral immune responses (measured by serum antibody ELISA, ex-vivo ASC ELISPOT and Bm ELISPOT) were more pronounced in individuals who developed typhoid and paratyphoid fever, as compared with those who did not develop disease after challenge. Previous exposure in the homologous re-challenge groups was not associated with a demonstrable booster response, as measured by circulating antibody concentration or increase number of ASCs at the time of diagnosis. In contrast, heterologous re-challenge with *S.* Typhi was associated with a stronger antibody and ASC response in participants who developed disease after challenge. Baseline antibody levels to O-, H- and Vi-antigens, and memory B-cell responses to O- and H-antigens were comparable between naïve and re-challenge participants. Overall, none of the baseline measures of humoral immunity measured here were associated with protection after challenge.

8.4.1. Antibody responses in naïve and re-challenge participants

Despite the moderate degree of protection observed after homologous *S.* Typhi and Paratyphi re-challenge, there was no demonstrable booster effect in terms of antibody levels when naïve and re-challenge groups were compared (Figure 8-11,Figure 8-15). In addition to comparing responses between groups, we were able to leverage data from earlier challenge studies to compare intra-individual primary and secondary antibody responses using paired samples (Figure 8-18,Figure 8-21). This represents the first individual-level longitudinal analysis of serum antibodies after typhoid exposure extending over up to 5 years (Figure 8-16). Consistent with the

between-group comparisons, re-challenge was not associated with boosting of the serum antibody responses after second challenge (Figure 8-18, Figure 8-22).

To a degree, these data contrast with the common interpretation of data from serosurveillance studies conducted in highly endemic areas, which describe an increasing prevalence of seropositivity to typhoid antigens associated with increasing age or peak exposure [29,290,411,412]. It is frequently assumed that increasing seropositivity is attributable to boosting by repeat environmental exposure over time, including exposure associated with sub-clinical infection[290]. Our data suggest that a single exposure in the absence of clinical disease is not associated with the development of detectable antibody responses and that repeated exposure to typhoidal *Salmonella* is not associated with an increased magnitude of antibody responses on re-exposure. However, these observations may be specific to the challenge model, which differs from field data with respect to frequency; infectious dose exposure; interval of repeated exposure and duration of infection.

The interpretation of these data is complicated by the limited number of timepoints, antigens, and antibody isotypes/subtypes that were studied. This limitation largely reflects the number of assays that could be run within the timeframe and budget of this study. In this study we focussed on studying antibody responses to common surface antigen, including O, H and Vi. This decision was made, in part, to provide a comparable data set with earlier studies, with the aim of studying the impact of re-challenge on antibody responses to these common antigens.

Surface polysaccharides and lipopolysaccharides represent only a small proportion of the potential antigenic targets exposed during acute infection. Several recent studies using antigen array platforms have described serodiagnostic protein antigens that are targeted during acute disease[158 159]. Antibody responses to different protein antigens may be able to distinguish

different stages of the infection life-cycle – for example, antibodies to HlyE and CdtB may be indicative of acute disease whereas strong antibody responses to the protein YncE may be indicative of carriage[413 414].

Additional work is planned to further characterise distinct patterns of antibody responses associated with primary or secondary infection. In particular, antibody responses may differ between naïve and re-challenge individuals with respect to antigenic targets, functionality or avidity, rather than simply in antibody concentration to surface antigens measured by ELISA. Additional studies could be undertaken to compare antibody functionality between naïve and re-challenge individuals using serum bactericidal activity assays[241,290], opsonophagocytic assays[366,415], assays of antibody dependent cellular cytotoxicity[416] or a combined approach of 'systems serology'[417]. In additions, measurement of secreted antibodies in gastrointestinal fluid, saliva or stool could be studied to better characterise the role of antibodies in protection.

The strongest antibody and ASC responses were observed in the heterologous re-challenge group previously exposed to *S.* Paratyphi (Figure 8-11 Figure 8-25 Figure 8-29). The factors underlying this observation are unclear but may relate to the lack of Vi-capsule expression by *S.* Paratyphi A resulting in cross-reactive responses to surface antigens priming for an enhanced response on re-exposure.

8.4.2. Antibody secreting cells

This is the first study to describe ASC responses to *S.* Typhi and Paratyphi infection in the context of controlled challenge and re-challenge. Consistent with antibody responses, we describe that O:9 antigen specific IgG and IgA producing ASCs can be detected in the circulation in the context of acute typhoid infection. Antibody-in-lymphocyte supernatant assays - which rely on the detection of specific antibodies after culture of ex-vivo ASCs - have demonstrated potential as

diagnostics for enteric fever[152,154]. In contrast, challenge with *S.* Typhi in the absence of invasion and bacteraemia, was not associated with a detectable ASC response in the blood. This contrasts with readily detectable circulating ASC responses after oral typhoid vaccination [314,366,402]. Sample collection constraints may have resulted in the peak ASC responses being missed in the non-diagnosed group, as only a single timepoint (day 7) was available for comparison. However, earlier studies have failed to detect ASC responses in undiagnosed participants measured at later timepoints after challenge (CJ/HTB – unpublished observations).

Consistent with antibody data, homologous re-challenge with *S*. Typhi or *S*. Paratyphi was not associated with a boosted ASC response on re-challenge (Figure 8-25,Figure 8-29). Repeated mucosal challenge with enteric pathogens may be associated with less robust ASC responses, as has been described with *Campylobacter* re-challenge[375] and following repeated Ty21a vaccination[418]. Studies to characterise the activation profile and homing markers expressed by ASCs after challenge using flow cytometry could provide additional insights into the role of ASCs in contributing to protection after re-challenge.

8.4.3. Bm responses in typhoid and paratyphoid infection

Humoral protection against reinfection with *S*. Typhi or *S*. Paratyphi could be mediated by pre-existing circulating or secreted antibodies produced by long-lived plasma cells and/or memory B-cells that are re-stimulated upon reinfection. A proportion of naïve B-cells stimulated during primary typhoid or paratyphoid infection would be expected to mature in memory B-cells, either through germinal centre-dependent or germinal-centre independent pathways[419,420]. This has been described for other enteric pathogens including *V. cholerae* [421], *Shigella flexneri* [422] and rotavirus[423]. In order to assess the contribution of memory B-cells to infection-derived immunity against S. Typhi and Paratyphi, we performed memory B-cell ELISPOT assays at multiple timepoint in naïve and re-challenged individuals. This approach has previously been applied to study

immunity to oral typhoid vaccines[409] [366], but – to my knowledge – has not been applied to study infection derived immunity following previous challenge.

Our data indicate that levels of anti-O9:LPS/O2 and anti-Hd/Ha memory B-cells in the circulation were comparable between naïve individuals and those who had previously been exposed to typhoidal *Salmonella* (Figure 8-30 Figure 8-34). It is plausible that Bm cells were not significantly increased in blood from re-challenge individuals at baseline, as those cells induced at primary infection likely underwent homing to the gastrointestinal tract[424,425]. We were able to detect anti-O9:LPS, O2 and Ha specific Bm cells in the circulation at 28 days after challenge, predominantly in participants who developed typhoid or paratyphoid after challenge (Figure 8-32, Figure 8-36). These cells may reflect recirculation of Bm cells activated in the gut associated lymphoid tissue. In keeping with antibody and ex-vivo ASC measurements, the magnitude of the Bm cell response after challenge was not enhanced in individuals with prior exposure compared with naïve participants.

Overall, the number of circulating anti-O9:LPS/O2 and anti-Hd/Ha Bm cells measured by ELISPOT at baseline was not associated with protection after challenge with *S.* Typhi and Paratyphi(Figure 8-31,Figure 8-35). A previous study has reported no difference in the frequency of different Bm subsets between individuals who went on to develop typhoid disease compared with those who were protected [284]. The same study demonstrated that key signalling proteins involved in B-cell activation (Akt, Erk1/2, NFkB and Btk) were phosphorylated in Bm cells during acute typhoid disease, suggesting that this cell type is activated during acute infection[284]. These data highlight the challenges of correlating memory B cell responses with protection against disease[423]. Alternative approaches to study memory B-cells – such as by flow cytometry of PBMCs or gut-resident B-cell populations – may be required to detail the role of this cell population (and their antigen specificity) in protection from disease.

8.5. Summary

Challenge-re-challenge studies offer a platform to identify correlates of protection to typhoidal *Salmonella*. The data presented in this chapter provide an overview of humoral immune responses following challenge and re-challenge with *S*. Typhi and Paratyphi, and whilst some differences were described depending on challenge outcome and between re-challenge groups none of the humoral measures presented in this chapter correlated with protection. Supplementation of these results with additional analysis of antibody functionality and measures of cellular immune responses will be required to identify correlates of protection.

Chapter 9 - The Impact of Vaccination and Prior Exposure on Stool Shedding of Salmonella Typhi and Salmonella Paratyphi A in Six Controlled Human Infection Studies

9. Introduction

Transmission of *Salmonella* Typhi and Paratyphi occurs primarily through consumption of contaminated food or water, via short-cycle or long-cycle transmission (Section 1.3). Short-cycle transmission is defined as the contamination of food and water in the immediate environment, whereas long-cycle transmission is defined as contamination of the broader environment, such as pollution of water supplies by sewage or inadequate treatment of piped water.[30] The relative contribution of each transmission mode may vary depending on the epidemiological context and may differ between *S*. Typhi and Paratyphi.[29] As these serovars are human-restricted, all modes of transmission involve shedding of the organism by infected individuals during incubation, acute disease or convalescence, or by chronic long-term carriers – ultimately resulting in contamination of food or water vehicles consumed by susceptible individuals. Disease control is therefore likely to require the integration of initiatives to improve water quality, sanitation and hygiene, coupled with the deployment of effective vaccines.[215]

Vaccines that both protect against clinical disease as well as reduce shedding would likely have enhanced effectiveness by interrupting transmission and providing indirect protection to unvaccinated individuals. Live attenuated oral typhoid vaccine, Ty21a, reduced stool shedding in the early Maryland challenge studies[296] and appears to induce herd-immunity in field trials[398]. However, there are conflicting data on the indirect protection conferred by Vi-polysaccharide

vaccines[426,427] and limited data on the impact of new Vi-tetanus toxoid conjugate vaccines on stool shedding[209]. Finally, the impact of previous exposure to typhoidal *Salmonella* on stool shedding after subsequent exposure has not been previously explored.

Experimental challenge studies of closely-monitored volunteers can be used to describe microbial dynamics in clinical and sub-clinical typhoid and paratyphoid infections, including the timing and pattern of stool shedding after challenge in naïve or vaccinated individuals[311]. Early experimental human challenge studies in Maryland indicate that shedding is more common in individuals who develop typhoid disease. However, individuals who fail to develop disease following challenge can continue to shed bacteria for several weeks[326]. Differences in stool shedding patterns between *S*. Typhi and Paratyphi A are poorly understood. Improved estimates of stool shedding dynamics in enteric fever are needed — particularly in the context of different immune-states — as these form important variables in models of typhoid transmission dynamics and estimates of vaccine impact[293,298].

The aims of this chapter are to model stool shedding after experimental challenge and to compare: i) those challenged with *S*. Typhi versus *S*. Paratyphi; ii) participants who did, or did not, develop enteric fever after challenge; iii) those receiving typhoid vaccines versus those who were unvaccinated; iv) re-challenged individuals previously exposed to *S*. Typhi or *S*. Paratyphi versus previously unexposed individuals; v) differences according to demographic variables, and vi) the relationship between antibody levels and stool shedding.

9.1. Methods

Data were available from six enteric fever human challenge studies conducted in Oxford between 2011 and 2017 (Table 7-3).

All challenge studies followed comparable protocols (Chapter 4 and[126,209,311,314,318]) using the Quailes strain of S. Typhi (genotype 3.1.0) and the NVGH308 strain *S.* Paratyphi A NVGH308 strain (Section 4.5).

9.1.1. Stool culture

Stool cultures were collected and processed as outlined in section 4.15.

9.1.2. Antibody Measures

Serum antibody levels were measured as detailed in section 8.2.1.

9.1.3. Statistical analysis

Stool culture data were combined in mixed effects logistic regression models, which included participant-specific random intercepts to account for the multiple samples per person. Models were adjusted for the vaccine received, study, and where applicable, challenge dose (high or low). 'Day' was included in the model as a categorical factor to allow the odds of stool shedding to vary by day.

An overall interaction term (day-by-vaccine) was tested to determine if vaccination altered the pattern of shedding over time. An interaction term for day-by-diagnosis status was used to compare the pattern of shedding in those who were diagnosed with enteric fever and those who remained undiagnosed at day 14.

The linear predictor from the model was exported for each participant for each day and converted into a probability by taking the inverse logit. These probabilities are presented in figures with a loess smooth to illustrate the findings from the logistic regression models. Odds ratios presented from logistic regression models represent the ratio of the odds of shedding in comparative groups on average across all 14 days. All models were fitted in SAS version 9.4 using PROC GLIMMIX.

9.2. Results

In total, 4,934 stool samples were analysed, from 430 participants. 3,698 samples were from *S.* Typhi challenge participants and 1,236 samples were from *S.* Paratyphi challenge (Table 9-1). 14.5% of stool samples from *S.* Typhi-challenged participants were positive compared with 7.5% of samples from those challenged with *S.* Paratyphi A.

9.2.1. Differences in S. Typhi stool shedding according to diagnosis status

There were 331 participants challenged with *S.* Typhi, of whom 186 (56.2%) met the diagnostic criteria for typhoid fever.[126] The highest incidence of shedding was observed on days 1 and 2 after challenge. The pattern of shedding over time was significantly different between those diagnosed, and those who did not develop enteric fever (P<0.0001). The odds of shedding 1 day after challenge were 2.5 times greater in those who were later diagnosed, than in those who remained undiagnosed (OR 2.49, 95%CI 1.32–4.69, p=0.0049). On day 2 odds were 9 times higher in those later diagnosed (OR 8.93, 95%CI 3.86–20.61, p<0.0001) and on day 3 were 23 times higher (OR 22.59, 95%CI 6.83–74.7, p<0.0001). In the second week after challenge, many diagnosed participants had commenced antibiotics and shedding ceased. In the undiagnosed participants, increased shedding was observed from day 10 onwards, until these also received treatment on day 14 (Figure 9-1 - A).

Table 9-1 : Stool culture results by challenge agent and study

Study	Number of participants	Number of participants with ≥1 positive stool sample	Number days shedding (median [IQR])	Number of samples from *S.* Typhi challenged participants		Number of samples from *S.* Paratyphi A challenged participants		TOTAL
				Negative	Positive	Negative	Positive	
OVG2009/10 (T1)	40	22 (55%)	1 [0 – 2]	520	54 (9%)	-	-	574
OVG2011/02 (T2)	92	72 (78%)	2 [1 – 4]	908	217 (19%)	-	-	1125
OVG2013/07 (P1)	40	17 (43%)	0 [0 – 1]	-	-	510	36 (7%)	546
OVG2014/08 (VAST)	103	64 (62%)	1 [0 – 2]	842	126 (13%)	-	-	968
OVG2014/01 (PATCH)	115	56 (45%)	0 [0 – 2]	562	84 (13%)	633	57 (8%)	1336
OVG2016/03 (TYGER)	40	24 (60%)	1 [0 – 2]	329	56 (15%)	-	-	385
TOTAL	**430**	**255 (59%)**		**3161**	**537 (14.5%)**	**1143**	**93 (7.5%)**	**4934**

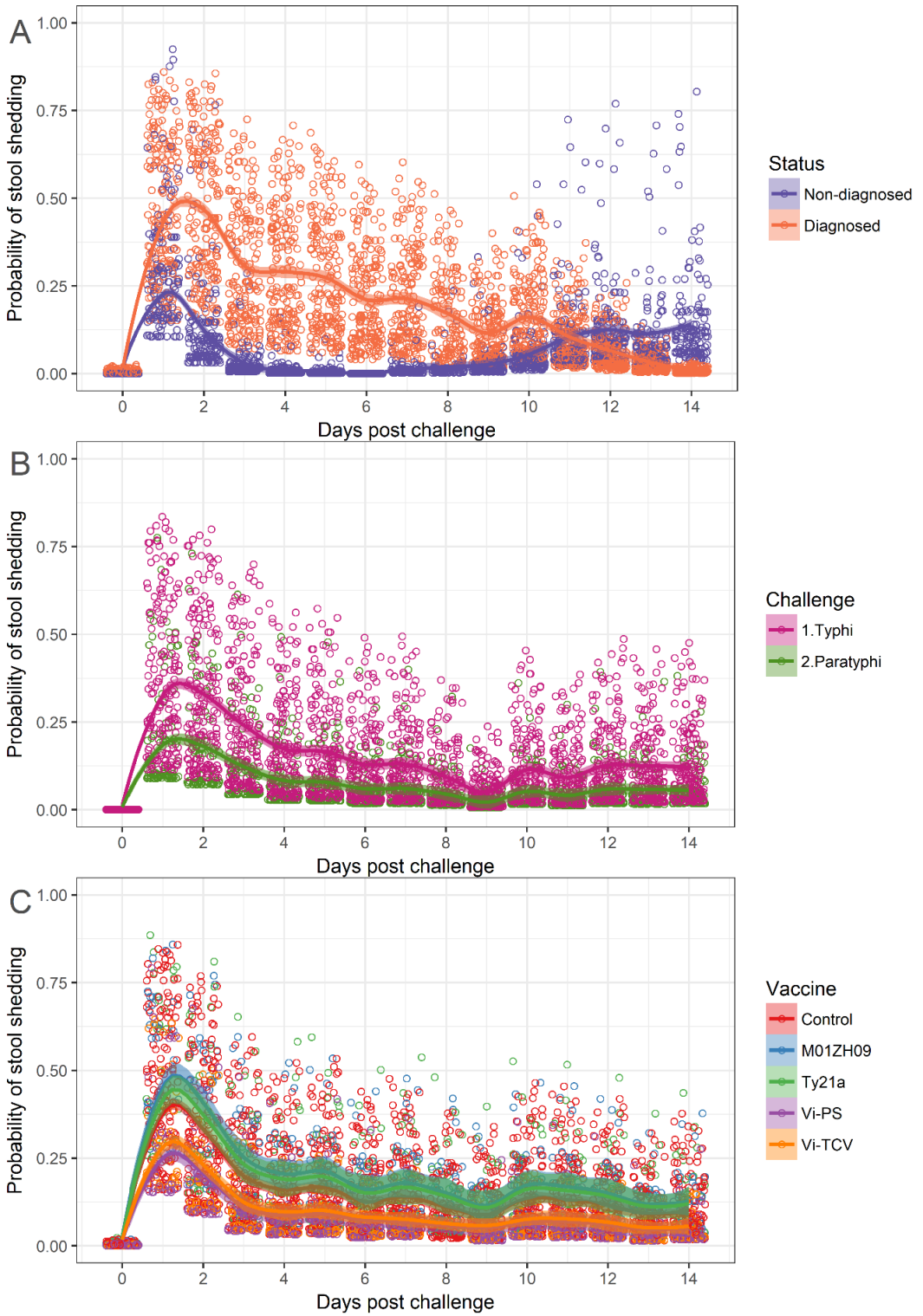


Figure 9-1 : Probability of bacterial shedding by day in stool in controlled human infection enteric fever studies.

(A) N=331 participants challenged with *S.* Typhi according to diagnosis status. Non-diagnosed N=145, diagnosed N=186; (B) Unvaccinated participants exposed to oral challenge with *S.* Typhi (N=197) or *S.* Paratyphi (N=109) bacteria; (C) Vaccinated and unvaccinated participants challenged with 1-5 x 10^4 CFU *S.* Typhi wild-type bacteria according to vaccine received. Control vaccine or no vaccine (N=158); M01ZH09: experimental typhoid vaccine (N=32); Ty21a: live attenuated oral typhoid vaccine (30); Vi-PS: Vi-polysaccharide typhoid vaccine (Typhim Vi ®, Sanofi Pasteur) (N=35); Vi-TT: Vi-tetanus toxoid conjugate vaccine (TypbarTCV®, Bharat Biotech) (N=37).

9.2.2. Differences in stool shedding after S. Typhi or S. Paratyphi challenge in unvaccinated participants

There were 197 unvaccinated participants exposed to *S.* Typhi and 99 unvaccinated participants exposed to *S.* Paratyphi. The odds of shedding in participants exposed to *S.* Typhi were twice as high as those exposed to *S.* Paratyphi (OR: 1.97, 95% CI 1.00–3.88, p=0.049). A sensitivity analysis excluding low dose challenge gave a very similar estimate (OR 1.97, p=0.044). The dose received was non-significant in the model (Figure 9-1 - B).

9.2.3. Effect of vaccination on stool shedding after S. Typhi challenge

Data were analysed from 5 *S.* Typhi studies. Participants challenged with low dose (10^3 CFU) or a typhoid toxin deficient *S.* Typhi were excluded.[352]

Participants who received Vi-PS or Vi-TT vaccine had lower rates of shedding than unvaccinated participants (OR: 0.34, 95%CI 0.15–0.77, p=0.010; and 0.41, 95%CI 0.19–0.91, p=0.029 for Vi-PS and Vi-TT respectively). In Ty21a vaccine recipients, there was a non-significant reduction in shedding compared with unvaccinated controls (OR 0.57; 95%CI 0.27–1.20; p=0.14) (Figure 9-1 - C). There were no differences between unvaccinated participants and those receiving the M01ZH09 vaccine, or between any other groups after adjusting for study-specific variation. The vaccine-by-day interaction term was non-significant, showing that the pattern of shedding over time was similar for all groups even though the amount of shedding differed.

9.2.4. Effect of previous exposure to *S.* Typhi and *S.* Paratyphi on stool shedding

The impact of prior exposure on stool shedding is detailed in Table 9-2. Participants previously exposed to *S.* Typhi had less stool shedding than previously unexposed individuals (OR 0.33; 95%CI 0.1–0.8; p=0.016) (Figure 9-2). For those participants receiving *S.* Typhi challenge, previous exposure to *S.* Paratyphi significantly increased stool shedding compared with previous *S.* Typhi

exposure (SPT-ST vs ST-ST, OR: 7.5, 95%CI 2.0–28.4, p=0.003), and non-significantly increased the rate of bacterial shedding compared with previously unexposed individuals (SPT-ST vs ST, OR: 2.5, 95% CI 0.8–8.0, p=0.125). For those receiving *S*. Paratyphi challenge, there were no differences between groups and a lower rate of shedding in general was observed in comparison with than those exposed to *S*. Typhi challenge.

Table 9-2 : Bacterial shedding in stool after S. Typhi or S. Paratyphi challenge, according to previous exposure.

SPT = S. Paratyphi naïve (n=39); SPT-SPT = Homologous S. Paratyphi re-challenge (n=13); SPT-ST = Heterologous S. Typhi re-challenge (n=10); ST = S. Typhi naïve (n=71); ST-SPT = Heterologous S. Paratyphi re-challenge (n=27); ST-ST = Homologous S. Typhi re-challenge (n=27).

Comparison	Odds Ratio	95% LCL	95% UCL	p value
SPT-SPT v ST-SPT	1.66	0.41	6.80	0.478
SPT-SPT v SPT	0.72	0.20	2.62	0.617
SPT-ST v ST	2.49	0.78	8.01	0.125
SPT- ST v ST-ST	7.52	1.99	28.4	0.003
ST-ST v ST	0.33	0.81	0.14	0.016
ST- SPT v SPT	0.43	0.15	1.24	0.120

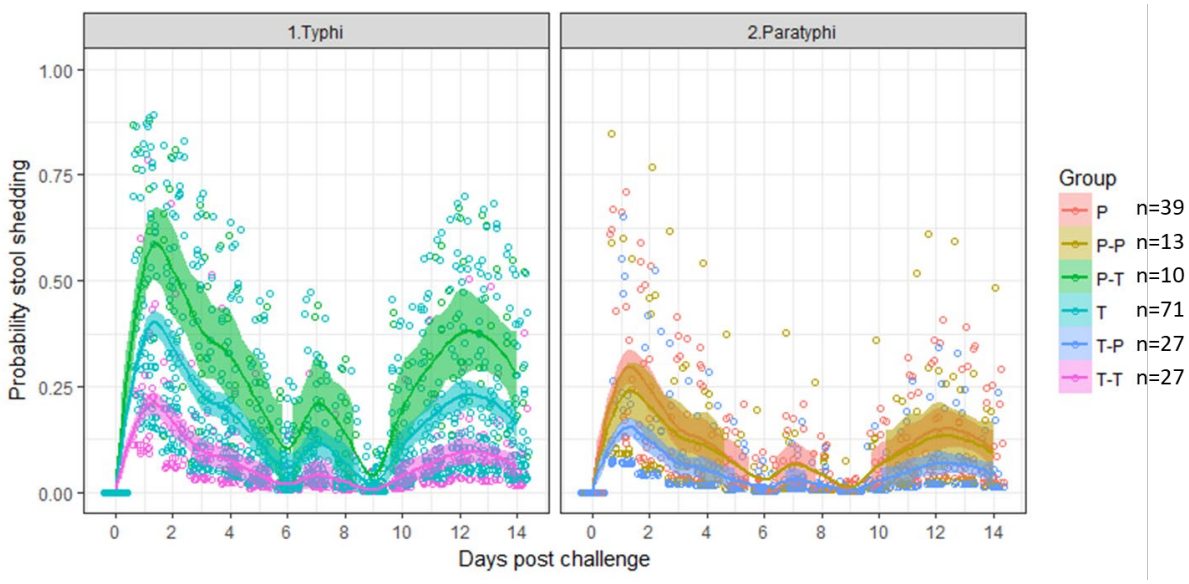


Figure 9-2 : Bacterial shedding in stool after S. Typhi or S. Paratyphi challenge, according to previous exposure.

SPT = S. Paratyphi naïve (n=39); SPT-SPT = Homologous S. Paratyphi re-challenge (n=13); SPT-ST = Heterologous S. Typhi re-challenge (n=10); ST = S. Typhi naïve (n=71); ST-SPT = Heterologous *S*. Paratyphi re-challenge (n=27); ST-ST = Homologous S. Typhi re-challenge (n=27).

9.2.5. Variation in shedding according to age and sex

In 197 unvaccinated participants (34% female), the median number of samples provided was 11 (IQR, 8–14) for women and 12 (IQR 9–15) for men. Men had proportionately more positive stool samples than women (OR 1.78, 95%CI 1.10–2.87, p=0.0187). Age was not significantly related to shedding (p=0.795).

9.2.6. Antibody levels prior to -challenge

Higher anti-Vi IgG antibody titres prior to challenge were associated with less bacterial shedding after challenge with *S*. Typhi (p<0.0001). There was no relationship between anti-LPS IgG or anti-H*d* IgG and shedding after challenge with either *S*. Typhi or *S.* Paratyphi (Figure 9-3). IgA and IgM responses to S. Typhi LPS and H*d* antigens were not-significantly associated with shedding (Figure 9-4).

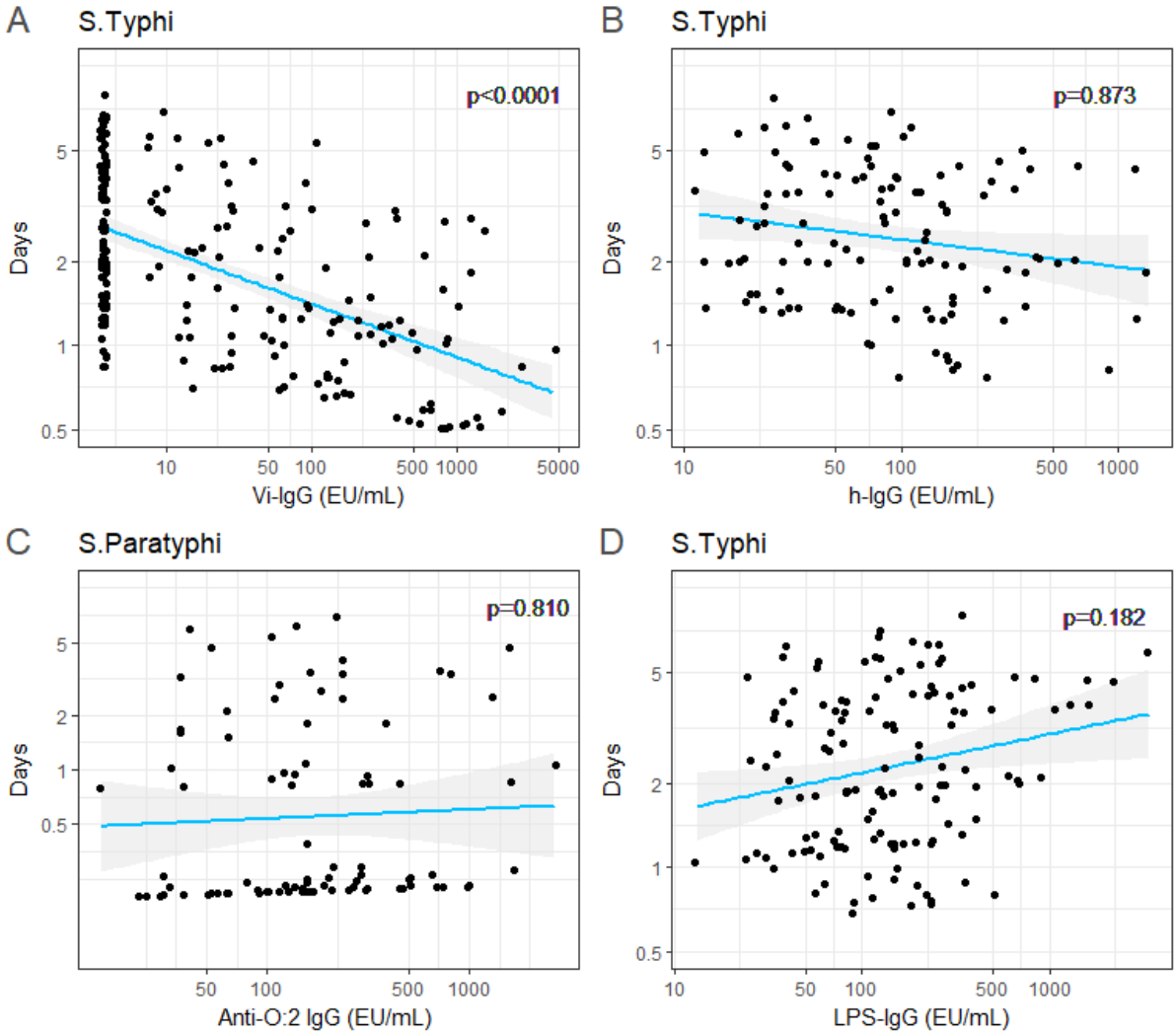


Figure 9-3 : Relationship between days of bacterial shedding in stool after *S.* Typhi or *S.* Paratyphi challenge and antibody levels prior to challenge.

(A) Anti-Vi IgG prior to challenge with *S.* Typhi; (B) Anti-Hd IgG prior to challenge with *S.* Typhi; (C) Anti-O:2 IgG prior to challenge with *S.* Paratyphi; (D) Anti-S. Typhi O9:LPS IgG prior to challenge with *S.* Typhi. Days: y-axis represents the predicted total number of days of stool shedding (out of 14). The total number of days was determined from the logistic regression model by summing across all 14 days the predicted probability for each day for each person.

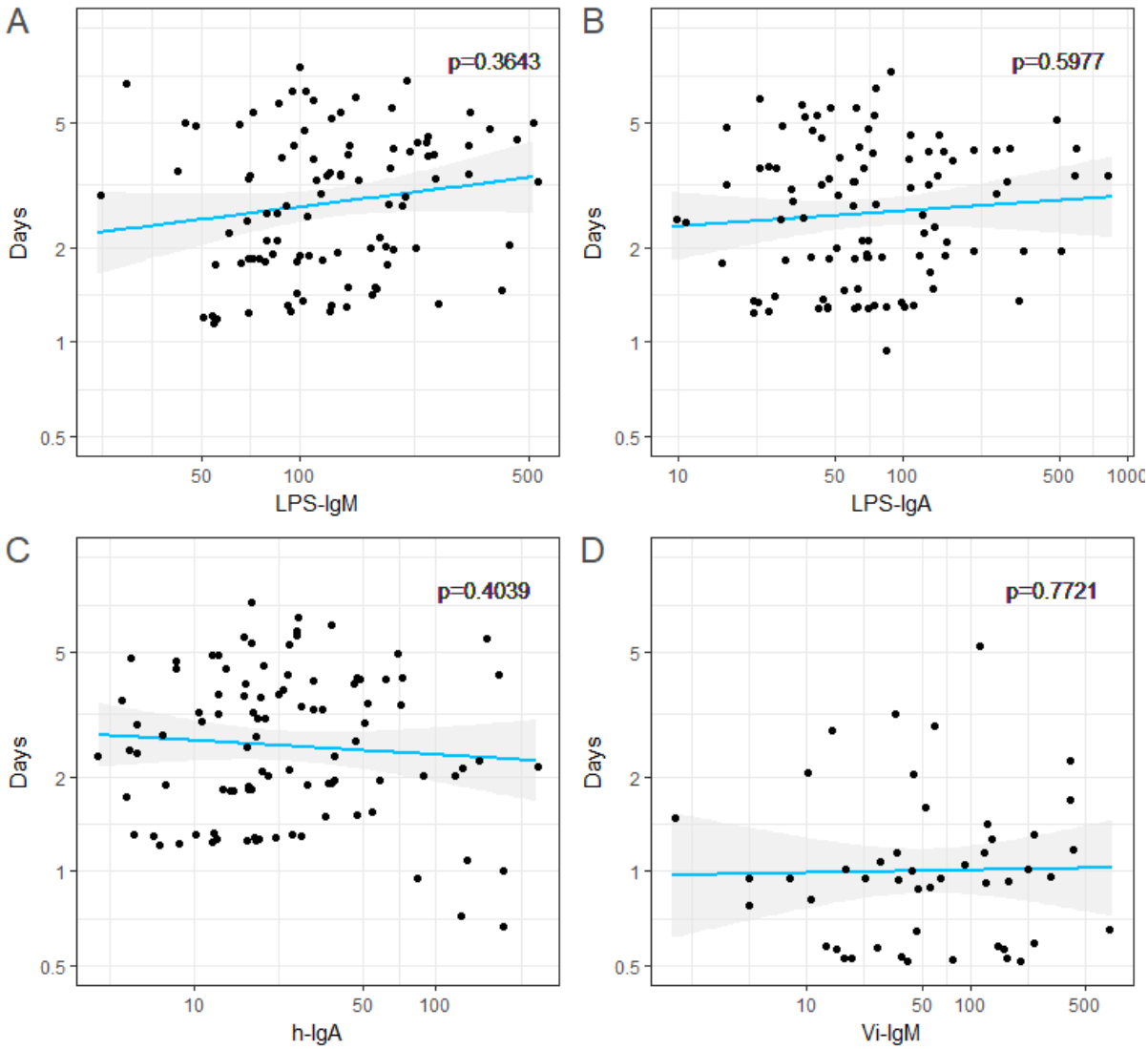


Figure 9-4 : Relationship between days of bacterial shedding in stool after *S.* Typhi challenge and antibody levels prior to challenge.

(A) Anti-*S*. Typhi O9:LPS IgM prior to challenge with *S*. Typhi; (B) Anti-*S*. Typhi O9:LPS IgA prior to challenge with *S*. Typhi; (C) Anti-Hd IgA prior to challenge with *S*. Typhi; (D) Anti-Vi IgM prior to challenge with *S*. Typhi. Days: y-axis represents the predicted total number of days of stool shedding (out of 14). The total number of days was determined from the logistic regression model by summing across all 14 days the predicted probability for each day for each person.

9.3. Discussion

Shedding of *S*. Typhi and *S*. Paratyphi bacilli is a pre-requisite for onward transmission in these human restricted infections. This is the first comprehensive analysis of bacterial shedding from almost 5000 stool samples taken after challenge of healthy volunteers with *Salmonella* Typhi or Paratyphi. This analysis demonstrates that stool shedding is more common in individuals meeting

the diagnostic criteria of enteric fever (fever 38°C for ≥12hrs and/or *S*. (Para)Typhi bacteraemia), but shedding can also occur in the absence of bacteraemia or clinical symptoms of disease. Vaccination with Vi-PS or Vi-TT significantly reduced stool shedding of *Salmonella* Typhi after challenge, suggesting that these vaccines are likely to reduce onward transmission of disease. The decreased shedding following vaccination with the live-attenuated Ty21a vaccine was not significant, possibly due to the small sample sizes available for these comparisons and moderate protective efficacy of Ty21a in the challenge model. In earlier challenge studies with Ty21a involving larger numbers of participants who received a freshly-harvested formulation of vaccine, shedding of *S*. Typhi was significantly reduced in recipients of the live oral vaccine[296].

The effects of Vi-PS or Vi-TT vaccination on indirect protection and stool shedding are poorly understood. A cluster randomised control trial in Kolkata, India, demonstrated that Vi-PS vaccination can result in indirect protection against typhoid fever in unvaccinated individuals resident in population clusters randomised to Vi-PS vaccine[426]. However, indirect protection of Vi-PS was not observed in another cluster randomised trial conducted in Karachi, Pakistan. One difference is that the trial in Kolkata vaccinated adults as well as children[427].

The reduction in stool shedding observed in individuals vaccinated with a Vi-TT conjugate vaccine is an important finding. To date, there are no completed cluster randomised trials of typhoid conjugate vaccines. However, one trial is ongoing in Bangladesh and individually randomised trials are ongoing in Nepal and Malawi[20]. A previous Vi-conjugate vaccine with *Pseudomonas aeruginosa* exotoxin A as a carrier protein showed high-efficacy in Vietnam (91.1, 95%CI 78.6–96.5).[27] Vi-TT vaccine is highly immunogenic in children with demonstrated efficacy of 54.6-87.1% in a controlled human infection model (depending on the diagnostic criteria used)[209] and vaccine efficacy of 85% estimated from serological data[210]. In October 2017 the WHO SAGE recommended the introduction of typhoid conjugate vaccines for children over 6 months of age

in typhoid-endemic countries[215]. It remains to be determined to what degree the reduction in shedding associated with Vi-TT vaccination will translate to indirect protection of unvaccinated persons in field-studies. If the reduction in shedding translates to indirect protection of non-vaccinees in field settings, the overall effectiveness of Vi-TT conjugate vaccines could be significantly higher than is estimated in challenge studies.

Current models predicting the potential impact of Vi-conjugate vaccines account for this potential indirect protection by assuming that transmission of *S.* Typhi is reduced in a manner that is proportional to the vaccine efficacy (i.e. by preventing infection and hence shedding among a proportion of vaccinated individuals). The results presented here support such assumptions. The protection afforded by Vi-TT against typhoid infection is similar to its effect against stool shedding. When both are expressed as odds ratios, the effects of Vi-TT against typhoid diagnosis and shedding have largely overlapping confidence intervals (typhoid diagnosis: OR 0.16, 95%CI 0.05 – 0.46; shedding: OR 0.41, 95%CI 0.19–0.91). However, more analysis is needed to examine the relationship between vaccination, stool shedding, and the development of clinical typhoid, and how this may vary between the human challenge model and field settings. Additionally, the mechanism by which anti-Vi antibodies prevent stool shedding requires further investigation.

The non-significant reductions in shedding with Ty21a vaccine contrast with data from early challenge studies, where Ty21a was associated with a reduction in any stool shedding of *S.* Typhi from between day 4 and day 30 post challenge (OR 0.08 [95%CI 0.02-0.29])[296]. The differences may result from methodological differences between the Oxford and Maryland challenge studies, including mode of administration, pre-treatment with sodium bicarbonate instead of milk, differences in challenge dose (Maryland:10^5 organisms; Oxford: 10^4) and criteria for initiating antibiotics. Of note, Ty21a appeared to provide herd-immunity in field-studies conducted in Chile[398].

As was observed in early typhoid challenge studies, early shedding increased was associated with an increased likelihood of developing typhoid fever[326]. Interestingly, we observed an increase in shedding in the undiagnosed group from day 10 onwards. It is possible that late shedding represents a harbinger of subsequent bacteraemia or fever, such that a proportion of the 'undiagnosed' group may have developed enteric fever had the infection not been halted by commencing antibiotics at Day 14. Conversely, late, asymptomatic, shedding has been described in undiagnosed participants from early challenge studies, where shedding peaked in the second week post-challenge before clearing spontaneously by six weeks (in the absence of clinical disease or antibiotic treatment)[326]. These data emphasise that a proportion of individuals exposed to typhoidal *Salmonella* will act as asymptomatic short-term carriers who transiently shed in the absence of overt clinical disease – a factor that should be considered when determining the target population for vaccination campaigns.

In individuals previously challenged with *S*. Typhi we detected a significant reduction in the rate of stool shedding compared with naïve controls. A single episode of typhoid exposure is thought to confer moderate protection against subsequent clinical disease (estimated at ~30% from historical challenge studies[295]) and modelling studies assume that multiple episodes of typhoid exposure are required to induce functional immunity. These are the first data suggesting that prior typhoid exposure significantly affects the pattern of shedding following re-challenge, albeit from a small sample size (n=27 re-challenged). Interestingly, we observed that individuals previously exposed to *S*. Paratyphi A had substantially higher rates of shedding when re-challenged with S. Typhi. The reasons for the increased risk of typhoid shedding on heterotypic re-challenge are unclear and are the subject of ongoing studies focussing on the role of secretory IgA targeted at cross-reactive antigens[428].

When shedding rates were compared between typhoid and paratyphoid challenge studies, rates of shedding following *S.* Typhi challenge were twice as high as those following *S.* Paratyphi A challenge. The higher rate of shedding following typhoid challenge may reflect the higher challenge dose administered in the typhoid model (10^4 vs. 10^3 CFU), or differences may exist between these serovars in host-pathogen interactions and transmission mechanisms. Several studies in areas co-endemic for *S.* Typhi and Paratyphi A have suggested that transmission dynamics and risk factors may differ between the two serovars.[29,49,110]4 For example, S. Paratyphi A cases appear to be more spatially dispersed than S. Typhi cases in an urban area,[110] and may be particularly associated with food-borne transmission[29,49]. Improved understanding of paratyphoid shedding and transmission dynamics will be an important consideration in the development of vaccines for paratyphoid fever[222].

There are several limitations to assessing stool shedding using data from human challenge studies. The population enrolled comprises adults from a non-endemic country; shedding dynamics may differ in individuals from endemic countries with prior immune priming or in children, who represent the majority of enteric fever cases (but not necessarily the majority of shedders). In this analysis, data were pooled across six studies conducted over six years. Whilst all samples were processed in the same laboratory using consistent protocols, study-to-study variation may still exist in the sensitivity of stool testing and thus 'study' was adjusted for in all models. Furthermore, challenge was conducted using only a single strain of *S.* Typhi (Quailes) or Paratyphi A (NVGH308) at a single dose, which may not mirror the shedding dynamics of contemporary circulating strains in Asia and Africa, such as the MDR-associated H58 (genotype 4.3.1) strain of S. Typhi[169]. These limitations primarily reflect safety considerations required for controlled human challenge and these data should be interpreted alongside emerging data from surveillance studies[19].

In summary, we have performed the first detailed model of shedding dynamics in the context of controlled typhoid and paratyphoid challenge and provide evidence for efficacy of new and existing typhoid vaccines (Vi-PS and Vi-TT) in reducing rates of shedding. These studies illustrate the value of challenge studies in obtaining novel insights into host-pathogen interactions and microbial dynamics, which can directly inform the disease control efforts for priority pathogens.

Chapter 10 - Discussion

10. Introduction

Controlled human infection models for enteric fever were first established more than 60 years ago. Over the intervening decades, these models have been applied in diverse ways, helping to accelerate vaccine development and yielding several insights into the pathophysiology of enteric fever. In this thesis, I have described a programme of work in two large controlled human infection studies of *S.* Typhi and Paratyphi infection, spanning over 30 months and involving challenge of over 150 volunteers. These include the first direct application of a *S.* Typhi challenge model to investigate the role of a specific virulence factor – the typhoid toxin – in the pathogenesis of acute typhoid. In addition, I have conducted the first *S.* Paratyphi challenge and re-challenge study, as well as providing the first description of heterologous re-challenge with *S.* Typhi and *S.* Paratyphi. These studies formed the basis for a series of preliminary studies of humoral mechanisms of immunity to typhoid and paratyphoid fever, as well as contributing to a combined analysis of factors associated with the susceptibility to enteric fever and shedding. Collectively, it is hoped that the data presented herein will contribute to the understanding of typhoid fever pathogenesis and immunity, and ultimately aid efforts aimed at disease control.

10.1. Summary of principal findings

10.2. Application of controlled human infection models to study virulence factors

In the first study described in this thesis (OVG2016/03), I began by reviewing the literature on the typhoid toxin, providing a rationale as to why this putative virulence factor was considered to have an important role in the pathogenesis of typhoid fever (Chapter 2). This was followed by a detailed characterisation of wild-type and typhoid toxin negative strains and description of the steps required to conduct a human challenge study involving a genetically modified challenge

strain, including manufacture to GMP standard and obtaining approvals from government regulatory agencies(Chapter 5). At the time of writing, relatively few controlled human infection studies involving deliberate release of genetically modified bacterial pathogens are ongoing, although this is predicted to increase in the coming years with the potential expanded use of controlled human infection models to study disease pathogenesis. The work presented herein provides one example of how to navigate the regulatory and practical steps involved in the setup of such models.

The principal finding of the OVG2016/01 study was a comparable rate of typhoid infection between participants challenged with wild type, or an isogenic typhoid toxin deficient strain of *S*. Typhi (Chapter 6). In a cohort of 40 challenged volunteers, the rate of typhoid infection following wild-type challenge was 15/21 [71%], compared with 15/19 [79%] following challenge with the toxin negative strain (p = 0.58 - Figure 6-5). Counterintuitively, there was a trend toward a more severe clinical syndrome in participants challenged with the typhoid toxin deficient strain, as evidenced by significantly prolonged duration of bacteraemia and a higher proportion of participants meeting the criteria for severe enteric fever. However, this trend was not observed for other surrogate measures of disease severity (e.g. fever clearance time, quantitative blood culture), suggesting that – overall - the clinical syndrome following wild-type or toxin-negative *S*. Typhi challenge was indistinguishable. Collectively these data suggest that expression of the typhoid toxin is not an absolute requirement for the establishment of acute typhoid fever.

The identification of pathogenicity genes is complicated by the redundancy of virulence factors expressed by *S*. Typhi[429]. A pathogen may expresses multiple effectors and virulence factors that modulate multiple distinct cellular process in synchrony to establish a common disease outcome - this has been referred to as 'systems level redundancy'[429] (Figure 10-1). The identification and screening of key virulence factors expressed by *S*. Typhi is further complicated by the lack of an

animal model in which putative virulence factors can be screened. Whilst the human challenge model offers the advantage of studying the bacteria and virulence factors within a biologically relevant system, the disease endpoints (bacteraemia and/or persistent fever) may lack the required granularity to dissect the specific role of the typhoid toxin in acute typhoid disease. Arguably, differences in these endpoints may only become apparent in the challenge model if the typhoid toxin was a central node in a complex network of host pathogen interactions [430,431]. Alternatively, early initiation of treatment within the context the challenge model may impact the natural course of typhoid disease masking any effect of typhoid toxin. Collectively these data suggest that the typhoid toxin may represent one of a number of redundant virulence factors expressed by *S.* Typhi in the establishment of typhoid infection, although this requires further study.

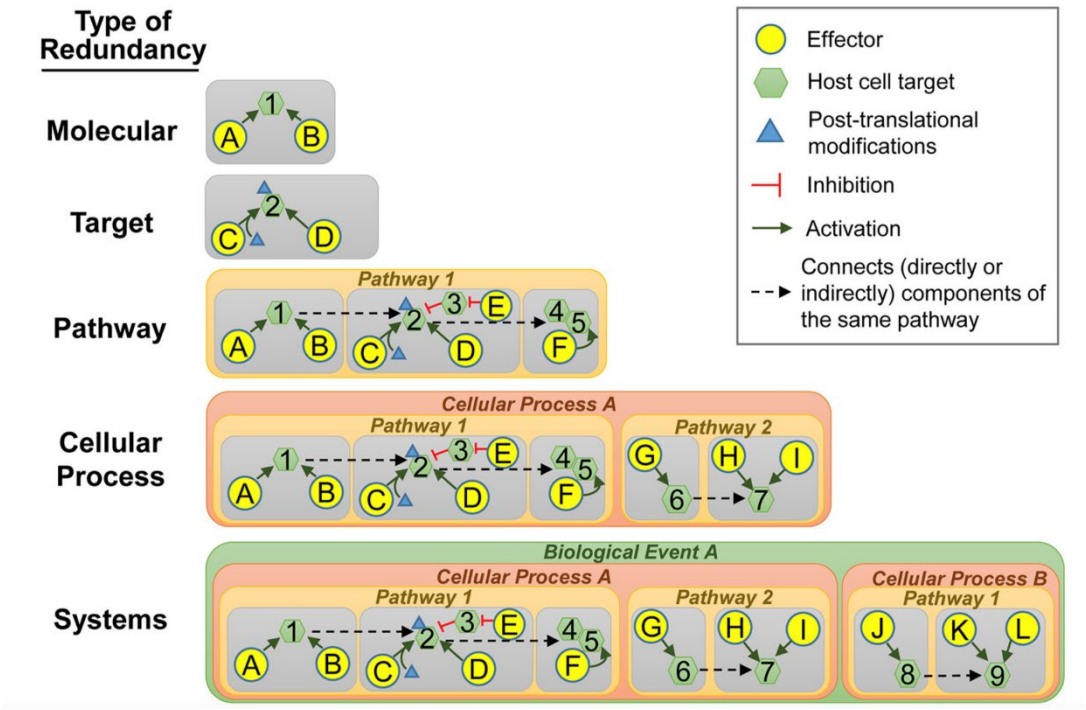


Figure 10-1 : Classification of redundancy for bacterial virulence factors.

As classified by Ghosh and O'Connor (2017) [429]. S. Typhi likely possess multiple effectors expressed at different stages of the life cycle, in different cellular and anatomical compartments, each of which contributes to the establishment a common 'biological event' i.e. typhoid infection. At a systems level, multiple redundant effector proteins may be expressed simultaneously, interacting with several distinct

cellular processes to achieve a 'common goal' of disease and/or onward transmission. Image adapted from ref [429]. Licensed under CC BY 4.0 [255].

10.2.1. Immune Responses to the Typhoid Toxin

The data from these studies raise intriguing question as to the utility of targeting the typhoid toxin with novel therapeutics or vaccines. Currently utilised typhoid vaccines – including Vi polysaccharide and Vi-conjugate vaccines – are capable of inducing protection against typhoid fever[203] despite uniquely targeting a virulence factor that is not strictly necessary for the establishment of enteric fever[71,240]. In the first instance, further studies are required to correlate host responses to typhoid toxin, with protection against disease. Antibodies to typhoid toxin components have been detected in the serum of patients with typhoid fever[158,159,276,277]. In a parallel study, we have also demonstrated that infection with *S*. Typhi and Paratyphi can induce T-cell responses to typhoid toxin components, measured by IFNγ ELISPOT[278]. Whilst the typhoid-toxin appears to be a strongly immunogenic antigen, it is unknown if these responses are simply bystander effects, or if humoral and cellular responses to the typhoid toxin contribute to protection against subsequent disease. To address this question, typhoid-toxin specific antibody and cellular responses can be measured in volunteers enrolled from the re-challenge study (Chapter 7), to determine if anti-toxin immunity correlates with protection on re-challenge. Serum from challenged volunteers could also be used within *in vitro* assays to determine if prior infection with *S*. Typhi can abrogate the activity of typhoid toxin[262 269].

10.2.2. Characterisation of the typhoid toxin in vivo

Additional studies are ongoing that attempt to further characterise the function of the typhoid toxin in the context of *S*. Typhi infection. Such studies are made possible by the collection, storage and biobanking of multiple longitudinal specimens from study participants after challenge (Supplementary Table 3).

In the first instance, the development of methods to directly detect typhoid toxin *in vivo* could identify the stage of the bacterial life cycle when the toxin is expressed and where it exerts its biological activity. Detection of the typhoid toxin, or toxin components, could theoretically be identified using mass spectrometry[432]; with aptamer based technology[433] or sensitive sandwich ELISA assays. Notably, a library of humanised monoclonal antibodies against the typhoid toxin have already been generated (Jorge Galan/Paul Kellam – personal communication) and could facilitate toxin detection. If successful, these approaches have potential utility as diagnostics for typhoid and paratyphoid fever, which could be validated using samples from patients with enteric fever recruited into ongoing surveillance studies.

It is feasible that levels of typhoid toxin in specimens collected from trial participants may be too low to be directly detected in the context of the challenge model. Indirect assays could instead be applied to describe how the typhoid toxin interacts with host cellular functions *in vivo*. For example, distinct transcriptional signatures of toxin activity in whole blood, and subsets of peripheral blood cells, could be detected using RNAseq. Whole blood transcriptional signatures following challenge with typhoid toxin deficient *S*. Typhi could be compared with pre-existing data sets from previous challenge studies[163] to identify 'toxin specific' transcriptional activity. Data generated from participant samples could then be cross-validated in a RNAseq on whole blood samples have been processed and are currently being analysed, with a particular focus on differential gene expression early after infection and during acute disease[163].

Gene expression analysis could be supplemented by further characterisation of host inflammatory response to infection in the presence or absence of the typhoid toxin, reflected by plasma cytokine measurements[434–437]. Contrary to our expectations, we observed a trend towards a more severe disease profile in the absence of the typhoid-toxin, as evidenced by significantly prolonged bacteraemia, increased frequency of severe typhoid fever and elevated acute-phase

markers. The underlying cause for these findings is unclear and is the subject of ongoing analysis. However, it is plausible that the typhoid toxin production stimulates early innate responses targeted at bacterial virulence factors, that result in clearance of the bacteria in a proportion of individuals (anti-virulence immunity). Similar observations have been made for other Gram-negative pathogens, including *E. Coli*[361]. Circulating cytokine levels have been associated with disease severity and response to treatment in typhoid fever patients. For example, reduced secretion of TNF-α in response to LPS stimulation is associated with prolonged fever[437] and elevated levels of IL-6 has been associated with an increased risk of treatment failure[435].

To further address this hypothesis, we have analysed plasma cytokine profiles at baseline and at multiple time points following challenge with wild-type or typhoid-toxin negative *S.* Typhi using a 62-plex Luminex human cytokine panel. Preliminary data are available, with expected results being the identification of a set of cytokines correlating with clinical outcome parameters and identification of distinct cytokine profiles between individuals challenged with either wild-type or typhoid-toxin negative strains of *S.* Typhi. Collectively, these analyses in conjunction with other planned analyses – such phenotyping of immune-cell subsets and detection of typhoid-toxin in vivo – could offer valuable insights into the role of typhoid toxin specifically, and the pathogenesis of invasive *Salmonella* disease more broadly.

In summary, the data from this study demonstrate the benefits – and some of the limitations – of applying controlled human infection models to study typhoid pathogenesis. This study adds to our knowledge of the typhoid toxin specifically, and typhoid pathogenesis more generally. Further studies are ongoing, using a combination of *in vitro* and *ex vivo* approaches to address the biological role of the typhoid toxin during acute typhoid disease.

10.3. Infection-Derived Immunity to Salmonella Typhi and Salmonella Paratyphi A

In the second study described in this thesis (OVG2014/01 - Chapter 7), I have described the impact of prior exposure to *S*. Typhi and Paratyphi on the rate of infection following homologous and heterologous re-challenge.

Previous exposure to *S.* Typhi was associated with a 36% relative reduction in the rate of typhoid disease after re-challenge, compared with naïve participants undergoing challenge for the first time (p=0.02 - Figure 7-7). In those participants who developed disease, previous exposure was associated with a non-significant delay to symptom onset compared with naïve control (median days [range] 7[4-14] vs 6.2 [5-10.1]; p=0.07). Previous exposure to *S*. Typhi was not associated with a demonstrable boosting of serum antibody levels and antibody secreting cell (ASC) responses to O-antigens following re-challenge, nor a significant difference in baseline *S*. Typhi specific Bm cells (Chapter 8). The observed reduction in the rate of typhoid disease on re-exposure was remarkably consistent with that described in previous studies[292,295] and is supportive of the hypothesis that prior exposure confers moderate, but incomplete, protection against subsequent infection. As predicted by modelling studies, it is conceivable that multiple exposure episodes result in boosting of immune responses that accumulate over time[293,294].

To my knowledge, no previous studies have directly presented data on immunity to *S*. Paratyphi following prior exposure, although it is frequently assumed that the extent of immunity conferred by prior infection mirrors that for typhoid infection. In this study, previous exposure to *S*. Paratyphi was associated with a 57% relative reduction in the rate of disease compared with naïve controls (Figure 7-12). The magnitude of the protection observed following previous *S.* Paratyphi re-challenge appeared to be greater than that following *S.* Typhi re-challenge, albeit not statistically significant. This may be related to lack of Vi-capsular expression by *S*. Paratyphi A, resulting in increased exposure of surface antigens to the immune system. Previous exposure was

also associated with a significant reduction in the time to diagnosis, suggesting that re-challenge is associated with a modified host response to infection in those who developed disease after re-challenge. Nonetheless, previous exposure to *S*. Paratyphi was not associated with a demonstrable boosting of serum antibody levels and antibody secreting cell (ASC) responses to O-antigens following re-challenge, nor a significant difference in baseline *S.* Paratyphi specific Bm cells, albeit from a small sample size. Whilst the mechanisms and determinants of immunity to paratyphoid infection require further study, collectively these data support the ongoing development of vaccines for paratyphoid fever.

In contrast with homologous re-challenge, prior exposure to *S*. Typhi was not associated with a reduced rate of infection on re-challenge with *S*. Paratyphi A. Patients with typhoid fever and individuals vaccinated with oral typhoid-vaccines have detectable cross-reactive humoral immune responses against Paratyphi A and B in vitro[365–367]. However, field studies in highly endemic areas have shown that Ty21a does not protect against S. Paratyphi A[368], whilst there is evidence for protection against S. Paratyphi B (49% efficacy; 95%CI 8-73%)[369].

The effect of previous *S.* Paratyphi infection on protection against *S*. Typhi disease has been less extensively studied. In contrast with homologous re-challenge, prior exposure to *S*. Paratyphi was not associated with a reduced rate of infection on re-challenge with *S*. Typhi. Overall, there was a trend to an altered humoral-response to infection in the heterologous re-challenge group, manifest as a greater magnitude of antibody and ASC responses to O-antigens compared with naïve control. Previous exposure to *S*. Paratyphi was also associated with increased likelihood of *S*. Typhi stool shedding compared with naïve and homologous re-challenge (Figure 9-2). Collectively, these data suggest that there is limited cross-protective immunity between *S*. Typhi and *S*. Paratyphi, within the context of a controlled human infection model, but that previous exposure may modify the host response to infection on heterologous re-challenge. Both *S*. Typhi

and *S.* Paratyphi are co-endemic and are thought to share common modes of transmission. However, the ecological interactions between the serovars, the reasons for distinct geographical distribution and the impact of typhoid vaccine on paratyphoid incidence are poorly understood[24]. Consequently, the interaction between these two serovars in terms of cross protection, host immune responses and their impact on transmission dynamics, is worthy of further study.

10.3.1. Infection derived Immunity following Natural Exposure

The overarching aim of the OVG2014/01 study was to investigate the mechanisms and determinants of immunity following natural infection with *S.* Typhi and Paratyphi. To address this within the context of the challenge model, the study incorporated a re-challenge group to approximate individuals with prior immunological priming. Re-challenged participants could be compared against ostensibly naïve individuals from a non-endemic country with no known prior exposure to typhoidal *Salmonella.* In reality, both the naïve and re-challenge groups are internally heterogenous with respect to baseline immune status, with a degree of overlap between groups (Figure 10-2). This was the illustrated by the elevated baseline anti-Vi IgG in the naïve cohort (Figure 8-3), presumably reflecting undisclosed/undocumented travel vaccination or cross-reactive immune responses from exposure to other serotypes. Re-challenge studies aim to approximate of the extent of immunity conferred by prior exposure to a pathogen. However, a single discrete exposure to a high pathogen load is unlikely to be representative of the exposure dynamics in typhoid and paratyphoid fever in endemic settings, where immunity is thought to be acquired through multiple exposure episodes over time. In addition, the age-distribution of participants enrolled in the challenge studies (Table 6-3 & Table 7-4) was not representative of those who acquire typhoid/paratyphoid fever in the field[170]. In order for findings from challenge studies to inform studies of transmission and vaccine development in countries with the highest burden of disease, it will be necessary to validate findings from challenge studies with naturally

occurring disease in an endemic setting. To that end, prospective studies comparing the response to natural infection in typhoid endemic countries are ongoing.

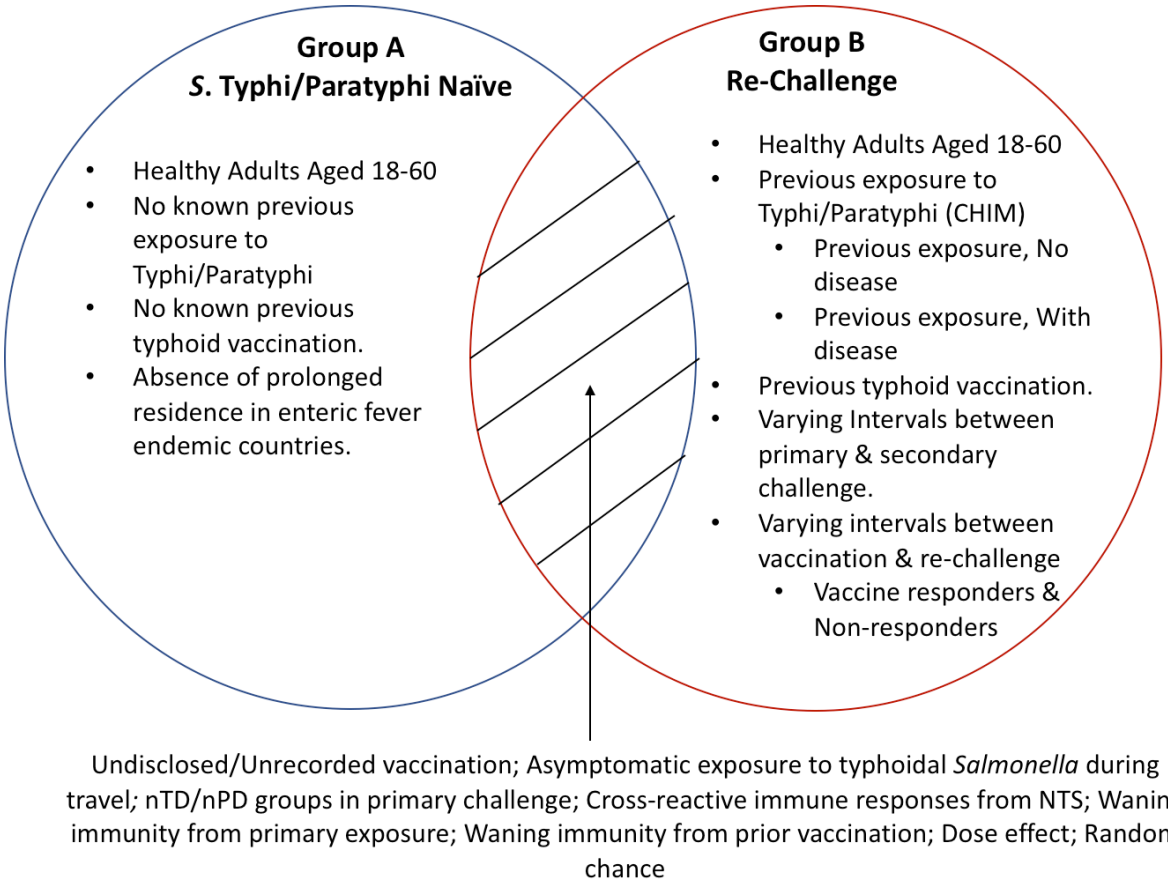


Figure 10-2 : Characteristics and overlap of naïve and re-challenge groups.

10.3.2. Expansion of challenge studies to Low & Middle-Income Countries?

Whilst *Salmonella* CHIM have proven successful to date, it remains unclear whether findings from challenge studies performed in non-endemic settings can be extrapolated to endemic settings where the burden of invasive *Salmonella* disease is highest (Figure 10-3). Volunteers in endemic countries are likely to differ from UK volunteers across a range of important variables such as previous *Salmonella* exposure, diet, intestinal microbiota, risk factors and genetic profile[438]. For findings from challenge studies to inform vaccine development, it may be necessary to validate

findings in endemic settings where vaccines will ultimately be deployed. Areas that could specifically benefit from conducting *Salmonella* CHIM in endemic settings include assessment of novel paratyphoid vaccines in South Asia; assessment of novel diagnostics (particularly those based on measuring host-response to infection) or even development of a challenge model for invasive non-typhoidal *Salmonella* (iNTS) in sub-Saharan Africa. These issues are particularly pertinent for invasive non-typhoidal *Salmonella* disease (iNTS), where chronic-malaria, sickle-cell disease, malnutrition and HIV infection represent major risk-factors[254].

There is a precedent for conducting challenge studies in low/middle income settings, including for malaria in East Africa[439 440] and enteric pathogens such as *Shigella* spp. in South East Asia[439,441]. The *Salmonella* model has an established safety profile in over 2000 volunteers and, theoretically, trial protocols could be readily transferred to new study sites. The *Salmonella* model could also provide a 'test-case' to establish feasibility for the future development of CHIM in low-and-middle income countries for other enteric pathogens of public health importance[442].

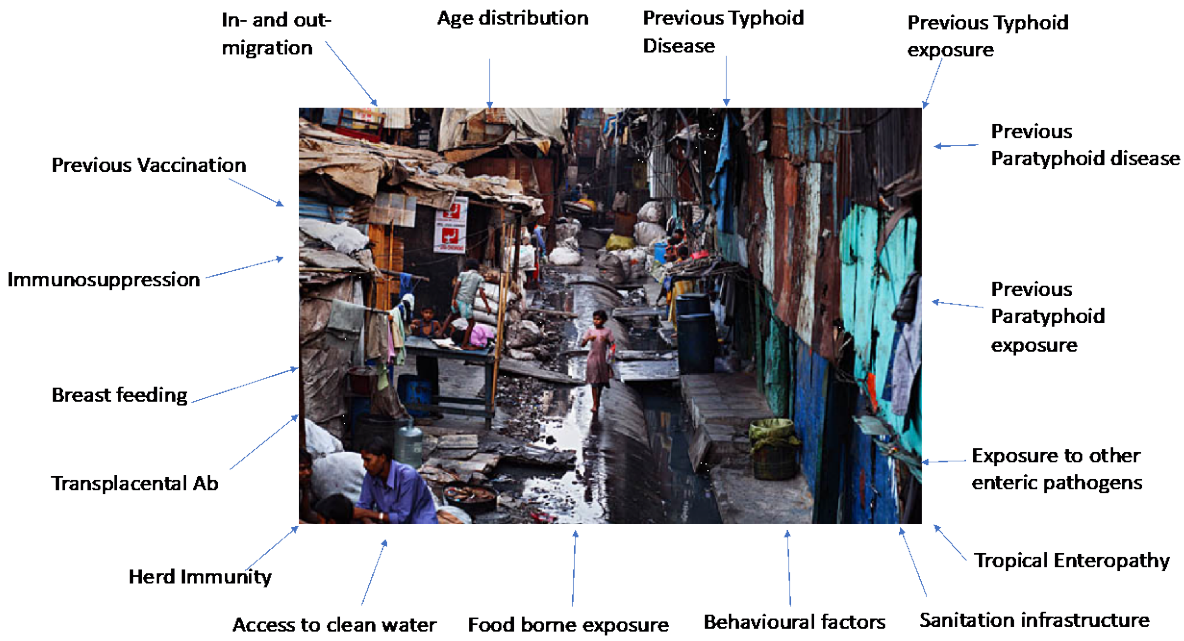


Figure 10-3 : Factors affecting susceptibility to enteric fever in endemic settings

As compared with the controlled human infection model.

10.3.2.1. Ethical Issues in conducting challenge studies in LMICs

10.3.2.1.1. Participant Safety and Benefit

In the absence of adequate medical care, *Salmonella* infection poses a theoretical risk to study participants. The mortality of typhoid fever in the pre-antibiotic era was estimated to be ~30%, although this is reduced to <1% with prompt antibiotic therapy. The mortality rate of iNTS disease (for which no CHIM currently exists) is estimated at 20%[443]. The symptoms of typhoid (such as fever, headache and abdominal pain) will reliably occur in most participants and - whilst these symptoms are undoubtedly burdensome - their occurrence is expected and predictable. Volunteers can therefore be clearly informed and unambiguously consented prior to enrolment, in contrast to the situation with phase 1 drug or vaccine trials, where the frequency and type adverse events are far less predictable.

Obtaining informed consent for challenge studies may also pose several potential challenges in resource limited settings. Adequate informed consent could be achieved by applying a multi-

staged consent process, supported by preliminary community engagement studies and community-based research advocates[346,439]. Whilst CHIM studies offer few direct benefits to participants, consideration could be given towards providing enhanced levels of care, or health insurance, for the duration of follow up.

The risks to study participants could be increased in low income settings, owing to – for example – worse pre-morbid health status, increased risk from prior immune priming and increased risk of carriage. In the Oxford challenge model, several safeguards are implemented to ensure safety of participants, including detailed screening assessments, appropriate hospital facilities with capacity for inpatient admission and intensive care; dedicated staffing with 24/7 contact; prompt access to treatment, combined with adequate hygiene and sanitation precautions. Such safeguards would need to be at least as stringent should *Salmonella* CHIM be performed in low-income countries, which may limit the number of sites where such studies could be performed or require large infrastructure investments.

10.3.2.1.2. Risk of transmission

In the absence of adequate personal hygiene, *Salmonella* and other enteric CHIM pose a theoretical risk of disease transmission to household contacts. The wider community may also be at risk in the absence of adequate sanitation infrastructure. These concerns are present in the UK but are potentially amplified in low-income settings. Household contacts could be consented at face-to-face screening or by provision of written material, although consideration should be given as to how to proceed should a household member decline consent. In contrast, it is unfeasible to consent an entire community who may be at theoretical risk of acquiring infection. In the UK consent to release potentially pathogenic bacteria into the environment is conducted under the observation of government bodies (e.g. Public Health England, DEFRA - 5.1), which might be

viewed as surrogate for community consent. Protection of communities would require a similarly stringent level of environmental protection oversight in low-and-middle income countries.

Transmission risks could be minimised by conducting these studies in an inpatient setting, where hand hygiene could be monitored and sanitation infrastructure guaranteed (e.g. flush toilets, waste treatment). In this scenario, participants would be confined to the inpatient facility until proven to be clear of infection. Whilst such an approach has been taken previously, [439,441] several ethical uncertainties remain. For example, in UK trials, a period of approximately 6 weeks is required before an individual is considered clear of *Salmonella* infection after challenge. It may be impractical or unethical to confine an individual for this length of time and would be complicated if participants expressed a wish to withdraw from the study.

10.3.2.1.3. Volunteer reimbursement

Safety monitoring in the *Salmonella* CHIM requires a large time commitment. Financial reimbursement in resource-rich countries is often set to a high level (e.g. £2000-£3000 for UK typhoid challenge studies), to account for potential loss of earnings. The time commitment required would increase even further if participants were to be confined as inpatients in low-income countries. The substantial reimbursement amounts inevitably raise concerns of undue inducement amongst financially vulnerable populations – a concern that is not confined to low-income countries. Some studies have suggested that volunteers who are financially motivated may pay particular attention to potential risks of participating in such studies. Nevertheless, reimbursement could be set at levels that achieve appropriate compensation for loss of earnings from time committed (e.g. equivalent to average local daily wage), but not necessarily proportional to perceived risks.

10.3.2.1.4. Engagement with local ethics committees

CHIM studies would likely attract intense scrutiny from research ethics committees in low-income countries, particularly in sites where such studies have never been previously undertaken. Such studies may be perceived as violating core ethical principles such as non-maleficence and may pose a reputational risk to the institution. Concerns may be allayed by open discussions between investigators involved with CHIM studies, independent study review by infectious diseases clinicians along with training and support for ethics committees, provided by independent bodies.

10.3.2.1.5. Regulatory requirements and scrutiny of challenge agents

The choice of challenge strain and dose is a key variable in *Salmonella* CHIM studies. Stocks of *Salmonella* challenge strain are typically manufactured to a GMP-like standard to ensure safety and reproducibility but are subject to different regulations between regions. For example, in the USA, microbial challenge agents are considered IMPs and, as such, are subject to scrutiny by the FDA. In contrast, UK legislation currently does not view challenge agents as IMPs (see section 5.1). Drug review boards in many resource-limited settings may have to develop country-specific legislation to cover regulation of challenge strains. Early engagement with investigators and regulators in other countries will be necessary to ensure consistent regulatory guidelines that are country specific, ensure participant safety, whilst simultaneously avoiding hindering research programmes.

Overall, the expansion of *Salmonella* challenge studies to low and middle-income countries are potentially both highly valuable and feasible, whilst also providing a platform to address an area of major global-health concern. In addition to addressing country-specific host and environmental factors relevant to vaccine testing, the establishment of *Salmonella* CHIM could increase research capacity, provide training of local staff in laboratory/research techniques and

provide a platform to undertake novel CHIM studies for other enteric diseases. High levels of safety (coupled with careful consideration of country-specific ethical issues) must be maintained to ensure confidence in the model and to avoid reputational damage. Whilst *Salmonella* CHIM studies are well established in the UK setting, trial protocols and regulatory processes must be adapted to local circumstances and only after a prolonged consultation period with local stakeholders to address any potential concerns and to ensure ownership of the model moving forward.

10.3.3. Identifying correlates of Protection

The development of vaccines for typhoid and paratyphoid fever could be aided by identifying correlates of protection following vaccination or prior infection. Whilst not being an all-encompassing definition, a correlate of protection has been defined as "an *immune response that is responsible for and statistically interrelated with protection*"[444]. Using this definition, very few correlates of protection exist for enteric pathogens and enteric vaccines. The reasons for this complex (reviewed in [445]), but could be partially addressed by expanding the use of controlled human infection models[445]. For example, serum vibriocidal antibody seroconversion following vaccination an oral cholera vaccine (CVD-103HgR) correlated with protection against mild and severe cholera in a controlled human infection model[446], supporting epidemiological data for vibriocidal antibodies as relative correlates of protection against cholera infection[447].

In this thesis, I have attempted to describe the relationship between serum antibody and memory B-cell responses with protection against typhoid and paratyphoid fever in naïve and previously exposed participants. As discussed in Chapter 8, no measures of humoral immunity studied here appeared to explain the moderate protection conferred after homologous re-challenge and further work is ongoing to determine if measures of antibody functionality – or antibodies targeted at distinct antigens – will be associated with protection.

As the principal barrier to infection, immune responses at the gastrointestinal mucosa likely have an important role in preventing the establishment of infection[201]. In this study, participants underwent an oesophagogastroduodenoscopy (OGD) prior to challenge and after recovery of illness, during which time intestinal biopsy and intestinal fluid samples were collected (Supplementary Table 4). Analysis of *S.* Typhi/Paratyphi specific gut resident T-cell populations - including CD4+,CD8+, γδ T-cells and MAIT cells – is ongoing and aims to describe the relationship between activation of different cell subsets and protection from disease. Analysis of secretory antibodies in intestinal fluid could supplement measures of gut cellular immunity.

The experience from these and other challenge studies suggests that identification of correlates of protection to typhoid and paratyphoid fever will most likely require integration of data from multiple studies, including a combination of 'hypothesis generating' and 'hypothesis driven' approaches. The programme of typhoid and paratyphoid challenge studies conducted in Oxford has enrolled a large, and growing, number of volunteers. For each individual enrolled, a vast amount of data is generated, using a number of different techniques and platforms. This offers the opportunity to apply an integrative approach to identifying correlates of protection, by combining data sets that have already been generated with those that are in the process of being generated. In turn, these can be related to clinically relevant outcomes, such as presence of disease, attenuation of symptoms, stool shedding. The wealth of data generated brings unique bioinformatic challenges. A challenge for future studies will be develop bioinformatic approaches for data integration and address challenges of curating, cleaning and storing data sets.

10.3.4. Innate Resistance to Infection

Paradoxically, an analysis of sub-groups within the re-challenge cohort indicated that individuals who did not develop disease on primary challenge were less likely to develop disease on re-

challenge (section7.2.14). One interpretation of these data is that the reduced rate of infection on re-challenge was driven by an increased resistance to infection in a proportion of participants, presumably driven by host innate factors. Additionally, the interval between primary challenge and re-challenge was not significantly associated with likelihood of developing disease on re-challenge (section). How this observation translates to the development of immunity following natural exposure is unknown and the factors underpinning this observation require further study, in particular to determine if this represents an example of trained innate immunity[385] or other non-specific host factors.

The data presented in this thesis could have implications for future typhoid vaccination strategies in the context of wider disease control efforts. The acquisition of immunity following natural exposure may account for the age-distribution of typhoid fever in endemic countries. Typhoid conjugate vaccines will likely be deployed in conjunction with routine infant immunisation, with or without a catch-up campaign in school-age children[211,212]. In the absence of improvements in water quality and sanitation infrastructure, this could result in an epidemiologic shift of the burden of disease to older age groups – depending in part on the susceptibility of older age groups (Supplementary Figure 3), duration of immunity conferred by vaccination, and characterisation of those age-groups persons responsible for transmission. The data supported in this chapter reinforce the importance of infection derived immunity in transmission dynamics of typhoid fever. Ongoing surveillance, coupled with parallel WASH interventions, will be required following the deployment of routine typhoid vaccination.

10.4. Limitations of These Data

The application of controlled human infection studies to study disease pathogenesis and immunity is accompanied by a number of intrinsic limitations. These limitations invariably stem

from ethical and safety considerations, and to protect the well-being of trial participants. Whilst specific limitations to these data are discussed in the relevant chapters, a consideration of general limitations – and the measures implemented to mitigate these limitations – can help to understand caveats to the data presented herein.

The studies presented in this thesis were limited by a small sample size, ranging from 10 to 27 participants per sub-group. As a consequence, these studies may be underpowered to detect differences between groups, particularly when the true effect size is small. Several factors are taken into consideration when determining the number of participants enrolled, beginning with ethical considerations. Challenge with *S.* Typhi and Paratyphi will result in illness in the majority of participants challenged. To minimise the number of individuals who will develop symptomatic illness, studies may be designed to enrol the minimum number of participants required to detect clinically meaningful differences between groups. Small sample sizes also may be an inevitable consequence of the costs and practicalities of running challenge studies. Some of these limitations can be mitigated by incorporating data from previous studies to act as historical controls (Chapter 9), which can be facilitated by maintaining consistent protocols from study-to-study (e.g. challenge stocks, challenge agent preparation, endpoint definitions). In the absence of increased capacity locally – or an increase in the number of groups conducting these studies – the number of participants enrolled in these studies is unlikely to increase for future studies.

As previously discussed, these studies apply a composite surrogate definition of *S.* Typhi/Paratyphi bacteraemia and/or fever ≥38°C for ≥12 hours as the primary endpoint. The disease endpoint is distinct to the WHO definition for typhoid fever (fever ≥ 38°C for ≥3 days, with laboratory-confirmed culture from blood, bone marrow, bowel fluid)[140] and from the disease definition used in the Maryland challenge studies (Oral temperature ≥39.4°C for 24-36h)[74]. It is unclear how closely the disease endpoint applied in the Oxford challenge model reflects the

presentation of disease in the field – close monitoring of temperature and regular blood culture sampling may detect mild cases that otherwise would not have reached medical attention in the field. Application of different endpoints can mitigate this limitation to a degree but is potentially susceptible to bias. An alternative approach would be to modify the primary endpoint definition to one that more closely mirrors clinical disease (e.g. persistent fever ≥24 hours with *S.* Typhi/Paratyphi bacteraemia) – however participant safety considerations perhaps makes this a less appropriate option.

As only one strain each of *S.* Typhi or *S*. Paratyphi was used in these studies (section 4.5), we cannot conclusively rule out a strain-specific effect for our observations. The NVGH308 strain of *S*. Paratyphi is, however, a recent clinical isolate from a symptomatic case with bacteraemia, and is closely related to currently circulating strains[311]. The Quailes strain was originally isolated from a chronic carrier of S. Typhi are there are some data to suggest that carriage isolates of S. Typhi evolve at a different rate to strains isolated from blood culture. However, recent data show that the Quailes strain of *S*. Typhi is also closely related to other known disease causing isolates, albeit not the most common circulating strains[126,234]. Both *S*. Typhi and *S*. Paratyphi are clonally monomorphic pathogens containing limited genomic variation[234], suggesting that the pathogenicity and immune response to both the Quailes and NVGH308 strains should translate to other wild-type strains. Establishment of new challenge stocks derived from currently circulating strains – such an antibiotic sensitive *S.* Typhi H58 strain – could help to validate the challenge model using contemporary strains, although the costs associated with establishment of new GMP challenge stocks may be prohibitive.

In summary, the general limitations of the studies described in this thesis are related to sample size, strain selection, endpoint definitions and participant characteristics. Each of these factors introduce caveats into challenge model data, although each can be mitigated to a greater or

lesser extent. These factors should be taken into account in the design of future challenge studies and when refining study protocols, but ultimately emphasise how challenge studies represent only one of several approaches to address the challenge of typhoid fever control.

10.5. Further Work

The studies presented in this thesis have given rise to several further lines of enquiry relating to the pathogenesis and immunity of enteric further. Many of these studies are currently ongoing and are beyond the scope of this chapter. Specific ongoing projects are detailed below.

10.5.1. Further characterisation of the typhoid toxin

As discussed in section 10.2 , further studies are ongoing to characterise the immunobiological role of the typhoid toxin during typhoid infection. These studies include:

- Analysis of plasma cytokine responses during early infection and acute typhoid fever following challenge with wild-type and typhoid toxin deficient *S*. Typhi.
- Analysis of whole blood gene expression measured by RNAseq during early infection and acute typhoid fever following challenge with wild-type and typhoid toxin deficient *S*. Typhi.
- Analysis of serum antibody responses to typhoid toxin components in i) participants challenged with wild-type and typhoid toxin deficient *S*. Typhi, and ii) participants in the re-challenge cohort, to correlate antibody responses with protection.
- Analysis of T-cell responses to typhoid toxin peptides measured by IFNγ ELISPOT in i) participants challenged with wild-type and typhoid toxin deficient *S*. Typhi, and ii) participants in the re-challenge cohort, to correlate IFNγ ELISPOT responses with protection.

10.5.2. Identifying correlates of protection

As discussed in section 10.3, further studies are planned to characterise the mechanisms underlying infection derived immunity, which aim to identify correlates of protection. These studies include:

- Analysis of functional antibody activity, including serum bactericidal assays and opsonophagocytic assays, and their relationship to protection in the challenge model.
- Analysis of *S.* Typhi and Paratyphi specific gastrointestinal T-cell responses, using biopsy specimens collected during the challenge study.
- Analysis of *S.* Typhi and Paratyphi specific T-cell responses in blood, measured by ELISPOT and flow cytometry, and their relationship to protection in the challenge model.
- Analysis of gastrointestinal microbiota at baseline and its relationship to protection in the challenge model.
- Comparison of humoral and cellular responses measured in the challenge model with natural typhoid and paratyphoid infection in endemic settings.

10.5.3. Application of Controlled Human Infection Models to Assess Typhoid and Paratyphoid vaccines

The application of controlled human infection models has arguably had a central role in the development of typhoid conjugate vaccines, leading to pre-qualification by the WHO and recommendations from their programmatic use[209,215,216]. The experience of typhoid vaccines is arguably an exemplar of how challenge models can accelerate vaccine deployment, and the momentum generated from this experience could be harnessed to address existing and future challenges in the global control of enteric fever.

The emergence of *S*. Paratyphi A is one such challenge. Data presented in this thesis indicate that prior infection with *S*. Paratyphi is associated with a reduced rate of disease on re-challenge. The

protective effect of prior exposure (~55%) is comparable to the degree of protection conferred by Vi-conjugate vaccines against typhoid fever[209]. This raises the possibility that *S.* Paratyphi vaccines could represent a viable strategy to control the emergence of paratyphoid diseases in South Asia.

It is hoped that these data will act as a catalyst for the development of paratyphoid vaccines. Paratyphoid vaccine candidates have been in development for several years, although few have progressed beyond pre-clinical or phase 1/2 trials[222]. A Paratyphi A O-specific polysaccharide-TT conjugate vaccine has completed phase 1 and 2 trials, which was reported in 2000[408]. Similarly a phase 1 trial of an oral live-attenuated Δ*guaBA*, Δ*clpX S*. Paratyphi vaccine (CVD-1902) completed recruitment in April 2013 (NCT01129453)[448], although these data are unpublished at the time of writing. The development of a *S.* Paratyphi challenge model provides a platform to test these vaccines, potentially accelerating their passage through to field efficacy trials. A study assessing the CVD-1902 vaccine in the paratyphoid challenge model is currently in set-up.

10.5.4. Considerations in vaccine efficacy testing using controlled human infection models

Vaccine efficacy trials using controlled human infection models require careful attention to study design, with particular reference to study power and pre-defined study endpoints. Robust and well-defined challenge studies can theoretically advance vaccines through to phase 3 efficacy trials, licensure and recommendations for widespread use – as illustrated by the CVD-103 cholera vaccine[446] and Vi-TT conjugate vaccines for typhoid fever[209,215]. However, overly stringent or underpowered challenge models have the potential to stall the development of promising vaccine candidates. Vaccine manufactures may therefore be reticent to subjecting promising vaccine candidates to efficacy testing in a challenge model if there is uncertainty in the robustness of the model or the relevance of the surrogate endpoints used. The uncertain role of challenge model in vaccine development pathways amplifies this problem[449]. Overall, challenge models

typically impose a high bar to demonstrate vaccine efficacy, meaning that only the most promising candidates progress to future testing. To my knowledge, there are no examples of vaccines that have demonstrated significantly greater efficacy in the field trials than demonstrated in challenge models[330].

10.6. Conclusion

Typhoid and paratyphoid fever are diseases of poverty. Whilst they have been virtually eradicated in the developed world, enteric fever remains a major public-health problem in resource-limited settings. It can be argued that a combination of global trends underpins the persistence of enteric fever in resource-limited settings, ranging from urbanisation and the growth of large urban slums; climate change and challenges to global water supply, through to the emergence and dissemination of antibiotic resistance. The global burden of enteric fever may eventually fall with economic development and rising wealth, but problems of antimicrobial resistance will continue to pose a challenge. Whilst there is considerable debate as to whether disease eradication represents an achievable goal in the medium-to-long term, a sustained reduction in the burden of disease is potentially achievable in the near future. Momentum to achieve control of enteric fever has been steadily building, driven by the availability of effective tools and support from key stakeholders.

Immunisation forms a central pillar of the global strategy to control enteric fever. The development, and impending roll-out, of cost-effective, scalable typhoid conjugate vaccines represents a major advance in typhoid control and could have a major impact on the global burden of disease. Nevertheless, several current and future challenges remain that threaten to limit anticipated gains, including the emergence of *S*. Paratyphi A in South Asia, as well as the potential for serovar replacement and emergence of Vi-negative *S*. Typhi following the

deployment of Vi-conjugate vaccines. These challenges will likely necessitate the development of new vaccine strategies, which ultimately forms the rationale for the studies presented herein.

The primary aim of this thesis has been to describe how the novel application of controlled human infection models can form part of efforts to achieve control of enteric fever and act as a catalyst for vaccine development. Specifically, I have aimed to describe how controlled human infection models can be applied to address fundamental questions relating to disease pathogenesis and immunity. These studies provide a unique opportunity to understand host-pathogen interactions from the time of initial exposure through to the development of infection. It is hoped that insights from these studies will form the basis for future studies to assess typhoid toxin as future vaccine or therapeutic target. In addition, an improved understanding of infection-induced immunity could provide valuable data to refine modelling of transmission dynamics and vaccine impact measures, in addition to aiding the identification of correlates of protection to expedite vaccine development for *S*. Typhi and Paratyphi.

In summary, controlled human infection models represent a valuable method to study and tackle typhoid and paratyphoid fever. Opportunities to achieve control of enteric fever have been missed in the past. The momentum generated from the availability of these studies and the development of new control strategies should be harnessed, to address outstanding challenges and to ensure opportunities for disease control are not missed again.

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Appendix 1 - Inclusion & Exclusion Criteria for the OVG2014/01(PATCH) and OVG2016/03 (TYGER) Studies

11. Study eligibility

Male or female participants aged 18-60 years inclusive who are in good health (as determined by a study doctor, medical investigation and review of medical history provided by their General Practitioner) and able to provide written informed consent will be eligible for inclusion in this study.

11.1. Inclusion Criteria

Participants must satisfy all of the following criteria to be considered eligible for the study:

- Agree to give informed consent for participation in the study.
- Aged between 18 and 60 years inclusive at time of challenge.
- In good health as determined by medical history, physical examination and clinical judgment of the study team.
- Agree (in the study team's opinion) to comply with all study requirements, including capacity to adhere to good personal hygiene and infection control precautions.
- Agree to allow his or her General Practitioner (and/or Consultant if appropriate), to be notified of participation in the study.
- Agree to allow study staff to contact his or her GP to access the participant's vaccination records and summary of medical history.
- Agree to allow Public Health England to be informed of their participation in the study.
- Agree to give his or her close contacts written information informing them of the participant's involvement in the study and offer them voluntary screening for S. Typhi carriage.
- Agree to have 24-hour contact with study staff during the four weeks post challenge and to be able to ensure that they are contactable by mobile phone for the duration of the challenge period until antibiotic completion.

- Agree to allow the study team to hold the name and 24-hour contact number of a close friend, relative or housemate who will be kept informed of the study participant's whereabouts for the duration of the challenge period (from the time of challenge until completion of antibiotic course). This person will be contacted if study staff are unable to contact the participant.
- Have internet access to allow completion of the e-diary and real-time safety monitoring.
- Agree to avoid antipyretic/anti-inflammatory treatment from the time of challenge (Day 0) until advised by a study doctor or until 14 days after challenge.
- Agree to refrain from donating blood for the duration of the study.
- Agree to provide their National Insurance/Passport number for the purposes of TOPS registration and bank account details for payment of reimbursement expenses.

11.2. Exclusion Criteria

The participant will not be enrolled if any of the following apply:

- History of significant organ/system disease that could interfere with trial conduct or completion. Including, for example, but not restricted to:
    - Cardiovascular disease
    - Respiratory disease
    - Haematological disease
    - Endocrine disorders
    - Renal or bladder disease, including history of renal calculi
    - Biliary tract disease, including biliary colic, asymptomatic gallstones or previous cholecystectomy
    - Gastro-intestinal disease including requirement for antacids, H_2-receptor antagonists, proton pump inhibitors or laxatives
    - Neurological disease
    - Metabolic disease
    - Autoimmune disease

- Psychiatric illness requiring hospitalisation or known or suspected drug and/or alcohol misuse (alcohol misuse defined as an intake exceeding 42 units per week)
- Infectious disease

- Have any known or suspected impairment of immune function, alteration of immune function, or prior immune exposure that may alter immune function to typhoid resulting from, for example:
  - Congenital or acquired immunodeficiency, including IgA deficiency
  - Human Immunodeficiency Virus infection or symptoms/signs suggestive of an HIV-associated condition
  - Receipt of immunosuppressive therapy such as anti-cancer chemotherapy or radiation therapy within the preceding 12 months or long-term systemic corticosteroid therapy.
  - Receipt of immunoglobulin or any blood product transfusion within 3 months of study start.
  - History of cancer (except squamous cell or basal cell carcinoma of the skin and cervical carcinoma in situ).
- Moderate or severe depression or anxiety as classified by the Hospital Anxiety and Depression Score at screening or challenge that is deemed clinically significant by the study doctors[3].
- Weight less than 50kg[4].
- Presence of implants or prosthesis.
- Anyone taking long-term medication (e.g. analgesia, anti-inflammatories or antibiotics) that may affect symptom reporting or interpretation of the study results.
- Contraindication to fluoroquinolone or macrolide antibiotics (e.g. ciprofloxacin or azithromycin).

[3] If elevated scores are due to temporary significant life events, the questionnaire may be repeated after resolution of the event with a view to inclusion if normal.
[4] Or a Body Mass Index (BMI) that, in the opinion of the study doctors, may adversely impair the interpretation of the study results or affect the safe performance of any study procedures.

- Female participants who are pregnant, lactating or who are unwilling to ensure that they or their partner use effective contraception 30 days prior to challenge and until three negative stool samples have been obtained after completion of antibiotic treatment.
- Full-time, part-time or voluntary occupations involving:
    - Clinical or social work with direct contact with young children (defined as those attending pre-school groups or nursery or aged under 2 years), or
    - Clinical or social work with direct contact with highly susceptible patients or persons in whom typhoid infection would have particularly serious consequences (unless willing to avoid work until demonstrated not to be infected with S. Typhi in accordance with guidance from Public Health England and willing to allow study staff to inform their employer).
- Full time, part time or voluntary occupations involving:
    - Commercial food handling (involving preparing or serving unwrapped foods not subjected to further heating)
- Close household contact with:
    - Young children (defined as those attending pre-school groups, nursery or those aged less than 2 years)
    - Individuals who are immunocompromised.
- Scheduled elective surgery or other procedures requiring general anaesthesia during the study period.
- Participants who have participated in another research study involving an investigational product that might affect risk of typhoid infection or compromise the integrity of the study within the 30 days prior to enrolment (e.g. significant volumes of blood already taken in previous study)[5].
- Detection of any abnormal results from screening investigations (at the clinical discretion of the study team).

[5] As assessed by both participant questioning and registration with The Over Volunteering Prevention System (TOPS) database.

- Inability to comply with any of the study requirements (at the discretion of the study staff and the participant's General Practitioner).
- Any other social, psychological or health issues which, in the opinion of the study staff, may
    - put the participant or their contacts at risk because of participation in the study,
    - adversely affect the interpretation of the primary endpoint data,
    - impair the participant's ability to participate in the study.
- Prior vaccination with an oral typhoid vaccines (e.g. Ty21a) or other investigational typhoid vaccine (e.g. Vi-conjugate vaccine, MO1ZH09)
- Prior vaccination with a Vi-polysaccharide typhoid vaccine administered within 5 years from the time of screening.
- Prior vaccination with a Vi-polysaccharide typhoid vaccine administered more than 5 years from the time of screening AND detectable Vi-antibody titre at screening (defined as IgG ≥7.4U/ml measured using the VaccZyme Salmonella Typhi Vi IgG kit, Binding Site ® UK).
- Having been resident in an enteric fever endemic country for 6 months or more.
- Have previously been diagnosed with laboratory-confirmed typhoid or paratyphoid infection or been given a diagnosis compatible with enteric fever.
- Have participated in previous typhoid or paratyphoid challenge studies (with ingestion of challenge agent).
- Have a prolonged corrected QT interval (>450 milliseconds) on ECG screening.

Appendix 2 – Construction of typhoid toxin deficient Salmonella Typhi strain

12. Introduction

Construction of the typhoid toxin deficient *Salmonella* Typhi strain was performed by Maria Lara-Tejero, Xuyaou Jiao, Shu-Jung Chan and Jorge Galán at the Department of Microbial Pathogenesis, Yale School of Medicine, New Haven, CT, USA.

12.1. Materials

12.1.1. Bacterial Strains

- *Salmonella* Typhi Quailes strain: Two vials of GMP-grade *S.* Typhi Quailes strain were supplied to the Galan laboratory (Department of Microbial Pathogenesis, Yale School of Medicine, New Haven, CT, USA). The Galán laboratory did not have this S. Typhi strain in its collection prior to its arrival as a frozen stock and the strain has been stored in a dedicated storage box at -75 °C.
- *Escherichia coli* strain CC118 λ pir was used to host different suicide plasmids during construction of deletions of the typhoid toxin genes cdtB, pltA, and pltB by standard recombinant DNA techniques.
- *Escherichia coli* strain ß2163 Δnic35 carries a conditionally lethal deletion mutation in dapA, which renders it dependent on diaminopimelic acid for its growth and therefore can readily be counter-selected even in rich media. In addition, this strain expresses the λ Pir protein thus making it a suitable host for the replication of the suicide vector pSB890 and its derivatives (see below). This strain also carries the RP4 conjugation machinery and is therefore capable of transferring the pSB890 suicide vector into S. Typhi by

conjugation. This strain is used as the donor of the suicide plasmids to generate mutant S. Typhi strains.

12.1.2. Plasmid vector:

- pSB890: a suicide plasmid vector to introduce DNA into the genome of S. typhi Quailes strain. This vector possesses a mutation in the replicon (R6K replicon) that results in plasmid replication being dependent on the Pir protein (5). When propagation of this vector is required, the E. coli host strains CC118 λ pir and ß2163 Δnic35 possessing a copy of the pir gene are utilized (see above). This plasmid also carries a tetracycline-resistance selectable marker and sacB, a gene that encodes for levansucrase, which converts sucrose into levansucrans that are toxic to E. coli and Salmonella and therefore renders host strains unable to grow in the presence of sucrose and thus can act as counter-selectable marker. In addition, pSB890 has the oriTRP4 and can therefore be transferred by conjugation from ß2163 Δnic35 into S. Typhi.

12.1.3. Growth media

In all cases, *Salmonella* Typhi or the strains of E. coli utilized during mutant construction (see below) were propagated in animal-product-free media. The composition and source of the specific media components were as follows:

- LB broth: BD Select APS LB media powder (BD Cat. No. 292438); Milli Q-purified Water c. s. p.
- LB agar: BD Select APS LB media powder (BD Cat. No. 292438); Milli Q-purified Water c. s. p.; Ultra Pure Agar (AmericanBIO Cat. No. AB01185-00500)
- LB agar plus sucrose: BD Select APS LB media powder (BD Cat. No. 292438) * Sucrose (AmericanBIO Cat. No. AB01900-01000); Milli Q-purified Water c.

Ultra Pure Agar (AmericanBIO Cat. No. AB01185-00500) 20 g 1 lt 20 g 1 lt 20% w/vol 20 g 20% w/vol 1 lt 20% w/vol .

To grow E. coli strain ß2163 Δnic35 the media was supplemented with DL-diaminopimelic acid (DAP) (Sigma Cat. No. D1377) at 50 µg/ml

12.1.4. Procedures

The strain was obtained from the Oxford Vaccine Group. Two vials labelled as vial #3 and vial #4 were received on dry ice. Upon arrival as a frozen stock, two vials were stored in a dedicated box, which was placed on a -75°C freezer for future reference. Material from the frozen vial #3 was plated on an APS-LB plate to obtain single colonies. A single colony was picked and cultured in LB broth overnight. The resulting culture was used for the first step in the construction of S. Typhi Δ*cdtB* Δ*pltA* Δ*pltB* mutant strain.

12.1.5. Genetic Construction

All plasmids were constructed using the Gibson DNA assembly protocol [450].

12.1.6. Construction of plasmid pSB5999 containing a complete deletion of the *cdtB* gene.

Two PCR reactions were performed using genomic DNA from *Salmonella* Typhi Quailes as a template using primers designed using the genome sequence of the S. Typhi strain CT18 (Accession number AL513382). One PCR (primers #777 and #761) amplified ~1,000 immediately 5' of the cdtB gene, the other PCR (primers #762 and #763) amplified ~1,000 nucleotides immediately 3' of the cdtB gene. The 5'-end of PCR product included in the 5' end 20 bp of homology to the multiple cloning site (MCS) of the suicide plasmid pSB890, while the 3'-end of the PCR product included this homology in its 3' end. Both PCR products will have 20 bp of homology in the *cdtB* deletion site. The resulting PCR products were mixed in equimolar concentrations with amplified pSB890 DNA (primers #566: and #567) then ligated using the Gibson assembly technique to yield the plasmid pSB5999, which carried a deletion of the coding sequence of cdtB bounded by ~1,000 bp of the 5' and 3' the immediately adjacent chromosomal

regions. The Gibson reaction was then electroporated into *E*. coli CC118 λ pir selecting on LB agar plates containing 20 µg/ml of tetracycline and eight isolated colonies were screened for the presence of the recombinant plasmid. Selected candidate plasmids were sequenced to verify the deletion of cdtB. The verified plasmid (pSB5999) was then transformed into the E. coli strain ß2163 Δnic35, selecting in LB agar plates containing 20 µg/ml of tetracycline as a selection marker, and 50 µg/ml of DAP to allow the growth of the host bacteria. Plasmid DNA was then isolated from the E. coli strain ß2163 Δnic35 and its nucleotide sequence determined to ensure that the sequence matches the expected sequence based on the construction scheme.

12.1.7. Construction of plasmid pSB6000 containing complete deletion of pltA and pltB genes

Nucleotide sequences consisting of 2,500 nucleotides immediately 5' and 3' from the coding sequences of the pltA and pltB genes, respectively, were amplified by PCR from the genome of *Salmonella* Typhi Quailes strain using primers obtained from the genome sequence of the S. Typhi strain CT18 (Accession number AL513382). One PCR product (primers #802: and #765:) amplified the region upstream of *pltB* whereas the other PCR product (primers #766 and #803) amplified the region downstream of *pltA*. The PCR amplification of the suicide vector pSB890 was identical to the PCR described in the construction of the Δ*cdtB* suicide plasmid. As with the previous plasmid, the 5'-end PCR product had 20 bp of homology at its 5'-end with the MCS of the suicide plasmid pSB890 while the 3'-end PCR product had 20 bp of homology with the MCS of pSB890 at its 3'-end. Both PCR products had a 20 bp homology at the *pltA* and *pltB* deletion site. The resulting PCR products were purified and mixed in equimolar concentration with amplified plasmid pSB890 and then ligated using the Gibson assembly technique to yield the plasmid pSB6000, which carries a deletion of the coding sequence of both pltA and *pltB* bounded by ~2,500 bp of the 5' and 3' immediately adjacent chromosomal regions. The Gibson reaction was electroporated into E. coli CC118 λ Pir selecting on LB agar plates containing 20 µg/ml of

tetracycline and eight isolated colonies were screened for the desired recombinant plasmid. A selected candidate plasmid was sequenced to verify the deletion of *pltA* and *pltB*. This plasmid (pSB6000) was transformed into the E. coli strain ß2163 Δnic35 selecting in LB agar plates containing 20 mg/ml of tetracycline as a selection marker and 50 µg/ml of DAP. Plasmid DNA was isolated from the E. coli strain ß2163 Δnic35 and its nucleotide sequence determined to ensure that the sequence matches the expected sequence based on the construction scheme.

12.1.8. Construction of S. Typhi SB5999 containing a complete deletion of the cdtB gene

The E. coli strain ß2163 Δnic35 carrying the suicide plasmid pSB5999 (see above) was used to introduce the deletion of the *cdtB* gene into the chromosome of the S. Typhi Quailes strain by conjugation. One ml cultures of both the donor (E. coli strain ß2163 Δnic35 carrying the suicide plasmid pSB5999) and recipient (*S.* Typhi Quailes) strains were mixed and filtered through a Nucleopore Track-Etch Membrane filter (Fisher Scientific Cat. No. 09-300-62) to facilitate the mating. The filter containing the mixed culture was placed on a LB-agar plate containing 50 mg/ml of DAP and incubated for 6 hours. Bacteria were recovered from the filter and plated on LB-agar plates containing 20 µg/ml of tetracycline and no DAP. These growth conditions selected for *S.* Typhi carrying plasmid pSB5999, which integrated into its chromosome by homologous recombination since this plasmid is unable to replicate in this strain. In addition, these growth conditions did not allow the growth of the E. coli ß2163 Δnic 35 donor strain due to the absence of DAP. After overnight growth, 5 independent tetracycline resistant colonies were picked and grown for 1 hour in independent LB broth cultures without antibiotics and subsequently plated on LB agar plates containing 20% sucrose to counter-select for the integrated plasmid, which excised from the chromosome by homologous recombination. Such recombination event resulted in *S.* Typhi carrying either a wild type copy or a complete deletion of *cdtB* with no plasmid derived sequences left remaining in the bacterial chromosome. Resulting colonies were screened by PCR and nucleotide sequencing to identify *S.* Typhi mutants carrying the expected deletion of

the cdtB gene. This procedure led to the identification of *S*. Typhi strain SB5999 carrying a deletion of the entire coding sequence of the *cdtB* gene.

12.2. Construction of S. Typhi SB6000 containing a complete deletion of cdtB, pltA and pltB genes

The E. coli strain ß2163 Δnic35 carrying the suicide plasmid pSB6000 (see above) was used to introduce a complete deletion of the *pltA* and *pltB* genes in the chromosome of the *S.* Typhi Quailes derivative strain SB5999, which carries a deletion of the *cdtB* gene. Introduction of the plasmid and selection of the transconjugates was carried out as described in Step 2. Resulting colonies were screened by PCR and nucleotide sequencing to identify S. Typhi mutants carrying the expected deletion of the *pltA* and *pltB* genes yielding the S. Typhi strain SB6000 (production date October 23, 2015), which carries deletions in *cdtB, pltA*, and *pltB* genes.

Supplementary Table 1 : Primers used in the construction of typhoid-toxin deficient *S.* Typhi

Primer #	Sequence
566	ggatcccccgggctgcagttcacttacaccgcttc
567	gcggccgccaccgcggtggggctttttattg
761	ctcattaacagcttcgaggttttttcataatatctactctcccgagcattac)
765	agaaagtataagttcaggtacatacttactcatatacatactactctcctgtacg
766	gagtaagtatgtacctgaacttatactttctaaataaatgaaacttacctatgttgcc
777	ctgcagcccgggggatccgaacgcattaaacagtgactgtacaaaaacc
802	ctgcagcccgggggatcctcctcaattcagaccgcaatagtagtccatcc
802	ctgcagcccgggggatcctcctcaattcagaccgcaatagtagtccatcc

Appendix 3 – Whole genome sequencing of typhoid toxin deficient S. Typhi SB6000 strain

13. Facility and Personnel

DNA extraction was performed at the Oxford Vaccine Group, University of Oxford by Dr Malick Gibani (Clinical Research Fellow and DPhil candidate, Oxford Vaccine Group) and Dr Jennifer Hill (Post-Doctoral research fellow, Oxford Vaccine Group).

Sequencing was performed at the Wellcome Trust Sanger Institute (Hinxton, UK) under the supervision of Dr Derek Pickard and Professor Gordan Dougan. Analysis of the sequence data was performed by Dr Eva Heinz (Senior Staff Scientist, Wellcome Trust Sanger Institute).

13.1. Methods

Sequencing and analysis was performed at the Wellcome Trust Sanger Institute (Hinxton, UK).
The samples were sequenced using MiSeq and on the PacBio RSII using P6/C4 sequencing chemistry, the library was made using the SMRTbell Template Prep Kit 1.0.

13.1.1. DNA Extraction and Sequencing

We extracted DNA from pre-GMP and post-GMP batches of both wild-type and knock-out strains to determine if any sequence changes were introduced during manufacture of the GMP batches. These were compared with the parent GMP-batches of *S*. Typhi Quailes strain from which the new challenge batches were generated. Whole genome sequencing was performed by the Wellcome Trust Sanger Institute using both the MiSeq (Illumina, San Diego, CA, USA) and PacBio (Pacific Biosciences, CA, USA) platforms.

DNA for MiSeq sequencing was extracted using the Wizard® Genomic DNA purification kit according to manufacturer's instructions[451].

DNA for PacBio sequencing was extracted using a protocol supplied by the Wellcome Trust Sanger institute. *Salmonella* Typhi from glycerol stocks were inoculated into 10mls of Lysogeny broth (LB) and cultured overnight in a shaking incubator at 220rpm and 37°C. Overnight cultures were spun down at 3,000 rpm for 10 min, and the pellet re-suspended in 2mls of 25% Sucrose in TE (25g Sucrose in TE consisting of 10mM Tris and 1mM EDTA pH8.0). 50μl of 100mg/ml Roche Lysozyme in 0.1M Tris pH8.0 was added to the re-suspension and left at 4°C for 30 minutes. After the elapsed time, we added 100μL of Proteinase K (20mg/ml), 30μl of RNase A (10mg/ml), 400μl EDTA (0.5M pH8.0) and 250 μl of 10% Sarkosyl NL30 and left on ice for 2 hours. Once the lysis was complete (slightly turbid but viscous) the samples were left at 50°C in a heat-block overnight.

After heating overnight, the DNA was made up to a volume of 12 mls with TE (10mM Tris, 1mM EDTA pH8.0) and mixed gently to equilibrate. To this was added 5ml phenol:chloroform:isoamyl alcohol (25:24:1) in a phase lock tube and mixed by gentle to an emulsion and subsequently centrifuged for 15 minutes at 4000 rpm to separate the phases. The aqueous phase was removed, and the phenol:chloroform:IAA extraction step and spins were repeated three times. After the third spin, the aqueous phase was removed into a fresh phase lock tube and extracted once with an equal volume of chloroform:IAA (24:1), mixed by gentle inversion to an emulsion and centrifuged for 5 minutes at 4000 rpm. After a second chloroform:IAA extraction, the final aqueous phase was transferred to a 50ml Falcon tube, to which 2.5 volumes of ethanol were added to precipitate the DNA. This was incubated at -20°C for 15 minutes (giving a 'cotton wool' like appearance) spun down at 4000 rpm for 10 minutes and the pellet washed with 25mls of

70% Ethanol. The pellet was re-suspended in 350ul of 10mM Tris pH7.4 buffer and then stored at 4°C prior to shipment.

13.1.2. Strains

Five samples were submitted for sequencing: 1) Salmonella enterica subspecies enterica serovar Typhi (S. Typhi) wild-type Quailes strain; 2) S. Typhi wild-type Quailes strain Pre-GMP WRAIR; 3) S. Typhi wild-type Quailes strain GMP WRAIR; 4) S. Typhi SB6000 typhoid-toxin k/o strain Pre-GMP; 5) S. Typhi SB6000 typhoid-toxin k/o strain GMP.

Strain 1 is a wild-type strain of S. Typhi, currently being used in a programme of human-challenge studies at the University of Oxford. Strains 3 and 5 will undergo deliberate release as part on an upcoming human-challenge study, also at the University of Oxford. Strains 2 and 4 represent the parent strains from which the GMP challenge-lots were derived. Comparisons of challenge strains with pre-GMP strain variants 2 and 4 was undertaken to assess if mutations were introduced as part of serial passage and GMP manufacture.

13.1.3. Analysis

The five samples were compared both on the level of small changes resulting in single nucleotide polymorphisms (SNPs) and small insertions/deletions (INDELs), as well as large-scale genome rearrangements. Both the raw (read) data as well as assembled data were analysed. The automated PacBio assembly pipeline resulted in one large contig per sample, as well as 1-3 additional small fragments, and the large fragment was not always circularised. The additional small fragments (additional contigs) were investigated for their potential as plasmids; all fragments were concluded to be fragments of the genome and encoded variable regions (bacteriophage proteins, discussed below) or parts of a large repeat region. Assemblies were resolved manually in samples 1, 2, 3, 5 into a single-chromosome assembly. Sample 4 included

contig breaks in highly variable regions and was therefore not manually polished into a single-chromosome assembly.

13.2. Results

Whole genome sequencing confirmed the expected absence of the typhoid-toxin pathogenicity islet in the SB6000 GMO strains (4 and 5) compared with the wild-type S. Typhi strains (1,2 and 3).

Other than the expected difference in the typhoid-toxin islet, we observed three differences supported in the assemblies and by ambiguous base calls in the SNP analysis/differences in the mapped reads between the strains, as described below. The described differences between wild-type Quails strain and the knock-out SB6000 strain variants occur in genomic regions known to be highly unstable and have no known association with virulence, persistence within the human host nor persistence in the environment.

- One stretch of 8-base pair repeats (CCCCAGTT) showed variation across the assembled genomes and in the raw (read) data, with 18 copies in strains 3, 4 and 5, compared with 17 copies in strains 1 and 2. These were located in an intergenic region, between positions 3290262 to 3290397 in the manually polished wild type assembly. This may represent sequencing error due to the repetitive nature of the sequence.

- Variable region encoding phage tail proteins - Located between position 2348074 and 2351741 in the manually polished wild type assembly. Focusing on strain 3 and 5, in the assemblies (where all variation is summed into a consensus sequence), the rearrangement results in the change/inversion of four proteins in strain 3 and three in

strain 5, all of which were annotated as phage tail proteins. The assemblies also differed within the wild type samples (1/2/3) in this region.

- Variable phage region – Located between positions 4585600 to 4587620 in the wild type assembly, encoding phage proteins. Variation was observed in the raw read data but not in the assembled sequence.

In addition, no differences between strains were observed in SPI-7, a region encoding genes required for expression of the Vi-capsule.

Using the SNP calling script to compare the MiSeq read data from all samples against the wild type (using both the automatically generated, as well as the manual assembly for this strain as reference) resulted in high-confidence base calls of 96.0%/97.2%, 96.0%/97.2%, 96.02%/97.21%, 95.9%/97.2%, 95.9%/97.2% for strains 1-5 for the automated and manually polished assemblies, respectively. The manual polishing was performed by using the one large contig and manually resolving a large repeat region, which was collapsed into a single copy in the automated assembly. The SNP calling excludes sites with ambiguous base calls and low mapping quality identical sequences of e.g. 16S/23S rRNA, multi-copy transposases or duplicated regions of the genome also result in low mapping quality. The only differences observed in the high-confidence base calls resulted in a single base deletion in an intergenic region in the knockout region in strains 4 and 5. The observed difference of mapped bases between wild type and knockout strains is not observed when mapping against the manually polished knockout strain (strain 5) as reference (96.02%, 96.02%, 96.02%, 96.03%, 96.03% for strains 1-5, respectively), where the only SNP call with high confidence relates to an insertion, which can be observed in strains 1-3 in the intergenic stretch in the knockout region.

13.3. Working Directories & Data Deposition

Pipeline files (raw read data, automated assembly/annotation outputs) are available on request from Wellcome Trust Sanger Institute, Hinxton, UK. The respective sample IDs are given in Supplementary Table 2 below.

13.4. Conclusions

Whole genome sequencing of challenge strains confirmed the expected absence of the typhoid-toxin pathogenicity islet in the SB6000 GMO strain. We observed no differences between the wild-type and SB6000 GMO strains in relation to key virulence factors, including genes required for Vi-capsule expression. Differences between strains supported by assemblies and raw data were confined to highly-variable regions encoding phage proteins, which are not predicted to impact on bacterial survival in the environment or persistence in the human host.

Supplementary Table 2 : Whole genome sequencing working directories and data deposition.

Strain Variant	Sample ID
S. Typhi wild-type Quailes strain Oxford	4316STDY6559668
S. Typhi wild-type Quailes strain Pre-GMP WRAIR	4316STDY6559669
S. Typhi wild-type Quailes strain GMP WRAIR	4316STDY6559670
S. Typhi SB6000 typhoid-toxin k/o strain Pre-GMP	4316STDY6559671
S. Typhi SB6000 typhoid-toxin k/o strain GMP.	4316STDY6559672

Appendix 4 - Environmental persistence testing of challenge strains

The wild-type and toxin- deficient SB6000 challenge strains of *Salmonella* Typhi were cultured in a variety of media and under different conditions to determine if the genetically modified (GM) strain of *S*. Typhi was associated with enhanced persistence in the environment compared with the wild-type strain. This work was performed in collaboration with the Food, Water and Environmental Laboratory, Public Health England, Porton Down. Raw data were provided to co-ordinate an application for deliberate release of the GM strain of *S*. Typhi.

14. Methods

14.1.1. Culture in river water

A two-litre sample of river water was collected from the River Adur (West Sussex, UK), of which 500ml was filter-sterilised and split into five 100ml aliquots. Aliquots were inoculated with the wild-type and typhoid-toxin deficient strains of *S*. Typhi grown to one of two starting innocula: 10^7 CFU/mL (high inoculum) or 10^5 CFU/mL (low inoculum). One river water aliquot was not inoculated and used as a negative control. Aliquots were statically incubated at either 21 °C or 4 °C. At each timepoint the viable cell count of the samples was determined by culturing a 100µl aliquot Xylose Lysine Deoxycholate agar (XLD) agar plates in duplicate. Samples were also inoculated onto nutrient agar at the final timepoint, when no growth was detected on XLD/PCA agar.

14.1.2. Culture in sea-water

A two-litre sample of sea water was collected from the Southampton Harbour (UK), of which 500ml was filter-sterilised and split into five 100ml aliquots. Aliquots were inoculated with the *S*. Typhi wild-type and knock-out strains grown to one of two starting innocula: 10^7 CFU/mL (high

inoculum) or 10^5 CFU/mL (low inoculum). Culture and sampling proceeded as for growth in river water.

14.1.3. Culture in topsoil

Topsoil was obtained from Porton Down (UK), sterilised by autoclaving and aliquoted into 1 g portions. Suspensions were created by adding 2 ml maximum recovery diluent to 1 g soil. Aliquots were inoculated with the *S*. Typhi wild-type and knock-out strains at 10^7 CFU/mL. Uninoculated soil aliquots were used as negative controls. Culture and sampling proceeded as for growth in river water and sea-water.

Appendix 5 - OVG2016/03 sampling time points and volumes

Supplementary Table 3 : OVG2016/03 sampling time points and volumes.

Exploratory samples not marked * may be collected in a subset of study participants only.

Investigation	Stool sample	Saliva sample*	Nasal Swab*	Urine sample*	Blood culture	Molecular Diagnostics*	Bacterial quantification	PBMC*	Serum	Plasma	Functional genomics	DNA samples (including	FBC	CRP, U+Es, LFTs	Screening Serology(1)	Glucose	
Sample tube	Stool Pot	Saliva pot	Nasal swab	Universal container	Aerobic BACTEC bottle	Heparin Vacutainer/Streck tube	Wampole™ Isostat® Isolator		Serum Vacutainer	Plasma vacutainer	Tempus™ Blood RNA	Taken from clot of serum tube	EDTA Vac	Serum Vacutainer		Glucometer	
Day																	
Screening		x	x										3	2	9	x	14
D-7		x	x			10											10
D0	x	x	x	x				60	15	6	3	x	1	2			87
D0 +12hrs (2)					10			10	10	3	3						36
D1	x			x	10			25	10	3	3		1	2			54
D2	x			x	10				10	3	3						26
D3	x				10												10
D4	x				10			25	10	3	3		1	2			54
D5	x				10												10
D6	x				10				10	3	3		1	2			29
D7	x			x	10			60									70
D8	x				10				10	3	3		1	2			29

D9	x				10												10
D10	x				10			25	10	3	3		1	2			54
D11	x				10												10
D12	x				10				10	3	3		1	2			29
D13	x				10												10
D14	x			x	10			60	10	3	3		1	2			89
TD	x			x	10	10	10		10	3	3		1	2			49
TD +12hrs	x				10												10
TD +24hrs [3]	x				10			60	5	3	3		1	2			84
TD +48hrs	x				10								1	2			13
TD +72hrs	x				10								1	2			13
TD +96hrs	x				10								1	2			13
TD Day 14	x				10			25	5	3	3		1	2			49
D28 +/- 5 days	x			x				50	15	3	3	x	1	2			74
Total for first 2 months (excluding screening)													If NO Typhoid Diagnosis				705
													If Typhoid Diagnosed				936
D90 +/- 14 days	x							40	5		3		1				49
D180 +/- 28 days	x							25	5								30

D365 +/- 56 days	x							40	5			x				45
Total in 12 months											If NO Typhoid Diagnosis					829
Total in 12 months											If Typhoid Diagnosed					1060

(1) Includes Vi-antibody, Hepatitis B/ Hepatitis C/HIV serology & coeliac serology; (2) PBMC samples at DO+12 samples to be collected in a subset of study participants; (3) PBMC samples may be collected at either the TD12, TD24 or TD48 visit to facilitate sample processing

Appendix 6 - OVG2014/01 sampling time points and volumes

Supplementary Table 4 : OVG2014/01 sampling time points and volumes.

Numbers in grid correspond to ml blood collected.

Investigation	Saliva sample	Stool sample	Enteric String Sampling	Wireless Video Capsule Endoscopy	Endoscopy, fluid aspirate & Mucosal biopsy	Blood culture	PCR	Bacterial quantification	Bacterial localisation/Exploratory assays	Antibody secreting cells	Blood to correlate with Mucosal Biopsy	Whole Blood Stimulation	CyTOF	Cell mediated immunity	Full blood count	CRP, U+Es, LFTs	Coeliac serology	Virology (HIV, Hep B, Hep C serology)	Serum bactericidal assay	Antibodies	Cytokines	Functional genomics	DNA samples (including epigenetics)	
Day																								Total
Screening															3	2	3	3						11
Endoscopy 1 (D-180 to -45)					x						20		5	60										85
-7 days		x	x				10								1	2								13
D0	x	x								20		5	5	50	1	2			3	10	2	3	x	101
D0+12 hrs						10															2	3		15

D1			x	x		10									1	2					2	3		18
D2		x				10									1	2				5				18
D3		x				10															2			12
D4	x	x	x			10							5	50	1	2				5		3		76
D5		x				10															2	3		15
D6		x				10									1	2					2	3		18
D7	x	x				10				20			5	50					3	5	2	3		98
D8		x				10									1	2								13
D9		x				10															2			12
D10	x	x				10							5	50	1	2				5	2	3		78
D11		x				10																		10
D12		x				10									1	2								13
D13		x				10																		10
D14	x	x				10					10		10	50	1	2				5	2	3		93
ED hr0	x	x				10	10	10	15						1	2			3	5	2	3		61
ED+12h						10															2			12
ED+24h		x				10							5	50	1	2						3		71
ED+48h		x	x	x		10				20					1	2				5	2			40
ED+72h		x				10																		10
ED+96h	x	x				10					10		10	50	1	2				5				88
D16			x	x																				0
D28	x	x											5	65	1	2				10		3	x	86
Endoscopy 2 (D21-48)					x						15	3												18
D90	x	x											5	50					3	5		3		66

D180	x	x																3	5		3		11
D365	x	x											80					3	5		3	x	91

Appendix 7 - Factors Associated with Resistance and Susceptibility to Salmonella Typhi and Salmonella Paratyphi infection

15. Introduction

In previous chapters I have described how the rate of infection in individuals with prior exposure to *S*. Typhi and Paratyphi differs from that in individuals who are naïve to exposure and described how prior exposure impacts humoral immune responses following re-infection. Human infection challenge models offer a platform to study a diverse range of host factors associated with susceptibility and resistance to infection, by detailed profiling of participants at baseline prior to exposure and by controlling as much as possible for important variables such as challenge strain, challenge dose and mode of infection. Identification of factors associated with protection against disease could facilitate the development of new vaccines by identification measurable correlates of protection.

In the absence of vaccination, challenge with *S*. Typhi at a dose of 10^4 CFU with sodium bicarbonate results in infection of approximately two-thirds of healthy volunteers (attack rate 69%; 95%CI 60-77%)[126 314]. The factors associated with the absence of disease in the remaining one-third of unvaccinated volunteers are poorly understood. In particular it is unclear if the lack of disease following controlled infection results from one of – or a combination of – i) a chance failure to establish infection following exposure; ii) a host-mediated control of infection through innate and/or adaptive immune effector mechanisms at any stage of the life cycle and/or iii) interruption of disease prior to clinical presentation by initiating treatment during the incubation period.

It is presumed that the lack of infection in ~1/3 of unvaccinated volunteers is largely a function of the infectious dose[303,331]. Indeed, a dose-response relationship has been described both in historical typhoid challenge studies (administered without sodium bicarbonate) and in the contemporary Oxford model (administered with sodium bicarbonate). Pre-treatment with sodium bicarbonate is thought to increase the 'effective infectious dose' of *S*. Typhi and Paratyphi that traverse the gastric acid barrier to reach the site of invasion in the small intestine[331] – for comparison a dose of 10^7CFU S. Typhi is required to achieve a 60-75% attack rate without sodium bicarbonate[303,331], compared with 10^4 CFU when sodium bicarbonate is administered[126].

Host factors have also been shown to impact disease susceptibility following controlled challenge. In historical typhoid challenge studies conducted by the University of Maryland, prior military service was associated with reduced risk of typhoid disease compared with challenge participants without a history of military service[297]. This was partly attributed to whole-cell typhoid vaccination on enlistment into the military and/or exposure to typhoidal *Salmonella* during military deployment in South-East Asia[297]. Other factors thought to be associated with protection against disease or delay to disease onset in the absence of known vaccination or exposure include age; baseline anti-H[452] and baseline anti-Vi IgG levels[314]. Improved understanding of factors associated with resistance and susceptibility to infection could help to refine protocols for controlled human infection models (e.g. by stratifying randomisation to vaccination or placebo) and help to adjust estimated attack rates between groups. More broadly, insights from these studies could help to refine models of transmission dynamics[293] and to identify measurable correlates of protection.

In this section I will aim to identify factors associated with resistance and susceptibility to infection within the controlled human infection model. `y. I will then perform a retrospective analysis of all typhoid and paratyphoid challenge studies conducted in Oxford between 2011 and 2017, focussing on demographic and anthropometric factors associated with susceptibility and

resistance to infection. Finally, I will assess the contribution of baseline humoral immunological measures with protection and susceptibility to enteric fever.

15.1. Methods

Data were available from six enteric fever human challenge studies conducted in Oxford between 2011 and 2017. A list of included studies is displayed in Table 7-3. Participant challenged with typhoid-toxin deficient S. Typhi (OVG2016/03) were not included in the analysis. All challenge studies followed comparable protocols, detailed in Chapter 4and elsewhere[126,209,311,314,318].

Participants were classified into one of two groups, as either having enteric fever (ED) or not having enteric fever (nED), according to whether they met the composite criteria for diagnosis (*S.* Typhi/Paratyphi bacteraemia ≥72 hours from challenge and/or temperature ≥38°C for ≥12 hours). Data were combined in mixed effects logistic regression models and adjusted for vaccine status, dose (high or low), age and study. The linear predictor from the model was exported for each participant for each day and these values converted into an odds ratio with 95% confidence interval by taking the inverse logit. Probabilities for continuous variable are presented in figures with a generalized linear model line to illustrate the findings of the logistic regression models. A combined analysis was performed on participants challenged with either *S.* Typhi or Paratyphi together, as well as analysing participants separately according to challenge agent. All models were fitted in R version 3.4.4. using the glm() function.

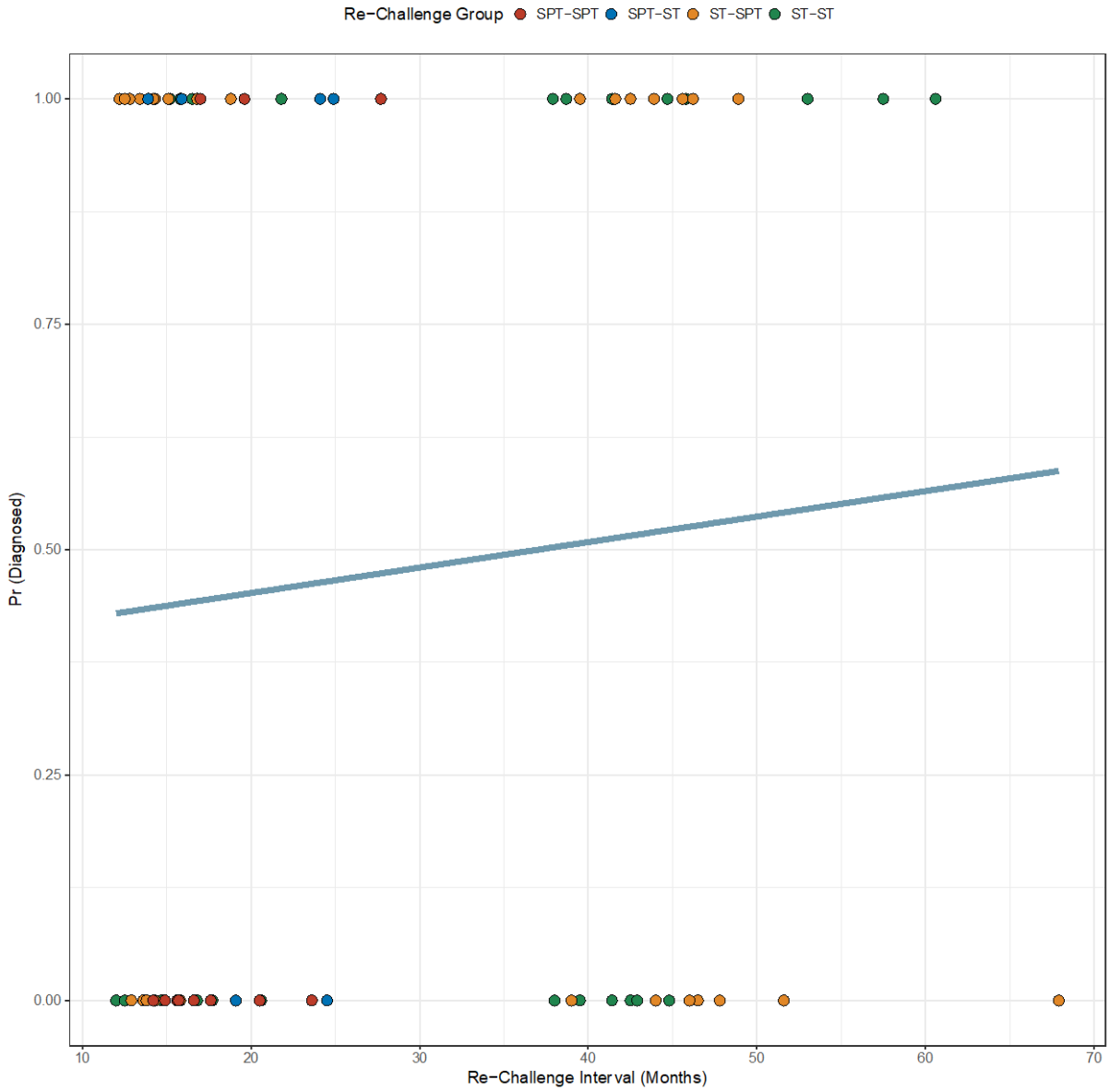
The subgroup analysis in the re-challenge study compared subgroups difference within the re-challenge cohorts using Fisher's exact test. In addition, the attack rates in individual re-challenge subgroups were compared with naïve controls from the OVG2014/01 study and pooled naïve controls as described in Chapter 7

15.1.1. Logistic regression modelling

In order to further characterise factors associated with protection in the re-challenge study, we performed a univariate and multivariate logistic regression analysis of a range of variables recorded at baseline. The dependent variable in the model was diagnosis status (disease =1, no disease = 0). The independent variables included within the model were outcome of primary challenge (disease vs. no disease), previous vaccination, interval from primary challenge and study. The unadjusted and adjusted odds ratios are presented in Supplementary Table 5.

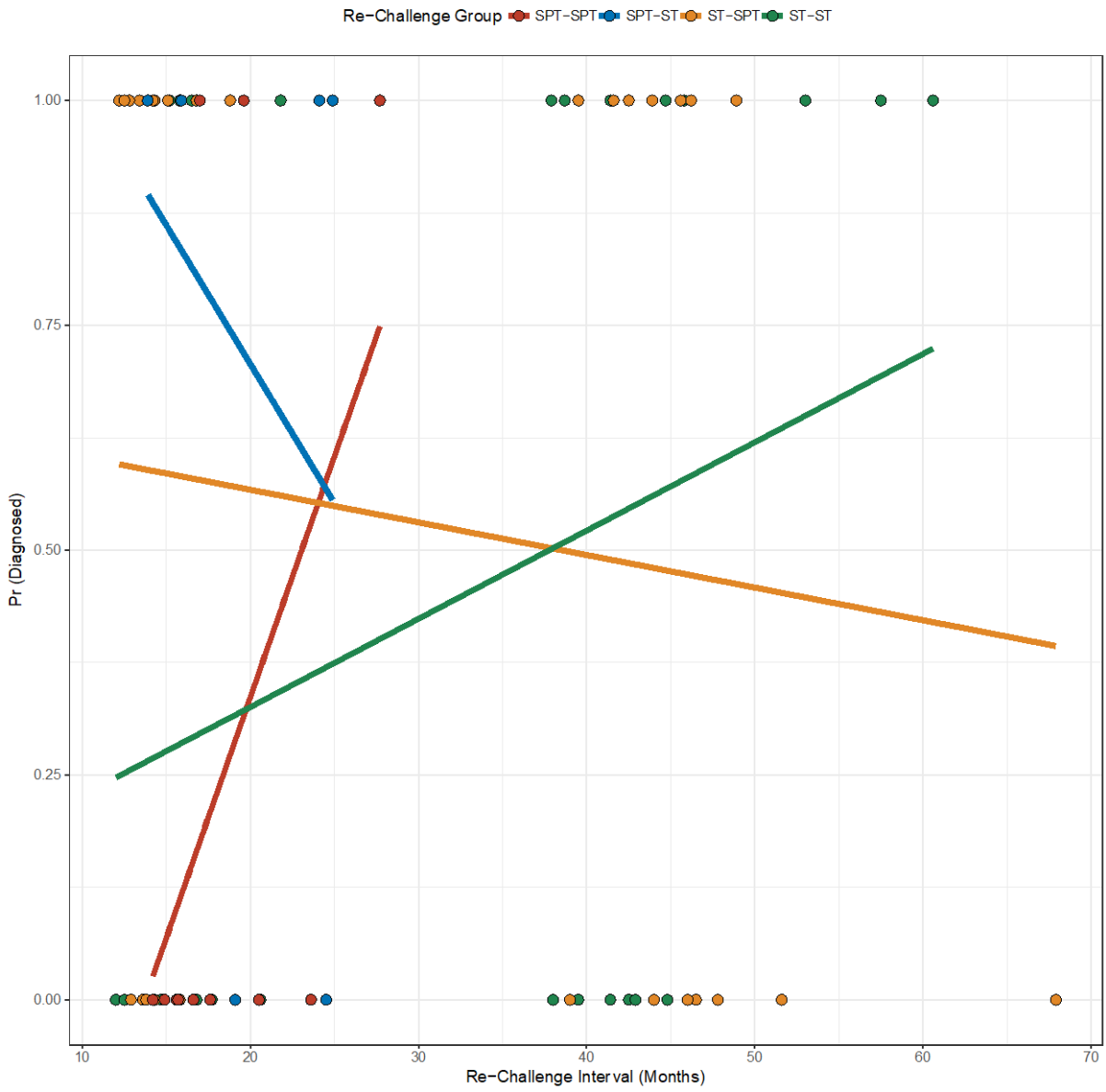
Supplementary Table 5 : Likelihood of enteric fever diagnosis in re-challenge group according to baseline participants characteristics.

	Attack Rate	Crude Odds Ratio (95%CI)	Adjusted Odds Ratio* (95% CI)	p
Previous Diagnosis				
Yes	25/37(68%)	1	1	
No	11/39 (28%)	0.26 (0.063-0.94)	0.17 (0.02-0.82)	0.043
Previous Vaccine (Any)				
No	22/48(46%)	1	1	
Yes	14/28(50%)	1.55 (0.43-5.78)	1.87 (0.11-36.1)	0.66
None	22/48(46%)	1	1	
Ty21a	2/4(50%)	-	-	0.99
MO1ZH09	3/5(60%)	1.56 (0.16-14.97)	-	
Vi-PS (All Challenged)	4/8(50%)	0.78 (0.03-9.33)	2.65 (0.04-128)	0.61
Vi-TT (All Challenged)	5/11(45%)	2.59 (0.51-15.33)	5.0 (0.17-159)	0.34
Interval (Months)				
12-23.9	9/24(38%)	1	1	
24-35.9	1/1(100%)	-	-	0.99
36-47.9	6/11(55%)	2 (0.47-8.89)	4.26 (0.54-41)	0.17
48+	1/3(33%)	0.83 (0.04-9.97)	1.98 (0.06-43)	0.66



Supplementary Figure 1 : Probability of diagnosis by re-challenge interval

Showing fitted logistic model (glm) and 95% confidence band for all re-challenged participants. Data point coloured according to re-challenge group.



Supplementary Figure 2 : Probability of diagnosis by re-challenge interval and re-challenge group.

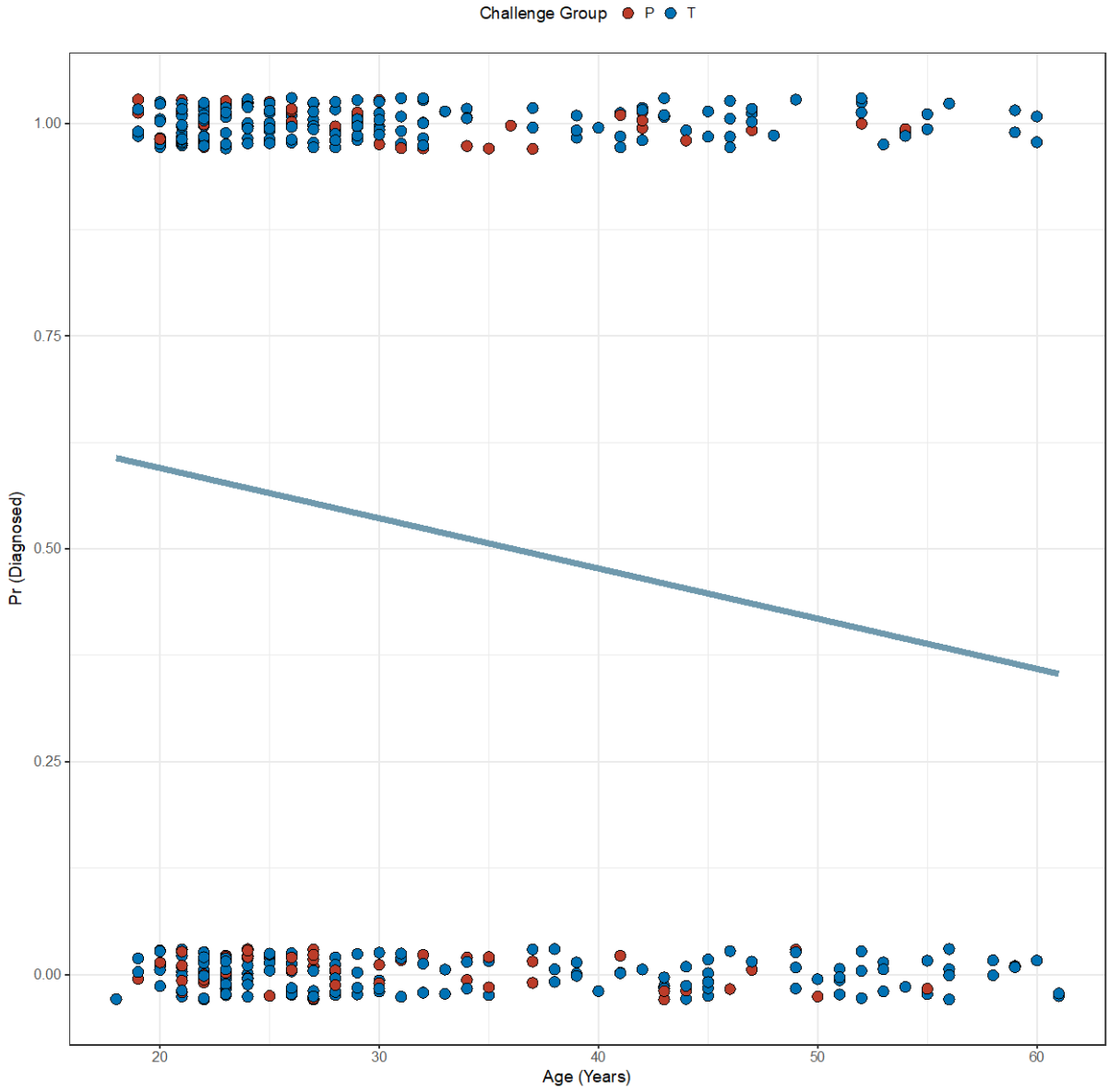
Showing fitted logistic model (glm) and 95% confidence band for all re-challenged participants. Data point coloured according to re-challenge group. The risk of enteric fever diagnosis increases with increasing interval for the homologous re-challenge groups (ST-S [green]T, SPT-SPT[red]), and decreased with increasing interval for the heterologous re-challenge groups (SPT-ST [Blue], ST-SPT [Yellow]).

15.1.2. Combined analysis of previous typhoid and paratyphoid challenge studies

Baseline demographic and anthropometric values were collated for all participants from previous typhoid and paratyphoid challenge studies, enrolled into six studies as previously described (Table 7-3) [126 314 311 209 380 352]. These data were inputted into a multivariate logistic regression

model to identify variables associated with susceptibility or resistance to typhoid or paratyphoid infection.

There was a trend towards decreasing risk of enteric fever diagnosis with increasing age, with a significant reduction in the 50-54 (0.27(0.09-0.77); p=0.017 and 55-59 (0.14(0.02-0.73; p=0.03)age group compared with the reference group (18-24 years -Supplementary Table 6). When analysed as a continuous variable, increasing age was significantly associated with decreasing odds of enteric fever diagnosis, after adjusting for challenge agent, vaccine status, ethnicity and dose (adjusted OR 0.97 [95%CI 0.95-0.99]; p = 0.004 - Supplementary Table 6). Overall, participants who had received any typhoid vaccination were less likely to develop enteric fever as well as those who classified their ethnicity as “white (other)” (Supplementary Table 6).



Supplementary Figure 3 : Probability of diagnosis by age at time of challenge.

Showing fitted logistic model (glm) and 95% confidence band for all challenged participants. Data point coloured according to challenge agent (T= *S.* Typhi, P= *S.* Paratyphi).

Supplementary Table 6 : Probability of enteric fever diagnosis in all participants challenged with wild-type S. Typhi and S. Paratyphi according to baseline participants characteristics.

	Attack Rate	Odds Ratio (95%CI)	Adjusted Odds Ratio (95% CI)	p
Challenge				
S. Typhi	172/310 (55%)	1		
S. Paratyphi	46/99 (46%)	0.71 (0.45-1.13)	0.33(0.15-0.75)	0.009
Sex				
Female	75/152 (49%)	1	1	
Male	143/261 (55%)	1.24 (0.83-1.86)	1.04 (0.60-1.79)	0.88
Age				
18-24	89/156 (57%)	1	1	
25-29	51/90 (56%)	0.98 (0.58-1.66)	1.17 (0.57-2.41)	0.66
30-34	26/46 (57%)	0.98 (0.51-1.91)	0.83 (0.36-1.96)	0.68
35-39	5/15(33%)	0.38 (0.11-1.11)	0.63 (0.11-2.96)	0.56
40-44	20/37 (54%)	0.89 (0.43-1.84)	1.47(0.53-4.28	0.46
45-49	11/21 (52%)	0.83 (0.33-2.10)	0.75(0.22-2.47)	0.63
50-54	9/26 (35%)	0.40(0.16-0.93)	0.27(0.09-0.77)	0.017
55-59	5/17 (29%)	0.31 (0.10-0.90)	0.14(0.02-0.73)	0.03
60+	2/5(40%)	0.50 (0.07-3.11)	-	0.98
Travel History <6 months				
No	128/235 (55%)	1	1	
Yes	66/137 (48%)	0.77 (0.51-1.19)	0.85 (0.47-1.51)	0.242
Ethnicity				
White British	157/284 (55%)	1	1	
Asian (Indian)	1/2(50%)	0.81 (0.03-20.6)	1.32 (0.04-38.6)	0.85
Mixed (Other)	5/13 (38%)	0.51(0.15-1.55)	0.57(0.13-2.29)	0.42
Other_Ethnic_Group	2/6 (33%)	0.40(0.06-2.11)	0.25 (0.03-1.65)	0.16
White_Other	28/63 (44%)	0.61 (0.35-1.05)	0.26 (0.09-0.80)	0.019
Place of Birth				

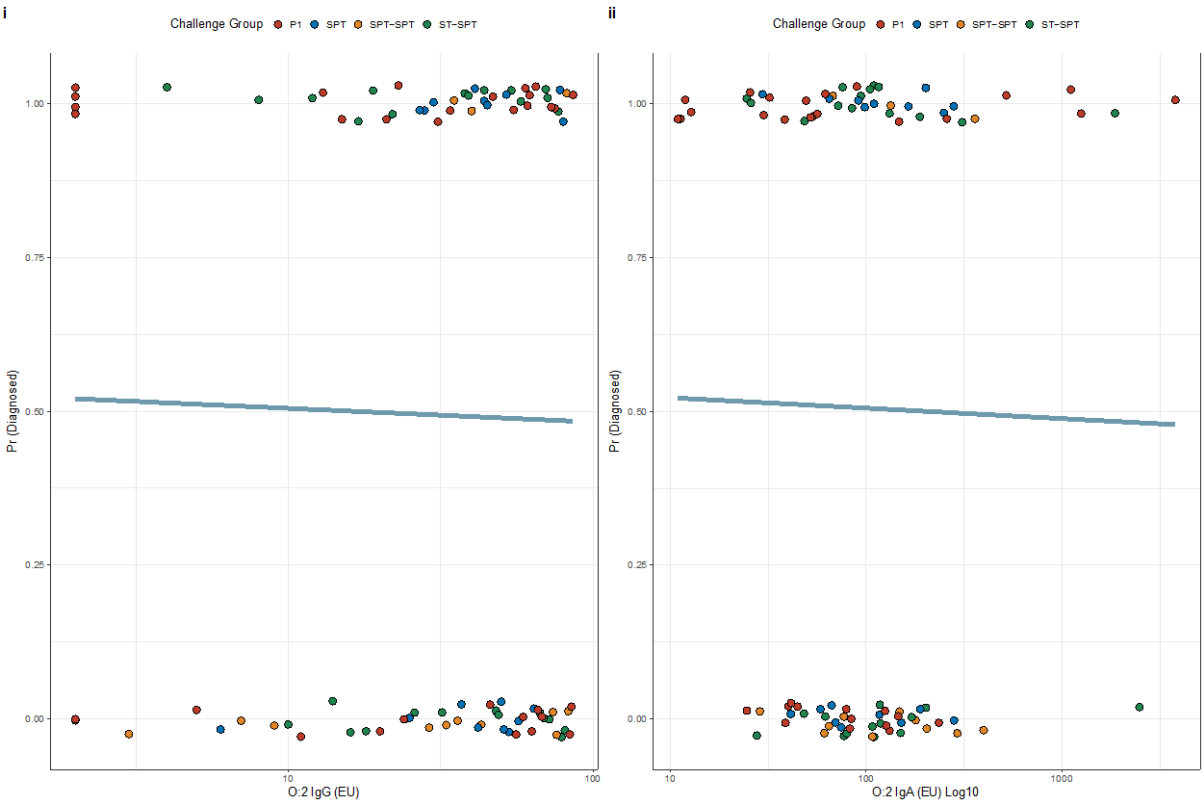
UK	108/277 (39%)	1	1	
Non-UK	34/77 (44%)	0.70 (0.40-1.18)	1.53 (0.57-4.29)	0.4
Previous Exposure				
Infection Naïve	182/336 (54%)	1	1	
SPT-SPT	3/12 (25%)	0.38 (0.1-1.18)	0.45(0.11-1.63)	0.24
SPT-ST	7/10 (70%)	2.12 (0.45-14.9)	0.91(0.16-7.20)	0.91
ST-SPT	14/27(52%)	0.786 (0.35-1.73)	1.04(0.38-2.81)	0.94
ST-ST	12/27 (44%)	0.68(0.30-1.49)	0.36(0.12-1.01)	0.05
Previous Vaccine (Any)				
No	154/277 (55%)	1	1	
Yes	64/136 (47%)	0.71 (0.47-1.07)	0.37 (0.15-0.85)	0.02
Vaccine Type				
None	154/277 (55%)	1		
Ty21a	13/30 (43%)	0.70 (0.32-1.49)		0.353
MO1ZH09	18/31 (58%)	1.03(0.49-2.18)		0.944
Vi-PS	13/35 (37%)	0.59(0.28-1.21)		0.155
Vi-TT	13/37 (35%)	0.63(0.32-1.20)		0.163

15.1.3. Baseline Measures of Humoral Immunity

I next performed a logistic regression model to determine how baseline measures of humoral immunity were associated with risk of enteric fever diagnosis. I began by focussing on baseline anti-O9:LPS IgG/IgA for participants challenged with *S.* Typhi and anti-O2:LPS IgG/IgA for participants challenged with *S*. Paratyphi. IgG and IgA producing memory B-cell measurements (measure by memory B-cell ELISPOT) were also included in the model.

Baseline anti-O:2 IgA and IgG measurements were collated for participants from all participants challenged with *S.* Paratyphi for whom data was available across two studied (n=92). After adjusting for age, study and challenge dose, baseline anti-O:2 IgA and IgG levels by ELISA were not associated with reduced odds of paratyphoid fever (adjusted odds ratio 1.0 [0.99-1.0] p=0.46 and 0.99 [95%CI 0.98-1.01],p =0.93 - Supplementary Figure 4). Baseline anti-O2 IgG/IgA and anti-Ha IgG/IgA producing memory B-cells ELISPOT data (n=48) were also included in the regression analysis and were not associated with risk of paratyphoid diagnosis.

Baseline anti-O9:LPS IgG data were available for 173 participants challenged across three studies. Similar to what was observed in the paratyphoid challenge group, baseline anti-O9:LPS IgG levels were not associated with reduced odds of typhoid fever (adjusted odds ratio 1.0 [0.99-1.0] p=0.26]. Baseline anti-O9 IgG/IgA and anti-Hd IgG/IgA producing memory B-cells ELISPOT data (n=34) were also included in the regression analysis and were not associated with risk of typhoid diagnosis.



Supplementary Figure 4 : Probability of diagnosis by baseline anti-O2 IgG and anti-O2 IgA

Showing fitted logistic model (glm) and 95% confidence band for all challenged participants. Data point coloured according to challenge group (P1/SPT=*S.* Paratyphi naïve, SPT-SPT = Homologous *S.* Paratyphi re-challenge, ST-SPT = Heterologous *S.* Paratyphi re-challenge).

15.2. Discussion

In this section I have described a series of factors associated with susceptibility to enteric fever within the context of the controlled human infection model. In a sub-group analysis of participants undergoing re-challenge, those participants who did no develop disease on primary exposure were significantly less likely to develop disease on re-challenge. This observation remained true after controlling for prior vaccination status in a multivariate analysis. To further characterise host factors associated with susceptibility or resistance to disease, I performed a further multivariate analysis of all participants enrolled into previous typhoid and paratyphoid challenge studies. Prior vaccination status and age were significantly associated with reduced

disease risk, whereas baseline serum antibody and memory B-cell ELISPOT against O:2 and O:9 antigens were not associated with altered risk of disease.

Increasing age was identified as a factor associated with moderate reduced risk of disease within the context of the challenge model. This reflects the distribution of disease by age in field settings[4,7,12], where declining incidence with age is thought to primarily reflect acquisition of immunity with increasing exposure. Whilst there was no correlation between antibody levels at baseline and age in the challenge model (see Figure 8-7), other unmeasured factors might be associated with declining risk with age. In future challenge studies involving randomised groups, consideration could be given to stratification by age.

When baseline measures of anti-O2 IgG/IgA serum antibodies and O2-specific IgG/IgA producing memory B-cells were included in the multivariate model, neither were associated with reduced risk of paratyphoid disease. In previous studies, we have described that individuals who did not develop paratyphoid disease after challenge had significantly higher levels of anti-O2 IgG at baseline than individuals who went on to develop disease[311]. Notably, the leading parenteral paratyphoid vaccine candidates are O2-specific polysaccharide (OSP) conjugates, which (if efficacious) would be predicted to function by targeting surface O2-antigens on *S*. Paratyphi. The apparent lack of association between baseline anti-O2 IgG/IgA and protection from paratyphoid disease in the multivariate model is of uncertain significance to future vaccine development. The absolute level of anti-O2 antibodies – and their functional activity – is likely to differ following vaccination with an OSP conjugate vaccine, compared with those found in naïve individuals. The role of anti-OSP (O2) antibodies in protection against paratyphoid disease could be studied further in field trial or by challenging participants with S. Paratyphi shortly after vaccination with an OSP conjugate.

15.3. Summary

In this section, I have applied data from human challenge studies to identify factors associated with susceptibility or resistance to enteric fever, including age and protection from disease on primary exposure. The ability to measure multiple participant factors at baseline, and combining data from multiple studies, represents an advantage of this approach, which can supplement classical epidemiological studies of enteric fever risk factors.