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**Kinetics of Natural and Acquired Immunity to
Typhoid Fever**

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LIST OF ABBREVIATIONS:

Ab: Antibody

Ag: Antigen

Vi: Virulence factor (of *Salmonella typhi*)

CDC: Centres for Disease Control, USA

DCA: Deoxycholate Citrate Agar

IgG: Immunoglobulin G

IgG1: Immunoglobulin G subclass 1

IgG2: Immunoglobulin G subclass 2

IgG3: Immunoglobulin G subclass 3

IgG4: Immunoglobulin G subclass 4

IgM: Immunoglobulin M

IgA: Immunoglobulin A

IgD: Immunoglobulin D

IgE: Immunoglobulin E

ELISA: Enzyme Linked Immunosorbent Assay

ELISpot: Enzyme Linked Immunospot Assay

PCR: Polymerase Chain Reaction

MDR: Multi-drug Resistant

LDC: Least Developed Countries

GMT: Geometric Mean Titre

GMC: Geometric Mean Concentration

SBA: Serum Bactericidal Assay

OPA: Opsonophagocytic Assay

NAR: Nalidixic Acid Resistant

CFU: Colony Forming Unit

IL-12: Interleukin-12

IFN- γ : Interferon- γ

PBS: Phosphate Buffered Saline

HBSS: Hanks Balanced Salt Solution

mHSA: methylated Human Serum Albumin

O.D: Optical Density

LB agar: Luria-Bertani agar

MH broth: Muller Hinton broth

TM Blue: Tetramethylbenzidine reagent

XLD: Xylose lysine deoxycholate agar (XLD agar)

PBMC: Peripheral blood mononuclear cells

XLT: Xylose Lisina Tergitol-4

UN: United Nations

WHO: World Health Organization

nm: nanometre

μ l: micro litre

μ g: micro gram

ml: milli litre

μ l: micro litre

M: Molar

“Before God and in the eyes of decent men, my name is Mary Mallon. I was christened and baptised Mary Mallon. I lived a decent, upright life under the name of Mary Mallon until I was seized. (Then I was) locked up in a pest-house and rechristened ‘Typhoid Mary’ the name by which the world has ever since known me.”

Mary Mallon, 1915

Chapter 1 INTRODUCTION

1.1. *Salmonella enterica* subspecies *enterica* serovar Typhi: Nomenclature & Classification

Salmonella enterica subspecies *enterica* serovar Typhi is the etiological agent of typhoid fever. This pathogen belongs to the genus *Salmonellae*, which includes other Gram negative bacilli that cause a group of human diseases known as salmonellosis. The genus is named after Daniel Elmer Salmon, an American veterinary pathologist, who discovered the genus in 1885. The most commonly accepted nomenclature for the classification of *Salmonella* consists of two species within this genus: *Salmonella bongori* and *Salmonella enterica* (previously denoted as *Salmonella choleraesuis*)¹. The latter is responsible for all *Salmonella* infections in mammals and consists of six subspecies: *Salmonella enterica* subspecies *enterica*, *Salmonella enterica* subspecies *salamae*, *Salmonella enterica* subspecies *arizonae*, *Salmonella enterica* subspecies *diarizonae*, *Salmonella enterica* subspecies *houtenae*, *Salmonella enterica* subspecies *indica*^{2,3}. Within each of these six subspecies there exists multiple serovars (serotypes) which, though evolutionarily similar, are found in different environments and are responsible for a spectrum of human and other mammalian diseases. The serovar Typhi and a closely related bacterium-serovar Paratyphi comes under the subspecies *enterica*. Conventionally, for ease of reference, a particular bacteria belonging to this genus is identified only by its genus and serovar nomenclature. Therefore *Salmonella enterica* subspecies *enterica* serovar Typhi is commonly referred to as *Salmonella typhi* (*S. typhi*). The taxonomical classification of *S. typhi* is illustrated in Table 1.1. The new terminology has replaced previous notations of typhoid bacillus, bacillus typhi, bacillus typhosa, *Eberthella typhi* and *Salmonella typhosa*.

Kingdom:	Bacteria
	↓
Phylum:	Proteobacteria
	↓
Class:	Gamma Proteobacteria
	↓
Order:	Enterobacteriales
	↓
Family:	Enterobacteriaceae
	↓
Genus:	<i>Salmonella</i>
	↓
Species:	<i>enterica</i>
	↓
Subspecies:	<i>enterica</i>
	↓
Serovar/Serotype:	Typhi
	↓
Strain (wild):	CT18 and Ty2

Table 1.1. Taxonomical classification of *Salmonella typhi* ⁴

For clinical classification of the *Salmonella* genus, the Kaufman-White serological classification is followed ⁵. Serovars of subspecies *enterica* are given a name, whereas the remaining serovars are designated by only their antigenic formula. The subspecies of *Salmonella enterica* are represented by Roman numerals in the scheme:

- . *S. enterica* subsp. *enterica* = I
- . *S. enterica* subsp. *salamae* = II
- . *S. enterica* subsp. *arizonae* = IIIa
- . *S. enterica* subsp. *diarizonae* = IIIb
- . *S. enterica* subsp. *houtenae* = IV
- . *S. enterica* subsp. *indica* = VI

Salmonella bongori is represented by the Roman numeral V.

The main division is first by the somatic 'O' antigen that relates to a lipopolysaccharide (LPS) associated with the bacterial polysaccharide and then by flagellar 'H' antigens. H antigens are further divided into phase 1 and phase 2. A full description of a *S. typhi* isolate is denoted by the O antigens, Vi (virulence factor associated with the polysaccharide capsule), H antigen phase 1, H antigen phase 2. Different antigens are numbered and each serotype is given an antigenic formula and classified into a group. Clinically relevant serovars are those that include the following antigens:

- O antigens: 2, 4, 6, 7, 8, 9 and 3, 10
- phase 1 H antigens: 1 2 3 4 5 6 7
- phase 2 H antigens: a b c d E G i r

1.2. Historical perspective

A translation of Thucydides' description of the Plague of Athens that devastated the city between 430-426 BC and heralded the end of the Golden Age of Athens reports thus ⁶:

“As a rule, however, there was no ostensible cause; but people in good health were all of a sudden attacked by violent heats in the head, and redness and inflammation in the eyes, the inward parts, such as the throat or tongue, becoming bloody and emitting an unnatural and fetid breath.

These symptoms were followed by sneezing and hoarseness, after which the pain soon reached the chest, and produced a hard cough. When it fixed in the stomach, it upset it; and discharges of bile of every kind named by physicians ensued, accompanied by very great distress.

In most cases also an ineffectual retching followed, producing violent spasms, which in some cases ceased soon after, in others much later.

Externally the body was not very hot to the touch, nor pale in its appearance, but reddish, livid, and breaking out into small pustules and ulcers. But internally it burned so that the patient could not bear to have on him clothing or linen even of the very lightest description; or indeed to be otherwise than stark naked. What they would have liked best would have been to throw themselves into cold water; as indeed was done by some of the neglected sick, who plunged into the rain-tanks in their agonies of unquenchable thirst; though it made no difference whether they drank little or much.

Besides this, the miserable feeling of not being able to rest or sleep never ceased to torment them. The body meanwhile did not waste away so long as the distemper was at its height, but held out to a marvel against its ravages; so that when they succumbed, as in most cases, on the seventh or eighth day to the internal inflammation, they had still some strength in them. But if they passed this stage, and the disease descended further into the bowels, inducing a violent ulceration there accompanied by severe diarrhoea, this brought on a weakness which was generally fatal.

For the disorder first settled in the head, ran its course from thence through the whole of the body, and even where it did not prove mortal, it still left its mark on the extremities; for it settled in the privy parts, the fingers and the toes, and many escaped with the loss of these, some

too with that of their eyes. Others again were seized with an entire loss of memory on their first recovery, and did not know either themselves or their friends."

The detailed but non-specific description have led to implications of multiple organisms including Ebola virus, *Rickettsia prowazekii*, *Bacillus anthracis*, *Yersinia pestis*, *Mycobacterium tuberculosis*, measles virus etc as a cause of these symptoms. The description of 'typhoid vigil' or 'muttering delirium' (violent heats in the head), weakness, diarrhoea (discharges of bile), nausea (ineffectual retching), 'rose spots' (small pustules), apathy and intestinal perforations (violent ulceration) were highly suggestive of typhoid fever. Recently a study using a PCR probe, for multiple microbiological agents, on DNA samples from dental pulp of skeletal material of corpses unearthed in the Kerameikos ancient cemetery and dated to around 430 BC demonstrated DNA sequences identical to that of *S. typhi* confirming the etiologic origin of the Athens plague ⁷. Suggestions have been made for further sequencing to determine if the causative agent was actually a precursor of *S. typhi* that could infect animals too, unlike *S. typhi* which is human adapted, because Thucydides' description included both humans and animals ⁸.

Alexander the Great, in his last days in 323 BC, had weakness, chills, recurring fever with increasing intensity, sweats and pain in the right upper abdominal pain which was initially tender to palpation ⁹. Preliminary diagnoses of alcoholic hepatitis, acute necrotizing pancreatitis, methanol toxicity, lead or arsenic poisoning, yellow fever and hepatitis were ruled out on the basis of negative symptoms. Osler's description of patients with untreated typhoid fever ¹⁰ suggests the diagnosis of typhoid fever complicated by bowel perforation and ascending paralysis possibly Guillain-Barre syndrome.

Perhaps the best recalled character with respect to typhoid fever is Mary Mallon, a single, lower-class, Catholic, Irish-American immigrant, surviving as a cook in the early twentieth century in New York, a Protestant-dominated city which was controlled by a quasi-police health authority; all of which were apparently factors contributing to her total forcible isolation for 26 years ¹¹. This was despite the fact that several male typhoid-carrier food handlers, who had caused more cases than the 54 with four deaths in nine epidemics in Mallon's case, were rehabilitated with different job training ¹².

More recently, *S. typhi* has been placed in the biological hazard group 3 on the Schedule 5 list (Anti-terrorism, Crime and Security Act 2001) in the UK ¹³. Similarly, in the US, *S. typhi* is categorised as a CDC Category B bioterrorism agent (defined as moderately easy to disseminate but with low mortality rates) ¹⁴. These followed at least two instances of bioterrorism attempts with *S. typhi*. The first was in 1939 when the biological warfare unit 731 of the Imperial Japanese Army unsuccessfully contaminated the rivers on the border of Chinese Manchuria and Soviet Union with *S. typhi* ¹⁵. The second attempt was in September 1984 when the Rajneesh cult in Oregon, USA contaminated salad bars in local restaurants with the closely related *S. typhimurium* in order to prevent the population from voting in a local election ¹⁶. Although 750 people were affected, no mortality was reported and as a consequence of the crime, the cult collapsed.

1.3. *Salmonella typhi* morphological characteristics and growth conditions

Morphologically *S. typhi*, like other bacteria in the genus *Salmonella*, is a Gram negative bacillus (Figure 1.3a). Electron microscopy reveals an encapsulated, flagellated and fimbriated pathogen (Figure 1.3b & c). The schematic structure of a typical Gram negative cell wall is shown in Figure 1.3d.

The most common selective culture media used for the growth and detection of *Salmonella* are MacConkey agar, Xylose lysine deoxycholate agar (XLD agar), Xylose Lysine Tergitol-4 (XLT) and Deoxycholate Citrate Agar (DCA). MacConkey agar is prepared from bile salts and crystal violet (to inhibit Gram positive bacteria), neutral red dye (to detect lactose fermenting bacteria), lactose and peptone. Unlike lactose fermenting genus like *Escherichia*, *Klebsiella* and *Enterobacter* which produce pink colonies on MacConkey agar, *Salmonella* and *Shigella* are non-lactose fermenters and therefore exhibit white colonies. XLD agar is another selective media that can be utilised to differentiate between *Salmonella* (red colonies with a black centre) and *Shigella* species (red colonies). Luria Bertani Agar (LB agar), consisting of sodium chloride, tryptone and peptone is a common non-selective agar for the growth of *Salmonella* species.

S. typhi, is a facultatively anaerobic bacterium capable of adapting to the anaerobic conditions in the small intestine. An increase in virulence, by means of increased hydrophobicity leading to increased adhesiveness to the intestinal mucosa and an increase in OMP expression particularly stress proteins, have been observed under anaerobic conditions ¹⁷.

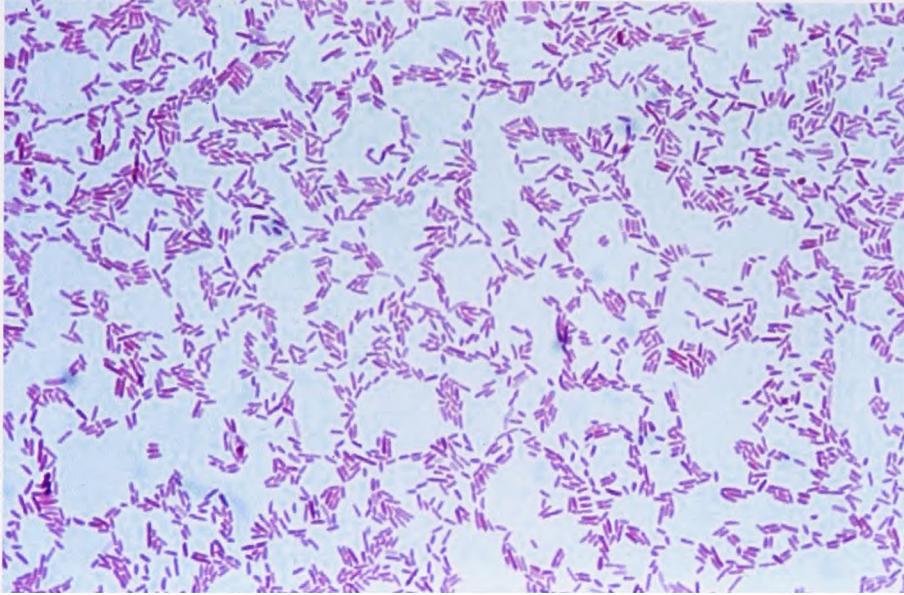


Figure 1.3a: Gram stain of *S. typhi* showing Gram negative rods



Figure 1.3b: Electron microscope image of *S. typhi* demonstrating flagellae and capsule ¹⁸

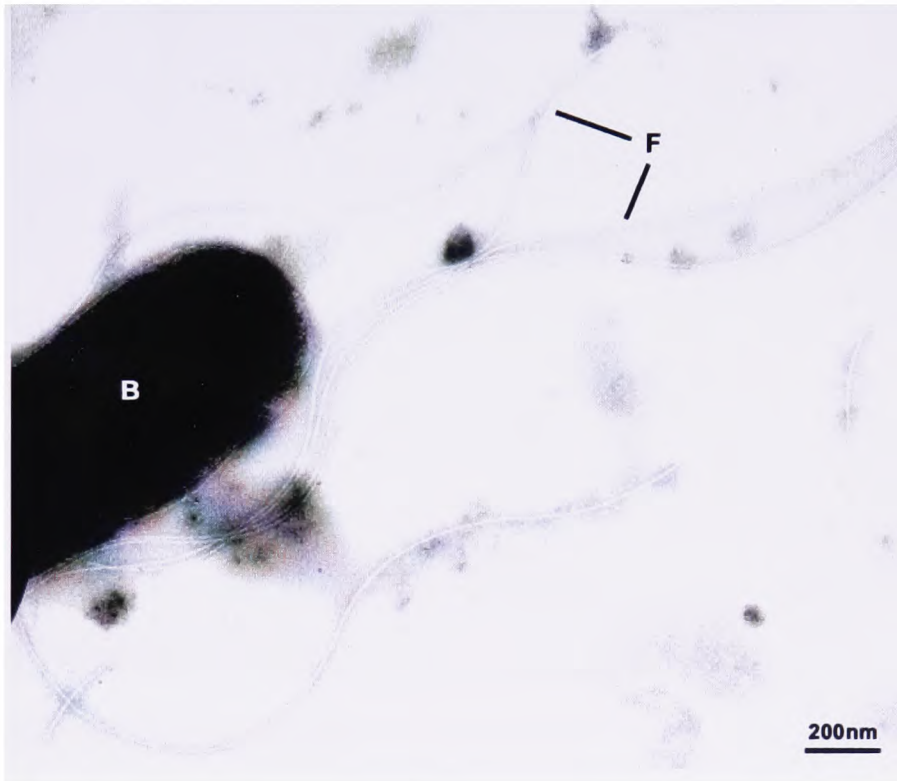


Figure 1.3c: Electron microscope image of *S. paratyphi* preparation demonstrating flagellae

Legend: B: bacteria, F: flagella, bar indicates 200nm
(EM photo by Dr. D. Ferguson, University of Oxford)

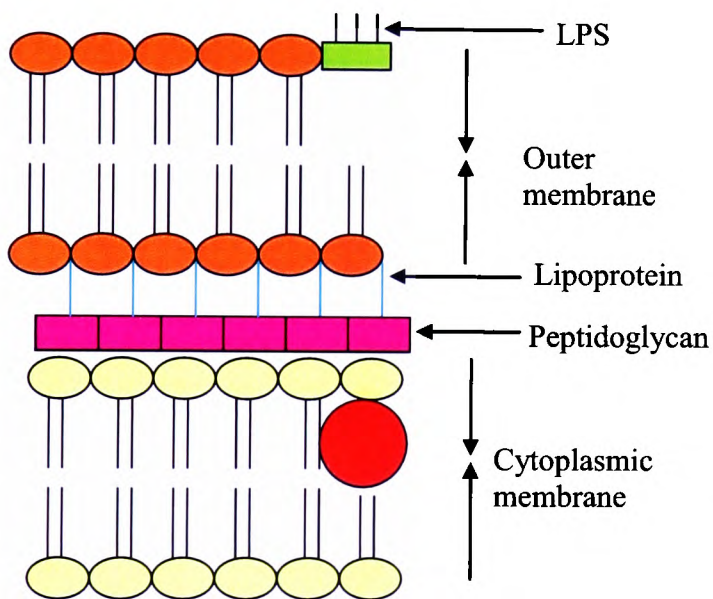


Figure 1.3d: Schematic diagram of a Gram negative cell wall illustrating the four layers of bacterial wall structures

1.4. Vi polysaccharide capsule

The capsule of *S. typhi* is a linear homopolymer of alpha (1-4) D-GalpANAc variably O acetylated at the C-3 position (Fig 1.4a) ¹⁹. Partial O deacetylation is reported to slightly increase immunogenicity while complete O deacetylation eliminates the immunogenicity of Vi ¹⁹. In the *S. typhi* serotype, the polysaccharide capsule is called the “virulence” (Vi) factor because it was historically considered important for the survival and pathogenicity of the bacteria. Early experiments demonstrated that the Vi polysaccharide from *S. typhi* was more potent at inducing antibody responses in mice as compared to *Citrobacter freundii* while less toxic than the whole cell vaccine ²⁰. Besides *S. typhi*, *S. paratyphi C*, *C. freundii* and *S. dublin* are capable of producing the Vi polysaccharide ^{21, 22, 23}. These species are characterised by the presence of a *viaB* locus in their genomes ²⁴. The Vi polysaccharide expressed by these species, while immunologically indistinct, have subtle differences in their structures, expression levels and sequences ²⁴. In view of the difficulty in the artificial synthesis of complex carbohydrates such as the ViPS, attempts were made to identify peptide mimotopes (epitope analogues) of the ViPS by phage display technology ^{25, 26}. 75% of the phage clones harboured the peptide sequence TSHHDSHGLHRV while the other clones harboured ENHSPVNIAHKL, obtained by panning sequences against a ViPS monoclonal antibody. These sequences were confirmed by panning against pooled patient sera; thereby offering the possibility of a peptide based diagnostic test and vaccine.

In *S. typhi*, *viaB* consists of 10 genes; 5 coding for the synthesis of the polysaccharide (*tviA*, *tviB*, *tviC*, *tviD* and *tviE*) and 5 coding for the polysaccharide transportation proteins (*vexA*, *vexB*, *vexC*, *vexD*, *vexE*) ^{27, 28}. The *viaB* in *S. typhi* is located on the Specific Pathogenicity Island-7 (SPI-7) ²⁷. It is argued that, on the basis of functional and bioinformatic analysis, that

SPI-7 has a mosaic structure and may have evolved as a consequence of several independent insertion events ²⁹. Sequence analysis of the SPI-7 region from *S. typhi* strain CT18 revealed significant synteny with a variety of saprophytic bacteria and phytobacteria, including *Pseudomonas aeruginosa* and *Xanthomonas axonopodis*, suggesting that SPI-7 may be a mobile element, such as a conjugative transposon or an integrated plasmid remnant ²⁹.

In addition to the genes on *viaB*, 3 other genes are thought to regulate the production of Vi polysaccharide: *rscB* and *rscC*, together comprising the *viaA* ^{30, 31} and the two-component regulator *ompR-envZ* ^{32, 33}. A defined deletion of 517 base pairs within the open reading frame of the *ompR* gene resulted in the *S. typhi ompR* mutants displaying a marked decrease in *ompC* and *ompF* porin expression and non-agglutination with Vi antiserum. Combined with the observation that the levels of Vi synthesis were sensitive to different concentrations of sodium chloride present in the growth medium, it was postulated that environmental osmolarity is a determinant of the *ompR* operon regulation of ViPS ³⁴.

The ViPS is believed to play an important role in the protection of *S. typhi* from multiple host defence mechanisms as follows: 1. Capsulate strains are more virulent than acapsulate strains ³⁵. 2. The expression of ViPS is associated with resistance to the action of the anti-O antibody and to phagocytosis and complement mediated killing; both of which are mediated by anti-Vi antibodies ³⁶. 3. Vi has been demonstrated to enhance survival of *S. typhi* in cultured macrophages in vitro ³⁷. 4. Capsulate *S. typhi* suppresses the production of TNF- α in macrophage cell lines in contrast to acapsulate strains that induced high levels of secretion ³⁸. 5. Capsulate *S. typhi* does not elicit the expression of pro-inflammatory, neutrophil chemoattractant IL-8 expression in human intestinal mucosa in contrast to mutant strains without capsular expression or *S. typhimurium* ³⁹. This is achieved by *tviaA* mediated

suppression of flagellin secretion, through TLR-5 receptors, inside human colonic epithelial cells⁴⁰. 6. *S. typhimurium* with a *viaB* insert, prevented the recognition of LPS via TLR-4 mediation in mice as evidenced by the expression of TNF-alpha and inducible nitric oxide synthase (iNOS) in contrast to the response to native *S. typhimurium*⁴¹. 7. ViPS is known to closely associate with the prohibitin family of molecules, leading to reduced IL-8 response, through reduced extracellular signal related kinase phosphorylation⁴². 8. In bovine ligated ileal loop model and streptomycin pre-treated mouse model, there was lower expression of chemokine growth related oncogene alpha (GRO α) and IL-17 in contrast to *S. typhi viaB* mutant⁴³.

Once thought to be a characteristic feature of *S. typhi*, the Vi polysaccharide was exploited in diagnostic and vaccine strategies⁴⁴⁻⁵². A rapid diagnostic test using agglutination with Vi antisera is based on the assumption that all *typhi* strains express the Vi polysaccharide⁵³. The current injectable Vi vaccine is based on the T-cell independent immunogenic characteristics of the polysaccharide^{54, 55} while the Vi conjugated to the inactivated Pseudomonas exotoxin A protein confers T-cell dependant properties on the same⁵⁶.

Reports of Vi negative isolates of *S. typhi* date back to the 1960s⁵⁷. In 1988, Hone and colleagues constructed a *galE*, Vi negative, mutant of *S. typhi* Ty2 which caused culture positive bacteraemia in two out of four human volunteers who had developed typhoid like symptoms after oral ingestion⁵⁸. Following that experiment which demonstrated that Vi negative mutants of *S. typhi* can indeed cause typhoid like illness, there has been continued interest in the existence of acapsulate *S. typhi* due to its implications for the use of Vi based diagnostic agglutination tests and Vi based vaccines. Reports of disease caused by Vi negative *S. typhi* were initially dismissed as improper identification as a result of poor microbiological

techniques or multiple sub-culturing or storage of isolates on nutrient media; all of which can cause the down-regulation of Vi expression resulting in a negative agglutination test ^{59, 60}. Spontaneous precise excision of the SPI-7 that contains genes for Vi synthesis and transportation, type IVB pili, putative conjugal transfer and *sopE* bacteriophage was postulated as a mechanism for the non-expression of capsule due to storage ⁶¹.

However following a 2000 report of a typhoid outbreak in India caused by acapsulate *S. typhi* ⁶², interest was renewed in the existence of acapsulate strains in Asia. Studies have demonstrated the absence of genes encoding the Vi polysaccharide in stored isolates and patient isolates in Pakistan ^{63, 71}. Based on these episodes, some experts have suggested a positive selection pressure on *S. typhi*, especially in the era of post-Vi vaccine introduction, to down-regulate or even abolish the expression of the Vi subunit. Adding to this hypothesis is evidence from laboratory experiments that although the Vi polysaccharide aids in the survival of *S. typhi* by the eight mechanisms described above, the rates of invasion into macrophages are comparable for both Vi positive and negative strains of *S. typhi* ^{64, 65}. More recently there are reports of Vi suppressed *S. typhi*, cultured in high osmolarity, being hyperinvasive in human tissues ^{66, 67}. Vi production may not be essential for the infection process in humans as evidenced by the disease caused and immune response induced by the Vi negative vaccine strains. This hypothesis could have implications not only for the implementation of the Vi polysaccharide vaccine in immunisation schedules in typhoid endemic countries but also for the future of the Vi polysaccharide protein conjugate vaccine that is undergoing clinical trials for licensure in paediatric populations ^{68, 69}.

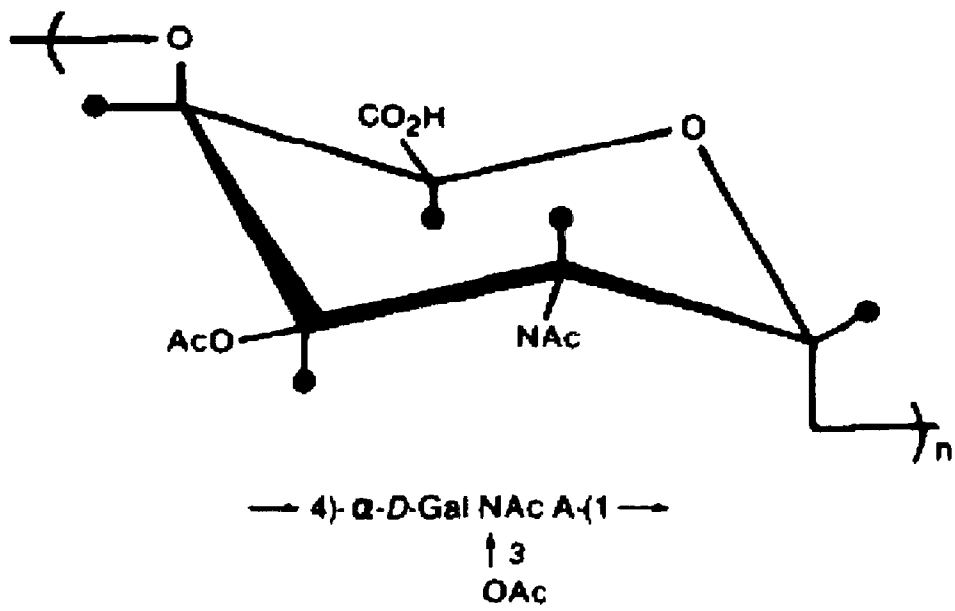


Figure 1.4: Schematic structure of the repeating unit of the typhoid capsular polysaccharide

1.5. Epidemiology of Typhoid fever

S. typhi is responsible for 17 million cases and 200,000 deaths annually ⁷⁰. These figures are conservative estimates as they exclude patients in regions where diagnostic bacterial culture facilities are unavailable and also patients with asymptomatic bacteraemia. These figures were revised downwards from a rate of 21.6 million and 600,000 deaths per year on the basis of regional data extrapolations ⁷¹. The highest incidence of typhoid fever is found in South Asia, Southeast Asia and Southern and Central Africa. These areas report an incidence of >100 cases per 100,000 population per year. Urban areas in endemic countries report more cases than rural areas ⁷². This is probably due to overcrowding and lower standards of water hygiene and human sewage disposal witnessed in urban slums in developing countries as a result of urban migration. The possibility of a better disease reporting system in urban areas accounting for higher disease rates cannot be discounted. Moderate incidence, defined as 10-100 cases per 100,000 people per year is found in other parts of Africa and Asia, Eastern Europe, Middle East and Latin America. The rest of the world reports a low incidence of typhoid fever (<10 cases per 100,000 population per year) ⁷². However sporadic outbreaks are not unknown and typhoid fever is important for travellers (Incidence of ~3 to 30 per 100,000 travellers) from these areas who visit endemic parts of the world ^{73, 74}. Figure 1.5a demonstrates the risk areas for acquiring typhoid fever. Figure 1.5b shows the number of enteric fever cases in the UK in the last two decades. Like in the UK, a majority of disease cases in industrialised countries are in travellers returning from visits to endemic regions or their contacts. Occasional cases are reported in laboratory personnel handling *S. typhi* ⁷⁵.

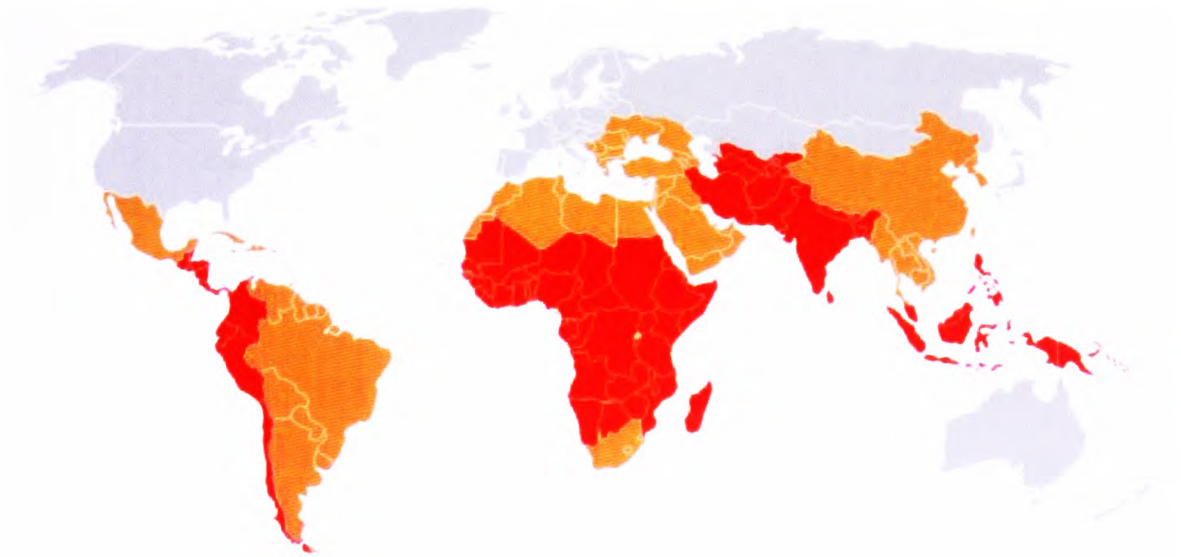


Figure 1.5a: Risk areas for typhoid fever. Areas in red represent the highest incidence areas with orange denoting moderate incidence areas ⁷⁶.

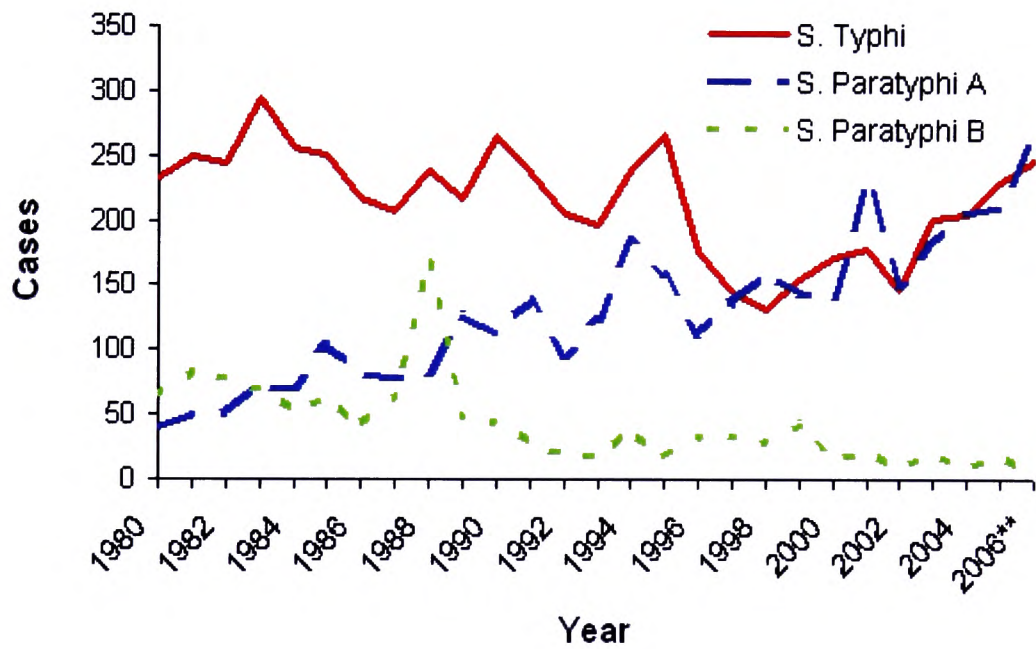


Figure 1.5b: Incidence of enteric fevers in the UK from 1980 to 2006 showing a ‘steady state’ of incidence of typhoid fever and an increasing incidence of paratyphoid A ⁷⁷.

Along with paratyphoid fever, a clinically indistinguishable disease caused by *S. paratyphi* (mostly *S. paratyphi A* and less commonly by *S. paratyphi B* (Schottmulleri) and *S. paratyphi C* (Hirschfeldii), a closely related serovar, typhoid fever is categorised under a group of diseases called enteric fevers^{78, 79}. These are characterised by a faeco-oral route of transmission through infected food or water. Because it is a food and water borne disease, poor standards of water supply, human waste disposal and sewage systems in developing countries are associated with risk of typhoid fever. Increasing standards of living in once endemic countries have now contributed to near eradication of typhoid fever in South-east Asia. Along with shigellosis and cholera that similarly affects young populations in developing and least developed countries (LDC), enteric fevers are classified under Diseases of the Most Impoverished (DOMI). Unlike high profile infectious diseases like tuberculosis, malaria and HIV, DOMI are often neglected not only in terms of defining the host and environmental determinants of disease, pathogenesis and micro and macro distribution but also in terms of funding for its preventive and curative measures. Reasons for the latter include these being outside the research horizon of public research funding bodies because of its near-eradication in industrialised countries and a lack of private entity investment mainly because of a perceived lack of an economic market for products targeting these diseases.

Conventionally thought to be a disease of school children and young adults there is increasing recognition of the occurrence of typhoid in infants and young children in endemic countries^{80, 81, 82} (Figure 1.5c). However a majority of cases are still seen in the 5 to 19 year age group (Table 1.5).

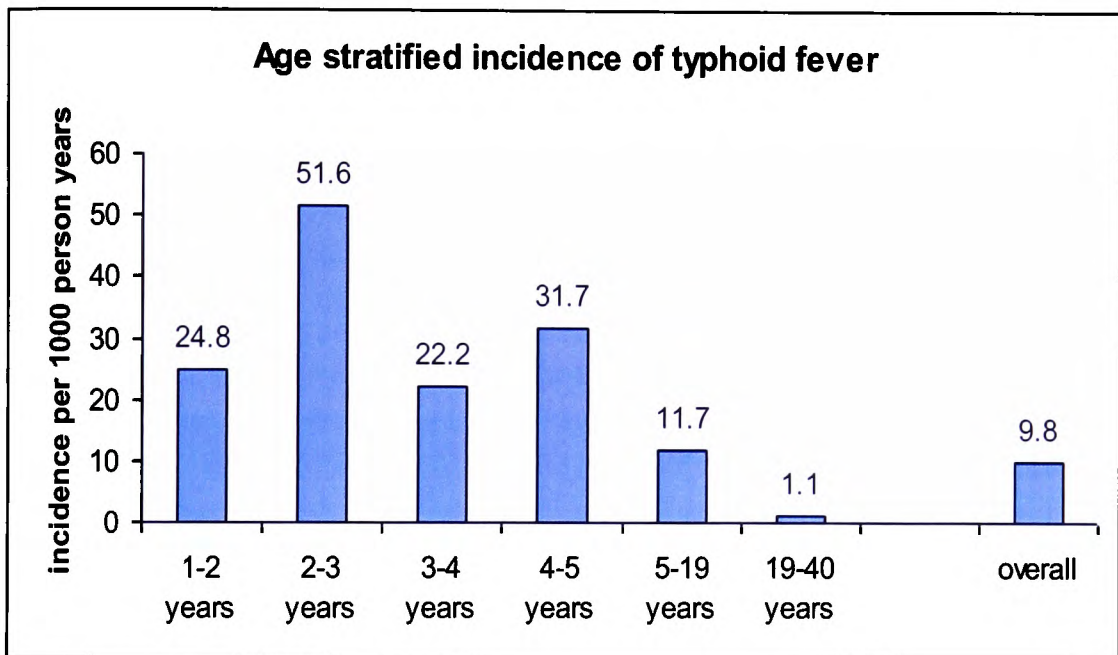


Figure 1.5c: Age stratified incidence of typhoid fever in an urban slum population in New Delhi ⁸⁰.

	Type of surveillance	Age (years)	Number	Incidence per 100,000 per year
Urban				
Indonesia	Passive	3-6	1592	1307
		7-19	4711	1172
India	Active	2-44	3965	182
		0-4	1027	2730
		5-19	2743	1170
		20-40	2684	110
Rural				
Vietnam	Passive	2-4	not reported	358
		5-9		531
		10-14		429
		15-19		153
		20-29		149
		30-39		51
Vietnam	Active	2-4	6017	414

Table 1.5: Age specific incidence of culture-proven typhoid fever from population based studies demonstrating highest incidence in population below five ⁷²

Risk factors for acquiring typhoid fever in endemic countries include drinking water from a contaminated source ⁸³, eating ice cream, having flavoured drinks or food from street vendors, poor housing ⁸⁴, eating raw fruits and vegetables grown in fields fertilised with sewage, a history of contact with patients before onset of illness, not washing hands with soap ⁸⁵, low education ⁸⁶, drinking unboiled water at home and eating papaya ⁸⁷. Chronic infection with the latter reduces gastric acidity and enhances the survival of *S. typhi* in the gastrointestinal tract ⁸⁸. It has been theorised that factors within the household i.e. recent typhoid fever in the household, sharing food from the same plate, no toilet in the household along with other factors like being female, young and using ice cubes are risk factors for typhoid fever in contrast to factors external to the household including consumption of food from vendors and flooding which are risk factors for paratyphoid fever ⁸⁵.

Instances of *S. typhi* isolated from HIV infected patients are infrequent and anecdotal ⁸⁹. However, in this population, there is a well documented risk of acquiring non-typhoidal salmonellosis including recurrent bacteraemia even in the era of HAART ^{90,91}. Despite the ViPS of *S. typhi* being known to be a T-cell independent antigen, antibody responses in HIV infected individuals are reported to be significantly lower than in healthy controls ⁹².

1.6. Typhoid fever: Clinical manifestations

Typhoid fever is exclusively found in humans, unlike salmonellosis caused by other species of the *Salmonella* genus. The incubation period following exposure to the onset of clinical symptoms is typically one week. This period could vary from 5 days to 9 days depending on the size of the ingested inoculum⁹³. The variability in the signs and symptoms makes the clinical diagnosis of typhoid fever a dilemma. Disease manifestations vary from mild infection which presents with low fever to life-threatening bacteraemia and death. Most common symptoms include mild to moderate fever lasting more than three days, generalised myalgia, headache, malaise, nausea, anorexia, vomiting, dry cough and constipation. Diarrhoea is more common in younger children and in HIV infected individuals. Progressive increase in the intensity and duration of fever is typically described as a step-ladder pattern although this is not commonly seen in clinical practice (Figure 1.6a). Signs on clinical examination could include a tender abdomen, hepatomegaly and splenomegaly. Occasionally central coating of the tongue and crepitations and rhonchi on chest auscultation are observed. Rarely pathognomonic 'rose spots' are seen (Figure 1.6b). Rose spots are small erythematous maculopapular rashes seen on the trunk, arms and legs seen in up to 25% of patients in the first week of fever. Relative bradycardia, at the height of fever, although inconsistent, is an indicator of typhoid fever. Differential diagnosis of typhoid fever in a tropical area or in a returning traveller includes malaria, viral fever, anicteric hepatitis, leptospirosis and dengue fever. Elevated levels of serum bilirubin and serum alanine transaminase in paediatric typhoid fever make typhoid hepatitis a differential diagnosis in a patient with fever and jaundice⁹⁴. Common clinical features of typhoid fever are shown in Table 1.6.

Neonatal typhoid is acquired as a result of vertical transmission from an infected mother. Clinical presentations include fever, vomiting, diarrhoea, abdominal distension and seizures usually within three days of delivery. Typhoid infection is now considered to be as severe in children as in adults, contributing to significant morbidity in both groups. Factors that affect the severity of disease in an adult include duration of fever before therapy, choice of antimicrobial drugs, virulence of the infective strain, inoculum size, host factors (such as AIDS and immunosuppressive conditions), HLA type and antacid consumption ⁷².

Chronic carriage

Chronic carriage is defined as excretion of *S. typhi* in stool for more than one year after infection or sub-clinical infection. The risk is higher in women, elderly and patients with schistosomiasis, cholelithiasis, carcinoma of the gall bladder and other gastro-intestinal malignancies ¹²⁶. Though a majority of chronic carriers are asymptomatic, they are responsible for contributing to the 'steady-state' of high infection rates in endemic regions.

MILD TYPHOID FEVER.

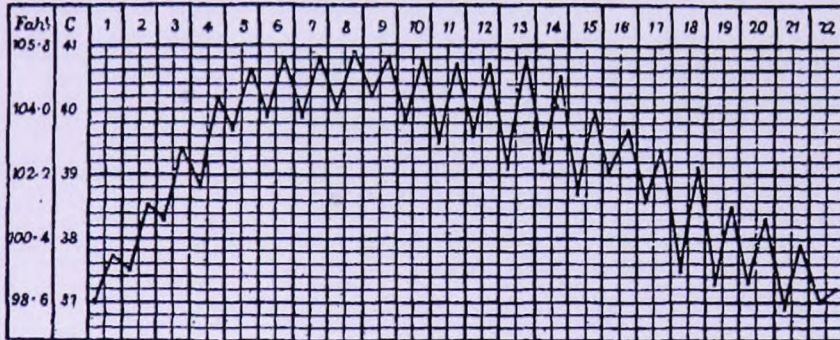


Fig. 3. Temperature chart of Typhoid Fever from Edward Seguin, *Medical thermometry and human temperature* (New York, 1876), p. 113. Although Seguin, who in 1871 had translated and edited an abridged version of Wunderlich's book, prepared a new chart of typhoid fever for his 1876 book, the result is so similar to Wunderlich's original chart that he might well have used it. The similarity of the two charts illustrates the consistency of pattern of typhoid fever.

Figure 1.6a: Graphical representation of fever pattern in typhoid fever illustrating the 'Step ladder' increase

	Hospital based patients (n=1158)	Community based cohort (n=340)
High grade fever	1044 (95)	338 (99)
Anorexia	811 (70)	11 (3)
Vomiting	451 (39)	43 (13)
Hepatomegaly	471 (41)	68 (20)
Diarrhoea	406 (35)	26 (8)
Toxicity	377 (33)	1 (0.3)
Abdominal pain	320 (28)	65 (19)
Splenomegaly	226 (20)	17 (5)
Constipation	127 (11)	1 (0.3)
Headache	138 (12)	26 (8)
Jaundice	23 (2)	0
Obtundation	23 (2)	1 (0.3)
Ileus	12 (1)	1 (0.3)
Intestinal perforation	58 (0.5)	1 (0.3)
Myalgia	174 (15)	15 (4.4)

Table 1.6 Common clinical features of typhoid fever in childhood in hospital and community settings in Karachi, Pakistan. Values are numbers (percentages) ⁷¹



Fig 1.6b: Rose spots in a typhoid fever patient ¹⁰²

1.7. Complications

Important complications of typhoid fever are summarised in Table. Three most common complications of typhoid fever are lower gastrointestinal bleeding, intestinal perforation and neurological complications ⁹⁵. The most common sites for gastrointestinal bleeding are the terminal ileum, ileocaecal valve, ascending colon and the descending colon ⁹⁶. Bleeding is usually a result of erosion of the infected lymph nodes (Peyer's patches), possibly as a result of the inflammation elicited by the mucosal immune response, and presents with a rapid fall in temperature which later rises with the onset of peritonitis. A rapid fall in blood pressure may also be suggestive of perforation and bleeding. Multiple punched out lesions with raised margins are seen on colonoscopy (Figure 1.7)

Intestinal perforations, most commonly in the ileum, occurs late during the course of disease and are associated with being male, leucopenic, having inadequate treatment and having a short duration of symptoms ⁹⁷. A study comparing three operative techniques (simple closure, wedge excision and anastomosis or segmental resection and anastomosis) demonstrated that the latter had the best success rate as evidenced by risk of re-perforation and mortality rate being zero and nine of 25 patients respectively in contrast to two and 13 of 21 patients respectively for wedge excision ⁹⁸.

Neurological manifestations of typhoid fever include convulsions in young children, typhoid meningitis, encephalomyelitis, Guillain-Barre Syndrome and cranial or peripheral neuritis in adults. 'Muttering delirium' or 'coma vigil' are neuropsychiatric features of severe disease. Rare complications include disseminated intravascular coagulation, pneumonia, multiple organ dysfunction syndrome, haemophagocytic syndrome, haemolytic uraemic syndrome, pseudotumor cerebri, glomerulonephritis, pyelonephritis, endocarditis and pericarditis ^{99, 100}.



Figure 1.7: Typhoid ulcers of the intestine ¹⁰¹

1.8. Laboratory Diagnosis

The gold standard for the diagnosis of typhoid fever is growth in culture of *S. typhi*. Specimen for culture could be blood, bone marrow, stool, urine, duodenal fluid and tissue from rose spots. With standard broth techniques for blood samples, *S. typhi* is isolated from 30-90% of clinical typhoid cases ⁷². However, 10 ml of whole blood is required to match the positivity rate of 1 ml of bone marrow. Bone marrow is the most sensitive specimen for culture, with a culture positive rate of 80-100% in typhoid patients ¹⁰³. In patients presenting with a long duration of fever and in whom antibiotic therapy has been initiated, bone marrow biopsy and culture may be preferred as the rate of bacterial culture from blood drops rapidly after the first week of fever and following antibiotic chemotherapy ¹⁰⁴. The volume of blood collected and the ratio of blood to broth determines culture yield. 10-15 ml of blood and a ratio of 1 to 12 are recommended ⁷². Culture of *S. typhi* from stool specimens confirms chronic carriage status following an episode of typhoid fever.

The Widal test, developed by F. Widal in 1896, is based on the identification of agglutinating antibodies against the O and H antigens. This can be performed as a simple slide test or a confirmatory tube test. This was the mainstay of serological diagnosis of typhoid fever for more than a century. Near eradication of typhoid fever in industrialised countries and better modalities of diagnosis have rendered this test obsolete in these areas although it is still commonly used in developing countries. However, this test requires analysis of paired acute and convalescent sera, obtained 2 to 3 weeks apart to demonstrate the classical 4 fold increase in agglutinating titre. Similarly, a lack of knowledge of the baseline antibody titre in the local

population makes the results of the test questionable. This is so because chronic sub-clinical exposure to *S. typhi*, as found in endemic regions, can confer high titres of agglutinating antibodies in the absence of clinical infection. The test is non-specific, as infection with a wide variety of organisms including non-*Salmonella* agents like malaria, dengue, miliary tuberculosis, endocarditis and brucellosis can render the test positive. The large number of false positive tests leads to over-diagnosis of typhoid fever and therefore, unnecessary treatment and is cited as a cause of the emergence of chloramphenicol resistant *S. typhi* ⁶⁸. False positive results in previous typhoid vaccinees and those who have had infection and poor reproducibility due to a lack of standardised commercial antigen preparation are other disadvantages of this test. False negative results can be obtained with previous antibiotic treatment, in typhoid carriers and with technical errors especially with the tube test ¹⁰⁵.

More sensitive and specific tests include immunoblot assays for anti-LPS IgG and IgM in typhoid fever that have demonstrated 100% sensitivity and 80% specificity in multiple studies which had low number of participants ^{106, 107}. However issues related to serology, described above for Widal test, exist for these assays too. PCR diagnosis is also sensitive and specific with approximately 70-100% sensitivity and specificity obtained from multiple studies ^{108, 109, 110, 111, 112} but its use is limited by the lack of universal availability of PCR facilities ^{113, 114}. PCR may aid in the detection of Vi negative *S. typhi* which may escape detection of serological assays based on the antigen ^{115, 116}. Currently there are no PCR assays to determine the presence of all 14 genes, *tviABCDE*, *vexABCDE*, *ompR-envZ*, *rscB-rscC*, that encode the expression of the ViPS capsule in *S. typhi*. Table 1.8 compares the sensitivity and specificity of common diagnostic tests for typhoid fever.

Diagnostic test	Sensitivity range (%)	Specificity range (%)
Microbiological tests		
Blood culture	40-80	NA
Bone marrow cultures	55-67	30
Urine culture	0-58	NA
Stool culture	30	NA
Molecular diagnostics		
Polymerase chain reaction	100	100
Nested polymerase chain reaction	100	100
Serological diagnosis		
Widal test (tube dilution and slide agglutination)	47-77	50-92
Typhidot	66-88	75-91
Typhidot-M	73-95	68-95
Tubex	65-88	63-89
Urine antigen detection	65-95	NA

Table 1.8 Comparison of laboratory diagnostic techniques for typhoid fever⁷¹

1.9. Treatment

The choice of antibiotic chemotherapy in a typhoid patient is based on the antibiotic sensitivity patterns of the pathogen at the geographical region of isolation. Historically, chloramphenicol, ampicillin and co-trimoxazole were used as first line drugs in the treatment of typhoid. However with reported resistance to these drugs and with the former being proven less effective than fluoroquinolones, ciprofloxacin and other fluoroquinolones are more commonly used in the first-line treatment nowadays.

The classification of *S. typhi* antibiotic resistance pattern is as follows:

1. Sensitive to most first line antibiotics
2. Multidrug resistant (MDR) but nalidixic-acid sensitive
3. nalidixic-acid resistant (NAR)

Nalidixic acid resistance is an indirect marker of quinolone resistance. Ofloxacin or gatifloxacin is used in the treatment of MDR *typhi*. Azithromycin and Cefixime (oral antibiotics; the latter a third generation cephalosporin) and Cefotaxime and Ceftriaxone (injectable third generation cephalosporins) are the drugs of choice in NAR strains and also MDR strains. Although cefixime is suggested by the WHO as an optimal drug in the treatment of multidrug resistance (Table 1.9a), a recent trial comparing gatifloxacin and cefixime in the treatment of typhoid patients in Kathmandu in Nepal has revealed that treatment with gatifloxacin is better ¹¹⁷. Pooled data from randomized controlled trials in the treatment of typhoid fever is shown in Table 1.9b.

Table 1.9a Recommended antibiotic treatment for typhoid fever ¹¹⁸.

Susceptibility	Optimal treatment			Alternative effective treatment		
	Drug	Daily dose(mg/kg)	Course (days)	Drug	Daily dose(mg/kg)	Course (days)
Uncomplicated typhoid fever						
Fully sensitive	Fluoroquinolone (such as ofloxacin or ciprofloxacin)	15	5-7*	Chloramphenicol	50-75	14-21
				Amoxicillin	75-100	14
				TMP-SMX	8-40	14
Multidrug resistance	Fluoroquinolone <i>or</i>	15	5-7	Azithromycin	8-10	7
	Cefixime	15-20	7-14	Cefixime	15-20	7-14
Quinolone resistance †	Azithromycin <i>or</i>	8-10	7	Cefixime	20	7-14
	Ceftriaxone	75	10-14			
Severe typhoid fever requiring parenteral treatment						
Fully sensitive	Fluoroquinolone	15	10-14	Chloramphenicol	100	14-21

		(such as ofloxacin)			
				Ampicillin	
					100
				TMP-SMX	8/40
					14
				Ceftriaxone <i>or</i>	60
Multidrug resistant	Fluoroquinolone	15	10-14		
				Cefotaxime	80
					10-14
				Ceftriaxone <i>or</i>	60
Quinolone resistant				Fluoroquinolone	
	Cefotaxime	80	10-14		20
					14

* Three day courses also effective, particularly so in epidemic containment.

†Optimum treatment for quinolone resistant typhoid fever has not been determined. Azithromycin, third generation cephalosporins, or a 10-14 day course of high dose fluoroquinolone is effective. Combinations of these are now being evaluated.

1.10. Drug resistance

The first typhoid epidemic caused by MDR *typhi*, resistant to all three first line drugs, was reported in China in 1987¹¹⁹. The next decade witnessed the emergence of similar strains in India¹²⁰. MDR strains are now reported from almost all continents¹²¹. Multi-drug resistance is mediated by the pHCM1 plasmid which has 99% sequence identity with the plasmid R27, an *inc* H1 plasmid¹²². MDR strains were followed by those strains resistant to ciprofloxacin and other quinolones. Isolates demonstrating susceptibility to ciprofloxacin have a minimum inhibitory concentration (MIC) of <0.03 mg/ml and are also susceptible to nalidixic acid. Isolates with MIC between 0.125 to 1.0 mg/ml, though susceptible to ciprofloxacin on disc diffusion testing are associated with clinical failure of treatment and are resistant to nalidixic acid. Single point mutations in the *gyrA* gene of *S. typhi* and *S. paratyphi* are responsible for fluoroquinolone resistance¹²³. Interestingly, a decreasing trend in the isolation of *S. typhi* strains resistant to first line antibiotics has recently been observed in India and Bangladesh^{124, 125}.

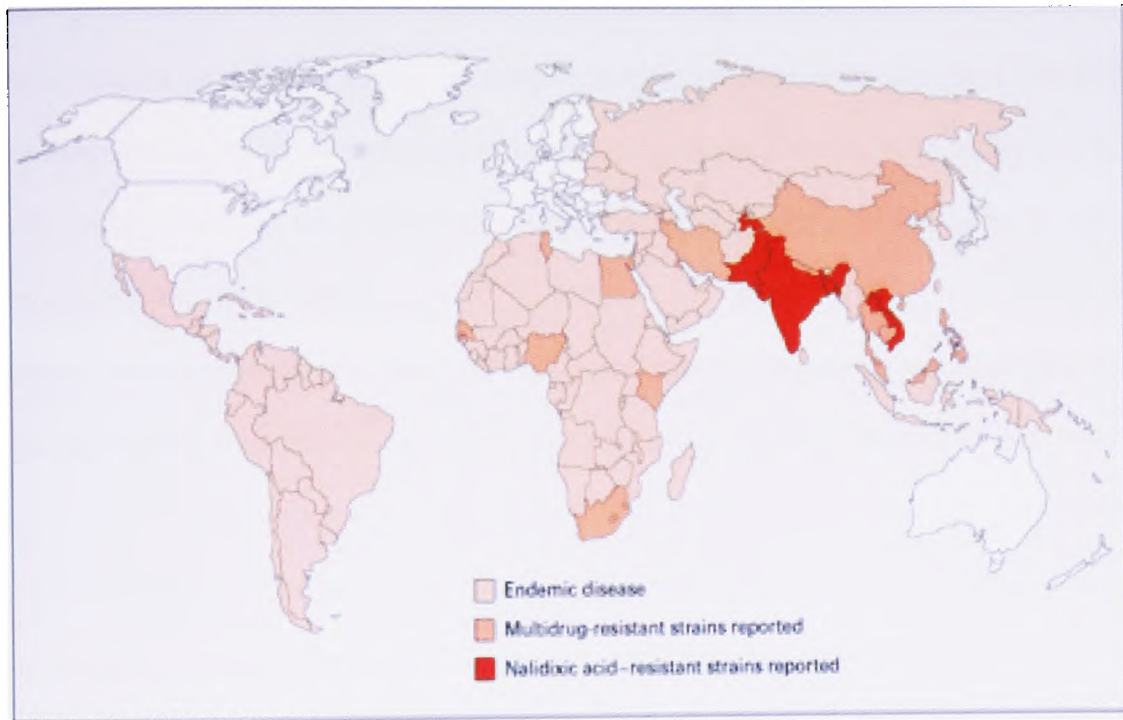


Figure 1.10: Drug resistance patterns of *S. typhi* worldwide showing geographical areas of multidrug resistance, NAR resistance and areas from where no drug resistance have been reported ⁹⁵.

1.11. Pathogenesis of *S. typhi*

The fact that typhoid fever is an exclusive human disease has hindered an understanding of the pathogenesis of *S. typhi* infection due to the lack of a suitable experimental animal model. Hence, our current understanding of typhoid fever pathology is based on the observation of the course of natural infection in human challenge models conducted in the seventies and eighties. While these studies revealed important information on typhoid fever, there remains a lack of data on pathogenesis, at the cellular and molecular level, in light of the rapid advances in immunology in the last twenty years.

A bacterial concentration greater than 1000 CFU is required to cause infection in healthy adults. Median incubation time and clinical attack rates are known to be dose dependant and are shown in Table 1.11. Hydrochloric acid is known to reduce or eliminate the number of bacteria that cross the stomach. Invasion of *S. typhi* involves rapid and effective translocation from the lumen of the intestine, through the M cells or enterocytes located in the intestinal mucosa, to the Peyer's patches that are specialized lymph tissue in the small intestine (Figure 1.11). Invasin, a protein produced by the bacteria enhances the bacterial translocation by altering the actin filaments of the enterocyte cytoskeleton thereby creating a passage for the bacteria. Secretory IgA, if induced following vaccination with oral vaccine, is believed to prevent the translocation of bacteria by binding to the bacilli in the intestinal lumen. *S. typhi* is known to multiply rapidly in the intestinal tract, as evidenced by the appearance of the bacilli in stool specimens within 24 hours of ingestion. Interestingly, unlike in other enteric infections like shigella and cholera, typhoid does not usually produce an intense inflammatory response in the intestinal epithelium. Indeed, constipation rather than diarrhoea is the more common feature in typhoid fever. This is attributed to both the immunomodulatory properties of the Vi polysaccharide capsule as well as

the relatively resistance-less entry into the epithelium by the 'ruffling' mechanism of the epithelial cells induced by the invasion protein.

Once in the Peyer's patches, *typhi* bacilli are thought to be carried intra-cellularly to other reticulo-endothelial organs within 24 hours of ingestion of bacilli. In animal experiments with chimpanzees that were inoculated with *S. typhi* intravenously and directly into the mesenteric lymph nodes, the bacilli were consistently identified in lymph tissue including the tonsils and mesenteric lymph nodes within days of the challenge. The mesenteric lymph nodes serve as a barrier to the entry of the bacilli into the systemic circulation but as a consequence, allows for additional multiplication of the bacilli. Entry into systemic circulation is from the mesenteric lymph nodes into the thoracic duct. Once in the blood the bacilli reside within macrophages, but proliferation results in spill over into the blood stream causing bacteraemia. The incubation period before reaching the blood stream and causing typical signs and symptoms of bacteraemia is long and could last 5 to 14 days depending on the ingested bacterial dose. Remarkable dissemination of the *typhi* bacilli were demonstrated in mice experiments where the bacilli were isolated in blood 20 seconds after oral challenge and from the spleen and liver shortly after.

The Vi polysaccharide capsule is thought to protect the bacilli in circulation by preventing phagocytosis by 'hiding' antigenic proteins. The febrile phase of the disease is thought to occur when the bacteria die and release endotoxin into the bloodstream. A major endotoxin of *S. typhi* is lipopolysaccharide or Lipid A that elicits an inflammatory response mediated by IL-1 that can lead to septic shock mediated by Tumor Necrosis Factor (TNF). Lipid A is a chemotactic factor for neutrophils that form the first line of non-specific human defence against bacterial infection. 0.25µg of purified endotoxin administered intravenously to healthy volunteers resulted in chills,

fever, headache, myalgia, anorexia, nausea, thrombocytopenia and leukopenia which were similar to the responses in typhoid fever.

Dose of <i>S. typhi</i>	Median incubation	Clinical attack rate
10 ⁹ CFU	5 days	95%
10 ⁷ CFU	7.5 days	50%
10 ⁵ CFU	9 days	28%
10 ³ CFU	-	0%

Table 1.11: Correlation between the dose of ingested *S. typhi* and median incubation time and clinical attack rate. (CFU-Colony Forming Unit) ⁹³

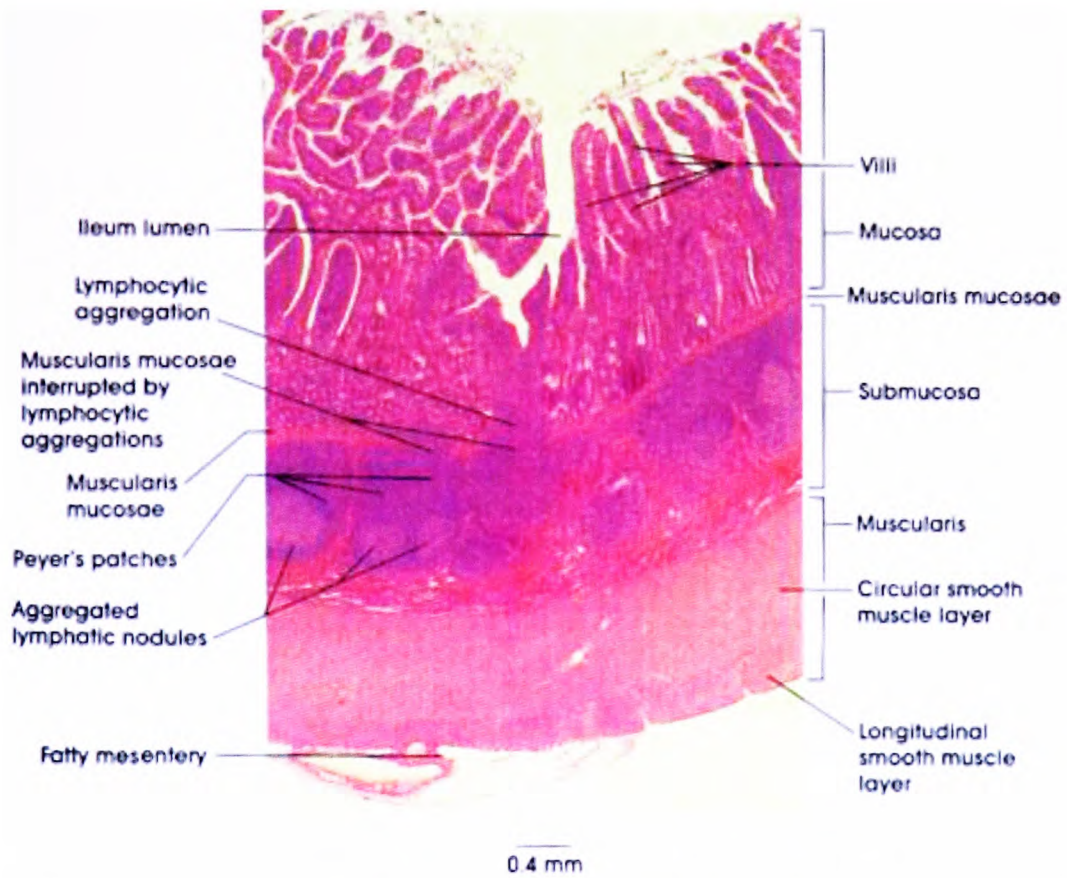


Figure 1.11: H & E cross-section of small intestine showing Peyer's patches

1.12. Human Genetics and Susceptibility to *S. typhi*:

Figure 1.12 illustrates the Interleukin 12 (IL-12) – Interferon- γ (IFN- γ) pathway that is essential for immunity against intracellular pathogens like *Salmonellae* and *Mycobacteriae*. Mutations in the genome could lead to a defect in one of the five sites (highlighted in black) in this cycle. The known mutations are:

1. Complete, loss-of-function deletion or insertion defect in the gene coding for the p40 subunit of the dimeric IL-12
2. Complete or partial defect in the gene coding for IL-12 Receptor β 1
3. Complete or partial defect in the gene coding for IFN- γ Receptor 1
4. Complete or partial defect in the gene coding for IFN- γ Receptor 2
5. Complete or partial defect in the gene coding for STAT1 protein

Patients with genetic defects in the IL-12 – IFN- γ pathway being susceptible to intracellular pathogens demonstrate the importance of cellular immunity in typhoid infection. HLA-DRB1*0301/6/8, HLA-DQB1*0201-3 and TNFA*2-308 are associated with susceptibility to typhoid fever while HLA-DRQB1*04, HLA-DQB1*0401/2 and TNFA*1(-308) are associated with lower risk. HLA-DRB1*12 is associated with protection against complicated typhoid ⁷². There was no association between mutations in the TLR-5, a toll like receptor that mediated innate immune response against bacterial flagellin, and susceptibility to typhoid fever ¹²⁷. However, an inverse relationship was observed between the most common mutation in the cystic fibrosis trans-membrane conductance regulator (CFTR) gene in a typhoid endemic population in Indonesia and the incidence of typhoid fever ¹⁹⁶. It was previously demonstrated that *S. typhi* uses CFTR to enter intestinal epithelial cells ¹⁹⁷.

Immunity against *S. typhi* can be categorised into non-specific mechanisms and specific defences; the latter divided into humoral and cellular compartments. Non-specific defence mechanisms include phagocytosis by granulocytes and macrophages. Opsonization of bacteria by complement and antibody aids phagocytosis; while *S. typhi* is relatively resistant to phagolysome, the engulfment by phagocytic cell is important for the initiation of a T-cell immune response. Complement activity through the different complement pathway play a role in the non-specific defence against typhoid fever by the formation of Membrane Attack Complex (MAC) that result in bacterial cytolysis.

Specific humoral compartment includes antibodies that are formed against antigens expressed on the surface of the bacterial cell wall. While IgM is the response to an acute infection, IgG reflect long-term immunity. Furthermore, IgG1 and IgG3 predominate in the antibody response to protein antigens whereas IgG2 is the predominant immunoglobulin formed against a polysaccharide antigen such the Vi polysaccharide. The role of antibodies in typhoid infection is two-fold. Firstly they are involved in agglutination reactions with bacterial antigens. This phenomenon is the basis of the Widal test utilising the somatic 'O' antigen and the 'flagellar 'H' antigen. Secondly, antibodies are involved in opsonisation, resulting in phagocytosis of bacilli by phagocytic cells and induction of 'T' cell immunity.

Specific cellular compartment include the T-cells to which antigen presenting cells (APC) including macrophages, B-cells and dendritic cells present engulfed bacteria. A unique phenomenon is that only protein or polysaccharide-protein conjugate antigens have T-dependant properties while a majority of polysaccharide antigens have a T-independent property.

Cellular immunity to *S. typhi* is important because intracellular infection is a mechanism for the pathogen to avoid complement mediated bactericidal activity. The role of opsonophagocytosis

in the clearing of infection is unclear. Evidence for the importance of humoral immunity comes from the fact that generation of bactericidal anti-Vi polysaccharide antibodies following immunization with the Vi polysaccharide vaccine is protective. The immune response to *typhi* bacilli therefore involves secretory immunoglobulins, circulatory antibodies and cell-immunity. Ideal protection against typhoid fever would involve all three components of the immune system. It has been argued that oral vaccination with live attenuated *S. typhi* vaccine induces all three components of immunity although the primary action is through the production of secretory immunoglobulin. The Vi polysaccharide subunit vaccine induces circulatory immunoglobulins alone.

Evidence from challenge studies of volunteers who have had an episode of typhoid reflects that immunity from previous typhoid infection is 23% efficacious in preventing re-infection⁹³. This is comparatively less than 70 to 100% protection offered by similar enteric organisms like *Vibrio cholerae* O1 and O139, enterotoxigenic *Escherichia coli* and *Shigella* species and approximately 70% protection offered by the live attenuated vaccine strain of *S. typhi*⁹³. The inference follows that wild strain *S. typhi* has immunomodulatory properties. However there is no data on humoral protection, if any, offered by sub-clinical exposure to the pathogen in the environment.

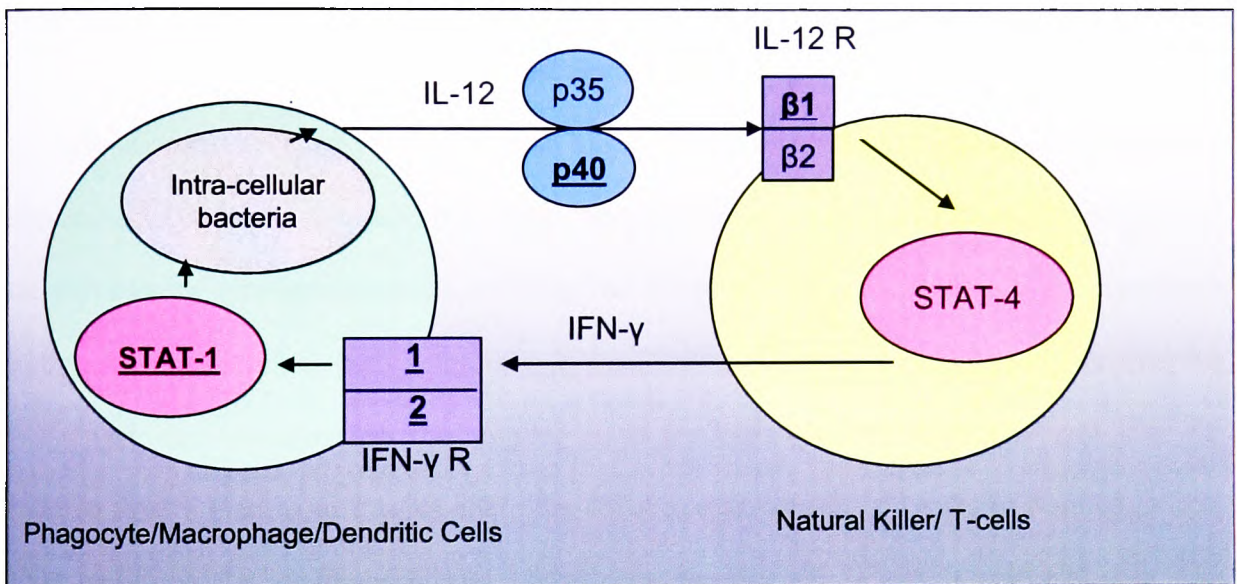


Figure 1.12: IL-12 – IFN- γ pathway. (IL-12-Interleukin 12 composed of p35 and p40 subunits, IL-12 R-Interleukin 12 receptor composed of $\beta 1$ and $\beta 2$ chains, STAT-1 & STAT-4: Second messenger molecules, IFN- γ : Interferon gamma, IFN- γ R: Interferon gamma receptor composed of 1 and 2 chains. Underlined areas denote possible defect sites in the cycle

1.13. Sero-epidemiology

Despite typhoid fever being a major public health problem causing more than 20 million new cases a year and being associated with significant mortality, there exists little data regarding sub-clinical exposure to *S. typhi* in endemic countries and the immune response associated with such sub-clinical exposure¹²⁸. There is also a lack of data regarding the age dependant immune response to sub-clinical exposure to *S. typhi*. Most studies have utilised the O and H antigens for the purposes of sero-epidemiology, utilising the Widal test with its disadvantages mentioned earlier. A cross-sectional study in India in 250 children aged 6 months to 15 years, utilising the Widal test, demonstrated that the baseline titres for both antigens were 1:40 and 1:80 for children aged 6 months to 2 years and older children respectively¹²⁹. A study from Nigeria in 2002 involving 175 children showed 14.9% were positive for O and H agglutinins at 1:80 dilution of Widal test¹³⁰. Yet another study on 310 healthy volunteers in Nigeria suggested that the baseline antibody titre to both antigens is 1:80 and that 1:160 be utilised as the diagnostic cut-off¹³¹. These titres were confirmed in another study of 364 healthy volunteers in Kenya¹³². All of the above studies utilized the Widal test for the estimation of somatic O and flagellar H antibodies. However, the Widal test is unreliable because of the cross-reactivity between antigens among members in the Enterobacteriaceae family which are common commensals. The determination of sero-epidemiological data with the Widal test is therefore unreliable for the same reasons. There are no data on the sero-epidemiology of typhoid fever as assessed by

bactericidal activity or by the Vi polysaccharide of *S. typhi* which is more specific for the organism as compared to other somatic antigens. Few studies on sero-epidemiology of sub-clinical exposure to typhoid are reported from South Asia, an area from where the highest rates of typhoid disease are found.

A better understanding of the sero-epidemiology of *S. typhi* and the humoral immune phenomena associated with sub-clinical exposure and age, to *S. typhi* could contribute to the implementation of an appropriate vaccination strategy for populations in typhoid endemic countries and could ultimately help in the design of an effective paediatric vaccine.

1.14. Typhoid vaccines

While provision of clean water and adequate facilities for sewage disposal is the ideal approach for the prevention of typhoid fever in the long term, protection by vaccination remains the most cost-effective strategy for prevention in the short to medium term ⁵⁶. Although there exist three vaccines licensed for use in adults among which only two are in clinical use and despite data being available on the mortality and morbidity patterns in endemic regions, no country has a uniform or universal immunisation programme for the prevention of typhoid fever. Non-availability of age specific natural immunity, that would be critical in determining the timing of vaccination, is one major impediment to vaccine implementation with either of the currently available vaccines ¹³³.

1.14.1. Whole cell inactivated vaccine:

The whole cell inactivated vaccine was the first vaccine to be licensed in the late nineteenth century but its efficacy was determined only in the latter half of the twentieth century. Various methods were employed for the preparation of the whole cell vaccine: acetone inactivation, alcohol inactivation, heat inactivation or phenol preservation. Two doses of the vaccine were reported to be 73% efficacious over three years (95% CI: 65-80%) ¹³⁴. To date, this is the only vaccine that prevents typhoid fever in all age groups. However the high rates of significant adverse effects associated with whole cell vaccination, with approximately 10% vaccinees

missing school or work after vaccination ¹³⁵ has limited the popularity of this vaccine and it is now rarely used in most parts of the world.

1.14.2. Vi polysaccharide vaccine:

The Vi polysaccharide vaccine was developed by John Robbins and colleagues at the National Institute of Health in the 1980s ¹³⁶ and first licensed by the FDA in 1994 ¹³⁷ following results from a study in 11384 children that demonstrated a protective efficacy of 60% calculated from the day of vaccination and 64% from 6 weeks after vaccination in comparison to a control group of children who received the meningococcal A + C CPS vaccine and 77.4% and 81.0% after 21 months, calculated immediately and 6 weeks after vaccination respectively, in comparison to unvaccinated children ¹³⁸.

The purified polysaccharide vaccine prevents *S. typhi* bacteraemia and confers moderate protection for up to 2 years following single dose vaccination. Immunity develops 7 days following vaccination and peaks 28 days after injection. Cumulative 3 year efficacy is 55% (95% CI 30-70%). Statistically significant protection was conferred in the first 2 years but not the third year after vaccination with vaccine efficacy rates of 68% (95% CI 50-80%), 60% (95% CI 31-76%) and 50% (-11-78%) in the first, second and third year following vaccination respectively ¹³⁹. An age dependant response to vaccine was observed with 46% (95% CI: 0-90%) of 5-9 year old children, 85% (95% CI: 77-97%) in 10-14 year olds and 60% (95% CI: 0-92%) in 15-19 year old adolescents ¹⁴⁰.

No difference in adverse event rate was observed for fever and erythema in the typhoid vaccine group as compared to the placebo group and while swelling at the site of injection was

significantly more common (RR: 8.13 (95% CI: 1.34-63.66) in the vaccine group, actual incidence was low at 2.9%¹³⁹.

Like other polysaccharide vaccines, this vaccine is ineffective in children below two years of age probably due to the immaturity of the toddler's immune system as a result of absence of a mature splenic marginal zone⁵⁶.

Advantages of the Vi vaccine, viewed in the context of public health measures, includes it comprising of a single dose and low rates of adverse effects. Because this vaccine is not patent protected, generic manufacturers are able to significantly reduce the cost of manufacturing making it more attractive for wide use in developing countries. Disadvantages include parenteral route of administration which requires additional equipments including needles and syringes along with technical expertise in intramuscular injection. Another disadvantage is the relatively short duration of protection of two years that would necessitate revaccination of large populations, more frequently raising the possibility of hyporesponsiveness. As mentioned before in the chapter on the Vi polysaccharide, many experts doubt the usefulness of the ViPS in the era of acapsulate *S. typhi*. Lastly, its ineffectiveness in children below two could handicap its implementation in a national programme in an endemic country where a significant number of cases are in children below five, with a proportion of them being under two.

1.14.3. Live oral vaccine:

Early reports of the use of the live oral vaccine date to the 1930s¹⁴¹. It was licensed in Europe in 1983 and in the USA in 1989. This vaccine, containing *Ty21a*, an attenuated strain of *S. typhi*, that has undergone non-specific chemical mutagenesis of several genes including the *viaB* locus, is licensed for use in people more than six years of age. Two formulations (enteric coated

capsule and liquid formulation) are available and at least three, and ideally four, doses on alternate days are required for optimal protection. Moderate protection is reported to last up to 3 years following vaccination. Following vaccination, immunity is elicited 10 to 14 days after the third dose and the current recommendation is to revaccinate travellers every year and residents in endemic regions every three years ¹³⁹.

Cumulative 3 year efficacy of three doses of the *Ty21a* (all formulations) was 51% (95% CI: 36-62%) with that of the liquid formulation being 74% (95% CI: 38-89%), enteric capsules being 47% (95% CI: 33-58%) and the less common gelatine capsule being 25% (95% CI: -10-49%). Pooled results of two trials comparing the liquid preparation with enteric capsules showed that 3 year cumulative risk of typhoid fever favoured the liquid preparation though the confidence intervals were wide and included one (RR=0.54, 95% CI: 0.23-1.23). Efficacy rates for three doses after vaccination are 49% (95% CI: 16-70%), 60% (95% CI: 44-71%), 59% (95% CI: 32-75%), 78% (95% CI: 35-93%) and 47% (-24-78) for Year 1, 2, 3, 4 and 5 after vaccination ¹³⁵.

The liquid formulation was associated with more nausea and abdominal pain (RR=3.18 (95% CI: 1.15-8.83) and vomiting (RR=2.48 (95% CI: 1.56-3.95). Enteric coated capsules were associated with milder adverse events (RR=1.78 (95% CI: 1.08-2.95) ¹³⁹.

Unlike injectable vaccines, *Ty21a* induces intestinal mucosal immunity, mediated by secretory IgA, in addition to cellular immunity. Also, as a result of bacterial shedding, it confers herd immunity among the local population as a result of natural infection replication. It also induces immunity against Vi negative *S. typhi*. Disadvantages include the need and facilities for storage at 2-4 degrees Celsius (the liquid formulation is stable for 48 hours and the enteric capsule for 7 days if not refrigerated), at least three doses for adequate immunity, contraindications in

immunocompromised populations including HIV infected persons, the highest concentrations of whom are incidentally found in typhoid endemic areas, poorly understood immuno-kinetics and a theoretical possibility of reversion to pathogenic strains.

1.14.4. Newer oral vaccines:

The lack of precise definitions of the attenuating gene mutations and multiple dose requirements of *Ty21a* led to efforts to develop a better oral vaccine. The new products include Ty800 with *phoP/phoQ* mutation, χ 3927 with *cya* and *crp* mutations and MO1ZH09 with *aroC* and *ssaV* mutations. CVD 908 had *aroC* and *aroD* mutations that interrupt the biosynthetic pathway for aromatic metabolites for *S. typhi*. However, a clinical study showed self limited vaccine bacteraemia in 100% of volunteers who ingested one dose of 5×10^8 organisms. This observation raised questions about the potential reactogenicity in vulnerable people and licensure issues leading to an improvement of the vaccine by the addition of a *htrA* (heat shock protein) mutation resulting in a CVD908-*htrA* vaccine. CVD908-*htrA* demonstrated an immune response in terms of anti-LPS IgG sero-conversion and ASC responses similar to CVD 908 but without vaccine bacteraemia. A third mutant, CVD 909, with CVD 908-*htrA* as parent strain had the highly regulated P_{viaA} replaced with the strong constitutive promoter P_{tac} to induce the expression of the Vi polysaccharide. Similar to the other mutants, the immune responses to the LPS and H antigen were robust. However, only 4% and 12.5% of volunteers who ingested 1 dose and 2 doses respectively had serum anti-Vi IgG although IgA anti-Vi ASC were detected in 80% of volunteers ¹⁴².

1.14.5. Newer parenteral vaccines:

Attempts to design new vaccines for the prevention of typhoid fever have continued because of the lack of an effective and safe paediatric vaccine. Vi polysaccharide vaccine conjugated to inactivated *Pseudomonas aeruginosa* exotoxin A has been shown to confer 91.1% protection in 2 to 5 year old Vietnamese children 27 months after vaccination ¹⁴³. Efficacy of protection over a period of 46 months was shown to be 89% ¹⁴⁴. Studies of vaccine efficacy in children below 2 years are continuing.

Proteins that have shown promise as vaccine candidates in animal experiments include the heat shock proteins *DnaJ* and *groEL* ^{145, 146} and Iron regulated OMP (IROMP) ¹⁴⁷.

1.15. Aims and Objectives

The aims and objectives of this study are:

1. To develop and validate an Enzyme Linked Immunosorbent Assay for the detection of antibodies to the Vi capsular polysaccharide of *S. typhi*
2. To develop and validate a Serum Bactericidal Assay for the detection of functional (bactericidal) antibodies against *S. typhi*
3. To develop and validate an Enzyme Linked Immunospot Assay for the detection of Vi polysaccharide antigen secreting cells
4. To develop and validate a Polymerase Chain Reaction for the detection of a known loss-of-function deletion in the IL-12 gene
5. To determine the effect of sub-clinical exposure to *S. typhi* on the natural immunity against acquisition of typhoid fever, utilising these assays
6. To determine the age related development of immunity against typhoid fever in populations in typhoid endemic regions, utilising these assays
7. To determine the kinetics of humoral immune response following vaccination with the Vi polysaccharide vaccine
8. To develop and validate a rapid, multiplex confirmatory, Polymerase Chain Reaction assay for the confirmation of *S. typhi* isolates
9. To determine the genotypic status of acapsulate *S. typhi* using novel PCR assays
10. To recommend appropriate vaccination strategies for the prevention of typhoid fever

Chapter 2 : DEVELOPMENT AND VALIDATION OF GENERAL LABORATORY ASSAYS

2.1 Development and Validation of a Vi polysaccharide ELISA

2.1.1 Introduction

Despite the existence of many in-house versions of the ELISA for the detection of antibodies to *Salmonella typhi* specific antigens especially the O and H antigen, those for the detection of the Vi polysaccharide antibodies are few. Most of them have been designed for the detection of antibody responses to the Vi polysaccharide vaccine ^{167,168,169}. Others have been utilized as urine diagnostic tests for the detection of acute typhoid fever ¹⁷⁰. Use of acute serum for the detection of Vi polysaccharide antibodies in the diagnosis of typhoid fever is not recommended due to the excessive amounts of immunoglobulin that can render the assay unreliable. One ELISA each has been reported for the diagnosis of chronic infection and for the detection of responders in participants vaccinated with the Vi polysaccharide protein conjugate vaccine ^{171,172}. However all ELISAs report either optical density or arbitrary units and are hindered by the absence of absolute antibody titres therefore making reproducibility of results difficult and the different relative cut-off values that they utilize for the determination of positive responders or for the diagnosis of typhoid fever results in the lack of a universal standard. The lack of a universal standard makes comparison of different preparation of the Vi polysaccharide or Vi polysaccharide-protein conjugate vaccine difficult. Determination of the antibody titre in populations with different rates of sub-clinical exposure to *Salmonella typhi* is also not possible

without the existence of a standard, accurate and precise Vi polysaccharide ELISA. In this chapter, the development and validation of a Vi polysaccharide ELISA, fit for use in sero-epidemiology, is described.

Immunoglobulin IgG Subclass ELISA

Of the 4 IgG subclasses, IgG1 and IgG3 are known to predominate the antibody response to protein antigens while IgG2 predominates in the response to polysaccharide antigen. While one study in 1993 reported that IgG1 subclass predominated the immune response to both the whole cell vaccine and the polysaccharide vaccine ¹⁷³, there exists no data on the subclass profile of the antibody response to the ViPS as a result of sub-clinical exposure. A modification of the ViPS ELISA was made to enable to determination of the IgG subclass following sub-clinical exposure and vaccination with the ViPS vaccine.

Anti-ViPS antibody Avidity

High antibody avidity is a correlate of vaccine protection against *H. influenza* ¹⁷⁴. Similarly for *N. meningitidis* Group C, higher avidity group C anti-capsular antibodies were associated with passive protection against the organism in the absence of protective bactericidal titres ¹⁷⁵. Avidity indices relating to the anti-ViPS antibody, as a result of natural immunity and following vaccination, are unknown. We derived an avidity ELISA for the determination of the same, based on the ViPS ELISA.

Reference ELISA

In the absence of a standard reference serum for anti-ViPS antibody, a reference ELISA was designed to establish the absolute antibody titre for a control serum obtained from a volunteer who had received the ViPS vaccine previously. This technique is based on the principle of equivalence of absorbance of 2 ELISAs performed in parallel under identical assay conditions representing equivalent amounts of antibodies when corrected for concentration ¹³. This technique is an adaptation of an experiment that quantitated concentrations of antibody to *Streptococcus pneumoniae* in human sera ¹⁷⁶. In that experiment, weight-based units were assigned to antibodies to 11 pneumococcal polysaccharide (PnPs) serotypes (1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F) by using enzyme-linked immunosorbent assay methodology and a human standard reference serum, USNRP IS 1644. The experimentally derived assignments for anti-PnPs antibodies of the immunoglobulin G (IgG), IgM, and IgA isotypes in lot 89-S correlated well to the separately determined immunoglobulin assignment. Based on the results, it was suggested that these assignments may be used to assess levels of antibody to PnPs serotypes in human serum.

2.1.2 Materials

a. Equipment:

Maxisorp ELISA plates (Nunc), ELISA reader (MRX Revelation), Electronic pipettes (Biohit), Pipette tips (Biohit) and an automated ELISA plate washer were used.

b. Reagents:

Salmonella typhi Vi polysaccharide (supplied by Sanofi-Pasteur), Phosphate buffered solution tablets (Sigma), Tween20 (BDH), 2% skimmed milk powder (BDH), Vi polysaccharide diluent solution for plate coating prepared with: 7.5g/l NaCl (Sigma), 383 mg/l KCl (Sigma), 318 mg/l MgSO₄ · 7H₂O (Sigma) and 305 mg/l CaCl₂ (Sigma) made to pH 7.4 0.01 M Tris-Cl (Sigma), Anti-human IgG conjugate, γ -chain specific, prepared in goat (Sigma), TM Blue tablets (Sigma), Citrate buffer for TM Blue tablets and 2 molar H₂SO₄ (BDH) were used.

2.1.3 Protocol for the Vi polysaccharide ELISA

Maxisorp plates were coated with 100 μ l of Vi antigen at a concentration of 5 μ g/ml in Vi polysaccharide diluent solution and left at 4 degrees Celsius overnight. Each well was washed thrice with 300 μ l of PBS/Tween20 (0.05%). Plates were then blocked with 200 μ l/well of 2% skimmed milk powder in PBS for 2 hours at room temperature. Wells were then washed thrice with 300 μ l of PBS/Tween 20 (0.05%). Serum to be tested was diluted 1:25 in PBS/Tween (0.05%) 2% skimmed milk powder, serially double diluted six times to a final concentration of 1:800 and left for two hours at room temperature. After washing each well thrice with 300 μ l of PBS/Tween 20 (0.05%), 100 μ l of anti-human IgG conjugate, diluted 1:1000 with PBS/Tween (0.05%) 2% skimmed milk powder was added. After one hour of incubation at room temperature, wells were washed six times with 300 μ l of PBS/Tween (0.05%). 100 μ l/well of

freshly prepared Tetramethylbenzidine reagent (TM Blue), to 10 ml of which preparation 2 μ l of H_2O_2 was added immediately prior, was added to each well. The reaction was stopped after 15 minutes with the addition of 25 μ l of 2 molar H_2SO_4 . The plate was then read at 450nm in the ELISA reader.

For the development of the Vi polysaccharide ELISA described above, the following experiments were done in order to determine the optimal antigen coating concentration, blocking solution, serum dilution, conjugate dilution and incubation time and temperature. A comparison was made between Vi polysaccharide alkaline phosphatase and peroxidase substrate ELISAs and ELISA with and without methylated Human Serum Albumin (mHSA). The specificity of the Vi polysaccharide ELISA was then confirmed by pre-adsorbance before validating it for intra-plate and inter-plate variation of results.

2.1.4 Determination of optimal antigen coating concentration

To determine the optimal concentration of Vi polysaccharide for coating the ELISA plates (Step 1 of the Vi polysaccharide ELISA protocol), 0.5 μ g per well, 1.0 μ g/well and 1.5 μ g/well concentrations of Vi polysaccharide were used for comparison. This was achieved by using an arbitrary concentration of 5 μ g/ml of Vi polysaccharide in the Vi polysaccharide diluent and pipetting 100, 200 and 300 μ l of this solution into the ELISA plates. The rest of the ELISA protocol was as described above. The experiment was performed in duplicate for both pre-vaccination and post-vaccination serum (Vi polysaccharide vaccine) and the mean O.D of the 2 experiments for pre-vaccination and post-vaccination serum was recorded.

2.1.5 Determination of optimal blocking agent

To determine the optimal blocking agent (Step 4 of the Vi polysaccharide ELISA protocol), 2% skimmed milk powder, 5% skimmed milk powder, 5% fetal calf serum, 80% fetal calf serum, 2% casein, 5% casein, 5% sucrose and 10% sucrose were compared for the serum control wells. The rest of the experiment was as per the above protocol. The experiment was performed in duplicate and the mean O.D of both experiments was recorded.

2.1.6 Determination of optimal serum dilution for post-vaccination samples

To determine the optimal starting dilution of serum (Step 6 of the Vi polysaccharide ELISA) 1:25, 1:50, 1:100, 1:200, 1:400 and 1:800 dilutions of an aliquot of post-Vi polysaccharide vaccination serum were compared. The rest of the experiment was as per the above protocol. The experiment was performed in duplicate and the results of both experiments were recorded.

2.1.7 Determination of optimal conjugate dilution

To determine the optimal concentration of conjugate (Step 8 of the Vi polysaccharide ELISA protocol), 1:1000, 1:2000, 1:5000, 1:8000 and 1:10,000 dilutions of anti-human IgG (γ -chain specific)(prepared in goat) were compared. The rest of the experiment was as per the above protocol. The experiment was performed in duplicate and the mean O.D of both experiments was recorded.

2.1.8 Determination of diluent concentration

For the reduction of O.D in the control wells, containing only post-vaccination serum samples, the effects of using 0.01% skimmed milk powder vs. 2% milk powder as the diluent for serum along with PBS/Tween (0.05%) was compared. (Step 6 of the Vi polysaccharide ELISA protocol). The rest of the experiment was as per the above protocol. The experiment was performed in duplicate and the mean O.D of both experiments was recorded.

2.1.9 Determination of serum incubation time and temperature

To determine the optimal incubation period and temperature of incubation after addition of serum (Step 6 of the Vi polysaccharide ELISA protocol), a comparison between incubating serum at 4 degrees overnight and at room temperature for 2 hours was made. The rest of the experiment was as per the above protocol. The experiment was performed in duplicate and the mean O.D of both experiments was recorded.

2.1.10 Determination of the incubation time for substrate reaction

This experiment was done to determine the difference in terminating the ELISA reaction 5 minutes and 15 minutes after the addition of the conjugate substrate (Step 11 of the Vi polysaccharide ELISA protocol). The experiment was performed in triplicate for both pre-vaccination and post-vaccination sample and the mean O.D of the three reactions was recorded for each of the two samples.

2.1.11 Alkaline phosphates vs. Peroxidase ELISA system

Alkaline phosphatase and peroxidase substrate are two commonly used ELISA substrate systems. After determination of the optimal antigen coating concentration, blocking agent, starting serum dilution, conjugate dilution and the serum incubation temperature and time, a comparison between alkaline phosphatase and peroxidase ELISA systems for the Vi polysaccharide (Steps 8, 10 and 11 of the Vi polysaccharide ELISA protocol) was made in order to determine the substrate system demonstrating the higher O.D with the same sample of post-vaccination serum.

2.1.12 mHSA vs. Non-mHSA ELISA

This experiment was done to determine if addition of methylated human serum albumin to the coating solution would increase its binding to the ELISA plate as evidenced by an increased O.D. This principle is used for Meningococcal C polysaccharide capsule ELISA. The following steps were done in this experiment: 50µl of 10µg/ml of Vi polysaccharide in Vi polysaccharide diluent solution and 50µl of 10µg/ml of mHSA were prepared. The latter was added drop by drop into the former with the former being constantly mixed. 100µl of the mix was then plated onto each ELISA plate well and incubated at 4 degrees Celsius overnight. The Vi polysaccharide ELISA was then performed as per protocol. This experiment was performed in quadruplicate and the mean value of all four experiments was recorded.

2.1.13 Specificity of Vi polysaccharide ELISA

Specificity of the Vi polysaccharide ELISA was determined by a pre-adsorbance assay. To account for pre-existing antibodies in the population for the use of human serum in the validation of experiments, a serum sample that had low antibody concentrations prior to vaccination with the Vi polysaccharide and high antibody concentrations thereafter was utilised. The principle of the specificity experiment was that pre-adsorbance of the post-vaccination serum, containing anti-Vi polysaccharide antibodies, with Vi polysaccharide would result in their binding further resulting in the reduction of unbound antibodies from the serum. This would then have been evidenced by a reduced O.D after ELISA with the pre-adsorbed serum. The following steps were used for the specificity assay:

Serum was diluted to double the required concentration i.e.1: 12.5 for the starting serum dilution of 1:25. The Vi polysaccharide was prepared to 50µg/ml and 25 µg/ml of sterile pyrogen free water. The serum was then mixed with an equal amount of 50µg/ml and 25µg/ml of Vi polysaccharide solution. The mix was incubated at 4 degrees Celsius overnight. The Vi polysaccharide ELISA was then performed as per protocol. The specificity assay was performed in duplicate and the mean value of both experiments was recorded.

2.1.14 Intra- plate validation

Following the development of the Vi polysaccharide ELISA, it was validated for intra-plate variability of O.D for both pre-vaccination and post-vaccination serum. The experiment was performed in triplicate on the same ELISA plate and the experiment repeated on three different days.

2.1.15 Inter- plate validation

This experiment was performed to determine the inter-plate variability of O.D on the same sample analysed on three different days. Each experiment was performed in triplicate and the O.D values, along with the confidence intervals, plotted on the graph.

2.1.16 Reference ELISA

In the absence of a standard reference serum for anti-ViPS antibody, a reference ELISA was designed to establish the absolute antibody titre for a control serum obtained from a volunteer who had received the ViPS vaccine previously. This technique is based on the principle of equivalence of absorbance of 2 ELISAs performed in parallel under identical assay conditions representing equivalent amounts of antibodies when corrected for concentration ¹³. In brief, serial two fold dilutions of a European Reference Material (ERM DA470) containing 9.68g/L of total IgG and starting at 1:100,000 dilution was run simultaneously with serial two fold dilutions of the test serum starting at 1:25 dilution. The rest of the protocol for the total IgG ELISA described below was followed. Optical density (O.D) obtained for both ELISAs were plotted concurrently and absolute antibody titre for the test serum estimated by correcting for the dilution factor.

2.1.17 Subclass ELISA

To determine the anti-Vi polysaccharide IgG subclass profile, the protocol for the Vi polysaccharide ELISA was modified to include conjugated IgG subclass secondary antibody (Sigma, UK), as per the manufacturer's instructions. 28 Nepali sera, collected from a clinically healthy population in Kathmandu in Nepal as detailed in Chapter 3, representing 4 random samples from each of the 7 age bands, starting from cord blood in lieu of newborn to 75 years, and tested to have a high antibody concentration in the total IgG ELISA, mentioned above, were analysed and compared to the IgG subclass profile in a post-ViPS vaccination serum described in Chapter 3.

2.1.18 Avidity ELISA

The avidity of anti-ViPS antibody was measured by a modified elution ELISA. In brief, the serum samples were allowed to interact with ViPS coated on the wells as per the protocol mentioned above. Subsequently, after washing, wells were incubated for 15 min with a variable molarity (range 0.5 -4.5 M) of the chaotropic agent ammonium thiocyanate (NaSCN) in PBS. The plates were then developed as per the protocol for ViPS ELISA described above. The relative avidity index was defined as the molarity of NaSCN at which 50% of the amount of antibody, as compared to a control well, remained bound to the coated ViPS antigen.

Results

2.1.4 Determination of optimal antigen coating concentration

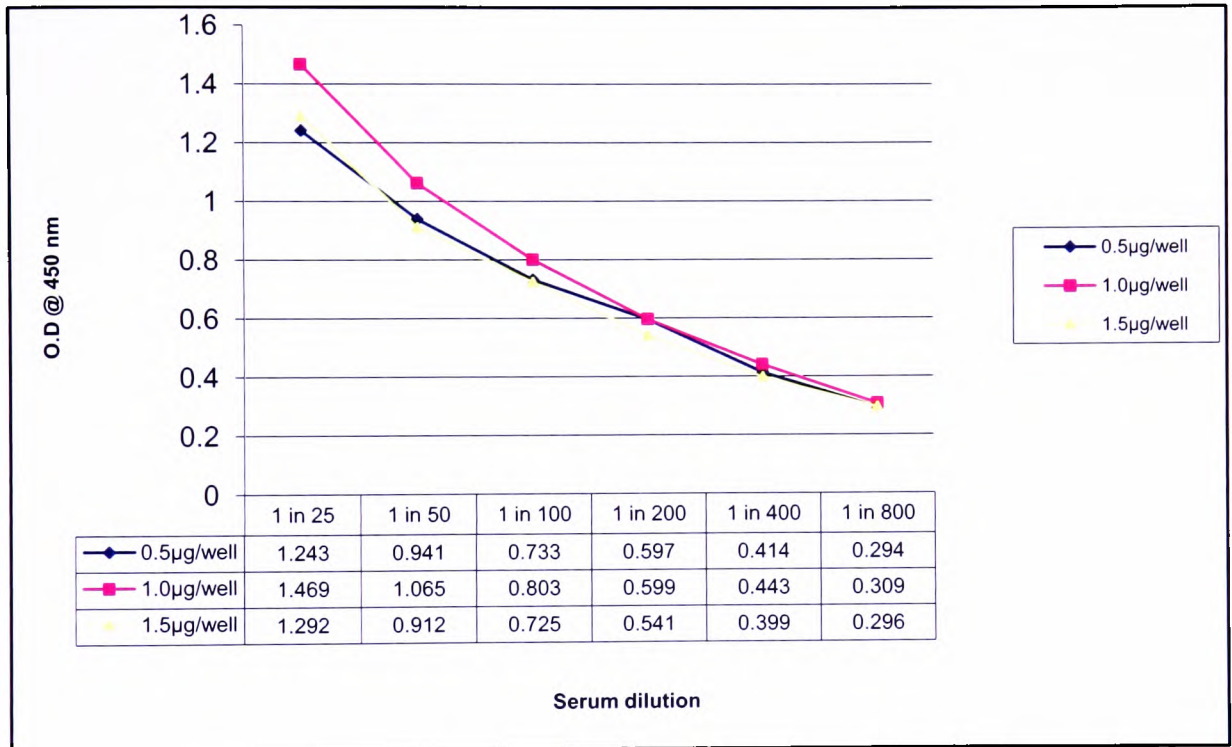


Fig 2.1.4a: Determination of optimal antigen coating concentration for pre-vaccination serum performed with three concentrations of Vi polysaccharide (0.5µg/ well, 1µg/well, 1.5µg/well)

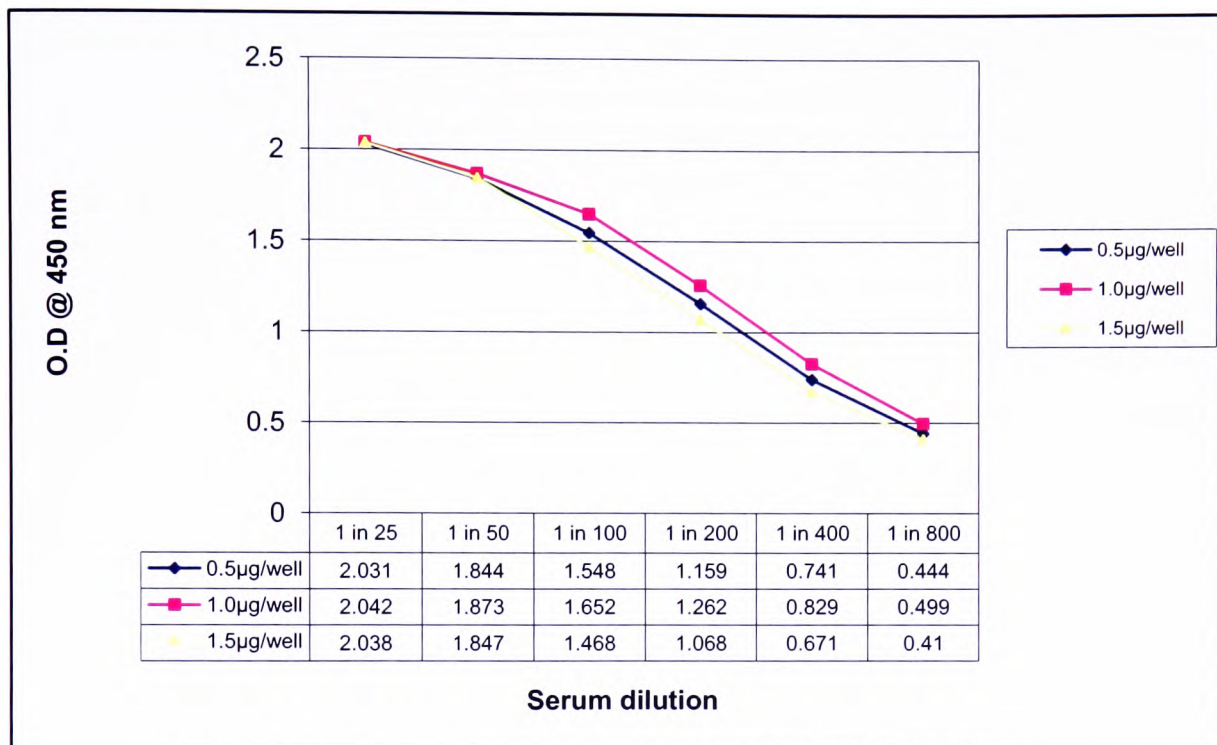


Fig 2.1.4b: Determination of optimal antigen coating concentration for post-vaccination serum performed with three concentrations of Vi polysaccharide (0.5µg/ well, 1µg/well, 1.5µg/well)

There appeared to be little difference between the 3 volumes and concentrations of Vi polysaccharide used. Hence the lowest concentration and volume of Vi polysaccharide i.e. 0.5µg/well or 100µl/well was determined to be optimal for use in future experiments.

2.1.5 Determination of optimal blocking agent

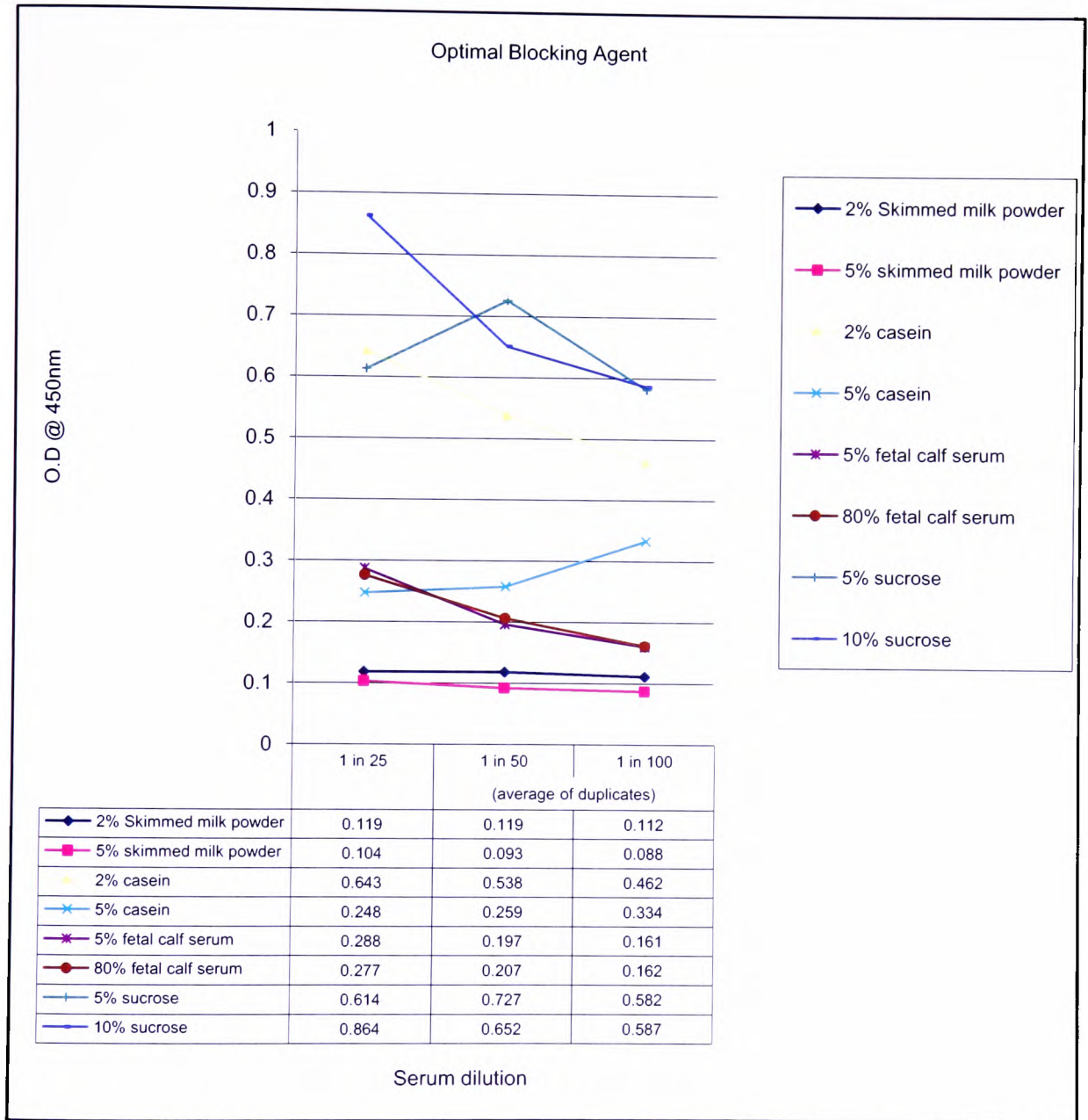


Fig 2.1.5: Determination of the optimal blocking agent in the Vi polysaccharide ELISA by comparing the lowest optical density at 450 nm for 2 concentrations each for 4 blocking agents

The best blocking agents, demonstrating lowest O.D for the serum control wells (no antigen coating), appeared to be 2% and 5% skimmed milk powder. There was little difference between these two agents. Hence the lower concentration blocking agent i.e. 2% skimmed milk powder was determined to be optimal for use in future experiments.

2.1.6 Determination of optimal serum dilution for post-vaccination samples

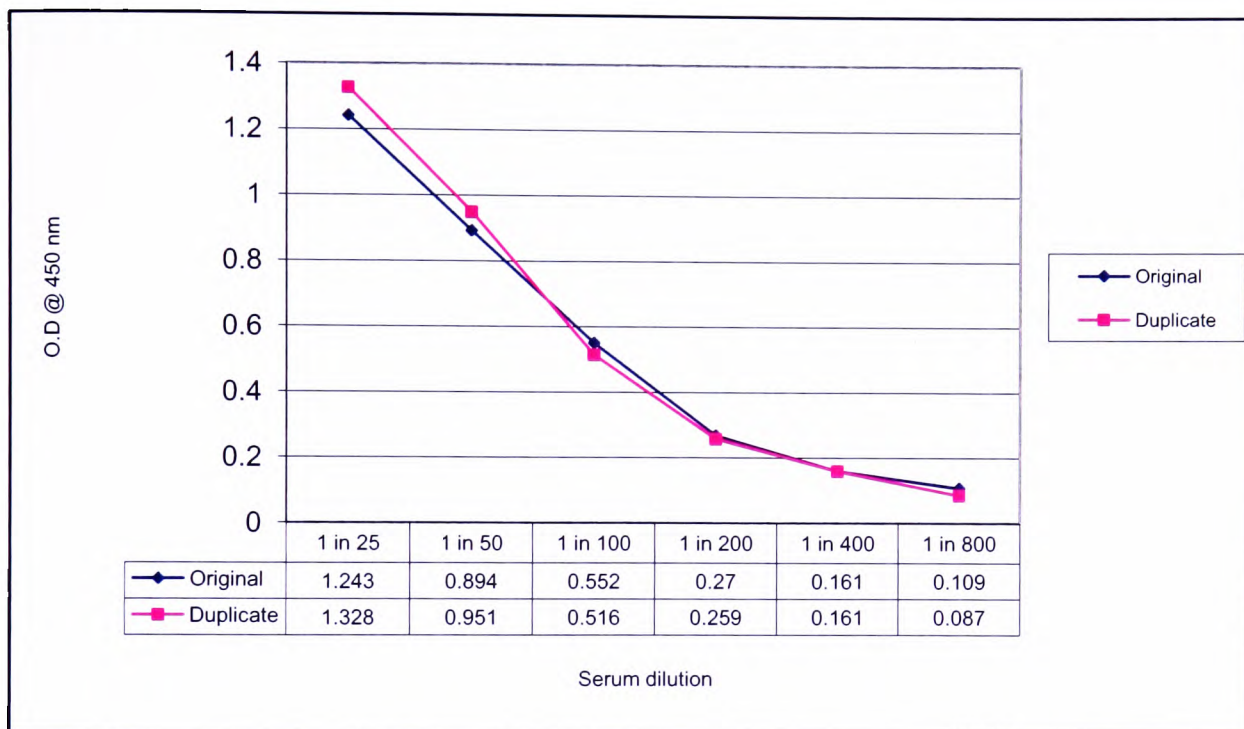


Fig 2.1.6: Determination of optimal serum dilution for post-vaccination serum by estimating the optical density at 450nm for 6 serial double dilutions of post-vaccination serum performed in duplicate

The starting serum dilution of 1:25 gave the highest O.D of 1.3 in the experiment. Hence 1:25 serum dilution was determined to be the optimal starting dilution of serum dilution for future experiments.

2.1.7 Determination of optimal conjugate dilution

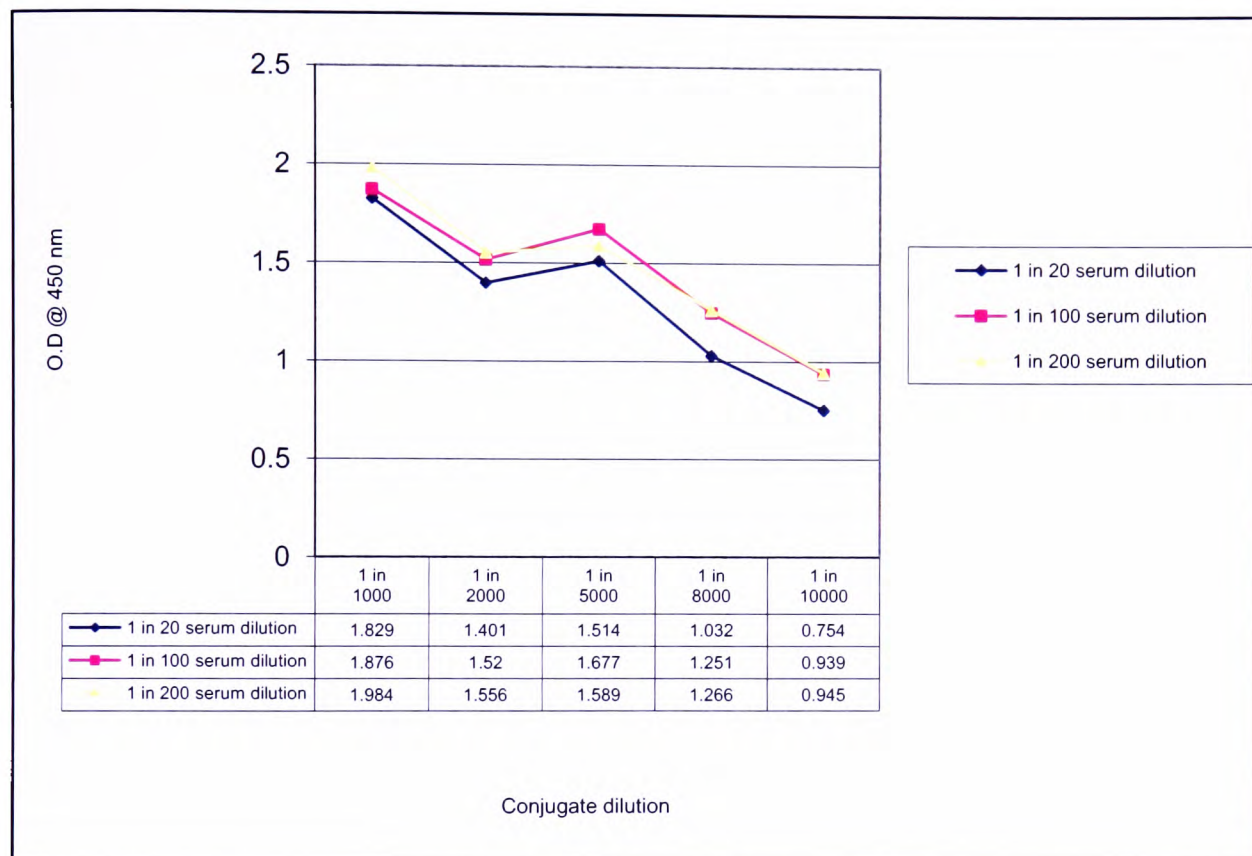


Fig 2.1.7: Determination of optimal conjugate dilution for the Vi polysaccharide ELISA by estimating the highest optical density at 450nm for 5 dilutions of conjugate using 3 dilutions of post-vaccination serum

A 1:1000 dilution of conjugate gave the highest O.D for all serum dilutions in this experiment. Hence 1:1000 conjugate dilution was determined to be optimal for future experiments.

2.1.8 Determination of diluent concentration

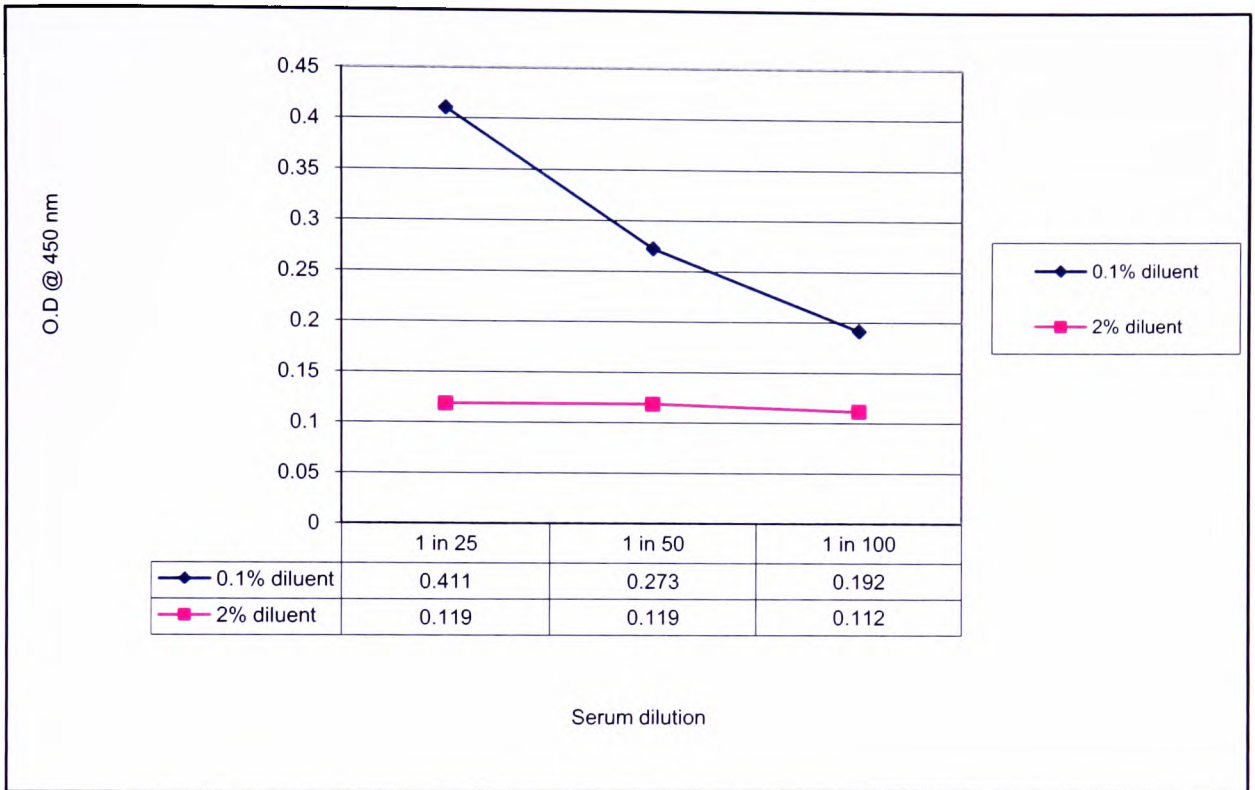


Fig 2.1.8: Determination of optimal serum and conjugate diluent concentration for the Vi polysaccharide ELISA by comparing the lower optical density at 450nm for 2 concentrations of the skimmed milk powder in PBS/Tween diluent

A 2% diluent concentration gave a lower O.D in the serum control wells compared to the 0.1% diluent concentration. Hence 2% diluent solution was determined to be the optimal diluent concentration for future experiments.

2.1.9 Determination of serum incubation time and temperature

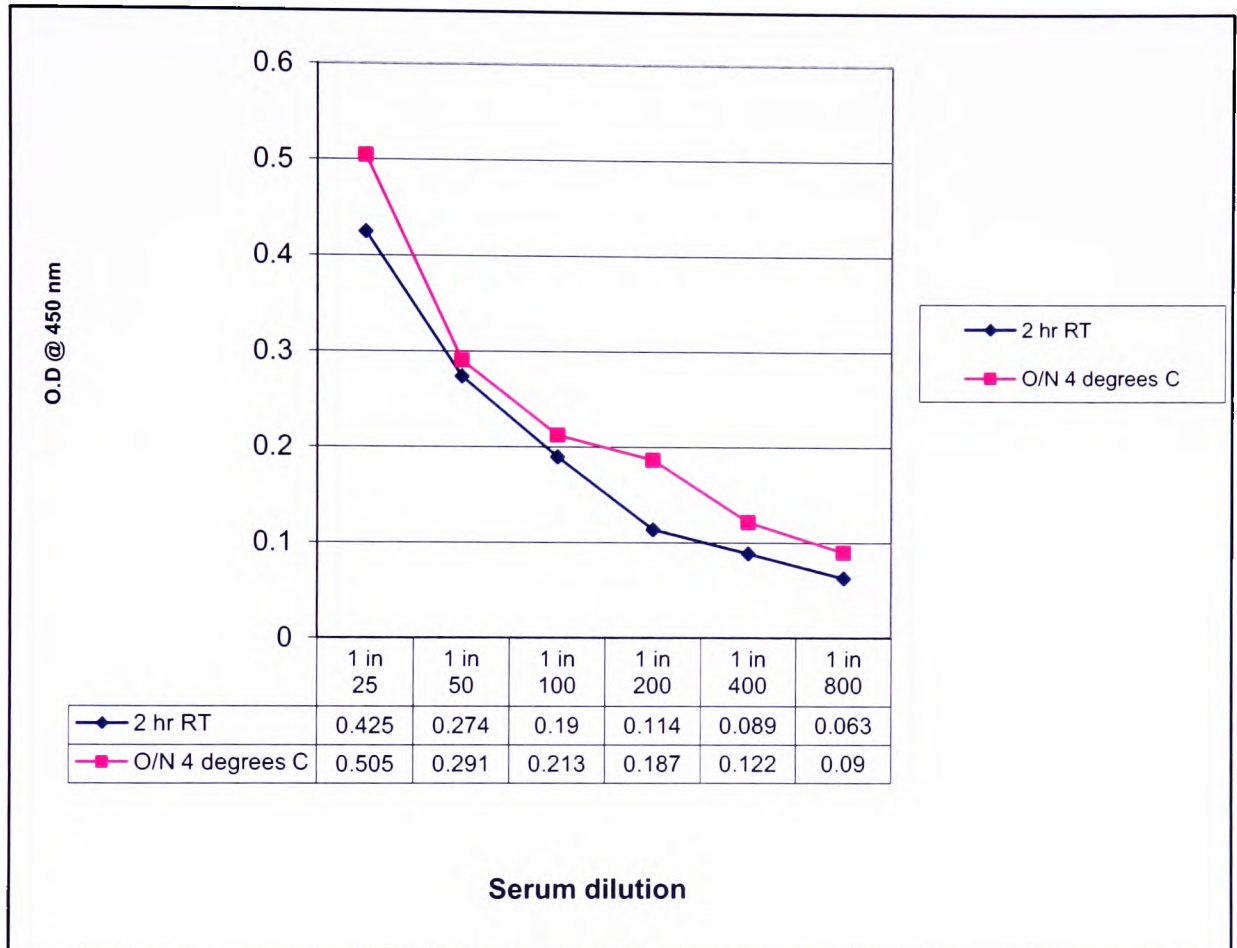


Fig 2.1.9a: Determination of optimal pre-vaccination serum incubation time and temperature for the Vi polysaccharide ELISA by comparing the higher optical density at 450nm for pre-vaccination sera incubated at room temperature for two hours and at 4 degrees Celsius overnight

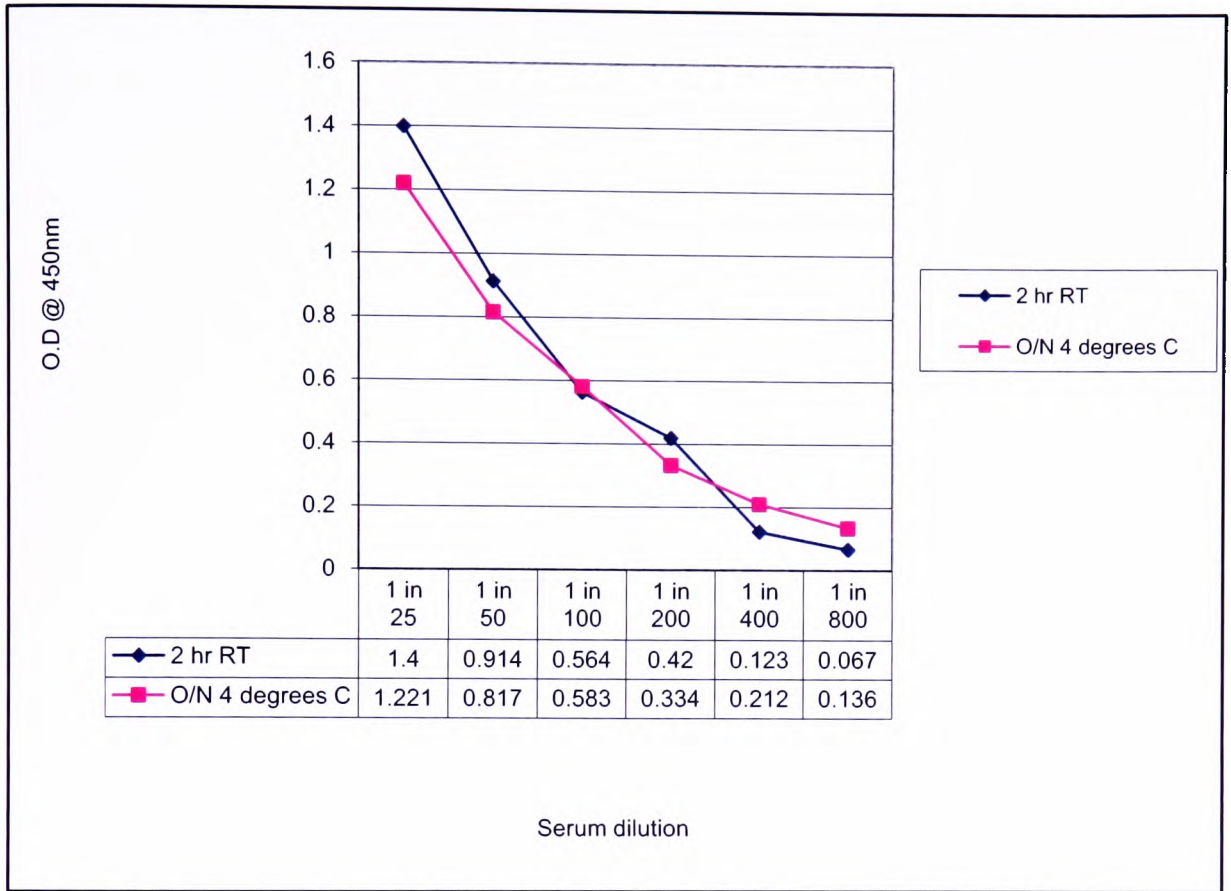


Fig 2.1.9b: Determination of optimal post-vaccination serum incubation time and temperature for the Vi polysaccharide ELISA by comparing the higher optical density at 450nm for post-vaccination sera incubated at room temperature for two hours and at 4 degrees Celsius overnight

There appeared to be little difference between the results of 2 hour incubation at room temperature and overnight incubation at 4 degrees Celsius. Hence, for a faster assay, the 2 hour incubation at room temperature was determined to be optimal for future experiments.

2.1.10 Determination of the incubation time for substrate reaction

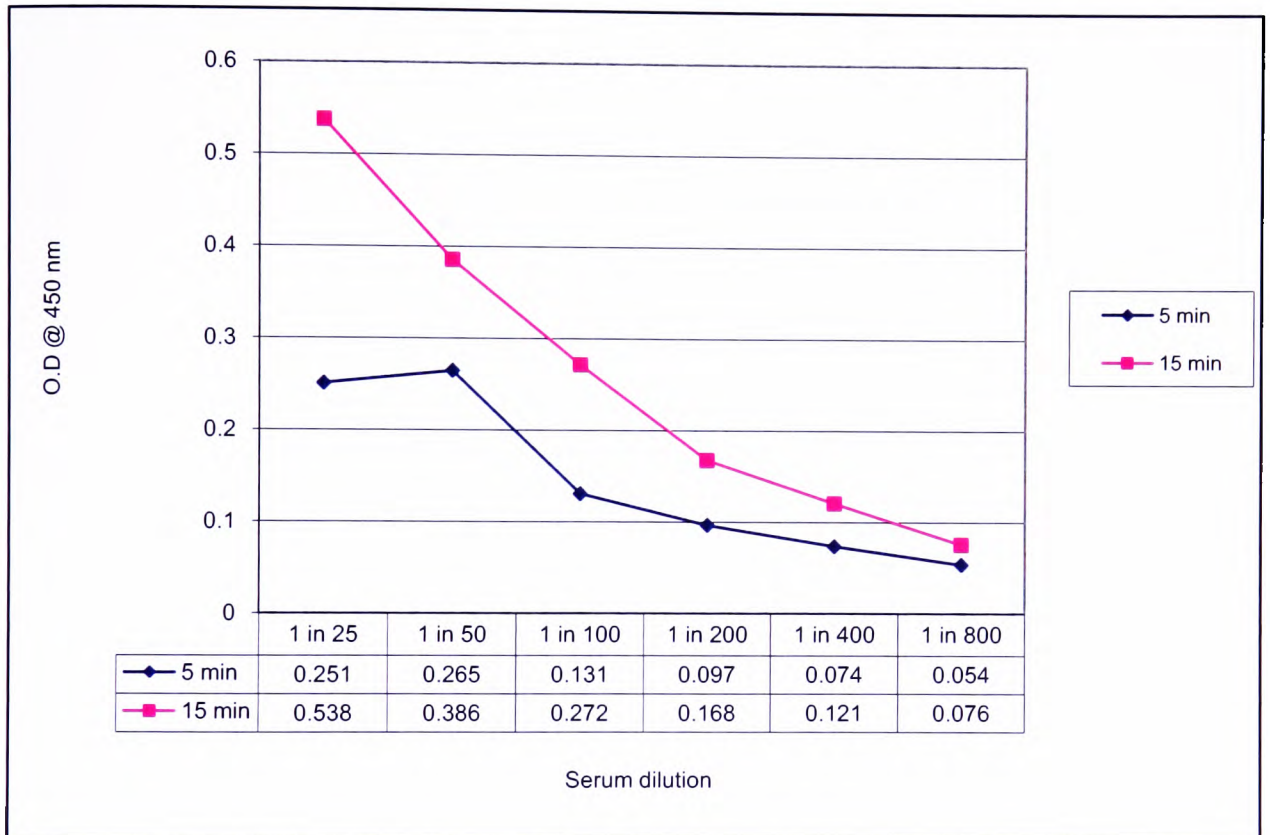


Fig 2.1.10a: Determination of optimal incubation time for substrate reaction for pre-vaccination serum by comparing optical density at 450nm for 5 and 15 minute incubation periods

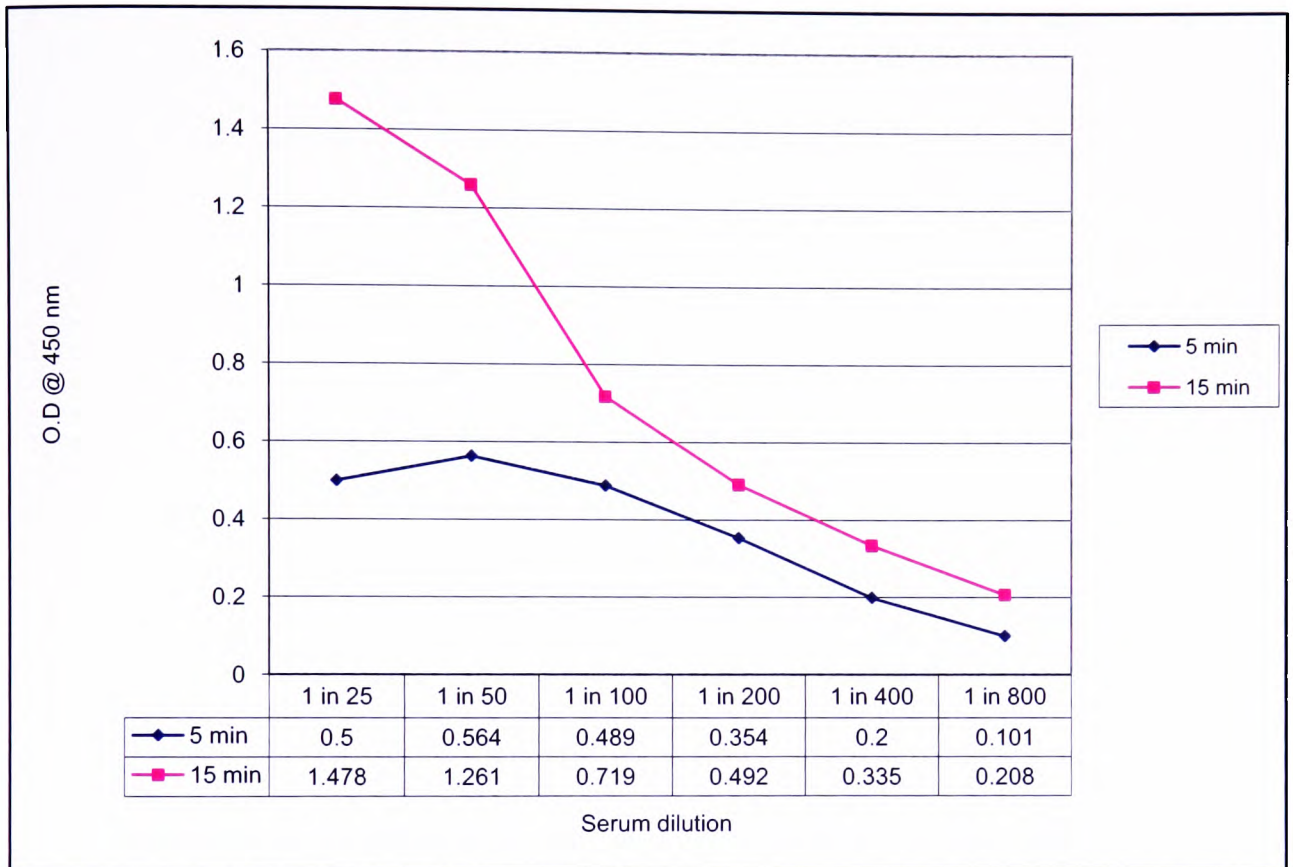


Fig 2.1.10b: Determination of optimal incubation time for substrate reaction for post-vaccination serum by comparing optical density at 450nm for 5 and 15 minute incubation periods

The 15 minutes incubation time gave a higher O.D as compared to the 5 minutes incubation time for both samples of serum. Hence 15 minutes was determined to be optimal for incubation in future experiments.

2.11 Alkaline phosphates vs. Peroxidase ELISA system

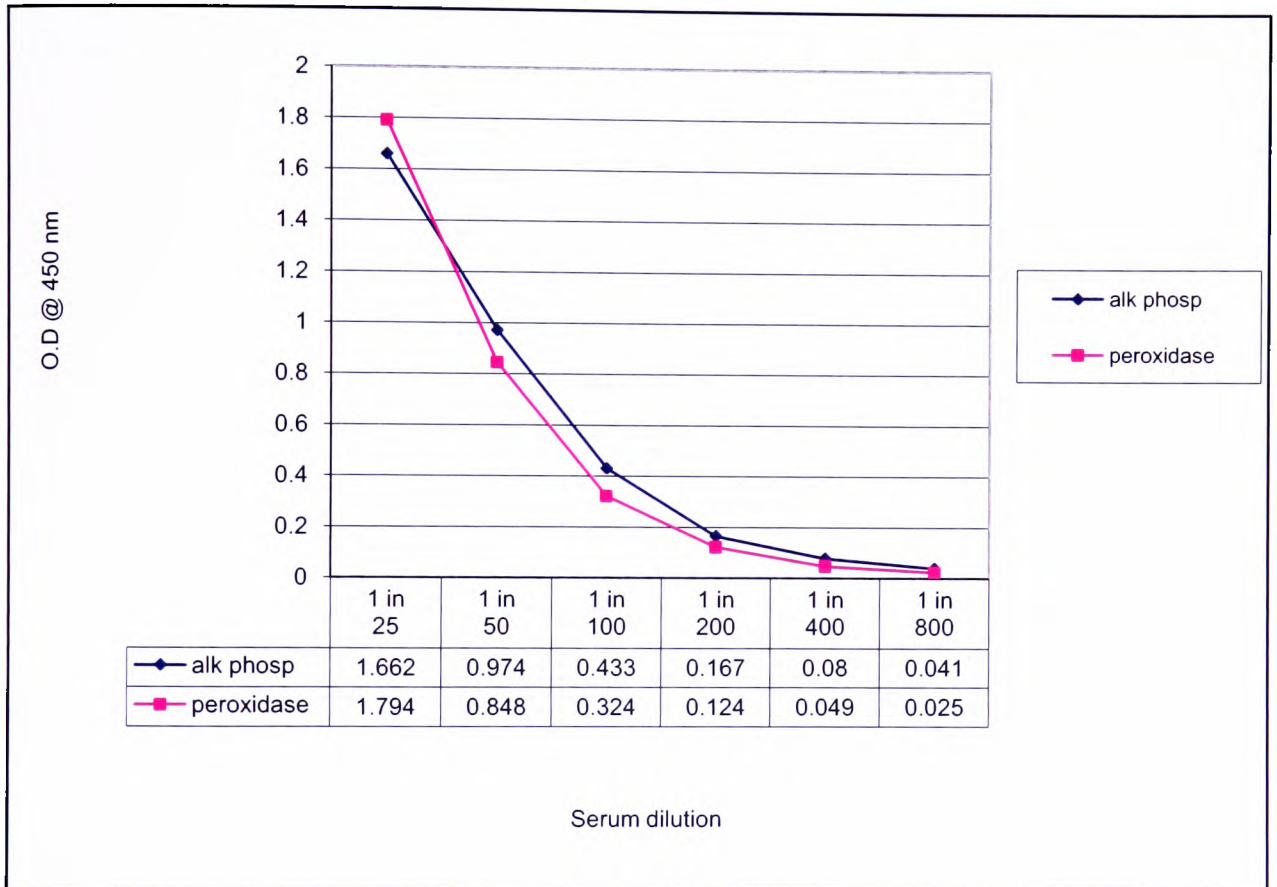


Fig 2.11: Comparison of the Alkaline phosphatase and Peroxidase ELISA systems for post-vaccination serum by estimating the higher optical density at 450nm

There appeared to be little difference between the results of the peroxidase ELISA and the alkaline phosphatase ELISA. Hence, for uniformity with other experiments conducted in the lab, peroxidase ELISA was determined to be optimal for use in future experiments.

2.1.12 mHSA vs. non mHSA ELISA

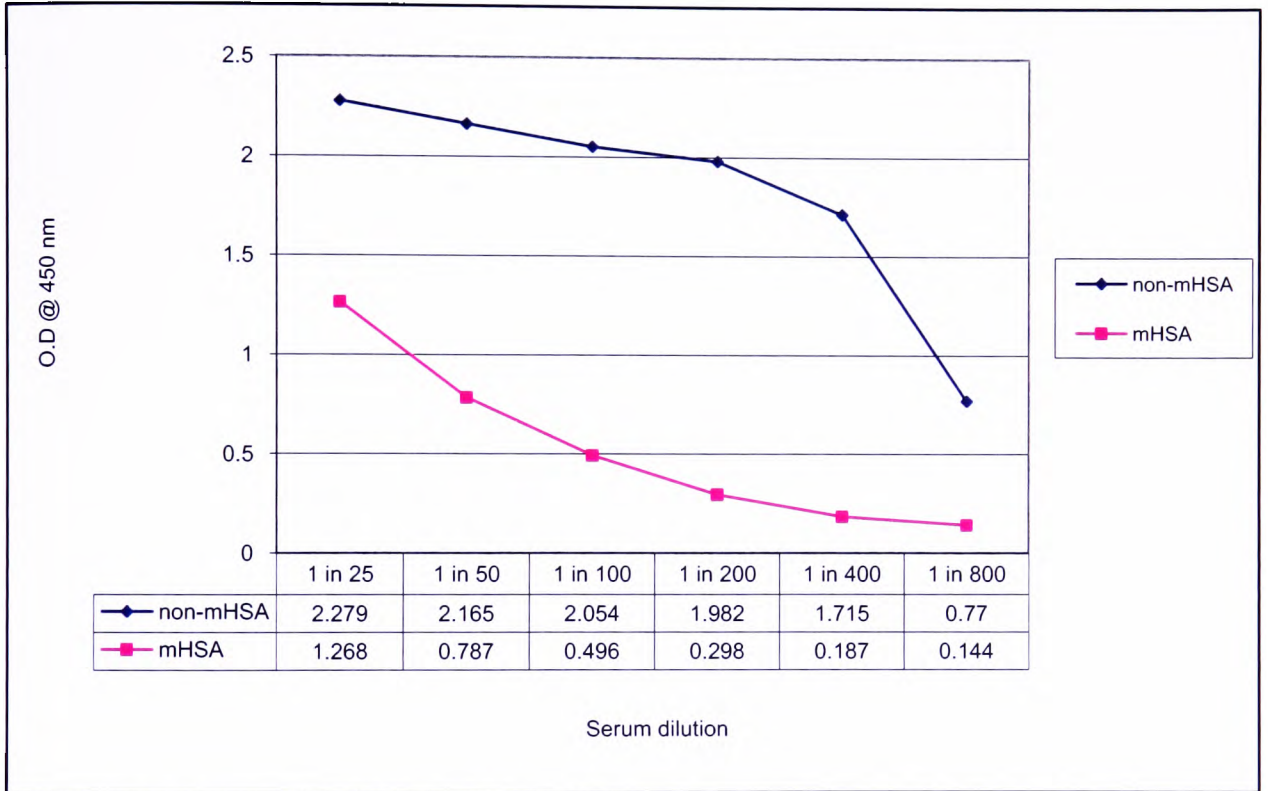


Fig 2.1.12: Comparison of ELISA with and without addition of mHSA to the coating solution by estimating the higher optical density at 450nm between the two solutions

O.D results in the ELISA without the addition of mHSA was higher than the one with the addition of mHSA to the serum. Hence an ELISA without mHSA was determined to be optimal for use in future experiments.

2.1.13 Specificity of Vi polysaccharide ELISA

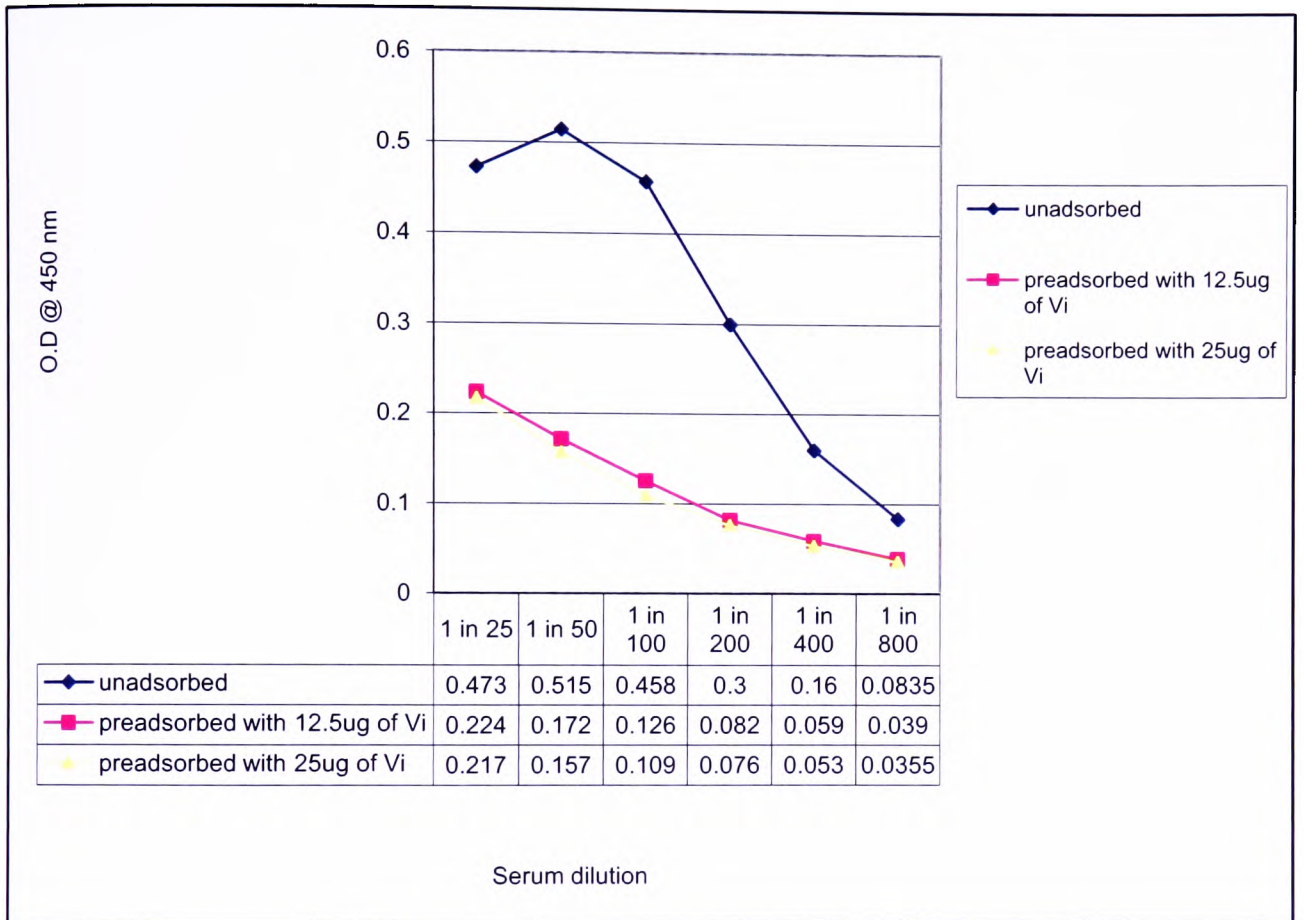


Fig 2.1.13: Specificity of the Vi polysaccharide ELISA determined by pre-adsorbance assay, incubating post-vaccination sera with 2 concentrations of Vi polysaccharide (12.5µg and 25µg) overnight, before estimating the decrease in optical density at 450nm in the Vi polysaccharide ELISA

O.D results of the ELISA using serum preadsorbed with both 12.5µl and 25 µl were lower than the unadsorbed serum, confirming the specificity of the Vi polysaccharide ELISA.

2.1.14 Intra- plate validation

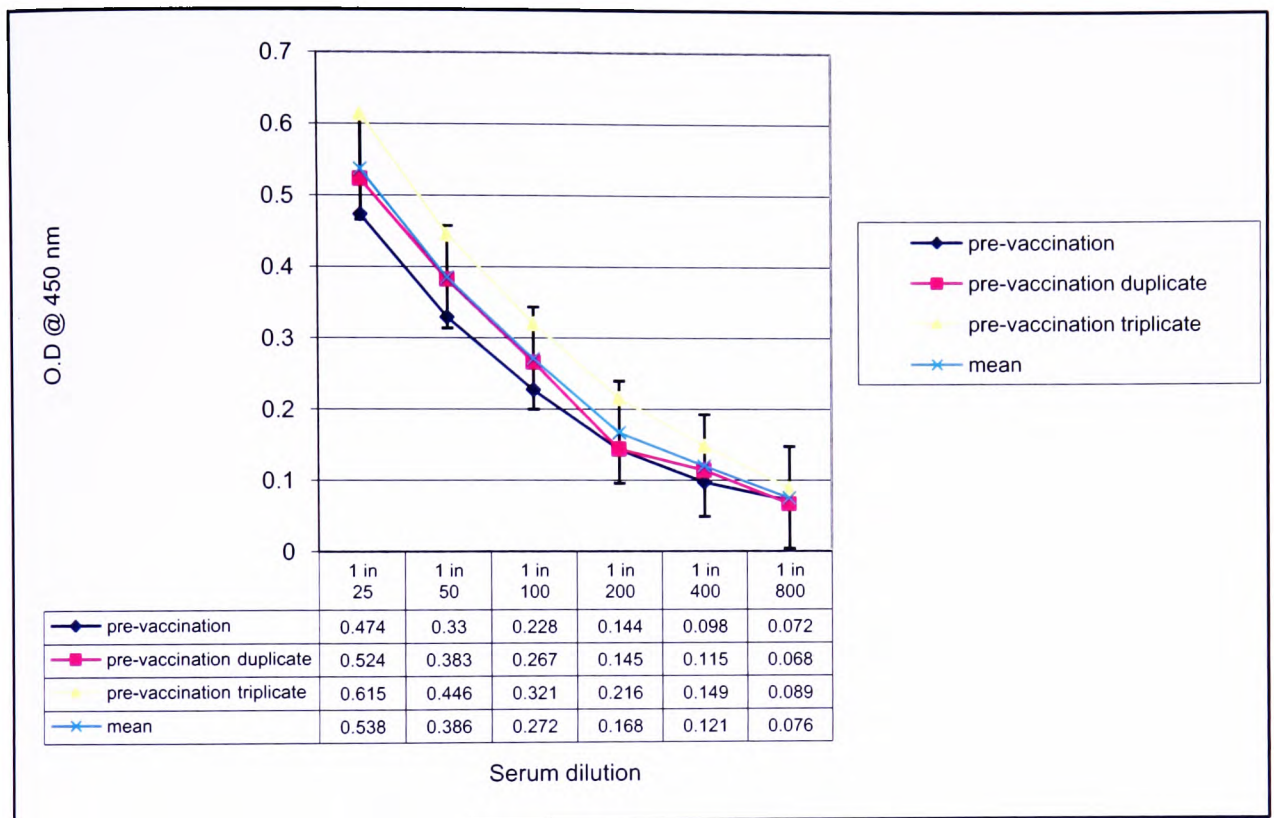


Fig 2.1.14a: Validation of intra-plate variability for results of pre-vaccination serum performed with three samples of sera on the same plate on the same day. The mean optical density at 450nm for each serum dilution is plotted with the standard error of mean bars

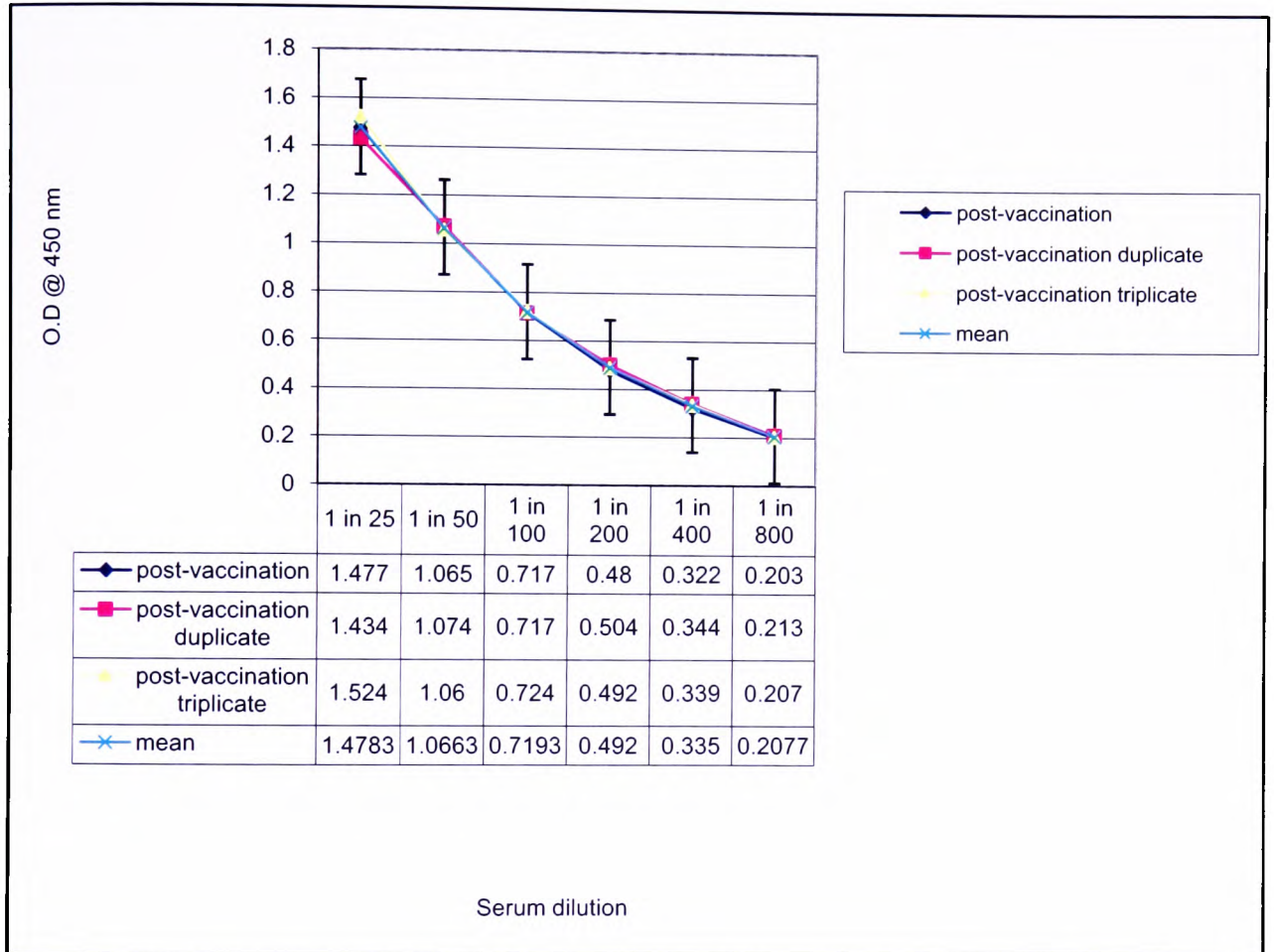


Fig 2.1.14b: Validation of intra-plate variability for results of post-vaccination serum performed with three samples of sera on the same plate on the same day. The mean optical density at 450nm for each serum dilution is plotted with the standard error of mean bars
 There was little intra-plate variability of results using triplicates of pre-vaccination and post-vaccination serum confirming the precision of the assay.

2.1.15 Inter- plate validation

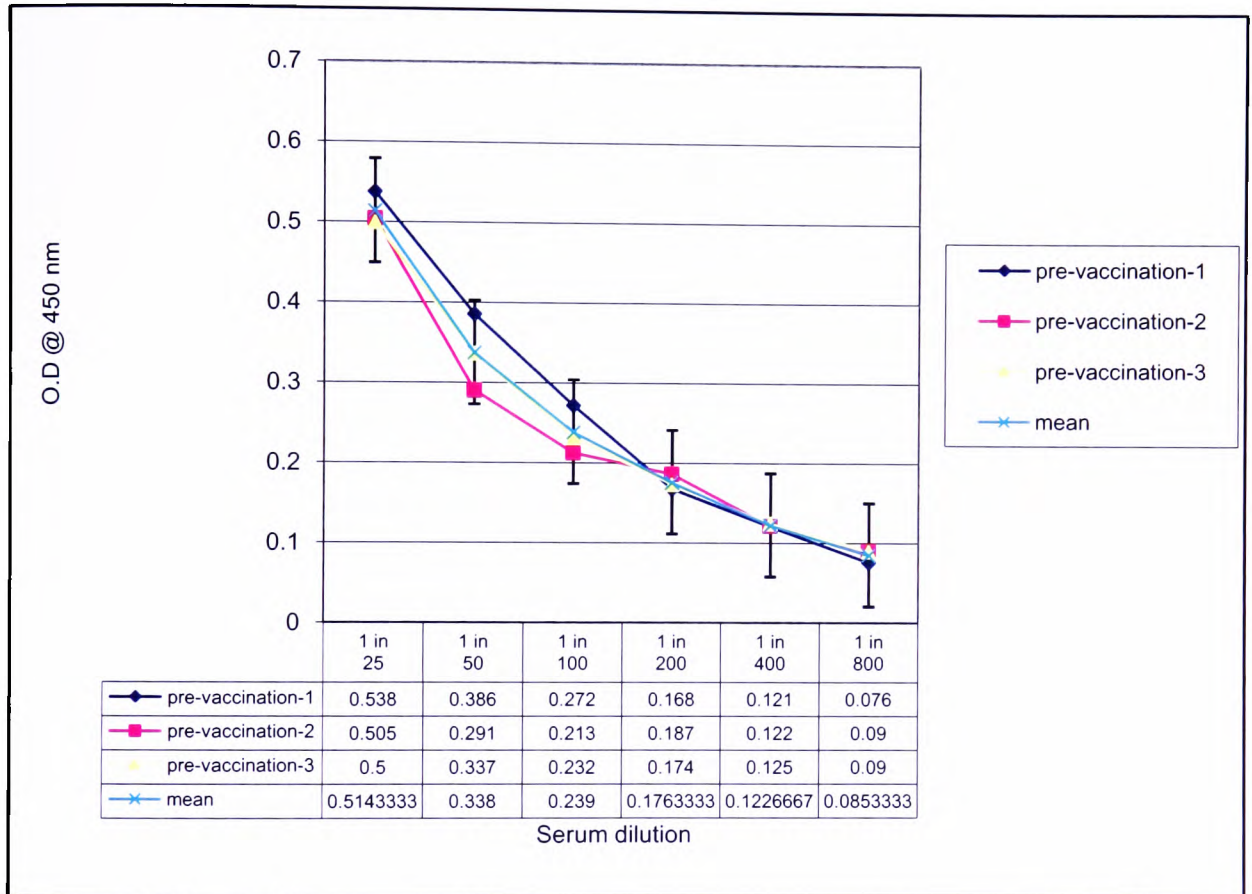


Fig 2.1.15a: Validation of inter-plate variability for results of pre-vaccination serum performed with three samples of sera on three different days. The mean optical density at 450nm for each serum dilution is plotted with the standard error of mean bars

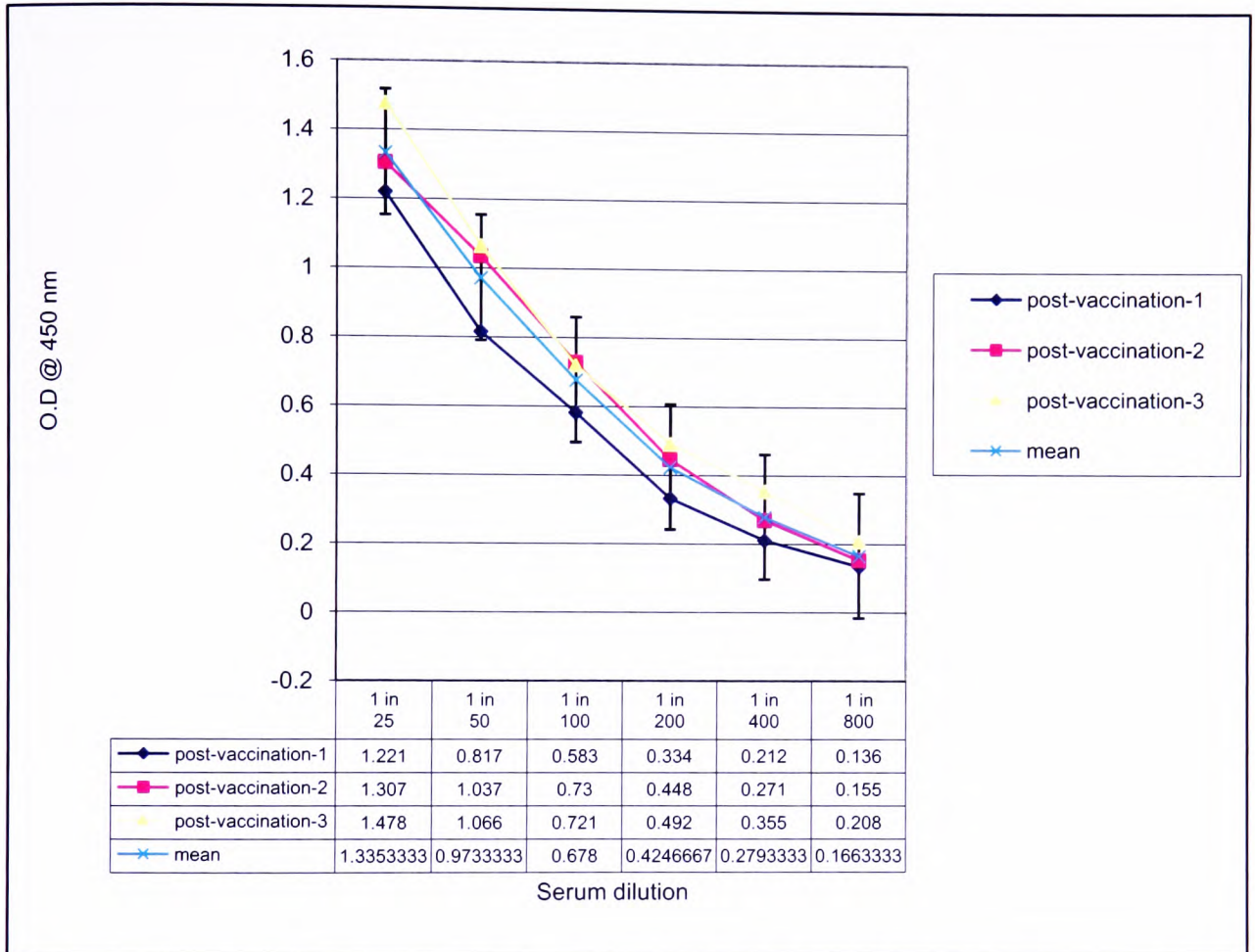


Fig 2.1.15b: Validation of inter-plate variability for results of post-vaccination serum performed with three samples of sera on three different days. The mean optical density at 450nm for each serum dilution is plotted with the standard error of mean bars

There was little variability of results in the inter-plate validation of the Vi polysaccharide ELISA confirming the precision of the assay.

2.1.16 Reference ELISA

At an O.D of 0.6, chosen to coincide with the approximate midpoint of the O.D curve for both test and reference sera, and correcting for the dilution factors of 100 and 800,000 for the test serum and reference serum respectively, the absolute antibody concentration in the test serum was determined to be 1.21mcg/ml (Fig 2.16) in relation to the total IgG concentration in the ERM reference serum which was 9.17g/L. This concentration, arbitrarily selected at a particular O.D factor to coincide with the mid-point of the linear phase of the O.D curve, and test sera served as the reference serum for estimating absolute concentrations of all other sera in the study.

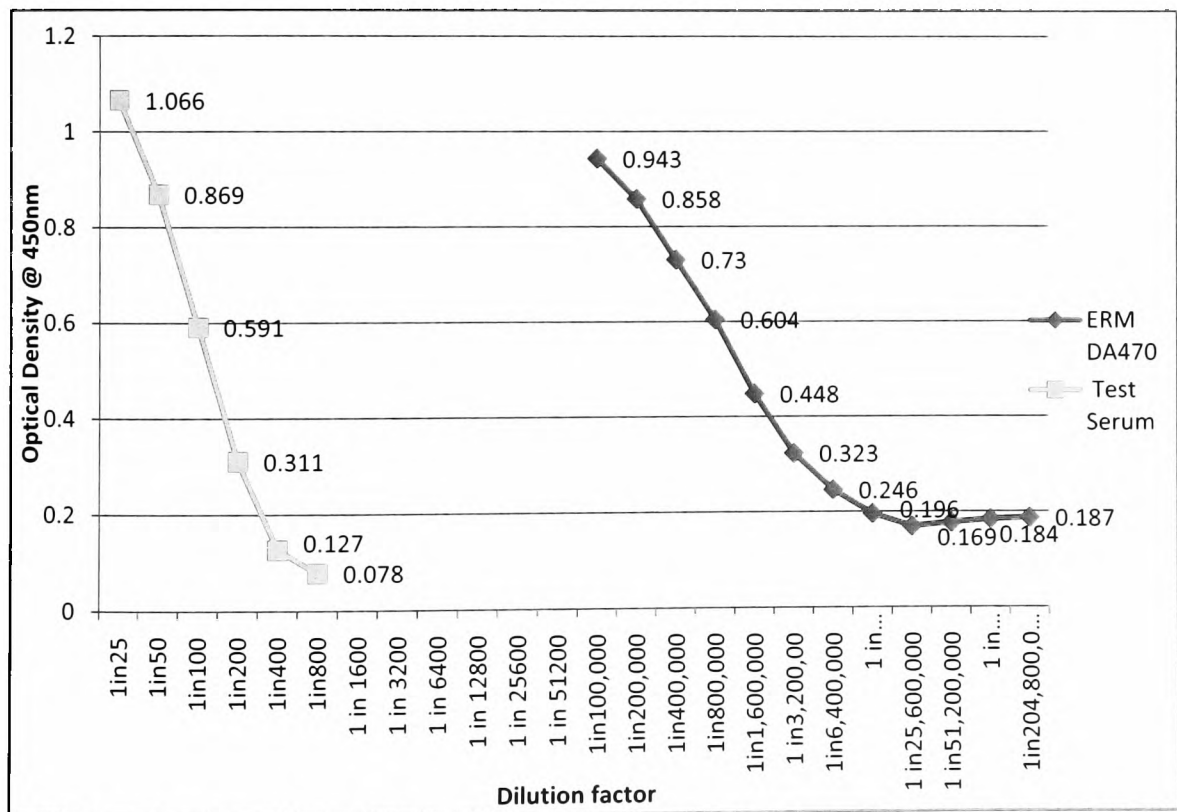


Figure 2.16. Reference ELISA comparing the optical density obtained with *S. typhi* anti-Vi polysaccharide IgG (Test Serum) and European Reference Material DA470

2.1.8 Discussion

For the development of the Vi polysaccharide ELISA the optimal antigen coating concentration, starting serum dilution, conjugate concentration and diluent concentration was determined to be 0.5µg/well, 1:25, 1:1000 and 2% respectively. The optimal blocking agent was determined to be 2% skimmed milk powder. Serum incubation at 37 degrees Celsius for 2 hours was determined to be the optimal incubation time and temperature. Optimal substrate incubation time was determined to be 15 minutes. Peroxidase ELISA was determined to be better than alkaline phosphatase. Results from ELISA without mHSA addition to serum gave better results than one with mHSA addition. Specificity of the Vi polysaccharide ELISA was confirmed by a pre-adsorbance assay. Validation of intra-plate and inter-plate variability of results demonstrated the assay to be accurate and precise.

2.2 Development and Validation of a *Citrobacter freundii* Serum Bactericidal Assay for *Salmonella typhi*

2.2.1 Introduction

The Vi polysaccharide capsule of *Salmonella typhi* is also found in a few other organisms including *Citrobacter freundii*, *Salmonella paratyphi C* and certain mutant strains of *Escherichia coli*. In contrast to *Salmonella typhi*, *Citrobacter freundii* is a relatively non-pathogenic organism. Case reports of *Citrobacter freundii* disease are anecdotal and are mostly in the immunocompromised^{198, 199, 200, 201}. In this experiment a novel bactericidal assay was designed to determine functional humoral immunity against *Salmonella typhi* utilizing the Vi polysaccharide antigenic similarity of *Citrobacter freundii*. For the development and validation of this assay, pre-vaccination and post-vaccination serum samples from volunteers who had been vaccinated with the Vi polysaccharide vaccine was used. The principle of this experiment was that the anti-Vi polysaccharide antibodies, in the serum of volunteers vaccinated with the *Salmonella typhi* Vi polysaccharide, would kill *Citrobacter freundii* (due to the Vi polysaccharide antigenic similarity between the two organisms) in the presence of an exogenous source of complement, thereby making *Citrobacter freundii* a reliable and safe surrogate for *Salmonella typhi* in the assessment of functional immunity against the latter involving the Vi polysaccharide capsule. For the development of the *Citrobacter freundii* serum bactericidal assay, the growth curves of the bacteria were initially determined, followed by the estimation of the optimal exogenous complement dilution, bacterial harvesting time and reaction volume. Specificity of the SBA was confirmed by a pre-adsorbance assay. The assay was finally validated by an intra-plate and inter-plate variability analysis using pre-vaccination and post-vaccination serum.

2.2.2 Determination of bacterial growth curves for *Citrobacter freundii*

The aim of this experiment was to construct a bacterial growth curve for *Citrobacter freundii* to determine the lag phase, log phase and stationary phase for the organism.

Materials & Equipment:

1. Nutrient agar plates (JR Microbiology Kitchen, Oxford)
2. Muller-Hinton broth (JR Microbiology Kitchen, Oxford)
3. 10µl flexible disposable microbiological loops (Copan)
4. Incubator with thermometer (Genlab)
5. Automatic colony counter (Anderman)
6. PBS solution, freshly prepared and autoclaved (Sigma)
7. *Citrobacter freundii* (NCTC strain 9750)
8. Spectrophotometer (Perkin-Elmer)

Day 1:

1. From the *Citrobacter freundii* stock (NCTC strain 9750) stored in glycerol at -80 degrees Celsius, an aliquot was defrosted and plated onto a nutrient agar plate using a disposable 10µl flexible microbiological loop. The plate was incubated at 37 degrees Celsius overnight (~18 hours).

Day 2:

One colony from the nutrient agar plate was sub-cultured in 20 ml of MH broth overnight (~18 hours)

Day 3:

MH broth tube was gently agitated to obtain a uniform mixture of bacteria. 75µl of broth was inoculated into each of the 24 bottles of 20 ml MH solution. (8 time points, in triplicate, hence 24 bottles). O.D of the overnight broth was checked at 540 nm. 500µl from each of the three '0' hours time period broth was diluted in 4.5 ml of PBS solution in a bijou container. The above solution (500µl in 4.5 ml) was serially diluted to the following concentrations: 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} . The first three dilutions were discarded. From the latter 5 dilutions, 100µl was plated onto a nutrient agar plate. The plates were incubated at 37 degrees Celsius overnight. Steps 3 to 7 were repeated at the following time periods: 0.5 hours, 1 hour, 2 hours, 5 hours, 8 hours, 12 hours and 24 hours. The colonies were counted after overnight culture on all plates. The following graphs were plotted:

- a. O.D vs. time points
- b. CFU vs. time points for each dilution
- c. CFU and O.D (primary and secondary axes) vs. time points

2.2.3 Serum Bactericidal Assay Protocol for *Citrobacter freundii*

Materials:

LB agar plates (JR Microbiology Kitchen, Oxford)

2. 10 μ l flexible disposable microbiological loops (Copan)

4. Incubator with thermometer (Genlab)

5. Automatic colony counter (Anderman)

6. PBS solution, freshly prepared and autoclaved (Sigma)

7. *Citrobacter freundii* (NCTC strain 9750)

8. Flat bottom, 96 well, tissue culture plates (Corning)

9. Hanks Balanced Salt Solution (Sigma)

10. Baby rabbit complement (Pel-Freeze Biologicals)

11. Bijou container

12. Spectrophotometer (Perkin-Elmer)

Day 1 (Preparation of target strain):

From the *Citrobacter freundii* stock (NCTC strain 9750) stored in glycerol at -80 degrees Celsius, an aliquot was defrosted and plated onto an LB agar plate using a disposable 10 μ l flexible microbiological loop. The plate was incubated at 37 degrees Celsius overnight, without CO₂ (~18 hours)

Day 2

Approximately one third of the colonies formed on the plate were picked and suspended thoroughly in 5 ml PBS solution in a bijou container. 500 μ l from the above suspension was pipetted and re-suspended thoroughly in 4.5 ml PBS solution in a bijou container. The optical density of the above suspension was measured on a spectrophotometer at 540 nm. The O.D was ensured to be between 0.15 and 0.25. Sterile water was added or aliquots from the above suspension were added to get the required O.D reading. 1 μ l from the above solution was pipetted and re-suspended in 5 ml of PBS solution in a bijou. This was the working solution of bacteria for the assay. In a flat bottom tissue culture plate 40 μ l of Hanks Balanced Salt Solution was added to each well. 20 μ l of HBSS was added to the column of wells for the Complement Independent Control (CIC). 40 μ l of heat inactivated (56 degrees Celsius for 30 min) test serum was added to the first column of the plate and serially diluted across the plate till the dilution factor required was reached. 40 μ l of the mix was discarded from the last column. 20 μ l of serum was added to the CIC column and none to the Complement Control (CC) and Viable Count (VC) columns. 20 μ l of the working solution of bacteria was added to each well. The plate was incubated at room temperature for 10 minutes. Baby rabbit complement diluted 1:8 in cold HBSS was added. Handling of complement was on ice. 20 μ l of the diluted complement was added to each well on the plate except the VC and CIC well. 10 μ l of heat inactivated complement (56 degrees for 30 minutes) was added to the VC and the CIC columns. The sealed tissue culture plate was incubated at 37 degrees Celsius for one hour.

10 μ l from each well was plated onto the labeled half plate of an LB agar plate using the agar overlay method. The plates were incubated at 37 degrees Celsius overnight, without CO₂ (~18 hours).

Day 3:

The colonies per half plate were counted. A graph with serum dilution factors on the x-axis and CFU counts on the y-axis was plotted. Another graph with serum dilution factor on the x-axis and percentage survival (calculated as {CFU count/VC for that sample} * 100) was plotted. The serum bactericidal titre was the inverse titre of the lowest dilution at which there was 50% killing of *Citrobacter freundii*.

2.2.4 Determination of optimal bactericidal reaction volume

To determine the optimal bactericidal reaction volume in the assay that gave maximum reproducibility and therefore standardisation of bacterial count at the end of the experiment, we compared a total reaction volume of 40 μl and 120 μl . For the former the following mix was used for the reaction:

HBSS buffer: 20 μl

Serum (1:60): 20 μl

After thoroughly mixing the above, 20 μl was discarded

Citrobacter freundii solution: 10 μl

Complement dilution: 10 μl

For the latter experiment the following mix was used for the reaction:

HBSS buffer: 60 μl

Serum (1:60): 30 μl

After thoroughly mixing the above, 40 μl was discarded

Citrobacter freundii solution: 30 μl

Complement dilution: 30 μl

The serum bactericidal assay protocol mentioned above was followed. Each experiment was repeated in 10 wells on the same plate. CFU counts from all 10 wells were plotted on the graph

2.2.5 Determination of optimal complement dilution and optimal harvesting time for *Citrobacter freundii*

To determine the intrinsic bactericidal effect of an exogenous complement source and to determine the optimal complement dilution for use in the SBA and harvesting time for *Citrobacter freundii* from the stock culture, 3 different concentrations of baby rabbit complement: a final concentration of 1:8 (starting dilution of 1:2), 1:16 (starting dilution of 1:4) and 1:32 (starting dilution of 1:8) were compared. 2 arbitrary time points (6 hours and 18 hours) to select bacteria in mid-log phase and in stationary phase after sub-culturing were chosen to harvest bacteria in the mid-log phase and in the stationary phase to compare the effect of harvesting time on the SBA. Quadruplicates of complement control wells and duplicates of viable count wells were maintained to estimate percentage of killing due to the exogenous complement source. The following steps were done in this experiment:

Citrobacter freundii was grown overnight (18 hours) at 37 degrees Celsius from glycerol stock on an LB agar plate. Another LB plate was plated with *Citrobacter freundii* from glycerol stock and incubated at 37 degrees Celsius for 6 hours. Working solution was prepared from both the above cultures according to the SBA protocol given above.

The reaction mix was as follows:

60 µl of HBSS

30 µl of working solution of *Citrobacter freundii* (6 hours and 18 hours)

30 µl of complement dilution (1:8, 1:16 and 1:32)

The plate was incubated for 45 minutes at 37 degrees Celsius. 10µl from each plate was inoculated onto half an LB agar plate, incubated overnight at 37 degrees Celsius and colonies formed were then counted.

2.2.6 Specificity of *Citrobacter freundii* SBA

Specificity of the *Citrobacter freundii* SBA was determined by a pre-adsorbance assay. The principle of the experiment was that pre-adsorbance of post-vaccination serum, containing anti-Vi polysaccharide antibodies, with Vi polysaccharide would result in the binding of anti-Vi polysaccharide antibodies to the Vi polysaccharide resulting in the reduction of unbound antibodies from the serum. This would then have been evidenced by a reduced titre of killing in the SBA with the pre-adsorbed serum. The following steps were used for the specificity assay:

Serum was diluted to double the required concentration i.e.: 1: 16 for the starting serum dilution of 1:32. The Vi polysaccharide was prepared to 100µg/ml, 50µg/ml and 25 µg/ml of sterile pyrogen free water. The serum was then mixed with an equal amount of 100µg/ml, 50µg/ml and 25µg/ml of Vi polysaccharide solution. The mix was incubated at 4 degrees Celsius overnight. Rest of the SBA was as per the protocol.

2.2.7 Validation for Intra-plate variability

Following the development of the *Citrobacter freundii* SBA, it was then validated for the intra-plate variability of CFU counts and percentage killing for a post-vaccination serum. The experiment was performed in triplicate on the same tissue culture plate and the experiment repeated on three different days.

2.2.8 Validation for Inter-plate variability

This experiment was performed to determine the inter-plate variability of CFU count and percentage killing on the same sample analysed on three different days. Each experiment was performed in triplicate and the percentage killing values, along with the standard error intervals, plotted on the graph.

2.2.9 Quality Control and Acceptance Criteria for SBA Results

1. Each tested sample had three control wells consisting of the following:
 - a. Complement Control: This control was included to detect any intrinsic bactericidal activity of the exogenous complement source
 - b. Viable Count: This control was included to determine the standard CFU count of bacteria in the absence of serum or complement
 - c. Complement Independent Control: This control was intended to detect any intrinsic bactericidal effect of serum, in the absence of complement, including intrinsic complement not inactivated by heating at 56 degrees for 30 minutes or antibiotic presence.
2. The inverse of the highest serum dilution (lowest concentration) demonstrating > 50% killing as compared to the viable count was considered the bactericidal titre if:
 - a. The Complement control count was >50% of the VC
 - b. The Complement Independent Control was >50% of the VC
3. All samples were tested in duplicates and the mean value was recorded for analysis
4. A Quality Control sample with a known bactericidal titre, donated by a volunteer who received the Vi polysaccharide vaccine was tested each day along with the test samples. The results were accepted if the bactericidal titre of the control sample was ± 1 known bactericidal titre.

Results

2.2.2 Determination of bacterial growth curves for *Citrobacter freundii*

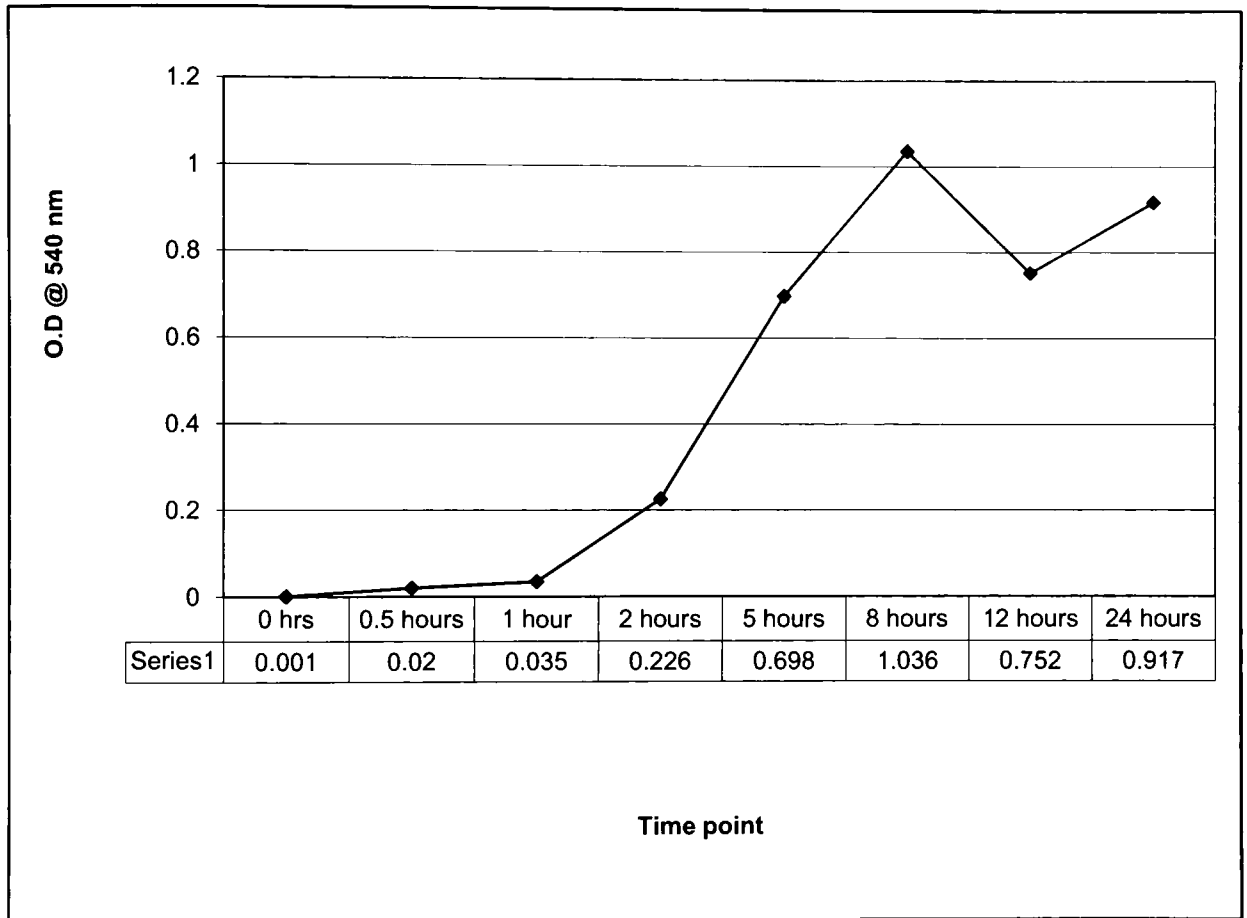


Fig 3.2a: Growth curve of *Citrobacter freundii* plotting optical density at 540nm over 8 time points of the broth culture

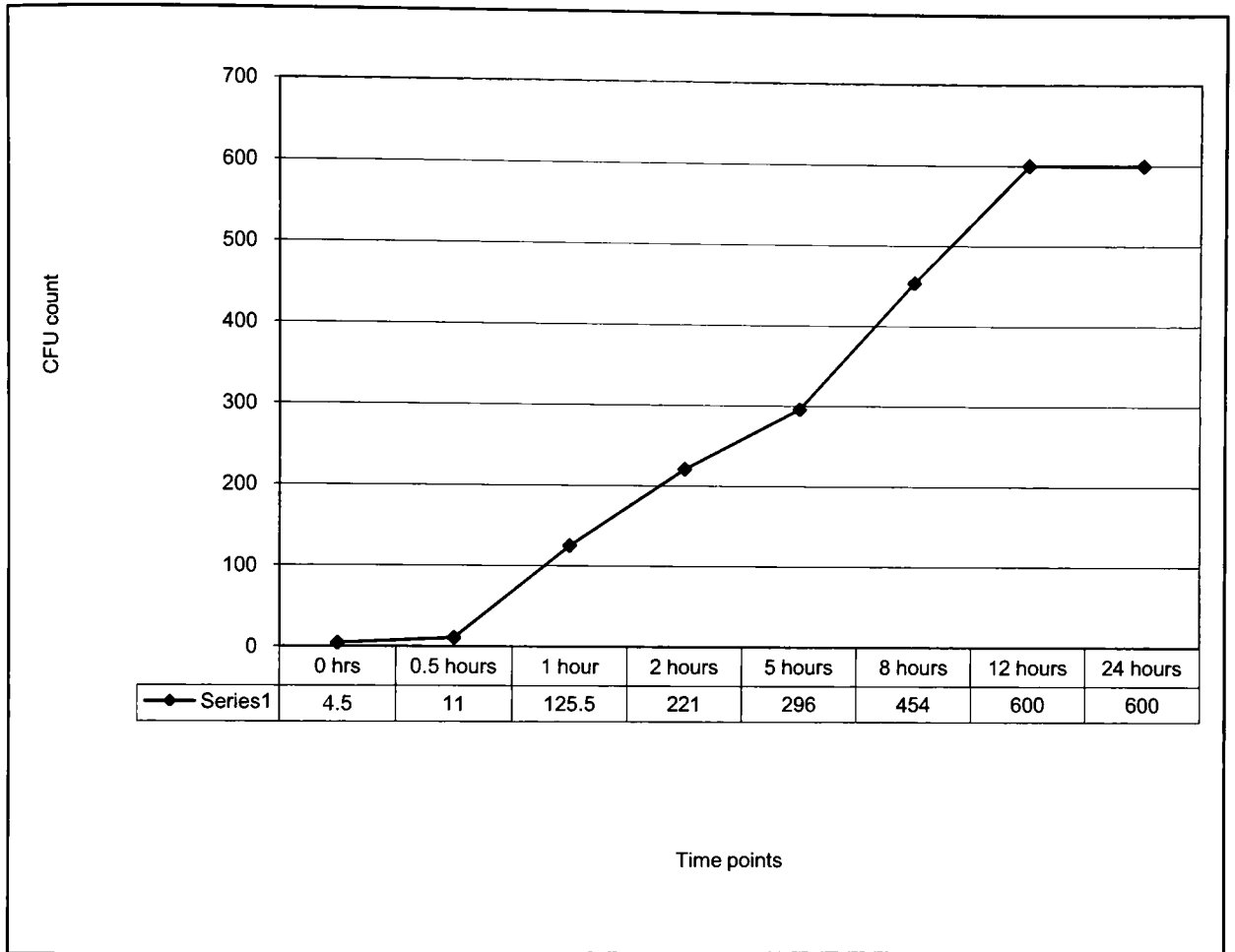


Fig 3.2b: Growth curve of *Citrobacter freundii* plotting colony forming unit count over 8 time points of the broth culture (At a dilution of 10^{-4})

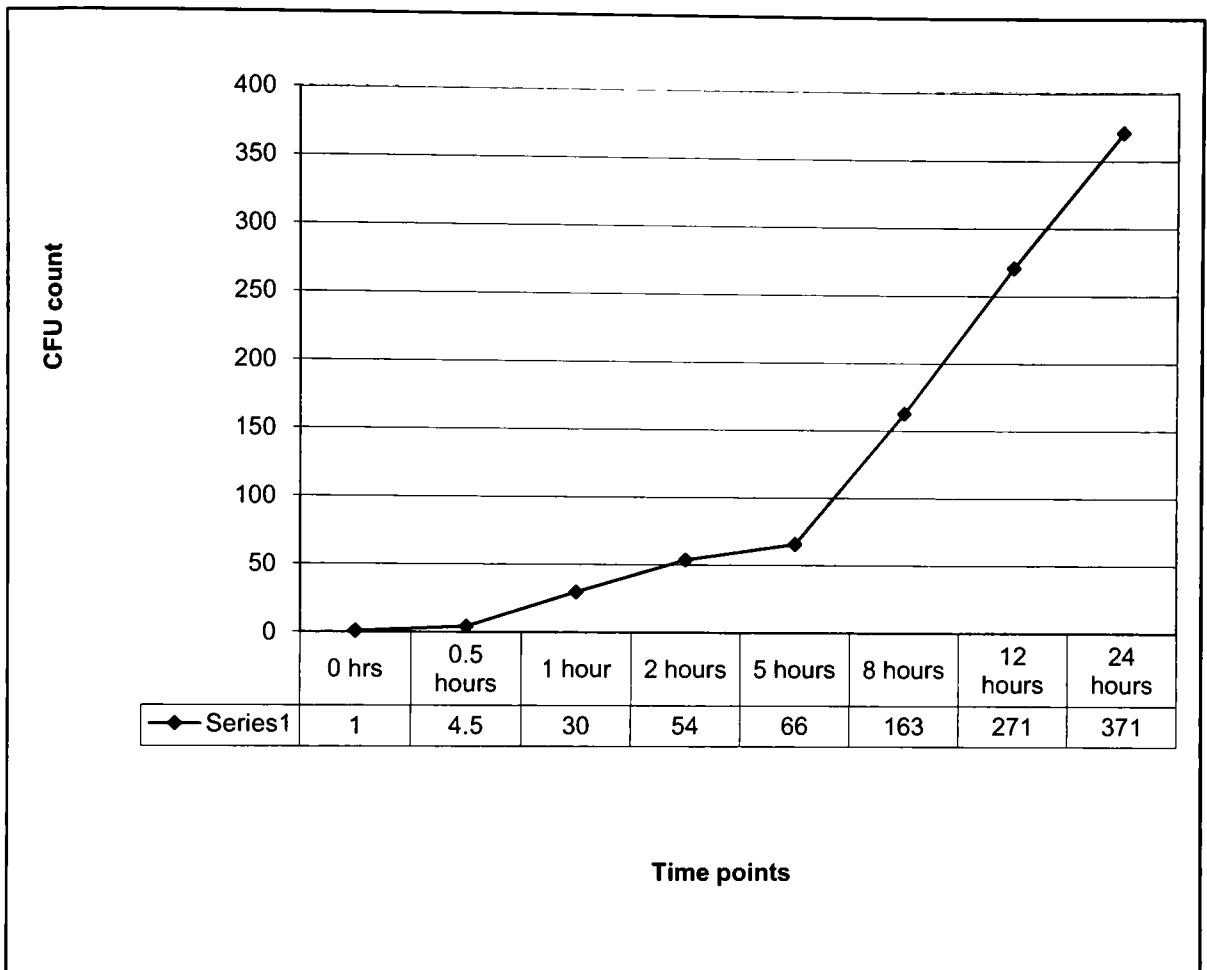


Fig 3.2c: Growth curve of *Citrobacter freundii* plotting colony forming unit count over 8 time points of the broth culture (At a dilution of 10^{-5})

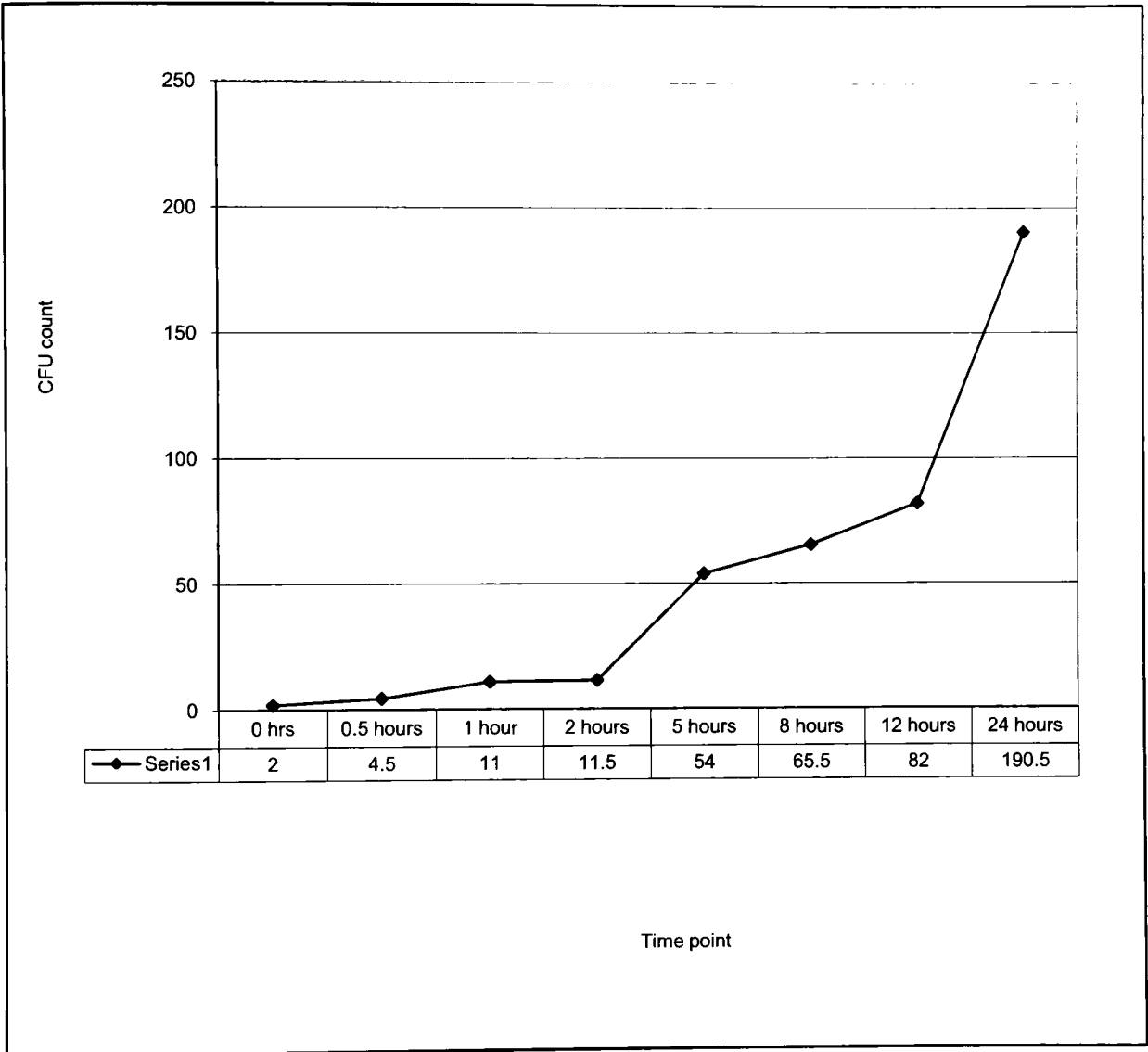


Fig 3.2d: Growth curve of *Citrobacter freundii* plotting colony forming unit count over 8 time points of the broth culture (At a dilution of 10^{-6})

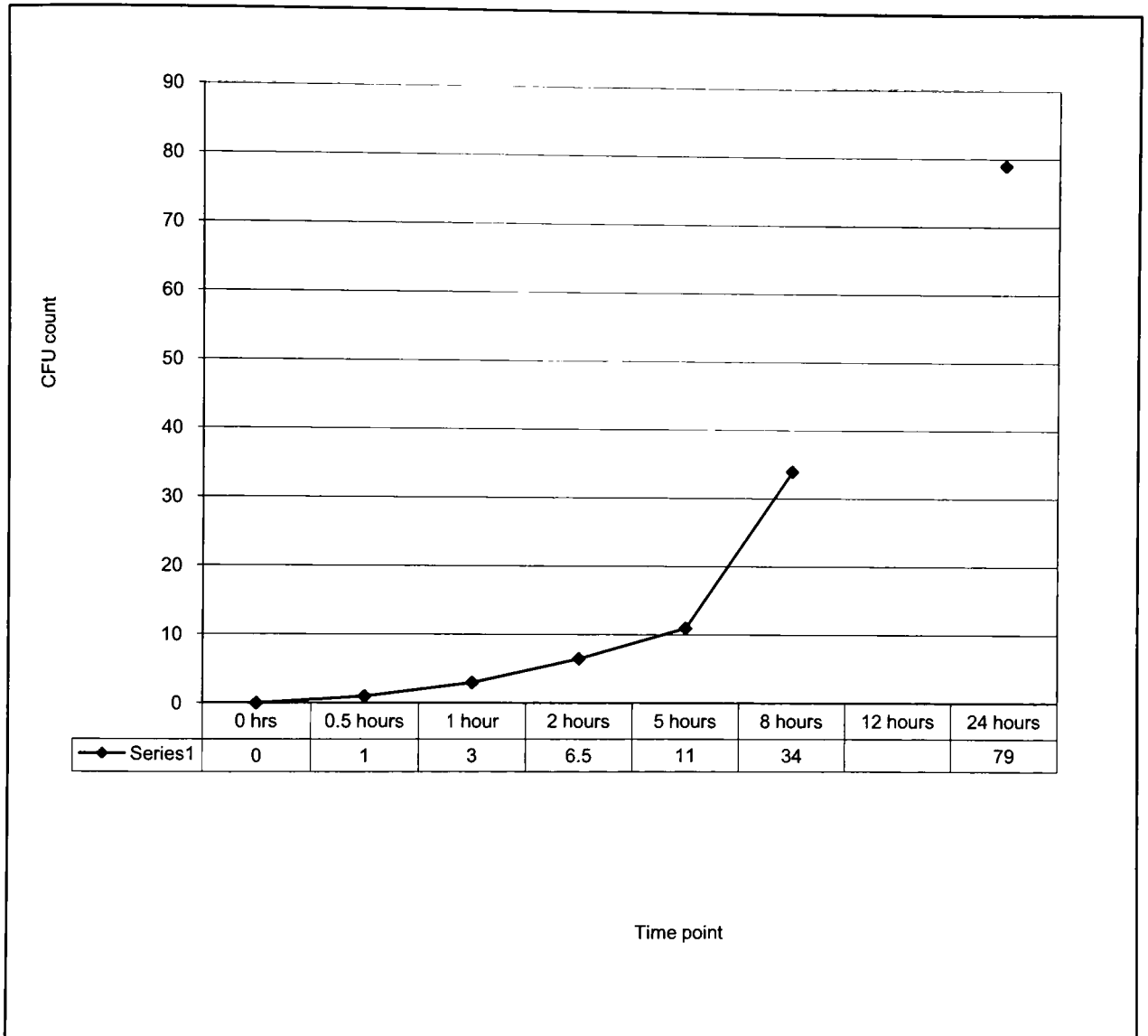


Fig 3.2e: Growth curve of *Citrobacter freundii* plotting colony forming unit count over 8 time points of the broth culture (At a dilution of 10^{-7})

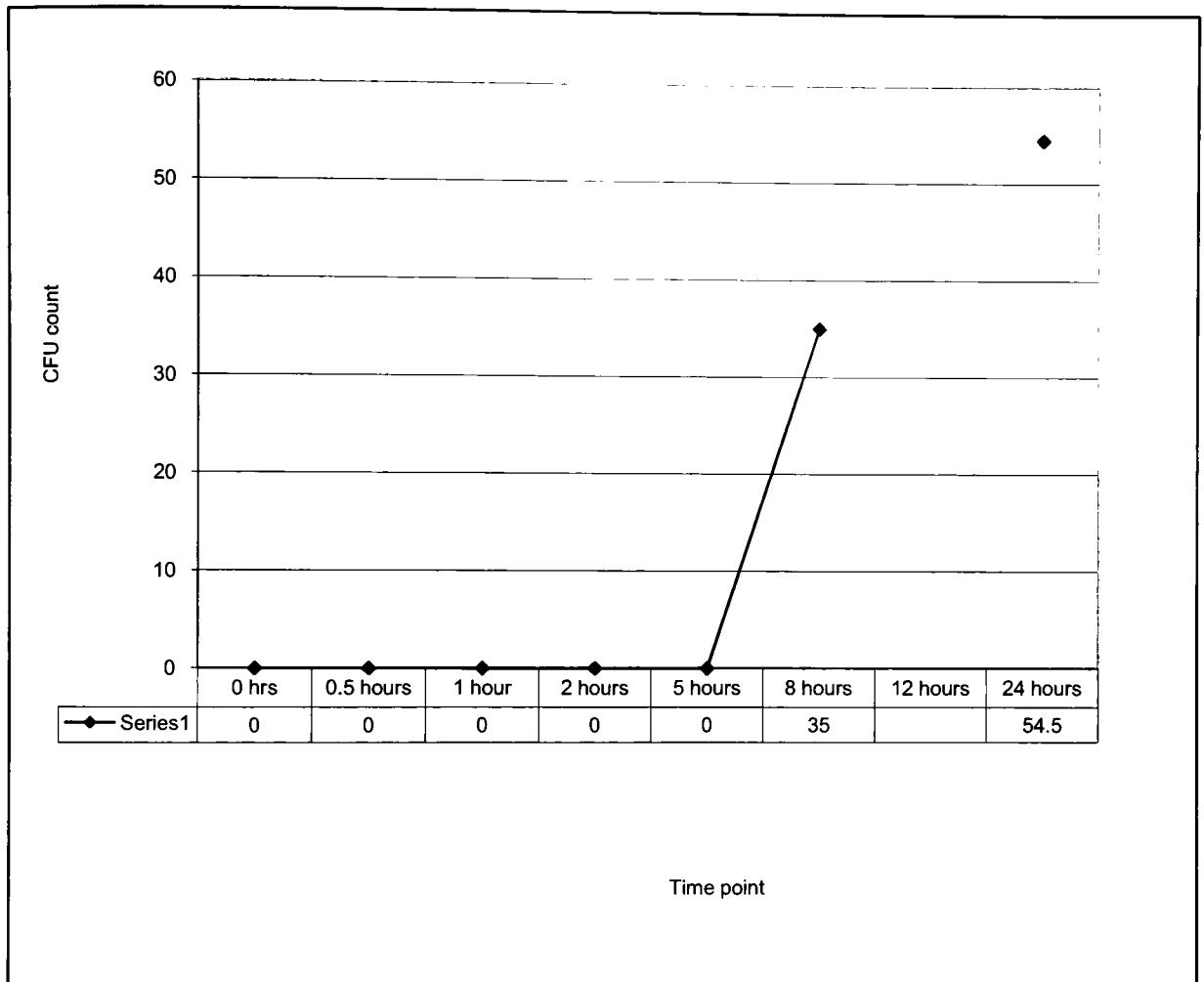


Fig 3.2f: Growth curve of *Citrobacter freundii* plotting colony forming unit count over 8 time points of the broth culture (At a dilution of 10^{-8})

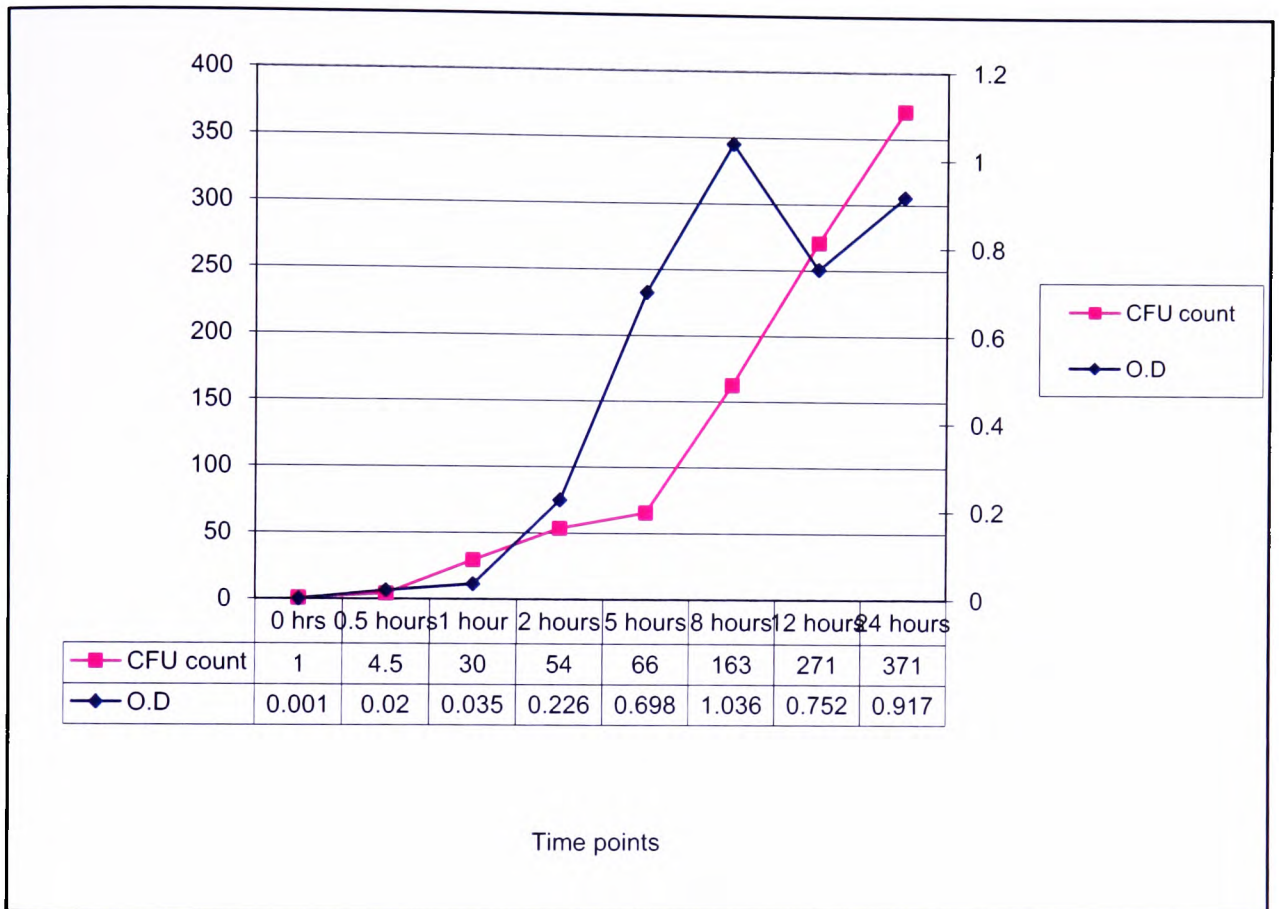


Fig 3.2g: Growth curve of *Citrobacter freundii* plotting colony forming unit count and optical density at 540 nm over 8 time points of the broth culture (At a dilution of 10^{-5})

The lag phase of bacterial growth was determined to be between 0 hours and 4 hours, the log phase between 4 hours and 12 hours and the stationary phase between 12 hours and 24 hours.

2.2.3. Determination of optimal bactericidal reaction volume

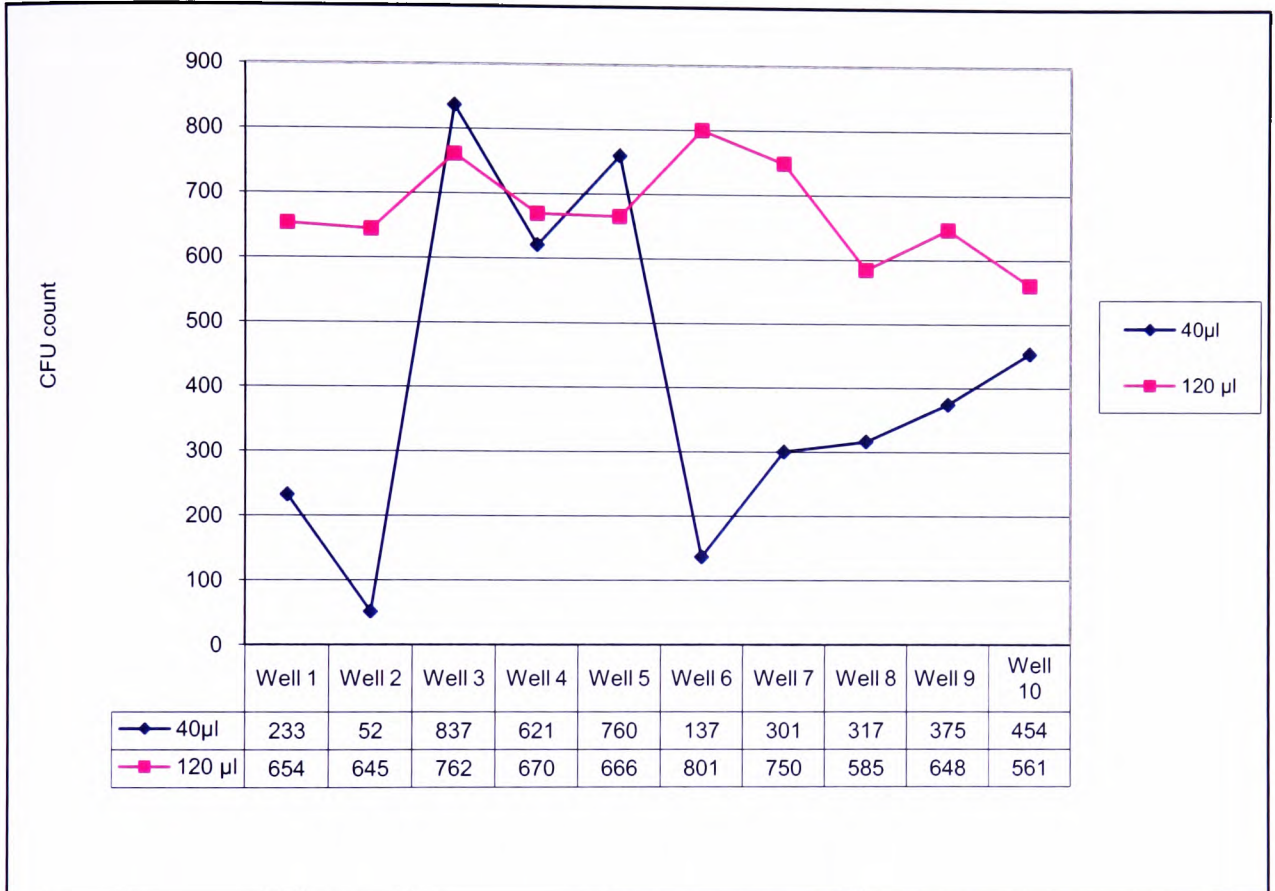


Fig 3.4: Determination of optimal bactericidal reaction volume for *Citrobacter freundii* SBA by comparing consistency in the colony forming unit count between experiments performed with 40µl and 120µl reaction volume

There was a better reproducibility of results and hence standardization of bacterial counts with a larger reaction volume. Hence 120 µl was determined to be the optimal reaction volume for future experiments.

2.2.4. Determination of optimal complement dilution and optimal harvesting time for *Citrobacter freundii*

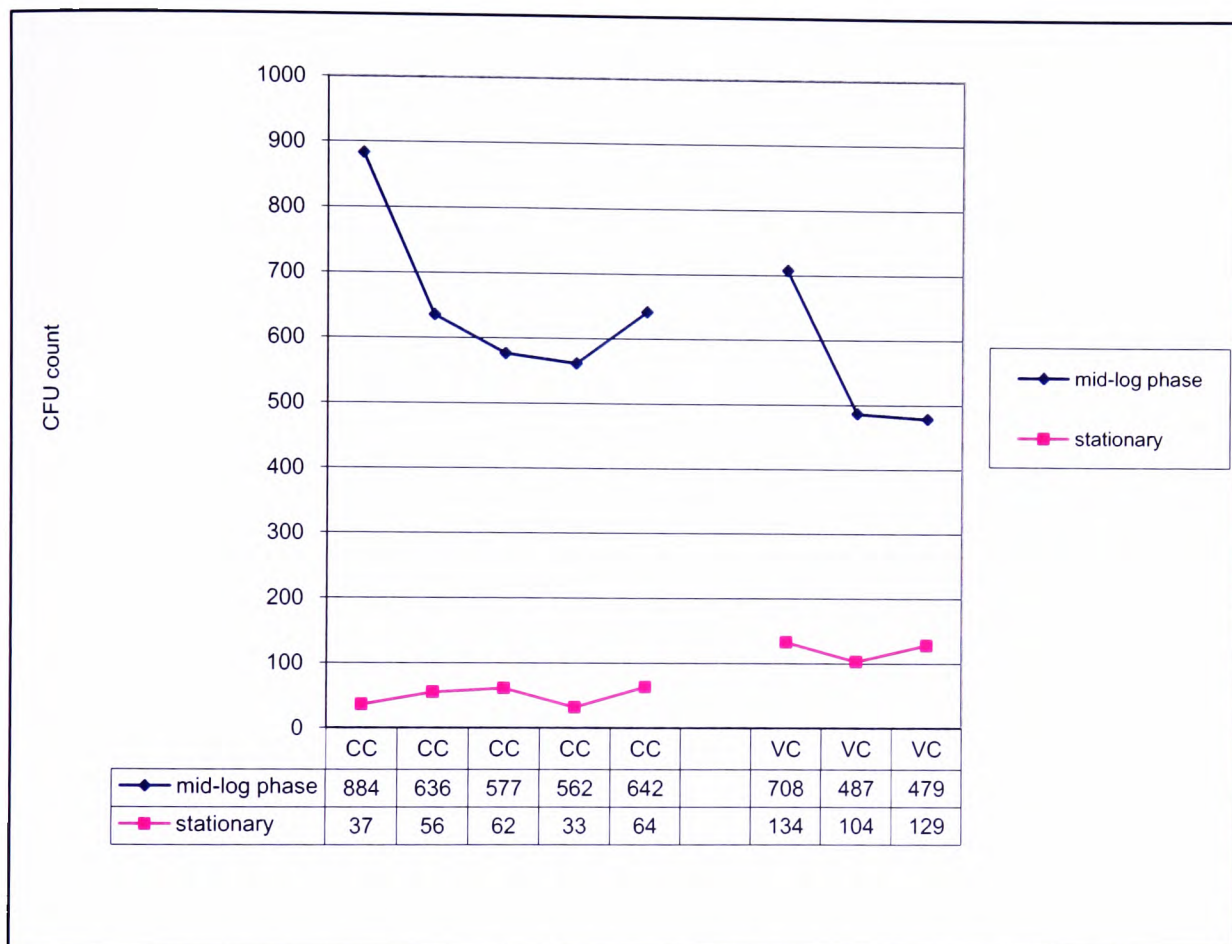


Fig 3.5a: Determination of optimal complement dilution and harvesting time for *Citrobacter freundii* using 1: 8 complement dilution by comparing the colony forming unit count using bacteria in mid-log phase (6 hours incubation) and stationary phase (18 hours incubation). CC-Complement control (bactericidal medium, bacteria and active complement), VC – viable count (bactericidal medium, bacteria and deactivated complement)

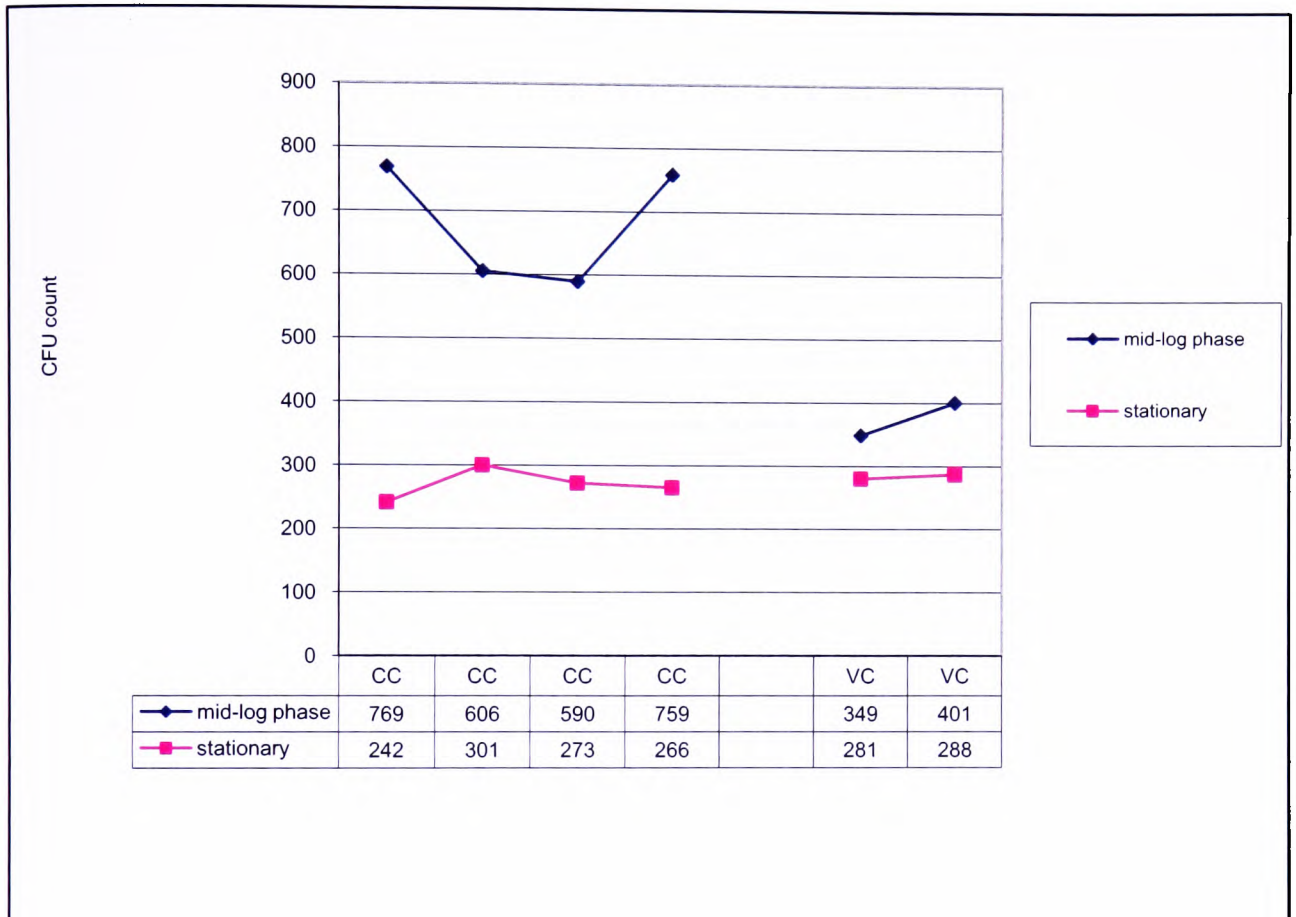


Fig 3.5b: Determination of optimal complement dilution and harvesting time for *Citrobacter freundii* using 1: 16 complement dilution by comparing the colony forming unit count using bacteria in mid-log phase (6 hours incubation) and stationary phase (18 hours incubation) CC-Complement control(bactericidal medium, bacteria and active complement), VC – viable count (bactericidal medium, bacteria and deactivated complement)

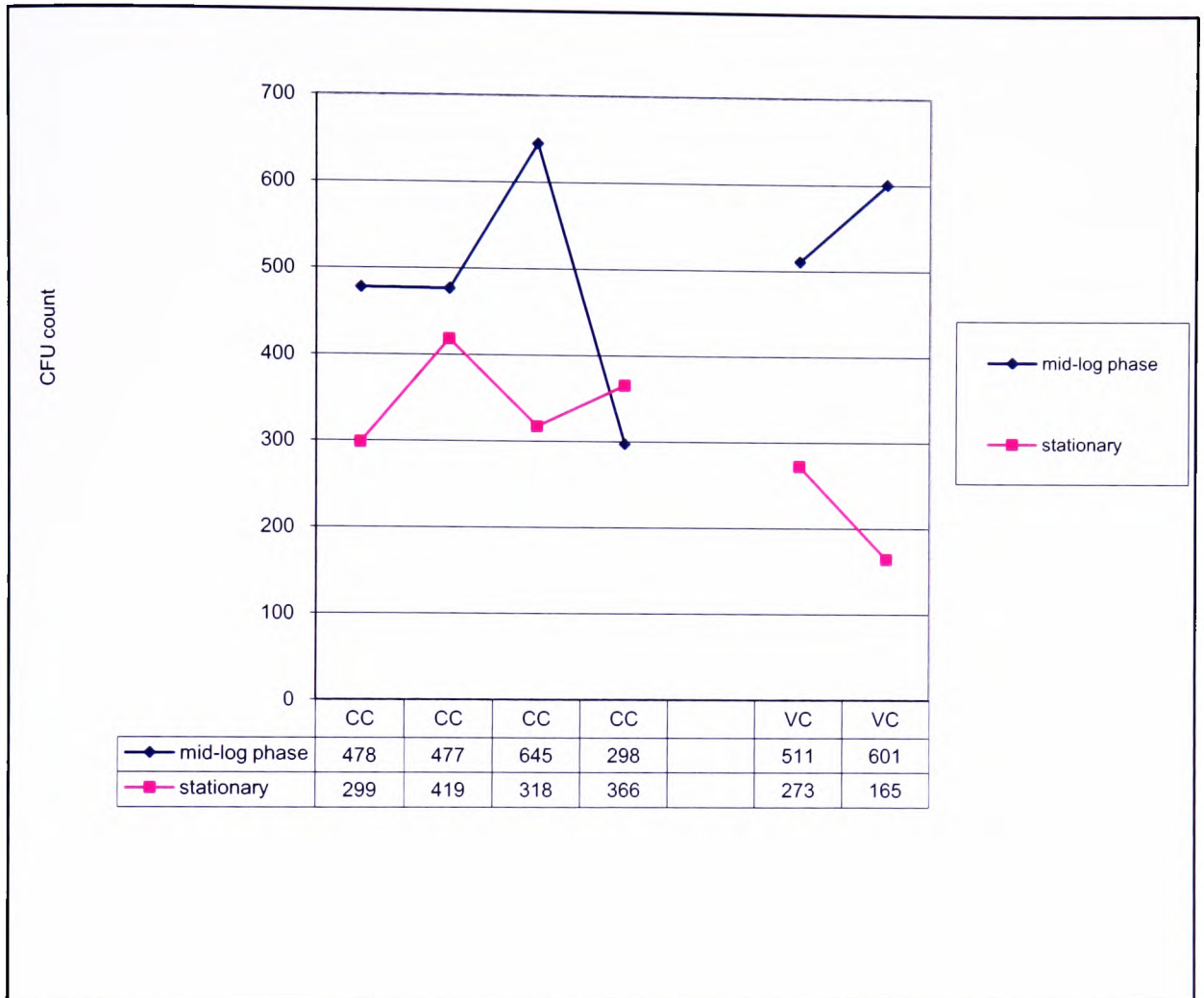


Fig 3.5c: Determination of optimal complement dilution and harvesting time for *Citrobacter freundii* using 1: 32 complement dilution by comparing the colony forming unit count using bacteria in mid-log phase (6 hours incubation) and stationary phase (18 hours incubation) CC-Complement control (bactericidal medium, bacteria and active complement), VC – viable count (bactericidal medium, bacteria and deactivated complement)

The 6 hour incubated *Citrobacter freundii* appears to be resistant to killing in the complement. 18 hour incubated bacteria was determined to be optimal for use in future experiments. Complement control colony counts appear to be similar to the viable counts at 1:32 dilution of complement. Hence, to avoid intrinsic bactericidal activity, a 1:32 dilution of complement was determined to be optimal for use in future reactions.

2.2.5. Specificity of *Citrobacter freundii* SBA

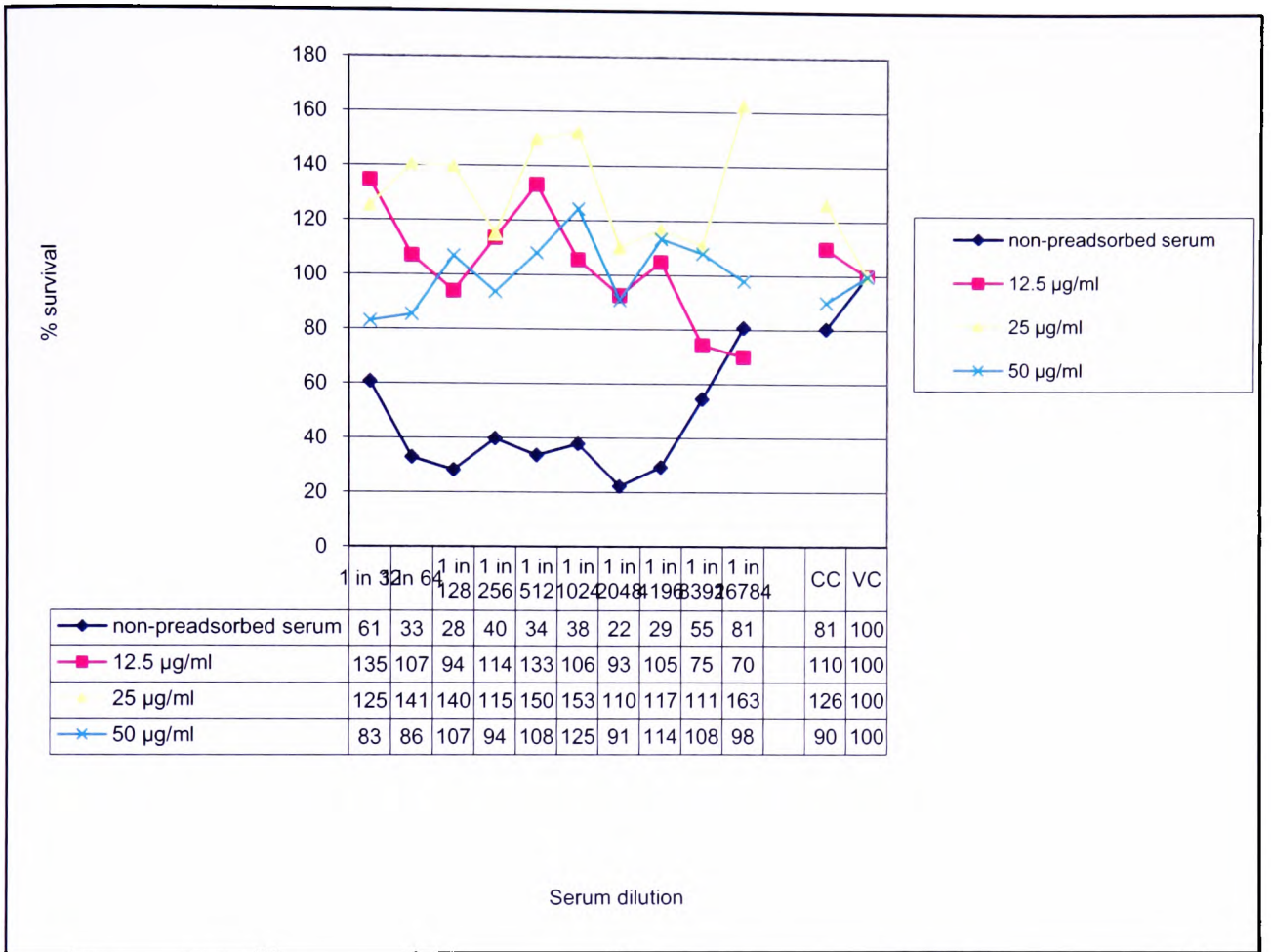


Fig 3.6: Specificity of *Citrobacter freundii* SBA as determined by pre-adsorbance assay, by incubating post vaccination serum with the Vi polysaccharide overnight and comparing the difference in percentage survival of bacteria with SBA using unadsorbed sera. CC- Complement control well (bactericidal medium, bacteria and active complement), VC – viable count, comparator (bactericidal medium, bacteria and deactivated complement)

Demonstration of higher CFU counts in the SBA of all three serum samples pre-adsorbed with the Vi polysaccharide compared to the non-preadsorbed serum sample confirms the specificity of the *Citrobacter freundii* SBA.

2.2.6 Validation for Intra-plate variability

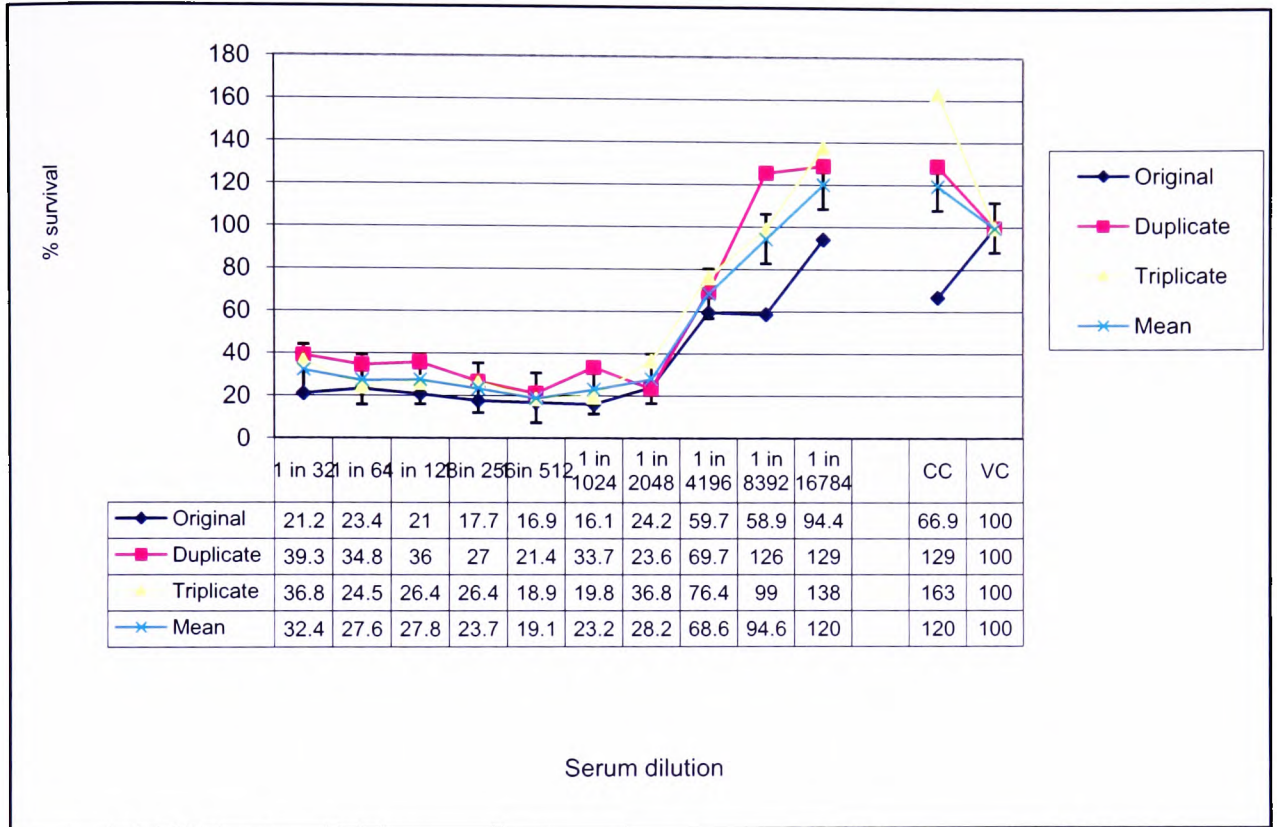


Fig 3.7: Validation for Intra-plate variability of results of post-vaccination serum in *Citrobacter freundii* SBA by comparing percentage survival of bacteria for three samples of sera tested on the same plate on the same day. The mean result is plotted along with the standard error of mean. CC-Complement control (bactericidal medium, bacteria and active complement), VC – viable count (bactericidal medium, bacteria and deactivated complement)

Results of intra-plate validation of the post-vaccination serum for the *Citrobacter freundii* SBA demonstrated the precision of the assay.

2.2.7 Validation for Inter-plate variability

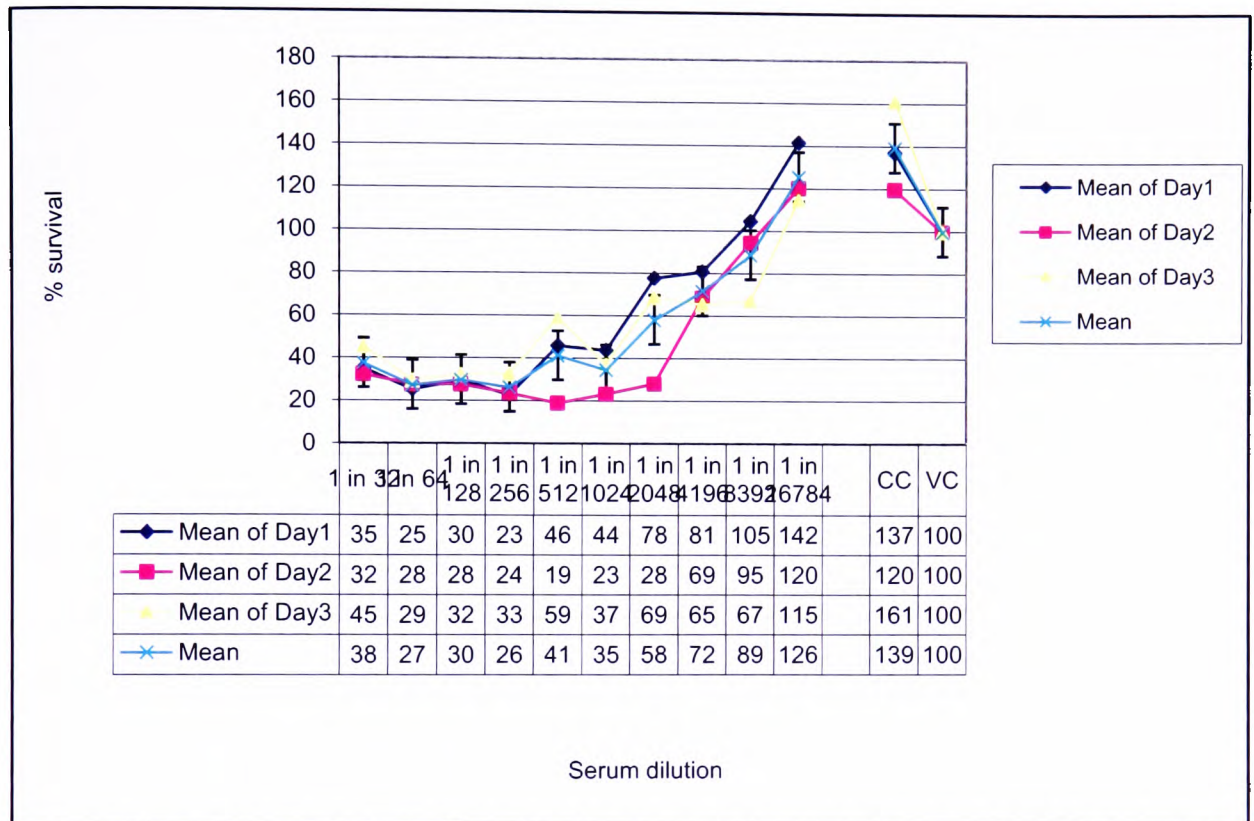


Fig 3.8: Validation for Inter-plate variability of results of post-vaccination serum in *Citrobacter freundii* SBA by comparing percentage survival of bacteria for three samples of sera tested on three different days. The mean result is plotted along with the standard error of mean. CC-Complement control (bactericidal medium, bacteria and active complement), VC – viable count (bactericidal medium, bacteria and deactivated complement)

CFU counts in the inter-plate validation assay for the *Citrobacter freundii* SBA using post-vaccination serum demonstrated the precision of the assay.

2.2.8 Discussion

Following the determination of the bacterial growth curves for *Citrobacter freundii*, the optimal bactericidal reaction volume, complement dilution and harvesting time were determined to be 120 μ l, 1:32 and 18 hours respectively. Specificity of the *Citrobacter freundii* SBA was confirmed by a pre-adsorbance assay. Validation of intra-plate and inter-plate variability of CFU counts demonstrated the assay to be accurate and precise.

Validation of the results obtained with pre-vaccination and post-vaccination serum using the *Citrobacter freundii* SBA will be validated against an SBA using *Salmonella typhi* to determine correlation between the results and to further confirm the validity of the results. Further validation of the assay would include testing the same using serum samples from patients with typhoid fever to understand the acquisition of functional antibodies against Vi-polysaccharide during acute infection

2.3 Serum Bactericidal Assay Protocol for *Salmonella typhi*

Materials:

1. LB agar plates prepared from LB agar powder (Sigma)
2. 10µl flexible disposable microbiological loops (Copan)
3. Incubator with thermometer (Genlab)
4. Automatic colony counter (Anderman)
5. 0.1M NaOH-1% SDS solution
6. *Salmonella typhi* (NCTC strain)
7. Flat bottom, 96 well, tissue culture plates (Corning)
8. Hanks Balanced Salt Solution (Sigma)
9. Baby rabbit complement (Pel-Freeze Biologicals)
10. Bijou containers
11. Spectrophotometer (Perkin-Elmer)

Day 1 (Preparation of target strain):

From the *S. typhi* stock (NCTC strain) stored in glycerol at -80 degrees Celsius, an aliquot was defrosted and plated onto an LB agar plate using a disposable 10µl flexible microbiological loop. The plate was incubated at 37 degrees Celsius overnight, without CO₂ (~18 hours).

Day 2

All individual colonies formed on the plate were picked and suspended thoroughly in 2 ml HBSS solution in a bijou container. 1ml from the above suspension was pipetted and placed in another bijou container after allowing for the settlement of cell debris in the former. 20 μ l of the mix was suspended in a 1 ml eppendorf containing 980 μ l of 0.1M NaOH-1% SDS lysis solution. The bacteria were lysed by gentle inversion. Blanks were prepared by adding 20 μ l of HBSS buffer to 0.1M NaOH-1% SDS. The optical densities of the above suspensions were measured on a spectrophotometer at 260 nm. The bacterial volume for further dilution was calculated as follows:

$$\text{Volume} = \frac{3}{(\text{OD}_{260} \times 250)} \times 250$$

The above volume of bacterial suspension was mixed with HBSS to make up a final volume of 250 μ l. 20 μ l of the mix was diluted in 180 μ l of HBSS and the process was repeated thrice. From the last mix, 100 μ l was pipetted into 2.5 ml of HBSS. This was the working solution of bacteria for the assay. In a flat bottom tissue culture plate 50 μ l of Hanks Balanced Salt Solution was added to each well. 25 μ l of HBSS was added to the column of wells for the Complement Independent Control (CIC). 50 μ l of decomplexed (56 degrees Celsius for 30 min) test serum was added to the first column of the plate and serially double diluted across the plate till column 9. 50 μ l of the mix was discarded from the last column. 25 μ l of serum was added to the CIC column and none to the Complement Control (CC) and Viable Count (VC) columns. 25 μ l of the working solution of bacteria was added to each well. The plate was incubated at room temperature for 10 minutes. Baby rabbit complement diluted to

a final concentration of 1:8 in cold HBSS was added. Handling of complement was on ice. 20 µl of the diluted complement was added to each well on the plate except the VC and CIC well. 25µl of heat inactivated complement (56 degrees for 30 minutes) was added to the VC and the CIC columns. The sealed tissue culture plate was incubated at 37 degrees Celsius for 45 minutes.

10µl from each well, six columns from each row at a time, was plated using a manual multichannel pipette onto the labelled plate of an LB agar plate using the tilt method. The plates were incubated at 37 degrees Celsius overnight, without CO₂ (~18 hours).

Day 3:

The colonies per column were counted. The serum bactericidal titre was the inverse titre of the highest dilution at which there was 50% killing of *S. typhi*.

2.3.1 Determination of optimal complement dilution for *S. typhi* SBA

To determine the intrinsic bactericidal effect of an exogenous complement source and to determine the optimal complement dilution for use in the SBA, 3 different concentrations of baby rabbit complement: 1:4, 1:8 and 1:16 were compared. Triplicates of viable count wells were maintained to estimate percentage of killing due to the exogenous complement source. Results of the experiment are shown in Fig 2.3.1. A concentration of 1:8 was demonstrated to be the optimal complement dilution for further experiments.

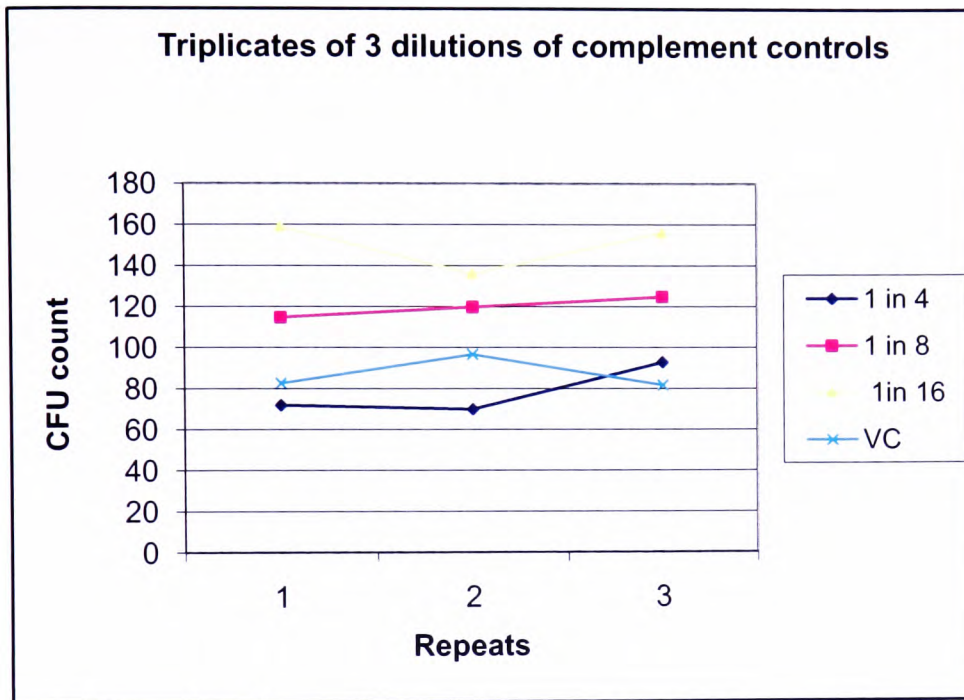


Fig 2.3.1: Optimal complement dilution validation experiment showing averages of triplicates of 3 dilutions of complement (1:4, 1:8 and 1:16) controls and viable count.

2.3.2 Validation for intra-plate and inter-plate variability

Following the development of the *S. typhi* SBA, it was then validated for the intra-plate variability of CFU counts and percentage killing for a post-vaccination serum. The experiment was performed in triplicate on the same tissue culture plate and the experiment repeated on two different days. Average results of triplicates on both days for both pre-vaccination and post-vaccination samples are shown in Fig 2.3.2. The consistency of results demonstrates that the assay is precise and accurate.

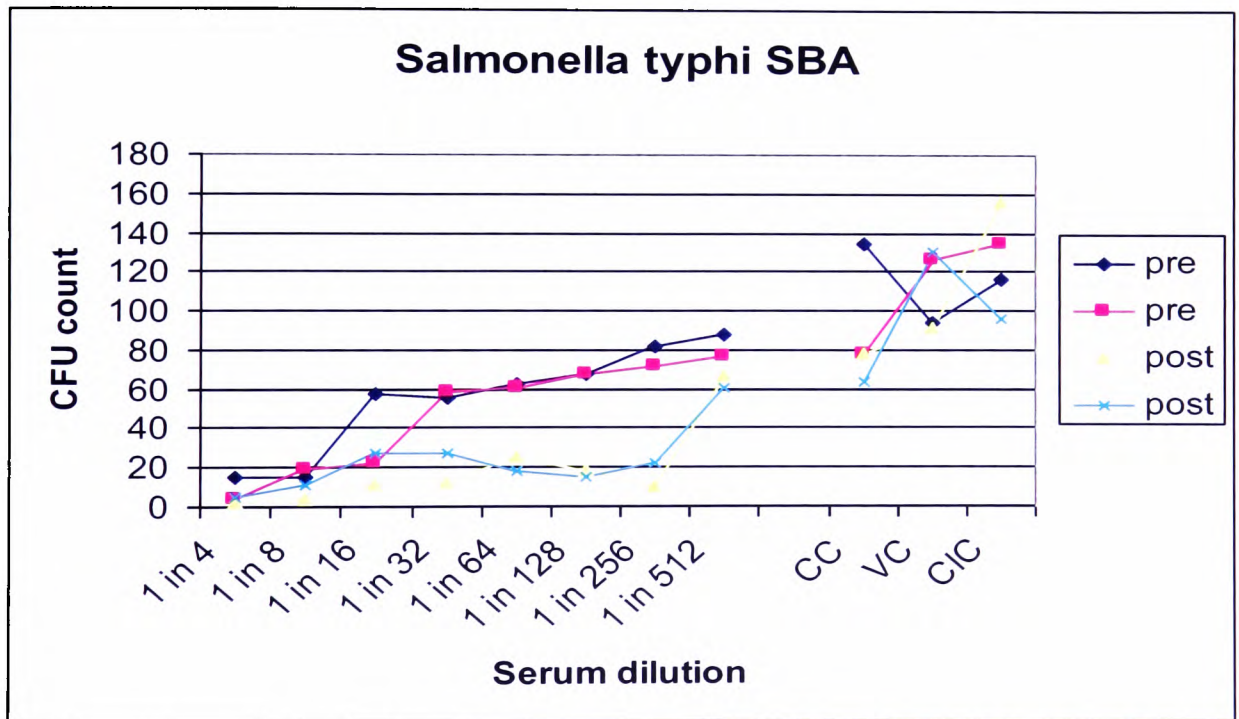


Fig 2.3.2: SBA with duplicates of pre and post –Vi PS vaccination sera. Colony Forming Units counts plotted against serum dilutions.

2.3.3 Correlation between *S. typhi* SBA & *C. freundii* SBA

In order to test whether *C. freundii* SBA could be used as a safe alternative to *S. typhi* SBA, a comparison was made between the results obtained with samples on which the two assays were performed. 90 serum samples from clinically healthy volunteers in Nepal, collected to test the sero-epidemiology of bactericidal antibodies against *S. typhi* in Kathmandu, Nepal (detailed in Chapter 3), and representing 30 random samples from low (<4 to 16), medium (32 to 128) and high (256 to >1024) *S. typhi* SBA titres, as determined by the *S. typhi* SBA protocol described above were subjected to *C. freundii* SBA, described above, and the paired results were analysed using Spearman ranked correlation. The results of correlation analysis (Fig 2.3.4) suggest that there was poor correlation between the two assays ($r=0.15$). *S. typhi* SBA assay was therefore used for all further experiments in the sero-epidemiology study in Chapter 3 and vaccine kinetics study in Chapter 4.

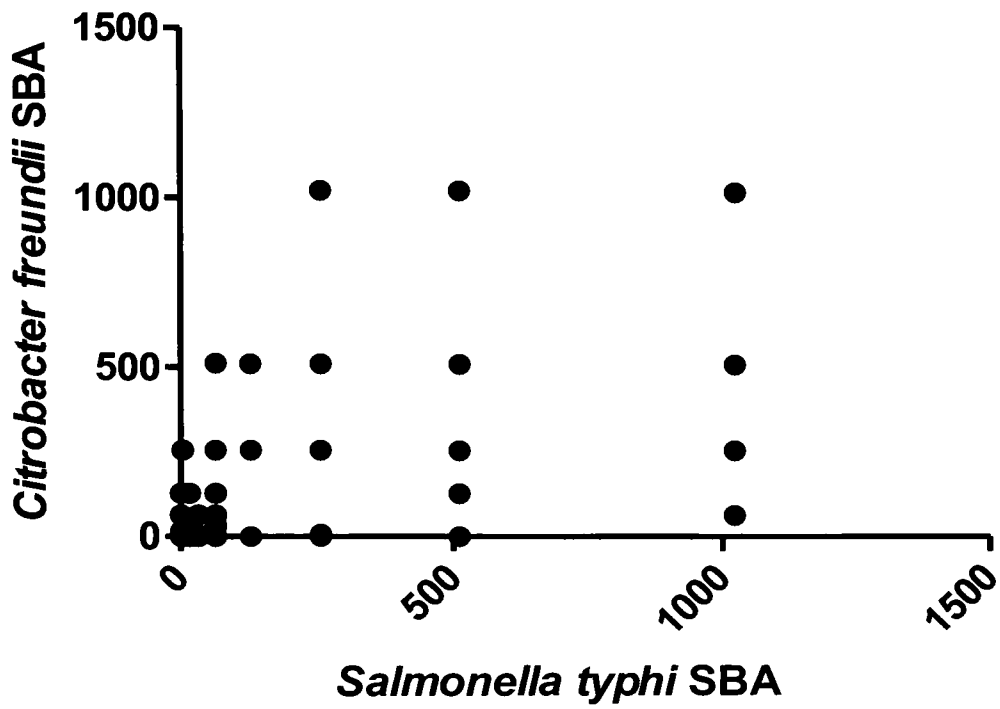


Fig 2.3.3. Correlation between *S. typhi* SBA and *C. freundii* SBA results of 90 serum samples demonstrating poor correlation between bactericidal titres

Chapter 3 : SERO-EPIDEMIOLOGY OF *S. TYPHI* IN KATHMANDU, NEPAL

3.1. Introduction

The typhoid sero-epidemiology study is based on the hypothesis that increasing age and sub-clinical exposure to *S. typhi* will result in the induction of natural immunity to typhoid fever. For the conduct of this study, two countries with different incidence rate of typhoid fever were selected. South Asia in general and Nepal in particular suffers from a large incidence of typhoid fever. A recent study demonstrated that 40% of culture confirmed bacteraemia in Nepal was caused by *S. typhi*¹⁴⁸. Similarly, a study in 876 febrile adults revealed that enteric fever accounted for 36.22% of pathogens identified in 323 cases¹⁴⁹. UK reports low incidence of disease with approximately 242 imported cases reported in 2006⁷⁷.

Nepal is a landlocked country in South Asia sandwiched between the Tibet Autonomous Region of the People's Republic of China to the North and the Republic of India to the South (Fig 3.1.1). Divided into 14 zones with 75 districts, the nation is the 40th most populous country in the world with a population of 29 million. It is listed by the United Nations as a Least Developed Country (LDC) on the basis of per capita income, human development indices and economic criteria.

Patan Hospital, located in Patan town (Fig 3.1.2) in Lalitpur District, Bagmati Zone, south of Kathmandu City is the research site for this study in Nepal. Patan Hospital, in Lagankhel (Fig 3.1.3) is a joint venture between the United Mission to Nepal (UMN), a non-political, non-sectarian, non-governmental, non-profit humanitarian Christian organisation comprised of organisations in 18 countries, and the Federal Democratic Government of Nepal (formerly the

Kingdom of Nepal). Currently it is a 204 bed general hospital with a large outpatient department that serves over 250,000 patients annually. It has a 24 hour emergency room as well as 16 other departments including a paediatric clinic for patients under 14, a male clinic and female clinic for adult patients, a diagnostic laboratory and a maternity ward.



Fig 3.1.1: Location of Nepal in South Asia, between China to the north and India to the South. Also shown is Kathmandu, the capital of Nepal

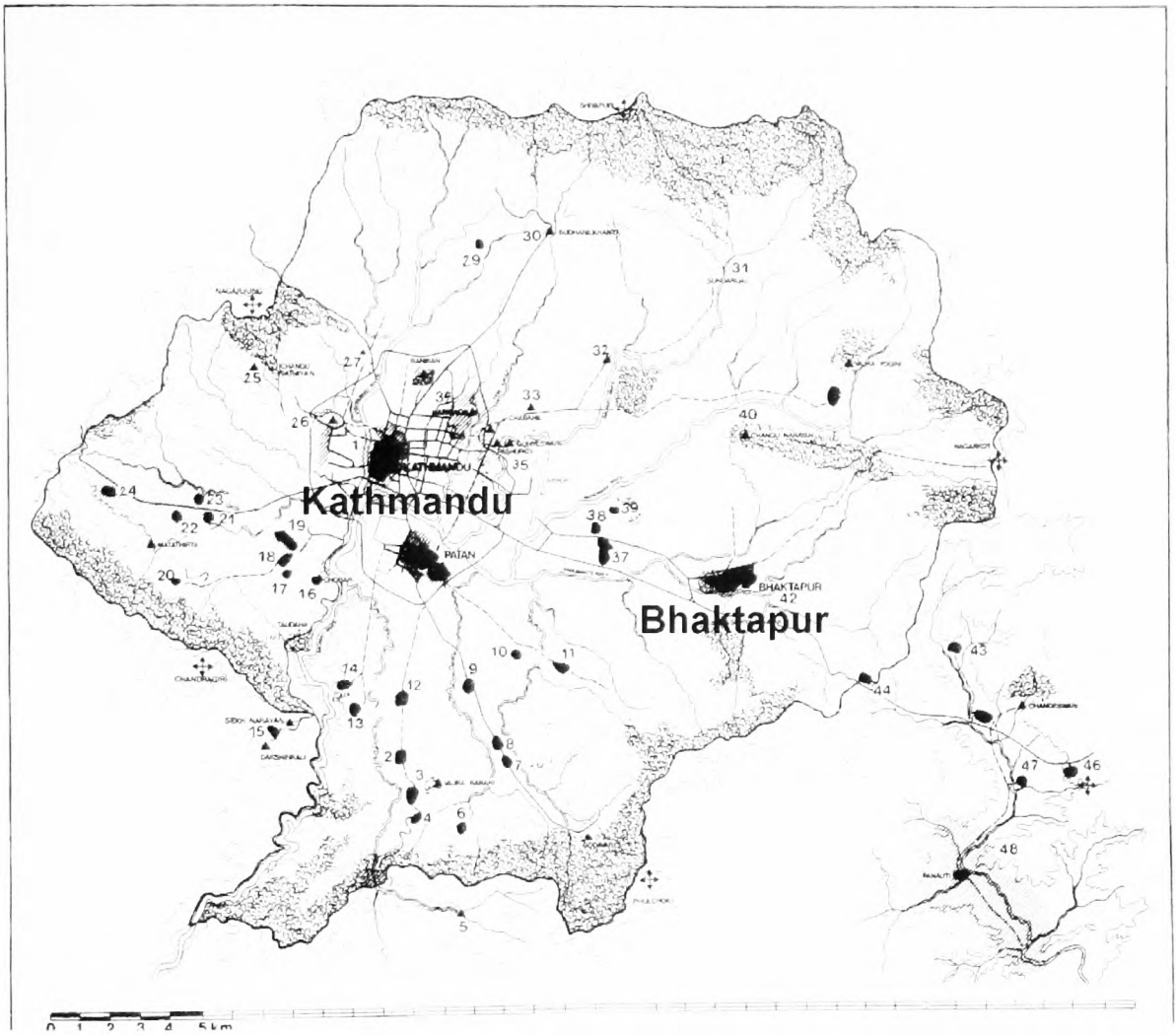


Fig 3.1.2. Location of Patan Town, south of the capital city of Kathmandu

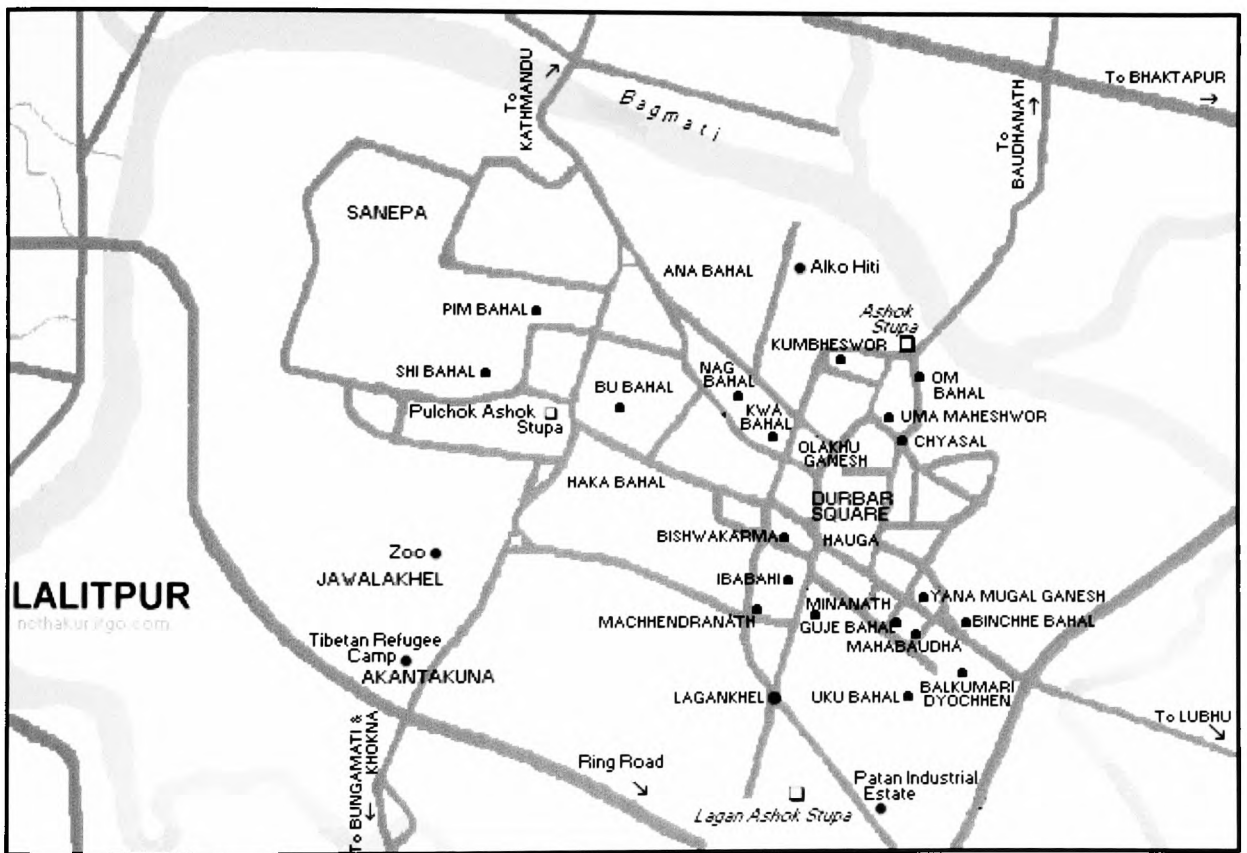


Fig 3.1.3. Location of Patan Hospital in the Lagankhel area of the Bagmati Zone in Lalitpur District.

3.2 Materials & Methods:

3.2.1 Study participants and Recruitment

Three recruitment methodologies were used for the collection of serum samples from healthy non-febrile volunteers in the 2 countries. In Nepal these included:

1. Recruitment of non-febrile patients attending the out-patient department of the hospital/laboratory
2. Recruitment of non-febrile volunteers in the community e.g. schools
3. Collection of cord blood in lieu of newborn sera (Age band 1 below)

In UK, stored, anonymised sera from healthy participants who had participated in one of the completed vaccine trials of the Oxford Vaccine Group were included after screening of the demographic database for age.

The seven age bands identified for the purpose of population age stratification for the determination of typhoid antibody sero-prevalence in Nepal were:

1. Cord blood (Age band 1)
2. Six months to Two years (Age band 2)
3. Three to Five years (Age band 3)
4. Six to Eight years (Age band 4)
5. Nine to Fifteen years (Age band 5)
6. Sixteen to Fifty-five years (Age band 6)
7. Fifty six to seventy five years (Age band 7)

Inclusion/Exclusion criteria

Participants who fulfilled all the conditions below were enrolled in the study:

Inclusion criteria:

1. Non-febrile outpatients (laboratory based) or apparently, clinically healthy subjects (community based)
2. Aged between newborn to 75 years
3. Able to provide written informed consent (legal guardians in case of subjects < 18 years) after the nature of the study had been explained.

Exclusion criteria:

1. Unwilling or unable to provide written informed consent (legal guardians in case of subjects <18 years)
2. Had received any preparation of typhoid vaccine previously
3. Had confirmed typhoid or paratyphoid disease previously
4. Suspected or known cases of immune disorders
5. Had any medical condition, which in the opinion of the investigator, might interfere with the evaluation of the study objectives
6. Had received any antibiotics within 14 days

3.2.2 Ethics

The study protocol was approved by the Oxford Tropical Research Ethics Committee (OxTREC) and the Nepal Health Research Council (NHRC). Ethics approval for the use of stored, anonymised sera in the UK was obtained from the Oxford Research Ethics Committee-B (OxREC-B)

3.2.3 Sample Handling

Whole blood was collected from participants, by standard venepuncture techniques and spun at 3000 rpm for 10 min. Sera were separated using sterile techniques and aliquots made and stored at -20°C. The samples were shipped in cryovials, on frozen pink ice, using express courier and under U.N guidance on the shipment of biological hazardous goods, to the Oxford Vaccine Group laboratory and stored at -20°C.

3.2.4 Laboratory Analyses

Sera (30 samples from each of the 7 age groups) from Nepal and 180 samples (30 samples from each of the 6 age groups) from UK were tested. All samples were subjected to the *S. typhi* SBA and anti-ViPS antibody ELISA described in Chapter 2. For anti-ViPS antibody avidity and anti-ViPS IgG subclass, 28 high titre samples, representing 4 samples each from the 7 age bands, from the Nepal cohort were tested

3.2.5 Statistical Analyses

Assuming a 5% significance level and 20% difference in antibody titre within the population, a sample size of 199 participants in the Nepal cohort would give 80% power to prove the study hypothesis. SBA titres and ELISA IgG concentrations are summarised as median titres and geometric means with corresponding 95% confidence intervals. Kruskal-Wallis test and one way ANOVA was employed to detect significant differences between the age groups' median titres and geometric mean bactericidal titre (GMT) respectively within each cohort, while Bonferroni's multiple comparison test was employed to detect inter-age band difference in GMT in the Nepal cohort. Pearson correlation was performed to detect correlation between the bactericidal GMT and anti-ViPS IgG expressed as geometric mean concentration (GMC). Anti-ViPS antibody avidity is represented as medians for each age band and IgG subclasses as a proportion of the total antibody concentration calculated from medians for each subclass within each age band.

3.3 Results

3.3.1 *S. typhi* SBA

The median bactericidal titres for in the Nepal cohort increased with age, from 8 in early childhood to 128 in adulthood (Table 3.3.1, Figure 3.3.1). Kruskal-Wallis (non-parametric) test for the inter-age group difference in median within the 7 age bands in the Nepal cohort was significant ($P < 0.0001$). The GMT of the Nepal age bands was also observed to increase with age

(Table 3.3.2, Fig 3.3.2). The GMTs were significantly different amongst the multiple age groups within the Nepal cohort by one way ANOVA ($p < 0.001$). Significant differences between the GMT, as determined by the Bonferroni's test, are shown in Table 3.3.3. The proportion of participants with low (<4, 4, 8, 16), medium (32, 64, 128) and high (256, 512, >1024) titres in each age band is shown in Fig 3.3.3.

For the UK cohort, the SBA titres were lower than in the Nepal cohort. The median bactericidal titres were 4 for each of the 6 age bands (Table 3.3.1, Fig 3.3.4). The bactericidal GMT did not increase with age unlike in the Nepal cohort (Table 3.3.2, Fig 3.3.5). The median titres and GMTs were not significantly different amongst the multiple groups by either Kruskal-Wallis (non-parametric) test or one way ANOVA respectively. The proportion of participants with low (<4, 4, 8, 16), medium (32, 64, 128) and high (256, 512, >1024) titres in each age band is shown in Fig 3.3.6.

Fig 3.3.7 compares the age distribution of natural immunity to *S. typhi* between the Nepal and UK cohorts, determined by SBA and expressed in median bactericidal titres for each age band.

Fig 3.3.8 compares the GMT of *S. typhi* bactericidal antibodies between the Nepal and UK cohort. Figure 3.3.9 and 3.3.10 compare the median bactericidal titres and bactericidal antibody GMT respectively between the Nepal and UK cohorts including all samples in the respective cohort.

3.3.2 Anti-ViPS antibody ELISA

GMC of the anti-ViPS antibody titres, with 95% CI, were 0.737 (0.69-0.79), 0.85 (0.79-0.92), 0.571 (0.49-0.66), 0.579 (0.53-0.64), 0.451 (0.41-0.50), 0.86 (0.77-0.96) and 0.69 (0.62-0.76) for cord blood, infants aged 6 months to 2 years, children aged 3 to 5 years, 6 to 8 years, 9 to 15

years, and adults aged 16 to 55 years and 56 to 75 years respectively in Nepal (Table 3.4.2, Fig 3.4.11) and 0.2 (0.16-0.24), 0.14 (0.11-0.18), 0.17 (0.14-0.2), 0.19 (0.16-0.23), 0.26 (0.23-0.3), 0.29 (0.26-0.35) for newborns, infants aged 6 months to 2 years, children aged 3 to 5 years, 6 to 8 years, 9 to 15 years, and adults aged 16 to 55 years respectively in UK (Table 3.3.2, Fig 3.3.12). A comparison of the GMC, with 95% C.I, of each age band between the Nepal and UK cohort is illustrated in Figure 3.3.13 and shows significant differences between the populations.

3.3.3 Anti-ViPS antibody avidity

The median avidity of the 28 samples tested from Nepal were 0.3, 0.4, 0.25, 0.4, 0.4, 0.25 and 0.1 for cord blood, infants aged 6 months to 2 years, children aged 3 to 5 years, 6 to 8 years, 9 to 15 years, and adults aged 16 to 55 years respectively (Fig 3.3.14).

3.3.4 Anti-ViPS antibody subclass

The proportion of each IgG subclass relative to the total anti-ViPS antibody in volunteers from Nepal is shown in Figure 3.3.15, demonstrating that the immunoglobulin in sera is predominantly IgG2.

3.3.5 Correlation between SBA GMT and ELISA GMC

A significant correlation between the *S. typhi* bactericidal GMT and anti-ViPS antibody GMC was observed for the UK cohort ($r = 0.918383$) (Fig 3.3.17) but not for the participants from Nepal ($r = 0.072472$) (Fig 3.3.16)

3.3.6 Relationship between SBA antibody titres and disease rates

An inverse relationship was observed between the bactericidal antibody GMT observed in this study and typhoid fever incidence rates in different age groups obtained from a prospective study in an urban slum region in New Delhi, India ⁴ (Fig 3.3.18).

Cohort	Age band (in years)	SBA GMT (95%CI)
Nepal	Cord blood	256
	0.5 to 2	8
	3 to 5	8
	6 to 8	12
	9 to 15	128
	16 to 55	128
	55 to 75	128
UK	Newborn	4
	0.5 to 2	4
	3 to 5	4
	6 to 8	4
	9 to 15	4
	16 to 55	4

Table 3.3.1. Median *S.typhi* bactericidal antibody titre in each age band of the Nepal and UK cohort

Cohort	Age band (in years)	SBA GMT (95%CI)	Anti-ViPS GMC (95%CI)
Nepal	Cord blood	150.47 (72.71-311.41)	0.737 (0.69-0.79)
	0.5 to 2	7.82 (4.25-14.38)	0.85 (0.79-0.92)
	3 to 5	11.31 (5.24-24.44)	0.571 (0.49-0.66)
	6 to 8	11.06 (6.32-19.34)	0.579 (0.53-0.64)
	9 to 15	84.45 (47.75-149.34)	0.451 (0.41-0.50)
	16 to 55	73.52 (35.66-151.58)	0.86 (0.77-0.96)
	55 to 75	75.24 (38.27-147.90)	0.69 (0.62-0.76)
UK	Newborn	2.4 (1.86-3.12)	0.2 (0.16-0.24)
	0.5 to 2	2.52 (2.01-3.16)	0.14 (0.11-0.18)
	3 to 5	2.7 (2.08-3.51)	0.17 (0.14-0.2)
	6 to 8	2.64 (2-3.47)	0.19 (0.16-0.23)
	9 to 15	3.48 (2.4-5.04)	0.26 (0.23-0.3)
	16 to 55	3.65 (2.34-5.68)	0.29 (0.26-0.35)

Table 3.3.2. *S.typhi* bactericidal antibody GMT and anti-ViPS antibody GMC in each age band of the Nepal and UK cohort

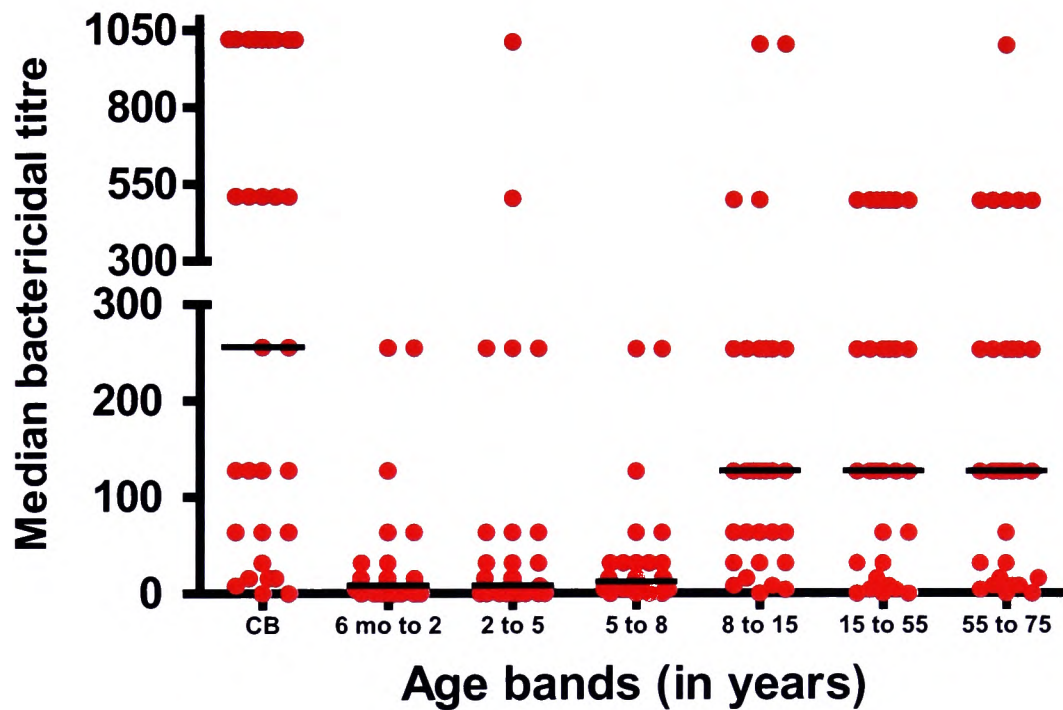


Fig 3.3.1. Median bactericidal titres and individual data points, expressed as the inverse of the highest dilution factor demonstrating >50% bactericidal activity in the SBA assay, of the seven age bands in the Nepal cohort. CB: Cord blood

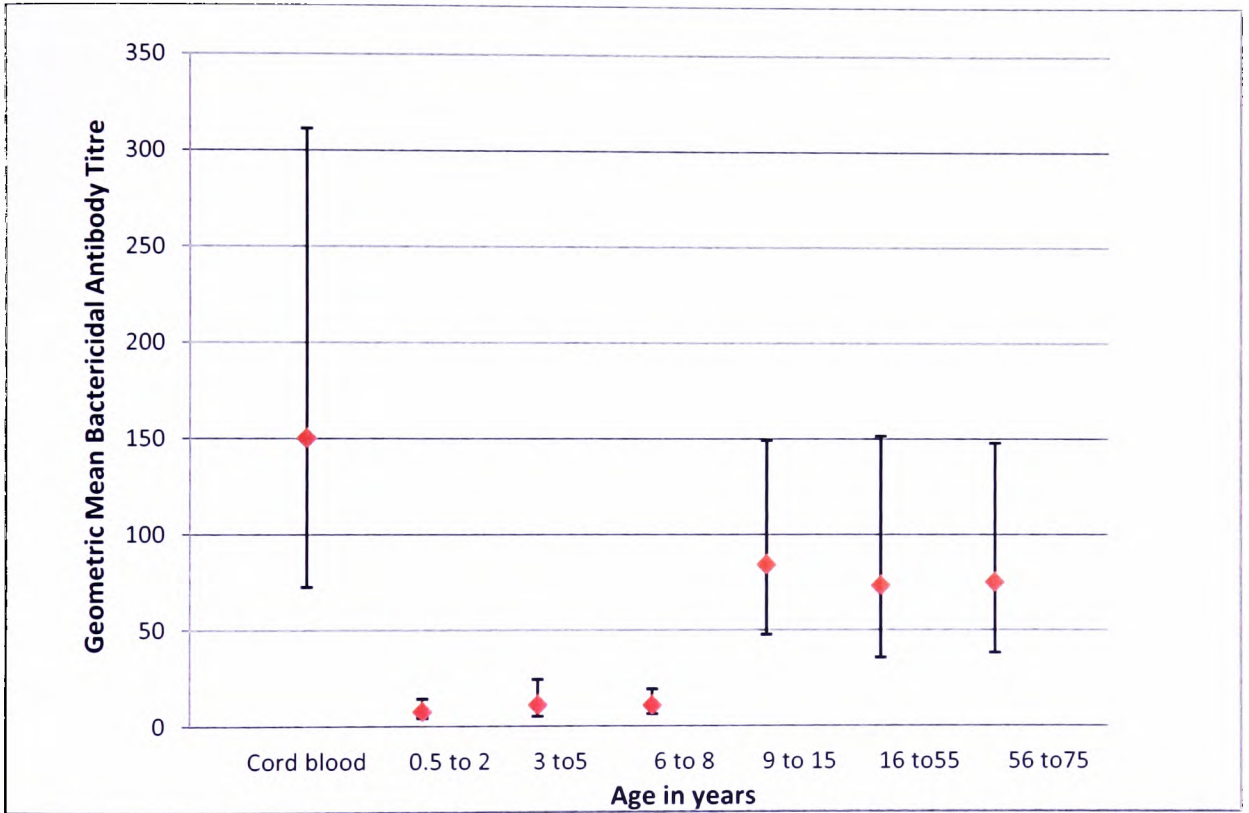


Fig 3.3.2. *S. typhi* bactericidal Geometric Mean Antibody Titre (95% CI) of the seven age bands in the Nepal cohort

	Cord blood	6months to 2 years	2 to 5 years	5 to 8 years	8 to 15 years	15 to 55 years	55 to 75 years
Cord blood	N.A	P<0.0001* (0.64 to1.9)	P<0.0001* (0.48 to1.8)	P<0.0001* (0.49 to1.8)	0.0944 (-0.39 to 0.89)	0.0915 (-0.33 to 0.95)	0.0873 (-0.34 to 0.94)
6months to 2 years	P<0.0001* (0.64 to1.9)	N.A	0.5936 (-0.8 to 0.48)	0.4024 (-0.79 to 0.49)	P<0.0001* (-1.7 to -0.39)	P<0.0001* (-1.6 to -0.33)	P<0.0001* (-1.6 to -0.34)
2 years to 5 years	P<0.0001* (0.48 to1.8)	0.5936 (-0.8 to 0.48)	N.A	0.8764 (-0.63 to 0.65)	0.0004* (-1.5 to -0.23)	0.0016* (-1.5 to -0.17)	0.0012* (-1.5 to -0.18)
5 years to 8 years	P<0.0001* (0.49 to1.8)	0.4024 (-0.79 to 0.49)	0.8764 (-0.63 to 0.65)	N.A	P<0.0001* (-1.5 to -0.24)	0.0002* (-1.5 to -0.18)	0.0001* (-1.5 to -0.19)
8 years to 15 years	0.0944 (-0.39 to 0.89)	P<0.0001* (-1.7 to -0.39)	0.0004* (-1.5 to -0.23)	P<0.0001* (-1.5 to -0.24)	N.A	0.7501 (-0.58 to 0.70)	0.8706 (-0.59 to 0.69)
15 to 55 years	0.0915 (-0.33 to 0.95)	P<0.0001* (-1.6 to -0.33)	0.0016* (-1.5 to -0.17)	0.0002* (-1.5 to -0.18)	0.7501 (-0.58 to 0.70)	N.A	0.9233 (-0.65 to 0.63)
55 to 75 years	0.0873 (-0.34 to 0.94)	P<0.0001* (-1.6 to -0.34)	0.0012* (-1.5 to -0.18)	0.0001* (-1.5 to -0.19)	0.8706 (-0.59 to 0.69)	0.9233 (-0.65 to 0.63)	N.A

Table 3.3.3. Inter age group differences in *S. typhi* bactericidal GMT for Nepal cohort by Bonferroni's Multiple Comparison Test. * denotes statistically significant differences in GMT

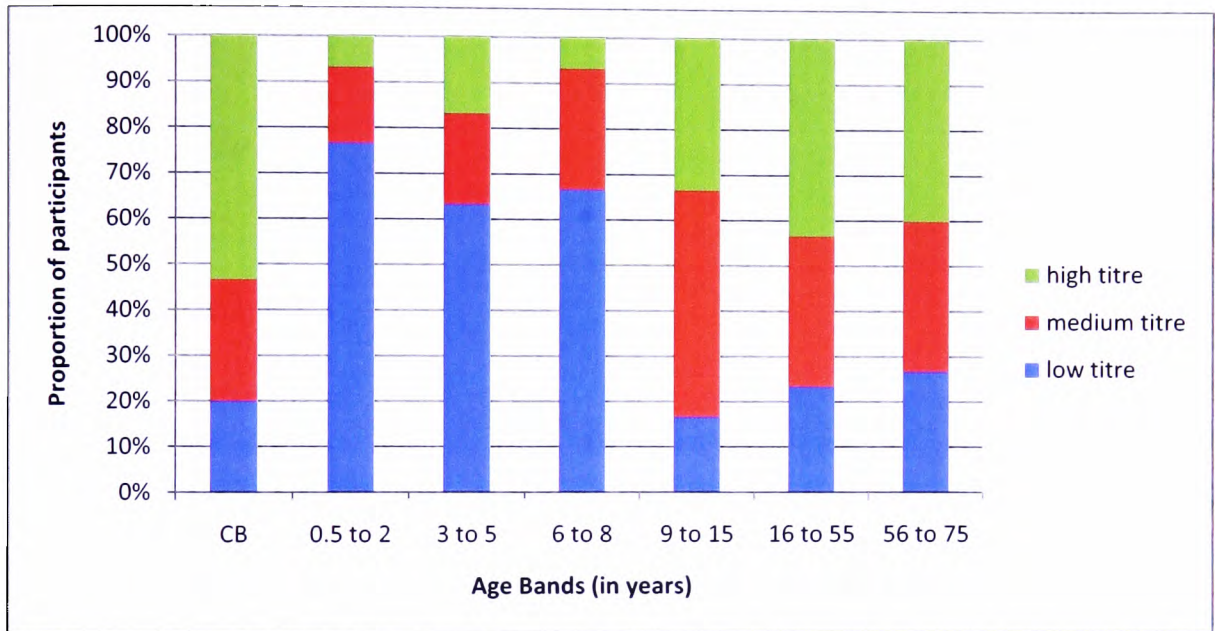


Figure 3.3.3. Age specific proportion of high (≥ 256), medium (32-128) and low (≤ 16) *S. typhi* bactericidal titres in the Nepal cohort. CB: cord blood

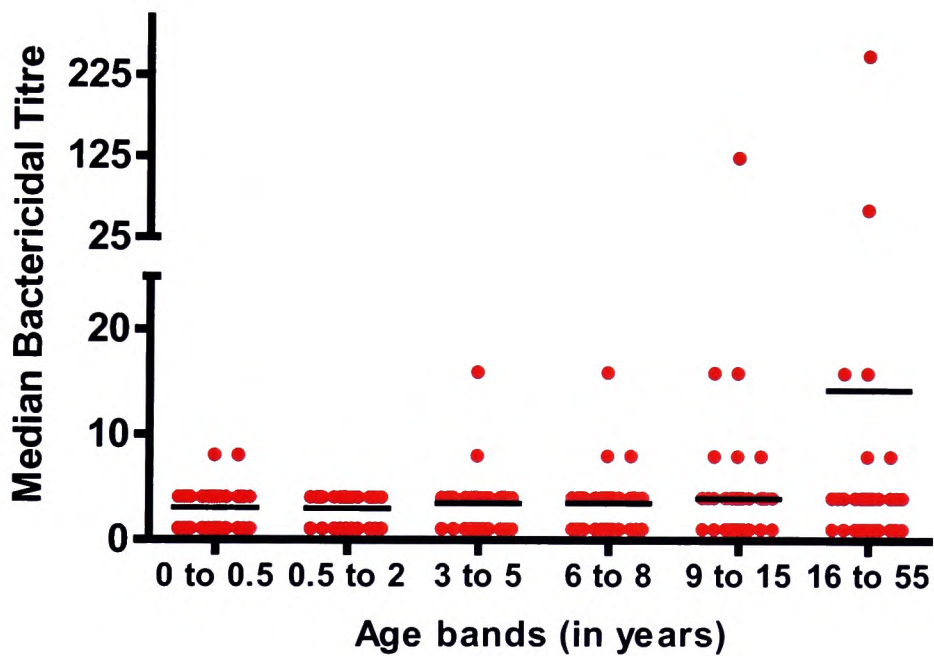


Fig 3.3.4. Median bactericidal titres, expressed as the inverse of the highest dilution factor demonstrating >50% bactericidal activity in relation to age in the UK cohort (note: y axis not the same as Fig 3.4.1)

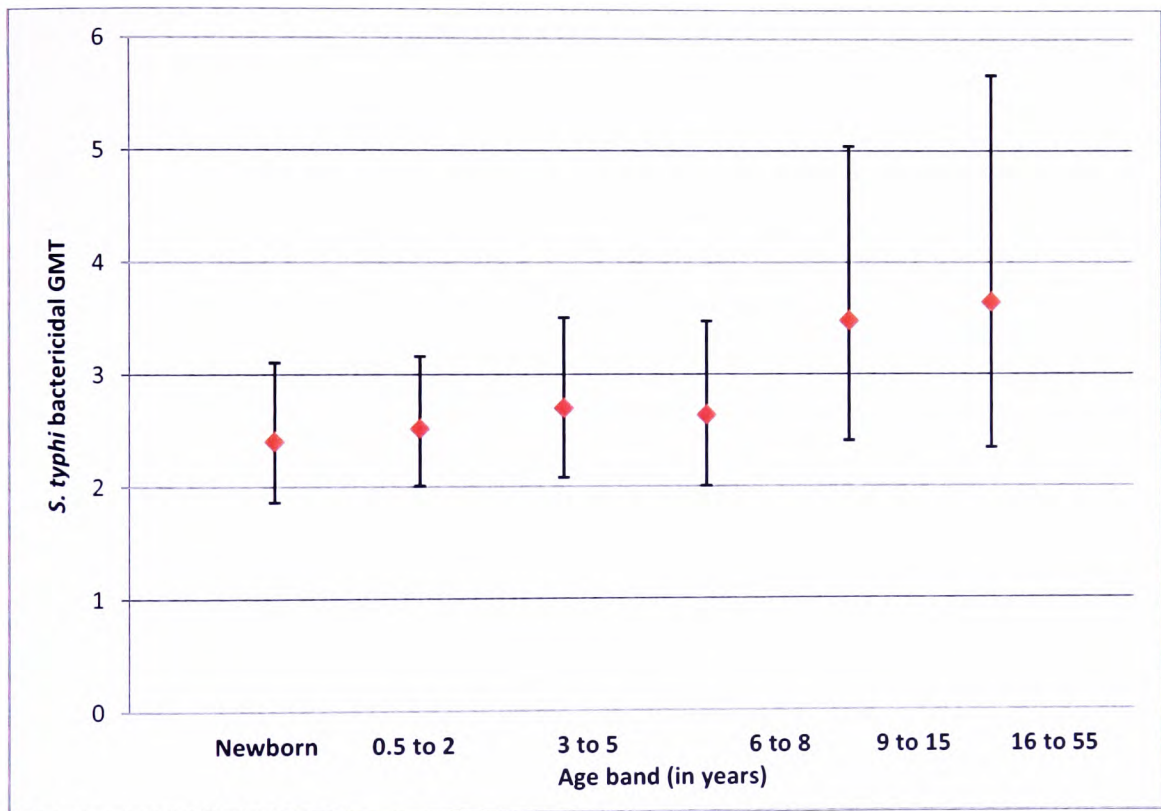


Fig 3.3.5. *S. typhi* bactericidal Geometric Mean Antibody Titre of the six age bands in the UK cohort (note: y axis not the same as 3.4.2)

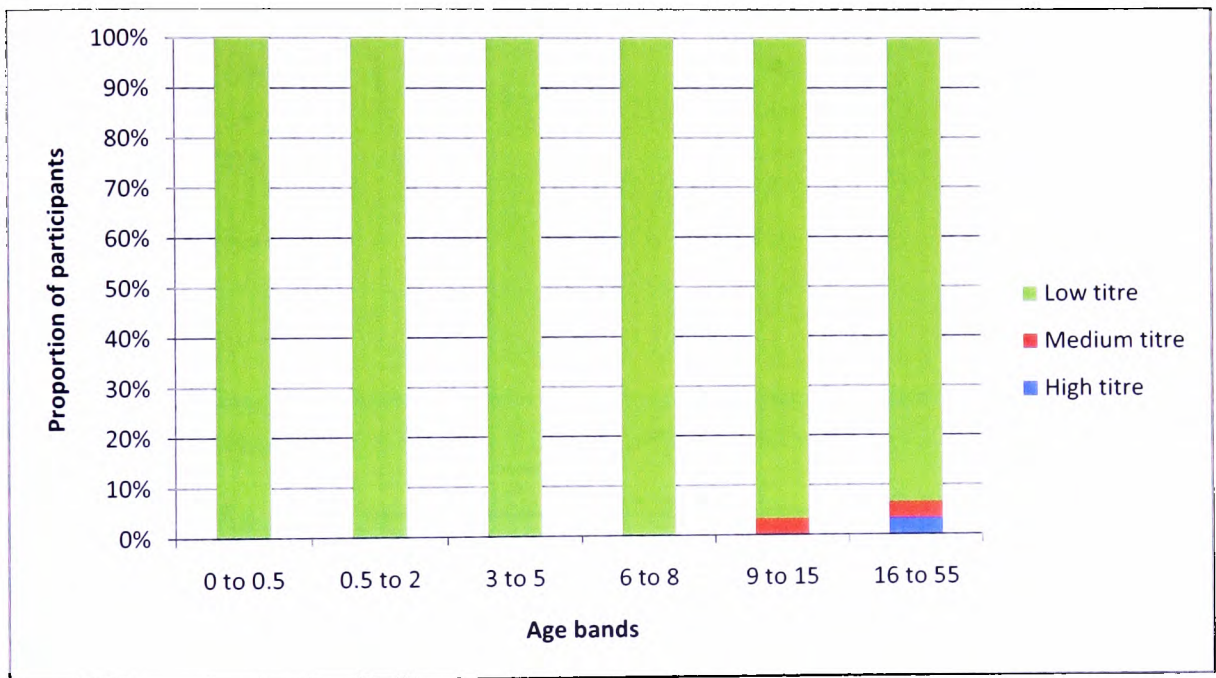


Figure 3.3.6. Age specific proportion of high (≥ 256), medium (32-128) and low (≤ 16) *S. typhi* bactericidal titres in the UK cohort

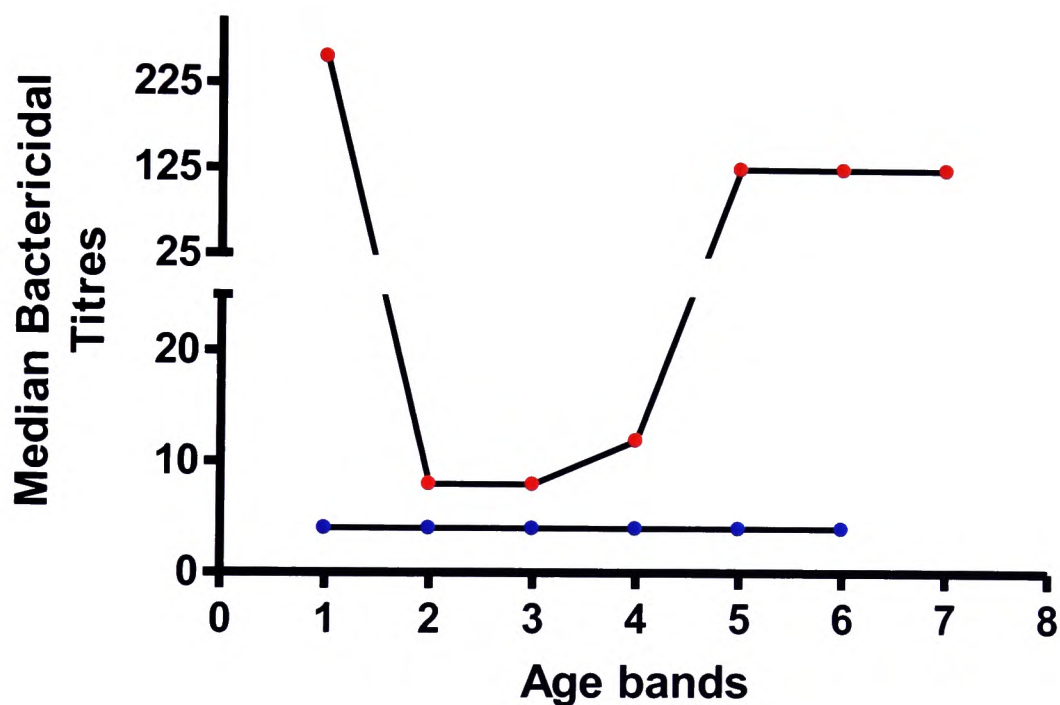


Fig 3.3.7. Comparison of the age distribution of natural immunity to *S. typhi* between the Nepal and UK cohorts determined by SBA and expressed in median bactericidal titres. 1 = newborn or cord blood, 2 = 6 months to 2 years, 3 = 3 years to 5 years, 4 = 6 years to 8 years, 5 = 9 years to 15 years, 6 = 16 to 55 years and 7 = 55 to 75 years (for Nepal cohort only)

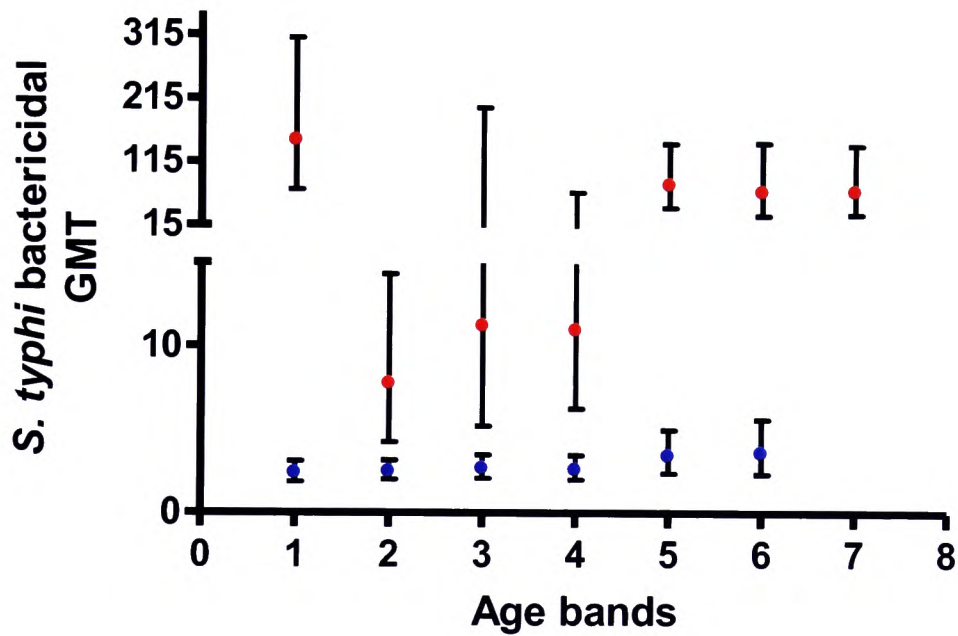


Fig 3.3.8. Comparison of the Geometric Mean Titre of *S. typhi* bactericidal antibodies (with 95% C.I) in the Nepal and UK cohort. 1 = newborn (UK cohort) or cord blood (Nepal cohort), 2 = 6 months to 2 years, 3 = 3 years to 5 years, 4 = 6 years to 8 years, 5 = 9 years to 15 years, 6 = 16 to 55 years and 7 = 55 to 75 years (for Nepal cohort only)

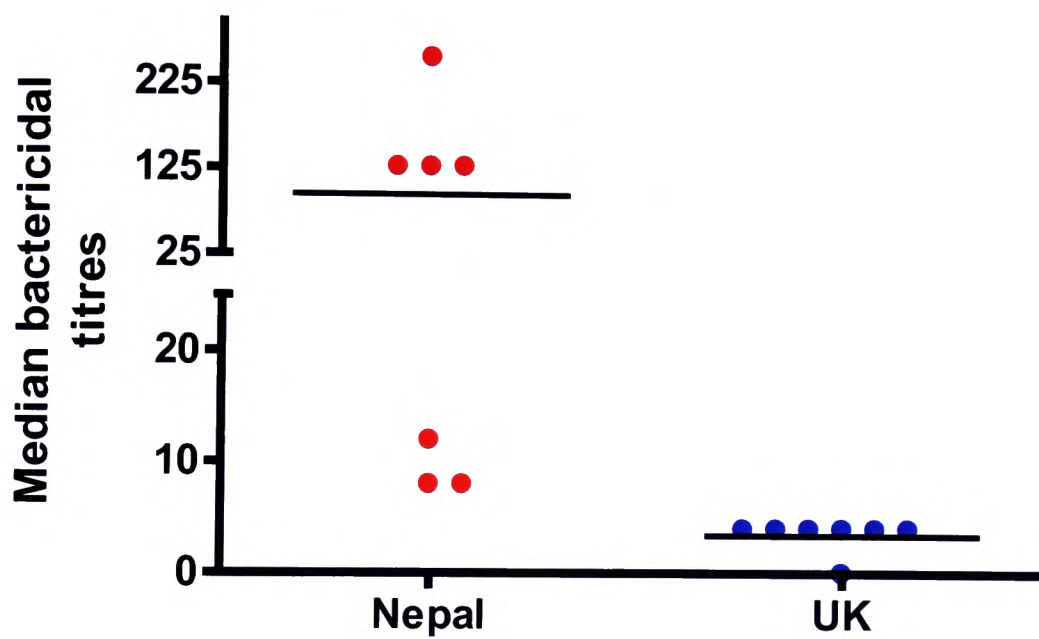


Fig. 3.3.9. Comparison of the median bactericidal titres of the Nepal and UK cohorts including all samples in the 7 and 6 age bands respectively

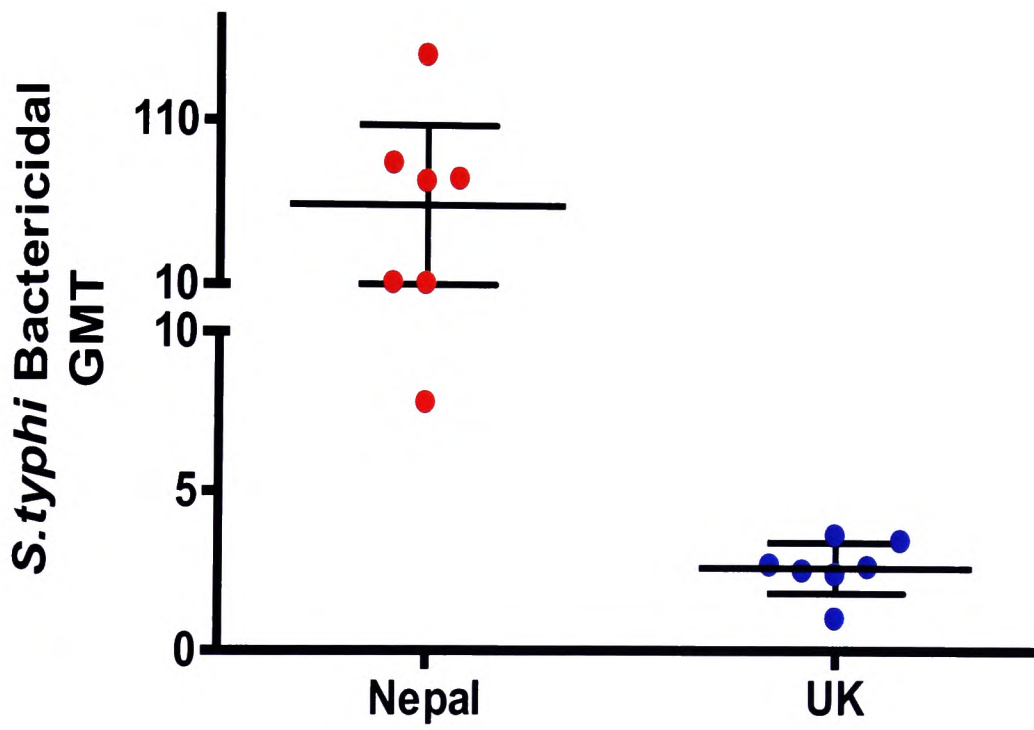


Fig. 3.3.10. Comparison of the *S. typhi* bactericidal GMT (with 95% C.I) of the Nepal and UK cohorts including all samples in the 7 and 6 age bands respectively

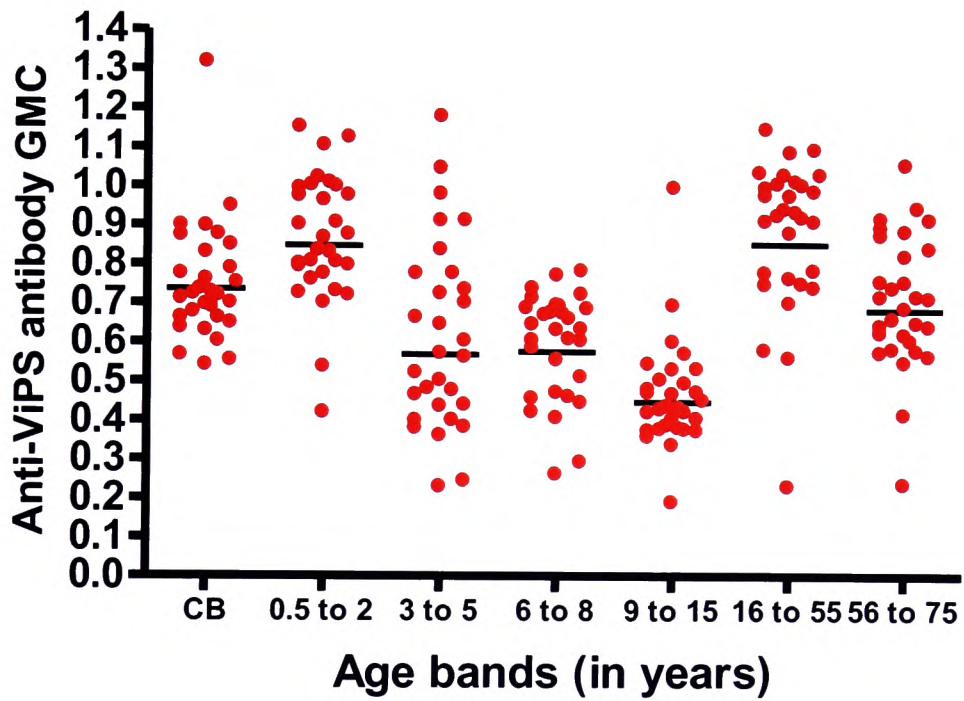


Fig 3.3.11 Anti-ViPS antibody Geometric Mean Concentration of the seven age bands in the Nepal cohort

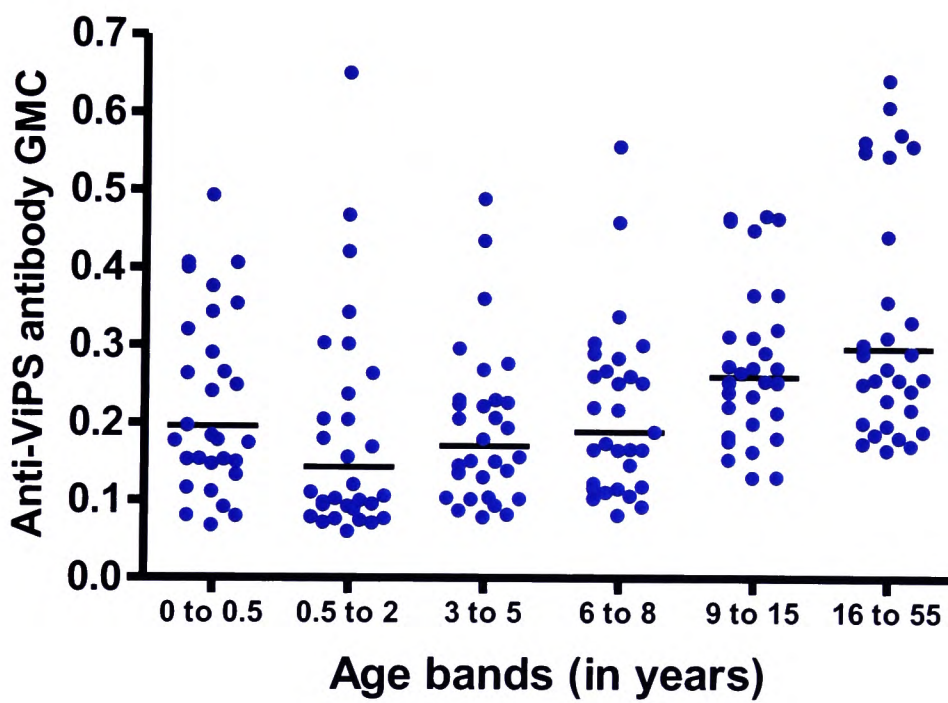


Fig 3.3.12 Anti-ViPS antibody Geometric Mean Concentration of the six age bands in the UK cohort

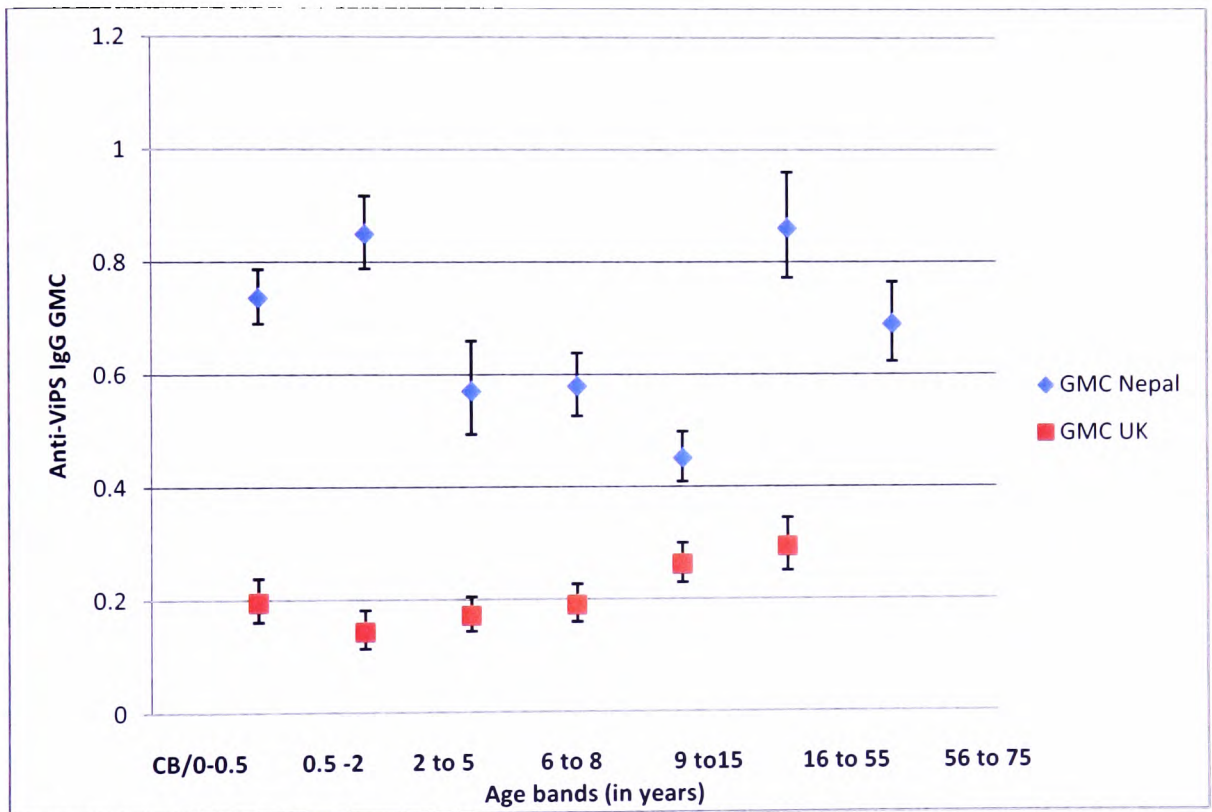


Fig 3.3.13 Comparison of the anti-ViPS antibody Geometric Mean Concentration between the Nepal and UK cohort. CB: cord blood

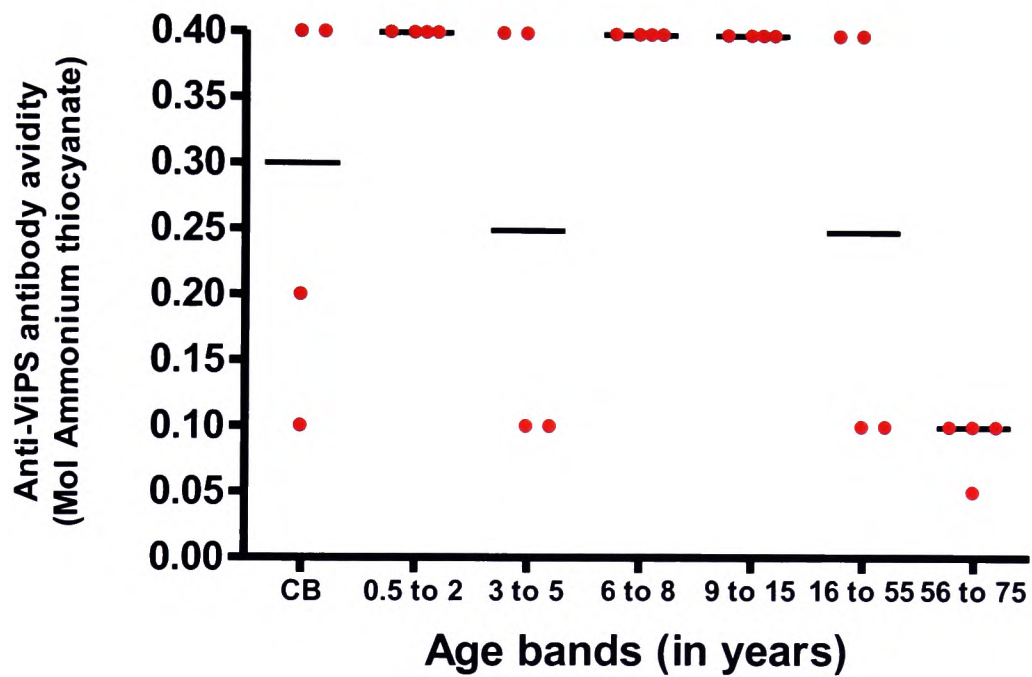


Fig 3.3.14. Median anti-ViPS antibody avidity of 28 samples in the Nepal cohort, representing 4 from each age band, expressed as Molar ammonium thiocyanate

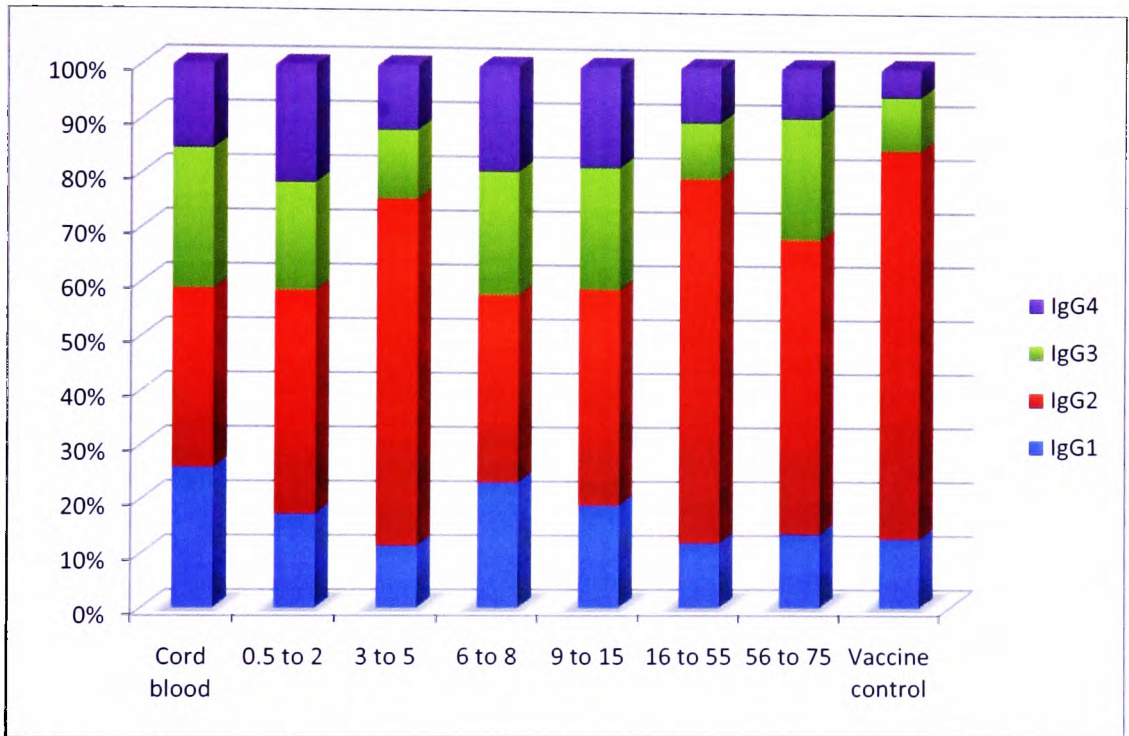


Fig 3.3.15. Proportion of IgG1, IgG2, IgG3 and IgG4 of the total anti-ViPS antibody calculated as median concentrations for each subclass within each age band

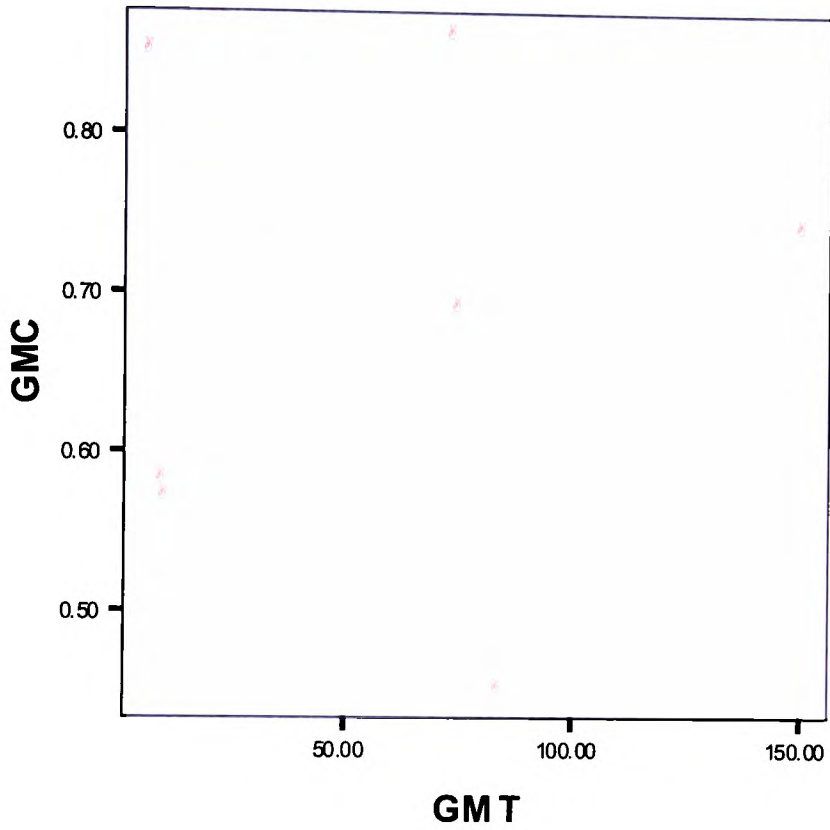


Fig 3.3.16. Correlation between the *S. typhi* bactericidal Geometric Mean Titre and the anti-ViPS antibody Geometric Mean Concentration of the 7 age bands in the Nepal cohort. $r = 0.07$

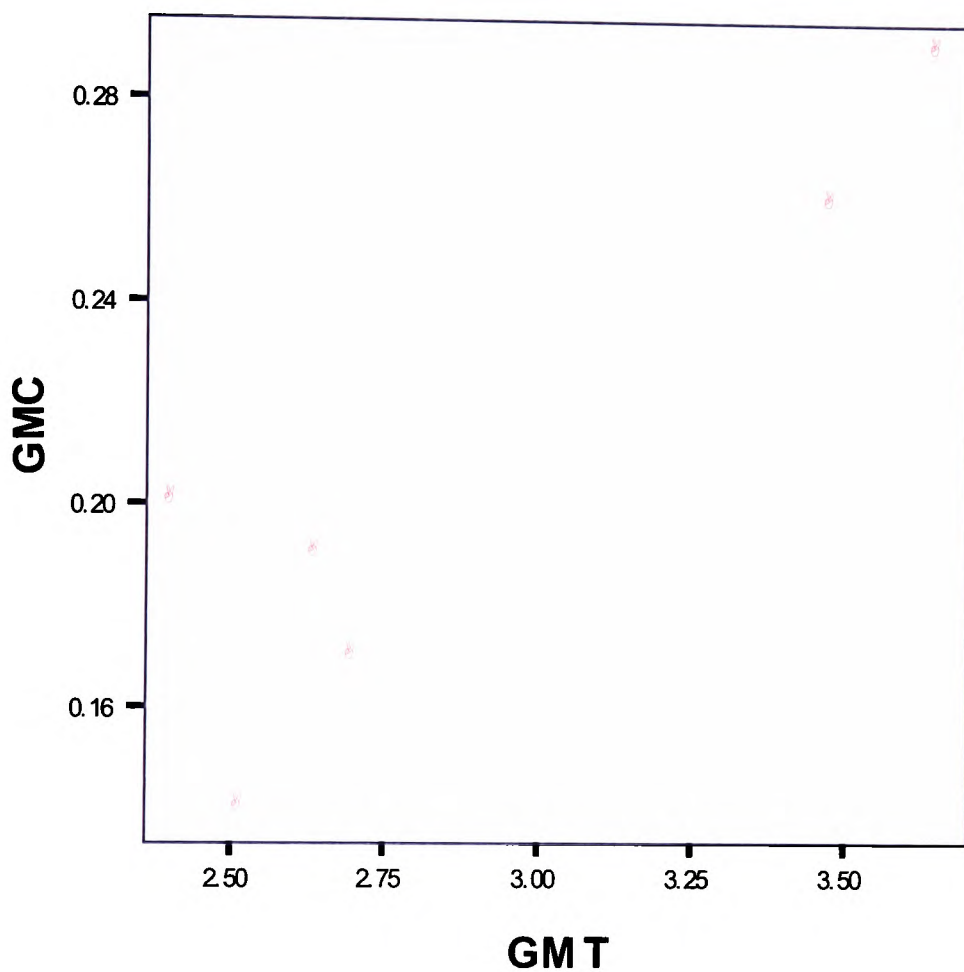


Fig 3.3.17. Correlation between the *S. typhi* bactericidal Geometric Mean Titre and the anti-ViPS antibody Geometric Mean Concentration of the 6 age bands in the UK cohort.

$$r = 0.92$$

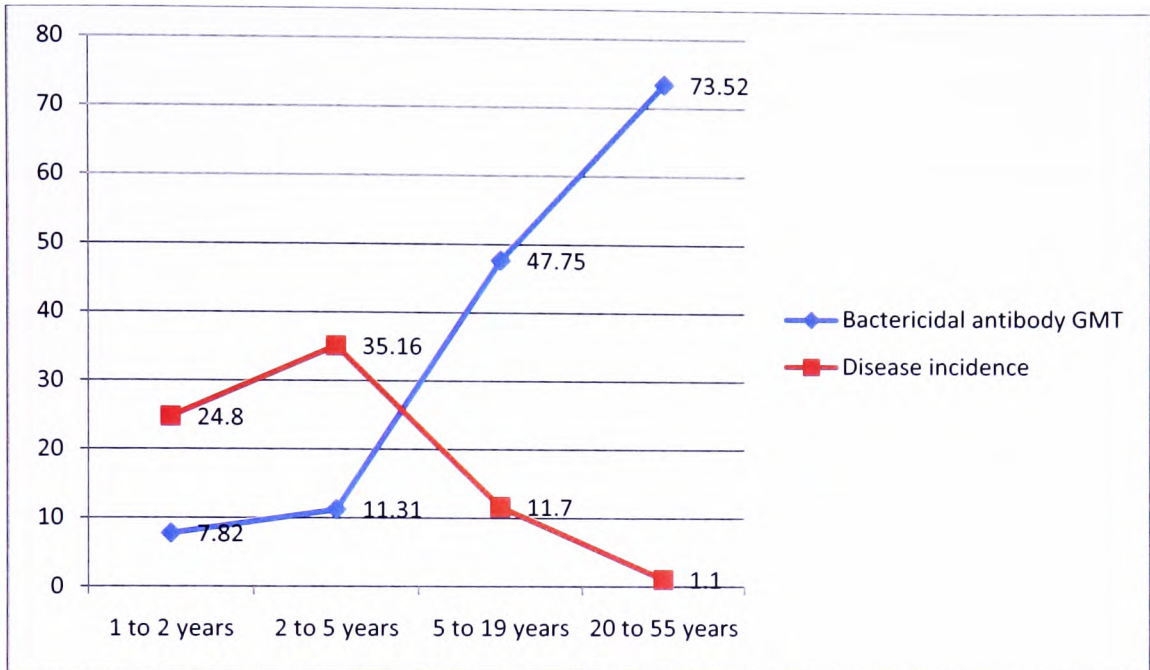


Figure 3.3.18. Inverse relationship between bactericidal antibody GMT in the Nepal cohort and typhoid fever incidence rates in different age groups in New Delhi, India ⁴

3.4 Discussion

This section describes the distribution of natural immunity in an age stratified population in a typhoid endemic region. Our understanding of the mechanisms of protection against typhoid fever, *in vivo*, is incomplete. Based on the protection offered by the Vi polysaccharide vaccine, it is believed that circulatory, bactericidal antibodies are important for immunity against typhoid fever¹⁵⁰. Similarly, secretory IgA and cell mediated immunity, induced by the live attenuated vaccine is also thought to be protective¹⁵¹. Therefore, a combination of different mechanisms appears to contribute to immunity against *S. typhi* following vaccination. Acquisition of natural immunity in response to clinical and sub-clinical exposure to *S. typhi* is however less well understood.

The hypothesis that natural immunity to typhoid fever is age dependant and related to sub-clinical exposure to *S. typhi* in populations in endemic countries was validated with the results of this study utilising a serum bactericidal assay targeting *S. typhi*. Significant differences were observed between the bactericidal titres in children and adults. Children between 6 months to 8 years had significantly lower bactericidal titres in contrast to children over 8, adults and cord blood sera. The high titres in cord blood and subsequent decrease after six months of age could be explained by the presence of maternal antibodies that were transferred transplacentally and its subsequent waning during infancy.

The inference that subsequent sub-clinical exposure to *S. typhi* induces bactericidal antibodies is strengthened by the increase in GMT in late childhood. It is reasonable to believe that because *S. typhi* is an enteric pathogen that is transmitted through contaminated food and water, maximal exposure occurs during early school age, due to reasons of relatively poor food and water hygiene in this age group and greater opportunities for the consumption of contaminated

food and water outside of home, unlike in infancy and during the toddler period. The presence of relatively high bactericidal titres in older children and adults, possibly as a consequence of sub-clinical exposure, could explain the lower incidence of typhoid fever in these age groups in endemic regions. Additionally, multiple field studies in endemic countries showed that the incidence rate of typhoid fever is the highest in children below five years, followed by young adults. These incidence data are substantiated with our observation of the prevalence of low bactericidal antibody titres in children below eight shown in this study.

Goldschneider, in a seminal paper in 1969, demonstrated that susceptibility to meningococcal disease is inversely related to the presence of serum bactericidal antibody ¹⁵². This was determined on the basis of a study in military recruits, using an SBA, which found that individuals with naturally acquired titres of ≥ 4 were protected from meningococcal serogroup C disease. In the absence of vaccination, such antibodies were generated naturally following periods of meningococcal carriage and also through transplacental acquisition. The nadir in protective bactericidal titre was at 12 months of age, the point at which the highest incidence of disease was found, from when it consistently increased to achieve adult titres by mid-teen age ¹⁵³.

Similarly, an inverse relationship between the increase in bactericidal antibodies against *H. influenza* between 2 and 3 years of age in children and a decrease in the incidence of the invasive Hib disease was first reported by Fothergill and Wright in 1933¹⁵⁴. A possible explanation for the increase in *H. influenza* and *N. meningitidis* bactericidal titres after the first year of life, in contrast to 8 years in children for *S. typhi*, could be that the former are air borne pathogens with which nasopharyngeal colonisation and carriage occurs earlier in life and hence

the resultant natural immunity, while exposure to *S. typhi* might be delayed till early school age due to the reasons mentioned earlier.

Unlike *N. meningitidis* serogroup C, for which the correlate of protection, in terms of bactericidal titre, has been estimated as ≥ 4 using human complement, the titre beyond which protection is afforded against *S. typhi* remain unknown. Hence, in the absence of an established threshold for protection, the determination of the proportion of the population that possesses protective antibody titres as a result of sub-clinical exposure is not possible. However, based on the results of this study that demonstrates median bactericidal titres of 128 for the age groups above 8 years and 12 for children below 8 years, combined with the low disease incidence data in the former age band it is speculated that the *S. typhi* protective antibody titre might be above 16. A weakness in this argument is that in the absence of disease data from the study population in Nepal, these numbers were derived from disease incidence rates from other studies.

Likewise for protective antibody concentrations, the anti-PRP antibody concentration for *H. influenza* is reported to be 0.15 $\mu\text{g}/\text{mL}$ and 1.0 $\mu\text{g}/\text{mL}$ as a correlate of short term and long term protection respectively ¹⁵⁵. Although similar putative protective antibody concentrations are unavailable for *S. typhi*, Robbins and colleagues in 1996 suggested, on the basis of a ViPS vaccine trial that computed vaccine protective efficacy and anti-ViPS antibody titre in vaccinees, that an absolute antibody concentration between 0.6 to 1.2 $\mu\text{g}/\text{ml}$ might be protective. Furthermore they suggested that by utilising this cut-off, 64% of vaccinees and 40% of controls at the mean age of 9 years had protective levels, the latter presumably due to natural acquisition of antibodies, in the study population in Cape Town, South Africa ¹⁵⁶.

A correlation between the bactericidal titre and anti-ViPS concentration in the Nepal population was not observed. Three reasons could explain this phenomenon. Firstly, antibodies against

other immunodominant antigens, e.g. the somatic 'O' antigen and iron regulated outer membrane proteins (IROMP), might account for the predominant bactericidal activity in SBA. Secondly, the expression of ViPS, strictly regulated by multiple operons, with its expression found in response to environmental stressors and non-expression in the bacterial intracellular niche could explain the discrepancy. This explanation is strengthened by observations from other studies that showed only 20% of patients with acute typhoid fever developing anti-Vi antibodies⁹³. Thirdly, although the absolute antibody concentrations in the Nepal population are higher than the UK population, other factors like antibody avidity and immunoglobulin subclass that determine functionality could explain its non-correlation with bactericidal titres. Relatively low geometric means of both antibody concentration and bactericidal titre might explain the correlation for the UK population.

Previous studies on the seroprevalence of typhoid antibodies have mostly dealt with the somatic 'O' antigen and the flagellar 'H' antigen, using the Widal test^{131,132}. A recent study in India that tested for the 'O' and 'H' antigen among 250 healthy children between the ages of 6 months and 15 years revealed that no child in the age group of 6 months to 2 years had a high titre of 1:80 while only 5.17%, 3.03%, 6.55% and 3.6% of children had the same titre for both antigens in the age groups of 3 to 5 years, 6 to 10 years and 11 to 15 years respectively¹²⁹. This study was accomplished by means of a sensitive and specific functional assay that is more reliable than non specific assays that target single antigens and determine total antibody titres in contrast to functional activity. This study establishes the kinetics of functional, natural immunity to *S. typhi* in an age stratified population resident in a typhoid endemic country.

CHAPTER 4: CHARACTERISTICS AND KINETICS OF HUMORAL IMMUNE RESPONSE TO THE TYPHOID Vi POLYSACCHARIDE VACCINE

4.1. Introduction

Typhoid fever, an enteric infection caused by *Salmonella enterica* subspecies *enterica* serovar Typhi, is responsible for 17 million new cases and 200,000 deaths a year in developing countries where clean drinking water is unavailable or human sewage disposal facilities are inadequate. The bacterium is characterised by a Virulence factor polysaccharide capsule (ViPS), a linear homopolymer of alpha (1-4) D-GalpANAc variably O acetylated at the C-3 position¹⁹. This feature has been exploited for serological diagnostic purposes and for the design of a polysaccharide vaccine.

Developed in the 1980s¹³⁶, the polysaccharide vaccine was licensed by the FDA in 1994¹³⁷ following results from a study in 11384 children that demonstrated a protective efficacy of 60% calculated from the day of vaccination and 64% from 6 weeks after vaccination in comparison to children who received the meningococcal A + C CPS vaccine. Similarly, 77.4% and 81.0% protective efficacy was observed, after 21 months, calculated immediately and 6 weeks after vaccination respectively, in comparison to unvaccinated children¹³⁸.

The purified polysaccharide vaccine confers moderate clinical protection for up to 3 years following a single dose vaccination. Cumulative 3 year efficacy is 55% (95% CI 30-70%). Statistically significant protection was conferred in the first 2 years but not the third year after

vaccination with vaccine efficacy rates of 68% (95% CI 50-80%), 60% (95% CI 31-76%) and 50% (-11-78%) in the first, second and third year respectively following vaccination ¹³⁹. Recent developments include the conjugation of the Vi polysaccharide vaccine to inactivated *Pseudomonas aeruginosa* exotoxin A that has been shown to confer 91.1% protection in 2 to 5 year old Vietnamese children 27 months after vaccination ¹⁴³.

Despite being in existence for more than two decades, the kinetics of the immune response following vaccination with the ViPS is poorly understood. Evaluation of the immune response to polysaccharide bacterial vaccines primarily relies on the measurement of antigen specific antibody. While antibody levels might be a reliable indicator of protection in the short-term, where the correlates of protection are known, the initial antibody response might not always correspond to the long-term protection induced by immunisation. Indeed, in young children, circulating antibody can drop rapidly and it has been shown that protective immunity can persist after immunisation when antibody is no longer detectable ^{181, 182}. Long-term humoral immunity may be better represented by the determinants of the B cell pool induced by immunisation from which long-lived plasma cells and memory B cells are derived.

Determining the magnitude and timing of the appearance of antibody, plasma cells and memory B cells in peripheral blood following vaccination could not only help in understanding the development and maintenance of long-term immunity to typhoid fever but is also critical to the design and licensure of new generation vaccines based on the ViPS. In this study, we evaluated the kinetics of anti-*S. typhi* bactericidal antibody, plasma cell, memory B-cell and anti-ViPS

antibody in response to a single dose of the ViPS typhoid vaccine in immunologically naïve adults.

4.2. Materials and Methods

4.2.1 Study population

This observational vaccine study was conducted in Oxford between June and December 2007. Ten, healthy, adult volunteers, aged 18-55 years, were enrolled after written informed consent was obtained. Exclusion criteria included having received any typhoid vaccine in the previous three years, having had typhoid fever, the presence of an immunological pathology or treatment with immunosuppressive drugs, a history of allergic reaction after previous vaccinations or known hypersensitivity to any vaccine component and a positive pregnancy test. The study was approved by Oxfordshire's Research Ethics Committees (approval number BO7/Q1605/29).

4.2.2 Vaccine

Typhim Vi[®], Typhoid Vi Polysaccharide Vaccine, produced by Aventis Pasteur SA, for intramuscular use, is a sterile solution containing the cell surface Vi polysaccharide extracted from *Salmonella enterica* serovar Typhi, *S typhi* Ty2 strain. The organism is grown in a semi-synthetic medium without animal proteins. The capsular polysaccharide is precipitated from the concentrated culture supernatant by the addition of hexadecyltrimethylammonium bromide and the product is purified by differential centrifugation and precipitation. The potency of the purified polysaccharide is assessed by molecular size and O-acetyl content. Phenol, 0.25%, is added as a preservative. The vaccine contains residual polydimethylsiloxane or fatty-acid ester-based antifoam. The vaccine is a clear, colorless solution. Each dose of 0.5 mL is formulated to contain 25 mg of purified Vi polysaccharide in a colorless isotonic phosphate buffered saline

(pH 7 ± 0.3), 4.150 mg of Sodium Chloride, 0.065 mg of Disodium Phosphate , 0.023 mg of Monosodium Phosphate and 0.5 mL of Sterile Water for Injection.

4.2.3 Immunisation and sampling protocol

0.5 ml of the typhoid polysaccharide vaccine, containing 25 μ g of ViPS, was given by intramuscular injection into the deltoid region at day 0 for each participant. After vaccination, the volunteers were observed for 15 minutes for any immediate adverse vaccine reactions. Blood samples (20 ml) were collected by venipuncture, prior to immunisation (Day 0) and on days 2, 4, 7, 10, 14 and 28. At every sampling, the volunteers were questioned on adverse events relating to the vaccine. 15 ml of whole blood was separated into a heparin tube for peripheral blood mononuclear cells (PBMCs) preparation within one hour and 5 ml was spun at 3000 rpm for serum that was stored at -20°C until further analysis.

4.2.4 Serum Bactericidal Assay

Bactericidal activity was determined by means of a novel SBA. In brief, two fold dilutions of decomplexed sera (56°C for 30 min), starting from 1:4 up to 1:1024, were incubated in Hanks Balanced Salt Solution (HBSS, Sigma, UK), with a working solution of *S. typhi* (NCTC Ty2 strain) and 1:8 dilution of freshly thawed baby rabbit complement (Pel-Freez Biologicals, USA) before overnight incubation on Luria-Bertani (LB) agar plates. The inverse of the highest dilution factor at which $>50\%$ bacteria were killed, as compared to a control well, was considered the bactericidal titre for that sample. Results of three assay controls for each sample including a viable count (containing no serum or complement but HBSS and *S. typhi*, to serve as bacterial colony count control), a complement independent control (containing no complement but serum and *S. typhi*, to detect intrinsic serum bactericidal activity) and a complement control

(containing no serum but complement and *S. typhi*, to detect bacterial complement sensitivity) confirmed the validity of the bactericidal activity. All samples were analysed in duplicate and the average results recorded.

4.2.5 Vi polysaccharide for ELISA & ELISpot

Lyophilised ViPS, manufactured according to the method described above and donated by Aventis-Pasteur, was reconstituted in sterile, distilled water to a concentration of 5µg/ml for use as coating antigen in the Vi polysaccharide ELISA and ELISpot.

4.2.6 Reference ELISA for IgG concentration

In the absence of a standard reference serum for anti-ViPS antibody, a reference ELISA was designed to establish the absolute antibody titre for a control serum obtained from a volunteer who had received the ViPS vaccine previously. This technique is based on the principle of equivalence of absorbance of 2 ELISAs performed in parallel under identical assay conditions representing equivalent amounts of antibodies when corrected for concentration ¹³. In brief, serial two fold dilutions of a European Reference Material (ERM DA470) containing 9.68g/L of total IgG and starting at 1:100,000 dilution was run simultaneously with serial two fold dilutions of the test serum starting at 1:25 dilution. The rest of the protocol for the total IgG ELISA described below was followed. Optical density (O.D) obtained for both ELISAs were plotted concurrently and absolute antibody titre for the test serum estimated by correcting for the dilution factor.

4.2.7 Vi polysaccharide ELISA

S. typhi Vi polysaccharide specific IgG concentrations were determined using a novel ELISA. In brief, Maxisorp plates (Nunc) were coated with 100µl of Vi polysaccharide (Sanofi-Pasteur, France). Following blocking with 2% skim milk powder (Sigma, UK), duplicates of a 1:25 dilution of serum were incubated for 2 hours. Additionally, 3 dilutions (1:25, 1:200, and 1:800) of an internal quality control serum were run on each plate to confirm that the O.D of these samples were in the high, medium and low range. A reference serum, the antibody titre of which was determined by the reference ELISA method described above, was used for the estimation of absolute IgG titres in the test sera. The plate was developed with anti-human IgG γ -chain conjugate (peroxidase) (Sigma, UK) (diluted in serum/conjugate buffer) for an hour followed by chromogenic substrate tetramethylbenzidine dihydrochloride monohydrate (Sigma, UK), and the reaction was stopped after 15 minutes with 2M sulphuric acid. The O.D of each well was then read at 450nm.

4.2.8 Preparation of PBMCs

15 ml heparinised blood was diluted 1:2 with RPMI-1640 medium (Sigma-Aldrich, England) to which penicillin-streptomycin solution (Sigma-Aldrich, England) and L-glutamine 200 mM (Sigma-Aldrich, England) had been added at a dilution of 1:100 (complete medium). The PBMCs were then separated by density gradient centrifugation over Lymphoprep (Axis-Shield, Diagnostics, England). PBMCs were washed once in complete medium prior to further preparation for ELISpot or cell culture.

4.2.9 Preparation of ELISpot plates

ELISpot plate (96 well PVDF membrane) (Millipore, England) wells specific for ViPS were first coated with 100 µl/well ViPS(5µg/ml) in KRT solution pH 7.4. Remaining ELISpot wells were coated with 10 µg/ml tetanus toxoid (Statens Serum Institut, Denmark), and 10 µg/ml goat anti-human Ig (Caltag laboratories, Burlingame, USA) in sterile PBS. PBS alone was added to the Ag blank wells. The ELISpot plates were stored at 4°C until use. Tetanus toxoid and immunoglobulin wells were used as positive controls and PBS wells as negative controls.

4.2.10 Detection of plasma cells

Washed PBMCs prepared from peripheral blood were re-suspended to a final concentration of 2×10^6 PBMCs/ml in complete medium with 10% foetal calf serum. 100 µl/well of the suspension was added to ELISpot plates, previously blocked with complete medium with 10% foetal calf serum, and incubated for 4 hours at 37°C in 5% CO₂ and 95% humidity. The cells were washed with PBS-Tween and bound IgG antibodies detected using a 1:5000 dilution of goat anti-human IgG, IgA, or IgM γ -chain specific alkaline phosphatase conjugate (Calbiochem, UK) in complete medium with 10% foetal calf serum, developed using a 5-bromo-4-chloro-3-indolyl phosphate in nitroblue tetrazolium dissolved in aqueous dimethylformamide (Bio-Rad Laboratories, England).

4.2.11 Detection of memory B-cells

PBMCs prepared from peripheral blood were re-suspended in complete medium with 10% foetal calf serum at a final concentration of 2×10^6 PBMCs/ml and 100 μ l was added to each 96-well round bottomed culture plates (Fisher, England). Medium, containing 1/5000 *Staphylococcus aureus* (Cowan strain, SAC) (Calbiochem, England), 1/6000, 83 ng/ml pokeweed mitogen (PWM) (Sigma-Aldrich, England) and 1/40 or 2.5 μ g/ml CpG oligonucleotide (ODN-2006) (Invitrogen, England) was added. The cells were incubated at 37°C in 5% CO₂ for 5 days before being re-suspended and washed. The cultured cells were plated onto pre-coated ELISpot plates at a cell concentration of 2×10^6 cells/well and then incubated and developed as for the ex-vivo ELISpot described above.

4.2.12 ELISpot counting

Spots were counted using an AID ELISpot Reader (Eltek-AID) and ELISpot software, version 3.2.3 (Cadama Medical Ltd, Stourbridge, UK). Spot-forming cells were counted and confirmed by visual inspection. Identical settings were used for all plates but different settings were used for the different antigens (Fig 4.2.1). Antibody forming spots were large, spherical in size with “fuzzy” granular edges (Fig 4.2.2). Spots that did not fit this description were not counted and considered as in vitro artefacts.

4.2.13 Statistical analyses

Statistical analyses were performed using Excel 2007 and Prism version 5. Median bactericidal titre besides geometric mean titre (GMT) and geometric mean concentration (GMC) were calculated for anti- *S. typhi* bactericidal antibody and anti-ViPS antibody respectively. Medians were calculated for the B-cell counts. Correlation between the log-transformed bactericidal antibody titre and log-transformed total antibody concentration was performed using Pearson correlation while Spearman's rank correlation method was used to correlate log-transformed antibody concentration and untransformed B cell counts.

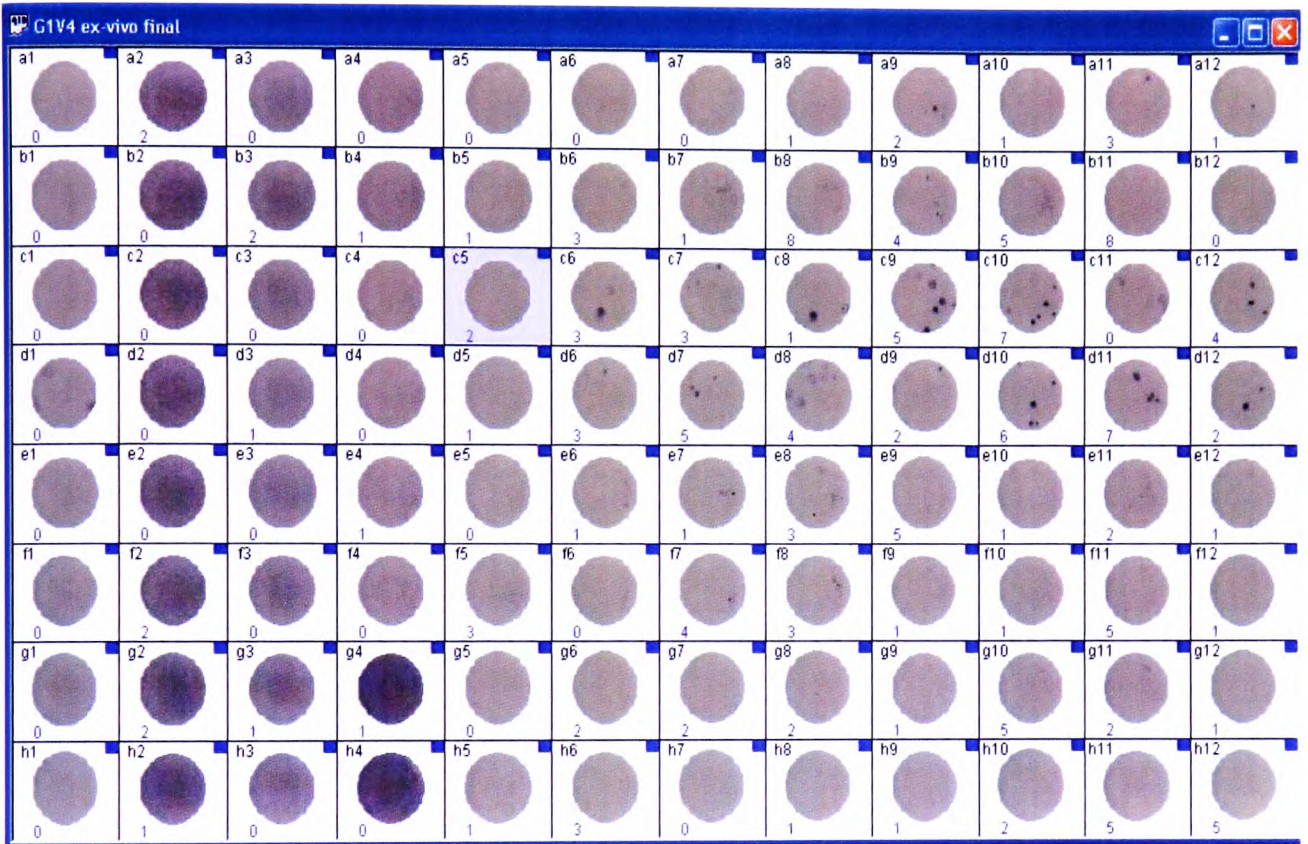


Fig 4.2.1. Sample ELISpot plate demonstrating spots formed by antigen secreting cells.

Column 1 is the negative control with PBS, column 2 and 3, with dark blue background, represent total IgG cells, column 4 and 5 represent tetanus and diphtheria antigen specific cells respectively, and rows 6 to 12 represent ViPS antigen specific plasma cells.

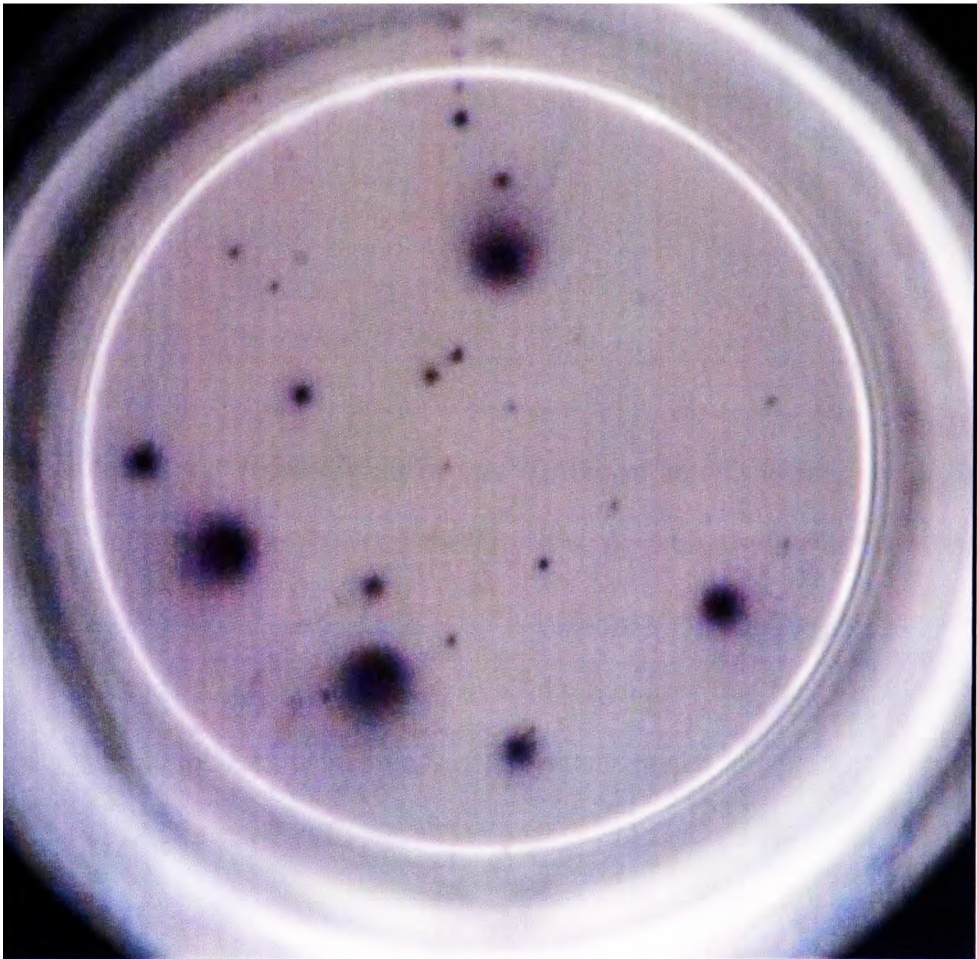


Fig 4.2.2. Sample well demonstrating spots formed by the ViPS specific memory B-cells

4.3. Results

4.3.1 Recruitment

All 10 participants who were enrolled in the study completed the participation.

4.3.2 Reactogenicity

No adverse reactions were reported at any visit by the participants.

4.3.3 SBA Assay

Prior to immunisation, the geometric mean titre (GMT) of anti-*Salmonella typhi* bactericidal antibody was 8. Following immunisation the titre was significantly higher 28 days following vaccination with a titre of 45.25 ($p < 0.001$). This represented a 4 fold rise in anti- *S. typhi* antibody GMT between day 0 and day 28 (Fig 4.3.1). Similarly, the median bactericidal titres demonstrated a six fold increase from 8 on Day 0 to 48 on Day 28 (Fig 4.3.2). The magnitude of change in bactericidal titre during this period is illustrated in Figure 4.3.3.

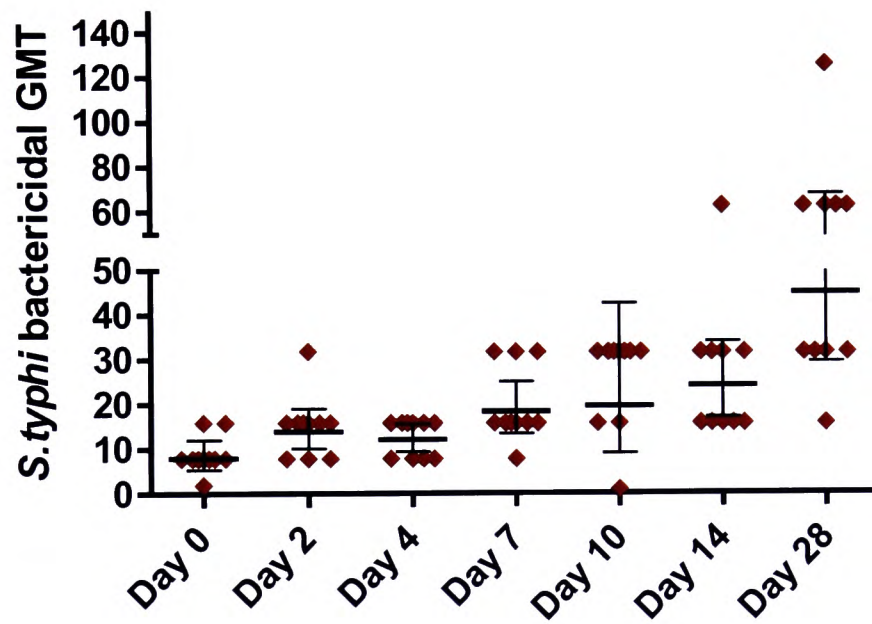


Fig 4.3.1 *S. typhi* bactericidal Geometric Mean Antibody Titre of the 10 participants at each of the seven study test points

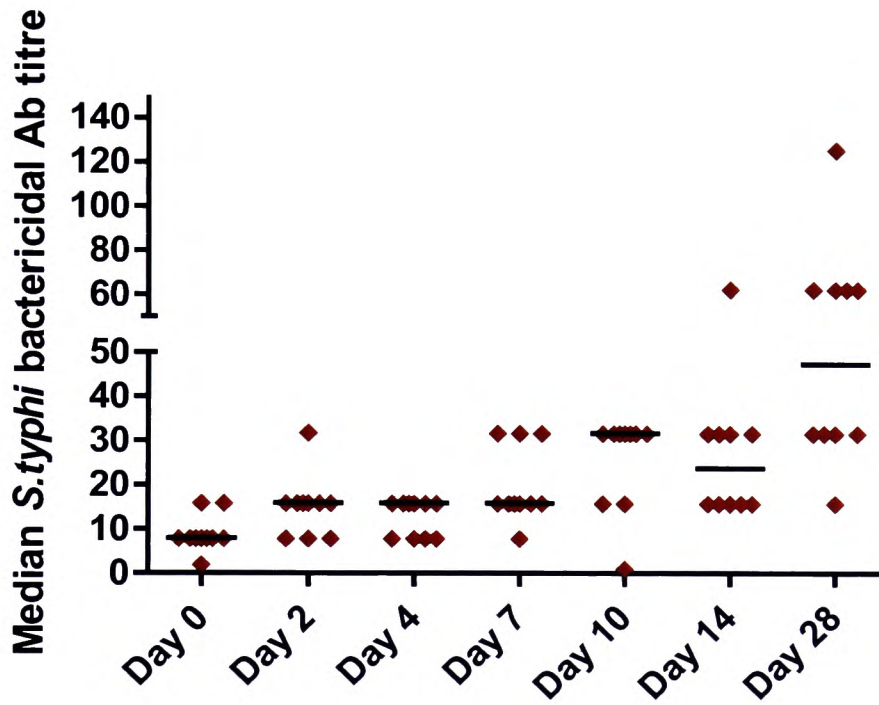


Fig 4.3.2. Median bactericidal titres, expressed as the inverse of the highest dilution factor demonstrating >50% bactericidal activity in the SBA assay, of the 10 participants at each of the study test points

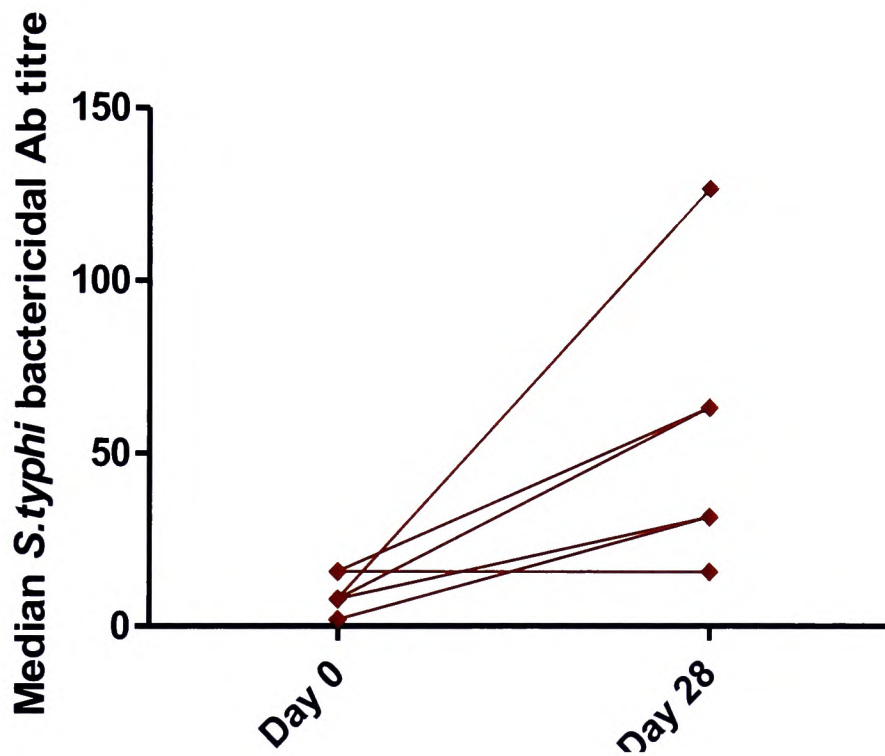


Fig 4.3.3 Magnitude of change in the median bactericidal titre between day 1 and day 28 for each of the 10 participants in the study.

4.3.4 Anti-ViPS ELISA

Prior to immunisation, the geometric mean concentration (GMC) of anti-ViPS IgG was 0.72 $\mu\text{g/ml}$. Following immunisation the IgG-GMC was 0.93 $\mu\text{g/ml}$ (Fig 4.3.4). The magnitude of change for each of the participants' absolute antibody concentration is illustrated in Figure 4.3.5.

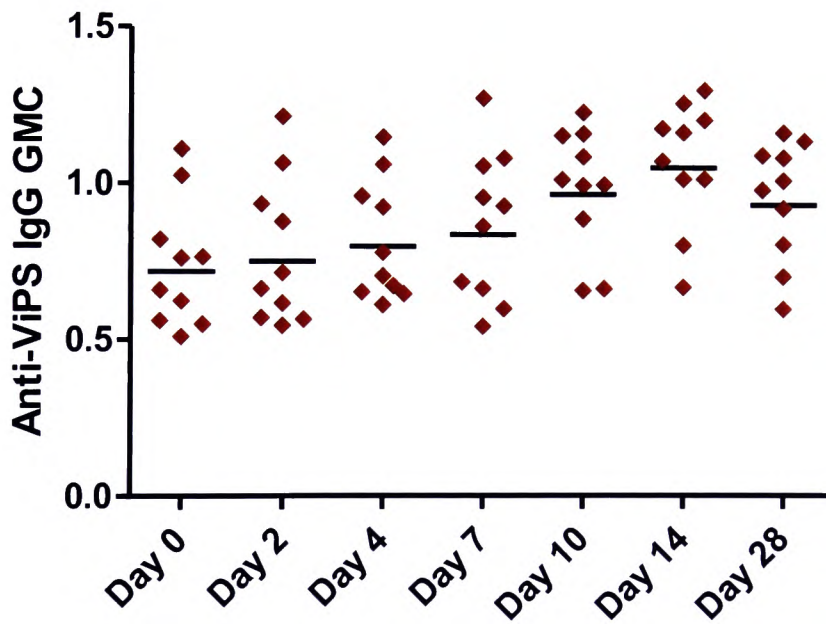


Fig 4.3.4 Anti-ViPS antibody Geometric Mean Concentration of the 10 participants at each of the seven test points in the study

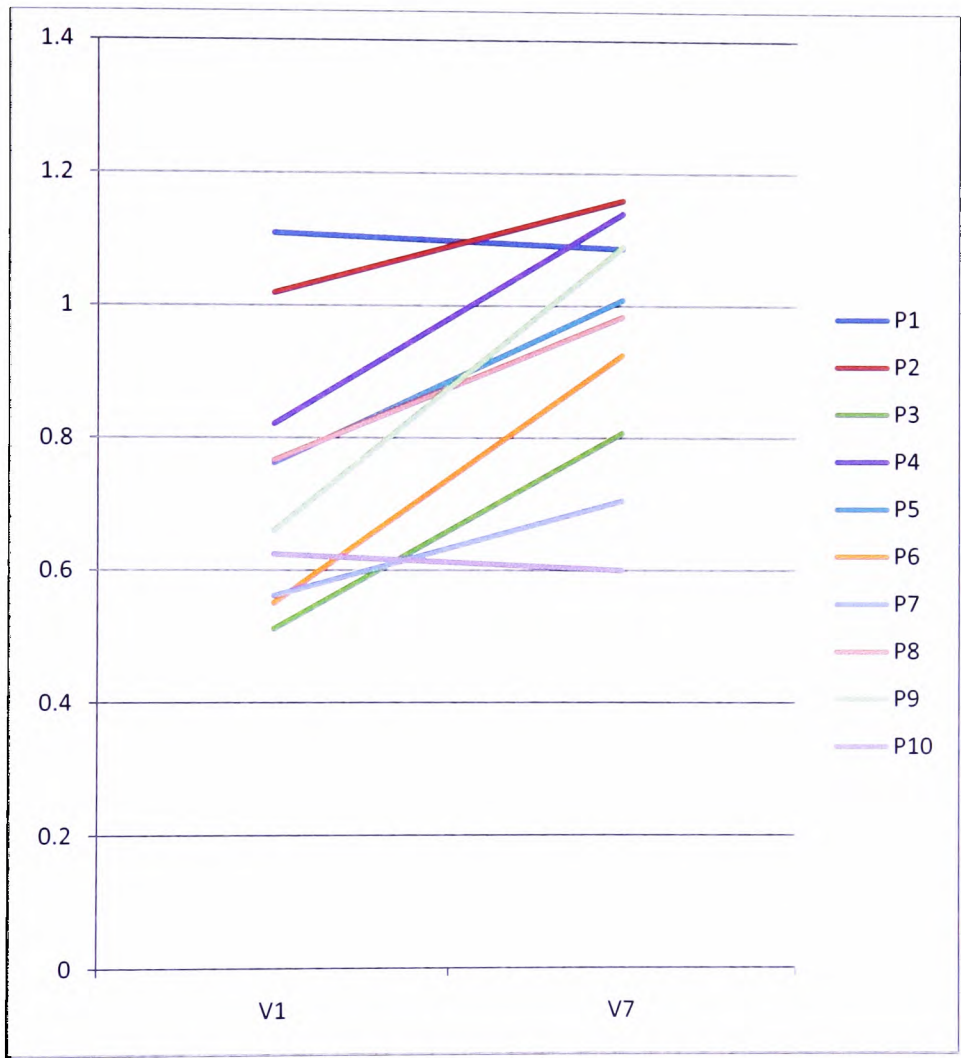


Fig 4.3.5 Magnitude of change in the absolute anti-ViPS antibody concentration between day 1 and day 28 for each of the 10 participants in the study.

4.3.5 Plasma cell response

Before immunisation, median frequency of plasma cells was zero in the peripheral blood of the participants. Following vaccination, a transient but non-significant ($p=0.53$) increase in the median plasma cell count was observed on Day 7 and Day 10 which returned to baseline on Day 14 (Fig 3.4.6).

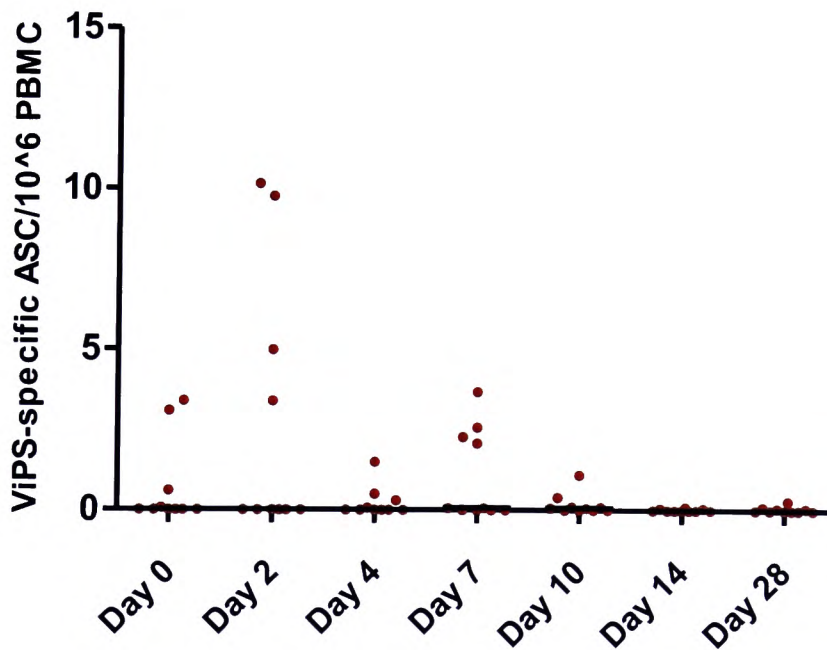


Fig 3.4.6 Median count of the absolute frequency of ViPS-specific plasma cells per 10^6 cultured lymphocytes for the 10 participants at each of the 7 test points in the study.

4.3.6 Memory B cell response

Before immunisation, the median frequency of ViPS-specific IgG memory B cells in peripheral blood of the naïve group was 0. Following vaccination, there was an increase in the median frequency of detectable ViPS-IgG-memory B cells starting on Day 2. Significant differences were observed in the median frequency of memory B cells between Day 0 and Day 7 ($p < 0.05$, Fig 4.3.7).

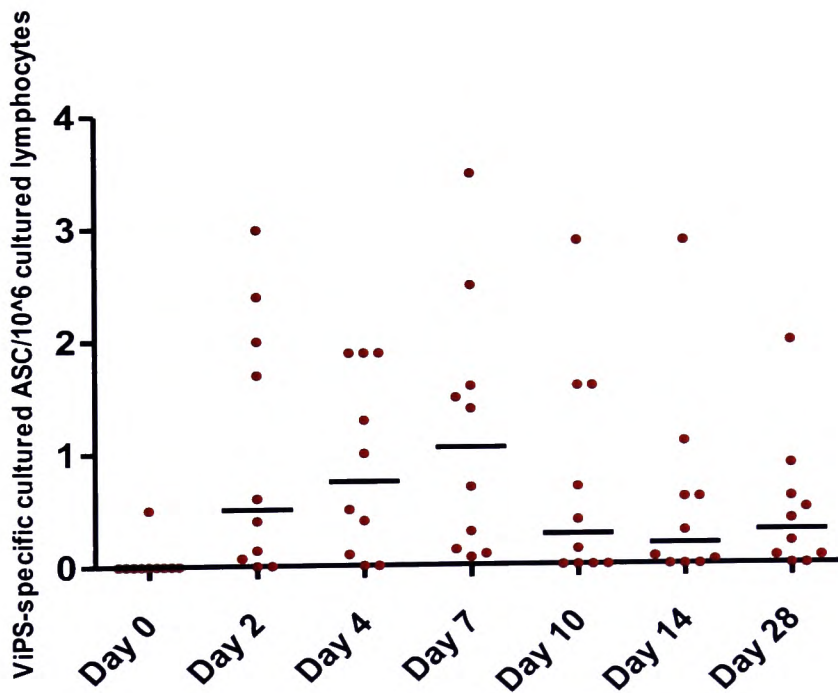


Fig 4.3.7 Median count of the absolute frequency of ViPS-specific memory B-cells per 10^6 cultured lymphocytes for the 10 participants at each of the 7 test points in the study.

4.3.7 Correlation between Antibody and B cell responses

Significant correlation was observed between the anti-*S. typhi* bactericidal GMT and total anti-ViPS IgG GMC on Day 0 ($r=0.80$, $p=0.031$) and on Day 28 following vaccination ($r=0.65$, $p=0.042$). However, no significant correlation was detected between bactericidal GMT and ASC frequency ($r=0.316$, $p=0.498$) or cultured ASC frequency ($r=0$). Similarly, no significant correlation was found between total IgG and ASC frequency ($r=0.316$, $p=0.498$) or cultured ASC frequency ($r=-0.1$, $p=0.84$)

4.4. Discussion

This chapter first describes the kinetics of the humoral immune response to the Vi polysaccharide vaccine. Advantages of the Vi vaccine, viewed in the context of public health measures, includes it being a single dose vaccine and having low rates of adverse effects. Because this vaccine is not patent protected, generic manufacturers are able to significantly reduce the cost of manufacturing making it attractive for wide use in developing countries. Disadvantages include the parenteral route of administration which requires the use of injection device and the skill to deliver an intramuscular injection. Another disadvantage is the relatively short duration of protection of two to three years that would necessitate revaccination of large populations, more frequently. As mentioned before in the chapter on the Vi polysaccharide, there has been concern raised about the usefulness of the ViPS in the era of the acapsulate *S. typhi*. Lastly, its ineffectiveness in children below two could handicap its implementation in a national programme in an endemic country where a significant number of cases are in children below five, with a proportion of them being under two. The last handicap could be overcome with the introduction of the ViPS conjugate vaccine. It is in the context of the large scale implementation of the ViPS vaccine or its derivatives that the findings of this study assume significance.

Protection offered by the ViPS vaccine is estimated to last between two to three years following a single dose. While the precise mechanisms of protection are not completely understood, it is believed, from previous vaccine trials, that circulatory antibody may play a crucial role in mediating protection against *S. typhi* which has both extra-cellular and intra-cellular phases during infection. In the previous chapter it was demonstrated that a lack of bactericidal activity

in children below eight in a typhoid endemic country correlated with the high incidence of disease in this age group. As a corollary to this hypothesis, it could be suggested that the induction of bactericidal antibody against *S. typhi* could account for the protective efficacy of the vaccine. In this study, the SBA assay showed a six fold increase in GMT from 8 prior to vaccination to 48 on Day 28 post-vaccination. In the last chapter it was suggested on the basis of a sero-epidemiology study in a typhoid endemic country that the protective titre may correspond to 1:16. All participants in the study achieved the titre by Day 28 post-vaccination.

The duration of protection offered by the vaccine exceeds the half life of antibody produced in response to vaccination. The persistence of protection could be explained by the presence of long lived plasma cells or memory B-cells that can contribute to the maintenance of circulatory antibody^{183, 184}. While, it is conventionally believed that polysaccharide antigens, with the exception of zwitterionic polysaccharide antigens, do not induce the production of memory B cells, there is increasing evidence to the contrary¹⁸⁵. Memory B cells contribute to long-term antibody persistence by continuously differentiating into plasma cells, which may be antigen independent and occur by polyclonal stimulation of B cells by microbial products through pathogen associated molecular patterns (PAMP) that stimulate B cells via toll like receptors, or through stimulation by cytokines secreted by T cells activated by another Ag (bystander effect), or in response to persistent Ag, intermittent Ag exposure or cross-reactive Ag which could include polysaccharide antigens of other bacteria^{183, 184, 186-189}.

Ag-specific plasma cells are not detectable in peripheral blood at steady state but these cells are thought to use the circulation to reach the bone marrow and they appear transiently in peripheral

blood after immunisation. Similarly, the majority of memory B cells are likely to reside in lymphoid tissues. An increase in memory B cell frequency is consistently observed shortly after immunisation and might represent newly generated memory B cells transiting through the circulation to other lymphoid tissues^{190, 191}. Understanding the timing of appearance of plasma cells and memory B cells in peripheral blood is important to define the kinetics following immunisation in order to design vaccine studies assessing the generation of long-term humoral immunity with improved ViPS vaccine.

In this study, the peak in the plasma B cell frequency was observed on Day 7 and 10 while the peak in the memory B cell was observed on Day 7. While the kinetics of the B cell response during secondary immunisation show a peak by day 6-7 post-immunisation, with a rapid return to baseline by day 10-12, the memory B cell frequency is known to increase by the end of the first week¹⁹⁰⁻¹⁹². The timing of appearance of both plasma cells and memory B cells resemble that of a secondary immune response. This could be explained by the phenomenon of cross-reactive antigens that induce natural immunity to *S. typhi* ViPS. The presence of plasma cells in a third of the participants on Day 0 and Day 2 could also be suggestive of immunity elicited by cross-reactive antigens of commensal bacteria.

The non-significant rise in anti-ViPS antibody following immunisation could be an artefact of the small study population. While a strong correlation was observed between *S. typhi* GMT bactericidal antibody and anti-ViPS antibody on Day 0 and Day 28, the non-correlation between the antibody concentration and B cell frequency suggest that these represent two components of humoral immunity. Persisting antibody may principally be produced by long-lived plasma cells

in the bone marrow, while circulating memory B cells are responsible for the rapid response to re-challenge with an antigen in the form of immunological memory^{193,194}.

This study has established the kinetics of humoral immune response following a single dose vaccination with the Vi polysaccharide vaccine. These findings shed light on the mechanism of protection induced by the current ViPS vaccine and are important for a better understanding of the immunity induced by the new generation derivatives of the ViPS vaccine.

Chapter 5: GENETIC BASIS OF ACAPSULATE *S. TYPHI*

5.1 Introduction

The Vi polysaccharide (ViPS) protects *S. typhi* *in vivo* through its resistance to complement mediated bactericidal activity mediated by anti-O antibody, and phagocytosis, suppression of TNF- α , IL-17 and IL-8 and the prevention of recognition of LPS via TLR-4. ViPS is encoded on a region of the bacterial genome termed *viaB* that consists of 10 genes; 5 coding for the synthesis of the polysaccharide (*tviA*, *tviB*, *tviC*, *tviD* and *tviE*) and 5 coding for the polysaccharide transportation proteins (*vexA*, *vexB*, *vexC*, *vexD*, *vexE*)^{27,28}. The *viaB* in *S. typhi* is located on the Specific Pathogenicity Island-7 (SPI-7)²⁷. In addition to the genes on *viaB*, 3 other genes are thought to regulate the production of Vi polysaccharide: *rscB* and *rscC*, together comprising the *viaA*^{30,31} and the two-component regulator *ompR-envZ*^{32,33}

Following an experiment by Hone and colleagues in 1988 using a *galE*, Vi negative mutant of *S. typhi* which demonstrated that these strains could indeed cause typhoid like illness, there has been renewed interest in the existence of acapsulate *S. typhi*⁵⁸. This is primarily due to its implications for the use of Vi based diagnostic agglutination tests and more importantly, the Vi based vaccines.

Laboratory techniques like multiple sub-culturing or storage of isolates on nutrient media can cause the down-regulation of Vi expression resulting in a negative agglutination test^{59, 60}. Spontaneous precise excision of the SPI-7 that contains genes for Vi synthesis and transportation, type IVB pili, putative conjugal transfer and *sopE* bacteriophage was postulated as a mechanism for the non-expression of capsule due to storage⁶¹. These mechanisms were

believed to be the reason for the detection of acapsulate *S. typhi*, including the isolates responsible for a typhoid outbreak in India in 2000 ⁶².

Recent studies have demonstrated the absence of genes encoding the Vi polysaccharide in stored isolates and patient isolates in Pakistan ^{63, 71}. Based on these episodes, some experts have suggested a positive selection pressure on *S. typhi*, especially in the era of post-Vi vaccine licensure, to down-regulate or even abolish the expression of the Vi subunit. Added to this hypothesis is evidence from laboratory experiments that although the Vi polysaccharide aids in the survival of *S. typhi* by the multiple mechanisms described above, the rates of invasion into macrophages are comparable for both Vi positive and negative strains of *S. typhi* ^{64, 65}. More recently there are reports of Vi suppressed *S. typhi*, cultured in high osmolarity, being hyperinvasive in human tissues ^{66, 67}. Vi production may not be essential for infection process in humans as evidenced by the disease caused and immune response induced by the Vi negative vaccine strains. This hypothesis could have implications not only for the implementation of the Vi polysaccharide vaccine in immunisation schedules in typhoid endemic countries but also for the future of the Vi polysaccharide protein conjugate vaccine that is currently undergoing clinical trials for use in paediatric populations ^{68, 69}.

Genetic analyses on acapsulate *S. typhi* in paediatric patients in Kathmandu, Nepal were performed, following serological tests, in order to determine the genetic basis for the non-expression of the capsule. This was achieved by the design of a novel, rapid, multiplex PCR assay to determine the identity, both genus and species, of the acapsulate strains followed by PCR analyses for the detection of the genes responsible for the synthesis, transportation and regulation of the polysaccharide capsule.

5.2 Materials and Methods

5.2.1 Sample collection

S. typhi isolates were obtained from febrile children presenting to the Department of Paediatrics at Patan Hospital, Lalitpur District, Kathmandu, Nepal. Positive blood cultures, on whole blood samples obtained on the day of presentation at the outpatient clinic, were identified with standard biochemical kits, *S. typhi* isolates were cultured on nutrient agar media, stored at -20°C till shipment and shipped on nutrient agar slopes, on dry ice using express courier and under U.N guidance on the shipment of biological hazardous goods, for serological testing at the Department of Pathology at Otago University in New Zealand and for genetic analyses at the Oxford Vaccine Group in UK.

5.2.2 Serological confirmation of *S. typhi*

The principle of the serological test is based on the characterisation of *Salmonella* species on their antigenic properties including the somatic (O) antigen, flagellar (H) antigen and the capsular (Vi) antigen. Preliminary serological grouping using commercially available polyvalent antisera was done on isolates which biochemically identified them as *Salmonella* sp. Thus the identity of these strains to at least genus level was confirmed. For slide agglutination tests, a suspension of the organism (taken from colonies grown on sheep blood agar plate) in saline was tested for auto-agglutination before antisera was added. 1 drop of bacterial suspension was mixed with 1 drop of saline on a glass slide, and manually rotated for 1-2 minutes. No agglutination or clumping should have been present.

For confirmation of an isolate as *Salmonella* sp. with polyvalent O antisera, a small drop of polyvalent O antisera next to a small drop of saline was placed on a glass slide. The bacterial colony was emulsified to a milky suspension in the saline and then mixed in the antisera. The slide was manually rotated for 1-2 minutes and agglutination observed. If polyvalent O was positive, each O antiserum was tested to determine the group O antigen. O antigen 0-4 was tested initially as these are the most common. Similar techniques were used for the determination of the flagellar H antigen using polyvalent H antisera- Phase 1 and Phase 2. If negative, the test was repeated after the organism was grown in a broth culture to enhance flagellar expression. Lastly, these isolates were tested for the Vi polysaccharide using Vi antisera. All isolates, biochemically determined to be *S. typhi*, but negative for agglutination with polyvalent sera were tested for Vi and a heavy suspension of the organism in saline was boiled for 15-30 minutes to unmask the effect of the Vi polysaccharide on the somatic antigen.

5.2.3 DNA extraction from bacterial isolates

The following protocol was used for isolating genomic DNA from *S. typhi*:

A suspension of an overnight culture of *S. typhi* was made in 1 ml of Hanks Balanced Salt Solution and spun at 13000 to 16000g to pellet the cells. The cells were gently pipetted till re-suspension after the addition of 600µl of Nuclei Lysis Buffer. After incubation at 80 degrees Celsius for cell lysis, the solution was cooled to room temperature. 3µl of RNase was added and the tube inverted several times. The mix was incubated at 37 degrees Celsius for an hour and cooled to room temperature. 200µl of Protein Precipitation Solution was added and the mix was vortexed at high speed for 20 seconds. The sample was incubated on ice for 5 minutes and centrifuged at 13000 to 16000g for 3 minutes.

Following gentle inversion, the mix was centrifuged at 13000 to 16000g for 2 minutes. The supernatant was poured off and the tube drained on clean absorbent paper. 600µl of ethanol at room temperature was added and the tube inverted multiple times to wash the DNA pellet. Following centrifugation at 13000 to 16000g for 3 minutes, the ethanol was drained and the tube was allowed to air dry for 10 to 15 minutes. 100µl of DNA rehydration solution was added and incubated at 65 degrees Celsius for an hour. The DNA was stored at 2 to 4 degrees Celsius.

5.2.4 Development and Validation of a Confirmatory Multiplex PCR Assay

A novel, rapid, multiplex PCR assay was designed to confirm the identity of the bacterial isolates. This assay included primer pairs to detect sequences within *aroC*, *fliC*, *vexB* and *tviB* genes. Potential oligonucleotide primer sequences in genes on *viaB* were chosen on the basis of a number of criteria. Oligonucleotide primers were designed to be approximately eighteen to twenty nucleotides in length; a base composition of approximately 50% GC in order to keep the melting temperatures within the range of 50 degrees Celsius to 70 degrees Celsius. Sequences with lengthy repeats of similar nucleotides were avoided as pyrimidine repeats might have allowed dissociation of template-primer interactions and led to sub-optimal priming, whereas purine repeats might have lead to difficulties in prevention of non-specific priming due to the stronger interaction between these bases. Primer sequences were designed to have a G or a C nucleotide at the 3' end to improve primer template interactions. The primer pairs designed for the multiplex PCR assay are shown in Table 5.1.

Genes	Forward primer	Reverse primer
<i>aroC</i>	aroCF1 (GGCACCAGTATTGGCCTGCT)	arocR1 (CATATGCGCCACAATGTGTTG)
<i>fliC</i>	fliCF1 (TATGCCGCTACATATGATGAG)	flicR1 (TTAACGCAGTAAAGAGAG)
<i>vexB</i>	vexCF1 (CTGCATACGCAACTGTGTGG)	vexAR1 (AATGACAATCTTGCCGATCC)
<i>tviB</i>	tviCF1 (ATGGGATCCTTTAGGGAACG)	tviAF1 (ATGTCAGTCAACACCAAAGTGG)

Table 5.1. Primer pairs for the 4 genes tested in the multiplex PCR assay used to confirm the identity of *S. typhi* isolates

The composition of the 50µl volume PCR reaction included 1µl of aroCF1 and aroCR1, 4µl of fliCF1, fliCR1, vexCF1, vexAR1, tviCF1 and tviAF1, premixed dNTPs (4 µl, 10mM, containing 2.5mM of each of dATP, dCTP, dGTP and dTTP), Taq DNA polymerase (6µl) with its reaction buffer (5µl used at 10 x working solution) and 10µl of the detergent Q solution. The volume of the PCR mix was adjusted to 46µl using milliQ water and 4µl of bacterial DNA was added. Reactions of 50µl were performed in 200µl PCR tubes and master mixes were prepared each time to prevent cross-contamination and to increase speed. PCR assay for each sample was performed in duplicate for the confirmation of results.

The cycling conditions for this PCR assay were as follows: 95 degrees Celsius for 3 minutes (to dissociate the DNA and oligonucleotide primers), followed by incubations of 95 degrees Celsius for 30 seconds, annealing of the primer and template at 55 degrees Celsius for 30 seconds and an extension of primer sequences at 68 degrees for 2 minutes and 15 seconds. This sequence of steps was repeated for a further 39 cycles after which the reaction was completed by holding at 68 degrees Celsius for a further 5 minutes before cooling to 4 degrees Celsius for storage. The total time for the PCR assay was 1 hour and 59 minutes.

5.2.5 Development and Validation of PCR Assays for Vi polysaccharide genes

Individual PCR assays were designed for the 14 genes responsible for the synthesis, transportation and regulation of the Vi polysaccharide. The primer design criteria described in section 4 above was followed for the design of the primers in this experiment. The primers for the genes are shown in Table 5.2

Table 5.2. Primer pair used for each of the 14 genes encoding the synthesis, transportation and regulation of the ViPS

Genes	Forward primers	Reverse primers
<i>vexA</i>	vexBF1 (GGAAACCGTTCAGACAGTGC)	tviER1 (TAGGCGATGGTGATTTACGC)
<i>vexB</i>	vexCF1 (CTGCATACGAACTGTGTGG)	vexAR1 (AATGACAATCTTGCCGATCC)
<i>vexC</i>	vexDF1 (CGAGTGGTGTTCAGACTCAGC)	vexBR1 (CATCGTTAGCGGTTGTCTCG)
<i>vexD</i>	vexEF1 (AGGCAAGATGACTTGCTCTGG)	vexCR1 (TGACCCAGCTTGAACAGTGC)
<i>vexE</i>	yjhpF1 (ACCTGACGCCAGTATGGTTC)	vexDR1 (GCTCTTCAGAAAGCCTGTCC)
<i>tviA</i>	tviBF1 (CAATGATCGCATCGTAGTGG)	STY4663 (AATCCAGCGCAACACACG)
<i>tviB</i>	tviCF1 (ATGGGATCCTTTAGGGAACG)	tviAR1 (AGCTCTTCCATACCACTTTCC)
<i>tviC</i>	tviDF1 (AGAACGCTGGTGAATCTTGG)	tviBR1 (TGAAACCGTTGGTACAGTGC)
<i>tviD</i>	tviEF1 (ACGTTCCCACCATATGTTCC)	tviDF1 (AGAACGCTGGTGAATCTTGG)
	tviDR1 (TAGCATGCTCATCCATCTGC)	tviCR1 (ACGGCTTACGAAGAACTACGG)

<i>tviE</i>	vexAF1 (CTCTGGTTCAGCGTCATTCC)	tviDR1 (TAGCATGCTCATCCATCTGC)
<i>rcsB</i>	rcsF1 (TGCGCAATACTTCTTTGTGC)	STY2494F2 (CTCAACGGACAGGATAACAGC)
<i>rcsC</i>	rcsBF1 (CATGCCGGGAGATAAATACG)	STY2499F1 (CCAGCTACAGGTTTCCTTCG)
<i>ompR</i>	STY4292R1 (CGTTAATCGCTTCGTCTTCC)	envZR1 (GGCTGCCATATGGTTAAACG)
<i>envZ</i>	ompRF1 (GGTGGTTGATGACGATATGC)	STY4296F1 (GCGCTTTATTACGGAAGTCG)

The PCR reaction composition for *vexA*, *vexB*, *vexC*, *vexD*, *tviA* was as follows:

Reagent	Volume (in μ l)
Forward primer	1
Reverse primer	1
Buffer	5
Q solution	10
Dntp	1
Sterile water	29.5
DNA polymerase taq	0.5

48 μ l of the above mix was mixed with 2 μ l of bacterial DNA to obtain the PCR reaction mix

The cycling conditions for this PCR assay were as follows: 95 degrees Celsius for 3 minutes (to dissociate the DNA and oligonucleotide primers), followed by incubations of 95 degrees Celsius

for 30 seconds, annealing of the primer and template at 55 degrees Celsius for 30 seconds and an extension of primer sequences at 68 degrees for 3 minutes (*vexB*, *vexC*, *tviA*) or 4 minutes and 30 seconds (*vexA*, *vexD*). This sequence of steps were repeated for a further 39 cycles after which the reaction was completed by holding at 68 degrees Celsius for a further 10 minutes before cooling to 4 degrees Celsius for storage. The total time for this PCR assay was 3 hours and 39 minutes (*vexB*, *vexC*, *tviA*) or 4 hours and 19 minutes (*vexA*, *vexD*).

The PCR reaction compositions for *vexE*, *tviB*, *tviC*, *tviE*, *rcsB*, *ompR* and *envZ* were as follows:

Reagent	Volume (in μ l)
Forward primer	1
Reverse primer	1
Buffer	5
Q solution	10
dNTP	1
Sterile water	29.5
DNA polymerase taq	0.5

48 μ l of the above mix was mixed with 2 μ l of bacterial DNA to obtain the PCR reaction mix. The cycling conditions for this PCR assay were as follows: 95 degrees Celsius for 3 minutes (to dissociate the DNA and oligonucleotide primers), followed by incubations of 95 degrees Celsius for 30 seconds, annealing of the primer and template at 55 degrees Celsius for 30 seconds and an extension of primer sequences at 68 degrees for 6 minutes. This sequence of steps were

repeated for a further 39 cycles after which the reaction was completed by holding at 68 degrees Celsius for a further 10 minutes before cooling to 4 degrees Celsius for storage. The total time for this PCR assay was 5 hours and 39 minutes.

The PCR assay for *tviD* was performed in two stages considering the difficulty in obtaining the correct size bands possibly due to the large base length. The composition of the mix was as follows:

Reagent	Volume (in μ l)
Forward primer (<i>tviEF1</i> and <i>tviDF1</i>)	1
Reverse primer (<i>tviDR1</i> and <i>tviCR1</i>)	1
Buffer	5
Q solution	10
dNTP	1
Sterile water	29.5
DNA polymerase taq	0.5

48 μ l of the above mix was mixed with 2 μ l of bacterial DNA to obtain the PCR reaction mix

The cycling conditions for this PCR assay were as follows: 95 degrees Celsius for 3 minutes (to dissociate the DNA and oligonucleotide primers), followed by incubations of 95 degrees Celsius for 30 seconds, annealing of the primer and template at 55 degrees Celsius for 30 seconds and an extension of primer sequences at 68 degrees for 6 minutes. This sequence of steps were repeated for a further 39 cycles after which the reaction was completed by holding at 68 degrees Celsius for a further 10 minutes before cooling to 4 degrees Celsius for storage. The total time for this PCR assay was 5 hours and 39 minutes.

The PCR reaction composition for *rscB* was as follows:

Reagent	Volume (in μ l)
Forward primer	2
Reverse primer	2
Buffer	5
Q solution	10
dNTP	1
Sterile water	27
DNA polymerase taq	1

46 μ l of the above mix was mixed with 4 μ l of bacterial DNA to obtain the PCR reaction mix

The cycling conditions for this PCR assay were as follows: 95 degrees Celsius for 3 minutes (to dissociate the DNA and oligonucleotide primers), followed by incubations of 95 degrees Celsius for 30 seconds, annealing of the primer and template at 55 degrees Celsius for 30 seconds and an extension of primer sequences at 68 degrees for 7 minutes. This sequence of steps were repeated for a further 39 cycles after which the reaction was completed by holding at 68 degrees Celsius for a further 10 minutes before cooling to 4 degrees Celsius for storage. The total time for this PCR assay was 6 hours and 19 minutes.

5.2.6 Determination of Genetic Basis of Acapsulate *S. typhi*

Following the development and validation of the novel, multiplex, confirmatory PCR assay and the individual PCR assays for each of the 14 genes influencing the expression of the Vi polysaccharide capsule, phenotypically acapsulate *S. typhi* isolates from paediatric patients in Kathmandu, Nepal along with phenotypically capsulate strains and the NCTC strain were tested.

5.3 Results

5.3.1 Sample collection

68 *S. typhi* isolates were obtained from febrile children presenting to the Department of Paediatrics at Patan Hospital, Lalitpur District, Kathmandu, Nepal.

5.3.2 Serological confirmation of *S. typhi*

4 out of the 68 *S. typhi* isolates (5.9 %) tested negative for capsular expression by slide agglutination tests.

5.3.3 Genetic determination of bacterial identity: Development and Validation of a Confirmatory Multiplex PCR Assay

Bands corresponding to the expected PCR product sizes for the 4 genes were detected and are illustrated in Fig 5.1.

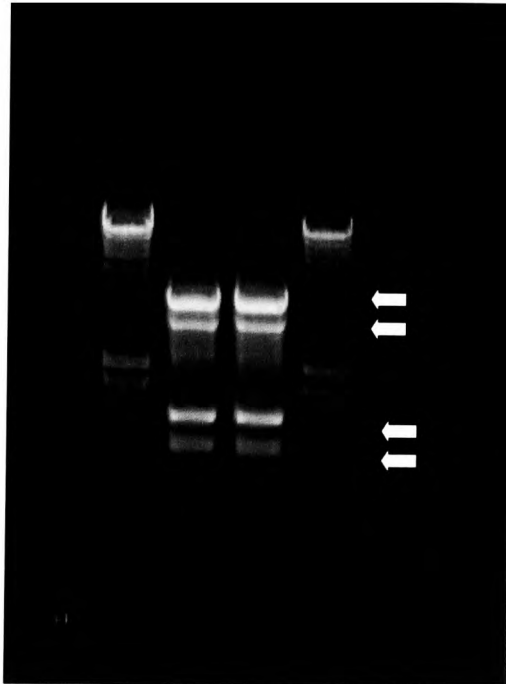


Fig 5.1. 1% Agarose gel image of multiplex PCR products demonstrating bands (indicated by arrows, in ascending order) for *fliC* (500b), *aroC* (638b), *vexB* (1546b) and *tviB* (1976b). Lane 1 and 4: 1 kb ladder, Lane 2 and 3: DNA from *S. typhi* (NCTC strain)

5.3.4 Development and Validation of PCR Assays for Vi polysaccharide genes

Fig 5.2 illustrates the PCR products corresponding to the expected sizes for *vexA*, *vexB*, *vexC*, *vexD*

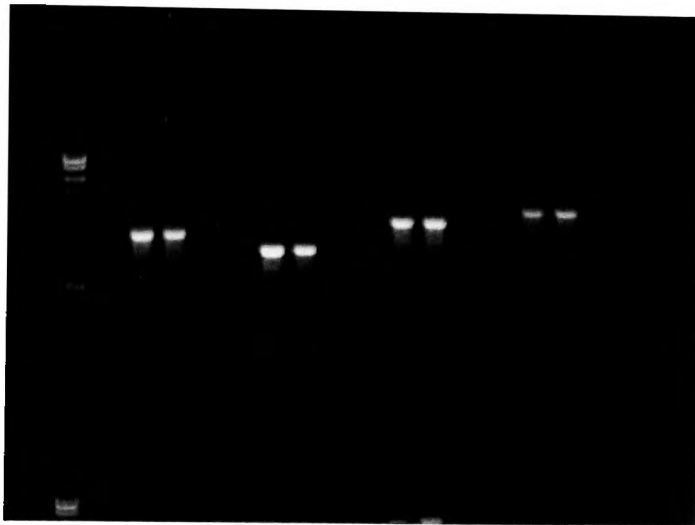


Fig 5.2. 1% Agarose gel image of PCR products demonstrating bands corresponding to *vexA* (2065b, Lane 2 and 3), *vexB* (1546b, Lane 4 and 5), *vexC* (2425b, Lane 6 and 7) and *vexD* (2876b, Lane 8 and 9). Lane 1: 1 kb ladder

Fig 5.3 illustrates the PCR products corresponding to the expected sizes for *vexE*, *tviB* and *tviC*

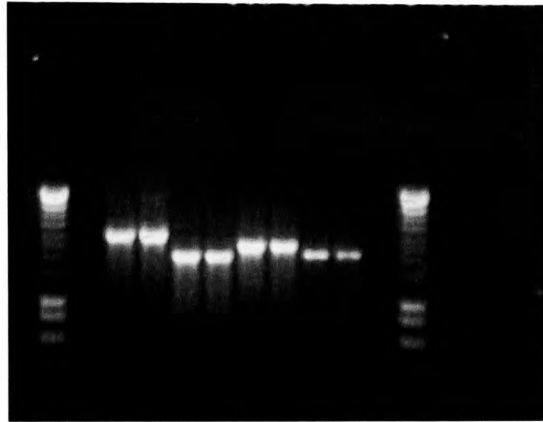


Fig 5.3. 1% Agarose gel image of PCR products demonstrating bands corresponding to *vexE* (3076b, Lane 2 and 3), *tviB* (2100b, Lane 4 and 5) and *tviC* (2564b, Lane 6 and 7).

Lane 1 and 10: 1 kb ladder

Fig 5.4 illustrates the PCR products corresponding to the expected sizes for *tvIA*

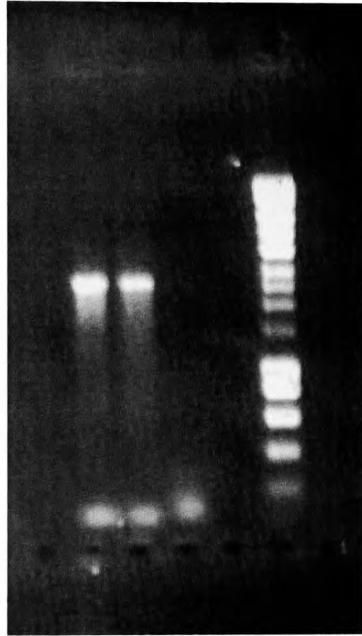


Fig 5.4. 1% Agarose gel image of PCR products demonstrating bands corresponding to *tvIA* (2625b, Lane 1 and 2), Lane 3: 1 kb ladder

Fig 5.5 illustrates the PCR products corresponding to the expected sizes for *tviE*



Fig 5.5. 1% Agarose gel image of PCR products demonstrating bands corresponding to *tviE* (3596b, Lane 2 and 3), Lane 1 and 4: 1 kb ladder

Fig 5.6 illustrates the PCR products corresponding to the expected sizes for *rcsB*, *rscC*, *ompR* and *envZ*

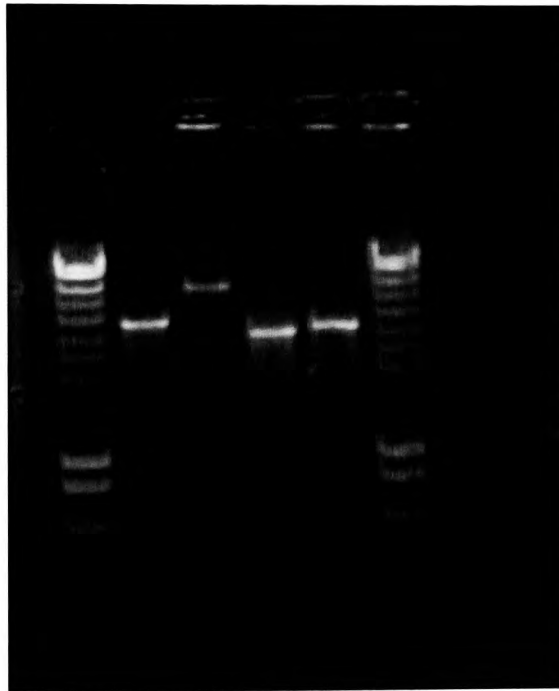


Fig 5.6. 1% Agarose gel image of PCR products demonstrating bands corresponding to *rscB* (3706b, Lane 2), *rscC* (5921b, Lane 3), *ompR* (3116b, Lane 4) and *envZ* (3339b, Lane 5). Lane 1 and 6: 1 kb ladder

5.3.5 Determination of the Genetic Basis of Acapsulate *S. typhi*

The multiplex PCR assay demonstrated the 4 bands corresponding to *aroC*, *fliC*, *vexB* and *tviB* in both acapsulate and capsulate *S. typhi* (Fig 5.7)

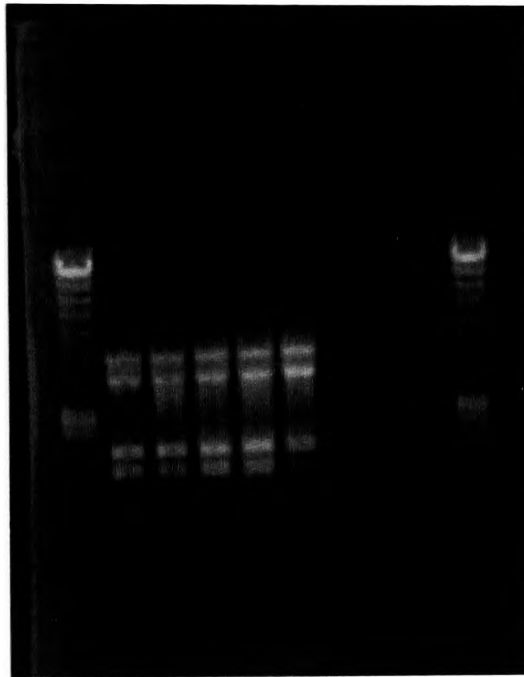


Fig 5.7. 1% Agarose gel image of multiplex PCR products demonstrating bands (indicated in ascending order) for *fliC* (500b), *aroC* (638b), *vexB* (1546b) and *tviB* (1976b) in Lanes 2 to 6. Lane 1 and 10: 1 kb ladder, Lane 2, 3, 4, 5: DNA from acapsulate *S. typhi*, Lane 6: DNA from capsulate *S. typhi* (NCTC strain), Lane 7: negative control.

Fig 5.8 illustrates the PCR products corresponding to the expected size for *vexA*

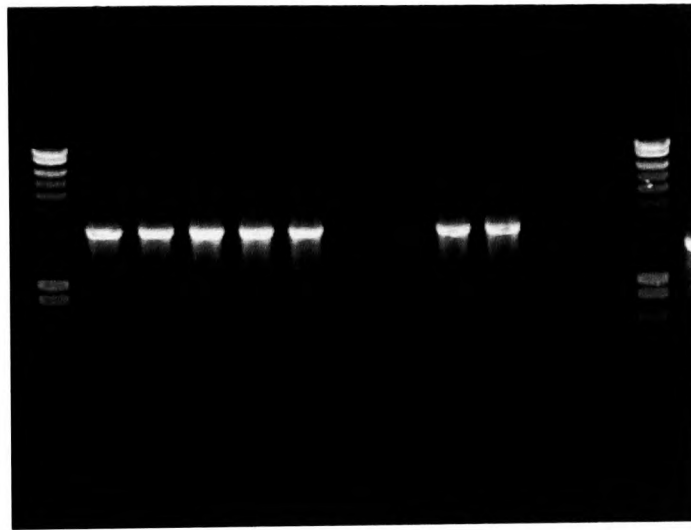


Fig 5.8. 1% Agarose gel image of multiplex PCR products demonstrating bands corresponding to the expected size for *vexA*. Lane 1 and 13: 1kb marker. Lane 2, 3, 4, 5: DNA from acapsulate *S. typhi*, Lane 6, 7, 8, 9: DNA from capsulate *S. typhi*, Lane 10: DNA from NCTC strain of *S. typhi*. Lane 11: DNA from *Neisseria meningitidis*, Lane 12: Negative control (water).

Fig 5.9 illustrates the PCR products corresponding to the expected size for *vexB*



Fig 5.9. 1% Agarose gel image of multiplex PCR products demonstrating bands corresponding to the expected size for *vexB*. Lane 1 and 13: 1kb marker. Lane 2, 3, 4, 5: DNA from acapsulate *S. typhi*, Lane 6, 7, 8, 9: DNA from capsulate *S. typhi*, Lane 10: DNA from NCTC strain of *S. typhi*. Lane 11: DNA from *Neisseria meningitidis*, Lane 12: Negative control (water).

Fig 5.10 illustrates the PCR products corresponding to the expected size for *vexC*

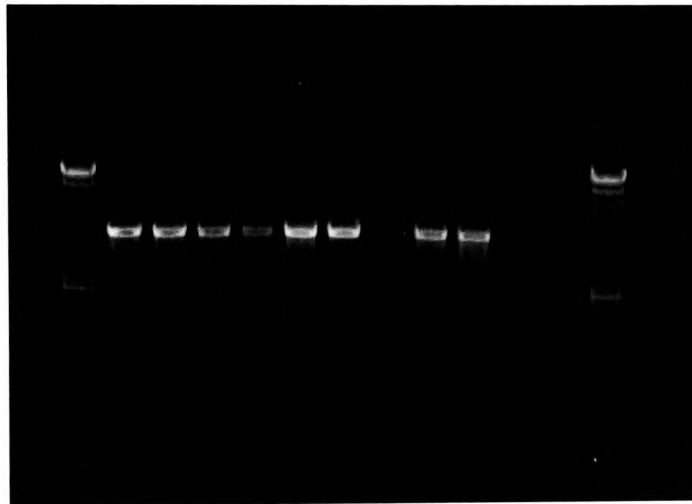


Fig 5.10. 1% Agarose gel image of multiplex PCR products demonstrating bands corresponding to the expected size for *vexC*. Lane 1 and 13: 1kb marker. Lane 2, 3, 4, 5: DNA from acapsulate *S. typhi*, Lane 6, 7, 8, 9: DNA from capsulate *S. typhi*, Lane 10: DNA from NCTC strain of *S. typhi*. Lane 11: DNA from *Neisseria meningitidis*, Lane 12: Negative control (water).

Fig 5.11 illustrates the PCR products corresponding to the expected size for *vexD*

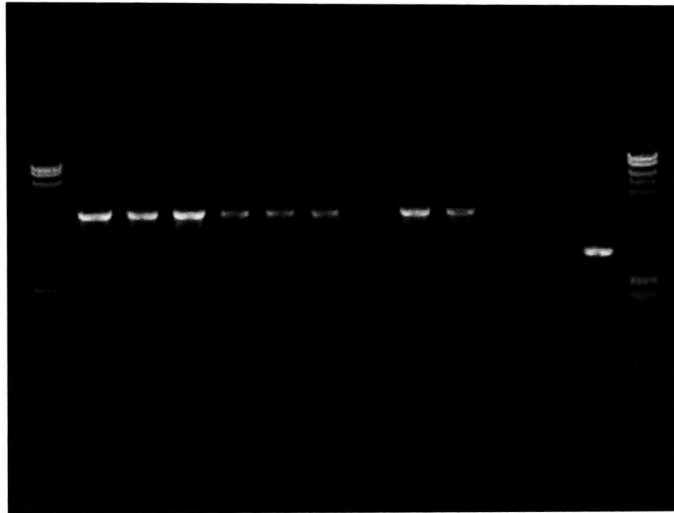


Fig 5.11. 1% Agarose gel image of multiplex PCR products demonstrating bands corresponding to the expected size for *vexD*. Lane 1 and 13: 1kb marker. Lane 2, 3, 4, 5: DNA from acapsulate *S. typhi*, Lane 6, 7, 8, 9: DNA from capsulate *S. typhi*, Lane 10: DNA from NCTC strain of *S. typhi*. Lane 11: DNA from *Neisseria meningitidis*, Lane 12: Negative control (water).

Fig 5.11 illustrates the PCR products corresponding to the expected size for *vexE*

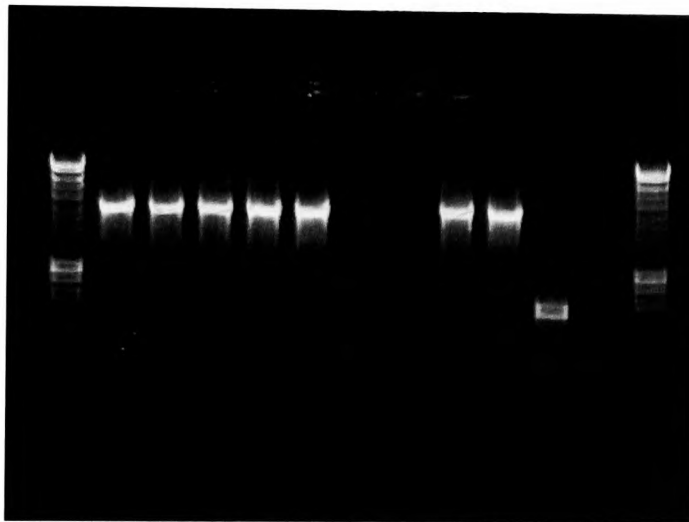


Fig 5.11. 1% Agarose gel image of multiplex PCR products demonstrating bands corresponding to the expected size for *vexE*. Lane 1 and 13: 1kb marker. Lane 2, 3, 4, 5: DNA from acapsulate *S. typhi*, Lane 6, 7, 8, 9: DNA from capsulate *S. typhi*, Lane 10: DNA from NCTC strain of *S. typhi*. Lane 11: DNA from *Neisseria meningitidis*, Lane 12: Negative control (water).

Fig 5.12 illustrates the PCR products corresponding to the expected size for *tviA*

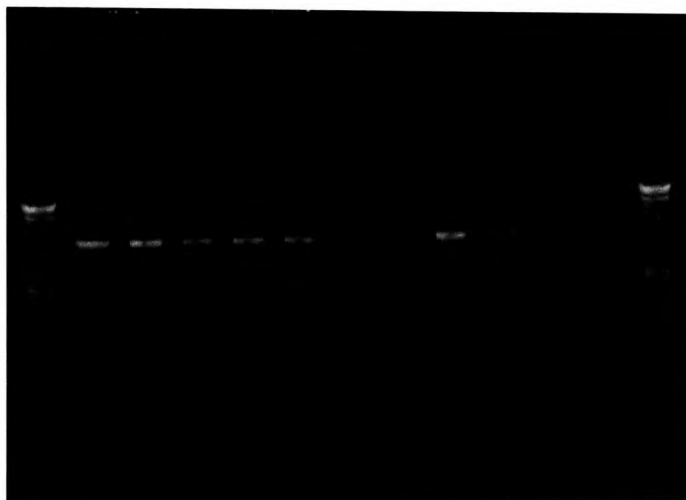


Fig 5.12. 1% Agarose gel image of multiplex PCR products demonstrating bands corresponding to the expected size for *tviA*. Lane 1 and 13: 1kb marker. Lane 2, 3, 4, 5: DNA from acapsulate *S. typhi*, Lane 6, 7, 8, 9: DNA from capsulate *S. typhi*, Lane 10: DNA from NCTC strain of *S. typhi*. Lane 11: DNA from *Neisseria meningitidis*, Lane 12: Negative control (water).

Fig 5.13 illustrates the PCR products corresponding to the expected size for *tvjB*

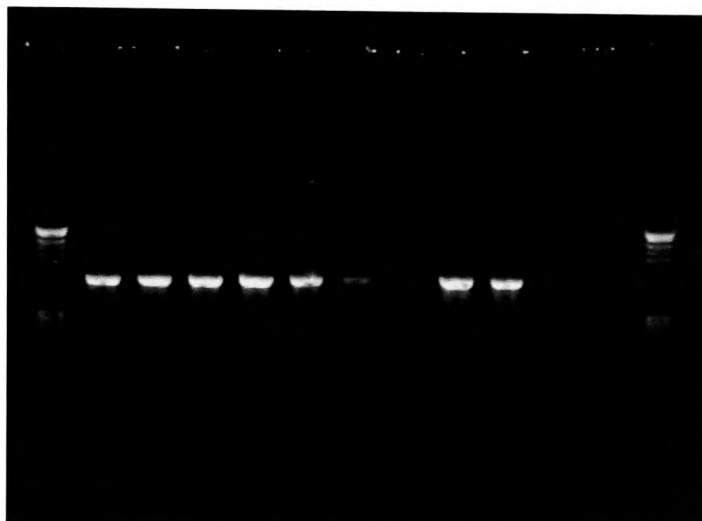


Fig 5.13. 1% Agarose gel image of multiplex PCR products demonstrating bands corresponding to the expected size for *tvjB*. Lane 1 and 13: 1kb marker. Lane 2, 3, 4, 5: DNA from acapsulate *S. typhi*, Lane 6, 7, 8, 9: DNA from capsulate *S. typhi*, Lane 10: DNA from NCTC strain of *S. typhi*. Lane 11: DNA from *Neisseria meningitidis*, Lane 12: Negative control (water).

Fig 5.14 illustrates the PCR products corresponding to the expected size for *tvfC*

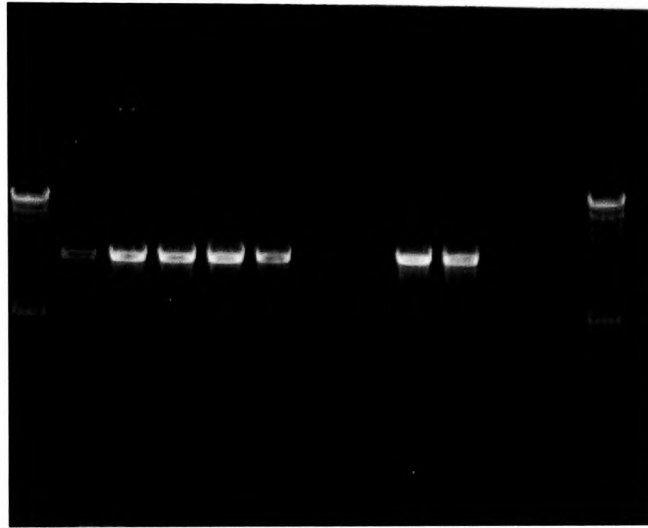


Fig 5.14. 1% Agarose gel image of multiplex PCR products demonstrating bands corresponding to the expected size for *tvfC*. Lane 1 and 13: 1kb marker. Lane 2, 3, 4, 5: DNA from acapsulate *S. typhi*, Lane 6, 7, 8, 9: DNA from capsulate *S. typhi*, Lane 10: DNA from NCTC strain of *S. typhi*. Lane 11: DNA from *Neisseria meningitidis*, Lane 12: Negative control (water).

Fig 5.15 illustrates the PCR products corresponding to the expected size for *tvfD*

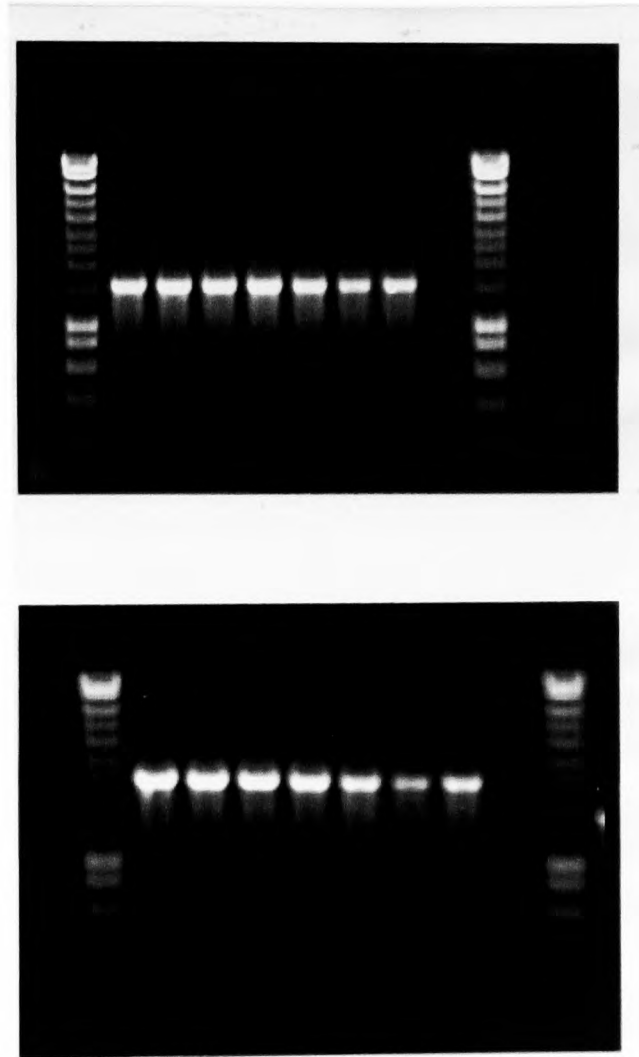


Fig 5.15. 1% Agarose gel image of multiplex PCR products demonstrating bands corresponding to the expected size for *tvfD*. Lane 1 and 13: 1kb marker. Lane 2, 3, 4, 5: DNA from acapsulate *S. typhi*, Lane 6, 7, 8, 9: DNA from capsulate *S. typhi*, Lane 10: DNA from NCTC strain of *S. typhi*. Lane 11: DNA from *Neisseria meningitidis*, Lane 12: Negative control (water).

Fig 5.16 illustrates the PCR products corresponding to the expected size for *tvfE*

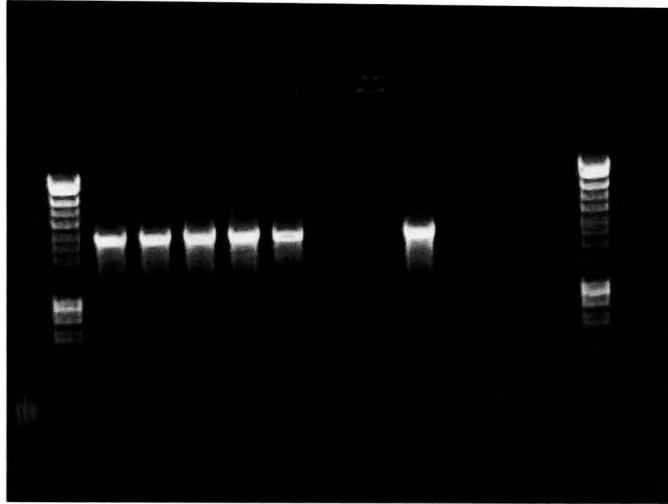


Fig 5.16. 1% Agarose gel image of multiplex PCR products demonstrating bands corresponding to the expected size for *tvfE*. Lane 1 and 13: 1kb marker. Lane 2, 3, 4, 5: DNA from acapsulate *S. typhi*, Lane 6, 7, 8, 9: DNA from capsulate *S. typhi*, Lane 10: DNA from NCTC strain of *S. typhi*. Lane 11: DNA from *Neisseria meningitidis*, Lane 12: Negative control (water).

Absence of expected bands in Lane 7 and 8 was determined to be due to poor quality of DNA preparation. DNA preparations from the 2 capsulate isolates were repeated and the assays performed showing expected results.

5.4. Discussion

This section of the thesis establishes that phenotypically acapsulate *S. typhi*, isolated from patients with typhoid fever in Kathmandu, Nepal have the 14 genes that are responsible for the expression of ViPS. The identity of both acapsulate and capsulate isolates were first confirmed by means of a novel, rapid, confirmatory PCR assay. The multiplex assay used in this study consisted of primers for *aroC*, *fliC*, *tviB* and *vexB*. The sequence for *aroC* amplified by the primer pair is specific for *Salmonella* genus; thereby confirming the identity of the organism. Similarly, primers for *fliC* amplified a sequence on the gene that is specific for serovar *typhi*. The presence of amplicons for these primers confirms the genus and the serovar of the isolate. The presence of *tviB* and *vexB* confirm the presence of the *viaB* locus in the SPI-7 of these isolates.

A recent study in Pakistan in 2005 tested 2222 stored samples of *S. typhi* for the presence or absence of the genetic loci required for Vi expression⁶³. Among these, 12 tested negative by slide agglutination. However, on multiplex PCR analysis only one was shown to be genetically acapsulate. This isolate had an excision of the SPI-7. This result was confirmed by immunofluorescence. However, the multiplex PCR in this study contained only 3 primer sets; to code for an internal region of *tviB*, an inter-genic region between *pheU* and *phoN* genes and *aroC*. Importantly, it did not test for the presence or absence of the 9 other genes that too are responsible for the expression of the capsule. Similarly, the presence or absence of regulators was not tested. Considering the lack of these data, the actual incidence of acapsulate *S. typhi* might be higher than the incidence of <0.1 reported in this study based on one of the 14 genes responsible for the capsular expression.

A follow up study in the same region wherein 60 stored isolates and 48 blood samples from patients with symptoms suggestive of typhoid fever demonstrated that while 9 strains in the former group tested negative for both *tviA* and *tviB*, only two lacked the SPI-7⁷¹. In the latter group, of the 42 that tested positive by *fliC* (confirming serovar *typhi* identity), 4 were negative for *tviA* and *tviB*. Three of these samples tested positive for SPI-7. While this study proved an important observation that *tviB* negative but SPI-7 positive serovar *typhi* is naturally occurring, the lack of information regarding the other capsular genes raise doubts about the actual magnitude of acapsulate *S. typhi* in nature.

In the study presented here, I have established the presence of all 14 genes in 4 phenotypically acapsulate *S. typhi* isolates among 68 isolates from a typhoid endemic country. Multiple factors might account for the phenotypical acapsulate status while possessing the genes that encode the same. Firstly, as these were stored isolates, sub-culturing on nutrient media might explain the down regulation of capsular expression. False negative results on slide agglutination could be another explanation for this phenomenon. Lastly, even in the presence of all genes that influence the expression of the capsule, the possibility of point mutations in these genes cannot be ruled out.

Existence of naturally occurring acapsulate *S. typhi* would have implications for the implementation of a Vi based vaccine. In view of the relative lack of data regarding the actual incidence of acapsulate *S. typhi*, it is difficult to comment on the potential usefulness of both the polysaccharide and the polysaccharide-protein conjugate vaccines. However, considering the extremely low incidence of disease caused by Vi negative strains it is unlikely that these will be an impediment to the implementation of a Vi based community vaccination programme. Indeed,

the rare occurrence of invasive disease caused by acapsulate meningococci has not prevented the successful implementation of the conjugate meningococcal vaccines.

Chapter 6: DEVELOPMENT AND VALIDATION OF A DIAGNOSTIC IL-12 PCR

6.1 Introduction

Interleukin-12 (IL-12) is a key cytokine in the human defence against intracellular pathogens including *Mycobacteria* and *Salmonella* species. Defects in the dimeric IL-12 structure (composed of p30 and p40 protein subunits) and IL-12 –IFN- γ pathway constitute a class of genetic immunodeficiencies that predispose to infections with these intracellular pathogens, collectively known as ‘Mendelian Susceptibility to Mycobacterial Disease’(MSMD). The first reports of *Salmonella* infections and BCGosis in patients with IL-12 deficiency and IL-12 receptor defects were in 1998^{157, 158}.

Subsequently, a number of genetic defects in the components of the IL-12- IFN- γ pathway have been discovered including the IFN- γ receptor ligand binding chain (IFN- γ R1), the IFN- γ receptor signalling chain (IFN- γ R2), the IL-12 receptor β 1 chain (IL-12 β 1) and STAT-1 (a transducer of IFN- γ mediated signals)^{159, 160, 161, 162, 163, 164}.

In this chapter, a description is made of a novel diagnostic genetic evaluation of a child with inherited IL-12 deficiency which is known to predispose to *Salmonella* and *Mycobacteria* species infection.

6.2 Clinical Case Description

A 6 month old male child presented with persistent left axillary lymphadenitis after BCG vaccination at birth. At the time of presentation, the patient was the youngest of three siblings, the elder two being girls, born to parents of Pakistani origin and of second degree consanguineous relationship. Subsequently, another child, a female, was born 2 years later. The elder siblings and the youngest sibling had no clinical complaints.

On the suspicion of BCGosis, a lymph node biopsy and culture was performed which revealed *Mycobacterium bovis*. The patient was initiated on isoniazid and rifampicin therapy for six months. Despite the treatment, the patient developed disseminated lymphadenopathy and failure to thrive. A repeat lymph node biopsy revealed *Mycobacterium tuberculosis* confirmed by DNA analysis and antibiotic sensitivity patterns. However, no TB contact was identified although the patient had visited Pakistan during infancy. On clinical suspicion of immunodeficiency, an immunological screen was performed that included HIV antibody, immunoglobulin profile, lymphocyte subsets and neutrophil oxidative burst; all of which were normal. A defect in the IL-12 – IFN- γ pathway was then suspected as a cause of cellular immunodeficiency in predisposing to multiple intracellular infections.

To identify a defect in this pathway, a whole blood stimulation assay was performed to compare IFN- γ production between the patient and a healthy Caucasian control. Whole blood was stimulated with the mitogen phytohaemagglutinin (PHA) and PHA supplemented with IL-12 and the supernatant IFN- γ concentration was measured with a standard ELISA. IFN- γ was detected in the whole blood of the control but in the patient only with the addition of PHA supplemented with IL-12. However, expression of IL-

12R β 1, IL-18R and IFN- γ R1 were determined to be normal. Together, these data indicated a deficiency of IL-12.

To determine the molecular genetic basis of this condition, a PCR assay was designed to rapidly detect a previously reported 4.6kb deletion in the IL-12 gene which had been observed in south Asian populations²⁰².

Functional tests on the infant female sibling of the patient also revealed a phenotypic IL-12 deficiency. Since this individual was clinically asymptomatic, this result prompted a genetic investigation of the inheritance of this condition in the immediate family members of the patient including both parents and two older female siblings.

Anti-tuberculous therapy in the patient was broadened with the addition of ciprofloxacin and clarithromycin. IFN- γ administration (30 μ g/kg subcutaneous thrice weekly) was associated with rapid clinical improvement. Three months later, chronic liver disease was evident in the form of pruritis, dilated abdominal veins and hepatosplenomegaly. An upper GI endoscopy revealed a Grade I varix at the gastro-oesophageal junction, which was banded, and early varices in fundus. Abdominal ultrasound revealed portal hypertension, cavernous transformation of the portal vein and intra-hepatic bile duct dilatation. Histological examination of liver tissue confirmed biliary cirrhosis. However, the histopathological findings were inconsistent with drug induced liver injury and in the absence of granulomata, of mycobacterial infection. Whether IL-12 deficiency accelerated the progression of the disease is unclear. In light of the advanced stage of cirrhosis, the patient underwent orthotropic liver transplantation with isoniazid prophylaxis. Post-transplantation immunosuppression was achieved with tacrolimus and prednisolone. Although there has been no relapse of mycobacterial infection post-surgery, the patient

continues to suffer from ongoing complications of solid organ transplantation including EBV and varicella infections.

6.3 Design of primers for PCR amplification of IL12B gene

Potential oligonucleotide primer sequences in genes surrounding the IL-12B loci and present in the gene were chosen on the basis of a number of criteria. Oligonucleotide primers were designed to be approximately eighteen to twenty nucleotides in length; a base composition of approximately 50% GC in order to keep the melting temperatures within the range of 50 degrees Celsius to 70 degrees Celsius. Sequences with lengthy repeats of similar nucleotides were avoided as pyrimidine repeats might have allowed dissociation of template-primer interactions and led to sub-optimal priming, whereas purine repeats might have led to difficulties in prevention of non-specific priming due to the stronger interaction between these bases. Primer sequences were designed to have a G or a C nucleotide at the 3' end to improve primer template interactions. The primers for the initial PCR reaction were:

1. Forward oligonucleotide primer IL12BF4: 5'AGA TGC TGG CCA GTA CAC CTG 3'
2. Reverse oligonucleotide primer IL12BR2: 5' CCC CTT GGC AAC ATA GTC AC 3'

6.4 PCR amplification of the IL-12B gene

The composition of the 50 μ l volume PCR reaction included 1.25 μ l of IL12BF4, 1.25 μ l of IL12BR2, premixed dNTPs (10mM, containing 2.5mM of each of dATP, dCTP, dGTP and dTTP), Taq DNA polymerase (0.5 μ l) with its reaction buffer (5 μ l used at 10 x working solution) and 10 μ l of the detergent Q solution. The volume of the PCR mix was adjusted to

46µl using milliQ water and 4µl of human genomic DNA was added. Reactions of 50µl were performed in 200µl PCR tubes and master mixes were prepared each time to prevent cross-contamination and to increase speed. PCR assay for each sample was performed in duplicate for the confirmation of results.

The cycling conditions for this PCR assay were as follows: 95 degrees Celsius for 3 minutes (to dissociate the DNA and oligonucleotide primers), followed by incubations of 95 degrees Celsius for 30 seconds, annealing of the primer and template at 65 degrees Celsius for 2 minutes and an extension of primer sequences at 72 degrees for 3 minutes. This sequence of steps were repeated for a further 39 cycles after which the reaction was completed by holding at 72 degrees Celsius for a further 4 minutes before cooling to 4 degrees Celsius for storage. The total time for this PCR assay was 12 hours and 31 minutes.

To confirm the heterozygous or homozygous status of the index case and of the family members and therefore to determine the pattern of inheritance of this genetic disorder, we designed a second PCR assay to detect a genomic region, absent in individuals homozygous for this condition. Primer design was done as discussed above. Primers for the second PCR assay were:

1. Forward oligonucleotide primer IL12BDF1: 5'GGA ACTCTCTCCCAATGTG3'
2. Reverse oligonucleotide primer IL12BDR2: 5'TGGAACAGCCAGGAGCTATC3'

The reaction mix for the second assay was as follows:

12. IL12BDF1: 2.5µl
13. IL12BDR2: 2.5µl
3. Buffer (10x) : 5µl
4. dNTP: 1µl
5. milliQ H₂O: 36µl

6. Taq DNA polymerase: 0.5 μ l

7. genomic DNA: 2.5 μ l

The cycling conditions for this PCR assay were as follows: 95 degrees Celsius for 3 minutes, followed by incubations of 95 degrees Celsius for 30 seconds, annealing of the primer and template at 60 degrees Celsius for 1 minute and an extension of primer sequences at 72 degrees for 1 minute and 30 seconds. This sequence of steps were repeated for a further 39 cycles after which the reaction was completed by holding at 72 degrees Celsius for a further 4 minutes before cooling to 4 degrees Celsius for storage. The total time for this PCR assay was 4 hours and 31 minutes.

6.5 Purification of PCR products

Amplicons were separated from unincorporated dNTPs and oligonucleotide primers by polyethylene glycol (PEG) purification, carried out as follows: Completed PCR reactions were added to a 60 μ l solution of PEG (20% w/v)/NaCl (2.5M) in 0.5 ml tubes, which was mixed by pulse vortexing and incubated at room temperature for 1 hour. The amplicons were sedimented by centrifugation at 13,500rpm for 10 minutes. The supernatant was removed using a micropipette. Unincorporated reaction components, including oligonucleotide primers, dNTPs and salt from the precipitation were removed by washing the pellets in ice-cold ethanol at a concentration of 70% (0.5ml) and performing centrifugation at the same speed for a further 10 minutes. The ethanol was removed with a micropipette; pellets were dried in an incubator for 10 minutes and then solubilised in water (15 μ l) at 4 degrees Celsius overnight. The presence of amplicons was determined by an agarose gel electrophoresis using 2 μ l of purified amplicon solution.

6.6 Nucleotide sequence determination of PCR products

6.6.1 Nucleotide Sequencing Reaction Protocol:

A nested sequencing protocol was used to reduce chances of detecting non-specific products. BigDye terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase containing premixed dideoxynucleotide triphosphate (ddNTP) terminators tagged with fluorescent dyes (each dye corresponding to each of the four nucleotide bases), dNTPs, buffer, MgCl₂ and Taq DNA polymerase were used. Each primer (2µl) was mixed with BigDye (0.5µl), 5 x Buffer (1.75 µl) and 1 µl of PCR product DNA. The reaction was made up to 10 µl with 4.75 µl of milliQ water. Cycle sequencing reactions were performed by incubation for 30 cycles of 96 degrees Celsius for 10 minutes, 50 degrees Celsius for 6 seconds and 60 degrees Celsius for 2 minutes and cooling to 4 degrees Celsius for short term storage.

6.6.2 Purification of Sequencing Reaction extension Products:

The sequencing extension products were separated from unincorporated ddNTPs and oligonucleotide primers by precipitation with a solution of absolute ethanol and sodium acetate made by adding sodium acetate solution (280µl) to 95% ethanol (7ml). This solution (52µl) was added to each sample and incubated at room temperature for 1 hour and 10 minutes. Extension products were separated from unincorporated dNTPs and oligonucleotide primers by centrifugation at 2750 x g at 4 degrees Celsius for 80 minutes. The supernatant was removed and pellets were washed as described before. The plates were sealed and stored at -20 degrees Celsius prior to electrophoretic separation of extension products. Automated Nucleotide Sequence Determination was done by an ABI 377 DNA analyzer or ABI 3700 DNA analyzer (Department of Zoology, University of Oxford). The nucleotide sequence of the extension products was detected by continual collection of the fluorescent light produced by excitation of

the ddNTP dyes by a laser emitting light at between 530 and 700nm. DNA sequence analysis of the deletion point and alignment against the normal human genome sequence was performed.

6.7 Results:

In a healthy Caucasian control, the PCR amplified a 6.4kb product (Figure 6.1). In the patient, a product of 1.8kb was detected, consistent with a 4.6kb deletion on both chromosomes. This was confirmed by DNA sequence analysis of the deletion point and alignment against the normal human genome sequence. In one older female sibling, a single 6.4kb PCR product indicated that the deletion was not present on either chromosome. In the parents, a younger female sibling and another older female sibling, a single 1.8kb band was observed, consistent with a 4.6kb deletion in at least one chromosome. Since neither parent yielded the 6.4kb product present in the older sibling, in the primary PCR, these data were inconsistent with the expected inheritance of the deletion from the heterozygote parents. This was suspected to be an experimental artefact caused by preferential amplification of the shorter, defective copy of the gene in the PCR due to the reduced amplification time when both were present in heterozygous samples. The secondary PCR, designed to detect a 1.3kb region of IL-12B which would be absent in true homozygotes, amplified the expected region in the Caucasian control, in one older sibling and the parents confirming their heterozygosity. These data, analysed in whole, indicated an autosomal recessive pattern of inheritance.

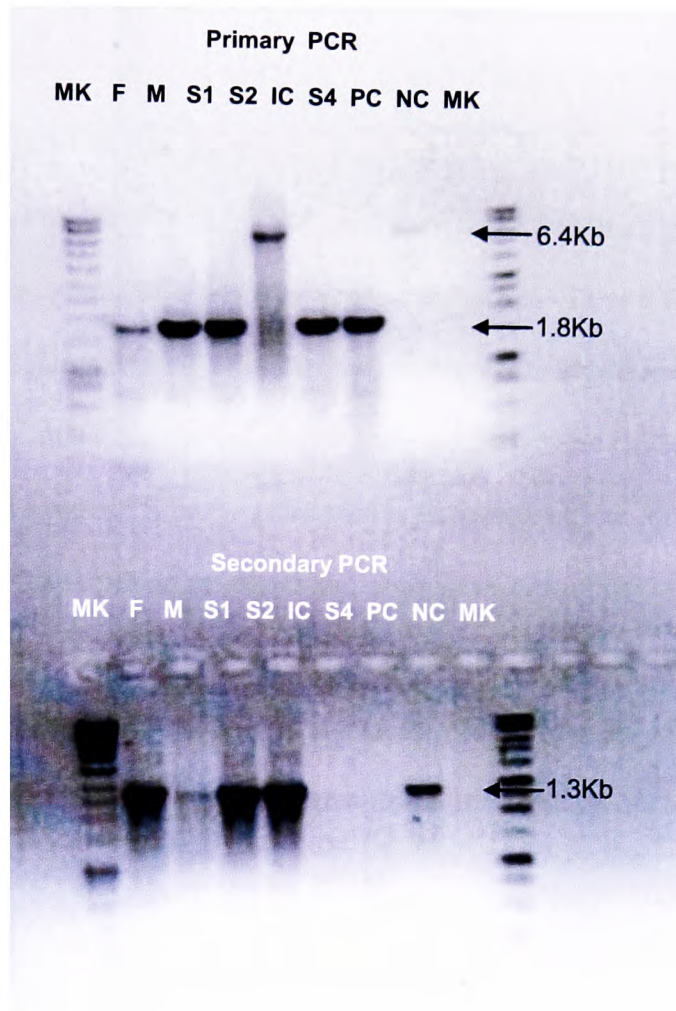


Figure 6.1: Agarose gel (1% agarose, negative gel image) showing results of primary and secondary PCR assays. Lane labels as follows: MK: 1kb DNA ladder (Promega Corp. Cat.# G571A); F: father of index case; M: mother of index case; S1: older female sibling 1, S2: older female sibling 2; IC: index case; S4: younger female sibling; PC: Caucasian control; NC: negative control (water). Loading: 10 μ l each sample except markers (3 μ l). 6.4kb, 1.8kb and 1.3kb bands indicated by arrows.

6.8 Discussion

This chapter describes a child with multiple, disseminated mycobacterial infection due to a loss-of-function deletion defect in the IL-12B gene encoding the p40 subunit of the IL-12 protein. This subunit also forms part of IL-23, a cytokine with similar functions as IL-12 but which is also responsible for the generation of distinctive memory T cells. Consequently, genetic defects in IL-12B can result in both IL-12 and IL-23 deficiencies ¹⁶⁵.

While infection with non-virulent or environmental mycobacteria and *Salmonella* species is common in MSMD, tuberculosis infection is rare and only one case has been reported in literature ¹⁶⁶. Furthermore, biliary cirrhosis was observed in this case, which is a previously unreported complication associated with this immunodeficiency.

Analysis of the immediate family members revealed an autosomal recessive pattern of inheritance. The parents were advised to avoid BCG vaccination of this individual and were provided with information on chemoprophylaxis for travel to tuberculosis endemic regions and she remains well.

In summary, a defect in the IL-12 – IFN- γ pathway should be suspected in patients presenting with multiple, repeated or persistent infection with intracellular pathogens notably mycobacteria and salmonella. IL-12 deficiency due to the loss-of-function deletion should be a differential diagnosis in children of South Asian origin and those born to consanguineous parents in view of the autosomal recessive patterns of inheritance. The diagnostic work-up and the immuno-genetic assay described here can aid in the quick and reliable diagnosis of this condition and its subsequent clinical management.

Chapter 7: DISCUSSION

This thesis describes the development of natural immunity to typhoid fever with increasing age in a population in a typhoid endemic country. Understanding the development of natural immunity to *Salmonella typhi* in typhoid endemic locations is important not only for a better understanding of the pathogenesis of infection but also for preventive purposes. It is already known that it requires 10^3 bacilli to cause clinical typhoid disease. In typhoid endemic regions like Nepal where the quality of drinking water supply, standards of food hygiene and sanitation facilities including sewage disposal are relatively poor, it could be assumed that there would be constant or regular exposure to a smaller number of typhoid bacilli; the numbers being inadequate to cause clinical typhoid fever. Understanding the immunological response of the body to this sub-clinical exposure over a period of time, established in this study, provides important data that can be used for typhoid prevention in public health.

It was established in Chapter 3 that natural immunity to typhoid fever in children above 6 months and up to 9 years was almost 10 fold less than that in adults. Combined with the fact that recent disease epidemiology studies in typhoid endemic countries demonstrating the highest incidence of disease in children below five, this observation would make a case for a vaccination policy targeting this age group in typhoid endemic countries where none currently exists. Indeed, this suggestion is at odds with the current practice where adult travellers and laboratory personnel are often the only vaccine recipients. Adding strength to this argument is that fact that childhood typhoid has been shown to be as clinically severe in children as it is in adults.

A suggestion of a vaccination policy targeting young children raises the issue of vaccine formulation and timing of vaccination, two critical aspects that determine immunogenicity. While it is known that plain polysaccharide vaccine does not induce immunity in children below two years presumably due to the latter's immunological immaturity, the polysaccharide-protein conjugate vaccine has demonstrated the potential to elicit immunity in children. Similarly, the new generation live attenuated oral vaccines could also hold promise in this context. Regarding the timing of vaccination, it could be debated that while introduction in the national immunisation schedule also known as the universal vaccination programme in most developing countries that include three doses of DTP and polio (sometimes with other vaccines) at 6 weeks (or 2 months), 10 weeks (or 4 months) and 14 weeks (or 6 months) might be the only opportunity for large scale population coverage in typhoid endemic countries where vaccine uptake is generally poor, immunogenicity for conjugate vaccines are known to be better when administered in late infancy. Addressing these issues will require further studies in the future.

In Chapter 4, the kinetics of humoral immunity to Vi polysaccharide vaccine were first described. Quantitative analysis of anti-Vi polysaccharide antibodies and anti-Vi polysaccharide B-cells were detailed. These findings shed light on the mechanism of protection induced by the current ViPS vaccine and can aid in the understanding of immunity induced by the new generation derivatives of the ViPS vaccine including the Vi polysaccharide-protein conjugate vaccines. In Chapter 3, it was suggested that the correlate of protection in terms of bactericidal titre might be 16. While a weakness of this suggestion include the fact that disease epidemiological data came from a population different, but in a neighbouring region, to the population that sero-epidemiology study was conducted, this cut-off if confirmed in future studies, could be used for the licensure of new generation polysaccharide-protein conjugate

typhoid vaccine based on bactericidal assays in a vaccine licensure strategy similar to that suggested for meningococcal conjugate vaccine which would result in an enormous saving of time, effort and resources otherwise spent on field efficacy studies.

For the determination of natural immunity and acquired immunity in Chapter 3 and Chapter 4 respectively, a Vi polysaccharide ELISA besides the *S. typhi* bactericidal assay were developed and validated, described in Chapter 2. These assays could be used for the estimation of the prevalence of total and functional anti-Vi polysaccharide antibodies in populations in typhoid endemic countries to gauge the extent of sub-clinical infection.

These assays have implications for the serological diagnosis of typhoid fever. While culture of *Salmonella typhi* is the gold standard for the diagnosis of typhoid fever, Widal test utilising the somatic 'O' antigen or the flagellar 'H' antigen is commonly used for the serological diagnosis of typhoid fever. However, its popularity has been rapidly waning due to the cross-reactivity of these two antigens with antigens from a variety of other organisms most notably *E. coli*. Besides requiring at least paired sera at different time points from the patient for a reliable diagnosis of typhoid fever, the test is also handicapped by the highly variable results of antibody response in serum of patients with typhoid fever confirmed by culture. This has brought about a need for alternate and reliable serological tests for typhoid fever. Knowledge of the baseline antibody titres in populations in different geographic locations and sero-prevalence data of *S. typhi* antigens would be imperative before the use of any serological assays in routine practice.

In Chapter 5, the development and validation of a novel, confirmatory PCR assay for *S. typhi* is described. Novel PCR assays for the detection of the 14 genes that are responsible for the synthesis, transportation and regulation of the Vi polysaccharide in *S. typhi* are described. These

assays can be utilised for the surveillance of the emergence of acapsulate *S. typhi* that would have implications for the implementation of Vi polysaccharide based vaccines.

In Chapter 6, the development and validation of the IL12B PCR which can be utilised to determine the genetic predisposition to typhoid fever in populations in typhoid endemic countries is described. This will be achieved by using this assay to detect the known deletion defect in the p40 subunit of the IL12 gene in the DNA of patients with typhoid fever as confirmed by growth in culture.

Ultimately, a better understanding of the protective mechanisms against typhoid fever, both post-vaccination and following sub-clinical exposure, could aid in the implementation of an appropriate vaccination strategy in typhoid endemic areas and could thereby contribute to preventing a disease responsible for a quarter of a million deaths annually.

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