Characterisation of the *Salmonella stk* fimbrial operon and examination of StkF, the putative adhesion protein, as a potential diagnostic and vaccine candidate

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Statement of originality

This accompanying thesis submitted for the degree of PhD entitled "**Characterisation of the *Salmonella stk* fimbrial operon and examination of StkF, the putative adhesion protein, as a potential diagnostic and vaccine candidate**" is based on work conducted by the author in the Department of Infection, Immunity and Inflammation at University of Leicester mainly during the period between May 2008 and May 2011.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of the work has been submitted for another degree in this or any other university.

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Abstract

Characterisation of the *Salmonella stk* fimbrial operon and examination of StkF, the putative adhesion protein, as a potential diagnostic and vaccine candidate

Mahmoud Mohamed Tawfick Abd Raboh

The StkF protein of *Salmonella enterica* serovar Paratyphi A, a putative adhesion-associated protein, was used to develop a candidate adhesin-based vaccine against *Salmonella* infections, particularly *S.* Paratyphi A-associated enteric fever. Subcutaneous immunisation of BALB/c mice with recombinant StkF (rStkF) showed that this protein was strongly immunogenic as revealed by the presence of high-titre (1:50,000) anti-StkF antibody in sera from immunized mice. Furthermore, as IgG1 was the major antibody isotype induced, it would appear that rStkF generates a strong Th2-type humoral immune response. The induction of a Th2-dominant immune response was further confirmed by splenocyte-associated cytokine profile analysis which demonstrated greater up-regulation of IL-4 than that of IFN-γ or IL-12-p70. In terms of the protective potential of this antigen, *in vitro* assays demonstrated that StkF-specific murine antiserum markedly enhanced opsonophagocytosis-mediated uptake of StkF-expressing *Salmonella* bacteria by human neutrophils. Augmented antibody-specific complement-mediated lysis targeting StkF-expressing *Salmonella* specifically was also shown. These data suggest a likely direct contribution of StkF-specific antibodies to *in vivo* killing and clearance of *Salmonella* and strongly support further investigation of StkF as a potential *Salmonella* vaccine candidate.

BlastN-based interrogation of the NCBI bacterial genome database and PCR investigation of a larger set of strains has shown that the *S.* Paratyphi A *stkF* gene and/or the whole *stk* fimbrial gene cluster is carried by ~1/3 of examined *Salmonella* serovars. Additionally, bioinformatics and phenotypic characterisation has revealed that the *stk* fimbrial operon belongs to the chaperone/usher-γ4-fimbrial clade and that it encodes a mannose-sensitive haemagglutinating fimbrial structure. The latter trait is typical of type 1 fimbriae.

ELISAs based on rStkF, rStaF and rSipA showed variable sensitivity and specificity for the serodiagnosis of invasive *Salmonella* infections depending on the particular UK or region-specific cut off applied for each antigen. Further refinement and/or merging of these assays may allow for development of simple and cost effective tests with higher sensitivity and/or specificity than the Widal test. Importantly, despite some minor reactivity with serum from patients with other Gram-negative bacterial infections, the rStkF-based ELISA exhibited no reactivity with serum from patients with dengue fever supporting its potential as a discriminatory diagnostic tool between fevers caused by *S.* Paratyphi A and dengue virus.

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Abbreviation

Abbreviation	Explanation
°C	degrees Celsius
Å	angstrom
amp	ampicillin
AP	alkaline phosphatase
BLAST	basic local alignment search tool
bp	base pair(s)
BSA	bovine serum albumin
CDC	centre for disease control and prevention
CDD	conserved domain database
cfu	colony forming unit
CML	chloramphenicol
Co-IP	complex-immunoprecipitation
ConA	concanavalin
C/U	chaperon/usher
dATP	deoxyadenosine triphosphate
dH_2O	deionised water
DNA	deoxyribose nucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate mix.
DR	direct repeat
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EG	ethylene glycol
ELISA	Enzyme Linked Immunosorbent Assay
FCA	Freund's complete adjuvant
FCS	foetal calf serum
FIA	Freund's incomplete adjuvant
GSH	glutathione (Reduced)
GSSH	glutathione (Oxidised)
g	gram
H antigen	flagellar antigen
HBSS	Hank's balanced salt solution
HCN	high copy number
HMM	hidden Markov model
h	hour
HPA	health protection agency
HRP	horseradish peroxidase
IFN-γ	interferon gamma
Ig	immunoglobin
IL	interleukin
IMAC	immobilised metal affinity chromatography
IPTG	isopropyl-β-D-thiogalactopyranoside
kan	kanamycin
kbp	kilobase pair(s)
kDa	kilodalton(s)
L	litre

LA	Luria Bertani agar
LB	Luria Bertani broth
LCN	low copy number
LPS	lipopolysaccharide
M	molar
mA	milliampere
M.W.	molecular weight
MBGD	microbial genome database for comparative analysis
min	minute
mM	millimolar
M	molar
ml	millilitre
MR	mannose resistant
MS	mannose sensitive
MSHA	mannose sensitive haemagglutination
MWCO	molecular weight cut off
NCBI	national centre for biotechnology information
NEB	New England Biolabs
nH_2O	NanoPure water
Ni-NTA	nickel-nitriloacetic acid
NP	nepalese
NPV	negative predictive value
NTS	non-typhoidal *Salmonella*
NT	non-typhoidal
O antigen	somatic antigen
o/n	over-night
OD	optical density
OD_{600}	optical density at 600 nm
OD_{595}	optical density at 595 nm
OD_{405}	optical density at 405 nm
OD_{450}	optical density at 450 nm
OPA	opsonophagocytosis
ORF	open reading frame
pBKS	pBluescript KS 11 (+).
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMNs	peripheral blood polymorphonuclear leukocytes
pNPP	p-Nitrophenyl phosphate
PPV	positive predictive value
PSLpred	protein sub-cellular localisation prediction
RBCs	red blood cells
rpm	rotation per minute
rSipA	recombinant SipA protein
rStaF	recombinant StaF protein
rStkF	recombinant StkF protein
RT	room temperature
s.c.	subcutaneous
SAT	salt aggregation test
SBA	serum bactericidal assay
SCOTS	selective capture of transcribed sequences
SD	standard deviation

SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulfate poly-acrylamide gel electrophoresis
SE	standard error
sec	seconds
sp.	species
SPI1	*Salmonella* pathogenicity island 1
SSH	suppressive subtractive hybridization
SSM	slipped-strand mispairing
Ta	annealing temperature
TAE	tris-acetate-EDTA
TBS	tris buffered saline
TEM	transmission electron microscopy
TEMED	N, N, N`, N`-tetramethylenediamine
Th	T helper cell
Th-1	T helper 1
Th-2	T helper 2
T_m	melting temperature
TMH	trans membrane helix
T3SS	type III secretion system
U	Unit
V	volt
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
λ	lambda
µl	microlitre
µg	microgram
µM	micromolar

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Chapter One

Introduction

1 Introduction

1.1 Salmonellosis and enteric fevers

1.1.1 *Salmonella* serovars and nomenclature

Salmonellae are Gram-negative bacteria belonging to the family Enterobacteriaceae. These bacteria are non-spore forming, flagellated, facultatively intracellular anaerobic bacilli (Porwollik & McClelland, 2003). *Salmonellae*, like other Enterobacteriaceae, they produce acid on glucose fermentation, reduce nitrates and do not produce cytochrome oxidase. All organisms except *S.* Gallinarum are motile because of peritrichous flagella, and most do not ferment lactose, thus the minority ferment lactose may not be able to be detected if only MacConkey agar is used to identify *Salmonella*. The differential metabolism of sugars can be used to distinguish many *Salmonella* serovars; serovar Typhi is the only organism that does not produce gas on sugar fermentation (Pegues & Miller, 2010). The *Salmonella* genus is currently classified into two main species: *Salmonella bongori* and *Salmonella enterica.* In addition, according to the traditional Kauffmann-White serotyping scheme, to date, there are over 2500 different known *Salmonella* serotypes (serovars). This serotyping scheme is depending on antigenic variation in the lipopolysaccharide somatic antigen (O-antigen) and flagellar antigen (H-antigen), which allows for classification of *Salmonella* by O-antigen into serogroups and further sub-classification by H-antigen into serovars. *S. enterica* species includes seven different subspecies: I (*enterica*), II (*salamae*), IIIa (*arizonae*), IIIb (*diarizonae*), IV (*houtenae*), VI (*indica*) and VII (Porwollik & McClelland, 2003, Jacobsen *et al.*, 2011). However, recent *Salmonella* typing methods including Pulsed Field Gel Electrophoresis (PFGE) and Multilocus Sequence Typing (MLST), have significantly improved discriminatory power (Foley *et al.*, 2007). For

example, *S. bongori* was originally designated as *S. enterica* subspecies V; however DNA-DNA hybridization based classification led to its assignment as distinct species (Brenner *et al*., 2000). Importantly, the vast majority (> 99 %) of most known *Salmonella* infections in humans and warm-blooded animals are caused by serovars associated with *S. enterica* subspecies *enterica* (subspecies I) (Porwollik & McClelland 2003, Lynch & Tauxe, 2009). In contrast, serovars of other subspecies are usually isolated from cold-blooded animals and the environment but rarely from humans (Brenner *et al*., 2000).

1.1.2 *Salmonella* nomenclature

The nomenclature for the genus *Salmonella* has evolved from the initial one serotype–one species concept proposed by Kauffmann classification to the current two species but a multitude of serovars definition. Historically, each serotype was given an individual species name that matched its serotype name. For example, *S. typhi*, *S. paratyphi A* were each regarded as distinct species suggesting a species count today of over 2500 species of *Salmonella*! The present classification system favoured by the Centre for Disease Control and Prevention (CDC) and recommended by WHO Collaborating Centres allows for identification of species, subspecies and serovar details as in the name *Salmonella enterica* subspecies *enterica* serovar Typhi, commonly abbreviated as *Salmonella* Typhi. The serovar is written in non-italics with the first letter capitalized. This latter abbreviation is more meaningful to physicians who are still accustomed to the historical names of these bacteria and the strong associations of particular *Salmonella* serotypes to disease-types (Brenner *et al*., 2000, Dougan *et al.*, 2011). This standardized CDC nomenclature has been used to refer to *Salmonella* serovars throughout this thesis.

1.2 Infection syndromes caused by *Salmonella enterica* serovars

The *Salmonella* genus is one of the most common human enteric pathogens. Infections caused by *Salmonella* are associated with considerable morbidity and mortality, especially in developing countries (Kumar *et al.*, 2009). The mortality rate remains as high as 30 to 50 % in these countries. The death from *Salmonella* infections mainly occurs in the very young, the very old and the immunocompromised (Chalkias *et al.*, 2008). Furthermore, the issue of greater resistance to antimicrobial agents in *Salmonella* has caused increasing concern (Lynch & Tauxe, 2009).

Salmonellae are generally described as causing either typhoidal (systemic, febrile) or non-typhoidal (gut-associated or gastroenteritis) salmonellosis, however this absolute divergence breaks down in particular hosts (Dougan *et al.*, 2011). When examined in closer detail, diseases caused by several *S. enterica* serovars were found to have significant variations in severity and characteristics. *Salmonella* infections can range from asymptomatic infection or mild gastroenteritis to severe systemic disease in the form of septicaemia and enteric or typhoid fever causing death of the host. *S. enterica* serovars have varying host ranges; some serovars have a broad-host range, for example, *S.* Typhimurium that can cause disease in humans, cattle, pigs, horses, poultry, rodents and sheep. On the other hand, there are a number of well-characterized host-restricted serovars, such as the avian-adapted serovar Gallinarum, the bovine-adapted serovar Dublin and the porcine-adapted serovar Choleraesuis. In addition, *S. enterica* serovar Typhi and *S. enterica* serovar Paratyphi A are human-restricted pathogens without a significant reservoir in poultry or other animals (Porwollik & McClelland 2003, Meltzer & Schwartz, 2010, Zav'yalov *et al.*, 2010).

Enteric fever, a potentially fatal multi-system illness, is a collective term that refers to typhoid and paratyphoid fevers. The most important causes of typhoid and paratyphoid enteric fevers are *S.* Typhi, and *S.* Paratyphi A and *S.* Paratyphi B, respectively. However, less frequently similar febrile diseases can be caused by *S.* Paratyphi C and some non-typhoidal *Salmonella* (NTS) serovars (Meltzer & Schwartz, 2010). Typhoid fever caused by serovar Typhi was thought in the past to have a more severe clinical course with life-threatening complications than the paratyphoid fever. On the contrary, the clinical appearance and illnesses caused by serovars Typhi and Paratyphi A are similar and very difficult to distinguish (Ochiai *et al.*, 2005, Sur *et al.*, 2007, Gupta *et al.*, 2008, Meltzer & Schwartz, 2010). As paratyphoid fever is indistinguishable from typhoid fever in its clinical course, *S.* Typhi, *S.* Paratyphi A, *S.* Paratyphi B and *S.* Paratyphi C are together named as the typhoidal *Salmonella* (Raffatellu *et al.*, 2008).

Non-typhoidal (NT) salmonellosis, a disease of significant clinical and public health importance, is caused by NTS serovars which belong to serovars other than Typhi and Paratyphi. NTS serovars typically cause self-limiting diarrhoeal disease and acute enterocolitis is the main presentation of non-typhoidal salmonellosis in immunocompetent adults. However, invasive, focal suppurative diseases and even an enteric fever like syndrome occur in some cases. These cases associated with high mortality are rather commonly observed in adults with underlying immunocompromising diseases (Lynch & Tauxe, 2009). It has been reported that NTS serovars are emerging as a major cause of fatal invasive bacteraemia among young children and HIV-infected adults in sub-Saharan Africa. However, the extent of the burden of this disease has been unappreciated until recently, in part, because of the

requirement for blood-culturing facilities required for diagnosis. The latter remains a major barrier, as these technologies and resources are generally unavailable in most areas with endemic NTS bacteraemia. Furthermore, at present, no vaccines against these *Salmonella* serovars are available (Gordon, 2008, Siggins *et al*., 2011).

1.3 *Salmonella* infections: transmission and risk factors

The most widespread route of transmission of *Salmonella* is ingestion of contaminated food and water (oral-faecal route), making both non-typhoidal and typhoidal (enteric fever) salmonellosis are mainly food and water-borne diseases. Other risk factors of *Salmonella* infections that have been established include recent close contact with acutely infected patients or with a chronic typhoid/paratyphoid carrier, eating ice cream, iced drinks or food from street vendors; and eating raw fruit and vegetables grown in fields polluted with sewage (Hosoglu *et al.*, 2006, Kothari *et al.*, 2008). *Salmonella* infections have also been shown to be sexually transmitted by anolingual sexual practices. In addition to flies carry the organism on their feet; have been incriminated indirectly as mechanical vectors that contaminate food (Levine, 2009).

Enteric fever is restricted to human hosts and human chronic carriers serve as a reservoir for these pathogens (Levine, 2009). Nevertheless, despite typhoid and paratyphoid fevers being clinically similar, there may be differences in the modes of transmission and/or risk factors associated with infection with *S.* Typhi and *S.* Paratyphi A, respectively (Vollaard *et al.*, 2004). A recent study from Indonesia found that infection caused by *S.* Typhi is transmitted within households by poor hand washing, sharing of food, and a recent typhoid fever in a household member. This contrasted with spread of *S*. Paratyphi A that was predominantly transmitted outside the household by

consumption of contaminated foods from street vendors and by flooding. Possible reasons for these differences have been proposed include a suggested higher infective dose necessary for paratyphoid fever, which is more likely to be present in contaminated food from street vendors. In addition to *S.* Paratyphi A can multiply in food to reach an infective dose. Thus, reduction strategies which are effective against *S.* Typhi may not protect against *S.* Paratyphi A (Vollaard *et al.*, 2004).

As contaminated food and water are the main sources of *Salmonella* infections, the highest risk of disease is in developing countries with poor sanitation. In addition, one of the major at-risk groups is travellers from developed countries contracting this disease whilst travelling in endemic destination countries. Similarly, military personnel stationed in endemic regions are also at risk (Ochiai *et al.*, 2005, Pandit *et al.*, 2008, Fangtham & Wilde, 2008, Meltzer & Schwartz, 2010). Other bacterial infections could also be a risk factor for *Salmonella* infections. Bhan et al. (2002) have proved that acute and chronic *Helicobacter pylori* infections are associated with a higher risk of enteric fever. The authors hypothesized that both acute and chronic *H. pylori* infections cause hypochlorhydria and thus compromise the gastric-acid barrier, which is an important protective mechanism against *Salmonella* infections (Bhan *et al.*, 2002). In addition, underlying pathology as kidney diseases and urinary schistosomiasis, which is endemic in some countries such as Egypt, must be considered as risk factors for *S.* Paratyphi A infections as the bacterium may establish itself in the damaged tissues and produce acute and/or chronic infection (Al-Otaibi, 2003).

1.4 Typhoidal and non-typhoidal salmonellosis epidemiology

Many *Salmonella* serovars show characteristic geographic distributions that make the epidemiology of *Salmonella* fascinating and complex (Lynch & Tauxe, 2009). The oral-faecal route of transmission of enteric fever results in the epidemiology of this illness strongly mapping to international socioeconomic status of subpopulations. For instance, enteric fever was a major cause of morbidity and mortality in the United States and Europe in the 19th century. However, due to the provision of safe and clean drinking water, good and modern sewage disposal systems as well as hygienic food, enteric fever incidence rates have dramatically decreased in developed countries since the early 20th century. Nevertheless, these illnesses remain a problem in underdeveloped countries without adequate sanitation and a safe water supply (Kothari *et al.*, 2008, Chalkias *et al*., 2008, Meltzer & Schwartz, 2010). On the contrary, NT salmonellosis appears to be a disease of industrialized nations, as internationally, the proportion of NTS infections appears to increase with increasing levels of economic development, while the proportion of typhoidal salmonellosis declines (Lynch & Tauxe, 2009). It is estimated that 1.4 million cases of NT salmonellosis occur each year in the United States, of which only a small fraction is reported (Lynch & Tauxe, 2009).

1.4.1 Enteric fever epidemiology

Enteric fever is a worldwide problem and extensively widespread in developing countries in the tropics. It is estimated about 21 million cases and 600,000 deaths caused by enteric fever occur throughout the world per annum. Along with a global estimation of enteric fever cases in 2000, the incidence of enteric fever was high (> 100 cases per 100,000 population per year) in regions of South-central Asia, Southeast Asia, and Southern Africa. However, it was medium (10 – 100 cases per 100,000) in the rest

of Asia, Africa and Latin America; and low in the other parts of the world (< 10 cases per 100,000). In Egypt, the estimated incidence of typhoid fever in 2001 was calculated as 13/100,000 persons / year in Bilbeis district (Lower Egypt). However, in Fayoum Governorate, the estimated incidence of typhoid fever in 2002 was 59/100,000 persons / year (Upper Egypt). The differing rates of typhoid fever may be the result of regional socioeconomic differences (Bhan *et al.*, 2005, Palit *et al.* 2006, Srikantiah *et al*., 2006, and Pandit *et al.*, 2008).

Typhoid fever caused by serovar Typhi is usually considered more widespread when compared to paratyphoid fever. Interestingly, some current data on the causative agent of enteric fever in different endemic countries points to *S.* Paratyphi A as an increasingly important cause of enteric fever. Several recent studies have proposed that *S.* Paratyphi A has become the most frequent cause of enteric fever in parts of south Asia, including hyper-endemic regions such in the Indian subcontinent, where more than a third of culture-confirmed cases are caused by this pathogen. Also, in Southeast China, *S.* Paratyphi A is more frequently isolated than *S.* Typhi. In consequence, it is no longer accurate to view *S.* Paratyphi A minor cause of enteric fever cases, particularly in Asia (Ochiai *et al.*, 2005, Sur *et al.*, 2007, Gupta *et al.*, 2008, Meltzer & Schwartz, 2010).

1.4.1.1 Paratyphoid A fever epidemiology

It was estimated that in the year 2000, 5.4 million cases of enteric fever caused by *S.* Paratyphi A (paratyphoid A fever) occurred throughout the world, and its occurrence was most frequent in developing countries frequented by international travellers (Gupta *et al.*, 2008). In addition, during the past four decades, both the incidence of *S.* Paratyphi A infection and its relative contribution to enteric fever cases have increased

in the United States and countries like China, India, Nepal, Pakistan, and Thailand. In some parts of China, the prevalence of paratyphoid A fever has surpassed that of typhoid fever (Gupta *et al.*, 2008). In a study by Wu et al. (2010), an increasing trend between 2002 and 2007 for *S.* Paratyphi A infections to overtake the levels of *S.* Typhi was noted at Shenzhen People's Hospital in Southern China (Wu *et al.*, 2010). Other studies from India and Nepal have also proposed that in some settings and times, paratyphoid A fever can contribute up to half of all cases of enteric fever (Bhan *et al.* 2005). There was a sudden rise in the incidence of *S.* Paratyphi A infections in the endemic focus of Kolkata, India, during the period 2004 – 2005 (Fig. 1.4.1) (Palit *et al.*, 2006). Though, in a study of enteric fever cases that occurred in Kathmandu, Nepal, it was found that both typhoid and paratyphoid A fevers could be highly prevalent at the same time (Kumar *et al.*, 2009).

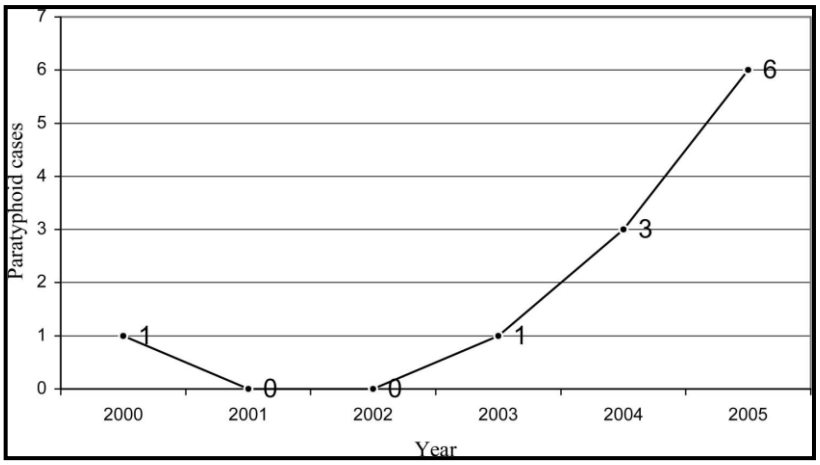

Figure 1.4.2: Incidence of enteric fever caused by *S.* Paratyphi A in Kolkata, India, during the period 2000 – 2005 (Palit *et al.*, 2006). A sudden rise in the occurrence of paratyphoid A fever cases was noted in a longitudinal community-based study in an endemic focus of Kolkata, during 2004 – 2005, compared to the previous three years. In addition, the rate of isolation of *S.* Paratyphi A was 1.5 times higher than that of *S.* Typhi. Reproduced from Palit et al. (2006).

The reasons for the emergence of paratyphoid A fever in Asia are unknown and cannot be readily explained by improved diagnostic methods, typhoid fever vaccination, or the effect of improved water and sanitation systems (Gupta *et al.*, 2008). Hence, the preceding statistics and results suggest that *S.* Paratyphi A represents an equivalent and potentially expanding public health risk as compared to that posed by *S.* Typhi. These data are also highlight the need for a safe and effective vaccine against *S.* Paratyphi A in a country like India (Kumar *et al.*, 2009).

Even in highly developed countries like UK and USA, there is an increasing incidence of *S.* Paratyphi A infections, most often among international travellers, whilst that of *S.* Typhi infections has fallen or remained relatively static over the last 2 – 3 decades. The following graphs (Figs. 1.4.3 and 1.4.4) show the incidence of infections caused by serovars Typhi and Paratyphi A according to national reports from these countries (Gupta *et al.*, 2008).

This shift in the epidemiology of *S.* Paratyphi A infections and the current absence of an effective vaccine against this pathogen has meant that prevention of paratyphoid A fever is increasingly challenging and has provoked interest in vaccines to prevent it. Therefore, the development of an effective vaccine should be a priority (Maskey *et al.*, 2006, Gupta *et al.*, 2008).

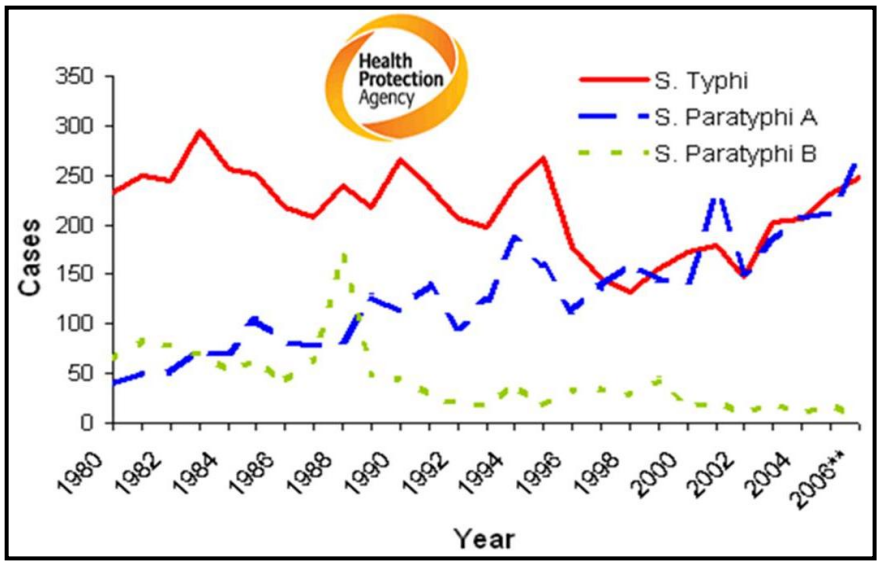

Figure 1.4.5: Number of cases of *S.* Typhi, *S.* Paratyphi A and *S.* Paratyphi B infections in England and Wales from 1980 – 2006 based on laboratory reports submitted to the Health Protection Agency (HPA), England and Wales. The incidence of cases of *S.* Paratyphi A infection was found to be significant among returning travellers to UK (mostly acquired in the Indian subcontinent) and exceed cases of *S.* Typhi infection. Adapted from (http://www.hpa.org.uk/).

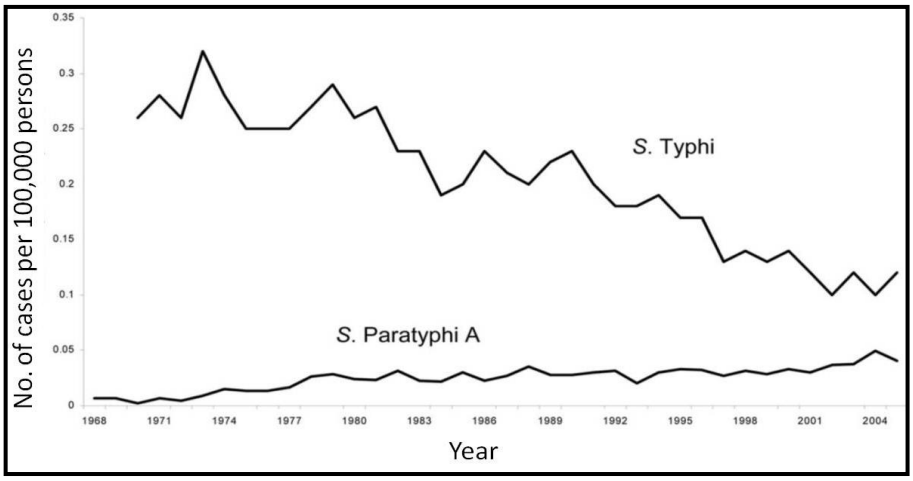

Figure 1.4.6: The incidence of *S.* Typhi and *S.* Paratyphi A infections in USA 1968 – 2005. According to National *Salmonella* Surveillance System, during the past 4 decades from 1968 – 2005, both the incidence of *S.* Paratyphi A infection and its relative frequency among enteric fever cases have increased in the US; most often among international travellers. Adapted from Gupta et al. (2008).

1.5 *Salmonella* infection pathogenesis: clinical features, immunity and host response pattern

1.5.1 Enteric fever and NT salmonellosis pathogenesis

The pathogenesis of *Salmonella* infections is not completely understood and extremely complex due to the diversity of serotypes and hosts involved. Briefly, following ingested organisms pass through the gastric acid barrier and multiply in the small intestine, the bacteria penetrate the intestinal mucosa are ingested by macrophages and may multiply in a limited fashion in mesenteric lymphoid tissues. However, in severe invasive infection, bacteraemia and focal infections in distant tissues occur. Thus, the *Salmonella* pathogenesis includes a number of distinct steps. One of these steps is the attachment and penetration of intestinal mucosal cells, which is facilitated by bacterial extracellular adhesins. The inflammation and tissue destruction step results in the fever, diarrhoea and colitis that usually accompany NT salmonellosis. The latter pathology results from a combination of the effects of bacterial endotoxin, specific cytotoxins that cause mucosal cell death, intracellular multiplication inside macrophages and apoptosis and necrosis of particular infected host cells (Levine, 2009, Lynch & Tauxe, 2009).

Looking at the *Salmonella* infection pathogenesis process in detail in enteric fever, subsequent to ingestion of food or water contaminated with *S.* Typhi or *S.* Paratyphi A, if sufficient numbers of bacteria are ingested, primary infection is established once surviving bacteria transit acidic pH of the stomach contents and compete successfully with the normal bacterial flora of the small intestine. Both *S.* Typhi and *S.* Paratyphi A are highly invasive bacteria, thus, having passed from the stomach through the pylorus into the small intestine, these organisms adhere to and rapidly and efficiently pass through the human intestinal mucosal barrier. There are

several possible mechanisms by which typhoidal bacilli penetrate the intestinal mucosa to arrive in the lamina propria. One mechanism involves uptake of the bacilli into endocytic vacuoles and their passage through the enterocytes to be ultimately released into the lamina propria without destroying the enterocytes. However, more recent studies suggest another more invasive mechanism involving invasion and spread via ileal Peyer's patches and possibly caecal lymphoid patches. Typhoid bacilli are actively taken up by M cells, dome-like epithelial cells, that cover Peyer's patches and other organized lymphoid tissue of the gut. From this initial focus of infection bacteria enter underlying lymphoid cells. There is a release of tumour necrosis factor-alpha (TNF-α), IL-2, IL-6 and other inflammatory cytokines triggered by interactions between macrophages and typhoidal bacilli. These cytokines are responsible for the marked inflammatory responses that occur in the distal ileum in the Peyer's patches and other organized lymphoid aggregations in *Salmonella* infections especially NT salmonellosis (Pandit *et al.*, 2008, Levine, 2009). Therefore, adhesion to the intestinal epithelium is considered the first and most important step in pathogenesis preceding invasion of *S. enterica* organisms and crossing of the mucosal epithelium (Fig. 1.5.1). Fimbriae are believed to mediate this process of adhesion (Althouse *et al.*, 2003).

In non-immune host, after penetration of the mucosal epithelium barrier, typhoidal bacilli reach Peyer's patches and draining mesenteric lymph nodes via the lymphatics, and eventually enter the thoracic duct, then gain access to the general circulation. Some bacilli may translocate via the blood stream to the reticuloendothelial cells of liver and spleen, where they replicate and after a 5 to 21 days incubation period precipitate a systemic illness (Pandit *et al.*, 2008, Levine, 2009). Other bacilli have been shown to remain within the macrophages of the small intestinal lymphoid tissue for extended

periods of time. A primary bacteraemia is believed to occur shortly after invasion of the intestinal mucosa resulting in extracellular circulating Typhi or Paratyphi A bacteria being filtered from the blood by fixed phagocytic cells of the reticuloendothelial system and ingested by circulating neutrophils, whilst intra-macrophage bacteria continue to disseminate in stealth (Pandit *et al.*, 2008, Levine, 2009).

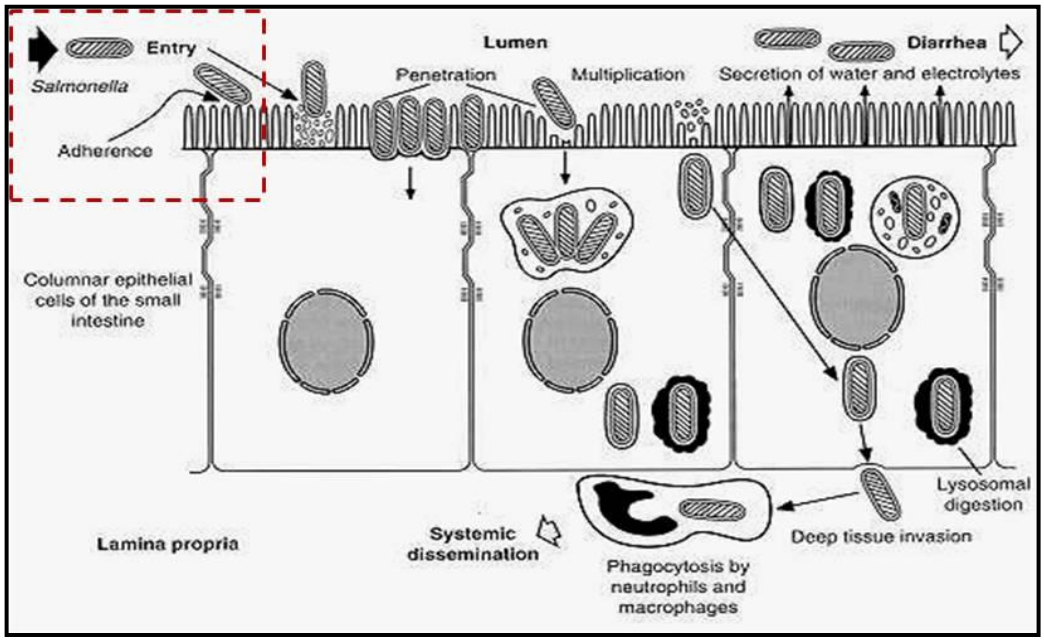

Figure 1.5.2: Invasion of intestinal mucosa by *Salmonella* bacteria. Adherence of bacterim to the intestinal epithelium is the first and most important step in pathogenesis preceding invasion of *S. enterica* organisms and crossing of the mucosal epithelium Adapted from, the Medical Microbiology online book, 4th edition (Giannella, 1996).

Enteric fever is usually described as severe systemic infection of reticuloendothelial system caused by typhoidal *Salmonella*, which can invade the cells of the reticuloendothelial system in the bone marrow, liver and spleen, unlike non-invasive *Salmonella* (Jones & Falkow 1996, Kudalkar *et al.*, 2004). However, Gordon (2008) has put forward a viewpoint that enteric fever and invasive NTS diseases have important differences in pathogenesis, particularly in the pattern of gastrointestinal

luminal involvement. Though, both typhoid and invasive NTS infections share an element of reticuloendothelial intracellular infection and persistence explaining recurrences (Fig. 1.5.3) (Gordon, 2008).

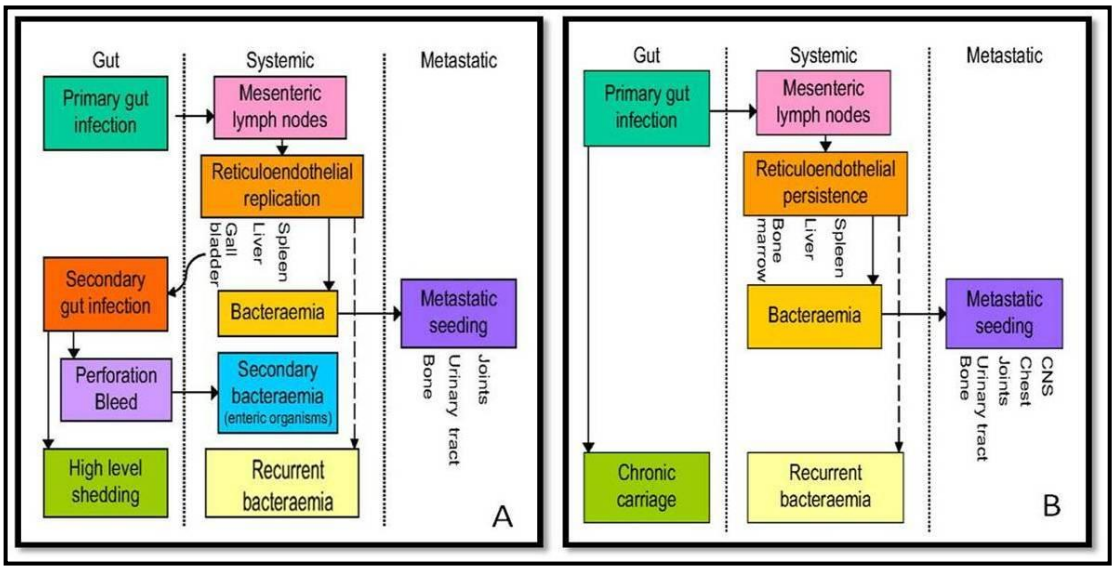

Figure 1.5.4: Scheme illustrating the difference in pathogenesis of enteric fever and NTS bacteraemia assumed by Gordon (2008). **A,** Pathogenesis of enteric fever caused by typhoidal *Salmonella* serovars. **B,** Possible pathogenesis of NTS bacteraemia in HIV patients. Both typhoid and invasive NTS infections share an element of reticuloendothelial intracellular infection and recurrences; however there may be a difference in the gastrointestinal involvement. Reproduced from Gordon (2008).

1.5.2 Clinical features of NT salmonellosis and enteric fever

Several factors greatly affect the incubation period, outcome and clinical manifestations of *Salmonella* infections. These factors include number of ingested bacteria that reach the intestine, the infecting serovar, the type of vehicle in which it is ingested and route of transmission. In addition to a number of host factors include age and the original health and immune status of the host (Levine, 2009, Lynch & Tauxe, 2009).

The most common clinical manifestation of NTS infections is enterocolitis or gastroenteritis. Initial symptoms often include nausea, vomiting, and headache, followed by the major manifestations of abdominal cramps and diarrhoea. Fever, often accompanied by chills, is present in about 50 % of NTS patients when larger dose of organisms ingested. Bacteraemia without enteric fever may also occur due to NTS. Enteric fever syndrome is characterized by a subacute onset with a potential long-lasting course with persistent fever and spleenomegaly. Headache is a prominent early feature. Dry cough, sore throat, chills, anorexia, weakness, dizziness and myalgias may be present at the onset of typhoid or paratyphoid fever. Typically, these symptoms resolve before the classic indicators of enteric fever appear (abdominal pain and fever). Enteric fever is usually accompanied by increasing the temperature in a stepwise fashion during the first week, reaching a peak of 39.4°C – 40°C. Late in the first week, careful inspection of the skin may reveal a few rose spots (slightly raised erythematous spots) in the abdominal area. *Salmonella* associated meningitis occurs primarily in infants and the mortality associated with this complication is usually high. Osteomyelitis and arthritis due to *Salmonella* may occur in normal hosts but are more common in immunocompromised persons. Other complications of *Salmonella* bacteraemia include pneumonia, empyema, endocarditis, intracranial abscess, urinary tract infections, and abscesses of spleen, liver and other soft tissues. Intestinal complications can occur in the later stages of untreated infection. These include bleeding and perforation that are the result of inflammatory responses in the Peyer's patch and consequent necrosis and ulceration of the intestinal epithelium. Relapses of enteric fever are observed in about 10 % of individuals who have apparently recovered from the original infection, and the organisms that initiate relapses appear to come from

within the reticuloendothelial system (Jones & Falkow 1996, Kudalkar *et al.*, 2004, Levine, 2009, Lynch & Tauxe, 2009).

1.5.2.1 S. Paratyphi A infection complications

Comparing the clinical phenotypes of *S.* Typhi and *S.* Paratyphi A infections of over 600 patients in a prospective study, verified that the two infections are clinically indistinguishable (Maskey *et al.*, 2006). Previously, *S.* Paratyphi A infections were considered more benign than those caused by *S.* Typhi. However, *S.* Paratyphi A is capable of causing serious complications such as infective endocarditis, pericarditis, empyema, sino-venous thrombosis, osteomyelitis, acute spondylitis (vertebral osteomylitis), meningitis, bone marrow infiltration, hepatitis, pancreatitis and even death in some instances (Pancharoen *et al.*, 2002, Pandit *et al.*, 2008, Kumar *et al.*, 2008 and D'Cruz *et al.*, 2009). There was a reported case in Nepal of *S.* Paratyphi A infection complicated with gastrointestinal bleeding, with progression to Acute Respiratory Distress Syndrome (ARDS), disseminated intravascular coagulation, and finally death (Pandit *et al.*, 2008). Sheng et al. (2011) described the first case report of uncommon neurological syndrome, Bickerstaff's brainstem encephalitis that developed following *S.* Paratyphi A infection with serious features of impaired consciousness, ophthalmoplegia and ataxia (Sheng *et al.*, 2011). Obviously, the latter described complications illustrate that *S.* Paratyphi A represents a major clinical challenge that is only likely to escalate given the fast emerging multi-resistance patterns associated with this pathogen and the absence of any available vaccine. However, *S.* Paratyphi A has been overshadowed by *S.* Typhi and neglected as an important pathogen by the medical and scientific society. Therefore, extra funds are needed to be directed toward understanding of the disease caused by this pathogen and toward developing a vaccine against it (Pandit *et al*, 2008).

1.5.3 Patterns of host response to *Salmonella* infections

The host response to *Salmonella* infections includes asymptomatic colonization, gastroenteritis, focal soft tissue infection, meningitis, bacteraemia, sepsis and death. However, the pattern of host response to *Salmonella* infections varies from person to person depending on many factors include the virulence of the infecting serovar, the size of the bacterial inoculum, and specific host factors including age, gastric acidity, immunologic competence, prior host exposure to antimicrobials and specific host illness. Some diseases, like the gastrointestinal tract disorder achlorhydria and esophageal, gastric or intestinal malignancies may predispose to *Salmonella* enterocolitis. By contrast, defects of cellular immunity (leukaemia, HIV infection) or reticuloendothelial cell function (sickle-cell anaemia, malaria) may predispose to bacteraemia and invasive illnesses. Thus, some infected individuals develop bacteraemia but without overt clinical signs of infection, whilst others develop severe and/or complicated disease (Levine, 2009, Lynch & Tauxe, 2009).

Since the phenotype of virulence of the infecting *Salmonella* serovar fully manifests only during *in vivo* host pathogen interactions, studying natural infection in an animal that develops signs of disease resembling those observed in humans is essential for a complete understanding of *Salmonella* pathogenesis. However, it may be useful to study the pathogenesis of different *Salmonella* serovars in different hosts as these may give rise to markedly different responses or disease manifestations. Thus, both the infecting serovar and the infected host determine the response to *Salmonella* infections in vertebrates. For example, the host-adapted *S.* Typhi causes typhoid fever in humans but is avirulent (or non-pathogenic) in mice and other mammals. *S.* Enteritidis causes severe but self-limiting gastroenteritis with occasional bacteraemia in humans, while

systemic disease develops in rats and mice. *S.* Typhimurium on the other hand infects a broad spectrum of mammalian hosts in which it usually causes a local and self-limiting gastroenteritis as the case typically in calves and humans. By contrast, the same serovar causes fatal typhoid fevers in mice despite the fact rodents constitute one of its natural hosts. Therefore, almost all *in vivo* experimental work aimed at studying the pathogenesis of typhoid fever has been done using a well-developed *S.* Typhimurium murine infection model that mimics typhoid fever in humans (Emmerth *et al.*, 1999, Rabsch *et al.*, 2001, Santos *et al.*, 2001).

1.5.4 Immunity and host defence to *Salmonella* infections

Several major barriers and mechanisms have been shown to contribute to the host defence against *Salmonella* infections. The general clearance mechanisms of mucosal barrier including mucus secretion, ciliary activity, gastric acidity, normal flora; and the innate immune system constitute the first non-specific line of defence against these infections by decreasing the number of bacteria to not be able to produce illness (Pandit *et al.*, 2008, Lynch & Tauxe, 2009).

The adaptive immune response represents a more advanced and pathogen-specific second line of defence, which sequentially leads to memory responses able to protect against subsequent challenges (Pandit *et al.*, 2008). In reality, a broad immune response is evident following *in vivo Salmonella* infections, with the appearance of serum antibodies (humoral response), mucosal secretory IgA responses, an array of cell-mediated immune responses, and a form of antibody-dependent killing of *S.* Typhi (Levine, 2009). Cellular immunity is one of most important defence barriers against *Salmonella* bacteraemia and invasive illness. Obviously, active T-cell immunity is one

requirement as HIV and other immunocompromised individuals are more susceptible to bacteraemia caused by NTS isolates. Additionally, it is possible that the general lack of a strong polymorphonuclear cell (PMN) influx in typhoid patients facilitates systemic spread of the organism. Nevertheless, this defence can be overcome in the normal host by certain highly invasive serovars, such as *S.* Paratyphi A, or by very large doses of other less virulent serovars. The humoral immune response to *Salmonella* infections has been characterized by demonstration of agglutinating antibodies that develop against the O and H antigens (Lynch & Tauxe, 2009). In conclusion, both humoral and cellular immune responses are being engaged against *Salmonella* infections. Thus, both humoral and cellular immunity are important in controlling these infections, but full protective immunity is not always induced following natural infection (Levine, 2009, Dougan *et al.*, 2011). In addition, much remains to be learnt about the mechanisms that prevent most *Salmonella* serotypes from causing systemic illness (Dougan *et al.*, 2011).

1.6 Diagnosis of *Salmonella* infections

The accurate identification and diagnosis of patients infected with *Salmonella* is extremely important to facilitate the appropriate treatment, to study the epidemiology of these infections and recognize asymptomatic carriers of these organisms (Chart *et al*., 2007). The diagnosis of *Salmonella* enterocolitis relies mostly on isolation of the bacterium from stool. Alternatively, in case of high fever or invasive disease, culturing blood, CSF or other relevant body fluids may be informative (Lynch & Tauxe, 2009). Clinical samples are typically cultured directly onto selective agar media, such as Xylose-Lysine-Desoxycholate (XLD) agar or Hekton agar, and incubated at 37^{o}C for 18-24 hours. Bismuth sulfite agar is the preferred medium for isolating *S.* Typhi. In addition, stool samples are usually inoculated into selective tetrathionat- and selenite-

based enrichment broths and incubated at 37^{o}C for 18-24 hours to facilitate the recovery of low numbers of organisms, before plating out onto selective agars (Pegues & Miller, 2010). After primary isolation, possible *Salmonella* isolates are then subjected to simple biochemical testing to reveal positive reactions typical of *Salmonella*.using to either triple sugar iron (TSI) or Kliger's iron (KI agar). Lysine iron agar (LIA) indicates the decarboxylation or deamination of lysine and the production of hydrogen sulfide, and is particularly helpful when used in conjunction with TSI agar for the presumptive identification of *Salmonella*. The usual reaction of *Salmonella* on TSI or KI will be an alkaline slant and acid butt, and they will produce gas and H_2S. Colonies with typical biochemical profile of *Salmonella* is then serougrouped with commercially available polyvalent O and H antisera (Lynch & Tauxe, 2009, Pegues & Miller, 2010).

1.7 Diagnosis of enteric fever: current state-of-play, challenges and progress

In most cases, suspicion of a typhoid-like *Salmonella* acute febrile illness is established based on clinical signs and symptoms, and thus such patients are typically treated presumptively. This approach can lead to a delayed or missed definitive diagnosis, or even to an incorrect diagnosis. Given that febrile illnesses can have many causes; patients with diseases other than enteric fever may receive inappropriate management or the wrong antimicrobial therapy because of misdiagnosis (Olsen *et al.*, 2004). Infections such as malaria, brucellosis, and dengue fever, may initially be confused because of similar symptoms associated with enteric fever (Levine, 2009). Additionally, enteric typhoid and paratyphoid fevers are often clinically similar and the pattern of onset of both illnesses is the same. Thus, precise diagnosis of enteric fever depends on laboratory confirmation permits differentiation not only between typhoid and

paratyphoid fever, but also identifies specifically other *Salmonella* serovars and other infectious agents that may cause an enteric fever-like syndrome (Haq *et al.*, 1997, Levine, 2009, Crump & Mintz, 2010).

1.7.1 Laboratory diagnosis of enteric fever

A diagnosis of enteric fever is confirmed by the recovery of bacterium from a suitable clinical specimen. Stool cultures become positive shortly after ingestion of the organism in only 45 – 65 % of cases. Typhoid bacilli can often be recovered from blood specimens as this pathogen appears in the blood coincidental within the onset of fever and remains circulating in the bloodstream for about 2 weeks in untreated bacteraemic patients before abating. However, positive blood cultures confirm bacteriological diagnosis in approximately 50 – 75 % of enteric fever patients (Levine, 2009). Typhoid bacilli can also be isolated from liver biopsies and bone marrow specimens obtained from patients even many months after the patient has fully recovered from the original symptoms (Levine, 2009). That because viable typhoidal *Salmonella* organisms can persist for months in human bone marrow through local intracellular replication. In addition, viable organisms count in the bone marrow are considerably less affected by antibiotic treatment than that corresponding to blood specimens and this contributes to the advantage of bone marrow isolation over blood culture in diagnosis of typhoidal *Salmonella* infections (Wain *et al.*, 2001). This concept was confirmed in a study that compared the recovery of *S.* Typhi from different specimens in 62 typhoid fever patients. *S.* Typhi was successfully isolated from culture of bone marrow in 56 patients (90 %) but was only recovered from blood in 25 (40 %), from stool in 23 (37 %), and urine in 4 (7 %) of the 56 patients (Gilman *et al.*, 1975). In addition, the study of Wain et al. (2001) demonstrated unambiguously that there were over 10 times more typhoidal

bacilli in a millilitre of bone marrow than in an equal volume of peripheral blood. It seems likely therefore that a large-volume blood culture (10 ml) would be needed to match the positivity rate of a 1-ml bone marrow culture. However, it may be difficult to collect this volume of blood from some patients (Wain *et al.*, 2001). This implies that bone marrow culture is the best method for recovery of typhoidal *Salmonella*, though as this is an invasive procedure, it is frequently a last resort specimen (Gilman *et al.*, 1975, Wain *et al.*, 2001). Blood culture, a less sensitive method than bone marrow culture, is often the most practical choice for both patient diagnosis and epidemiologic evaluation of *S.* Typhi and *S.* Paratyphi burden (Crump & Mintz, 2010). However, unfortunately, most enteric fever cases occur in low- and middle-income countries where blood cultures are often unavailable, as it is too costly, requires both laboratory and clinical expertise and hence is very inconsistently performed (Crump & Mintz, 2010).

1.7.2 Serological diagnostic methods (Antibody detection tests)

The 'gold standard' to confirm diagnosis of enteric fever is the isolation and identification of the causative agents (*S.* Typhi or *S.* Paratyphi A) from the faeces, bone marrow, or blood specimens of patients. However, in the absence of culturable organisms, immunodetection of pathogen-specific antibodies in serum samples from patients based on well-characterized antigens might give definitive evidence of infection with these *Salmonella* serovars (Chart *et al.*, 2007, Nakhla *et al.*, 2011).

Serodiagnosis of enteric fever has been attempted since the late 19th century when Widal and Sicard (1897) showed that the sera of patients with typhoid fever agglutinated typhoidal bacilli. This agglutination method formed the basis of serodiagnostic tests for typhoid for over 100 years, with some refinement over the years

(Olsen *et al.*, 2004). It was found that serum antibodies level to O and H antigens present on *Salmonella enterica* rise at the time of onset of illness in enteric fever patients. The Widal agglutination test used to diagnose *Salmonella* infections depends mainly on measuring agglutinating antibodies (agglutinins) to somatic (O) and flagellar (H) antigens usually appear on days 6 to 8 and days 10 to 12 after the onset of the disease, respectively, and the virulence-associated capsular polysaccharide 'Vi' antigen of *S.* Typhi. However, this test has been restricted because of its low sensitivity and/or specificity. Non-specific cross-reactivity with other *Salmonella* serovars antigens can occur as *S.* Typhi shares O and H antigens with other *Salmonella* serotypes. Equally, elevated antibody titres may result from other nonspecific inflammatory diseases, undefined cross reacting antibodies, and cross-reacting epitopes with other Enterobacteriacae or antibodies due to past history of immunisation by killed whole cell vaccines or even infection in the distant past, which can lead to false-positive results. On the other hand, suppression of O and H agglutinins by early treatment may result in a false-negative Widal test (Olsen *et al.*, 2004, Wain & Hosoglu, 2008, Levine, 2009, Lynch & Tauxe, 2009). Like typhoid, paratyphoid A fever has been commonly diagnosed by Widal test based on the specific O and H antigens of *S.* Paratyphi A (Tam *et al.*, 2008).

Many trials have been done to develop serological diagnostic methods for enteric fever able to improve on the performance of Widal test, which remains in widespread use in the developing world. Despite newer serological tests performing better than the century-old Widal test, many of these still continue to offer unacceptable sensitivity and specificity. Furthermore, none of these new tests has been successfully validated or proved to be of practical value to be used in areas where this disease is endemic (Olsen

et al., 2004, Chart *et al.*, 2007, Meltzer & Schwartz, 2010). Hence, there is an urgent need for a practical, well evaluated, effective and appropriate diagnostic method for enteric fever, which must include detection of infections due to the emerging threat of *S.* Paratyphi A (Wain & Hosoglu, 2008).

The recent attempts to improve the diagnosis of enteric fever have focused on two approaches. The first is to improve serological diagnosis of enteric fever by using new sensitive immunoassays (ELISAs) and the second relies on nucleic acid testing for *S.* Typhi and *S.* Paratyphi A (Meltzer & Schwartz, 2010). An attempt to improve the serodiagnosis was recently undertaken by Chart et al. (2007) who developed ELISAs to detect human serum antibodies to the O and H antigens of *S.* Typhi, *S.* Paratyphi A, *S.* Paratyphi B and *S.* Paratyphi C as well as detection of antibodies against 'Vi' antigen of *S.* Typhi and *S.* Paratyphi C. These assays represented a viable replacement for the Widal assay for the screening of sera. However, ELISA successfully detected serum antibodies to Vi antigen may be of limited value in the serodiagnosis of acute *S.* Typhi and *S.* Paratyphi A infections due to the kinetics of serum antibody production against the Vi antigen (Chart *et al.*, 2007). Thus, they are excellent assays to screen for chronic *S.* Typhi carriers as most of these individuals have highly elevated levels of Vi antibody (Levine, 2009). The *Salmonella* serodiagnosis protocols described in this study are the new standard operating procedures used by the Health Protection Agency's National *Salmonella* Reference Centre based in the Laboratory of Enteric Pathogens, Colindale, UK (Chart *et al.*, 2007). Another recent ELISA assay uses a mixture of O and H antigens for diagnosis of *S.* Typhi infection has been developed by Fadeel et al. (2011). The developed ELISA showed superior sensitivity and specificity as diagnosed 93 % compared to 71 % using Widal test (Fadeel *et al.*, 2011).

On the contrary, over the years, antigen detection tests have not been well investigated. In addition, there has been very little commercial interest in developing antigen detection kits (Wain & Hosoglu, 2008). Attempts have been made to develop a rapid diagnostic test for typhoid fever that detect typhoid bacilli O or Vi antigens in blood, urine and other body fluids using agglutination or ELISA; with few exceptions these tests have failed to perform satisfactorily (Olsen *et al.*, 2004).

DNA dependent diagnosis, the other major approach, based on the Polymerase Chain Reaction (PCR) using *S.* Typhi and *S.* Partyphi A specific primers showed good sensitivity and specificity. Recently, a multiplex PCR for the detection of both pathogens has been reported to be highly sensitive and even can differentiate between them (Ou *et al.*, 2007, Kumar *et al.*, 2010, Yin Ngan *et al.*, 2010). Despite the elegance and apparent simplicity of these PCR-based methods, none of these tests are commercially available. This could be due to the expense of these methods, thus, they have only been amenable to research and reference laboratories that are associated with clinical facilities, however, they are impractical for routine use in most laboratories in endemic areas (Levine, 2009, Meltzer & Schwartz, 2010).

1.7.3 Serologic rapid diagnosis of enteric fever (Rapid immunoassays)

Enteric fever as a severe and potentially life-threatening disease requires prompt medical intervention. However, laboratory investigations required for the confirmation of the diagnosis requires laboratory equipments and trained technicians that are not available in most primary health care facilities in the developing world. In addition, Widal test appears of limited value, especially in endemic areas. Thus, there is an urgent

need for simple, rapid, and affordable point-of-care tests for enteric fever in these regions (Nakhla *et al.*, 2011).

Recently, investigations of potentially more sensitive and specific markers in the blood and urine of patients with enteric fever have enabled the development and manufacture of practical and affordable rapid diagnostic tests for this illness. Some of these tests constitute currently available commercial kits trialled in developing countries. The TyphiDot rapid assay is one such example that can be used even in areas with limited resources. TyphiDot is a DOT enzyme immunoassay (Typhidott and Typhidot-Mt; Malaysian Biodiagnostic Research SDN BHD, Kuala Lumpur, Malaysia) that detects either IgM or IgG antibodies against a specific outer membrane protein antigen of serotype Typhi. Several studies have showed that both Typhidott and Typhidot-Mt gave better diagnostic sensitivity and specificity as compared to the Widal test; however batch to batch variation has caused some limitations. Another trial with a dipstick assay, Multi-Test Dip-S-Ticks (PANBIO INDX, Inc., Baltimore, Md.), which detects anti-O, anti-H and anti-Vi IgG and IgM antibodies in patient serum, plasma, or heparinised whole blood, has suggested several advantages for this assay. Using this latter method, results can be obtained on the same day allowing for prompt treatment of patients, only a small volume of serum is needed, and no special laboratory equipment is needed to perform the assay. Although superior to the Widal, this sassy yet again lacks sufficient sensitivity and specificity. The former two tests are qualitative assays. The third test is the TUBEX-TF (IDL Biotech, Sollentuna, Sweden), a semi quantitative test that uses polystyrene particle agglutination to detect IgM antibodies to the O9 group-specific and immunodominant antigen of *S.* Typhi. TUBEX-TF detects antibodies from a patient's serum by the ability of these antibodies to inhibit the binding

between an indicator antibody-bound particle and a magnetic antigen-bound particle. However, as the TUBEX-TF test uses a colorimetric reaction which may be masked in haemolysed samples, sensitivity may be impaired. Also, false positive results may occur in persons with recent *S.* Enteritidis infection (Olsen *et al.*, 2004, Wain & Hosoglu, 2008). In the study by Olsen et al. (2004), these commercial rapid diagnostic kits as well as the Widal test have been evaluated for serovar Typhi infections with sera collected from patients who presented acute febrile illness with more than four days of fever in Vietnam. Overall, the sensitivity of the Multi-Test Dip-S-Ticks was quite high (89 %), however specificity was low (50 %). The Typhi-Dot and the TUBEX-TF both had high sensitivities of 79 % and 78 %, respectively, and specificities of 89 % and 94 %, respectively, strongly supporting the utility of these assays. The Widal test was the least sensitive and specific of the assays (64 % sensitive and 76 % specific). The sensitivity of TyphiDot was high during the first week of illness, presumably as this assay is biased towards detection of IgM antibodies that occur earlier in the course of the illness. However, this was not seen with the TUBEX assay, which also detects IgM antibodies preferentially (Olsen *et al.*, 2004). In summary, of the currently available commercial kits trialled in developing countries, TUBEX seems to perform the best (Wain & Hosoglu, 2008).

Two other rapid diagnostic field tests, a latex agglutination Dri-Dot assay and an IgM Lateral Flow assay developed by the Royal Tropical Institute (KIT) in Netherlands, were evaluated in typhoid fever patients in Egypt. The IgM Lateral Flow assay designed for the detection of IgM antibodies against LPS of *S.* Typhi in serum showed 80 % sensitivity and 71.4 % specificity. In comparison, the sensitivity and specificity of the *S.* Typhi Dri-Dot assay, for the detection of *S.* Typhi LPS-specific agglutinating antibodies

in serum, were 71.4 % and 86.3 %, respectively. These two rapid assays had previously been evaluated in Indonesia but showed different sensitivities and specificities. The sensitivity of the Dri-Dot assay increased from 31 % for samples collected during the first 4 to 5 days after the onset of illness to about 85 % for samples collected after more than 9 days and the assay specificity was calculated to be 97 %. The sensitivity of the IgM Lateral Flow assay was with a range from 41 % to 90 %, depending on the duration of illness (Nakhla *et al.*, 2011). Thus, the major limitations of these rapid point-of-care assays for typhoid fever is the limited sensitivity at the early stage of disease and are most useful in patients presenting one week after symptom onset (Nakhla *et al.*, 2011).

TUBEX-PA, the first rapid (5-min colorimetric) test for diagnosis of paratyphoid A fever, was described by Tam et al. (2008), which detects antibodies against O2, the group-specific and immunodominant antigen of *S.* Paratyphi A by inhibiting the binding between two types of reagent particles similar to TUBEX-TF except that *S.* Paratyphi A LPS is used instead of *S.* Typhi LPS, and an IgM anti-O2 mAb is used instead of the IgM anti-O9 mAb. Both of these assays were evaluated in China, TUBEX-PA showed a sensitivity of 81.0 % to 93.3 % for the diagnosis of *S.* Paratyphi A infections and was 98.1 % specific. However, TUBEX-PA also detected 50 % to 81.8 % of typhoid patients, and conversely, TUBEX-TF detected 46.7 % to 73.3 % of paratyphoid A cases. These unexpected results of mutual cross-detection of typhoid and paratyphoid A fevers were due to anti-O12 antibodies in typhoid and paratyphoid A fever patients (Tam *et al.*, 2008).

In conclusion, the rapid field tests are attractive diagnostic tools in distant and rural clinical settings for enteric fever where access to laboratory facilities is limited or not available. However, the increasing numbers of these tests have been developed;

none has yet found widespread application. This could be explained by many factors including the availability and quality of assays, the routine empirical use of antibiotics, patterns of disease severity, duration and phase of the disease at first presentation to the clinic, besides the prevalence of cross-reactive antibodies in endemic populations that may influence the sensitivity and specificity of these assays (Nakhla *et al.*, 2011).

1.8 Vaccination against *Salmonella* infecions

Ever since the advent of the antibiotic era, enteric fever has been a treatable condition that responds in the vast majority of cases to appropriate antimicrobial therapy. However, we are currently facing a threat to this situation as successful treatment of enteric fever is becoming increasingly difficult due to the global emergence of multi-drug resistant *Salmonella* strains (Guzman *et al.*, 2006). Frequent exposure to *Salmonella* infection in endemic areas results in the acquisition of partial protection, however, activation of primary immunity often takes longer than is necessary to contain first episode infection (Levine, 2009). In addition, as the provision of safe water and appropriate sanitation is inadequate in endemic areas and as these services are usually linked with economic development status (Bhan *et al.*, 2005), enteric fever is likely to pose an ongoing challenge for decades, if not centuries, to come. Thus, vaccination against enteric fever is still considered an essential tool for the effective management of this disease alongside other preventive strategies as provision of safe drinking water. Definitely, the establishment of population wide protective immunity against *S.* Typhi infection through vaccination has been of major benefit in controlling the disease in endemic regions and for travellers to these areas however has not provided significant protection against other *Salmonella* serovars infections (Jones & Falkow 1996, Bhan *et al.*, 2005).

As large number of persons may become infected when commercial kitchens serve *Salmonella*-contained foods that have not been suffiently cooked, such as raw or undercooked eggs, or that have been mishandled. In developed countries, vaccination of feed animals and improved food safety practices has reduced the burden of foodborne NT salmonellosis (Pegues & Miller, 2010). Several studies with live attenuated *Salmonella* vaccines in pigs, cattle, and chickens have shown them to be effective in stimulating a strong cell-mediated immune response and protecting animals against disease. A live attenuated *S.* Choleraesuis vaccine that has been licensed for use was found to protect pigs from the disease as well as protectd calves against experimental challenge with *S.* Dublin. Live *S.* Enteritidis vaccine has also been effective in significantly reducing the infection of laying hens challenged with *S.* Enteritidis (Kahn *et al.*, 2011).

1.9 Available vaccines against enteric fever

Three types of active vaccines are available to protect against typhoid fever, while there is no licensed vaccine against paratyphoid fever. These vaccines include the early, inactivated whole cell parenteral vaccines, which are no longer being manufactured and have been replaced by two modern licensed vaccines: (Vi) polysaccharide antigen based (TyphiViM) and the oral live attenuated bacteria (Vivotif) vaccines. These two vaccines offer comparable protection and are both well-tolerated (Guzman *et al.*, 2006, Levine, 2009).

1.9.1 Inactivated whole cell vaccines against typhoid fever

Inactivated whole-cell parenteral vaccines against typhoid were prepared by heat or chemicals inactivation of virulent bacteria. These vaccines included heat-inactivated,

phenol-preserved or acetone-inactivated vaccines. Parenteral whole cell inactivated vaccines have been widely used for many years. One of these historic vaccines comprised inactivated *S.* Typhi, *S.* Paratyphi A and *S.* Paratyphi B and was claimed to be effective against both typhoid and paratyphoid fevers. While these early parenteral vaccines conferred protection for up to seven years against enteric fever, all were unsatisfactory because of a high frequency of significant associated adverse reactions, caused them are no longer being used (Bhan *et al.*, 2005, Guzman *et al.*, 2006, Levine, 2009).

1.9.2 Live oral attenuated Ty21a vaccine against typhoid fever (Vivotif)

The use of attenuated live bacterial vaccines constitutes a recent approach for typhoid vaccination. Ty21a is an attenuated mutant strain of *S.* Typhi strain Ty2, which is safe and protective vaccine against typhoid fever. This vaccine was developed in the early 1970s by chemical mutagenesis of the pathogenic *S.* Typhi Ty2. The distinctive mutations in this strain include an inactivation of the *galE* gene which encodes an enzyme involved in LPS synthesis leading to the inability to express Vi ("virulence") capsular polysaccharide. It is now the only licensed live oral vaccine against typhoid fever and is available as enteric-coated capsules or a liquid formulation. The orally administered Ty21a attenuated vaccine mimics the mucosal and systemic immune responses elicited by natural infection and is also associated with fewer and less severe side effects. Vaccination with Ty21a induces strong serum IgG and mucosal IgA antibodies responses allowing for increased O-specific faecal IgA to be observed one to eight months post-immunisation. Theses IgG and IgA responses mediate protection against *S.* Typhi infection. Ty21a also triggers cell-mediated immunity, which is crucial for protection against intracellular bacterial pathogens like *Salmonella*. This vaccine is

approved for use in people that are six years of age and above and three doses are recommended, each given two days apart. A booster dose is recommended every three years in endemic areas and travellers should be revaccinated annually (Bhan *et al.*, 2005, Guzman *et al.*, 2006, Levine, 2009).

1.9.3 Vi-based subunit vaccine against typhoid fever (TyphiViM)

Another approach in typhoid vaccination is the use of a subunit vaccine that is generated using the purified Vi capsular polysaccharide antigen of *S.* Typhi Ty2. Immunisation with Vi-antigen is safe and well tolerated. The Vi polysaccharide vaccine functions in the form of a T-cell-independent vaccine. It is administered as a single intramuscular or subcutaneous dose containing 25 µg of Vi-antigen. The antibodies elicited by a single dose persist for a minimum of 3 years. However, revaccination is recommended every 2 years in the US. The main drawbacks of the parenterally administered Vi-based vaccines are the inability to stimulate mucosal immunity and revaccination does not elicit any booster effect, as shown in clinical trials. This absence of a booster effect is because immune responses against polysaccharides do not involve T cells; therefore, immunological memory cannot be established. Furthermore, these vaccines are not effective in infants who are unable to mount an adequate T cell-independent response. However, the linkage of the T-independent Vi polysaccharide antigen to a T-dependent protein carrier molecule results in a T-dependent conjugate vaccine that can overcome these limitations. This approach has been employed successfully for the development of a vaccine for *S.* Typhi by covalent binding of Vi polysaccharide to recombinant *Pseudomonas aeruginosa* exoproteinA (rEPA) (Guzman *et al.*, 2006, Levine, 2009).

Table 1.9-1: Comparison of available vaccines against typhoid fever. Reproduced from Guzman et al. (2006).

	Whole cell vaccine	Vi vaccine	Ty21a vaccine
Route of administration	Perenteral	Perenteral	Oral
Adverse systemic reactions	10-20%	2%	<1%
Adverse local reactions	10-50%	10-40%	NA
Protection rate	~60-80%	64-72%	~60-80%
Duration of protection	7 years	>17-12 months	4-7 years
Booster doses/revaccination	3 years	2-3 years	1-7 years
Medical professional needed for vaccination	Yes	Yes	No

One major disadvantage of these newer typhoid vaccines is that they do not provide significant protection against the emergence of *S.* Paratyphi A infections (Fangtham & Wilde 2008, Sheng *et al.*, 2011). Ty21a live oral typhoid vaccine has recently been shown to confer partial protection against *S.* Paratyphi B (Gupta *et al.*, 2008). From an immunological perspective, parenteral Vi-based vaccines contain only purified Vi antigen of *S.* Typhi and both serovars Paratyphi A and Paratyphi B lack this Vi antigen which means that a Vi-based *S.* Typhi vaccine is ineffective at preventing paratyphoid fever (Fangtham & Wilde, 2008). In fact, cases of paratyphoid A and B fevers were reported for travellers to India and Morocco even though they were immunised with the Vi-based subunit vaccine (Guzman *et al.*, 2006). In addition, Rodrigues et al (2003) noticed a worrying trend of a steady increase in the total numbers of *S.* Paratyphi A isolates at tertiary care centre in India while patients managed at this centre had received typhoid immunisation with both recent vaccines (Rodrigues *et al.*, 2003). As serovars Paratyphi A and Paratyphi B share the somatic O12-antigen with serovar Typhi, potential partial cross-protection against both of these other serovars

may arise from the Typhi-specific vaccine triggering immune responses against the O12-antigen. Accordingly, some immunologic studies have shown that volunteers vaccinated with the Ty21a vaccine displayed cross-reactive cell-mediated immunity against *S.* Paratyphi A and *S.* Paratyphi B (Guzman *et al.*, 2006).

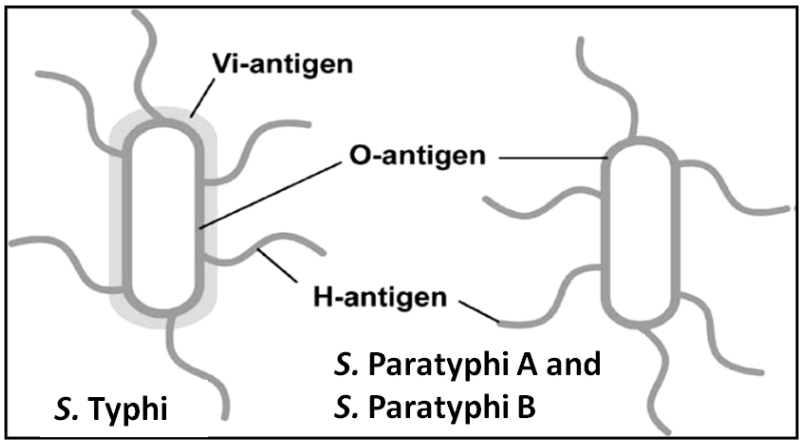

Figure 1.9.1: A schematic diagram represents the surface structures of serovars Typhi, Paratyphi A and Paratyphi B. *S.* Typhi expresses the surface antigens LPS (O-antigen), flagella (H-antigen) and polysaccharide capsule (Vi antigen), while the serovars Paratyphi A and Paratyphi B do not express Vi antigen. The most important surface antigen of *S.* Paratyphi A and *S.* Paratyphi B is the O12 antigen, which is shared with serovar Typhi. Adapted from Guzman et al. (2006).

1.9.4 Future enteric fever vaccines

One of these next-generation rationally designed vaccines is the M01ZH09 (*S.* Typhi Ty2 Δ*aroC* Δ*ssaV*) vaccine which is administered as a single-oral-dose and is known to be well-tolerated, currently in human trials. Lindow et al. (2011) studied the role of humoral immune response and function of *S.* Typhi-specific antibodies following vaccination with this new vaccine in an attempt to recognise the mechanism of vaccine-induced protection against *Salmonella* infections. They reported that post-vaccination antibodies function as opsonins, thus increasing the uptake and killing of wild-type *S.*

Typhi by human macrophages by up to 2.3-fold relative to pre-vaccination samples. In addition, bactericidal assays indicated that antibodies generated by vaccination were recognized by complement factors and assisted in killing *S.* Typhi. This study on vaccine correlates of protection against typhoid fever, demonstrated at least two mechanisms by which vaccine-elicited antibodies facilitate killing of *S.* Typhi, opsonophagocytosis and complement fixation (Lindow *et al.*, 2011).

Efforts to develop a vaccine against *S.* Paratyphi A started in 1915 with attempts to improve the old parenteral whole-cell typhoid vaccine. It has been hypothesized that as these whole-cell vaccines have been discontinued and as the current typhoid vaccines have been used minimally and/or been ineffective in preventing paratyphoid fever. In addition, the latter has become an increasing problem in many developing parts of the world. This has led to calls for the development of a vaccine to provide protection against *S.* Paratyphi A infection or a bivalent vaccine that protects against both *S.* Typhi and *S.* Paratyphi A (Fangtham & Wilde, 2008).

1.10 *Salmonella* virulence factors

Salmonellae have many factors responsible for its viruelce. The role of the distinct expressed fimbriae in adherence and in initital intestinal infection has been cited, and fimbriated strains appear more viruelnt than non fimbriated strains (Walker *et al.*, 2007, Pegues & Miller, 2010). Another factor is its ability to traverse and invade intestinal mucosa mediated by type III secretion system (T3SS), which is encoded within Salmonella pathogenicity island 1 (SPI-1 T3SS) (Walker *et al.*, 2007, Pegues & Miller, 2010). Entrotoxin produced by certain strains that cause gastroenteritis has been implicated as a significant virlence factor (Walker *et al.*, 2007). A second T3SS and

PhoP/PhoQ two-component regulatory system are required for intracellular adaptation and replication within host cells. In addition, flagella and fimbriae are also involved in accessing and utilizing the intracellular niche (Pegues & Miller, 2010).

1.10.1 Bacterial adhesion and role of bacterial adhesins in pathogenesis

The first and the most important step in colonization, establishing of successful infection and initiation of pathogenesis, is the adhesion of bacteria to the human mucosa. While epithelial mucosal surfaces exhibit many defence mechanisms to prevent adhesion, the colonizing bacteria have developed number of means to overcome these hurdles. One strategy is production of specific adhesive structures such as fimbriae assemblages which extend from the surface of bacteria and frequently facilitate attachment to host cells (Giannella, 1996, Soto & Hultgren, 1999).

In general, the surface-exposed macromolecules involved in bacterial adhesion are referred to as adhesins. However, this is a generic designation describing different extracellular structures, ranging from surface polysaccharides to a number of monomeric or polymeric proteinaceous surface appendages secreted by different export pathways. In many instances, adhesins are assembled into and locate at tips of fimbriae. In other cases, the adhesins are directly associated with the microbial cell surface, so-called non-pilus adhesins or stand-alone adhesins (Soto & Hultgren, 1999). Bacterial genomic analysis shows that most bacteria have the potential to express surprisingly high numbers of distinct adhesin molecules, which mediate the binding of the bacterial cell to the epithelial extracellular surface through recognition and binding to specific receptor moieties (mostly carbohydrate compounds) of host cells during the pathogenesis process (Soto & Hultgren, 1999, Korea *et al.*, 2011).

1.11 Bacterial fimbrial adhesins and colonization

The words, fimbriae and pili, are interchangeable terms used to describe short hair-like, multi-unit peritrichous filamentous structures on the surfaces of many (particularly Gram-negative) bacteria (Edwards *et al.*, 2000). Fimbriae are only demonstrable with the electron microscope as these are much smaller structures than flagella (Duguid *et al.*, 1966). There are two types of pilus. A specialized type of pilus, the F or sex pilus, mediates transfer of DNA between mating bacteria. The smaller more numerous common pili are often involved in adherence of bacteria to surfaces in nature. Fimbriae are major determinants of bacterial virulence because they allow bacteria to colonise, interact and invade host tissues; and form biofilms on inanimate surfaces such as those associated with medical equipments. Type 1 fimbriae constitute the most common of these structures and are found on many Gram-negative bacteria in which these appendages have been shown to mediate adherence of the bacterium to the epithelial cells of the pharynx, intestine and bladder (Edwards *et al.*, 2000).

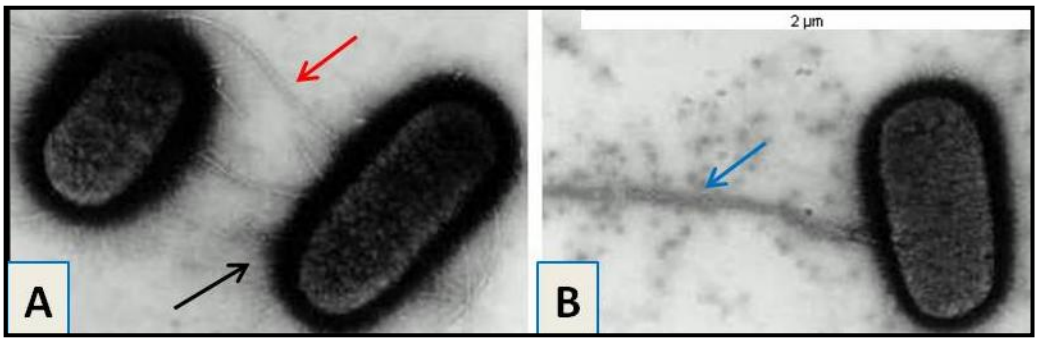

Figure 1.11.1: Electron micrograph of *E. coli* showing: A, The wavy flagella (red arrow) and numerous, short, thinner common pili (black arrow). B, The long sex pilus (blue arrow). Adapted from, the Medical Microbiology online book, 4th edition (Salton & Kim, 1996).

1.12 Types of fimbriae

Generally, different cells of the same clone within a culture population may carry from one up to 1000 fimbriae. These fimbrial appendages vary in length between 0.2 µm and approximately 20 µm. In addition, the width of these structures ranges between 30 and 140 Å, some having a diameter of up to 250 Å (Ottow, 1975). Fimbriae can be phenotypically classified into six groups (or types). Group 1 fimbriae function as adhesive organelles play an important role in the initial stages of many bacterial infections. These are peritrichously arranged and are typically present at between 100 to 300 copies per organism. Fimbriae of this group are further classified into four subtypes, as described by Duguid (1968), based on morphology, ability to haemagglutinate RBCs, mannose haemagglutination inhibition sensitivity and other specific adhesive characteristics (Ottow, 1975). Subtype 1 fimbriae, also called type 1 fimbriae, are peritrichously arranged, approximately 7 nm in width, and responsible for adhesive properties in general and haemagglutination in particular. Enterobacteriaceae with subtype 1 fimbriae are characterized by their ability to adhere to fungal and animal cells, including erythrocytes of the guinea pig and horse. However, this interaction is inhibited by D-mannose. Thus, these adhesion events are described as mannose sensitive (MS). Additionally, many studies have suggested that fimbrial phase variation, the ability of a bacterium to alternate between a fimbriated and non-fimbriated phase, is very common with this type of fimbriae (Clegg & Gerlach, 1987). Subtype 2 or type 2 fimbriae, are found in a few *Salmonella* serovars, but are thought to be mutants of subtype 1 fimbriae. They differ from subtype 1 in that they lack adhesive and haemagglutinating properties. However, these organelles morphologically resemble type 1 fimbriae (Clegg & Gerlach, 1987). Filaments of subtype 3 are specific to

Klebsiella spp. and *Serratia marcescens*. These fimbriae are antigenically and morphologically distinct from type 1 fimbriae. Typically, these structures are thinner (4 to 5 nm diameter), lack an axial hole when examined by electron microscopy and are more numerous. A key feature is the strong mannose resistant (MR) adhesiveness to fungal cells and the failure to mediate attachment to erythrocytes and animal tissue cells without prior tannic acid treatment (Clegg & Gerlach, 1987). Subtype 4 fimbriae, found on *Proteus* spp., are extremely thin (~4.0 nm) and peritrichously inserted filaments that present on the cells with MR haemagglutinating and adhesive features (Ottow, 1975). The subtypes 5 and 6 fimbriae described by Duguid (1968) include the non-flagellar filamentous structures observed on *Caulobacter*, the K88 filaments of *E. coli*, and the PF pili of *Pseudomonas aeruginosa*, which together form a group with functions similar to those of sex pili (Ottow, 1975). Group 2 sex pili are specialized filamentous appendages generally encoded by sex factors. These pili include the F pili and are thought to act as specific bridges during bacterial conjugation. Compared to the fimbriae, these filaments are rare as usually numbering not more than 1-10 per organism, in most cases longer and wider than fimbriae (Ottow, 1975). Group 3 filamentous appendages, are thick hollow tubes observed on *Agrobacterium* sp. and various unidentified soil bacteria. These appendages measure 400-600 Å wide and up to 3 µm long, and are attached to the cell surface as infundibulate structures. However, the nature and function of these structures need more investigation (Ottow, 1975). Group 4 fimbriae are flexible, rod-like pili arranged in a polar position on members of the genus *Pseudomonas* and *Vibrio*. These non-peritrichous fimbriae exhibit the ability to promote bacterial motion as opposed to acting as attachment organelles (Ottow, 1975). Group 5 fimbriae are attached at the bacterial poles and comprise contractible tubules. These structures have been observed on star-forming soil inhabitants such as *Pseudomonas*

rhodos, and *Rhizobium lupine* (Ottow, 1975). Group 6 fimbriae are found on cells of the Gram-positive bacterium *Corynebacterium renale* which are responsible for bovine pyelonephritis and cystitis. Neither haemagglutination nor pellicle formation could be detected with these latter structures (Ottow, 1975).

1.13 Phenotypic properties of fimbriated bacterial cells

1.13.1 The relationship between type 1 fimbriae expression and haemagglutinating ability of bacterial cells

Fimbriated bacteria expressing type 1 fimbriae were mainly found to strongly adhere to the surfaces of RBCs and bind them together in clumps (Duguid *et al.*, 1966). Consequently, bacterial cultures that contain type 1 fimbriated bacilli can be easily recognised by a simple haemagglutination test (Duguid *et al.*, 1966, Korhonen *et al.*, 1981). Fimbriae, particularly Enterobacteriaceae fimbriae, possess carbohydrate-binding activities and their major function is the binding to host epithelial glycoconjugates (glycolipids and glycoproteins). A number of these epithelial glycoconjugates fimbrial receptors had been characterized at the molecular level. The glycoprotein receptors for type 1 fimbriae, the most commonly occurring fimbrial type in Enterobacteriaceae, were detected on erythrocytes, consistent with the fact that type 1 fimbriae are defined by their ability to bind to the oligomannoside chains of glycoproteins (Kukkonen *et al.*, 1993). Accordingly, the attachment of bacteria to mammalian cells can be measured directly as binding of bacterial cells to receptors on mammalian cells or indirectly as induced haemagglutination of erythrocytes. However, Enterobacteriaceae bacteria were found to present two main patterns of agglutination: agglutination that is inhibited by D-mannose or MS haemagglutination and agglutination unaffected by D-mannose or MR haemagglutination. Mostly, fimbrial haemagglutinins (adhesins) are MS; especially the

MS type 1 fimbrial haemagglutinins. In contrast, non-fimbrial haemagglutinins are mainly MR (Korhonen *et al.*, 1981).

The fimbrial protein responsible for mannose binding was purified in 1988 and later identified as the product of the *fimH* gene (Korea *et al.*, 2011). FimH is the mannose-specific fimbrial adhesin subunit that binds specifically to target molecules such as sugars or glycolipids of host cells (Hori & Matsumoto, 2010). Considering that type 1 fimbriae of all Enterobacteriaceae bacteria share the property of mannose sensitivity, the gene(s) encoding the receptor-binding subunit may be more highly conserved (Gerlach *et al.*, 1989).

1.13.2 Pellicle formation of type 1 fimbriated bacteria in a standing culture

A feature of adhesiveness attributed to the fimbriated organisms especially those bearing type 1 fimbriae is the formation of a surface pellicle in an undisturbed, aerobic static liquid medium incubated for 48 − 72 hours. Many fimbriated bacteria were found to be capable of forming a thin layer of packed cells on the surface of the liquid medium (pellicle) as these bacteria gather at liquid-air interface either by adhesion or by flotation. The surface pellicle allows increased access of bacterial cells to atmospheric oxygen and thus fimbriated bacteria grow to higher population densities in aerobic static broth than non-fimbriated and non-pellicle forming bacteria. In a study, this culture characteristic belonged to bacteria such as *E. coli*, *S.* Typhimurium and *Shigella flexneri*, all of which also exhibited MS haemagglutination attributed to type 1 fimbriae (Ottow, 1975).

1.13.3 Fimbriae and its effect on hydrophobicity of bacteria

Some Enterobacteriaceae bacteria have been showed to be hydrophobic due to the presence of hydrophobic residues on their surfaces. Several structures contribute to the bacterial hydrophobicity including fimbrial subunits. This hydrophobic effect may help the organism to adhere to surfaces if the associating sites possess sufficiently high densities of apolar areas. There is also a little doubt that hydrophobic interactions contribute to initial adhesion of pathogens to tissues, leading to colonization and invasion. In addition, a correlation between hydrophobicity and virulence has been suggested as virulent strains were found to be hydrophobic, whereas avirulent strains lacked this trait (Doyle, 2000). Owing to surface hydrophobicity, fimbriated bacteria stick to both inorganic and organic surfaces better. It has also been demonstrated that *Salmonella* fimbriae may be involved in non-specific adhesion to mineral particles and adherence of *S.* Typhimurium to surface teguments of schistosomes may partly explain why schistosomiasis is a risk factor for certain *Salmonella* infections (Falkowski *et al.*, 1986, Clegg & Gerlach, 1987).

1.14 Assembly of fimbrial structures

Fimbriae are complexes consisting of several kinds of protein subunits. A single protein subunit called the major fimbrial subunit is stacked by hundreds and arranged into a helix to form a long rod-shaped body. The tip of the fimbriae contains a number of proteins capped by several different adhesin subunits. At least four distinct mechanisms have been put forward to explain the assembly of fimbrial adhesins which can form a base of its classification: (i) the chaperone/usher (C/U) pathway, (ii) the general secretion pathway, (iii) the extracellular nucleation-precipitation pathway and (iv) the alternate C/U pathway. Examples of fimbriae that constitute the best-characterized

systems for each assembly classification are as follows: P and type 1 fimbriae (C/U pathway), type IV pili (general secretion pathway), curli (extracellular nucleation precipitation pathway) and CS1 pili (alternate chaperone pathway) (Soto & Hultgren, 1999, Nuccio & Baumler, 2007).

1.14.1 Biogenesis of chaperone/usher fimbrial adhesins

The most common fimbrial gene clusters found in genomes of Gram-negative bacteria, with the exception of the atypical type IV pili, are associated to the C/U biogenesis pathway. The initial translocations of fimbrial subunits, that have signal peptides direct their transport across the cytoplasmic membrane to periplasm, occurs by the general secretory system. However, further export from the periplasm to the cell exterior is mediated by a specific two-component system consisting of two accessory proteins. These two proteins are a periplasmic chaperone and an outer membrane usher (Fig. 1.14.1). Basically, C/U biogenesis leads to an ordered non-covalent polymerisation of the periplasmic chaperone-bound subunits into an assembly platform composed by the outer membrane usher (Korea *et al.*, 2011). Subunits that constitute the fimbriae are initially bound by the chaperone protein in the periplasm by a mechanism called donor-strand complementation to allow for the correct folding of the subunits, thus preventing subunit–subunit interactions and the premature aggregation of these subunits. Fimbrial subunits possess an incomplete Ig-like fold missing the final antiparallel beta strand. In the periplasm space, each fimbrial subunit is bound by a chaperon inserts a donor beta strand in the parallel orientation thereby completing the Ig-like fold of the bound subunit. Then, the chaperone-subunit complexes in the periplasm are targeted to the outer membrane usher, which serves as assembly platform for translocation of fimbrial subunits across the outer membrane and the construction of the fimbrial shaft through a

donor-strand exchange process. This process is energised through a donor-strand exchange reaction that replaces the parallel donor beta strand of the chaperon with more energetically favourable antiparallel donor beta strand of another fimbrial subunit thereby connecting fimbrial subunits into a filamenmt that is anchored to the cell surface. In all fimbrial systems that have been studied, the genes encoding the chaperone, usher and major fimbrial subunit are located in the same operon (Edwards *et al.*, 2000, Korea *et al.*, 2011).

The majority of the characterized linear multi-subunit fimbriae exported through the C/U pathway are found in Enterobacteriaceae such as *E. coli* and *Salmonella enterica* serovars (Korea *et al.*, 2011). There are two types of adhesive structures assembled by the C/U pathway; homopolymers are composed of a single protein subunit (e.g., Dr fimbriae), while heteropolymers are composed of a few different protein subunits (e.g., type 1 and P fimbriae). The heteropolymeric organelles typically comprise a rigid fimbrial filament and a thin tip-fibrillum with the adhesin subunit at the filament tip. The structural fimbrial subunits of type 1 and P fimbriae in *E. coli* and *Salmonella* serovars are encoded by the best studied *fim* and *pap* gene clusters, respectively. The major fimbrial subunit of type 1 pili and P pili are FimA and PapA, respectively. An adhesin subunit, FimH in type 1 fimbria or PapG in P fimbria, is located at the distal end of each fimbria. These fimbriae are assembled through the C/U in a top-down fashion, with the adhesin subunit incorporated first (Fig. 1.8) (Piatek & Zalewska, 2005, Hori & Matsumoto, 2010). The fimbrial adhesin subunits FimH, PapGII, F17G, FedF, and CfaE are two-domain adhesins (TDAs), which consist of an N-terminal carbohydrate receptor-binding domain and a conserved C-terminal pilin domain. Both domains have Ig-like folds and are joined via a short interdomain linker.

However, the single-domain adhesins (SDA) with a single domain conferring receptor-binding properties can also assemble into thin and flexible fibers (De Greve *et al.*, 2007).

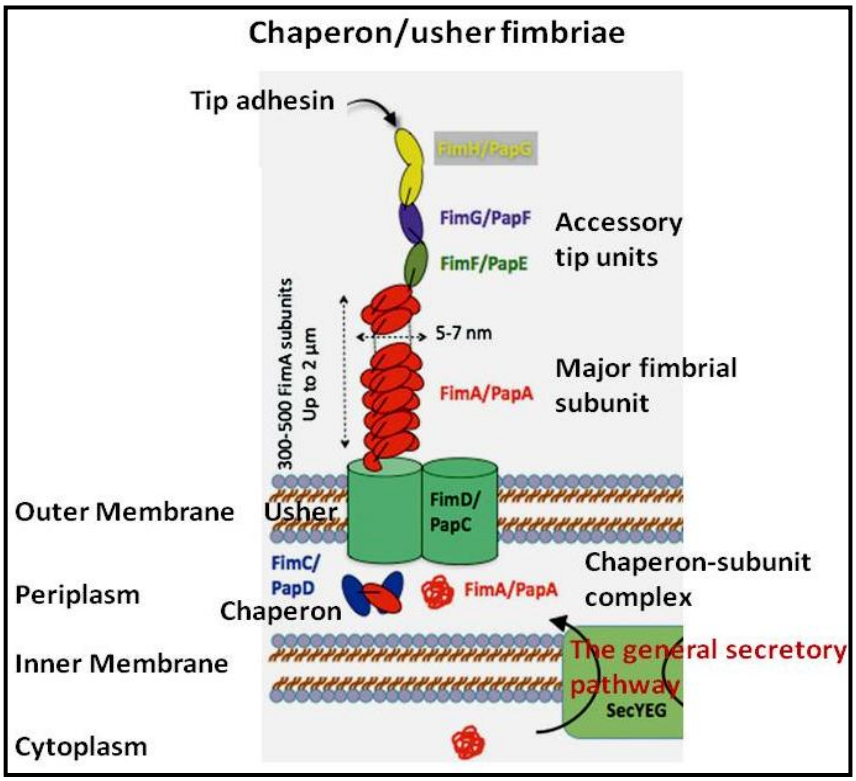

Figure 1.14.2: An overview of fimbrial adhesins assembly via the chaperon/usher pathway. The *E. coli* Fim and Pap systems are represented as examples. During fimbriae assembly, subunits (pilins) are secreted into the periplasmic space via the general secretory pathway and bind to a chaperone that assists in protein folding and prevents premature assembly of the subunits. The subunit/chaperone complex is then delivered to the outer membrane usher, which serves as a platform for fimbriae assembly. The usher protein forms a pore in the outer membrane, allowing the passage of individual pilins. Adapted from Korea et al. (2011).

1.15 Bacterial adhesins (fimbrial adhesins) as a candidate for vaccine development (Anti-adhesive vaccines)

Conventional antibiotics kill bacteria by interfering with vital cellular functions, like cell-wall synthesis or DNA replication, an approach that imposes selection pressure for emergence of resistant bacteria. However, targeting bacterial virulence functions directly is an attractive alternative approach. An obvious target is the bacterial adhesion to host surfaces mediated by cell surface adhesins (Kelly & Younson, 2000, Klemm *et al.*, 2010). Thus, bacterial adhesion is increasingly gaining attention as a target for preventing and treating major infections (Smyth *et al.*,1996). Consequently, bacterial adhesins have recently received considerable attention as potential vaccine candidates (Pointon *et al.*, 2010).

Anti-adhesive strategies aimed at blocking this interaction offer an attractive means of preventing infections at an early stage. In general, three classes of adhesion-blocking agents have been investigated: anti-adhesin antibodies, adhesin analogues and receptor analogues. The effectiveness of a number of these adhesion-blocking compounds has been demonstrated in human and animal models and these three types of anti-adhesion agents have protected against a variety of infections at mucosal surfaces (Kelly & Younson, 2000). However, the most appealing approach to inhibit bacterial adhesion is to block the adhesin receptor interaction by the production of adhesin-specific antibodies in the host after immunization with the adhesin molecule, which ideally would prevent bacterial adhesion and colonization (Klemm *et al.*, 2010).

Surface structures, such as LPS and fimbriae, were found to not only affect the virulence of the bacteria but were also key targets of the host immune system (Fierer & Guiney, 2001). Fimbrial adhesins have recently attracted the attention of vaccine

researchers (Smyth *et al.*,1996). Fimbriae are attractive candidates for epitope display for several reasons: (1) they are present in extremely high numbers at the cell surface, (2) they are strong immunogens, and (3) they possess natural adhesive properties (Zhang & Zhang, 2010). Many attempts have been made to make anti-adhesive vaccines using fimbrial adhesin molecules (Klemm *et al.*, 2010). The majority of fimbriae assisted peptide display work has been focused on the development of recombinant vaccines. While, vaccines based on wild type fimbriae have been highly successful. It is believed that vaccines inducing anti-adhesin immunity to block bacterial adherence to host receptors would provide broad-spectrum protection against broad range of pathogenic bacteria (Zhang & Zhang, 2010). Indeed, an anti-diarrhoea vaccine based on K88 (or F4) fimbriae from enterotoxigenic *E. coli* (ETEC) strains has been highly successful in piglets. Van den Broeck et al. (1999) used experimental oral vaccines carrying the entire purified F4 fimbriae, which play an important role in the pathogenesis of ETEC, a major cause of diarrheal disease in humans and animals. Protection was conferred against colonization by ETEC strains expressing the same or homologous adhesins. These adhesins were strong antigens for oral and systemic immunizations in piglets. Oral administration of purified F4 was found to stimulate the intestinal mucosal immunity as it induced antigen-specific antibody-secreting cells in the Peyer's patches, mesenteric lymph nodes, blood and lamina propria after immunisation. Therefore, the F4 antigen was considered as an important component in the development of oral vaccines against ETEC infections (Van den Broeck *et al.*, 1999).

Vaccination with the adhesin subunit of fimbriae alone has also been successful, particularly when infection is dependent on a specific interaction between host receptors

and bacterial adhesins (Xia *et al.* 2000, Piatek & Zalewska, 2005). One such example was the attempt to make a vaccine against cystitis based on the adhesion moiety of type 1 fimbriae (FimH adhesin) of uropathogenic *E. coli* (Klemm *et al.*, 2010). The immunisation of animal models with this adhesin subunit demonstrated that antibodies specific to the FimH adhesin could successfully block uropathogenic *E. coli* colonizing the bladder in mice and monkeys models of urinary tract infection (Piatek & Zalewska, 2005, Xia *et al.*, 2000). Hence, available data support the notion that fimbrial adhesins are good immunogens both in the context of live vaccines and as purified, native or recombinant proteins (Klemm & Schembri, 2000). In addition, the most recent Ghosh et al. (2011) study showed that a 27-kDa T2544 adhesin protein of *S.* Typhi is strongly immunogenic. T2544-specific antiserum enhanced *in vitro* uptake and clearance of *Salmonella* by macrophages and augmented complement-mediated lysis, indicating a contribution of T2544-specific antibodies to the killing process. In addition, the mice immunised with recombinant T2544 or passively with T2544 antiserum were protected against subsequent bacterial challenge. As T2544 is widely distributed and conserved in different *Salmonella* serovars, this protein adhesin qualifies as a potential broad-range *Salmonella* vaccine candidate (Ghosh *et al.*, 2011).

1.16 *Salmonella* fimbrial adhesins

The existence of fimbrial structures on the surface of *Salmonella* has been recognized for many years. Three main types of fimbriae have been characterized in *Salmonella* serovars based on their morphology and erythrocytes agglutination ability. Rigid type 1 fimbriae that mediate MS agglutination of erythrocytes are ubiquitous among *Salmonella* serovars. Type 2 fimbriae, which are morphologically similar to type 1 fimbriae but lack the ability to agglutinate erythrocytes, have only been found on a few

serovars. In addition, the smaller and more flexible type 3 fimbriae, which mediate the agglutination of tanned animal erythrocytes in the presence of D-mannose, have been found on a few serovars (Thorns *et al.*, 1990).

Edwards et al. (2000) study of the role of *Salmonella* fimbriae in *in vivo* infections of mice has characterised the function of fimbriae in intracellular infection. This study indicated that fimbriae are essential for *Salmonella* systemic infections after the initial colonization of host epithelial surfaces (Edwards *et al.*, 2000). The *in vivo* biology of some serovars such as Paratyphi A is difficult to study because of the lack of an animal model that replicates human infection. However, in a recent microarray-based transcriptional analysis (SCOTS and SCOTS/microarray technologies) to identify *S.* Paratyphi A transcripts expressed by bacteria in the blood of three naturally infected bacteraemic humans in Bangladesh. From the set of transcripts, forty-three expressed genes were located within known *Salmonella* pathogenicity islands including SPI 1-4, 6, 10, 13, and 16. SPI-1 encodes a T3SS, via its *sip* and *spa* gene clusters, which is involved in intestinal epithelial cell invasion as well as eukaryotic cell invasion outside of the intestinal epithelium. In addition, SPI-6 encodes a number of fimbrial proteins involved in adherence and virulence in a number of *Salmonella* animal models. Transcripts of *tcfBC* and *safC* which encode fimbrial proteins were also identified. Detection of fimbrial proteins in blood samples of infected patients further supports the proposal fimbrial structures play a role at various stages of *Salmonella* infection (Sheikh *et al.*, 2010).

In Harris et al. (2006) study, immunogenic bacterial proteins expressed during human infection with serovar Typhi were identified in the sera of infected individual using immunoscreening techniques. Proteins implicated in fimbrial structures and

biogenesis, were among 25 proteins identified with potential roles in the pathogenesis of *S. enterica.* This was the largest functional category recognised and included six fimbrial antigens derived from three separate fimbrial operons, *tcf, stb,* and *agf*. Of these fimbrial antigens, TcfB, the major structural subunit of fimbrial *tcfABCD* operon, is found in serovar Typhi but is not extensively distributed in other serovars. Specific serum anti-TcfB IgG responses were also documented in patients with Typhi bacteremia. This further highlights the significance of fimbrial antigens both in the pathogenesis of *Salmonella* infections and in host innate and adaptive immune responses to this pathogen. In addition, the *tcf* operon is found in serovar Paratyphi A, which like Typhi organisms, are restricted to human hosts. This distribution of Tcf fimbriae has also been suggested as a possible contributing factor towards the restricted host range of these two typhoidal *Salmonella* serovars (Harris *et al.*, 2006).

According to Bronowski and Winstanley (2009) study where Suppression Subtractive Hybridization (SSH) was used to identify 41 sequences within the accessory genome of an invasive strain of *S.* Heidelberg from Malawi, six of the SSH sequences were within fimbrial operons. The *tcf* operon, potentially associated with the host specificity of *S.* Typhi, and the *stk* operon reported in *S.* Paratyphi A, were both present in either all (*tcf*) or most (*stk*) isolates of *S.* Heidelberg examined. However, both of these operons had restricted distributions among the other serovars tested. These observations were consistent with the idea that fimbrial gene clusters make a major contribution to the accessory genome of *Salmonella* serovars. The *stk* gene cluster had been reported to be specific for *S.* Paratyphi A, but based on analysis of the distribution of SSH sequences *S.* Heidelberg and *S.* Kentucky were also shown to harbour the *stk* cluster. However, limited reverse transcription PCR data suggest that this operon may

not be carried by all *S.* Heidelberg isolates as it has also been shown that *stkD* expression could only be detected in some but not all *S.* Heidelberg isolates tested. Given the proven role of fimbriae in *Salmonella* pathogenicity, the variable carriage of this (*stk*) fimbrial operon, and the variable expression even among those strains carrying the gene, further investigation of the *stk* operon is indicated (Bronowski & Winstanley, 2009). Furthermore, a greater understanding of the nature and types of these surface structures expressed by different *Salmonella* serovars can be valuable for the development of new diagnostic assays and for the identification of protective antigens for use in future vaccines (Thorns *et al.*, 1990).

1.16.1 *Salmonella enterica* fimbrial operons

Based on analysis of the available genome sequences of various *S. enterica* serovars, at least 15 operons predicted to encode fimbrial adhesins have been identified. In addition, the most common fimbrial operons found in these genomes belong to the C/U assembly class. Each of these operons is composed of 3 to 10 genes. Comparative genomics and microarray studies have shown that most serovars are characterised by a unique combination of these putative fimbrial operons (Table 1.16-1) (Townsend *et al.*, 2001, Edwards *et al.*, 2000, Porwollik & McClelland, 2003). For example, *S.* Typhimurium LT2 has eleven different chromosomal fimbrial operons, *bci, sti, stf, saf, stb, fim, stc, std, lpf, stj* and *sth*, and an additional plasmid encoded fimbrial locus, *pef*. Functionally, it was found that the existence of at least four of these clusters is important for its virulence. *S.* Typhi CT18 has a subset of twelve C/U fimbrial operons and the *agf* locus which encodes a type IV fimbrium. Some of these fimbrial operons are distributed between different *S. enterica* serovars while others are not. Fimbrial operons specific for one or a limited number of serovars include *stl* (Dublin) and *stk* (Paratyphi A)

(Porwollik & McClelland, 2003). Edwards et al. (2002) suggested that broadly distributed fimbrial operons provide general adhesive functions but fimbrial operons whose distribution is limited may provide serovar-specific functions that have yet to be defined (Edwards *et al.*, 2000).

Table 1.16-1: Distribution of the fimbrial operons in different *Salmonella* serovars. Adapted from Edward et al. (2002).

Fimbrial operon[1]	Dublin	Enteritidis	Partyphi	Typhi	Typhimurium
agf	+	+	+	+	+
bcf	+	+	+	+	+
fim	+	+	+	+	+
lpf	+	+	-	-	+
pef	-	+	-	-	+
saf	+	+	+	+	+
sef	+	+	+	+	-
sta	-	-	-	+	-
stb	+	+	+	+	+
stc	+	+	+	+	+
std	+	+	+	+	+
ste	+	+	+	+	-
stf	+	+	+	-	+
stg	-	-	-	+	-
sth	+	+	+	+	+
sti	-	+	-	-	+
stj	-	-	-	-	+
stk	-	-	+	-	-
stl	+	-	-	-	-
tcf	-	-	+	+	-

[1]Distribution of number of predicted fimbrial clusters according to genome comparisons study of the closely related *Salmonella enterica* serovars (Edwards *et al.*, 2002).

Genetic differences that could explain variation in the host ranges of different *Salmonella* serovars have been only partly explained. A few virulence factors, such as fimbrial structures, known to differ between serovars Typhimurium and Typhi, were

found to be involved in early stages of infection. *S.* Typhimurium contains several different fimbrial biosynthetic operons that are absent in *S.* Typhi and data exists which seems to link serovar-specific fimbrial repertoires with host-adaptation. For example, the *lpf*-encoded long polar fimbriae appear to mediate tropism of serovar Typhimurium towards mouse small intestinal Peyer's patches, facilitating the preferential invasion of Peyer's patch-embedded M cells by Typhimurium over Typhi (Emmerth *et al.*, 1999). Thus, the varying distribution of fimbrial operons is likely to reflect adaptation of various serovars to different niches (Humphries *et al.*, 2003). However, the viewpoint that a simple correlation exists between hosts ranges of *Salmonella enterica* serovars and the presence of specific fimbrial operons needs more investigations (Edwards *et al.*, 2000).

1.17 Phase variation

1.17.1 Antigenic variation

Microorganisms have developed different mechanisms for avoiding destruction and producing a balanced host–pathogen interaction. One strategy is to evade the host immune system by antigenic variation, which is defined as the capacity of an infecting organism to alter the proteins displayed to the host immune system over the course of an infection. The term antigenic variation is generally used to include both phase variation, the on–off expression of a particular antigen, and true antigenic variation, the expression of alternative forms of a particular antigen (Deitsch *et al.*, 2009).

1.17.2 Phase variation and its biological significance

A simple form of antigenic variation arises when a reversible switch triggers one of two alternative phenotypes (phases) or 'on' and 'off' states, resulting in variations in the

level of expression of one or more proteins between individual cells of the same clonal population (van der Woude & Baumler, 2004). Phase variation is hypothesized to help the bacterium to evade the host immune system and to cope with different environmental conditions and recurrent infections. This view is supported by the fact that mostly the structures that are known to exhibit phase variation are on the cell surface, where these would be exposed to the immune system (van der Woude & Baumler, 2004, Deitsch *et al.*, 2009).

Bacterial phase variation and an antibody immune selection by the host of escape variants constitute an interesting model system to reflect host-pathogen co-evolution (Brunham *et al.*, 1993). Phase variation in bacteria was first noted with the expression of flagella in *Salmonella* and pilin expression in *Moraxella bovis*, where variations were found to be governed by DNA inversion mechanism. However, these variations occur reversibly in nature at relatively low-frequency events (Brunham *et al.*, 1993, Deitsch *et al.*, 2009). The most studied organisms that undergo phase variation are the pathogenic *Neisseria* spp. These species produce surface proteins that mediate adherence to host cells and direct tissue tropism, in particular pili and the Opa proteins. Phase variation mechanisms in these bacteria involving slipped-strand mispairing (SSM) during DNA replication and gene conversion, has provided a theoretical framework for understanding of the mechanisms responsible for phase variation in other prokaryotes (Stern & Meyer, 1987, Deitsch *et al.*, 2009).

1.17.3 Molecular mechanisms of phase variation

There are two general types of mechanisms creating the antigenic or phase variation in microorganisms: genetic and epigenetic. Genetic actions, occurring in the form of

mutation and recombination, change the DNA sequence of an antigen-encoding gene or its regulatory elements, thereby altering the level of expression of the gene or the amino acid sequence of its product. In contrast, epigenetic mechanisms affect the expression of a gene and are often associated with DNA modifications, in particular DNA methylation, without alteration of primary nucleotide sequence. Both genetic and epigenetic mechanisms occur at specific loci and are readily reversible (Deitsch *et al.*, 2009).

1.17.3.1 Phase variation through transcriptional regulation

Phase variation can be regulated at the level of transcription of the gene product through SSM of DNA short sequence repeats. Multiple adjacent repeats of DNA sequence also referred to as simple sequence repeats (SSRs) can be subjected to expansion or contraction of the number of unit repeats as they are subjected to SSM mutations during either DNA replication or DNA repair. During DNA replication, SSRs can 'slip', leading to changes in the number of repeats and consequent changes in the transcription of the gene product. This is most likely to happen when repeat sequences are located in the promoter region between -10 and -35 RNA polymerase binding sites. For example, genes encoding subfamilies I (*tprC, tprD, tprF* and *tprI*) and II (*tprE, tprG* and *tprJ*) of the *tpr* family in *Treponema pallidum* have homopolymeric guanosine (poly-G) repeats of lengths 7 bp to 12 bp immediately upstream of the transcriptional start sites. Loci with poly-G tracts of 8 or fewer Gs are transcribed efficiently, whereas transcription of genes with longer poly-G tracts is reduced by ~95 – 100 % (Fig. 1.17.1A). Changes in the number of G repeats are thought to occur through SSM during DNA replication. In addition, phase variation can be regulated at the transcription level through epigenetic changes, such as the transcription of *pap* operon of UPEC which is regulated by

methylation of specific sites in the regulatory region of the locus (van der Woude & Baumler, 2004, Deitsch *et al.*, 2009, Kumar *et al.*, 2011).

1.17.3.2 Phase variation through translational regulation.

Phase variation can also occur at the translational level in pathogens by different mechanisms, including SSM, early ribosome dissociation and mRNA instability. Translation of a protein can be affected by SSM if the tandem repeats are located within its coding sequence leading to disruption of the open reading frame and synthesis of a non-functional and typically truncated protein due to a premature stop codon. Phase variation regulated at the level of translation was first described as a consequence of SSM of CTCTT repeat units in the *opa* genes of *Neisseriae* (Fig. 1.17.2B) (Stern & Meyer, 1987, van der Woude & Baumler, 2004, Deitsch *et al.*, 2009, Kumar *et al.*, 2011).

1.17.4 Phase variation of fimbriae

Most of the genes that are putatively thought or known to undergo phase-variable expression encode surface-exposed proteins like fimbrial antigens or proteins that modify or regulate surface proteins. Phase variation of fimbriae is regulated mostly by transcription regulation mechanisms originating at the major promoter of the operon, resulting in variable (on/off) expression of most or all genes in the fimbrial operon, as with phase variation of *hif* encoded fimbriae in *H. influenza.* Phase-variable type 1 fimbriae are common in most *E. coli* isolates. In addition, phase variations of the structural subunits and phase-variable modification have been described in type IV pili. In *Salmonella*, phase-variable expression has been identified for Pef, Ipf, and Fim fimbriae (van Ham *et al.*, 1993, Clegg *et al.*, 1996, van der Woude & Baumler, 2004).

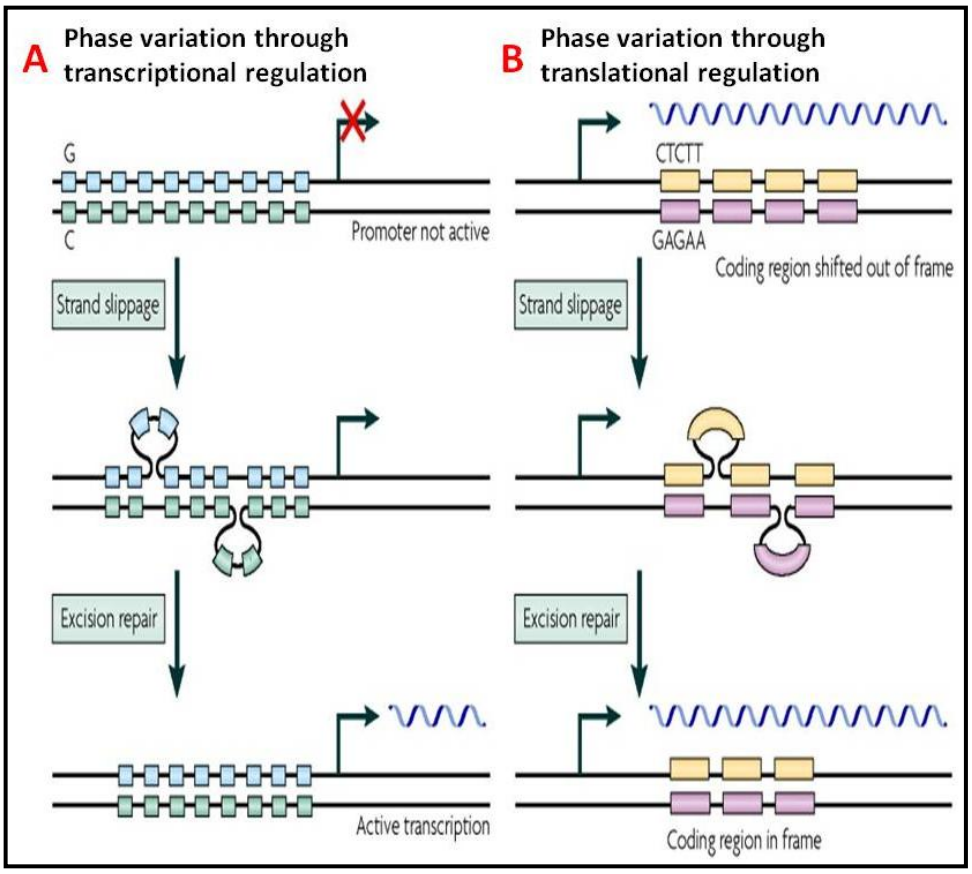

Figure 1.17.3: Schematic diagram of phase variation through slipped-strand mispairing. During DNA replication, tandem DNA repeats can 'slip' leading to changes in the number of repeats and consequent changes in the transcription or translation of the gene product. **A,** Some *tpr* genes of *Treponema pallidum* are regulated at the level of transcription through changes in the number of Gs found upstream of the transcription start site; **B,** Similar mispairing of CTCTT repeats in the open reading frame can regulate Opa expression in *Neisseria* spp. at the level of translation. Adapted from Deitsch et al. (2009).

1.18 Aim of the work

Vaccines offer a safe, successful, and cost-effective tool for protection against pathogenic microorganisms (Yang *et al.*, 2011). However, one major disadvantage of the commercially available typhoid vaccines is that they do not provide significant protection against other *Salmonella* serovars, especially the emergence of *S.* Paratyphi A infections (Fangtham & Wilde 2008, Sheng *et al.*, 2011). Therefore, the development of an effective vaccine is now a major priority. While, a better understanding of the nature and types of surface structures, such as fimbriae, expressed by *Salmonella* genus will be valuable for developing new diagnostic assays and for identification of protective antigens as potential vaccines (Thorns *et al.*, 1990). Given that several *Salmonella* fimbrial adhesins have been shown to be involved in the initial adhesion step of pathogenesis and that fimbriae are frequently abundant on the cell surface and hence exposed to the immune system. In addition, production of anti-adhesin antibody in the host to obstruct bacterial adhesion is a recent approach for developing vaccines against bacterial infections (Soto & Hultgren, 1999). This project aimed to characterise the putative *Salmonella stk* fimbrial operon and the Stk fimbrial structure encoded by this operon as well as to develop a recombinant StkF, putative adhesion protein, based vaccine against *Salmonella* infections, which would appear to be a good candidate as immunogen.

Aims:

- PCR profiling of *Salmonella* serovars to investigate carry of the *spa0180* (*stkF*) gene which encodes a putative fimbrial adhesin protein, and/or the whole *stk* specific fimbrial gene cluster (*stkABCDEFG*) of *S.* Paratyphi A ATCC 9150.

- Isolation of the *stkF* gene from *S.* Paratyphi A SARB 42, cloning of *stkF* into an expression vector, expressing and purifying recombinant StkF protein. This component of my project mapped perfectly to a request of my sponsor, the Egyptian Government, to acquire specific experience and skills in the production of recombinant biomolecules.

- Bioinformatics analysis of the *stk* fimbrial operon, and heterologous expression of the Stk fimbriae in *E. coli* HB101 for phenotypic and functional characterisation.

- Generation of polyclonal antibody against recombinant StkF and testing immunogenicity in mice to assess its efficacy as a potential vaccine against *Salmonella* serovars carrying this antigen. In addition, the protective efficacy of the mouse anti-StkF antibody was assessed by different *in vitro* assays.

- PCR amplification, cloning and expression of StaF, a *S.* Typhi-specific protein, and SipA, a protein encoded by all genomically characterized *Salmonella* serovars, and evaluation of the diagnostic use of recombinant StkF, StaF and SipA proteins for sero-diagnosis of different *Salmonella* serovars infections using an ELISA to detect antigen-specific antibodies in serum samples from human patients.

Chapter Two

Materials and Methods

2 Material and Methods

2.1 Organisms, media and growth conditions

All bacterial strains used in this study were routinely grown at 37°C on Luria Bertani agar (LA) plates aerobically. Routine growth in liquid culture was carried out in 5 ml Luria Bertani broth (LB) using 20 ml tubes and incubated at 37°C in a rotary shaking incubator at 200 rpm. Media was supplemented with antibiotics as indicated in the text and in Table 2.2−1.

2.2 Bacterial strains, plasmids and genomic DNA

Non-typhoidal *Salmonella* and *E. coli* strains; and plasmids that were used for this work are listed in Table 2.2−1. In addition, important properties and the sources of these are listed.

Table 2.2-1: List of bacterial strains and plasmids

Strains[1] and Plasmids	Relevant characteristics	Resistance	Source or reference
***E. coli* strains**			
Top10	F– mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15ΔlacX74 recA1 araD139Δ(ara leu)7697 galU galK rpsL (StrR) endA1 nupG		Invitrogen®
DH5α	F–Φ80lacZΔM15Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoAsupE44 λ– thi-1 gyrA96 relA1		Lab 212
BL21(DE3)	F– ompT hsdSB (rB–, mB–) gal dcm (DE3)		Invitrogen®

BL21(DE3) pLysS	F– ompT hsdSB (rB–, mB–) gal dcm (DE3) pLysS	Cm^R	Invitrogen®
HB101	F^- mcrB mrr hsdS20(r_B^- m_B^-) recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20glnV44 λ^-		(1)
Typhoidal and non-typhoidal *Salmonella* strains[2]			LRI (2)
***S.* Paratyphi A strains[3]**			HPA (3) & LRI
Vectors and Plasmids			
pCR®4-TOPO®	High copy number (HCN) PLAC *lacZα*, TOPO-TA Cloning vector	Kan^R & Amp^R	Invitrogen®
pBluescript II KS(+)	HCN PLAC *lacZα* vector	Amp^R	Stratagen®
pGEM®-T Easy	HCN T7and SP6 RNA polymerase *lacZα*,TA Cloning vector	Amp^R	Promega®
pET-23a(+)	low copy number (LCN) T7 promoter expression vector contains C- terminal His-tag coding sequence	Amp^R	Novagen®
pET-28a	low copy number (LCN) T7 promoter *LacI* expression vector His Tag coding sequence (C and N terminal)	Kan^R	Novagen®
pUCP-20	(HCN) PLAC *lacZα*, general cloning vector	Amp^R	(4)
pTstkF	TOPO plasmid harbouring full length of *stkF* gene with parts of flanking genes amplified from *S.* Paratyphi A (SARB 42) using stkF-F and stkF-R primers, prepared by TA cloning	Kan^R & Amp^R	This study
pBstkF	pBlueScript KS(+) plasmid harbouring full length of *stkF* gene with parts of flanking genes prepared by restriction digestion from	Amp^R	This study

	pTstkF plasmid		
pGEMstkF	pGEM-T easy vector harbouring full length of *stkF* gene only amplified from *S.* Paratyphi A SARB 42 by primers PStkF-F and PstkF-R and prepared by TA cloning	Amp^R	This study
pGEMsipA	pGEM-T easy vector harbouring full length of *sipA* gene amplified from *S.* Paratyphi A (SARB 42) by primers sipA-F & sipA-R, prepared by TA cloning	Amp^R	This study
pGEMstaF	pGEM-T easy vector harbouring full length of *staF* gene amplified from *S.* Typhi (Ty2) by primers staF-F & staF-R, prepared by TA cloning	Amp^R	This study
pGEMstkFOff	pGEM-T easy vector harbouring $stkF^{211}$ gene (OFF form) amplified from *S.* Paratyphi A strains PA42 by primers PstkF-F and PstkF-R, prepared by TA cloning	Amp^R	This study
pGEMstk	pGEM T easy vector harbouring whole *stk* operon carries $stkF^{159}$-ON form amplified from *S.* Paratyphi A SARB 42 by primers stk-F & stk-R, prepared by TA cloning	Amp^R	This study
pGEMstkOff	pGEM T easy vector harbouring whole *stk* operon carries $stkF^{211}$-OFF form amplified from *S.* Paratyphi A PA42 using primers stk-F and stk-R, prepared by TA cloning	Amp^R	This study
pETStkF	pET23a expression vector harbouring full length of *stkF* gene (600 bp) prepared by restriction double digestion from pGEMStkF clone	Amp^R	This study
pETStkFOff	pET28a expression vector harbouring full length of $stkF^{211}$ gene (OFF form) prepared by restriction double digestion pGEMstkOff clone	Kan^R	This study
pETSipA	pET28a expression vector harbouring full length of *sipA* gene (2055 bp) prepared by restriction double digestion from pGEMSipA	Kan^R	This study

pETStaF	pET28a expression vector harbouring full length of *staF* gene (597 bp) prepared by restriction double digestion from pGEMStaF	Kan^R	This study
pUCstk	pUCP20 vector harbouring whole *stk* operon (~7.2 kb) carries $stkF^{159}$-ON form prepared by restriction double digestion from pGEMstk	Amp^R	This study
pUCstkOff	pUCP20 vector harbouring whole *stk* operon (~7.2 kb) carries $stkF^{211}$-OFF form prepared by restriction double digestion from pGEMstkOff	Amp^R	This study

[1]Bacterial and transformed *E. coli* strains with these plasmids were stored in 30 % glycerol at – 20°C and – 80°C.**[2]**59 different *Salmonella* starins (47 different serovars), 11 standard typhoidal strains and 48 clincal NT isolates; Takoradi (2), Typhi (8), Typhimurium (2), Enteritidis (2), Schwarzengrund (2) Montevideo (2).**[3]**121 *S.* Paratyphi A isolates were analysed for the presence of the ON and OFF forms of *stkF* gene. These isolates were obtained from LRI (13 isolates) and HPA (107 isolates). The available epidemiological information is that 102 strains originated from South East Asia (India, Pakistan, Nepal, Bangladesh, Cambodia and Indonesia), 5 from the Middle East (Kuwait and Bahrain) and 2 from Africa (Kenya); 43 strains were blood culture isolates, 17 stool isolates and one isolate from urine.**[3]**All NT strains are stool isolates except *S.* Corvallis. **1**, HB101 strain was obtained from Department of Genetics, University of Leicester; **2**, Non-typhoidal *Salmonella* strains were obtained from Department of Clinical Microbiology, Leicester Royal Infirmary, University Hospitals of Leicester, Leicester, UK; **3**, *S.* Paratyphi A strains were provided by *Salmonella* Reference Unit, Health Protection Agency (HPA), Colindale and the Department of Clinical Microbiology, Leicester Royal Infirmary (LRI), University Hospitals of Leicester, Leicester, UK; **4**, West et al. (1994).

I - DNA-related techniques and methods

2.3 Genomic and plasmid DNA extraction

2.3.1 Genomic DNA extraction

Standard Typhoidal *Salmonella* strains (7) extracted genomic DNA was obtained from Leicester Royal Infirmary, Leicester, UK. The genomic DNA of these strains was prepared using QIAamp mini kit (QIAGEN) in 2008 by Dr Richard Spence. Non-typhoidal *Salmonella* strains originated as routine clinical isolates obtained at the LRI over three years (2008 – 2010). The genomic DNA from these was extracted using ArchivePure DNA Cell/Tissue Kit (5 PRIME- 2300820) following manufacturer's instruction protocol for isolation of genomic DNA from Gram-negative bacteria. Firstly, 500 µl of each bacterial overnight culture was added to a microfuge tube on ice and centrifuged at 13,000 – 16,000 ×g for 20 sec to pellet cells. The supernatant was carefully removed by pipetting and 300 µl of cell lysis solution added and pipetted up and down until cells were suspended. The re-suspended pellet was incubated at 80°C for 5 min to lyse the cells. Following the complete lysis of cells, 1.5 µl RNaseA solution (4 mg/ml) was added and the tube was inverted 25 times for mixing followed by incubation at 37°C for 15 – 60 min. The sample was then cooled to room temperature and 100 µl of protein precipitation solution added. The protein precipitation solution was mixed uniformly with the cell lysate by vigorous vortexing at high speed for 20 sec, followed by centrifugation at 13,000 – 16,000 ×g for 3 min until the precipitated protein formed a tight pellet. The supernatant containing DNA was transferred into a clean microfuge tube containing 300 µl of 2-propanol 100 % (Sigma, UK) to trigger DNA precipitation. The sample was mixed by gentle inverting for 50 times. After

centrifugation at 13,000 – 16,000 ×g for 1 min, the DNA was visible as a small white pellet. The tube was inverted and drained on clean absorbent and allowed to 5 – 10 min air dry. Finally, 50 µl of DNA hydration solution was added and the sample incubated in this hydration solution for 1 h at 65°C and/or overnight at room temperature. Genomic DNA samples were labelled and stored at – 20°C for long-term storage.

2.3.2 Plasmid DNA extraction

Plasmid DNA (vector and recombinant plasmid) was extracted from transformed bacterial cells using Wizard® *Plus* Miniprep DNA purification kit (Promega- A7510) following manufacturer's instructions and taking in consideration special precautions for low copy number plasmids and *endA*$^+$ *E. coli* strains. Firstly, transformed bacteria were grown overnight in 5 ml (for high copy number plasmids) or 10 ml (for low copy number plasmids) of LB broth containing selective antibiotic at 37°C with shaking at 200 rpm. On the next day, bacterial cells were centrifuged at 1,400 ×g for 10 min and cell pellet was completely re-suspended in 250 µl of cell re-suspension solution (50 mM Tris-HCl (pH 7.5), 10 mM EDTA and 100 µg/ ml RNase). Cell suspension was lysed by incubation with 250 µl of Cell Lysis solution (0.2 M NaOH and 1 % SDS), inverted four times and left for 1 – 5 min. The endonucleases and other released proteins from the lysed *E. coli* cells were inactivated by adding 10 µl of alkaline protease to the sample, which inverted 4 times and incubated for more 5 min. Cell lysate was then neutralized by adding 350 µl of neutralization solution (4.09 M guanidine hydrochloride, 0.759 M potassium acetate and 2.12 M glacial acetic acid) and the tubes were inverted 4 times to mix. Alternatively, if using an *endA*+ strain, 700µl of neutralization solution was added, mixed by inverting the tube 4 times and incubated at room temperature for 10 min. At this stage, a white precipitate of protein was formed that was separated from the DNA-

containing supernatant by centrifugation at maximum speed for 10 min. The clear supernatant without any white precipitate contaminant was carefully transferred into the spin column. The column was centrifuged for 1 min at maximum speed to allow DNA binding. Column was washed twice using 750 µl and 250 µl of column wash solution (162.8 mM Potassium acetate, 22.6 mM Tris-HCl, pH 7.5 and 0.109 mM EDTA pH 8.0), diluted with 95 % ethanol with centrifugation at maximum speed for 1 min. Then the column was centrifuged for 2 min at maximum speed to remove any remaining of wash buffer. Finally, the spin column was transferred to clean 1.5 ml microcentrifuge tubes where bound DNA was eluted by adding 50 µl of nuclease-free water to centre of the membrane of spin column and centrifuged at maximum speed for 1 min. Plasmid DNA at this stage was ready for downstream applications and was stored at – 20°C for long-term storage.

2.4 DNA concentration determination

DNA concentration was determined using a NanoDrop 8000 (ThermoFisher, UK), or approximately quantified by comparison to a known DNA standards (Generuler and λ-*Hin*dIII ladders (Fermentas, UK) run along samples in agarose gels.

2.5 Polymerase Chain Reaction (PCR)

PCR is a powerful tool used to amplify certain fragments of DNA *in vitro* in a reasonably short time. In order to amplify certain sequence from DNA template (genomic DNA, plasmid or bacterial colony-derived crude DNA), set of two oligonucleotides or primers (forward and reverse primers) is required. The forward primer will bind to a specific sequence on the anti-sense strand while the reverse primer will bind to a specific sequence on the sense strand through complimentary bases. In

PCR, template is incubated with specific molar ratio of the primers and deoxynucleotide triphosphates (dNTPs) in the presence of optimised molar concentration of $MgCl_2$ that is required for DNA polymerase activity. The standard PCR reaction consists of 5 steps. The initial denaturation step in which complimentary DNA strands separate from each other by high temperature. The second is cycle denaturation step. The third step is primers annealing step, where primers bind specifically to DNA strands. The fourth is cycle elongation step, in which new DNA strands are being synthesized by thermophillic DNA polymerase. The fifth step is the final elongation step to allow complete synthesis of DNA strands. The 2nd, 3rd and 4th steps are repeated from 30 – 35 times to allow exponential amplification of the sequence of interest, as in each cycle the newly synthesized DNA strands serve as templates for synthesis of new strands.

2.5.1 PCR reactions and conditions

PCR products in this study were generated by using GoTaq® DNA polymerase, KOD Xtreme Hot Start DNA high fidelity polymerase and Extensor Hi-Fidelity PCR enzyme mix, as directed by the manufacturer instructions (Fig. 2.5.1). The PCR reactions were prepared on ice in total volumes of 20 or 50 µl. PCR reactions using using GoTaq® DNA polymerase contained 0.4 pmol of each primer and dNTPs (Bioline, UK) at a final concentration of 0.2 mM. The Extensor Hi-Fidelity PCR enzyme mix is a blend of Thermoprime *Taq* DNA polymerase and thermostable proofreading DNA polymerase. The two enzymes form a mix act synergistically to amplify DNA templates with four times higher fidelity than standard *Taq* DNA polymerase. The KOD Xtreme™ Hot Start DNA Polymerase kit (Novagen, 71975) is an optimized PCR system for the amplification of long or GC-rich DNA templates. KOD Xtreme Hot Start DNA

polymerase quickly and accurately amplifies genomic and phage/plasmid DNA targets up to 24 and 40 kbp, respectively.

2.5.2 PCR oligonucleotides (primers)

The sequences of the PCR oligonucleotides (primers) used throughout this study are listed in Table 2.5-1. These have an average length of about 19 bases with some exceptions having 30 – 40 bases, the G/C content of over 45 %, and the Tm of about 55°C – 65°C. Designing of PCR primers, secondary structures and dimmers formations were controlled by using PCR primer programme (Primer 3plus software available at http://www.bioinformatics.nl/cgibin/primer3plus/primer3plus.cgi) (Untergasser *et al*., 2007). The 5` ends of the forward and the reverse primers might be modified by inserting certain restriction sites that will serve to ensure unidirectional cloning of PCR products into certain vectors.

2.5.3 PCR primer synthesis and DNA sequencing

PCR primers were synthesised by company VH Bio (Gateshead, UK) or Sigma (UK) and DNA sequencing was carried out by MWG Biotech (Germany) or GATC Biotech (Germany), using the technology of Applied Biosystems ABI 3730xl for Sanger sequencing. All primers were dissolved in dH_2O and stored in a concentration 100 pmol/µl at – 20°C.

PCR reaction with Go Taq DNA Polymerase

Component	Final volume	Final Conc.
5X GoTaq® Reaction Buffer 10µl	4µl	1X (1.5mM MgCl2)
dNTP Mix, 10mM each	0.4µl	0.2mM each dNTP
upstream primer	Xµl	0.1–1.0µM
downstream primer	Yµl	0.1–1.0µM
GoTaq® DNA Polymerase (5u/µl)	0.25µl	1.25u
template DNA <0.25µg	Zµl	
Nuclease-Free water to	20µl	

Thermal Cycling Conditions for GoTaq® DNA Polymerase

Step	Temperature	Time	Number of Cycles
Initial Danaturation	95°C	2 minutes	1 cycle
Denaturation	94°C	0.5–1 minute	
Annealing	42–65°C (lowest Tm -2 to 5)°C	0.5–1 minute	25–35 cycles
Extension	72°C	1min/kb	
Final Extension	72°C	7 minutes	1 cycle
Cooling down	4°C	Indefinite	1 cycle

PCR reaction with Extensor Hi-Fidelity PCR Enzyme Mix

Component	Final volume
Master Mix 1:	
10X Extensor Buffer 1 (22.5mM $MgCl_2$)	2.5µl
Extensor high-fidelity PCR enzyme mix (5U/µl)	0.125-0.25µl
Distilled sterilized water to	25µl
Master Mix 2:	
20mM dNTPs (5mM of each dNTP)	1- 2.5µl
Forward primer (to final conc. of 0.2µM)	X µl
Reverse primer (to final conc. of 0.2µM)	X µl
DNA template	X µl
Distilled sterilized water to	25µl

Mix both master mixes just prior to reaction cycling.

PCR reaction with Extensor Hi-Fidelity PCR Enzyme Mix

Step	Temperature	Time	Number of Cycles
Initial Danaturation	92-94°C	2 minutes	1 cycle
Denaturation	92-94°C	10 seconds	
Annealing	50-68°C (lowest Tm -2 to 5)°C	30 seconds	25 cycles
Extension	68°C	X minutes (1)	
Final Extension	68°C	7 minutes	1 cycle
Cooling down	4°C	Indefinite	

Amplicon size (kb)	3	6	10	20
Extension time (min)	2	4	8	15

PCR reaction with KOD Xtreme™ Hot Start DNA Polymerase

Component	Final volume	Final Conc.
2x Xtreme Buffer	25µl	1X
dNTPs (2mM each)	10µl	0.4µM each dNTP
Upstream primer	1.5µl	0.3µM
Downstream primer	1.5µl	0.3µM
KOD Xtreme Hot Start DNA Polymerase (1u/µl)	1µl	0.02u
Template DNA	Yµl	
Nuclease-Free water to	50µl	

PCR reaction with KOD Xtreme™ Hot Start DNA Polymerase

Step	Temperature	Time	Number of Cycles
1. Polymerase activation	94°C	2 minutes	1 cycle
2. Denaturation	98°C	10 seconds	
3. Annealing	lowest primer Tm°C	30 seconds	20-40 cycles
4. Extension	68°C	1min/kb	

Figure 2.5.1: Polymerases used in the PCRs. Go *Taq* DNA polymerase, Extensor Hi-Fidelity PCR enzyme and KOD Xtreme Hot Start DNA polymerase were used in this study and matching reaction mixtures and temperature cycling conditions were used as shown above.

Table 2.5-2: List of PCR oligonucleotides used in this study and corresponding features

Name	Sequence (5' – 3') and description	Amplicon size	Source
stkF-F **stkF-R** (External primers)	5` ATCTGTCGTGTATCGCCATT 3`**(Forward)** 5` GTGCGGGTACCTACCCAAT 3`**(Reverse)** Forward and reverse primers used to amplify *stkF* gene full-length with parts from flanking genes (*stkE* and *stkG*) from *S.* Paratyphi A SARB 42.	849 bp	This study
stkF-L2 **stkF-R2** (Internal primers)	5' CCCGGTCTTGTGTTGCTTATG 3' **(Forward)** 5' CGTGACGTTGGGAAGGTAGA 3' **(Reverse)** Forward and reverse primers used for amplification of specific amplicon inside *stkF* gene of *S.* Paratyphi A SARB 42.	159 bp	1
PstkF-F (Forward) **PstkF-R** (Reverse)	5'TACTTCCAATCCATATGCAACAATTACTGTTTC TTTTAC 3'**(*Nde*I restriction site)** 5'TATCCACCTTTGGATCCTCATTCGTAGCTGACG TCAA 3'(***Bam*HI restriction site)** **Stop codon mutated** Forward and reverse primers used for amplification of full-length of *stkF* gene from *S.* Paratyphi A SARB 42. These primers containing two restriction digestion sites to ensure forced cloning to the expression vector pET23a.	600 bp	This study
stk-F (Forward) **stk-R** (Reverse)	5' AAGCGCGAATTCAATGGATGGCTTCATTCC 3' **(*Eco*RI restriction site)** 5' AAGCGCAAGCTTTAATCCGATGCTGCTCAA 3' **(*Hin*dIII restriction site)** Forward and reverse primers used for amplification of full-length of *stk* gene cluster with extra ~100 bp from both sides from *S.* Paratyphi A SARB 42 and S. Paratyphi A PA42. These primers containing two	7210 bp	This study

	restriction digestion sites to ensure forced cloning to pUCP20 vector.		
staF-F (Forward) **staF-R** (Reverse)	5'TACTTCCATCCATATGGTGATACCAATGAGAC GTTTGC 3' **(*Nde*I restriction site)** 5' TATCCACCTTCTCGAGTTCATAGGAGACATT CACCAGT 3'**(*Xho*I restriction site)** **Stop codon deleted** Forward and reverse primers used for amplification of full-length of *staF* gene from *S.* Typhi Ty2. These primers containing two restriction digestion sites to ensure forced cloning to the expression vector pET28a.	597 bp	This study
sipA–F (Forward) **sipA–R** (Reverse)	5'TACTTCCATCCATATGGTTACAAGTGTAAGAA CTCAGC 3'**(*Nde*I restriction site)** 5'TATCCACCTTCTCGAGACGCTGCATGTGCAAG CCATCA 3'**(*Xho*I restriction site)** **Stop codon deleted** Forward and reverse primers used for amplification of full-length of *sipA* gene from *S.* Paratyphi A SARB 42. These primers containing two restriction digestion sites to ensure forced cloning to the expression vector pET28a.	2055 bp	This study

Under lined bases is restriction sites were engineered in the forward and reverse primers sequences at 5'. **1**, Ou et al. (2007).

2.5.4 Gradient PCR

A gradient PCR was done to optimize the PCR and to determine the optimum annealing temperatures (Ta) for each pair of olignucleotides. A range of annealing temperatures, 50°C – 65°C was usually examined and thermocycler was setup on this range of temperatures. The best working annealing temperature for each pair was determined by agarose gel electrophoresis.

2.5.5 Colony PCR

For preliminary screening of large number of clones (transformants), it is inefficient and costly to screen all clones by extracting plasmid DNA by minprep. Thus, crude total DNA obtained directly from colonies of interest was used as template in colony PCR reactions. A large single colony was picked from a freshly grown plate, using a sterile toothpick or pipette tip, the bacterial material on the end of the toothpick / pipette tip was then transferred to 25 µl of sterile nH_2O in a sterile eppendorf microfuge tube. The eppendorf tube was then heated to 95°C for 5 min in a heat block then centrifuged at 14,000 rpm for 2 min. After that, 2 µl of the supernatant was used as template in a standard PCR reaction.

2.5.6 *In silico* PCR amplification software

In silico PCR amplification software, available at http://insilico.ehu.es/PCR/, was used to simulate and examine the specificity of the primers against up-to-date sequenced prokaryotic genomes before ordering.

2.6 TAE-agarose gel electrophoresis

DNA agarose gel electrophoresis was used for analysing PCR products, plasmid miniprep and digestion reactions. It was carried out using concentration range of 0.8 – 2.0 % of molecular biology grade agarose (Bioline, UK) in 1× TAE buffer depending on the size of the DNA fragment. It was prepared by mixing the specific weight of agarose with 100 ml of 1× TAE buffer. The agarose mixture was heated in microwave until the powder completely dissolved, then left to cool. After that 50 µl ethidium bromide (10 mg/ml) diluted 1:10 was added for 100 ml agaros solution which helps in DNA visualisation. The agarose solution was poured into the gel cast tray, containing a sample comb, sealed with autoclaving tape and left at room temperature until solidification. The gel tray, after removing tape and comb, was put into the agarose gel electrophoresis tank full with the running buffer (1× TAE). DNA samples were applied to wells in 6× gel loading buffer for colourless samples (see Appendix 1) in order to monitor migration of the DNA. DNA bands stained with ethedium bromide were visualized under UV light using UV illumination and were photographed using a gel documentation system (Olympus digital camera). For sizing of the separated DNA fragments, λ-*Hind*III marker (Fermentas) and GeneRuler™ 1 kb ladder (Fermentas) were used.

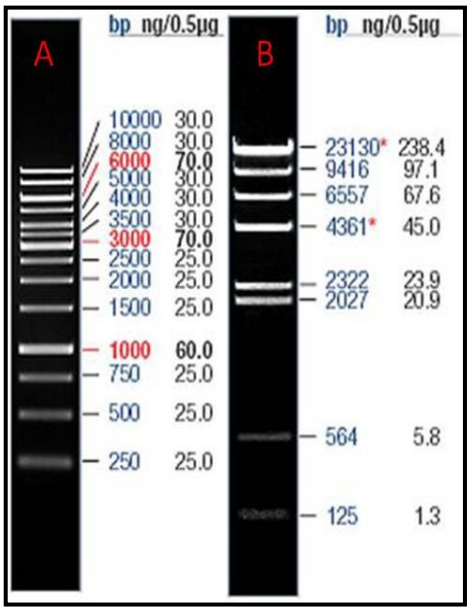

Figure 2.6.1: DNA Markers. DNA markers used in this study, the left hand columns of numbers represent the band sizes in bp of the markers. The right hand columns of numbers represent the equivalent ng per 0.5 µg marker approximately quantifying DNA bands; **A**, 1 kb GeneRuler™ DNA molecular weight marker (100 – 10,000 bp); **B**, lambda *Hind*III ladder (Fermentas, UK).

2.7 Extraction of DNA from TAE-agarose gel

After running TAE-agarose gel electrophoresis, DNA band of interest was excised from gel under indirect exposure to UV light using sterile sharp scalpel avoiding excess gel. Extraction and purification of DNA from the gel slice was carried out using E.Z.N.A.® Gel Extraction and DNA Purification Kit (Omega Bio-Tek, USA) to get rid of DNA contaminants that could interfere with subsequent cloning and sub-cloning processes such as salts, enzymes, dNTPs and others. This kit was used following manufacturer's instructions. Briefly, gel slice weight was determined and equal volume of binding buffer was added to the gel in centrifuge tube. The gel slice in binding buffer was incubated at 55^{o}C – 65^{o}C for 7 min or until gel was completely melted. The melted solution was applied to HiBind® extraction column assembled in 2 ml collection tube

and centrifuged at maximum speed for 1 min at RT. The column was washed with 300 µl binding buffer and the column was then washed twice with 700 µl SPW buffer. The empty column was centrifuged for 1 min at maximum speed to dry out. At last, the column was placed into clean 1.5 ml microfuge tube and DNA was eluted in 30 – 50 µl sterile nH_2O by centrifugation for 2 min at maximum speed. The eluted DNA was analysed by agarose gel electrophoresis and kept at – 20^{o}C for subsequent use.

2.8 A-tailing of PCR products synthesized by high-fidelity DNA polymerase

The use of high-fidelity DNA polymerases, such as KOD Polymerase, in PCR is associated with the production of blunt-end amplicon as these polymerases have intrinsic 3` – 5` exonuclease activity. That amplicon cannot be ligated directly into cloning vectors, which depends on TA cloning, such as pGEM-T Easy vector system except after an additional A-tailing step prior to ligation reaction. According to Promega protocol; 3 µl of gel-purified PCR product (200 – 500 ng) generated by a proofreading polymerase was mixed in a total of 10 µl reaction with 2 µl of *Taq* DNA polymerase 5× buffer, 1 µl of 1mM dATP to achieve a final concentration of 0.2 mM and 1 µl of *Taq* DNA polymerase (5 U). Sterile nH_2O was then added to a final reaction volume of 10 µl. The reaction was incubated for 15 – 30 min at 70°C. The A-tailed PCR products were stored at – 20°C until use and usually 1 – 2 µl was used in the ligation reaction with pGEM-T easy vector.

2.9 Ligation reactions of DNA fragments into plasmid vectors

Ligations were carried out using T4 DNA ligase and reaction buffers purchased from Promega (UK). In general, the reaction final volume was 10 – 20 µl with 3:1 molar ratio of insert : vector DNA. Two different reaction buffers were used dependent on the incubation time and temperature: 2× rapid ligation buffer for rapid ligation (~1 h) at RT (~22°C – 25°C) or 10× buffers for o/n (~16 h) ligations at 4°C. Following incubation time, ligase enzyme was heat inactivated at 70°C for 10 min. Then, 1 – 2 µl of ligation reaction was transformed into (chemical or electro) competent *E. coli* cells. If the ligation was to be used downstream in electroporation the ligation was cleaned up by vacuum drying followed by two cycles of washing with ~ 500 µl 70 % (v/v) ethanol via centrifugation at 14,600 rpm for 10 min. The sample was then dried again and eluted in 5 – 10 µl nH_2O to be directly used in electroporation.

2.10 Restriction enzymes and restriction digestion reactions

DNA constructs prepared by miniprep were subjected to restriction analysis through digestion with certain restriction enzyme(s) to yield specific DNA bands on TAE-agarose gel electrophoresis. Digested DNA bands will be of certain expected sizes according to the sequence of both vector and insert that confirm the success of ligation of DNA fragment to the vector. Restriction enzymes were purchased from New England Biolabs (NEB) (UK) and Roche (UK). Reactions were performed according to manufacturer instructions, using appropriate buffers at 1× dilution and when required 1× BSA (bovine serum albumin). Restriction endonucleases were added in a proportion of 8 – 10 U enzyme/µg DNA. The reaction volume depending on enzyme and DNA amount was 20 – 100 µl. If two enzymes were used for restriction digestion (reaction double digestion), a compatible reaction buffer was chosen by double digest NEB

finder. Following incubation for the appropriate time and temperature, restriction enzymes were heat inactivated as indicated by manufactures. When required by downstream applications, restriction digests were processed using commercially available DNA clean up kits, after TAE-agarose gel electrophoresis and cutting the band, then elution in 20 – 50 µl of nH_2O.

2.11 Transformation

Heat shock (chemical) and electroporation transformations were used in this study to transform plasmid DNA or ligation reactions into chemically competent or electrocompetent *E. coli* (DH5α, BL21 (DE3), BL21 (DE3)pLysS, HB101) cells.

2.11.1 Heat shock transformation into chemically competent *E. coli* cells

2.11.1.1 Preparation of chemically competent E. coli cells

Chemical competent (heat shock competent) cells were made by using the following method. A single colony of *E. coli* cells was inoculated in 5 ml of LB broth o/n at 37°C and 200 rpm (no antibiotic was added). The next day, 500 µl of the overnight culture was used to inoculate 1 L of sterile fresh LB broth. The cells were then grown at 37°C with shaking at 200 rpm until they reached an OD_{600} of 0.3 to 0.4. The cells were harvested by centrifugation at 3,000 ×g for 10 min at 4°C. The supernatant was removed and the bacterial pellets were then gently re-suspended in 50 ml of ice cold 100 mM $MgCl_2$ (Sigma, UK) and incubated for 10 min on ice. The cells were then centrifuged again at 3,000 ×g for 10 min at 4°C. The supernatant was then discarded and the cells gently re-suspended in 50 ml of ice cold 100 mM $CaCl_2$ (Sigma, UK) and incubated for 10 min on ice. The cells were then centrifuged at 3,000 ×g for 10 min at 4°C, the supernatant removed and the cells re-suspended in 4 ml of ice cold, sterile

100 mM $CaCl_2$ (Sigma, UK) in 15 % (w/v) glycerol (Fisher, UK). Cells were frozen down in 50 µl aliquots at – 80°C.

2.11.1.2 Heat shock (chemical) transformation procedures

For heat shock or chemical transformation, the plasmid DNA or the ligation mixture (1 – 4 µl), was added to 50 µl aliquot of thawed chemical competent *E. coli* cells. The cells and DNA / ligation were mixed gently and then incubated on ice for 20 min. The heat shock was performed by placing the cells in heat block at 42°C for 45 sec, then immediately returned to ice, and incubated for 2 min. Fresh 250 µl SOC broth (see Appendix 1) was added and the mixture was incubated at 37°C with 200 rpm for 1.5 h. Transformed cells were then plated onto LA plates containing the appropriate antibiotic and were incubated o/n at 37°C.

2.11.2 Electroporation into electrocompetent *E .coli* cells

2.11.2.1 Preparation of electrocompetent E. coli cells

The method used for preparing electrocompetent *E. coli* cells was as follows. A single colony was inoculated into a 5 ml LB broth, incubated o/n at 37°C and 200 rpm (no antibiotic was added). In the next day, 1 ml of the overnight culture was used to inoculate 100 ml of pre-warmed sterile fresh LB broth (dilution 1:100). The cells were then grown at 37°C with shaking at 200 rpm until they reached an OD_{600} of 0.5 to 0.6. The cells were then chilled on ice for 10 min. The cells were then harvested by centrifugation for 10 min at 4,000 rpm and 4°C. The supernatant was removed carefully and the bacterial cell pellet was then gently re-suspended in 50 ml of ice-cold 10 % glycerol and repeated centrifugation at 4,000 rpm for 10 min at 4°C. The supernatant was then discarded and the cells gently re-suspended the in 20 ml of ice-cold 10 %

glycerol and repeat centrifugation at 4,000 rpm for 10 min at 4°C. The supernatant was then again discarded and the cells gently re-suspended the in 10 ml of ice-cold 10 % glycerol and repeat centrifugation at 4,000 rpm for 10 min at 4°C. Finally, the supernatant was then again discarded and the cells gently re-suspended the in 1 ml of ice-cold 10 % glycerol and repeat centrifugation at 4,000 rpm for 10 min at 4°C. Cells were frozen down in 50 µl aliquots at – 80°C (~20 aliquots). In total, there are three 10 % glycerol washes to get rid of salt. The cells were thus concentrated ~100 fold compared to the density in the initial growth media. All steps were done on ice.

2.11.2.2 Electropration transformation procedures

To carry out the electroporation, 50 µl aliquot of electrocompetent cells was taken from the – 80°C freezer and thawed on ice. The plasmid DNA was added to cells and mixed gently. The cells/plasmid mix then transferred to a pre-chilled electroporation cuvette (0.2 cm gap) (Geneflow, UK). The cells were electroporated in a Genepulser II system (Biorad, UK) with following settings 25 µF, 200 Ω, 2.5 kV. Immediately, after the application of the pulse, 950 µl of sterile SOC medium was added to the cuvette and the contents transferred to 1.5 ml eppendorff tube. The cells were incubated for 1.5 h at 37°C and 200 rpm to allow recovery and then plated onto LA plates containing appropriate antibiotics and incubated o/n at 37°C.

II - Protein related techniques and methods

2.12 Recombinant protein expression with IPTG

2.12.1 Preliminary protein expression

A pilot experiment was carried out for expression of each recombinant protein (rStaF, rSipA, and rStkF) with isopropyl-beta-D-thiogalactopyranoside (IPTG) inducing agent. Transformed bacteria subjected to various preliminary protein induction or expression conditions (incubation temperatures, time intervals and IPTG concentrations) were examined by SDS–PAGE and Coomassie blue staining. The expression hosts, *E. coli* BL21 (DE3) and *E. coli* BL21 (DE3)pLysS, transformed with the constructs containing *orfs* of *staF, sipA* or *stkF* genes cloned into the pET28a and/or pET23a expression vectors were grown o/n at 37°C on LA-agar containing the appropriate antibiotic(s). A single colony was used to inoculate 5 ml LB broth containing the appropriate antibiotic(s) and incubated o/n at 37°C and 200 rpm. The overnight culture was then used to inoculate 15 ml fresh sterile LB broth supplemented with the appropriate antibiotic(s) in 50 ml Falcon tubes with cell density adjusted to an OD_{600} of 0.1. Cells were grown in shaker incubator at 37°C and 200 rpm. When the culture reached an OD_{600} of 0.5 – 0.6, expression of cloned proteins was induced by addition of filter-sterilised IPTG to final concentrations of 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM and 1 mM and incubated at temperatures of 20°C, 25°C, 30°C and 37°C.

2.12.2 Protein expression detection

At the cell culture OD_{600} of 0.4 – 0.6, aliquot of 0.5 ml was taken before addition of IPTG and the cell pellets were harvested by centrifugation at 4,000 rpm at 4°C for 5 min using bench centrifuge (Eppendorf, centrifuge 5415R), the cell pellet was kept at – 20°C

(zero time sample). Further 1 ml aliquots were taken every hour until 9 h then 16 h (o/n) after addition of IPTG then were harvested and cell pellets were kept at – 20°C. After that, each pellet was re-suspended in 50 µl of 20 mM phosphate buffered saline (PBS), pH 7.0 with vigorous mixing using micropipette tip to disrupt the cells as much as possible. Then, the suspension was subjected to 3 – 4 cycles of freezing and thawing in methanol/dry ice and 42°C. The samples were centrifuged at 4°C for 10 min at the maximum speed and the supernatant was transferred to a new tube, and mixed with appropriate volumes of 5× SDS–PAGE loading buffer contains 5 % of 2-mercaptoethanol added freshly to it before gel loading. The protein sample was denatured by boiling at 100°C for 5 min. The protein samples were then analysed with SDS–PAGE, gel staining with Coomassie blue and/or Western blot analysis.

2.12.3 Large-scale production of recombinant proteins

To produce recombinant StkF, StaF and SipA proteins in large amounts, the basic steps of protein expression described in the section 2.12.1 were followed after optimisation with increasing the volumes of bacterial cultures. The large-scale production was usually done in 2 litre volume of LB broth containing the appropriate concentrations of antibiotic(s) and IPTG, and incubated for the optimum expression time associated with maximum recombinant protein production. The culture was then split into 100 ml lots and cells were harvested by centrifugation at maximum speed at 4°C for 20 min and the cell pellets were stored at – 80°C until use.

2.13 Protein solubility examination

After furthur breakdown of the bacterial cell pellet suspension in 20 ml lysis buffer used in native conditions (see Appendix 1) by sonication on ice using a sonicator (Soniprep

150, MSE, UK) equipped with a microtip 3 – 5 times for 20 sec each time with 10 sec pause between or more until getting a translucent cell suspension. The cell lysate was then cleared by centrifugation at 4,400 rpm for 10 min at 4°C to pellet the cellular debris. An aliquote of 40 µl of the clear supernatant was kept for SDS–PAGE. The cell debris was re-suspended in another 1 ml of lysis buffe and a sample of 40 µl was also kept for SDS–PAGE. To check the protein solubility, the amount of proteins in both the supernatant (soluble fraction) and cell debris (insoluble fraction) was examined by loading both samples into SDS–PAG and staining of gel with Coomassie blue.

2.14 Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

An OmniPAGE mini System (Geneflow- G9-0370) apparatus was used to carry out SDS–PAGE gel electrophoresis. Resolving gel (12 % or 15 %) was prepared by using the reagents in Table 2.14-1 which produced sufficient mix for two mini-gels. SDS–PAGE was used to examine expression of recombinant proteins and the subsequent purification steps. Protein samples of 20 µl in loading dye were loaded into gel wells. Gels were run at 120 V until the dye band reached the end of gel. Gels were carefully removed from the supporting glass plates used for gel casting and placed in Coomassie blue solution with gentle shaking for 60 min. After soaking period, gels were then washed using several changes of de-staining solution until the gel background became clear. Protein bands were compared to the protein markers in order to determine approximate molecular weight and quantity of protein.

Table 2.14-2: SDS-PAGE gel components

SDS-PAGE Resolving Gel		
The material	**12 % gel (15 ml)**	**15% gel (15 ml)**
H_2O	4.9 ml	3.4 ml
30 % Acrylamide/bis-Acrylamide Mix	6.0 ml	7.5 ml
1.5 M Tris (pH 8.8)	3.8 ml	3.8 ml
10 % SDS	0.15 ml	0.15 ml
10 % Ammonium persulphate	0.15 ml	0.15 ml
TEMED (N,N,N',N'-Tetramethylethylenediamine)	0.006 ml	0.006 ml
SDS-PAGE Stacking Gel		
The material	**6 ml**	
H_2O	4.1 ml	
30 % Acrylamide/bis-Acrylamide Mix	1.0 ml	
1 M Tris (pH 6.8)	0.75 ml	
10 % SDS	0.06 ml	
10 % Ammonium persulphate	0.06 ml	
TEMED (N,N,N',N'-Tetramethylethylenediamine)	0.006 ml	

TEMED was mixed immediately before pouring the gel into the glass plate mould. The mentioned amounts are appropriate to make two gels.

2.14.1 Preparation of gel cassettes

The resolving gel was loaded in the glass plate until it reached a mark of one centimetre below the level of pre-placed comb teeth. Then, one ml of 2-propanol was added on the top of the gel to prevent air bubble formation and make straight surface. The gel was left for 45 – 60 min at RT until completely polymerized. The 2- propanol layer was dried by a piece of filter paper. The stacking gel was poured on the surface of the resolving gel until reaching the upper border of the glass plate. The clean comb was then placed to construct the wells. The gel was left for 30 – 45 min until occurrence of complete polymerization. The comb was removed and the gel cassettes were placed in the electrode assembly inside the tank filled with SDS–PAGE running buffer.

2.14.2 Protein sample preparation for SDS–PAGE

To prepare protein sample (cell lysate supernatant or purified protein) for loading into SDS–PAGE gel, 16 µl from each protein sample was mixed with 4 µl of 5 % 2-mercaptoethanol loading buffer (5×) and boiled at 100°C for 5 min. The 20 µl of protein samples in loading dye were loaded into gel wells. In addition to the proteins samples, 10 µl of protein marker PageRuler™ Prestained Protein Ladder (Fermentas-SM0671) was loaded in the first well. The power was applied and electrophoresis was conducted at voltage of 100 V until the samples pass the stacking gel when the voltage was raised to 120 V and left at this level until the dye front reached the lower end of the cassette.

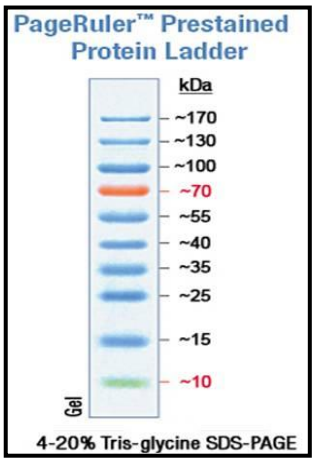

Figure 2.14.1: Protein Marker (PageRuler™ Prestained Protein Ladder, Fermentas-SM0671) used in this study. The sizes are given in kDa.

2.14.3 Coomassie blue staining and de-staining of the SDS–PAG gel

After completing the SDS–PAGE electrophoresis, gels were immersed immediately in the Coomassie blue solution in a plastic box with a lid and left for 1 h on a rocking platform at RT. The excess stain was removed and the de-staining solution (30 % methanol and 10 % acetic acid) was poured over the gel. The de-staining solution was

changed at regular intervals until all background staining was removed. Then the gel was kept in 50 % de-staining solution/water o/n on the rock platform in a tightly sealed box to prevent the gel from drying. The gel was examined for seeing the band of increasing intensity at the expected size range for each protein and then scanned to get photos.

2.15 Purification of His-tagged fusion proteins using Immobilised Metal Affinity Chromatography (IMAC)

The recombinant proteins were produced in this study as fusion proteins with a hexameric histidine-tag (His-tag) added on the N-terminal and/or C-terminal end to facilitate purification. These His-tagged recombinant proteins were purified by immobilised metal affinity chromatography (IMAC) technology using Ni-NTA (nickel-nitriloacetic acid) column. IMAC has been widely employed as a powerful separation approach in the purification of His-tagged recombinant proteins expressed in bacteria. The principle of affinity chromatography is that there is a ligand (certain transitional metal ion) that is bound to the chromatography matrix and protein binds specifically and reversibly to this ligand. The protein is then eluted by using another competitive ligand, such as imidazole. The Ni-NTA purification system is designed for purification of recombinant proteins depending on the high affinity and selectivity of binding of Ni-NTA agarose for recombinant fusion proteins tagged with six tandem histidine residues. Thus, the polyhistidine sequence will bind to nickel, which is in turn bound by the nitroloacetic acid. This immobilizes the recombinant protein in the column for later extraction. Proteins bound to the resin were eluted by competition with range of concentrations of imidazole. The native conditions were used in purification process when the protein was soluble (found in the supernatant after bacterial cell lysis), as was

the case with purification of recombinant StkF protein. However, protein denaturing conditions had to be used when the target protein was insoluble (found in cell debris), as was the case with purification of the recombinant StaF and SipA proteins.

2.15.1 His-tagged proteins purification using IMAC Ni SepFast BG Resin

IMAC Ni SepFast BG resin (Geneflow, UK, P7-0024) was used in this study in the purification of His-tagged StkF, StaF and SipA recombinant proteins and the process was run in IMAC BG-30 gravity flow column (Geneflow, UK) following the protocol of gravity flow purification. The base matrix is made of heavily cross-linked agarose with immobilised metal (Ni^{+2}) ligands.

Figure 2.15.1: His-tagged Protein purification materials. A, IMAC Ni SepFast BG resin (Geneflow, UK); **B**, IMAC BG-30 Gravity flow column (Geneflow, UK).

2.15.2 Protein purification process in gravity flow mode

2.15.2.1 Preparation of cell lysate prior to purification under native conditions

The cell pellet of 100 ml culture was taken out from – 80°C and kept on ice. The pellet was then re-suspended in 10 ml lysis buffer containing the same concentration of imidazole as the binding buffer (see Appendix 1) and supplemented by DNase I (1 mg/ml) and the protease inhibitor, Pefabloc SC (10 mg/ml) (Roche), in the case of

BL21 (DE3)pLysS. When BL21 (DE3) was the expression host lysozyme was added to final concentration up to 1 mg/ml to facilitate lysis of cells, however, was not needed in case of lysis of the *E. coli* BL21 (DE3)pLysS cells due to lysosyme produced by this strain upon thawing. In addition, cell suspensions of both strains were subjected to mechanical lysis by sonication (Soniprep 150, MSE, UK) on ice using a sonicator equipped with a microtip to improve cell lysis. The sonication was done four times for 20 sec each time with 10 sec pause between for BL21 (DE3)pLysS, however BL21 (DE3) needed longer timer until getting a translucent cell suspension. The cell lysate was then cleared by centrifugation at 4,000 for 20 min at 4°C to pellet the cellular debris. These native conditions were used later in purification process when the protein was soluble. The pH of cell lysate was adjusted to 7.4 prior to starting the purification procedures.

2.15.2.2 Preparation of cell lysate under denaturing conditions for insoluble proteins purification

Cell lysates were prepared using denaturing condition to lyse cell pellets containing the insoluble rStaF and rSipA proteins expressed in *E. coli* BL21 (DE3). Cell pellets obtained from 100 ml culture lots were removed from – 80°C and kept on ice. The pellet was then re-suspended in 10 ml lysis buffer used in native conditions supplemented by DNaseI (1mg/ml), protease inhibitor, Pefabloc SC (10 mg/ml) and lysozyme enzyme to final concentration 0.2 mg/ml. The cell suspension was then agitated for 1 h at room temperature. The suspension was then subjected to mechanical lysis by sonication on ice using a sonicator equipped with a microtip to improve cell lysis 10 times for 20 sec each time with 10 sec pause between or more until getting a translucent cell suspension. The cell lysate was then cleared by centrifugation at 2000

×g for 20 min at 4°C to pellet the cellular debris. The supernatant was removed and the cell pellet debris was washed 3 times with PBS by centrifugation. After that the cell debris was re-suspended in the denaturing binding buffer containing 8 M urea (20 mM Na_2HPO_4, 0.5 M NaCl and 8M Urea, pH 7.4) which was used to solubilise the protein. The cell debris suspension was subjected again to sonication (4 times for 20 sec each time with 10 sec pause between), followed by centrifugation at maximum speed for 20 min at 4°C. Finally, the solubilised protein was recoverd in the clear supernatant which was collected and used in the further purification steps.

2.15.2.3 Preparation of column before use

When preparing the BG gravity flow column, the snap-off cap at the bottom of the column had to be remained intact. The small chamber under the sintered mesh of the BG column of choice was pre-filled with water and had to be air bubble free. One ml of distilled water was transferred into the column firstly by pipetting, and then the bottom lid was removed to let a few drops of liquid come out. One ml of the resin slurry (50 % (v/v) concentration in 20 % ethanol) was transferred into the 30-ml BG purification column, which is equal to 500 µl resin. The column was hold in vertical position to a proper stand and the bottom lid was removed to allow the resin to settle completely by gravity (5 min).

2.15.2.4 Steps of protein purification in gravity flow mode

The protein purification was carried out using the gravity flow mode (Fig. 2.15-2). Firstly, the resin was equilibrated by gently adding 10ml of binding buffer and discharging under gravity until the liquid was fully passed through the column. The bottom lid was put on. The protein cell lysate was loaded into the column in the

following manner; the first 2 ml of protein sample was slowly added into BG-30 column using a pipette to minimise the disturbing of the settled resin particles. Rest of the protein sample was then gently poured in. The bottom lid then removed to let the feedstock pass through under gravity. The flow through was collected for future analysis. After the binding process was finished as observed by passage of the whole protein sample through the column, washing buffer was gently added to remove the weakly bound impurities. At least 5-bed volume of washing liquid was used. The washing waste was collected to a suitable container for further analysis. The elution buffer was then added to the column at 10 times of the resin volume to be sufficient to recover the bound protein. The eluate was collected to a suitable container. The elution might be repeated once or twice to maximise the recovery yield. The starting standard binding buffere (20 mM Na_2HPO_4, 500 mM NaCl, 10 mM imidazole, pH 7.4) and washing buffer (20 mM Na_2HPO_4, 500 mM NaCl, 20 mM imidazole, pH 7.4) was usually used in the first trials of purifying the His-tagged recombinant protein in this study, with the addition of 8M urea in case of denaturing buffers. The elution buffers composed of 20 mM Na_2HPO_4, 500 mM NaCl, and gradient concentrations of imidazole form 20 – 500 mM were used in the elution step. However, further modifications in the imidazole concentrations in binding and washing steps were needed to achieve the optimum conditions giving the highly purified proteins.

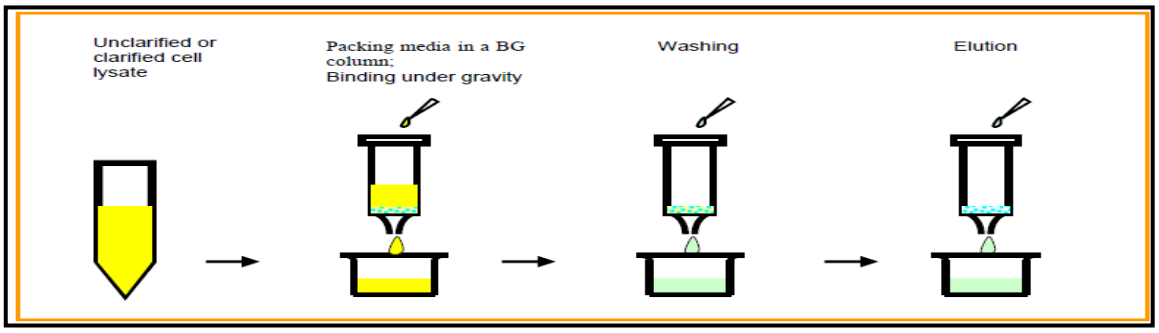

Figure 2.15.2: Diagram showing the purification process using gravity flow mode.

2.15.2.5 Imidazole concentration and optimization of the purification

Imidazole is included in the native and denaturing binding, wash and elution buffers to minimize the binding of untagged, contaminating proteins and increase the purity of the target protein with fewer wash steps. His-tagged protein did not bind well while using standard buffers in pilot purification experiments, as was the case with the weak binding of rStkF protein, thus, additional optimization steps were performed. All purification steps were done in 4°C (cold room) to increase the binding, while reducing the imidazole concentration to 0.5 mM in lysis, binding and washing buffers. A detergent 2 % Tween 20 was added to the wash buffer to disrupt non-specific interactions. In addition, the imidazole concentration was lowered in the elution buffers using a concentrations range starting from 5 mM to 50 mM imidazole. The imidaole concentration was also lowered in the binding and washing steps in purifying rStaF and rSipA proteins to optimise binding, however, the purified proteins were recovered at 250 mM and 500 mM imidazole concentrations.

2.15.3 Detection of the purified protein

The purified protein was verified by SDS–PAGE (12 %) followed by Coomassie blue staining. The proteins were further confirmed by Western blot analysis using monoclonal anti-His-tag antibody.

2.16 Protein refolding

Purification of insoluble proteins under denaturing conditions was conducted in this study with 8 M urea containing buffers. Cell lysis, column washing, and protein elution were performed with same buffer containing different concentrations of imidazole. Denatured rStaF and rSipA were eluted with 250 mM and 500 mM imidazole. The

eluted fractions containing the recombinant proteins were collected and pooled. Then, these antigens were refolded using particular refolding conditions by the drop dilution method.

2.16.1 Screening of the basic buffer composition for protein refolding

The identification of the conditions needed to refold the protein properly remains an empirical science. The folding of proteins in solution is affected by a number of physiochemical parameters. These parameters include ionic strength, pH, temperature, oxidation state and protein concentration as well as the presence of hydrophobic, polar, chaotropic agents and other proteins. Thus, the first step to develop a method for refolding proteins purified from inclusion bodies is to determine the composition of the refolding solution. In this study, the QuickFold™ protein refolding screening kit (AthenaES, USA-0600) was used to simplify the process of identifying the buffer composition that is best suited for the refolding of insoluble proteins (rStaF and rSipA). The QuickFold™ Protein refolding screening kit contains 15 different buffer compositions, which permit the rapid identification of the right conditions, or optimum buffer formulation, for protein refolding. Five different techniques are employed to exchange the denaturant buffer with the refolding buffer including dilution, dialysis, diafiltration, gel filtration and immobilization on a solid support. For screening purposes and large-scale production, dilution is the simplest approach. However, the diluted protein solutions would subsequently need be concentrated.

2.16.1.1 Protein refolding buffer compositions screening procedures

The dilution method was used in this study for screening of the 15 different refolding buffer compositions of the QuickFold™ Protein refolding kit. These buffers were

numbered from 1 to 15 (Fig. 2.16.1). Firstly, dithiothreitol (DTT), reduced glutathione (GSH), and oxidized Glutathione (GSSH) solutions were prepared by dissolving the powder content of each vial of these agents in 1 ml of dH_2O. DTT solution was stored at 4°C; however, GHS and GSSH solutions were stored at – 20°C. Secondly, the buffers were prepared following the manufacture's protocol. Buffers 1, 3, 5, 7, 9, 11, 13 and 15 were prepared by adding 1 µl of DTT solution to 950 µl of the respective buffer. However, Buffers 2, 4, 6, 8, 10, 12 and 14 were prepared by adding 1 µl of GSH solution and 1 µl of GSSH solution to 950 µl of the respective buffer. The solubilised protein in neutral buffered 8M urea was adjusted to the concentration 1 mg/ml. Then, 950 µl of each buffer was dispensed into each of 15 in 1.5 ml microfuge tubes. In addition, 950µl of the denaturation buffer was dispensed into tube number 16. Slowly, 50 µl of the protein solution was add to each tube while vortexing the solution gently. The tubes were incubated at 4°C for 1 h. After that, the mixtures were centrifuged for 5 min and the liquid that should contain the refolded, soluble protein was carefully transferred into a clean tube, while the pellet was reserved.

2.16.1.2 Assessment of successful refolding

Successful refolding was assessed in this study by SDS–PAGE as following, 40 µl of the soluble fraction was mixed with 10 µl 5× SDS–PAGE loading dye and heated at 100°C for 5 min and 20 µl was used in loading per lane of a gel. After the electrophoresis, the gel was stained with Coomassie blue and successful refolding is evidenced by the presence of the target protein in the liquid fraction.

2.16.2 Large-scale protein refolding

The denatured purified proteins were refolded on large-scale by the drop dilution technique, as the denatured purified protein was diluted 10 or 20 times with the

refolding buffer (Dames, 2008). The refolding of denatured SipA and StaF proteins was achieved by drop wise dilution at 4°C for 16 h. That was carried out by gravity flow of the denatured protein solution in small drops into the refolding buffer from a syringe with a fine needle (BD Microlance™ 3, 25 G × 5/8″, 0.5 mm × 16 mm) into a screw cap bottle on a magnetic stirrer that was kept at 4°C. The whole volume of the denatured protein had been injected into the refolding buffer. Then the solution continued to be stirred slowly o/n at 4°C. Next, any precipitated material was removed by centrifugation at 20,000 ×g for 20 min and 4°C. That was followed by concentrating the very diluted protein solution using Millipore's Amicon Ultra-15 centrifugal filters have pore sizes with molecular-weight cut off (MWCO) suitable for proteins smaller than 10 kD for rStaF and 50 kD for rSipA according to the manufacturer's manual. The purified final product was confirmed by SDS–PAGE analysis.

QuickFold™ Protein Refolding Buffer Formulation			
Component	Formulation after Preparation (Addition of DTT, GSH, and GSSH)	DTT	GSH/GSSH
Buffer 1	50 mM MES pH 6.0, 9.6 mM NaCl, 0.4 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.75 M Guanidine HCl, 0.5% Triton X-100, 1 mM DTT	●	
Buffer 2	50 mM MES pH 6.0, 9.6 mM NaCl, 0.4 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5 M arginine, 0.05% polyethylene glycol 3,550, 1 mM GSH, 0.1 mM GSSH		●
Buffer 3	50 mM MES pH 6.0, 9.6 mM NaCl, 0.4 mM KCl, 1 mM EDTA, 0.4 M sucrose, 0.75 M Guanidine HCl, 0.5% Triton X-100, 0.05% polyethylene glycol 3,550, 1 mM DTT	●	
Buffer 4	50 mM MES pH 6.0, 240 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5 M arginine, 0.5% Triton X-100, 1 mM GSH, 0.1 mM GSSH		●
Buffer 5	50 mM MES pH 6.0, 240 mM NaCl, 10 mM KCl, 1 mM EDTA, 0.4 M sucrose, 0.75 M Guanidine HCl, 1 mM DTT	●	
Buffer 6	50 mM MES pH 6.0, 240 mM NaCl, 10 mM KCl, 1 mM EDTA, 0.5 M arginine, 0.4 M sucrose, 0.5% Triton X-100, 0.05% polyethylene glycol 3,550, 1 mM GSH, 0.1 mM GSSH		●
Buffer 7	50 mM MES pH 6.0, 240 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.75 M Guanidine HCl, 0.05% polyethylene glycol 3,550, 1 mM DTT	●	
Buffer 8	50 mM Tris-Cl pH 8.5, 9.6 mM NaCl, 0.4 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.4 M sucrose, 0.5% Triton X-100, 0.05% polyethylene glycol 3,550, 1 mM GSH, 0.1 mM GSSH		●
Buffer 9	50 mM Tris-Cl pH 8.5, 9.6 mM NaCl, 0.4 mM KCl, 1 mM EDTA, 0.5 M arginine, 0.75 M Guanidine HCl, 0.05% polyethylene glycol 3,550, 1 mM DTT	●	
Buffer 10	50 mM Tris-Cl pH 8.5, 9.6 mM NaCl, 0.4 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5 M arginine, 0.4 M sucrose, 0.75 M Guanidine HCl, 1 mM GSH, 0.1 mM GSSH		●
Buffer 11	50 mM Tris-Cl pH 8.5, 9.6 mM NaCl, 0.4 mM KCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM DTT	●	
Buffer 12	50 mM Tris-Cl pH 8.5, 240 mM NaCl, 10 mM KCl, 1 mM EDTA, 0.05% polyethylene glycol 3,550, 1 mM GSH, 0.1 mM GSSH		●
Buffer 13	50 mM Tris-Cl pH 8.5, 240 mM NaCl, 10 mM KCl, 1 mM EDTA, 0.5 M arginine, 0.75 M Guanidine HCl, 0.5% Triton X-100, 1 mM DTT	●	
Buffer 14	50 mM Tris-Cl pH 8.5, 240 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5 M arginine, 0.4 M sucrose, 0.75 M Guanidine HCl, 0.5% Triton X-100, 0.05% polyethylene glycol 3,550, 1 mM GSH, 0.1 mM GSSH		●
Buffer 15	50 mM Tris-Cl pH 8.5, 240 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.4 M sucrose, 1 mM DTT	●	

Figure 2.16.1: QuickFold Protein refolding buffers composition used in protein refolding

2.17 Protein dialysis and concentration

2.17.1 Protein dialysis

Dialysis was used for exchanging the buffer to one suitable for the intended use of the protein by allowing diffusion of only the buffers selectively through permeable membranes. The solution to be dialyzed is placed in a sealed dialysis semi-permeable membrane (cellulose membranes with pore sizes designed to exclude molecules above a selected molecular weight) and immersed in a selected buffer. The pore size of the membrane determines the MWCO at which the protein is unable to pass through the membrane and is thus the protein of interest is completely retained in the solution by the dialysis membrane.

The dialysis membrane with a MWCO of 10 kD (10,000 MWCO, Snakeskin Pleated Dialysis Tubing- Thermo Scientific-68100) was used for dialysis and buffer exchange in this study. The dialysis membrane was rinsed with distilled water and a clamp (Spectra/Por Closures) was secured to one end of the membrane. The membrane was filled with buffer, the unclamped end was held closed and the membrane was squeezed. The protein-containing sample to be dialyzed was poured into the membrane bag and the open end clamped. The membrane was squeezed again to check the integrity of the membrane and clamps. Then, the dialysis membrane was immersed in a flask containing 500 ml of the appropriate dialysis buffer. For example, the physiological buffer that used for rStkF to be used for mice immunisation was 20 mM Tris HCl, 145 mM NaCl, pH 7.4, and the coating buffer for the ELISA diagnostic assays was 15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6. The protein sample was dialyzed against three buffer changes for 16 h at 4°C with gentle stirring of the buffer. Finally, the dialysis membranes were removed from the buffer and were held vertically and the

excess buffer trapped in the end of membrane outside the upper clamp removed. The upper clamp was released and the sample was removed with a pipette.

2.17.2 Protein concentration

The proteins samples were concentrated using Millipore's Amicon® Ultra-15 centrifugal filter devices for volumes up to 15 ml (Millipore, UK). Ultracel regenerated cellulose membrane of different MWCO (3 kD, 10 kD, 50 kD) were used for the centrifugal concentration. The most appropriate membrane cut-off was selected for each protein. The concentrator was firstly washed with the dialysing buffer by centrifugation for few minutes at 4,000 ×g and 4°C. The dialysed protein sample was then added in the concentrator, which then was inserted into centrifuge and centrifuged at 4,000 ×g at 4°C for 30 – 45 min. The time and speed was changed according to the column used. Once the desired concentration was achieved, the protein was recovered from the bottom of the unit by pipetting.

Figure 2.17.1: Amicon® Ultra Centrifugal Filter Device

2.17.3 Alternative dialysis method

Combined buffer exchange (dialysis) and concentration was done using Millipore's Amicon Ultra-15 centrifugal filter devices to reconstitute the retentate to the original sample volume (15 ml) within the appropriate buffer and then concentrated the sample as required. This process was repeated four times until the protein sample buffer was completely exchanged.

2.18 Estimation of protein concentration

Bio-Rad Protein Assay Kit (Bio-Rad- 500-0006) was used for measuring the protein concentration in samples using Microtiter Plate Protocol. The Bio-Rad Protein Assay, based on the method of Bradford, is a simple and accurate procedure for determining concentration of solubilised protein. It involves the addition of an acidic dye to protein solution, and subsequent measurement of OD at 595 nm with a spectrophotometer. Comparison to a standard curve provides a relative measurement of protein concentration in samples. According to the manufacturer's protocol, six dilutions of the standard bovine serum albumin solution (BSA) were prepared in the diluent buffer provided (50 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml, 600 µg/ml and 800 µg/ml) and a standard curve drawn. Aliquots of 10 µl of samples and BSA standard dilutions were added in duplicate into wells of a flat bottom 96-well plate (Nunc). After that, 200 µl of Coomassie dye reagent was added to each well. The sample and reagent was mixed well and incubated for 5 min at room temperature. Absorbance was measured at OD_{595} for the plate and blank absorbance (protein diluents plus Coomassie reagent) was subtracted from the reading of each protein solution. After establishing the protein standard curve by plotting protein concentration against the corresponding absorbance value, unknown

protein concentration was determined from the curve using its measured value at OD_{595} from the equation generated from that curve (Fig. 2.18.1).

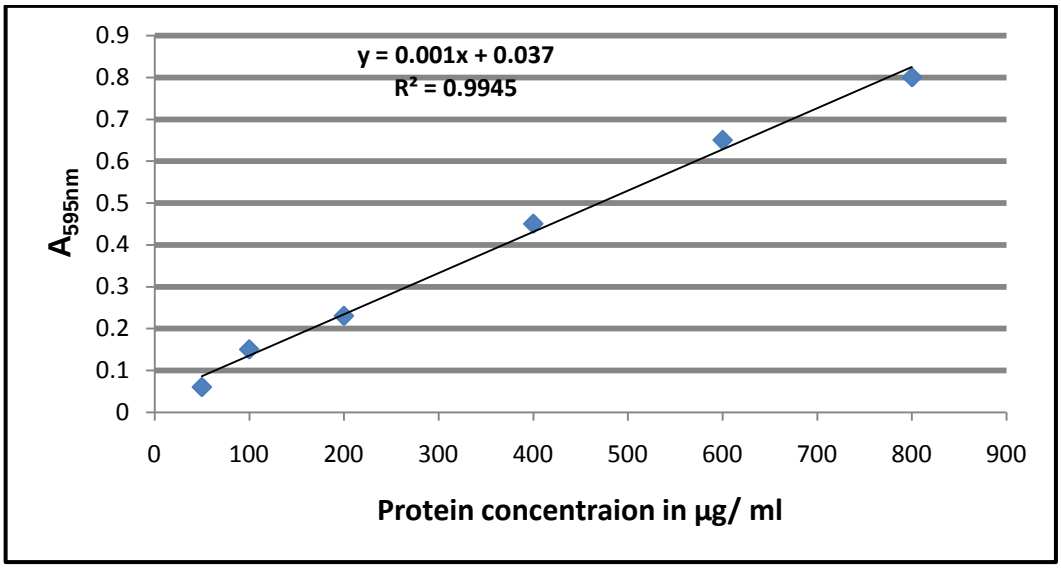

Figure 2.18.1: Protein concentrations standard curve. Calculation of protein concentration using Bio-Rad protein assay kit (Bio-Rad- 500-0006); OD_{595} value for each protein was used for calculation of its concentration using the formula y = 0.001x + 0.037, where x is the protein concentration in µg/ml and y is the OD measured at λ_{595}nm.

2.19 Purified recombinant protein storage

For long-term storage, the final solution of the proteins was kept in 0.5 ml aliquots. The tubes containing proteins were at first put in dry ice briefly, to prevent protein precipitation, and then stored at - 80°C until required.

III – Immunological assays-techniques and methods

2.20 Immune-blotting techniques

2.20.1 Western Blot

Western blot analysis was performed in this study to confirm expression and purification of the recombinant His-tagged proteins. Briefly, proteins were transferred from the SDS–PAGE into nitro-cellulose membrane using the semi-dry transfer system (Trans-Blot SD cell – Bio-Rad Laboratories – 170-3940) under the effect of an electric current according to manufacturer's instruction. Semi-dry blotting was carried out without cooling with minimal transfer time (15 – 30 min) and small amount of buffer confined to the stacks of filter paper. In the semi-dry transfer system, the gel and membrane sandwich was placed horizontally between two stacks of wetted filter papers in direct contact with closely spaced plate electrodes. Membranes were then incubated with a protein-specific antibody or serum to detect the protein or antibody of interest, respectively.

In details, the protein samples were subjected to 15 % SDS–PAGE by running the gel in 1× Tris/Glysine/SDS running buffer. The separated proteins were electrophoretically transferred to nitrocellulose membranes (Hybond-C Super, 0.2 μm pore size, Amersham) by the semi-dry blotting system in 1× Tris/Glycine blotting buffer. Membranes were then incubated with dry 5 % fat-free milk powder (Oxoid) diluted in 1× TBS for 1 h at RT to block residual protein binding sites. The membranes were then incubated with the antibody diluted in the blocking buffer. Commercial murine monoclonal anti-polyhistidine peroxidase conjugate was used at a dilution of 1:4,000 in blocking buffer (Sigma, A7058) for detection of His-tagged proteins, while

StkF murine antiserum, generated in this study, was used at a dilution of 1:1,000 in blocking buffer. Membranes were then incubated o/n at 4°C with gentle shaking on a shaking platform. The membranes were washed 3 times for 10 min each time with 1× TBS–0.05 % Tween 20 (TBS–Tween buffer). Immunodetection of proteins was performed by Chemiluminescence ECL Blotting Kit (PIERCE, 32106). The detection reagents 1 (peroxide) and 2 (luminal) were mixed in equal volume and added to the membrane and incubated for 2 min. After incubation for 2 min, the excess reagents were removed and the membrane was completely covered with clean plastic sheet. In a dark room, the membrane with its previously marked protein side was incubated and exposed to Hyperfilm^{TM} ECL Western in a film cassette for 3 min, 10 min and 15 min. Then, the film was put in the developer solution (AGFA, G153A) and shaken until the signal appeared, then it was transferred to the fixer solution (AGFA, G153B). The film was washed with water and left at RT until dried. Finally, the film was aligned against the membrane in the light to mark the locations of the pre-stained standard protein marker on the film. In the anti-StkF detection experiment, immunodetection was performed by incubation with the goat anti-mouse HRP IgG secondary antibody diluted 1:5,000 for 1 h at RT. The membranes were then washed in TBS-Tween three times for 10 min each time with shaking. The protein bands were visualised using Chemiluminescence ECL Blotting kit for 2 min and exposed to Hyperfilm^{TM} ECL Western (Amersham) for 5 min, 10 min, and 15 min.

2.20.1.1 Assembly of gel and membrane sandwich

Gel was carefully removed from the supporting glass plates used in gel casting and 6 to 10 sheets of 10 MM Whatman paper and nitro-cellulose membrane were cut to size of gel. All were pre-soaked in transfer buffer. The sandwich was assembled in the

following order, 3 – 5 sheets of wet Whatman papers, then the nitrocellulose membrane, then the gel, and then 3 – 5 sheets of wet Whatman papers. After that, a roller was used to roll gently over the sandwich to remove trapped air bubbles. Few ml of 1× transfer buffer was applied on top of the sandwich to avoid drying out during membrane transfer. The transfer was carried out at 220 V and a current of 250 mA for 10 min, then 270 mA for 10 min, and 310 mA for other 10 min.

2.20.2 Protein dot blot

Protein dot blot analysis is a fast and useful technique used for detecting, analyzing, and identifying the presence of certain protein in a sample on a membrane within a short time using an antibody specific for the protein of interest. It is similar to the Western blot technique but the protein samples are not separated electrophoretically but are spotted through circular templates directly onto the membrane. The spotted protein samples on a membrane are hybridized with an antibody that acts as a probe.

2.20.2.1 Dot Blot for detection of native StkF protein expression

This method was used for the primary screening of native StkF protein expression in *S.* Paratyphi A strain (PA 105) which carries an intact *stkF* gene predicted to code for a 199 aa protein. A second strain of *S.* Paratyphi A (PA 42), which carries an 'OFF' form of the *stkF* gene predicted to code for a truncated and partially missense 60 aa protein, was also examined. Murine polyclonal StkF antiserum was used as the primary antibody and HRP–conjugated anti-mouse IgG as the secondary antibody. Cell lysates for typhoidal *Salmonella* were prepared in Cat3 lab in the Leicester Royal Infirmary by Dr Andrew Rosser using lysis buffer containing lysozyme enzyme through thawing and freezing four times. Lysis of samples was further confirmed by sonication on ice using

a sonicator equipped with a microtip 5 times for 20 sec each time with 10 sec pause between. An aliquot of 20 µl of cell lyaste was spotted on a nitrocellulose membrane (Hybond-C Super, 0.2-µm pore size, Amersham) and allowed to dry in air. Membrane was incubated in blocking buffer (5 % (w/v) fat-free milk powder (Oxoid) diluted in 1× TBS) for 1 h at RT to block residual protein binding sites. The membrane was then incubated with shaking o/n at 4°C in anti StkF antiserum diluted 1:1,000 in blocking buffer. Membrane was then washed with shaking three times with 1× TBS – 0.05 % Tween 20 (TBS-Tween buffer) (10 min for each wash). Then, the membrane was incubated with goat anti-mouse HRP IgG secondary antibody diluted 1:5,000 for 1 h at RT. The membrane was then washed in TBS-Tween buffer 3 times (10 min for each wash) with shaking. After washing, the membrane was treated with the peroxidase substrate from the ECL Kit (PIERCE) and the remaining detection steps performed as described previously for immunoblots.

2.20.2.2 Dot Blot for detection of expression of Stk fimbriae

Protein dot blot analysis was also used to detect expression of the putative Stk fimbriae on the surface of afimbriated *E. coli* HB101 carrying an intact *stk* operon. Bacterial pellets were re-suspended in sterilized PBS and the cell concentration was adjusted by optical density to 1.5×10^8 cfu/ml. Cell suspensions (3 µl) were then applied directly onto the nitro-cellulose membrane alongside a PBS only control and the membrane left to dry at RT for 20 min. The membrane was then incubated in blocking buffer (5 % skimmed milk in 1× TBS) on a shaker for 1 h at RT. The blocking buffer was discarded and the membrane was incubated with shaking at RT in anti-StkF antiserum (1:1,000) diluted in blocking buffer for 2 h. The primary antibody solution was discarded and the membrane was washed in 1× TBS–0.05 % Tween 20 three times, 10 min for each wash

with shaking. The washing buffer was discarded and the membrane was incubated with goat anti-mouse IgG –AP secondary antibodies (1:30,000) diluted in blocking buffer for 2 h at RT. The secondary antibody solution was discarded and the membrane was washed three times as before. Finally, the dots were detected by incubating the membrane in BCIP/NBT (Sigma, UK–B5655) substrate alkaline phosphatse substrate solution (BCIP/NBT, pH 9.5) prepared by dissolving one tablet in 10 ml dH_2O until the signal was clearly visible.

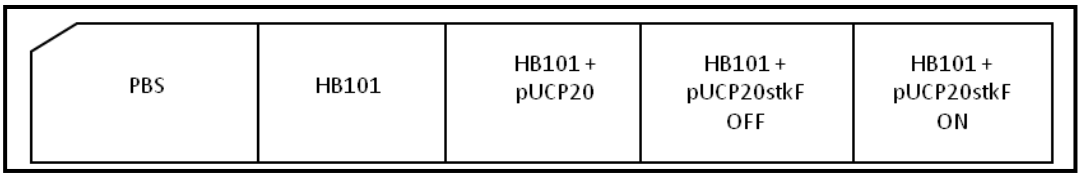

Figure 2.20.1: Sample orders in dot blot assay of *E. coli* HB101 clones

2.21 Complex-Immunoprecipitation (Co-IP)

Co-IP is one of the most widely used methods for antigen detection and analysis. Co-IP can be used to confirm a specific antigen–antibody interaction even when using whole cell extract, where proteins are present in a native conformation within a complex mixture of cellular components. The principle of Co-IP involves addition of an antibody (monoclonal or polyclonal) against a specific target antigen to a sample such as whole cell extract to form an immune complex with that target. The antibody–protein immune complex is then captured or precipitated on a solid support to which either Protein A or Protein G can be used. Both Protein A and Protein G bind specifically to antibodies, in turn are bound to the target antigen. Protein A agarose was used in this study with the Protein A component binding to Fc domain of IgG (Fc receptor). Protein A agarose resin is a high capacity, low leakage, stable affinity medium for rapid capture of

immunoglobulin from cell culture. The process of capturing this complex from the solution is referred to as precipitation. Any proteins not "precipitated" by the immobilized Protein A support are washed away by a series of washes. Finally, components of the bound immune complex (both antigen and antibody) are eluted from the support, dissociated by SDS sample buffer and analyzed by SDS–PAGE, and the co-precipitated protein can then be identified by Western blot detection using a specific antibody for that antigen.

2.21.1 Native StkF protein Co-IP procedures

Co-IP was done in this study according to the following procedure. About 350 µg in 500 µl of the total proteins lysates of *Salmonella* cells, were lysed and prepared under native conditions and incubated with one µl of murine StkF antiserum o/n with rotation at 4°C in the cold room. The next day 50 µl of the protein A-agarose beads was added to the tubes and incubated for 2 h with rotation at 4°C. Then the mixture was centrifuged at 2500 rpm for 5 min at 4°C. Carefully, the supernatant was aspirated and discarded. The pellets were washed four times with 500 µl with the sample buffer. After final wash, the supernatant was aspirated and discarded (using syringe) and then 20 µl of 2× SDS–PAGE sample loading buffer was added. The mixture was boiled for 5 min at 100°C and 30 µl of supernatants were loaded on the gel. Then, Western blot analysis was performed as before using anti-StkF antiserum in dilution 1:1,000.

2.22 Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA), also known as an enzyme immunoassay (EIA), is a technique used to detect the presence of an antibody or an antigen in a sample. The ELISA has been used as a diagnostic tool in medicine and there are many types of ELISA. In a direct ELISA, an antigen is affixed to a surface, and then a specific antibody is applied over the surface so that it can bind to the antigen. This antibody is linked to an enzyme, and in the final step, a substance is added that the enzyme can convert to a detectable signal, most commonly a colour. In an indirect ELISA, the signal is detected by adding a secondary antibody which binds to the primary antibody that is not linked to an enzyme. Instead, it is the secondary antibody that is conjugated to the enzyme. A variant of this technique is the "sandwich" ELISA used to detect a sample antigen. In this type of assay, a known quantity of capture antibody is bound to the surface of wells. Antigen in the sample is then bound to this antibody. In the final step, an enzyme linked antigen-specific antibody is applied and the enzyme substance added to produce a detectable signal. ELISA results are often reported as a numbers though determination of an appropriate "cut-off" point between positive and negative results remains controversial. The indirect ELISA technique was used in this study to measure anti-StkF antibody titres in serum from mice immunized with rStkF. Indirect ELISA was also used to evaluate an ELISA based serodiagnostic assay employing rStkF, rStaF and rSipA antigens as diagnostic tools for diagnosis of invasive *Salmonella* infections in human sera. In addition, the sandwich ELISA technique was used for estimation of cytokine concentrations in culture supernatants of mouse splenocytes recovered from rStkF-immunized mice.

2.22.1 Detection of specific antibodies against rStkF antigen in mice sera and measuring the antibody titre by ELISA

2.22.1.1 Preliminary detection of anti-StkF specific antibodies in the immunised mice sera

For coating an ELISA plates (Nunc Maxi-Sorp Immuno Plate-469264), the rStkF protein was diluted in carbonate/ bicarbonate coating buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6) to a concentration 10 µg/ml and 100 µl from rStkF antigen solution was dispensed per well in duplicate. The plates were wrapped in cling film and were incubated o/n at 4°C. The next day, wells were blocked for 2 h at RT to prevent non-specific binding using 250 µl/well of 1% BSA/1× TBS (1 gm/100 ml 1× TBS). The plates were washed four times with 250 µl/well of washing buffer (1× TBS/0.05 % Tween-20). Each well was filled with 250 µl then flicking the contents of the plate into the sink and then hit the plate strongly on a tissue until being dry and then another 250 µl was added again and so on for four times. After that, 100 µl of negative or positive standard sera serially diluted in 1× TBS/5mM $CaCl_2$ starting from 1/100 was added to appropriate wells. Two neighbouring columns were incubated with dilutions of pre-immunization serum, while the neighboured two columns carried equivalent post-immunization samples (dilutions from 1/100 to 1/12,800). The last row received all reagents except sera. The plates were incubated for three h at RT. After that, the plates were washed four times as before; the secondary anti-mouse AP-conjugated IgG in dilution of 1:10,000 (Sigma-Aldrich-A 3688) was added to the wells 100 µl /well and left at RT for 90 min. The plate was washed for the last time and then 100 µl/well of SIGMAFAST™ p-Nitrophenyl phosphate (Sigma-Aldrich-N1891) substrate was added. The yellow colour developed and plates were read within 20 min at λ_{405}nm using an ELISA plate reader.

2.22.1.2 Antibody titre calculation

The anti-StkF antibody titre was calculated by plotting absorbance vs. antiserum dilution (pooled sera from three mice) using the mean for each duplicate set. The inflection point of the post-immunisation sera graph was calculated. The titre was interpolated by drawing a line down to the x-axis. In this experiment, higher antiserum dilutions were used (1/100 to 1/100,000).

2.22.1.3 Detection of antibodies against rStkF protein in the mice sera at various time points

The goal of this ELISA experiment was to determine the immunogenicity of the rStkF antigen related to the time of appearance of specific antibodies in immunised mice. From the previous experiment, the pooled pre-immunisation, first-bleed and post-immunization sera was used at a dilution of 1:1,000 as this value lays on top of the linear part of the sigmoid curve of the standard positive serum against the rStkF antigen.

2.23 Using ELISA for IgG isotyping and IgG1/IgG2a ratio detection

For detection of IgG subclass (IgG1 and IgG2a), indirect ELISA was performed using goat anti-mouse IgG1-AP (Southernbiotech-1070-04) and goat anti-mouse IgG2a-AP (Southernbiotech-1080-04) secondary antibodies. One coated ELISA plate with 10 μg / ml of the rStkF protein was incubated with pooled mouse sera at dilution 1:1,000 for 1 h. After washing, goat anti-mouse IgG1-AP and goat anti-mouse IgG2a-AP at 1:2,000 dilution was incubated with the mouse sera for 2 h. After washing for the last time, p-nitrophenyl phosphate substrate was added and when the yellow colour developed, the absorbance measured at OD_{405} within 20 min.

2.24 Detection of specific antibodies against the recombinant StkF, StaF and SipA antigens by ELISA in sera from human patients and controls

ELISA plates (Nunc Maxi-Sorp Immuno Plate-469264) were coated the rStkF, rStaF, rSipA protein diluted in carbonate/ bicarbonate coating buffer (15 mM Na_2 CO_3 and 35 mM $NaHCO_3$, pH 9.6) to a concentration 10 µg/ ml for StkF and StaF and 5 µg/ ml for SipA based on standard checkerboard titrations (Crowther, 2001). Of each antigen solution 100 µl was dispensed per well in duplicate. The coated plates were then wrapped by cling film; and incubated o/n at 4°C. Further processing was as described previously. Then, 100 µl of a 1/200 dilution in TBS / 5 mM $CaCl_2$ of each serum sample was then added to these antigen-coated ELISA plates' appropriate wells and the last row received all reagents except sera. In the next day, block non-specific binding was done by 250 µl/ well of 1 % BSA/TBS and incubated 2 hours at RT. The plates were washed four times with 250 µl/well of washing buffer. The plates were incubated for three hours at RT (or at 4°C o/n for storage of large number of pe-coated plates). After that the plate was washed four times as before. Detection of antibodies of the IgG class was carried out using a goat anti-human IgG conjugated with alkaline phosphatase (Sigma-Aldrich, A 1543) in dilution of 1:10,000 was added as 100 µl/well and left at RT for 90 min. The plate was washed for the last time and 100 µl/well of SIGMAFAST™ p-Nitrophenyl phosphate (cat. no. N1891) substrate was added. When the yellow colour developed, plates were read at λ_{405} nm using an ELISA plate reader. The readings were recorded when the ODs of the standard positive control sera attained a value of 1.5 ± 5 %. The first two columns (1 and 2) of each coated plate were incubated with a number of standard positive control sera, which gave the highest responses to each antigen (preliminary examined before) and the last two wells of the

first two columns (H1 and H2) received all the reagents except sera as blank. Each plate also contained a number of sera from the healthy donors as a negative control. The quality control positive and negative sera were incubated in the same locations on all plates. The rest of the plate received the individual test sera. The variation from plate to plate of quality control sera (also intra-samples variation on same plate) was asseesd by calculating the the coefficient of variation, which is the ratio of the standard deviation to the mean. This ratio is expressed as percentage and was set for ELISA results in this study to be not more than 5 %.

2.25 Cytokine estimation in splenocytes culture supernatant by sandwich ELISA

The levels of interleukins 12 and 4 (IL-12 and IL-4) and interferon-γ (IFN-γ) in culture supernatants seeded with splenocytes from mice immunized with rStkF were determined using ELISA kits (eBioscience, CA) as per manufacturer's instructions. The kits, (Mouse IL-4 ELISA Ready-SET-Go! 88-7044-22), (Mouse IFN-γ ELISA Ready-SET-Go! 88-8314), (Mouse IL-12-p70 ELISA Ready-SET-Go! 88-7121) were purchased from eBioscience, Ireland, UK. Briefly, 100 µl/well of anti-mouse IL-12 or anti-mouse IL-4 or anti-mouse IFN-γ capture antibody in coating buffer were added in 96 well microtiter plates (Corning® Costar-9018). The plates sealed and incubated o/n at 4°C. The wells were aspirated and washed 5 times with > 250 µl/well wash buffer (1× PBS, 0.05 % Tween-20) for 5 times allowing time for soaking (~1 min) during each wash step (blot plate on absorbent paper to remove any residual buffer) and then blocked with 200 µl/well 1× assay diluents for 1 h at room temperature. Using 1× assay diluent, standards were diluted as noted on the certificate of analysis, 100 µl/well of each standard was added to the appropriate wells. Two-fold serial dilutions of the top

standards were performed to make the standard curve. Then 100 µl of culture supernatant was added to the appropriate well. The plates were covered, sealed, and incubated overnight at 4°C. The plates were washed as before followed by the addition of 100 µl of biotin conjugated detection antibody/well diluted in 1× assay diluent and plates were incubated at RT for 1 h. After washing as before, 100 µl of avidin–HRP was added to the wells and incubated at RT for 20 – 30 min. The plates were washed 5 times as before and 100 µl /well of TMB substrate solution to each well was added and incubated for 15 min in the dark. Reaction was stopped by adding 50 µl of 2N H_2SO_4, absorbance was read at λ_{450} nm, and data was analyzed.

2.26 Mice immunization and production of polyclonal anti-StkF antibodies (carried out by Dr. Roger James)

The rStkF purified protein was used for mice immunisation following a 35 day immunization protocol by Harlan UK Ltd Hillcrest**,** Leicestershire as follows:

1- Immunization route: subcutaneous (s.c.)
2- Antigen quantity used per immunization: 100 µg/mouse.
3- Concentration of prepared antigen was 1 mg/ml.
4- Adjuvant: Freund's adjuvant **(**Freund's complete adjuvant (FCA) / Freund's incomplete adjuvant (FIA)
5- Mice: Balb/c strain, male gender
6- Number of mice: 3 (mouse 493, mouse 494, and mouse 495)

Each mouse received four subcutaneous injections of rStkF protein (25 µg/dose) at the start and then on the 7th, 14th and 28th day of the immunisation course. The first dose was mixed with equal volume of FCA at 0 day of immunisation. Then, the subsequent three booster doses were mixed with an equal volume of FIA.

Day No.	Action
0	Pre-bleed + rStkF immunization + FCA (0.1 ml)
7	Boost 1 FIA (0.1 ml)
14	Boost 2 FIA (0.1 ml)
21	Test bleed 1
28	Boost 3 FIA (0.1 ml)
35	Terminal bleed + Spleen removal

2.27 Mouse serum storage and spleen freeze down

2.27.1 Serum storage

The mice sera were stored in 50 µl aliquots at – 80°C. Aliquots were thawed on ice once only with any remainder kept at 4°C.

2.27.2 Spleen freeze down (Done in Dr. Roger James laboratory with the help of Mr Simon Byrne)

Each mouse spleen was cut into several small pieces using a scissors. The spleen cells were isolated by mashing the spleen and passing the material through a BD Falcon mesh (cell strainer 70 µm nylon mesh, BD Biosciences-352350) using the plunger from a one ml syringe on top of a 50 ml Falcon tube (containing 10ml medium RPMI) to remove connective tissue and other debris. This was done, while applying fresh media onto the spleen to keep the cells moist and promote transfer through the sieve. The sieves were then rinsed over falcon tubes while adding media to remove remaining cells, and the resulting cell suspension centrifuged at 2,000 rpm for 5 min. The cell pellet was re-suspended in freezing media (10 % DMSO in FCS) with a pipette and one ml was transferred to each vial. The vials were stored at – 80°C for 24 h, and then transferred to liquid nitrogen for long term storage.

2.28 Cytokine production assays

Splenocytes from rStkF-immunized and non-immunized mice were cultured following the protocol of Khan et al. (2006). The spleen cells were treated with Tris-ammoinium chloride buffer pH 7.6 on ice to lyse contaminating red blood cells. The splenocytes were then re-suspended in RPMI 1640 medium supplemented with 10 % FCS. After that, cell suspension (~1 × 10^9 cells/well) were incubated in the presence of StkF protein (5 µg/well), ConA (+ve T cell inducer) and mitomycin C (-ve T cell inducer) in 24 well microtiter plates in triplicate for 72 h in 5 % CO_2 incubator at 37°C. After incubation for 72 h at 37°C, the culture supernatants were collected and stored at – 80°C until used for the cytokines assays. The pooled supernatant from triplicate cultures stimulated with each of StkF, ConA and mitomycin C were assayed for IL-12 p70, IL-4 and IFN-γ using sandwich ELISA kits (eBiosciences) as per manufacturers' instructions.

2.29 *In vitro* evaluation of protective efficacy of StkF-specific antibodies

Due to the lack of mouse model which replicates the systemic infection of *S.* Paratyphi A, the potential function of StkF-specific antibodies in protection against *Salmonella* infection was evaluated by using *in vitro* serum bactericidal (SBA) and opsonophagocytosis assays (OPA). A murine oral *S.* Typhimurium infection model which mimics enteric fever has been widely used to study the human disease caused by typhoidal *Salmonella* serovars (Santos *et al.*, 2001). However, this model is not applicable in our study because *S.* Typhimurim does not possess the StkF protein based on bioinformatics analysis and PCR investigation. In addition to the limitation of Category 3 laboratory facilities, these *in vitro* opsonophagocytosis assay (OPA) using

human neutrophils and an *in vitro* complement-mediated serum bactericidal assay (SBA) were proformed using the non-typhoidal *Salmonella* strains HP0204 (StkF-positive) and HP0174 (StkF-negative) belonging to the serovars Kentucky and Enteritidis, respectively. The StkF status of these strains had been verified by Western blot. It was hypothesized that these assays would mirror a potential direct contribution of StkF antibodies to *in vivo* killing and clearance of the StkF-positive bacteria by host during systemic salmonellosis.

2.29.1 *In vitro* opsonophagocytosis assay (OPA) using human neutrophils

2.29.1.1 Preparation of human polymorphonuclear leukocytes

Human peripheral blood polymorphonuclear (PMN) leukocytes, used in this experiment, were isolated according to Polymorphprep™ (AXIS-SHIELD PoC AS, Norway) protocol with help of Kashif Halim (Lab 232). Polymorphprep™ is a ready-made, sterile and endotoxin tested solution for the isolation of pure PMNs from whole blood of healthy adult donors. In brief, 30 ml of blood was collected on K-EDTA as anticoagulant from a healthy donor according to health and safety regulations of the University of Leicester. Using a syringe, a layer 20 ml of blood was carefully layered over 20 ml of Polymorphprep™ in a 50 ml centrifuge tube followed by centrifugation at 500 ×g for 30 min at RT. The plasma and mononuclear cells (upper band of cells) were removed and the lower band of PMNs was harvested. An aliquot of solution B was diluted with an equal volume of water and one volume of this half-concentration saline was mixed with the PMN suspension. The PMNs were harvested by centrifugation at 400 ×g for 10 min and re-suspend in Solution B. Any residual erythrocyte contamination of the granulocytes was removed by re-suspending the cell pellet in 3 ml of Solution C and incubated at 37°C for 7 min. Finally, the PMNs were harvested by

centrifugation and re-suspended in HBSS at a final concentration of ~5.4 × 10^7 cells/ml counted by tryphan blue staining. The viability of neutrophils was checked at the beginning and end of the experiments by trypan blue exclusion. More than 98 % of the neutrophils were viable and were used within 2 h of isolation.

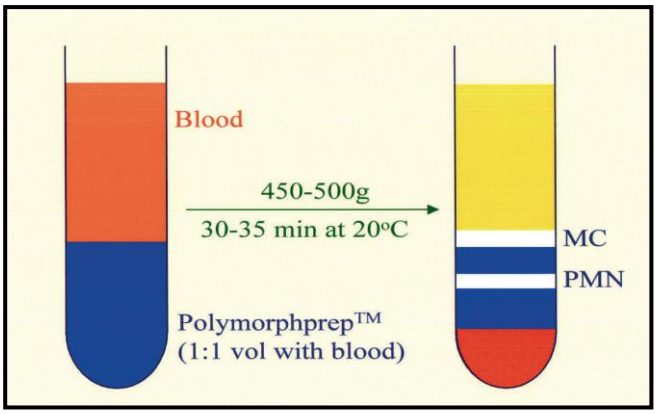

Figure 2.29.1: Schematic diagram of procedure used to purify peripheral polymorphnuclear leukocytes from a freshly collected blood sample.

2.29.1.2 Opsonisation of *Salmonella* Kentucky and *Salmonella* Enteritidis

S. Kentucky (HP 0204) and *S.* Enteritidis (HP 0174) was cultured o/n in LB broth at 37°C and 200 rpm. In the next day, the cell pellet was harvested by centrifugation at 4,400 rpm and washed 3 times in PBS. Opsonisation was carried out in 1.5 ml eppendorf tubes using StkF murine antiserum (pooled pre- and post-immunization sera) where 400 µl of microbial suspension (~1 × 10^8 cfu/ml) in HBSS and 100 µl of StkF murine antiserum to achieve 20 % (vol/vol) final serum concentration were incubated for 30 min at 37°C with shaking in horizontal position and 80 rpm.

2.29.1.3 Phagocytosis assay procedures

The phagocytosis assay was carried out as described in Jounblat et al. (2004). In phagocytosis assay, the reaction mixture composed of 100 µl of prepared PMNs

(~5.37 × 10^7cell/ml) was incubated with 100 µl of pre-opsonised bacteria (~1 × 10^8 cfu/ml) in 1.5 ml eppendorf tubes at 37°C and 80 rpm. The assay was designed to measure the viable count of *Salmonella* at different time points following incubation with human neutrophils. Samples from each phagocytosis reaction were taken at time points 0 min, 30 min, 60 min, and 90 min for analysis. Phagocytosis was stopped by incubating samples on ice. Ten-fold serial dilutions of samples were prepared in Hank's balanced salt solution (HBSS) and then plated onto LA agar. Viable count was determined by plating 10 µl aliquots in triplicate for cfu count on LA plates using the surface drop method (Miles & Misra, 1938). The number of viable bacteria was calculated using this following equation:

cfu (average per triplicate) × dilution factor × 100 = Number of cells/ml

2.29.2 Complement-mediated serum bactericidal assay (SBA) of anti-StkF polyclonal antibody

In this assay the *in vitro* complement-fixing bactericidal activity of the anti-StkF mouse serum against two non-typhoidal *Salmonella* strains was investigated based on the method described in Igumbor and Osayande (2000). Cultures of *S.* Kentucky (HP 0204), positive StkF protein, and *S.* Enteritidis (HP 0174), negative for StkF, were used in this study. Initial bacterial suspensions were adjusted to ~1.5 × 10^8 cfu/ml by diluttion in HBSS/BSA 0.5 %. Anti-StkF mouse serum was inactivated (de-complemented) at 56°C for 30 min and subjected to a series of two-fold dilutions (from 1:2 to 1:256) in Hank's balanced salt solution (HBSS) which contains calcium and magnesium ions (14025-050-invitrogen). Each dilution was performed in duplicate. For each two fold dilutions of StkF antiserum, 20 µl was incubated with 10 µl of 1.5 × 10^8 cfu/ml of *Salmonella* and 10 µl freshly thawed human complement source (healthy donor human serum) to achieve 25 % of total assay mixture at 37°C for 1 h in duplicate wells of a round-bottomed microtiter plate. Next, aliquots from each well were suitably diluted and plated onto LA agar for o/n culture. The number of surviving bacteria was determined and counted by the spot plate method. Bactericidal titre was calculated as the lowest serum dilution at which ≥ 50 % killing of bacteria was observed.

Table 2.29-1: Test and control reaction mixtures of *S.* Kentucky and *S.* Enteritidis

Test mixture	Components of mixture
***S.* Kentucky (or *S.* Enteritidis) + De-complemented antiserum**	10 µl *S.* Kentucky (or *S.* Enteritidis) cell suspension 20 µl De-complemented StkF murine antiserum 10 µl HBSS
***S.* Kentucky (or *S.* Enteritidis) + De-complemented antiserum + Intact complement**	10 µl *S.* Kentucky (or *S.* Enteritidis) cell suspension 20 µl De-complemented StkF murine antiserum 10 µl Intact complement
***S.* Kentucky (or *S.* Enteritidis) + Intact complement**	10 µl *S.* Kentucky (or *S.* Enteritidis) cell suspension 20 µl HBSS 10 µl Intact complement
***S.* Kentucky (or *S.* Enteritidis) + Inactivated complement**	10 µl *S.* Kentucky (or *S.* Enteritidis) cell suspension 20 µl HBSS 10 µl Inactivated complement
***S.* Kentucky (or *S.* Enteritidis) only**	10 µl *S.* Kentucky (or *S.* Enteritidis) cell suspension 30 µl HBSS
De-complemented antiserum + Intact complement	10 µl De-complemented StkF murine antiserum 10 µl Intact complement 20 µl HBSS

2.30 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.02 software package. Student's *t*-test (paired and unpaired) was used where appropriate to assess significance. One-way ANOVA was used to compare the immune response in the three immunised mice using different anti serum dilutions. Differences were considered significant at $P < 0.05$ and 95 % confiednece interval. A one- or two-tailed *t-test* was used when the result turned out in a particular direction or would be interesting in either direction. The sensitivity, specificity, PPV and NPV percentages of serodiagnosis of invasive *Salmonella* infections were calculated based on culture confirmed infections as the gold standard using mean + 2SD algorithm.

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Chapter Three

Cloning, expression and purification of recombinant StkF protein and examination of the distribution of *stkF* in different *Salmonella* serovars

3 Cloning, expression and purification of recombinant StkF protein and examination of the distribution of *stkF* in different *Salmonella* serovars

3.1 Introduction

The *stkF* (*spa0180*) gene (GeneBank accession CP000026.1) is a part of the *S.* Paratyphi A ATCC 9150 *stk* fimbrial gene cluster (*stkABCDEFG*) which is predicted to encode Stk fimbriae. The *stkF* gene encodes a putative fimbrial protein, StkF (GenBank accession AAV76213), which is an orthologue of *E. coli* MG1655 YadK that has been labelled as a predicted fimbrial adhesin-like protein (Ou *et al.*, 2007). Ou et al. (2007) used *stkF* as a specific target in developing a multiplex PCR based method for the diagnosis of *S.* Papartyphi A. However, the authors found the presence of two distinct alleles of this gene (ON and OFF forms) (Ou *et al.*, 2007). The StkF protein was chosen for vaccine development against *S.* Paratyphi A because like many other fimbrial adhesins, it was predicted to be a good immunogen. Given that several *Salmonella* fimbrial adhesins have been shown to be involved in the initial adhesion step of pathogenesis and that fimbriae are frequently abundant on the cell surface and hence exposed to the immune system, StkF would appear to be a good candidate immunogen.

The studies presented in this chapter attempts to characterise the location and function of StkF protein in *S.* Paratyphi A using bionformatics analyses and describes the cloning, expression and purification of recombinant StkF (rStkF) protein. The highly purified protein was then used to raise polyclonal antibody in mice and examined as a potential diagnostic agent; will be described in following chapters. This chapter also describes results of a PCR survey for *stkF* homologues in different *Salmonella* serovars.

3.2 Bioinformatics characterisation of the *stkF* gene and StkF protein

BLAST functions and other bioinformatics analyses of the *stkF* nucleotide sequence and StkF protein amino acid sequence were performed using available online bioinformatics tools and servers, such as NCBI (http://www.ncbi.nlm.nih.gov/).

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                      M  Q  Q  L  L  F  L  L  R  Q  P  G  L
                    ATGCAACAATTACTGTTTCTTTTACGCCAGCCCGGTCTTG
V  L  L  M  G  L  T  G  G  F  W  S  G  I  S  Q  A  G  S  N
TGTTGCTTATGGGTCTGACAGGCGGGTTTTGGTCTGGCATCAGCCAGGCGGGTTCCAATT
L  D  I  N  L  T  A  S  I  V  N  S  T  C  K  L  S  L  V  N
TGGATATCAATCTGACCGCCAGTATTGTTAACAGCACCTGCAAACTCAGTCTGGTAAATG
G  G  E  V  Y  L  P  N  V  T  R  A  W  F  Y  N  N  D  G  S
GGGGGGAAGTCTACCTTCCCAACGTCACGCGGGCATGGTTTTACAATAACGACGGCAGCA
S  K  Y  A  P  T  D  E  A  G  G  T  P  F  N  V  R  L  E  D
GTAAATATGCGCCTACCGACGAGGCGGGCGGCACGCCTTTTAACGTGCGTCTCGAAGACT
C  A  D  N  S  S  I  K  Q  L  M  F  S  F  T  P  Q  S  G  F
GTGCAGATAACAGTAGCATCAAGCAGTTGATGTTCAGCTTTACGCCGCAATCCGGTTTTT
W  S  N  Q  N  Q  V  F  K  N  D  A  T  T  G  A  A  Q  N  V
GGAGCAACCAAAACCAGGTTTTCAAAAATGATGCCACAACAGGCGCGGCGCAAAACGTTG
G  I  V  I  F  S  A  D  Y  K  T  N  V  L  N  S  D  G  S  S
GTATCGTCATTTTCTCGGCAGACTATAAAACTAACGTCTTAAACAGCGATGGCAGTTCTA
N  V  V  Y  D  V  S  G  K  S  S  S  L  Y  L  T  D  Y  Q  F
ACGTAGTTTACGACGTCAGCGGAAAAAGCAGTTCGCTGTATTTAACGGATTATCAGTTTT
Y  A  R  Y  Q  N  T  G  A  V  V  S  G  V  V  T  S  N  V  L
ACGCGCGCTATCAGAATACGGGCGCAGTTGTCAGCGGCGTTGTCACCAGCAACGTGTTGG
V  D  V  S  Y  E  *
TTGACGTCAGCTACGAATAA
```

Figure 3.2.1: Nucleotide sequence of *stkF* gene (600 bp) and the translated amino acid sequence of StkF protein (199 aa) from *S.* Paratyphi A strain ATCC 9150. The open reading frame of StkF protein is in red capital letters.

3.2.1 BLAST functions of *stkF* gene and StkF protein sequences

BLAST search of both DNA sequence of *stkF* gene and amino acid sequence of StkF protein against the non-redundant database of GenBank was performed using NCBI blastn and blastp functions, respectively. Blast results showed significant alignments to *stkF* gene and StkF protein within the sequenced genomes of *S. enterica* serovars.

Table 3.2-1: DNA nucleotide sequences producing significant alignments to *stkF* gene of *S.* Paratyphi A strain ATCC 9150

Accession No.	Description	Query coverage	Alignment percentage[1]
NC_006511.1	*S.* Paratyphi A str. ATCC 9150	100%	600/600 (100%)
NC_011147	*S.* Paratyphi A str. AKU_12601	100%	600/600 (100%)
ABFH02000001.1	*S.* Virchow str. SL491	100%	594/600 (99%)
ABFG01000012.1	*S.* Hadar str. RI_05P066	100%	594/600 (99%)
ABEL01000008.1	*S.* Heidelberg str. SL486	100%	593/600 (98%)
NC_011083.1	*S.* Heidelberg str. SL476	100%	593/600 (98%)
ABEI01000003.1	*S.* Kentucky str. CDC 191	100%	593/600 (98%)
ABAK02000001.1	*S.* Kentucky str.CVM29188	100%	593/600 (98%)
ACBF01000005.1	*S.* Tennessee str. CDC07-0191	100%	589/600 (98%)

[1]Using blastn function, *stkF* DNA sequence showed homologues within five *S. enterica* serovars other than Paratyphi A. All hits were annotated as encoding putative fimbrial proteins.

Table 3.2-2: Amino acid sequences producing significant alignments to the StkF protein of *S.* Paratyphi A strain ATCC 9150

Accession No.	Description	Query coverage	Alignment percentage[1]
YP_149525.1	*S.* Paratyphi A str. ATCC 9150	100%	199/199 (100%)
YP_002141021.1	*S.* Paratyphi A str. AKU_12601	100%	199/199 (100%)
ZP_02655532.1	*S.* Kentucky str. CDC 191	100%	197/199 (99%)
ZP_03076139.1	*S.* Kentucky str. CVM29188	100%	197/199 (99%)
ZP_03214101.1	*S.* Virchow str. SL491	100%	197/199 (99%)
ZP_04655078.1	*S.* Tennessee str. CDC07-0191	100%	195/199 (98%)
ZP_02668325.1	*S.* Heidelberg str. SL486	100%	196/199 (98%)
YP_002044159.1	*S.* Heidelberg str. SL476	100%	196/199 (98%)
ZP_02685939.1	*S.* Hadar str. RI_05P066	100%	196/199 (98%)

[1]Using blastp function, StkF protein amino acid sequence showed significant alignments (homologues) within proteins predicted to be coded for by five *S. enterica* serovars other than Paratyphi A. All hits were annotated as putative fimbrial proteins.

```
ParatyphiA    ATGCAACAATTACTGTTTCTTTTACGCCAGCCCGGTCTTGTGTTGCTTATGGGTCTGACA 60
Virchow       ATGCAACAGTTACTGTTTCTTTTACGCCAGCCCAGTCTTGTGTTGCTTATGGGTCTGACG 60
Kentucky      ATGCAACAATTACTGTTTCTTTTACGCCAGCCCGGTCTTGTGTTGCTTATGGGTCTGACG 60
Heidelberg    ATGCAACAGTTACTGTTTCTTTTACGCCAGCCCAGTCTTATGTTGCTTATGGGTCTGACG 60
Tennessee     ATGCAACAATTACTGTTTCTTTTACGCCAGCCCGGTCTTGTGTTGCTTATGGGTCTGACG 60
Hadar         ATGCAACAATTACTGTTTCTTTTACGCCAGCCCAGTCTTATGTTGCTTATGGGTCTGACG 60
              ******** ************************ ***** ******************

ParatyphiA    GGCGGGTTTTGGTCTGGCATCAGCCAGGCGGGTTCCAATTTGGATATCAATCTGACCGCC 120
Virchow       GGCGGGTTTTGGTCTGGCATCAGCCAGGCGGGTTCCAATTTGGATATCAATCTGACCGCC 120
Kentucky      GGCGGGTTTTGGTCTGGCATCAGCCAGGCGGGTTCCAATTTGGATATCAATCTGACCGCC 120
Heidelberg    GGCGGGTTTTGGTCTGGCATCAGCCAGGCGGGTTCCAATTTGGATATCAATCTGACCGCC 120
Tennessee     GGCGGATTTTGGTCTGGCGTCAGCCAGGCGGGTTCCAATTTGGATATCAATCTGACCGCC 120
Hadar         GGCGGGTTTTGGTCTGGCATCAGCCAGGCGGGTTCCAATTTGGATATCAATCTGACCGCC 120
              ***** ************ ****************************************

ParatyphiA    AGTATTGTTAACAGCACCTGCAAACTCAGTCTGGTAAATGGGGGGGAAGTCTACCTTCCC 180
Virchow       AGTATTGTTAACAGCACCTGCAAACTCAGTCTGGAAAATGGGGGGGAAGTCTACCTTCCC 180
Kentucky      AGTATTGTTAACAGCACCTGCAAACTCAGTCTGGAAAATGGGGGGGAAGTCTACCTTCCC 180
Heidelberg    AGTATTGTTAACAGCACCTGCAAACTCAGTCTGGAAAATGGGGGGGAAGTCTACCTTCCC 180
Tennessee     AGTATTGTTAACAGCACCTGCAAACTCAGTCTGGAAAACGGGGGGGAAGTCTACCTTCCC 180
Hadar         AGTATTGTTAACAGCACCTGCAAACTCAGTCTGGAAAATGGGGGGGAAGTCTACCTTCCC 180
              ********************************** *** ********************

ParatyphiA    AACGTCACGCGGGCATGGTTTTACAATAACGACGGCAGCAGTAAATATGCGCCTACCGAC 240
Virchow       AACGTCACGCGGGCATGGTTTTACAATAACGACGGCAGCAGTAAATATGCGCCTACCGAC 240
Kentucky      AACGTCACGCGGGCATGGTTTTACAATAACGACGGCAGCAGTAAATATGCGCCTACCGAC 240
Heidelberg    AACGTCACGCGGGCATGGTTTTACAATAACGACGGCAGCAGTAAATATGCGCCTACCGAC 240
Tennessee     AACGTCACGCGGGCATGGTTTTACAATAACGACGGCAGCAGTAAATATGCGCCTACCGAC 240
Hadar         AACGTCACGCGGGCATGGTTTTACAATAACGACGGCAGCAGTAAATATGCGCCTACCGAC 240
              ************************************************************

ParatyphiA    GAGGCGGGCGGCACGCCTTTTAACGTGCGTCTCGAAGACTGTGCAGATAACAGTAGCATC 300
Virchow       GAGGCGGGCGGCACGCCTTTTAACGTGCGTCTCGAAGACTGTGCAGATAACAGTAGCATC 300
Kentucky      GAAGCGGGCGGCACGCCTTTTAACGTGCGTCTCGAAAACTGTGCAGATAACAGTAGCATC 300
Heidelberg    GAGGCGGGCGGCACGCCTTTTAACGTGCGTCTCGAAGACTGTGCAGATAACAGTAGCATC 300
Tennessee     GAAGCGGGCGGCACGCCTTTTAACGTGCGTCTCGAAAACTGTGCAGATAACAGTAGCATC 300
Hadar         GAGGCGGGCGGCACGCCTTTTAACGTGCGTCTCGAAGACTGTGCAGATAACAGTAGCATC 300
              ** ********************************* ***********************

ParatyphiA    AAGCAGTTGATGTTCAGCTTTACGCCGCAATCCGGTTTTTGGAGCAACCAAAACCAGGTT 360
Virchow       AAGCAGTTGATGTTCAGCTTTACGCCGCAATCCGGTTTTTGGAGCAACCAAAACCAGGTT 360
Kentucky      AAGCAGTTGATGTTCAGTTTTACGCCGCAATCCGGTTTTTGGAGCAACCAAAACCAGGTT 360
Heidelberg    AAGCAGTTGATGTTCAGCTTTACGCCGCAATCCGGTTTTTGGAGCAACCAAAACCAGGTT 360
Tennessee     AAGCAGTTGATGTTCAGTTTTACGCCGCAATCCGGTTTTTGGAGCAACCAAAACCAGGTT 360
Hadar         AAGCAGTTGATGTTCAGCTTTACGCCGCAATCCGGTTTTTGGAGCAACCAAAATCAGGTT 360
              ***************** ********************************** ******

ParatyphiA    TTCAAAAATGATGCCACAACAGGCGCGGCGCAAAACGTTGGTATCGTCATTTTCTCGGCA 420
Virchow       TTCAAAAATGATGCCACAACAGGCGCGGCGCAAAACGTGGGGATCGTCATTTTCTCGGCA 420
Kentucky      TTCAAAAATGATGCCACAACAGGCGCGGCGCAAAACGTGGGTATCGTCATTTTCTCGGCA 420
Heidelberg    TTCAAAAATGATGCCACAACAGGCGCGGCGCAAAACGTGGGGATCGTCATTTTCTCGGCA 420
Tennessee     TTCAAAAATGATGCCACAACAGGCGCGGCGCAAAACGTGGGTATCGTCATTTTCTCGGCA 420
Hadar         TTCAAAAATGATGCCACAACAGGCGCGGCGCAAAACGTGGGTATCGTCATTTTCTCGGCA 420
              ************************************ ** ******************
ParatyphiA    GACTATAAAACTAACGTCTTAAACAGCGATGGCAGTTCTAACGTAGTTTACGACGTCAGC 480
Virchow       GACTATAAAACTAACGTCTTAAACAGCGATGGCAGTTCTAACGTAGTTTACGACGTCAGC 480
Kentucky      GACTATAAAACTAACGTCTTAAACAGCGATGGCAGTTCTAACGTGGTTTACGACGTCAGC 480
Heidelberg    GACTATAAAACTAACGTCTTAAACAGCGATGGCAGTTCTAACGTAGTTTACGACGTCAGC 480
Tennessee     GACTATAAAACTAACGTCTTAAACAGCGATGGCAGTTCTAACGTGGTTTACGACGTCAGC 480
Hadar         GACTATAAAACTAACGTCTTAAACAGCGATGGCAGTTCTAACGTAGTTTACGACGTCAGC 480
              ******************************************* ***************
ParatyphiA    GGAAAAAGCAGTTCGCTGTATTTAACGGATTATCAGTTTTACGCGCGCTATCAGAATACG 540
Virchow       GGAAAAAGCAGTTCGCTGTATTTAACGGATTATCAGTTTTACGCGCGCTATCAGAATACG 540
Kentucky      GGAAAAAGCAGTTCGCTGTATTTAACGGATTATCAGTTTTACGCGCGCTATCAGAATACG 540
Heidelberg    GGAAAAAGCAGTTCGCTGTATTTAACGGATTATCAGTTTTACGCGCGCTATCAGAATACG 540
```

```
Tennessee      GGAAAAAGCAGTTCGCTGTATTTAACGGATTATCAGTTTTACGCGCGCTATCAGAATACG 540
Hadar          GGAAAAAGCAGTTCGCTGTATTTAACGGATTATCAGTTTTACGCGCGCTATCAGAATACG 540
               ************************************************************

ParatyphiA     GGCGCAGTTGTCAGCGGCGTTGTCACCAGCAACGTGTTGGTTGACGTCAGCTACGAATAA 600
Virchow        GGCGCAGTTGTCAGCGGCGTTGTCACCAGCAACGTGTTGGTTGACGTCAGCTACGAATAA 600
Kentucky       GGCGCAGTTGTCAGCGGCGTTGTCACCAGCAACGTGTTGGTTGACGTCAGCTACGAATAA 600
Heidelberg     GGCGCAGTTGTCAGCGGCGTTGTCACCAGCAACGTGTTGGTTGACGTCAGCTACGAATAA 600
Tennessee      GGCGCAGTTATCAGCGGCGTTGTCACCAGCAACGTGTTGGTTGACGTCAGCTACGAATAA 600
Hadar          GGCGCAGTTGTCAGCGGCGTTGTCACCAGCAACGTGTTGGTTGACGTCAGCTACGAATAA 600
               ********* **************************************************
```

Figure 3.2.2: Alignment of nucleotide sequences with significant similarities to *stkF* gene of *S.* Paratyphi A ATCC 9150. Using online programme "ClustalW2" available at (http://www.ebi.ac.uk/Tools/clustalw2/index.htmlMuscle), the alignment showed nucleotide polymorphism in these sequences. The sequences showed are in 5' to 3' direction.

3.2.2 Prediction of trans-membrane regions in StkF amino acids sequence

Trans-membrane proteins contain at least one trans-membrane alpha helix (TMHs) domain. Trans-membrane helices (TMHs) in StkF protein sequence were predicted using TMHMM server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) (Sonnhammer *et al*., 1998) which is based on a hidden Markov model **(**HMM). The number of predicted TMHs sequences was zero, which indicates that the full length of StkF amino acid sequence is probably located outside the cell membrane (Fig. 3.2.3).

3.2.3 Prediction of signal peptide

Prediction of the presence and location of signal peptide cleavage site in StkF amino acid sequence was performed using SignalP 3.0 server available at http://www.cbs.dtu.dk/services/SignalP; based on combination of several artificial neural networks and HMMs (Emanuelsson *et al*., 2007). By analysing the sequence from position 1 to 70, the highest prediction value of signal peptide was for the sequence from 1 to 30 as the most likely signal peptide cleavage site (maximum probability) was between positions 30 and 31 (Fig. 3.2.4).

3.2.4 Prediction of StkF protein sub-cellular localization

StkF protein sub-cellular localization was predicted using PSLpred server (http://www.imtech.res.in/raghava/pslpred/). PSLpred server predicts the five major sub-cellular localizations (cytoplasm, inner-membrane, outer-membrane, extracellular and periplasm) of Gram-negative bacteria based on different features of the proteins such as physicochemical properties of amino acids and evolutionary information of PSI-Blast (Bhasin *et al*., 2005). Based on this analysis, StkF was predicted to be an extracellular protein**.**

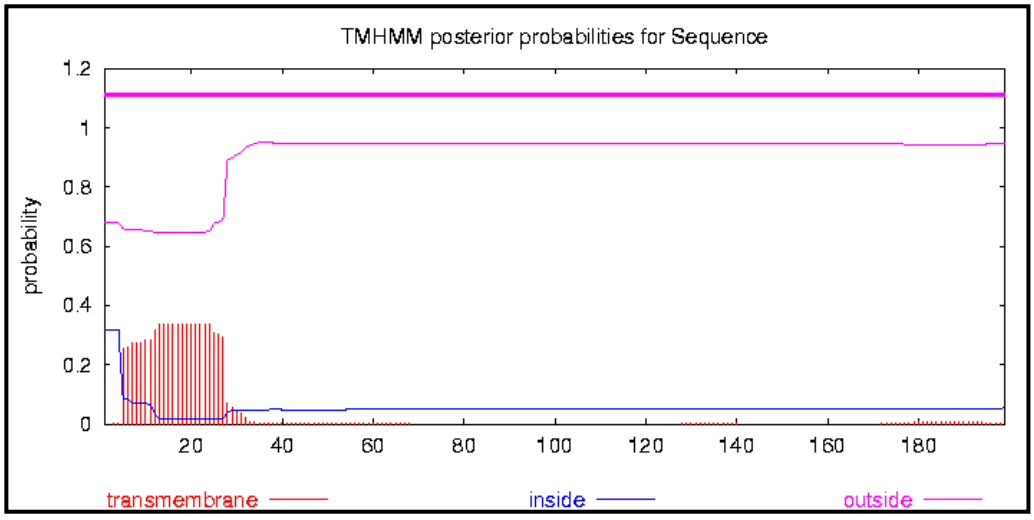


Figure 3.2.3: TMHs prediction of StkF protein using TMHMM Server v. 2.0. The diagram shows that the whole 199 aa sequence of StkF protein is outside the membrane as indicated by the best prediction pink coloured line at the top of the plot (between 1 and 1.2), that sums over all paths through the hidden Markov model. The red lines at N-terminal region could be predicted as a signal peptide not TMH.

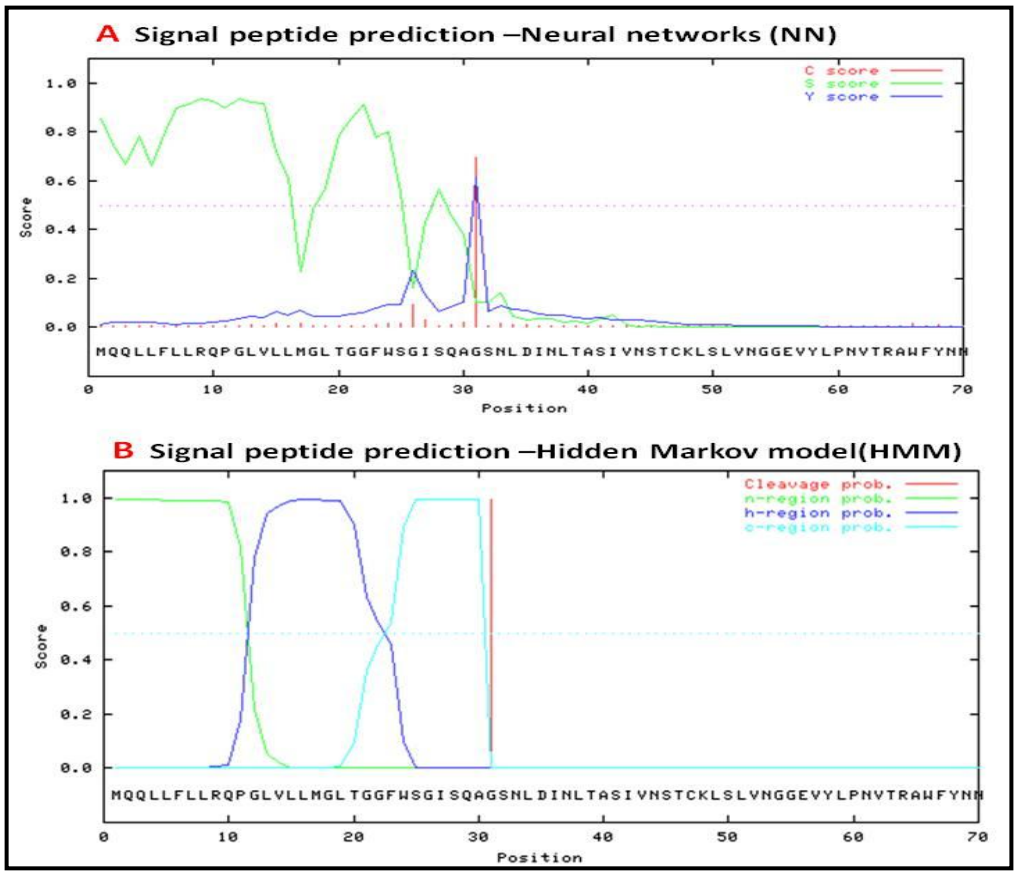


Figure 3.2.4: Prediction of signal peptide in StkF amino acid sequence by neural networks (NN) (Fig. A) and by HMM (Fig. B). Both diagrams show that the maximum probability score of signal peptide matches residues 1 – 30 and this is consistent with the most likely predicted cleavage site located between residues 30 and 31 (red line).

3.2.5 Prediction of StkF protein domain superfamily

The Superfamily, HMM server http://supfam.cs.bris.ac.uk/SUPERFAMILY/index.html, was used to predict whether the StkF protein contained domains related to one of the recognized domain superfamilies. This analysis revealed that StkF contains one domain related to the bacterial adhesin superfamily. In addition, the closest structure in the database for this domain was the mannose binding-specific domain of the fimbrial adhesin FimH of *E. coli* (1qun B: 159 - 279) (Fig.3.2.5).

Sequence	gi|56412450|ref|YP_149525.1 | StkF
Domain Number 1	**Region: 45 - 199**
Classification Level	**Classification**
Superfamily	Bacterial adhesins
Family	Pilus subunits

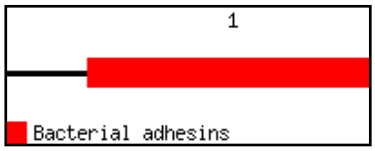
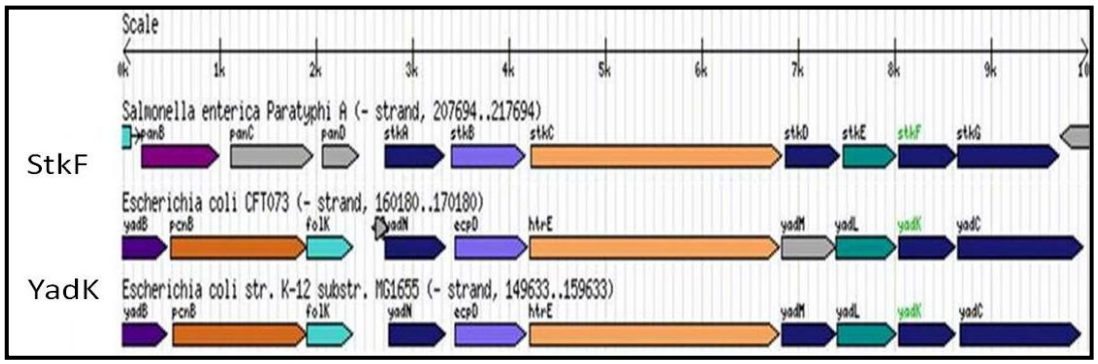


Figure 3.2.5: Results output of StkF domain related superfamily prediction. The prediction of StkF superfamily using Superfamily HMM library and genome assignments server suggested that StkF contains one recognizable superfamily protein domain that belongs to bacterial adhesins superfamily and pilus subunit family. Mannose-specific domain of FimH 1qun B (159-279) of *E. coli* was the closest structure to StkF in database.

3.2.6 Identification of StkF protein orthologue

Identification of StkF protein orthologue was performed using MBGD server, http://mbgd.genome.ad.jp/ (Uchiyama *et al.*, 2010), which is a comparative genomics tool of completely sequenced microbial genomes for orthologues identification. This analysis revealed that the predicted fimbrial-like adhesin YadK protein of Yad fimbriae in *E. coli* is an StkF orthologue (Fig. 3.2.6).



sp|P37016|YADK_ECOLI: yadK

```
QUERY  35 DINLTASIVNSTCKLSLVNGGEVYLPNVTRAWFYNNDGSSKYAPTDEAGGTPFNVRLEDC 94
P37016 31 DIDLVANVKNSTCKSGISNQGNIDLGVVGVGYFSGNVTPESYQP----GGKEFTITVSDC 86

QUERY  95 ADNSSIKQLMFSFTPQSGFWSNQNQVFKNDATTGAAQNVGIVIFSADYKTNVLNSDGSSN 154
P37016 87 ALQGTLNQLHIDFRALSGVMAGSRQIFANEISSGAS-NVGVVIFSTQDSANVLNASGGSR 152
                \               \                               \
                |               |                               |
              GDV               A                             NTF

QUERY  155 VVYDVSGKSSSLYLTDYQFYARYQNTGAVVSGVVTSNVLVDVSYE 199
P37016 153 SVYPV--MSDDMNGSSWKFSTRMQKIDSVTSGQLMSHVLVDIYYE 198
                                       \

                                       |
                                     PAL
```

Figure 3.2.6: StkF orthologue identification using MBGD server. YadK, predicted adhesin-like protein of *E. coli*, was identified as an StkF orthologue using MBGD server available at http://mbgd.genome.ad.jp/, thus strengthening the hypothesis that StkF is a fimbrial adhesin. The alignment of YadK amino acid sequence, which showed the highest similarity in the database to the query amino acid sequence of StkF protein.

3.3 PCR amplifications of the *stkF* gene

3.3.1 PCR amplification of the *stkF* gene from *S.* Partyphi A strain SARB 42

The *stkF* gene together with parts of the flanking *stkE* and *stkG* genes was amplified by PCR from *S.* Paratphi A SARB 42 genomic DNA using the primers stkF-F and stkF-R (external primers). Based on *in silico* PCR amplification, these external primers should produce an amplicon of 849 bp from the sequenced *S.* Paratyphi A str. ATCC 9150. In addition, the internal primers, stkF-L2 and stkF-R2 used by Ou et al. (2007) would be expected to produce an amplicon of 159 bp if the ON form of $stkF^{159bp}$ was present and a 180 bp amplicon if the $stkF^{180bp}$ OFF form was present (Ou *et al*., 2007). The PCR reactions were done using Go*Taq* DNA polymerase as follows:

DNA template	1.0 µl
dNTPs mix (10 mM)	0.4 µl
5× Buffer	4.0 µl
Go*Taq* polymerase	0.25 µl
Forward primer (10 pmol/ µl)	1.00 µl
Reverse primer (10 pmol/ µl)	1.00 µl
Sterile nH_2O	12.35 µl
Total	20.00 µl

The PCR program for amplification with both pairs of primers was as follows:

Step 1: Initial denaturation: 95°C for 4 min

Step 2: Denaturation at 94°C for 30 sec

Step 3: Annealing at 57.4°C (internal primers) and 58°C (external primers) for 30 sec

Step 4: Extension at 72°C 1 min

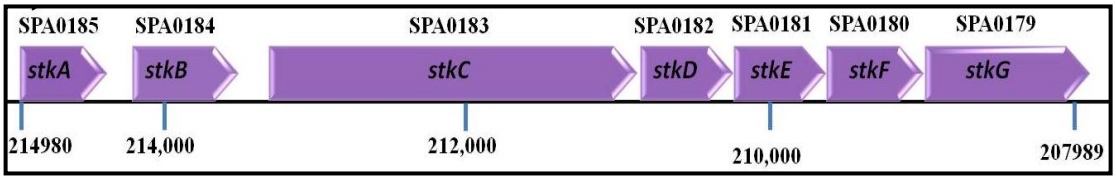
Step 5: Final extension at 72°C for 7 min

Steps 2-4 were repeated for 35 cycles

Step 6: Cooling down at 4°C for ∞

Reaction aliquots were resolved through a 1.5 % agarose gel for the small amplicon and 0.8 % gel for the large amplicon. The fragments were then confirmed by sequencing which showed 100 % matching to GenBank published *stkF* nucleotide sequence of *S.* Paratyphi A ATCC 9150.

(A)



(B)

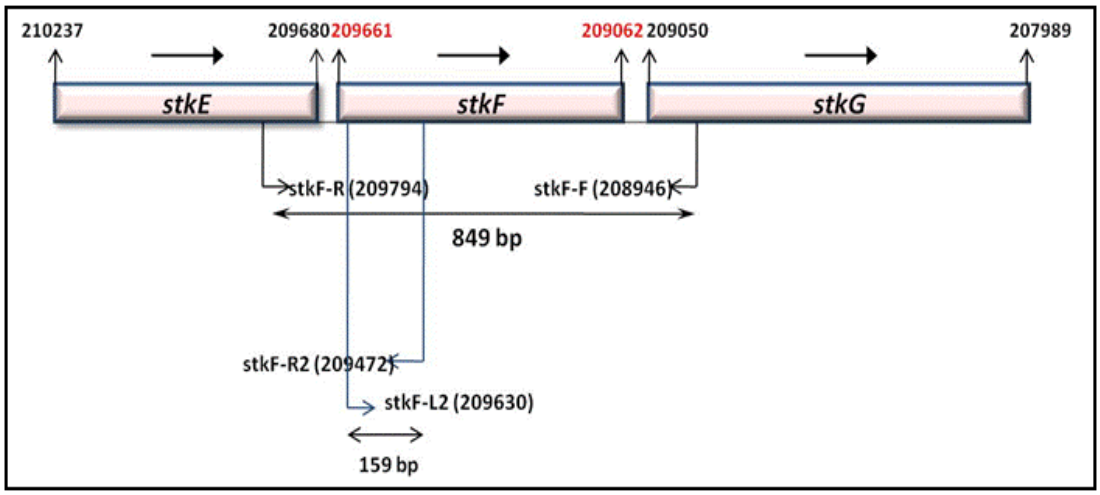


Figure 3.3.1: Schematic diagram of *stk* gene cluster. A, Genetic organisation of *stk* gene cluster in *S.* Paratyphi A ATCC 9150; **B,** Position of internal (stkF-L2 and stkF-R2) and external (stkF-F and stkF-R) primer pairs used in *stkF* PCR amplifications.

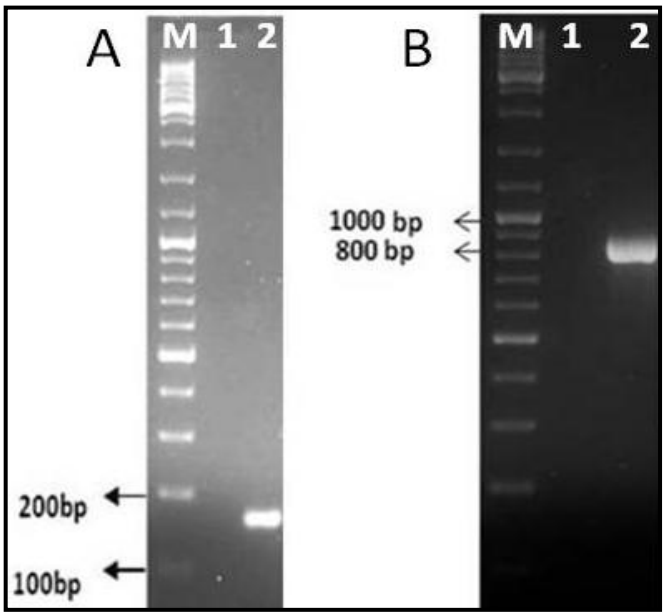


Figure 3.3.2: Agarose gel electrophoresis of *stkF* PCR products. A, Agarose gel (2 %) electrophoresis of PCR amplified product (159 bp) using stkF-L2 and stkF-R2 primers; **B,** Agarose gel electrophoresis (0.8 %) of PCR amplified product (849 bp) using stkF-F and stkF-R primers. Lane M, GeneRuler™ DNA M.W. marker; Lane 1, nH_2O (-ve control); lane 2, PCR amplicon. GeneRuler™ DNA molecular weight marker (100 − 10,000 bp).

3.3.2 PCR examination of different *Salmonella* serovars for *stkF* gene

Over a period of three years 2008 – 2011, 48 clinical non-typhoidal *Salmonella* isolates were obtained from the Leicester Royal Infirmary (LRI). In addition, the genomic DNA from 11 typhoidal *Salmonella* isolates was also obtained. Genomic DNA from all 59 of these clinical isolates, archived as new strains, was examined by PCR with both the internal and external *stkF* primer pairs. *stkF* was amplified with both pairs of oligonucleotides from 15 of the 59 strains examined (25.4 %) or ~30 % of *Salmonella* serovars examined (Table 3.3-1). However, all *stkF*-positive strains produced a 159 bp amplicon with the internal primers, highly suggestive of an ON form of $stkF^{159bp}$.

Table 3.3-1: List of *Salmonella* strains investigated by PCR for *stkF* gene

***Salmonella* serovar**[1]	**Strain number**	***stkF***
S. enterica serovar Anatum	MG505387W	Negative
S. enterica serovar Agona	MG503116A	Negative
S. enterica serovar Arizonae	4693	Negative
S. enterica serovar Augustenbora	MG506546X	Negative
S. enterica serovar Bareilly	105491H	Positive
S. enterica serovar Blockley	126277K	Negative
S. enterica serovar Braenderup	123311A	Positive
S. enterica serovar Colindale	MG505706D	Positive
S. enterica serovar Corvallis	014252K	Negative
S. enterica serovar Dare es salaam	MG178936Y	Negative
S. enterica serovar Derby	MG500913A	Negative
S. enterica serovar Diarizonae	4692	Negative
S. enterica serovar Dublin	109612S	Negative
S. enterica serovar Durham	124808A	Negative
S. enterica serovar Dwban	124809F	Negative
S. enterica serovar Ealing	119959G	Negative
S. enterica serovar Enteritidis	MG507000G	Negative
S. enterica serovar Enteritidis LK5	LK5/3820	Negative
S. enterica serovar Haifa	126217D	Negative
S. enterica serovar Hvittingfoss	MG506386L	Negative
S. enterica serovar Ibadan	116781X	Negative
S. enterica serovar Indiana	132615X	Negative
S. enterica serovar Infantis	MG508923W	Negative
S. enterica serovar Kentucky	MG504780F	Positive
S. enterica serovar Kottbus	109529M	Positive
S. enterica serovar Mbandaka	116588M	Positive
S. enterica serovar Montevideo	MG507272V	Negative
S. enterica serovar Montevideo	114933J	Negative
S. enterica serovar Muenchen	MG503839F	Negative
S. enterica serovar New-kaw	121110T	Negative
S. enterica serovar Newport	MG512574A	Negative
S. enterica serovar Ohio	114649C	Positive
S. enterica serovar Oranienburg	121030R	Negative
S. enterica serovar Paratyphi A	SARB 42	Positive
S. enterica serovar Paratyphi B	S181	Positive
S. enterica serovar Paratyphi C	SARB 48	Positive
S. enterica serovar Poona	MG507475A	Negative
S. enterica serovar Rubislaw	MG504710X	Negative
S. enterica serovar Saintpaul	531179J	Positive
S. enterica serovar Schwarzengrund	MG175772H	Negative
S. enterica serovar Schwarzengrund	531224Z	Negative
S. enterica serovar Stanley	MG50899T	Negative
S. enterica serovar Taautoa	106865C	Negative
S. enterica serovar Takoradi	MG502664B	Positive
S. enterica serovar Takoradi	MG178922E	Positive

S. enterica serovar Tennessee	106383W	Positive
S. enterica serovar Thompson	119350D	Negative
S. enterica serovar Typhi	26T25	Negative
S. enterica serovar Typhi	ST24A	Negative
S. enterica serovar Typhi	3125	Negative
S. enterica serovar Typhi	In15	Negative
S. enterica serovar Typhi	SARB 63	Negative
S. enterica serovar Typhi	SARB 64	Negative
S. enterica serovar Typhi	Ty2	Negative
S. enterica serovar Typhi	CT18	Negative
S. enterica serovar Typhimurium	MG178140H	Negative
S. enterica serovar Typhimurium	LT2/1412	Negative
S. enterica serovar Virchow	503984K	Positive
S. enterica serovar Weltevreden	MG507349Z	Negative

[1]59 different *Salmonella* starins (of 47 different serovars), 11 standard typhoidal strains and 48 clincal NT isolates were examined by PCR using both internal and external primers for the presence of *stkF* gene. The serovars (more than on strain of the same serover) examined are Takoradi (2), Typhi (8), Typhimurium (2), Enteritidis (2), Schwarzengrund (2) Montevideo (2). All 15 strains (14 serovars) shown as positive for *stkF* (red coulour) produced amplicons of indistinguishable sizes with each of the primer pairs used (~ 849 bp and 159 bp). Strains shown as negative failed to produce amplicons with either primer pair.

3.3.3 PCR examination of *S.* Paratyphi A strains for *stkF* gene alleles (this work was done by Dr Andrew Rosser)

One hundred and twenty one *S.* Paratyphi A isolates were analysed for the presence of the ON and OFF forms of *stkF* gene. Genomic DNA was extracted in LRI and the *stkF* PCR performed in Lab 212. PCR examination of these strains using the internal primer pair showed that 120 strains carried the ON form of $stkF^{159bp}$ and only one strain PA 42, a stool culture isolate from a 50-year-old female with travel history to Indonesia, carried a new version of $stkF^{211bp}$ (Table 3.3-2 and Fig. 3.3.3). PCR amplicons obtained from *S.* Paratyphi A strains PA42 (211 bp) and PA105 (159 bp), the latter chosen at random, were sent for sequencing.

Table 3.3-2: Internal primer-based PCR investigation for *stkF* gene alleles

Gene	Number of strains assessed	"ON form"	"OFF form"
stkF	121	120 (~159 bp)	1 (~211 bp)

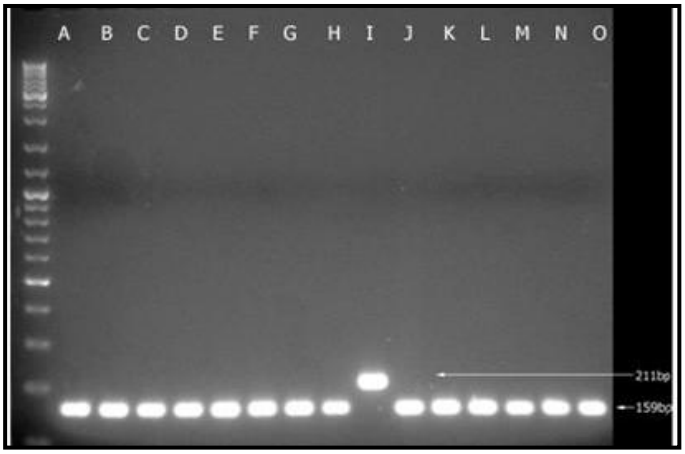


Figure 3.3.3: Agarose gel electrophoresis (2 % agarose) of *stkF* PCR investigation of 121 *S.* Partyphi A strains. First lane, GeneRuler DNA M.W. marker; Lanes A – H and Lanes J – O are *S.* Partyphi A strains carry the ON form ((*stkF*^{159bp}); Lane I, strain PA42 *stkF* PCR amplicon 211bp (*stkF*^{211bp}).

Sequencing of the 159 bp amplicon amplified from strain PA105 showed the native 26 base pair sequence and exhibited no polymorphisms relative to the *stkF* sequence of ATCC 9150 within the available data. However, sequencing of the 211 bp fragment from strain PA42 showed the presence of two additional identical 26 bp sequences in addition to the native 26 bp sequence (Fig. 3.3.4), all three of which were located tandemly end-to-end. This resulted in a frame shift introducing a premature stop codon and the production of a truncated protein of only 60 amino acids long.

A) 159 bp *stkF* amplicon (*stkF*^{159bp})

CCGGTCTTGTGTTGCTTATGGGTCTGACAGGCGGGTTTTGGTCTGGCATCAGCCAGGCGGGTTCCAATTT
GGATATCAATCTGACCGCCAGTATTGTTAACAGCACCTGCAAACTCAGTCTGGTAAATGGGGGGGAAGTC
TACCTTCCCAACGTCACGA

***stkF*^{159bp} ON form nucleotide sequence**

ATGCAACAATTACTGTTTCTTTTACGCCAGCCCGGTCTTGTGTTGCTTATGGGTCTGACAGGCGGGTTTT
GGTCTGGCATCAGCCAGGCGGGTTCCAATTTGGATATCAATCTGACCGCCAGTATTGTTAACAGCACCTG
CAAACTCAGTCTGGTAAATGGGGGGGAAGTCTACCTTCCCAACGTCACGCGGGCATGGTTTTACAATAAC
GACGGCAGCAGTAAATATGCGCCTACCGACGAGGCGGGCGGCACGCCTTTTAACGTGCGTCTCGAAGACT
GTGCAGATAACAGTAGCATCAAGCAGTTGATGTTCAGCTTTACGCCGCAATCCGGTTTTTGGAGCAACCA
AAACCAGGTTTTCAAAAATGATGCCACAACAGGCGCGGCGCAAAACGTTGGTATCGTCATTTTCTCGGCA
GACTATAAAACTAACGTCTTAAACAGCGATGGCAGTTCTAACGTAGTTTACGACGTCAGCGGAAAAAGCA
GTTCGCTGTATTTAACGGATTATCAGTTTTACGCGCGCTATCAGAATACGGGCGCAGTTGTCAGCGGCGT
TGTCACCAGCAACGTGTTGGTTGACGTCAGCTACGAATAA

StkF total protein sequence (199 aa)

MQQLLFLLRQPGLVLLMGLTGGFWSGISQAGSNLDINLTASIVNSTCKLSLVNGGEVYLPNVTRAWFYNN
DGSSKYAPTDEAGGTPFNVRLEDCADNSSIKQLMFSFTPQSGFWSNQNQVFKNDATTGAAQNVGIVIFSA
DYKTNVLNSDGSSNVVYDVSGKSSSLYLTDYQFYARYQNTGAVVSGVVTSNVLVDVSYE*

B) 211 bp *stkF* amplicon (*stkF*^{211bp})

CCGGTCTTGTGTTGCTTATGGGTCTGACAGGCGGGTTTTGGTCTGGCATCAGCCAGGCGGGTTTTGGTCT
GGCATCAGCCAGGCGGGTTTTGGTCTGGCATCAGCCAGGCGGGTTCCAATTTGGATATCAATCTGACCGC
CAGTATTGTTAACAGCACCTGCAAACTCAGTCTGGTAAATGGGGGGGAAGTCTACCTTCCcAACGTCACG

***stkF*^{211bp} OFF form nucleotide sequence**

ATGCAACAATTACTGTTTCTTTTACGCCAGCCCGGTCTTGTGTTGCTTATGGGTCTGACAGGCGGGTTTT
GGTCTGGCATCAGCCAGGCGGGTTTTGGTCTGGCATCAGCCAGGCGGGTTTTGGTCTGGCATCAGCCAGG
CGGGTTCCAATTTGGATATCAATCTGACCGCCAGTATTGTTAACAGCACCTGCAAACTCAGTCTGGTAAA
TGGGGGGGAAGTCTACCTTCCCAACGTCACGCGGGCATGGTTTTACAATAACGACGGCAGCAGTAAATAT
GCGCCTACCGACGAGGCGGGCGGCACGCCTTTTAACGTGCGTCTCGAAGACTGTGCAGATAACAGTAGCA
TCAAGCAGTTGATGTTCAGCTTTACGCCGCAATCCGGTTTTTGGAGCAACCAAAACCAGGTTTTCAAAAA
TGATGCCACAACAGGCGCGGCGCAAAACGTTGGTATCGTCATTTTCTCGGCAGACTATAAAACTAACGTC
TTAAACAGCGATGGCAGTTCTAACGTAGTTTACGACGTCAGCGGAAAAAGCAGTTCGCTGTATTTAACGG
ATTATCAGTTTTACGCGCGCTATCAGAATACGGGCGCAGTTGTCAGCGGCGTTGTCACCAGCAACGTGTT
GGTTGACGTCAGCTACGAATAA

StkF version 2 protein sequence (60 aa)

MQQLLFLLRQPGLVLLMGLTGGFWSGISQAGFGLASARRVLVWHQPGGFQFGYQSDRQYC*QHL
QTQSGKWGGSLPSQRHAGMVLQ*RRQQ*ICAYRRGGRHAF*RASRRLCR*Q*HQAVDVQLYAAIRFLEQP
KPGFQK*CHNRRGAKRWYRHFLGRL*N*RLKQRWQF*RSLRRQRKKQFAVFNGLSVLRALSEYGRSCQRR
CHQQRVG*RQLRI

Figure 3.3.4: *stkF* ON and OFF amplicons sequencing results. A, The *stkF* nucleotide sequences shown are based upon sequencing of the 159 bp amplicon of strain PA 105 with one 26 bp tandem repeat. The *stkF* allele (*stkF*^{211bp}) shown with additional two 26 bp tandem repeats is based upon sequence data from 211 bp amplicon of strain PA 42. The predicted amino acid changes are shown and the predicted numbers of amino acid residues for each gene are shown in brackets.* is stop codon.

3.3.4 Detection of the expression of native StkF protein

Dot blot technique was used for primary screening of native StkF protein expression in cell lysates of *S.* Paratyphi A strain PA105 (carries the ON frame of *stkF*) and *S.* Partyphi A strain PA42 (carries the OFF frame of *stkF*). The cell lysates were prepared in Cat3 lab in LRI by Andrew Rosser. StkF mouse antiserum (1:1,000), produced in this study, was used as primary antibody and HRP-conjugated anti-mouse IgG (1:5,000) used as secondary antibody. The dot blot showed that there was a clear difference between the two cell lysates, indicative of variable expression of StkF protein between the two strains. Furthermore, to identify and confirm the native StkF protein–anti-StkF antibody interaction, immunoprecipitation (IP) followed by Western blot analysis of precipitated protein was performed. Murine anti-StkF (1:1,000) was used as the primary antibody and HRP-conjugated anti-mouse IgG (1:5,000) as the secondary antibody. CO-IP clearly identified the native StkF protein (~21.5 kDa) in the PA105 cell lysate but not in the PA42 cell lysate. Using CO-IP, anti-StkF reactive StkF protein was also identified in selected representatives of *S.* Kentucky (HP 0204) and *S.* Virchow (HP 0206) but not in *S.* Enteritidis (HP 0174). The former two serovars had been shown to be *stkF*-positive by PCR assays, whilst *S.* Enteritidis was known to be *stkF*-negative.

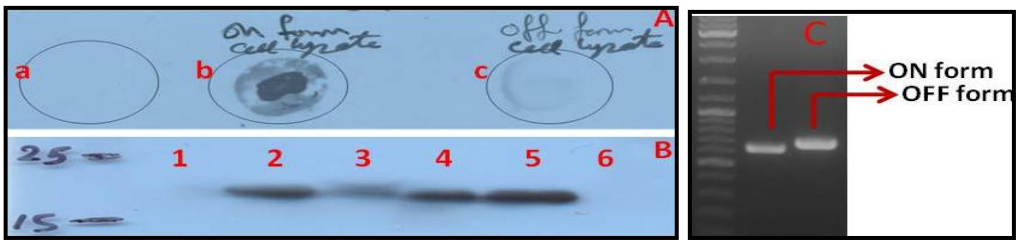


Figure 3.3.5: Immunoblotting techniques for native StkF detection using StkF mouse antiserum. A, Dot blots assay: a, lysis buffer; b, PA 105 cell lysate; c, PA42 cell lysate. **B,** CO-IP of *Salmonella* strains cell lysates; 1, PA42; 2, PA105; 3, Typhi Ty2;4, Virchow (*stkF*-positive); 5, Kentucky (*stkF*-positive); 6, Enteritidis (*stkF*-negative). **C,** Agarose gel (0.8 %) resolved PCR amplicons of the ON and OFF forms of *stkF* generated using Pstkf-F and Pstkf-F primers producing 600 bp amplicon.

3.4 Cloning of *stkF* with flanking genes fragment into pBKS

The target *stkF* gene with parts of flanking genes (*stkG* and *stkE*) was cloned intermediately into TOPO 4.0 plasmid and finally into HCN plasmid pBKS to be used as a template for further *stkF* amplifications as well as positive control in *stkF* PCR amplifications instead of genomic DNA.

3.4.1 Cloning of *stkF* with parts of flanking genes fragment into TOPO 4.0

The *stkF* together parts of its flanking genes PCR amplified fragment of ~849 bp was cloned into an intermediate vector TOPO 4.0 TA cloning kit. 2 µl of the ligation mix was transformed to TOPO 10 chemically competent *E. coli* cells by heat shock transformation. Positive clones were selected by white/blue screening on LA plates containing kan 50 µg/ml and amp 100 µg/ml + X-gal 40 µg/ml. The positive colonies were analysed by colony PCR followed by plasmid DNA (pTstkF) purification and *Eco*RI restriction analysis.

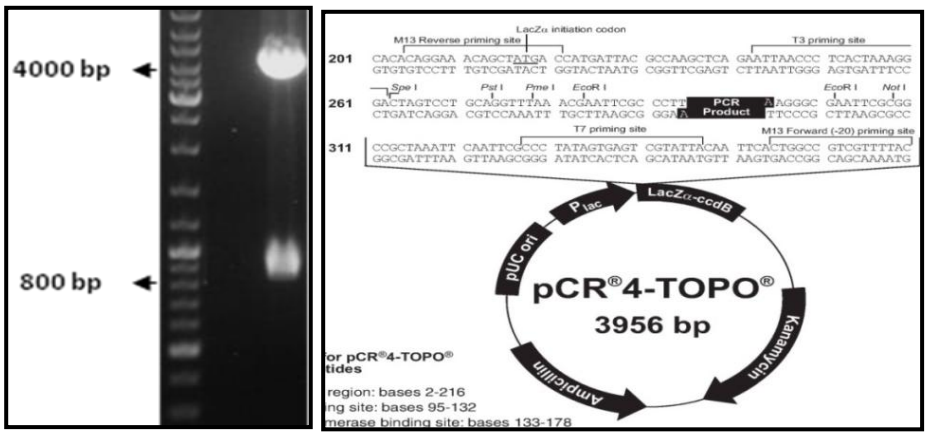


Figure 3.4.1: Agarose gel (0.8 %) electrophoresis of *Eco*RI-restricted pTstkF (TOPO plasmid harbouring *stkF* with flanking genes fragment). The cloning was confirmed as appearance of two bands after digestion, the vector band at 3956 bp and the insert corresponding to its size (~849 bp). Topo 4 plasmid map is on the right side. GeneRuler™ DNA molecular weight marker (100 − 10,000 bp).

3.4.2 Sub-cloning of *stkF* with flanking genes into pBKS

Following *Eco*RI digestion of pTstkF, the small fragment containing *Eco*RI restriction sites on both ends was recovered from the gel and sub-cloned into *Eco*RI-digested pBluescript KS 11 (+) (pBKS) vector by ligation reaction using T4 DNA ligase enzyme, then 2 µl of ligation mix was transformed by heat shock into DH5α chemically competent *E. coli* cells. Positive clones were selected by white/blue screening on LA plates containing amp 100 µg/ml + X-gal 40 µg/ml. The positive clones were confirmed by colony PCR followed by pBstkF plasmid DNA purification and analysed for insert by PCR amplification using T3 and T7 vector primers. The recombinant plasmid was then sequenced using T3 and T7 primers and the sequence obtained was 100 % matching with *stkF* nucleotide sequence of *S.* Paratyphi A ATCC 9150.

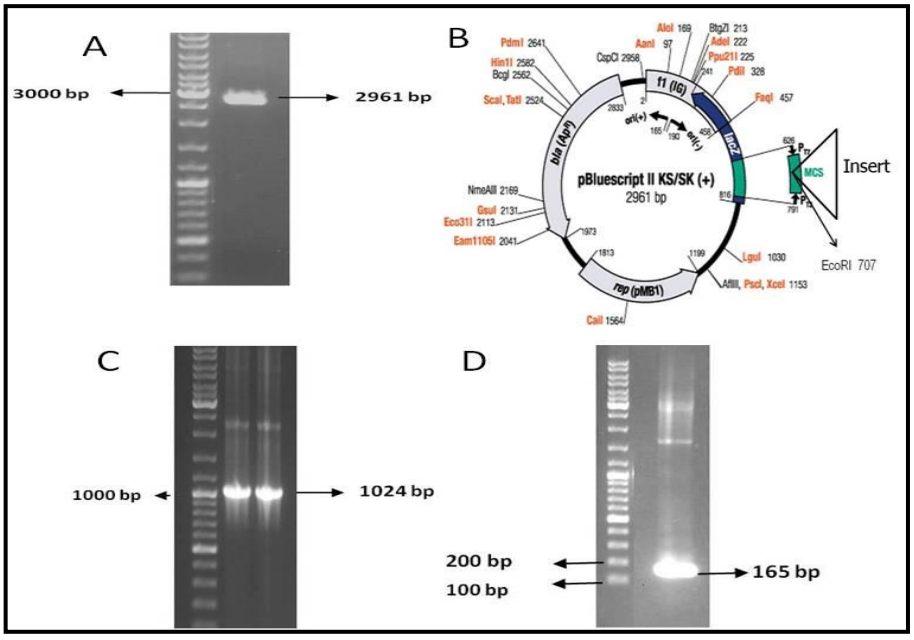


Figure 3.4.2: Sub-cloning of fragment bearing *stkF* and partial flanking genes into pBKS. Agarose gel electrophoresis (0.8 % agarose) of: **A,** *Eco*RI restriction analysis of pBKS vector giving one band at the expected size; **C,** PCR amplicon (1024 bp) of insert amplified from pBstkF using T3 and T7 primers; **D,** PCR amplicon (165 bp) of vector only amplified by T3 and T7 primers; **B,** pBluescript KS 11 (+) plasmid map.

3.5 Cloning of full length of *stkF* gene into pET23a expression vector

Briefly, the full-length of *stkF* gene (600 bp) was cloned intermediately into pGEM-T easy plasmid and finally into expression plasmid pET23a (+), then expressed in *E. coli* BL21 (DE3)pLysS.

3.5.1 *stkF* full length PCR amplification

The full sequence (*orf*) of *stkF* gene was amplified from the pBstkF by PCR using the extensor high-fidelity PCR enzyme mix. The primers, Pstkf-F and Pstkf-R used in the amplification, were generated by adding *Nde*I and *Bam*HI restriction sites at the 5' ends of the forward and reveres primers, respectively. The forward primer sequence contains an ATG start codon and the reverse oligonucleotide has a mutated stop codon to allow fusion to the C-terminal His-tag. The PCR reaction was setup in 50 µl and the temperature cycling conditions were as follows.

Master Mix 1 (25 µl):	
10× Extensor Buffer 1	5.0 µl
Extensor high-fidelity PCR enzyme mix	0.25 µl
nH_2O	19.75µl
Master Mix 2 (25 µl):	
20mM dNTPs (10 mM)	2.0 µl
Forward primer (10 pmol/µl)	4.0 µl
Reverse primer (10 pmol/µl)	4.0 µl
DNA template	1.0 µl
nH_2O	14.0 µl
Both master mixes were just mixed prior to reaction cycling	

PCR conditions for cycling comprised a 4 min at 95°C initial denaturation, followed by 30 cycles of 94°C for 30 sec (denaturation), 57.6°C for 30 sec (annealing), and 72°C for 1 min (extension), and a final extension at 72°C for 7 min.

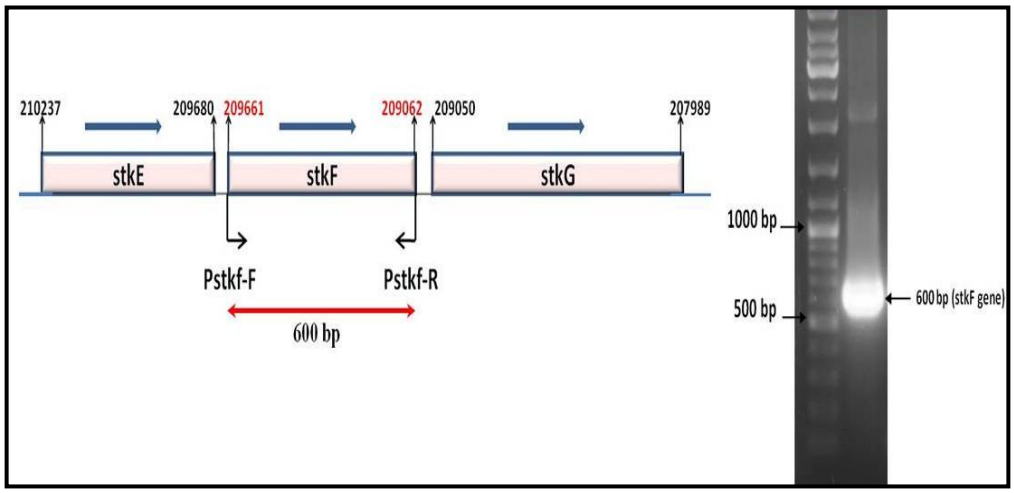


Figure 3.5.1: *stkF* gene full-length PCR amplification. Agarose gel (0.8 %) electrophoresis of PCR amplicon of full-length of *stkF* gene (600 bp). The schematic diagram showing the position of expression primers Pstkf-F and Pstkf-R used in *stkF* PCR amplification. GeneRuler™ DNA molecular weight marker (100 − 10,000 bp).

3.5.2 Cloning of *stkF* into pGEM®-T Easy vector

The pGEM®-T easy vector is a HCN intermediate plasmid, which is linear and fitted with a 3' terminal T base overhang on either end to facilitate cloning of an A-tailed PCR product (TA cloning). The PCR-amplified fragment bearing *stkF* was recovered from the agarose gel and subjected to A-tailing following the Promega protocol. The A-tailed PCR amplified *stkF* sequence was then cloned into pGEM-T easy vector by ligation reaction using T4 DNA Ligase enzyme. Two µl of the ligation mix was then used to transform chemically competent *E. coli* DH5α. Positive clones were selected by white/blue screening on LA plates containing amp 100 µg/ml + X-gal 40 µg/ml. Putative positive colonies were confirmed by colony PCR followed by isolation of the recombinant pGEMstkF plasmid and *Eco*RI restriction analysis.

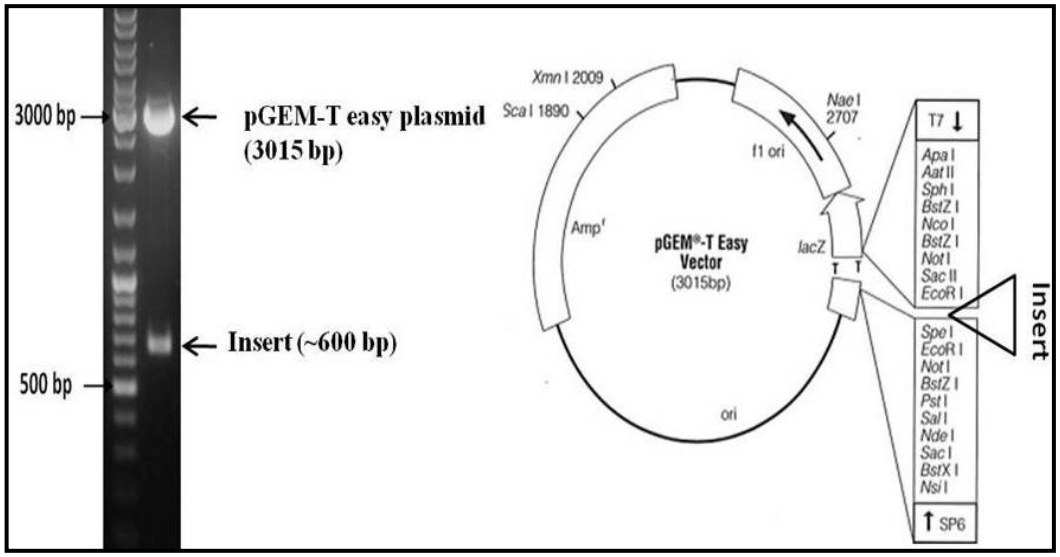


Figure 3.5.2: Agarose gel (0.8 % agarose) electrophoresis of *Eco*RI restriction analysis of pGEMstkF plasmid. The restriction digestion confirmed the cloning as the vector contains the insert appeared in two bands after digestion, the vector at 3015 bp and the insert at ~600 bp. pGEM®-T Easy plasmid map is on the right side. GeneRuler™ DNA molecular weight marker (100 − 10,000 bp).

3.5.3 Construction of rStkF over-expression plasmid

The pET23a is a pBR322, f1-derived expression vector that induces high protein expression under control of T7 promoter which is activated by binding to T7 RNA polymerase, which in turn is induced by isopropyl-D-thiogalactopyranoside (IPTG). This vector allows expression of the desired insert as a fusion protein with a C-terminal poly-His tag to facilitate detection of the expressed protein as well as purification. The *orf* of *stkF* amplified from *S.* Paratyphi A SARB 42 was sub-cloned from pGEMstkF into pET23a vector between *Nde*I and *Bam*HI restriction sites originating the corresponding pETStkF expression plasmid.

3.5.3.1 Preparation of insert (*stkF*) and vector (pET23a)

Both pGEMstkF plasmid and pET23a expression vector were subjected to restriction double digestion using the restriction enzymes *Bam*HI and *Nde*I to release *stkF* fragment and prepare pET23a, respectively. Both the insert (600 bp) and linearized pET23a vector (3666 bp) fragments were recovered from agarose gel and used in ligation reaction.

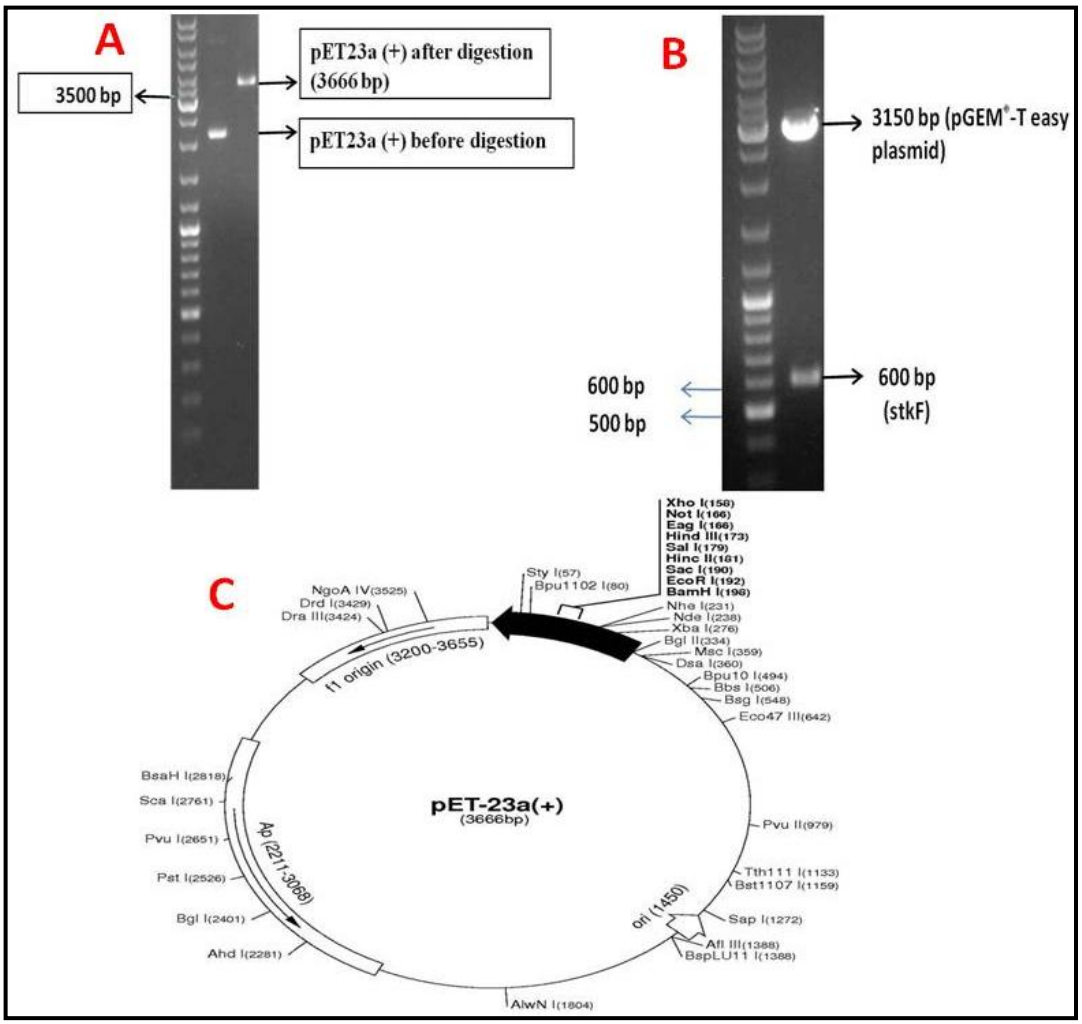


Figure 3.5.3: Preparation of the fragments bearing the *orf stkF* gene and the pET23a expression vector for ligation. Agarose gel (0.8 %) electrophoresis of *Bam*HI and *Nde*I restriction double-digestion of: **A,** pET23a vector, the digested linearised plasmid appeared in one band at the accurate size of vector (3666 bp); **B)** pGEMstkF recombinant plasmid, the insert band appeard at 600 bp; **C)** The pET23a (+) plasmid map. GeneRuler™ DNA molecular weight marker (100 − 10,000 bp).

3.5.3.2 Sub-cloning of *stkF* into pET23a (+) expression vector

The fragment recovered from the gel after restriction double digestion of pGEMstkF was ligated into the corresponding cloning sites in linearised pET23a vector using T4 DNA ligase enzyme. The ligation mix was then used to transform chemically competent *E. coli* DH5α. Positive clones were selected by antibiotic selection on LA plates

containing amp 100 µg/ml The positive clones were identified by colony PCR, plasmid isolation and restriction analysis using *Bam*HI and *Nde*I. DNA sequencing by T7 primer showed that the sequence obtained was 100 % matching the published *orf* of *stkF* gene in *S*. Paratyphi A str. ATCC 9150 (GeneBank database) downstream to T7 promoter.

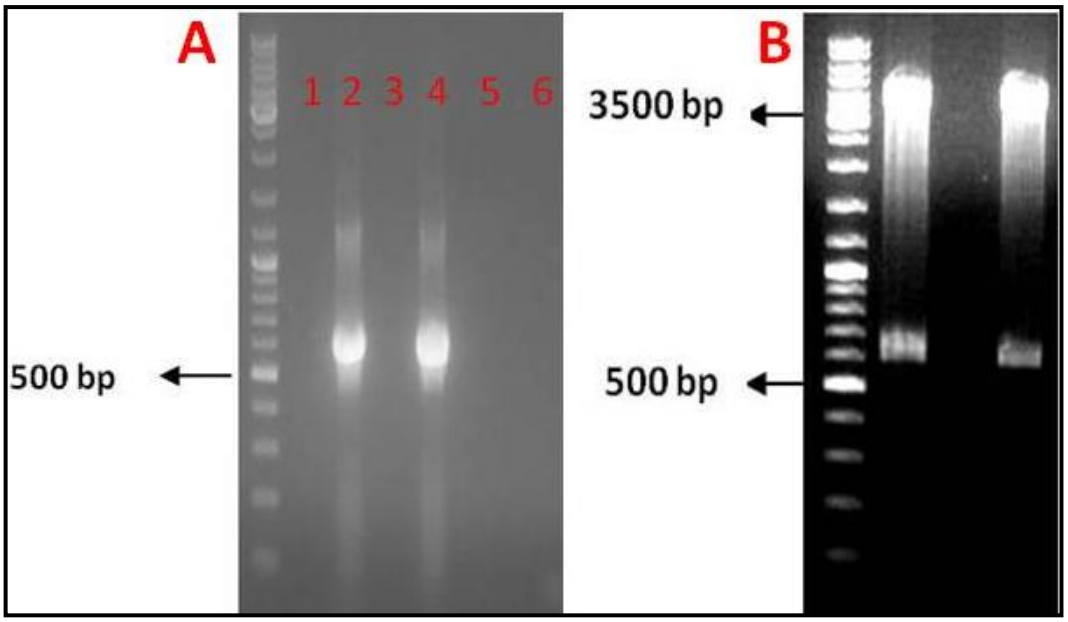


Figure 3.5.4: Analysis of pETStkF transformants (antibiotic selection). A, Colony PCR of six colonies using specific insert primers (Pstkf-F&Pstkf-R), two colonies showed the expected amplicon (600 bp); **B**, Plasmid miniprep for the two PCR positive colonies and restriction analysis using *Bam*HI and *Nde*I. GeneRuler™ DNA molecular weight marker (100 − 10,000 bp).

3.6 Recombinant StkF (rStkF) protein expression

3.6.1 Pilot protein expression experiment

The pETStkF expression plasmid (C-terminal His_6-tagged *stkF* gene (ORF) in a pET-23a (+) vector, amp resistant) was used to transform chemically competent *E. coli* BL21 (DE3)pLysS and *E. coli* BL21 (DE3) expression cells by heat shock transformation; however no expression occurred in *E. coli* BL21 (DE3). The bacterial cells were grown o/n on LA plates containing amp 100 µg/ml + CML 34 µg/ml at 37°C. The preliminary protein expression experiment was carried out using the pETStkF carrying *E. coli* BL21 (DE3)pLysS in LB broth contains antibiotics (amp 100µg/ml + CML 34 µg/ml), incubated at different temperatures (20°C, 25°C, 30°C, and 37°C) and 200 rpm. When the OD_{600} of the bacterial culture reached 0.5 – 0.6, the cells were induced by adding 1 mM IPTG inducing agent, the optimum concentration for expression. In addition, 37°C was the optimum temperature giving high yield of protein expression. In addition, the expression of rStkF was detected 6 h after IPTG induction and the amount of the recombinant protein increased in correlation with duration of induction as shown by the thickness and intensity of protein bands in Coomassie blue stained SDS–PAGE gels. The optimum time yielded the highest amount of the expressed protein was 16 h after IPTG addition (overnight expression). In addition, to confirm the identity of the expressed protein in the cell pellets samples, Western blot analysis was performed using anti-polyhistidine (HRP-conjugated) monoclonal antibody (Sigma-A7058). The recombinant StkF protein (rStkF) band appeared at its expected molecular weight after 6 h of IPTG induction (Fig. 3.6.1).

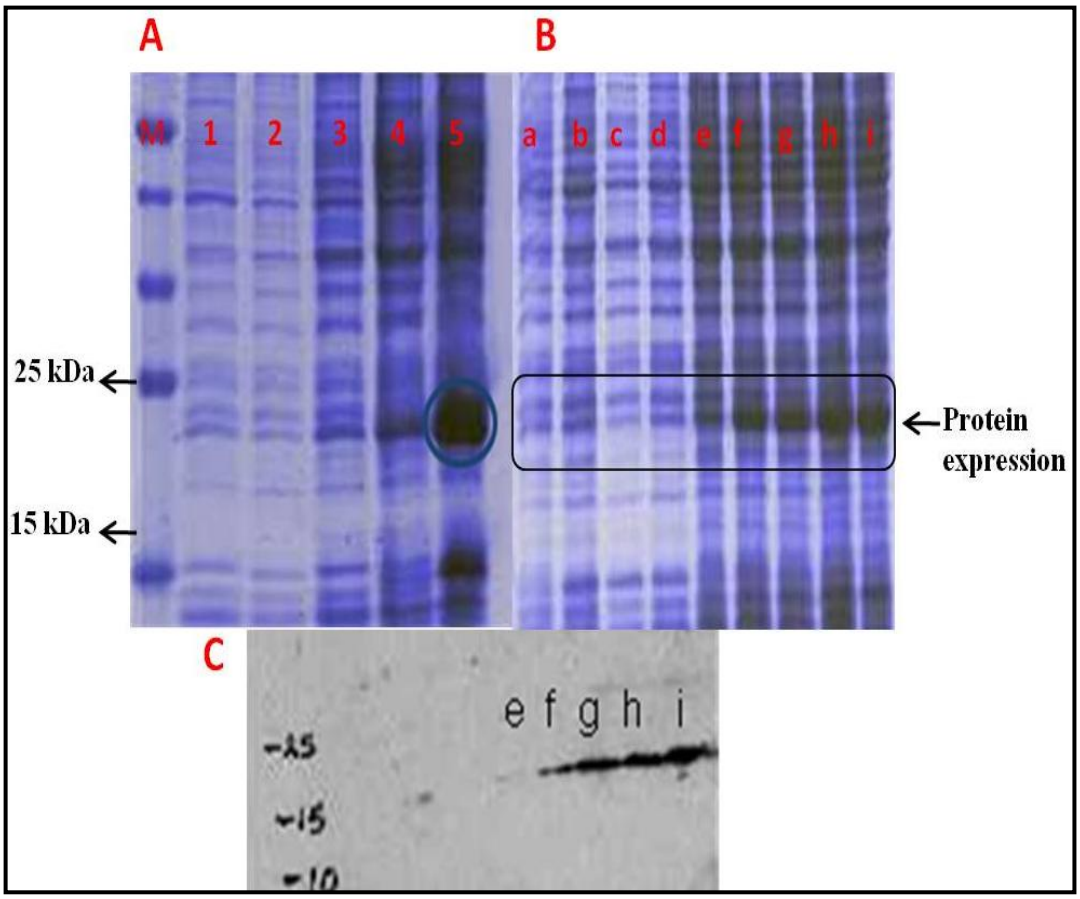


Figure 3.6.1: Pilot expression of rStkF protein. The ORFof *stkF* gene was expressed in BL21 (DE3)pLysS cells using 1 mM IPTG. An aliquot of 20 µl of each sample (re-suspended cell pellet) was mixed with 5 µl of 5× SDS–PAGE loading dye and boiled for 5 min at 100°C, then 20 µl of each protein cell pellet sample was loaded onto SDS–PAG (12 %) for analysis of expression by SDS–PAGE and gel staining with Coomassie blue. **A,** Expression of the recombinant protein at different temperatures using 1 mM IPTG: lane 1, pre-induction, lanes 2 – 5, induction at 20°C, 25°C, 30°C, and 37°C, respectively. **B,** Expression of the recombinant protein at 37°C: protein samples of cell pellets harvested at different time points. Lane a, pre-induction; lanes b - i, expressions at 1, 3, 5, 6, 7, 8, 9 and 16 h after 1 mM IPTG induction. The head of the black arrow points to over expression bands of rStkF protein (~23 kDa). **C,** Western blot analysis of expressed rStkF in *E. coli* cell pellets using monoclonal anti-polyhistidine HRP conjugate antibody. The expression started 6 hours after IPTG induction and increased with time. Lane M, PageRuler™ Prestained protein M.W. marker.

3.6.2 Large scale production of rStkF protein

The rStkF protein was produced on large scale for purification following the steps of preliminary protein expression and optimum conditions for its expression, however using larger volumes of transformed bacterial culture.

3.6.3 Protein solubility examination

A cell pellet obtained from *E. coli* BL21 (DE3)pLysS / pETStkF after o/n IPTG-induced expression was treated following the steps mentioned at Chapter 2 (section 2.13) to check expressed StkF protein solubility. The Coomassie blue stained SDS–PAGE gel showed that the rStkF protein was soluble as the protein was found in the supernatant aliquot but not in the insoluble cell debris aliquot.

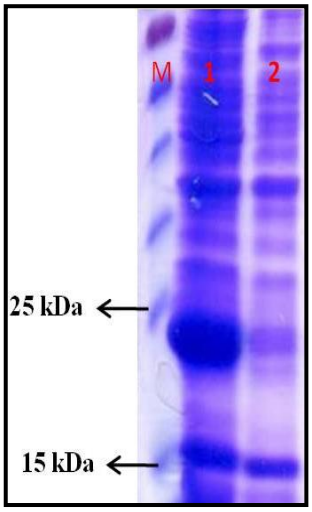


Figure 3.6.2: Coomassie blue stained SDS–PAGE (12 %) gel loaded with cell lysate supernatant (soluble rStkF protein fraction) and cell debris (insoluble rStkF protein fraction) samples. Lane M, PageRuler™ Prestained protein M.W. marker; lane 2, cell lysate supernatant sample (20 µl); lane 3, re-suspended cell debris sample (20 µl).

3.7 Purification of His-tagged rStkF

The rStkF-six-His-tagged protein purification was performed by immobilised metal affinity chromatography (IMAC) technology using Ni-NTA column. rStkF protein was purified as soluble protein form the the clarified cell lysate supernant under native conditions using IMAC Ni SepFast BG Resin (Geneflow, UK, P7-0024) and the process was run in an IMAC BG-5, 30 ml Gravity Flow Column (Geneflow, UK) following the gravity flow purification protocol. The clarified cell lysate supernant was loaded onto Ni-NTA affinity column which had been pre-equilibrated with binding buffer (20 mM Na_2HPO_4, 500 mM NaCl and 0.5 mM imidazole, pH 7.4). The resin was washed using washing buffer (20 mM Na_2HPO_4, 500 mM NaCl and 0.5 mM imidazole, 2 % Tween 20, pH 7.4). For elution of the protein, elution buffers with varied imidazole concentratins (5 - 50 mM) were used. Highly purified rStkF-six-His protein was eluted in 20 mM imidazole containing elution buffer (20 mM Na_2HPO_4, 500 mM NaCl and 20 mM imidazole, pH 7.4). A single band at approximately 23 kDa corresponding to the predicted M.W. of the rStkF was verified by SDS–PAGE (12 %) followed by Coomassie blue staining. The purified protein was further confirmed by Western blot analysis using anti-His-tag monoclonal antibody (Fig. 3.7.1).

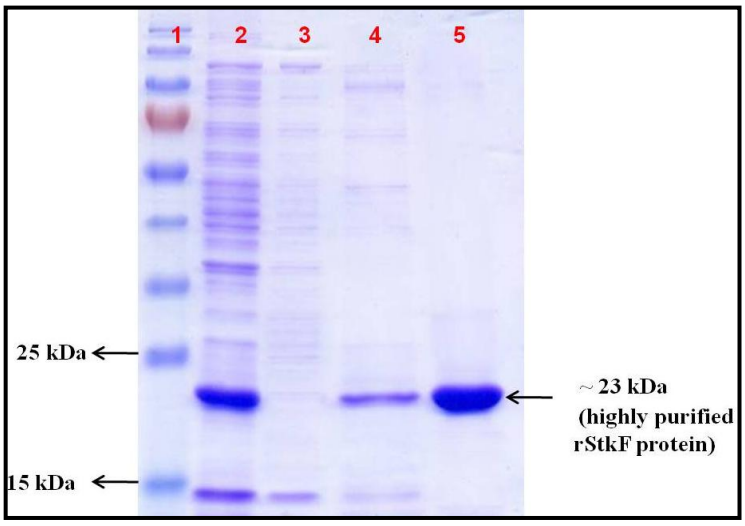


Figure 3.7.1: SDS–PAGE analysis of protein purification process of rStkF-6-His-tagged using IMAC. Coomassie blue stained SDS–PAGE gel (12 %) loaded with 20 µl of the eluted fractions from the purification steps. Lane 1, PageRuler™ Prestained Protein M.W. marker; lane 2, BL21 bacterial clarified cell lysate before purification steps; lane 3, cell lysate after passing through the column; lane 4, column wash with washing buffer containing 0.5 mM imidazole; lane 5, highly purified eluted rStkF protein with 20 mM imidazole containing buffer.

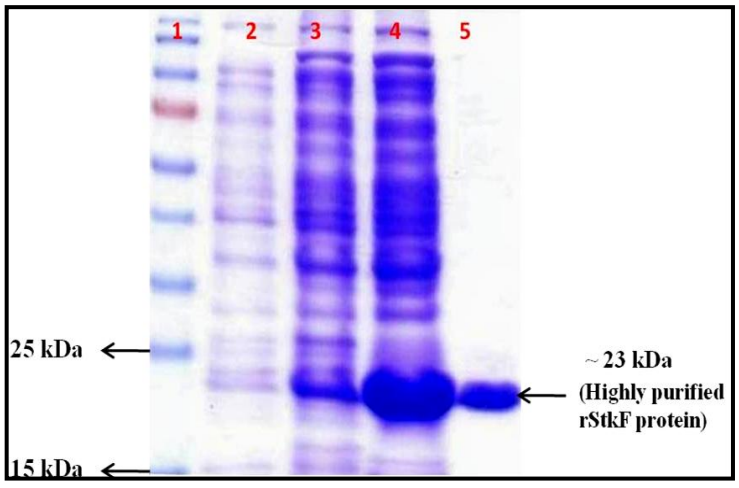


Figure 3.7.2: Collective SDS–PAGE (12 %) of rStkF protein expression and purification (optimum conditions). Lane 1, PageRuler™ Prestained Protein M.W. marker; lane 2, BL21 bacterial cell lysate before induction; lane 3, BL21 bacterial cell lysate 6 h after IPTG induction; lane 4, BL21 bacterial cell lysate o/n after IPTG induction; lane 5, highly purified rStkF protein.

3.7.1 Protein dialysis and concentration of highly purified rStkF

The rStkF protein was dialysed against a physiological buffer (20 mM Tris HCl and 145 mM NaCl, pH 7.4) for use in mice immunisation using two different procedures. Protein dialysis using a Snakeskin pleated dialysis membrane (Thermo Scientific-68100-cut-off of 10 kD), followed by protein solution concentration using Amicon® Ultra-15 centrifugal filter (Millipore, UK- cut off 3 kDa). Alternatively, the Amicon® Ultra-15 column was used for buffer exchange and concentration as well. The two methods were comparable as loss of protein is insignificant with both methods. Then, the protein concentration was determined by a Bradford assay using BSA as standard and adjusted to the required concentration for immunisation (1 mg/ ml).

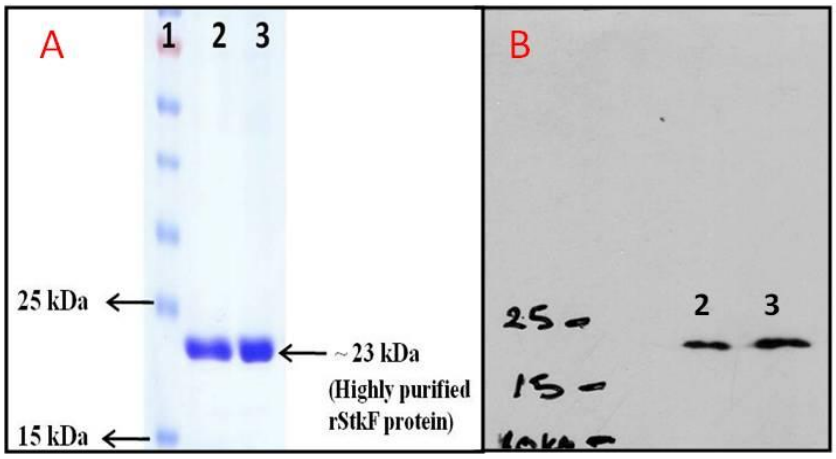


Figure 3.7.3: rStkF protein dialysis and concentration. **A,** Coomassie blue stained SDS–PAGE (12 %); Lane 1, PageRuler™ Prestained Protein M.W. marker; lane 2, 20 µl of protein solution dialysed using dialysis membrane then the concentration using Amicon® Ultra centrifugal filter column (Millipore); lane 3, 20 µl of protein solution dialysed and concentrated using Amicon® Ultra centrifugal filter column; **B,** Western blot analysis of purified rStkF dialysed and concentrated by the two procedures using anti-His-Tag® HRP conjugate monoclonal antibody; Lane 2 and 3 are parallel to these in A.

3.8 Cloning of full length of *stkF*^{211bp} allele into pET28a vector

Briefly, the full-length of *stkF*^{211bp} gene OFF form was PCR amplified from *S.* Paratyphi A PA 42, then cloned intermediately into pGEM-T easy plasmid (pGEMstkFoff) and finally into expression plasmid pET28a (+), which express recombinant protein with N-terminal His-tag. The construct pETStkF-Off was then transformed in *E. coli* BL21 (DE3) expression hosts; however there was no detectable protein expression, which could indicate that this form of *stkF* does not produce an intact full length StkF protein.

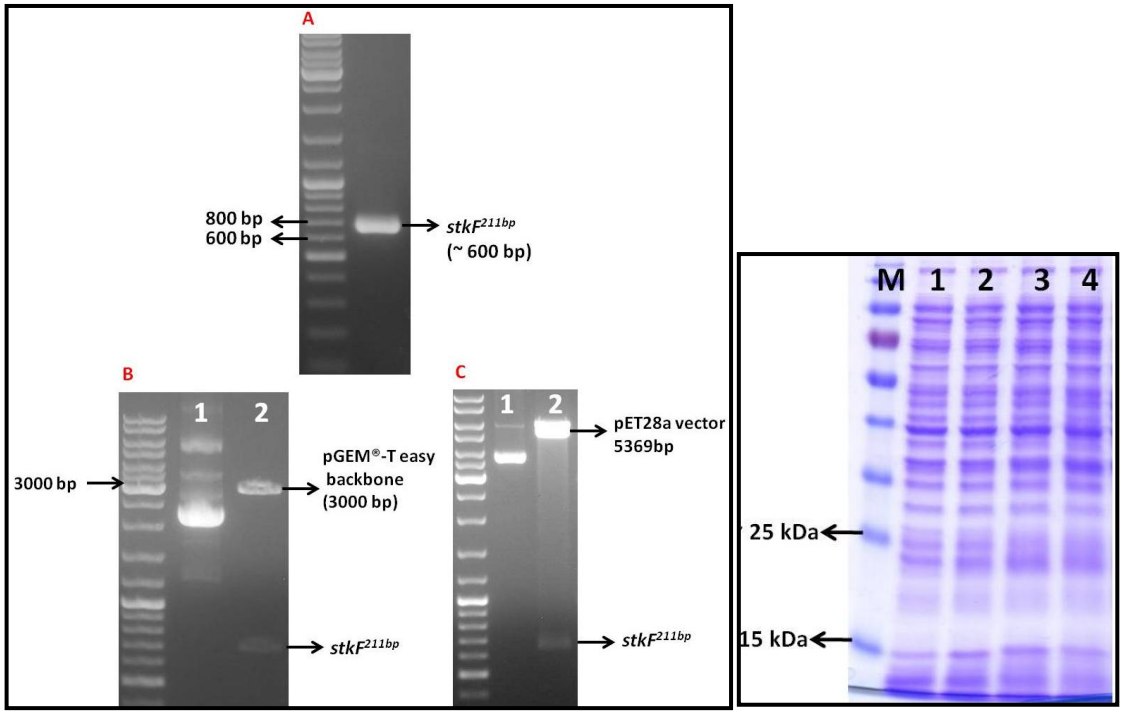


Figure 3.8.1: *stkF*^{211bp} gene full-length PCR amplification and cloning. Agarose gel (0.8 %) electrophoresis of: **A,** PCR amplicon of full-length of *stkF*^{211bp} gene (~600 bp); **B,** *Eco*RI restriction analysis of pGEMstkFoff plasmid; **C,** Analysis of pETStkFoff transformants by restriction analysis using *Bam*HI and *Nde*I. The SDS–PAGE (12 %) shows no protein expression: lane 1, pre-induction; lane 2, 1h; lane 3, 6 h; lane 4, 16 h after IPTG induction. GeneRuler™ DNA molecular weight marker (100 − 10,000 bp).

3.9 Discussion

A better understanding of the nature and types of surface structures, such as fimbriae, expressed by *Salmonella* genus will be valuable for developing new diagnostic assays and for identification of protective antigens as potential vaccines (Thorns *et al.*, 1990). *stkF* gene is a part of *stk* fimbrial gene cluster (*stkABCDEFG*) which was reported to be conserved to *S.* Paratyphi A (Edwards *et al.* 2002, Porwollik & McClelland, 2003). On the contrary, a recent study showed that several other *Salmonella* serovars, including *S.* Kentucky and *S.* Heidelberg, also possessed *stk* homologue (Bronowski & Winstanley, 2009). However, experimental studies to identify the putative Stk fimbriae were lacking, emphasizing the need for investigation and characterisation of the predicted Stk fimbrium and its subunits (Bronowski & Winstanley, 2009).

The work in this chapter focused on further characterisation of the StkF subunit of the hypothesized Stk fimbrial structure. StkF had been annotated as a predicted fimbrial adhesin-like protein or a fimbrial subunit protein (Ou *et al.*, 2007). Cloning, expression and purification of this protein in *E. coli* (BL21) was achieved by PCR amplification of s*tkF* from *S.* Paratyphi A SARB 42 and introduction of this gene into the pET23a expression vector. The recombinant His-tagged StkF protein was then expressed in *E. coli* BL21 (DE3)pLysS at 37°C and purified using Ni affinity chromatography under native conditions as it was expressed as soluble protein. The expressed and purified protein were identified by Western blot using anti-His-Tag® HRP conjugate antibody. However, there was no expression when the recombinant pETStkF expression plasmid transformed into *E. coli* BL21 (DE3) as expression host or when the PCR product was cloned into pET28a, followed by transformation into both *E. coli* BL21 expression hosts. This is consistent with the recognized observation that efficient expressions of

several recombinant proteins require particular combinations of vector, host strain, and culture conditions. Consequently if a high yield of a recombinant protein is needed, it is worth testing several different vector / host and culture combinations to find the optimal combination. In case of StkF, optimum expression was achieved using pET23a expression vector and *E. coli* BL21 (DE3)pLysS as a host, which has the added advantage of preventing basal expression of protein, a particular advantage if the protein is toxic to host cells. In addition, o/n IPTG induction and growth at 37°C was found to yield the highest amount of protein (i.e. overnight expression). The highly purified protein was then examined as a potential diagnostic tool and vaccine candidate against *Salmonella* infections. In addition, there was no expression when the full sequence of *stkF*[211] gene from *S.* Paratyphi A strain PA42 was cloned intermediately into pGEM-T easy plasmid (pGEMstkFOff) and finally into pET28a expression vectors to produce pETStkFOff, then transformed and expressed in *E. coli* BL21 expression hosts following addition of IPTG which could indicate the expression of truncated unstable protein.

Both *stkF* DNA sequence and StkF protein amino acid sequence were characterised based on bioinformatics analyses in an attempt to identify both the function and cellular location of this protein. The BLAST (blastn and blastp) analyses of DNA and amino acid sequences against the non-redundant database of GenBank identified a number of identical and/or highly similar hits in the genomes of several *Salmonella enterica* serovars, such as Virchow, Hadar, Kentucky, and Tennessee (Table 3.2-1). In addition, PCR investigation showed that *stkF* and associated immediate flanking sequences in *stk* operon were present in 15 of the 59 *Salmonella* strains (~25 %) or ~30 % of total serovars examined (Table 3.3-1). Collating data from GenBank,

the Ou et al. (2007) study and the present work, *stkF*, and by likely extrapolation the *stk* operon, is now known to be found in ~30 % of the *S. enterica* serovars for which genome sequence and/or PCR data are available.

Homologuos proteins often share similar three-dimensional shapes and/or perform identical or related functions. There are two subclasses of homologes: orthologes (homologues proteins in different species) and paraloges (homologous proteins in the same organism) (Eddy, 2009, Margelevicius & Venclovas, 2010). Based on bionformatics analysis, StkF (GenBank accession AAV76213, *S.* Paratyphi A ATCC 9150), was annotated as a predicted fimbrial adhesin-like protein. Similarly, the *E. coli* MG1655 YadK which was identified as an orthologe of StkF protein (Fig. 3.2.6) had also been annotated as a predicted adhesin-like protein. Prediction of the sub-cellular localisation of StkF strongly suggested that it is an extracellular protein but its amino acid sequence has no recognizable trans-membrane helices. In addition, the protein domain superfamily prediction revealed that StkF contains one domain related to the bacterial adhesin superfamily and the closest structure in that database for this domain was the mannose-specific binding domain of the *E. coli* type 1 fimbrial adhesin FimH. These bionformatics analyses outputs on the predicted function and sub-cellular localisation of StkF strongly support its assignment as a fimbrial adhesin subunit. Thus, StkF could contribute to host adhesion of *S.* Paratyphi A and other serovars carrying this protein through binding to mannose containing receptors.

3.9.1 The stkF gene phase variation hypothesis

Phase variation can occur at a translational level by different mechanisms, including slipped-strand mispairing (SSM). Translation of a protein can be affected by SSM if the tandem repeats are located within its coding sequence leading to disruption of the open reading frame and production of a non-functional, usually truncated protein because of formation of a premature stop codon (van der Woude & Baumler, 2004, Deitsch *et al.*, 2009). Ou et al. (2007) used the *stkF* gene as a specific target in developing a multiplex PCR based method for identification of *S.* Papartyphi A. They designed internal *stkF* primers, which were used in this study as well, that produced a 159 bp PCR product. However, when these primers were tested in multiplex PCR against 52 Paratyphi A strains, 21 % of these strains produced a fragment of ~185 bp instead. One example of this larger amplicon was sequenced and found to be 185 bp in length as a result of a duplication of the 16 bp spacer and an additional 10 bp DR. This additional 26 bp insertion would mean the gene was in an OFF form that was predicted to produce a truncated (non-functional) protein of 46 aa only (Spa0180v1. The observed *stkF* polymorphism suggested the possibility of two distinct lineages ($stkF^{159bp}$ and $stkF^{180bp}$ alleles) (Figs. 3.9.1, 3.9.2).

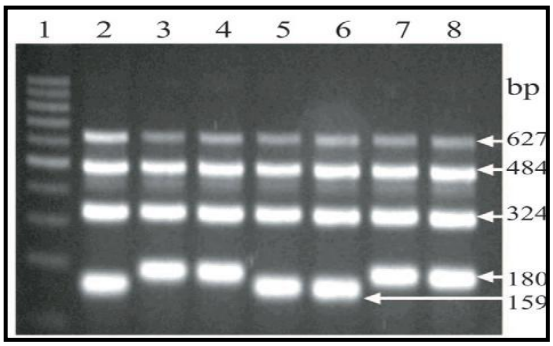


Figure 3.9.1: *S.* Paratyphi A specific multiplex PCR assay. Reproduced from Ou et al. (2007) Agarose gel electrophoresis of mPCR targeting *stkF* (*spa0180*), *spa2473*, *spa2539*, and *spa4289* of *S.* Paratyphi A strains that produces amplicons of 159 or ~180 bp, 324 bp, 484 bp, and 627 bp, respectively (Ou *et al*., 2007).

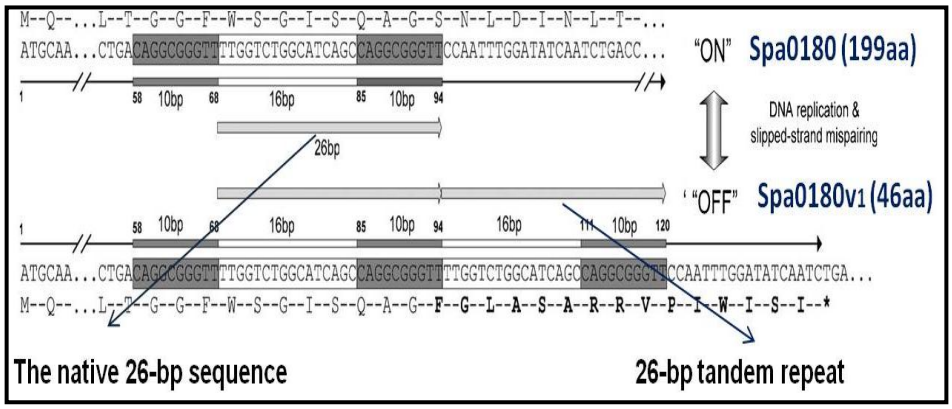


Figure 3.9.2: StkF (Spa0180) polymorphism and the hypothesized model for low frequency fimbrial phase switching (Ou *et al*., 2007). Adapted from Ou et al. (2007).

Based on the observed *stkF* PCR results, Ou et al. (2007) hypothesized that there were two forms of this gene, an ON and OFF form. The ON form had a single 26 bp native sequence that comprised a 16 bp spacer and one of the two flanking 10 bp perfect direct repeats (DRs). By contrast the OFF form possessed a perfect tandem repeat of this 26 bp sequence and consequently an additional 16 bp spacer sequence and a third 10 bp DR. The predicted translational products of the ON and OFF forms of the *stkF* gene corresponded to a full-length 199 aa functional StkF protein and a truncated,

almost certainly non-functional 46 aa protein, respectively (Ou *et al*., 2007)**.** However, in this study, the *stkF* PCR investigation of 121 *S.* Paratphi A strains as performed by Andrew Rosser failed to detect any *stkF*^{180bp} OFF form but identified a single instance of a third form which had three perfect 26 bp tandem repeats. Of 121 *S.* Paratphi A strains in this study, 120 possessed the ON form *stkF*^{159bp} allele and one possessed this new allele (*stkF*^{211bp}). The *stkF*^{211bp} would also constitute an OFF form allele as its predicted product would be a truncated 60 aa protein (Spa0180v2). Given the lack of evidence that these *stkF* alleles mapped to distinct *S.* Paratyphi A lineages, the closely spaced 10 bp direct repeats in *stkF* (Figs. 3.3.4, 3.9.2 and 3.9.3) would suggest that slipped-strand mispairing and/or recombination would seem to be a likely mechanism for independent mutation events instead. Since this type of mutation is frequently reversible, an alternative hypothesis would be that the observed polymorphism reflected low frequency switching between the three *stkF* alleles. The observed 26 bp intragenic duplication or triplication event may be mediating a form of phase switching at the translational level resulting in a premature stop codon, as was originally suggested by Ou et al. (2007). Indeed, such a phenomenon may underpin immune evasion by turning OFF a key fimbrial structural protein (Ou *et al.*, 2007). In accordance with the above idea, whole cell lysates of *S.* Paratyphi A strains PA105 and PA42 were shown via IP followed by Western blot analysis using murine StkF antiserum to produce or not to produce surface-exposed native StkF protein, respectively (Fig. 3.4.5).

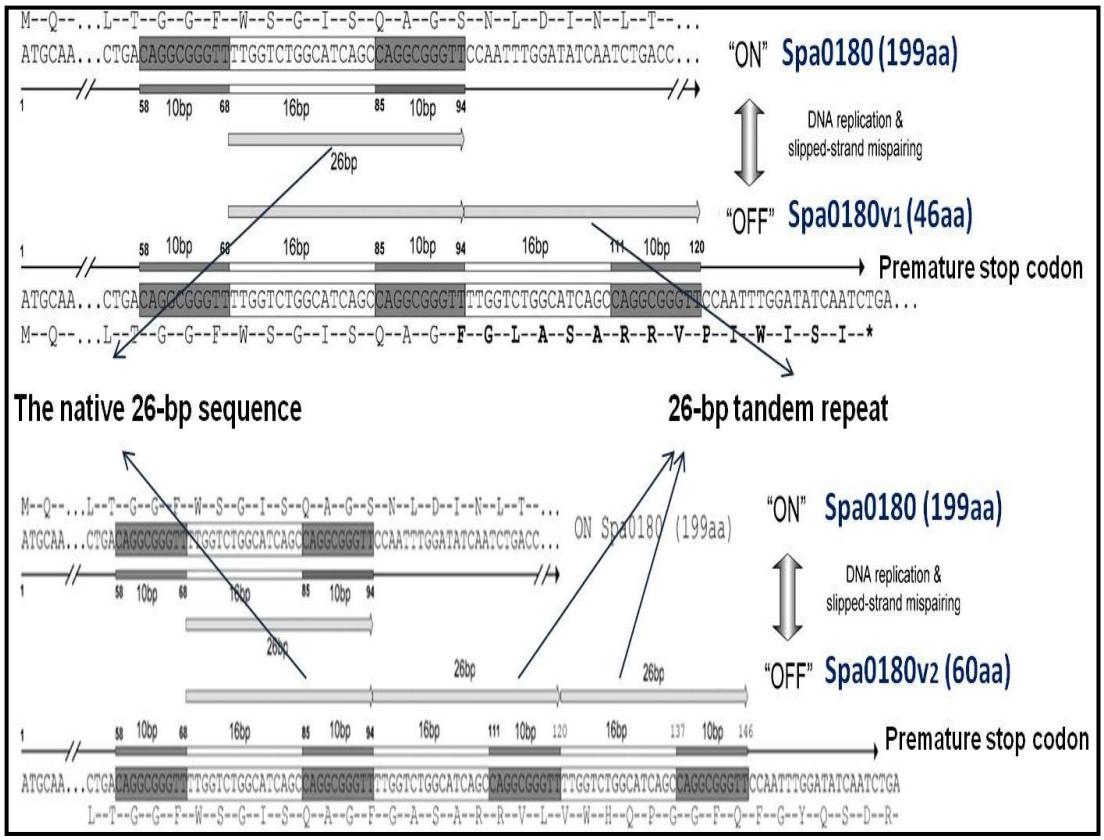


Figure 3.9.3: The *stkF* polymorphism and the hypothesized model for low frequency fimbrial phase switching. Adapted from, Ou et al. (2007) with some modifications according to the data of Andrew Rosser. The observed polymorphisms of *stkF* and the proposed low frequency switching between the three *stkF* alleles. The ON form ($stkF^{159bp}$) would be predicted to produce a native full-length StkF protein (199 aa), while the two OFF forms, $stkF^{180bp}$ and $stkF^{211bp}$, would produce truncated proteins of 46 aa and 60 aa, respectively.

Chapter Four

Use of recombinant StkF in mice immunisation and vaccine development

4 Use of recombinant StkF in mice immunisation and vaccine development

4.1 Introduction

Vaccination and immunization are often used interchangeably. However, by strict definition the former denotes only the administration of a vaccine or toxoid, whereas the latter describes a state of inducing or providing immunity whether by active or passive means. Vaccines may contain attenuated or killed microorganisms, bacterial toxoid or antigenic material from particular parts of the infecting organism, which can be derived from the organism itself or produced by recombinant DNA technology (Kishore *et al.*, 2008). Vaccines offer a safe, successful, and cost-effective tool for protection against pathogenic microorganisms. Several vaccination programs have been able to prevent or reduce the morbidity and mortality caused by diverse infectious diseases. Immunization against smallpox has eliminated the causative virus from the human population, while efforts directed at polio have markedly reduced the incidence of this disease, although completely eradicated it in many parts of the world (Yang *et al.*, 2011). Identification of protective antigens remains the key challenge for vaccine development against infectious diseases. Protective antigens are those antigens that are specifically targeted by the acquired immune response of the host, and when introduced into the host are able to stimulate the production of antibodies and/or cell-mediated immunity against the target pathogen (Yang *et al.*, 2011).

This chapter describes the immunogenicity of rStkF protein in mice and evaluates its efficacy as a potential protective antigen against *Salmonella* infections, especially those caused by serovar Paratyphi A.

4.2 Testing the immunogenicity of rStkF in BALB/c mice

The immunogenicity of the highly purified rStkF protein was tested in three BALB/c mice, numbered 493, 494 and 495, following the immunisation protocol mentioned in Chapter 2 (section 2.26). The StkF antiserum raised was used to determine the immunogenicity of the purified rStkF by indirect ELISA. Serum samples were collected from the three immunised mice before the course of immunisation (pre-bleeding or pre-immunisation serum) to serve as negative controls in subsequent experiments. In addition, serum samples were collected at three weeks (first bleeding serum) and five weeks (terminal bleeding or post-immunisation serum) after the first immunisation. StkF specific IgG immune response was assayed in these serum samples by ELISA using serial dilutions of the mice sera ranging from 1/100 to 1/12,800 as described in Chapter 2 (section 2.22). These ELISA results showed that StkF-specific IgG antibodies were detectable in the sera of the immunised mice as early as three weeks after the first dose of rStkF and the intensity of the immune response against rStkF immunisation increased following the subsequent booster doses. In addition, the ELISA results showed that post-immunization mice sera (or terminal bleeding serum samples) reacted against rStkF even at the higher serum dilutions as still giving high detectable signal at the highest serum dilution used, denoting a good immunogenicity of this antigen. There was significant difference between the three collected serum samples for each mouse indicated by three asterisks $P < 0.0001$ using one-way ANOVA.

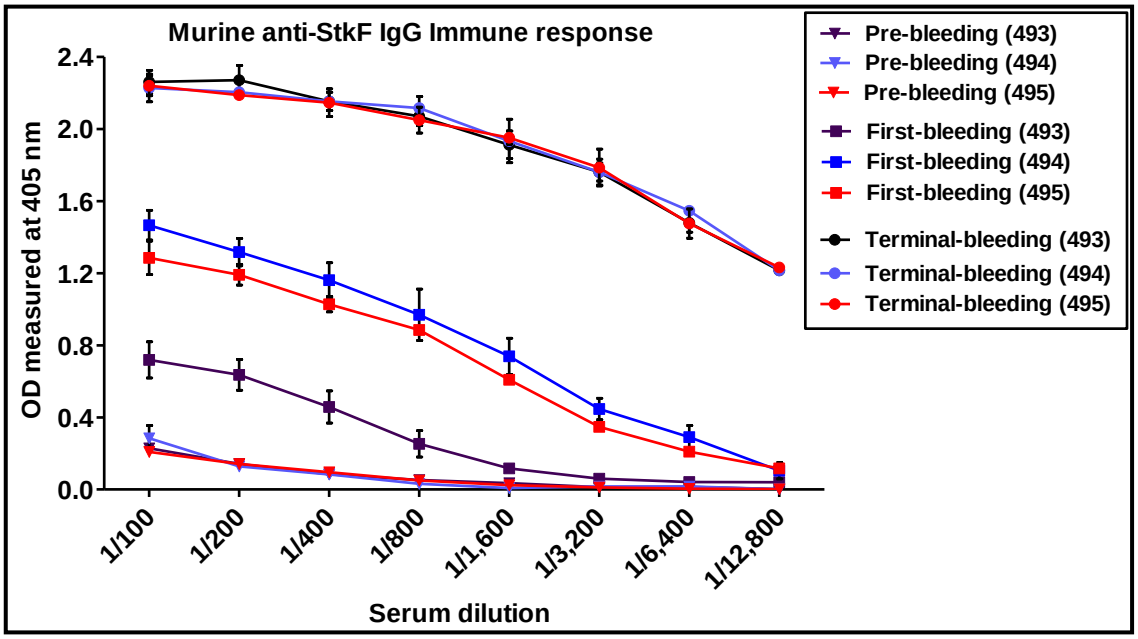


Figure 4.2.1: Preliminary anti-StkF specific IgG immune response detection in the immunised mice sera. Indirect ELISA results comparing the immune IgG response of the three rStkF Balb/c immunised mice in pre-bleeding (pre-immunisation), first bleeding and terminal-bleeding (post-immunization) mice sera collected at 0 day and on three weeks and five weeks after first dose of immunisation with rStkF, respectively. There was a good IgG response in two mice and a moderate response in the third one in the first bleeding sera. However, in the terminal-bleeding sera, results showed an equal good IgG response in the three mice and higher dilutions were still giving high detectable signal. Each point represents the mean of three independent assays $\pm$ SE of each serum dilution. Using one-way ANOVA of repeated measures, there was significant difference between the three point time samples for each mouse indicated by three asterisks $P < 0.0001$. ELISA plates (Nunc Maxi-Sorp Immuno Plate-469264) were coated with rStkF protein and incubated with different dilutions of pre-, first- and terminal- bleed sera in duplicates. The anti-mouse IgG–AP conjugate was used at a dilution of 1:10,000 (Sigma-Aldrich, A3688) as the secondary antibody. Plates were read by adding the SIGMAFAST™ p-Nitrophenyl phosphate substrate and the OD was measured at λ_{405nm}.

4.2.1 Estimation of anti-StkF IgG titre in mouse serum

The antibody titre of serum is a measure of the quantity of antibody that recognizes a particular epitope or antigen. ELISA titration, a simple method to determine the antibody titre, was performed to detect the anti-StkF IgG titre using serial dilutions of the pre- and post-StkF mouse antiserum. The titre, usually expressed as a ratio, is defined as the highest dilution that binds significantly to the antigen as evidenced by a detectable signal in an ELISA. There was no significant difference in the immune responses of the three mice based on preliminary ELISA results of post-immunisation sera. Thus, the anti-StkF antibody titre was determined by indirect ELISA using pooled pre- and post-immunisation sera of the three mice. The titre was calculated as described in the booklet 'Antibodies: from design to assay practical guide' (Van Regenmortel, 1988), available online at http://www.jenobiotech.com/techsupport/AbDesign/ABI-ChIntro.htm. Fundamentally, this involved the plotting of absorbance at 405 nm (Y-axis) vs. antiserum dilution (from 1/100 to 1/00,000) using the mean ± SE for each triplicate measurments. Then, the anti-StkF antibody titre was calculated from the post-immunisation curve by estimating its inflection point, the point at which the curvature of the curve changes, using GraphPad prism 5 equation (Inflection point = LogXb = LogEC50 + (1/HillSlope)*Log ((2^ (1/S)) -1 of non linear fit five parameters curve analysis) and reading off the OD value on the Y-axis which was 1.029. The titre was then interpolated by drawing a line down to the X-axis. The dilution nearest the inflection point of the standard post-immunisation serum curve was taken as the titre value for the antiserum. Based on this analysis the StkF-specific IgG titre in the pooled post-immunisation sera, obtained one week after the fourth StkF immunisation dose was 1:50,000 which indicated that the StkF antigen was highly immunogenic in mice.

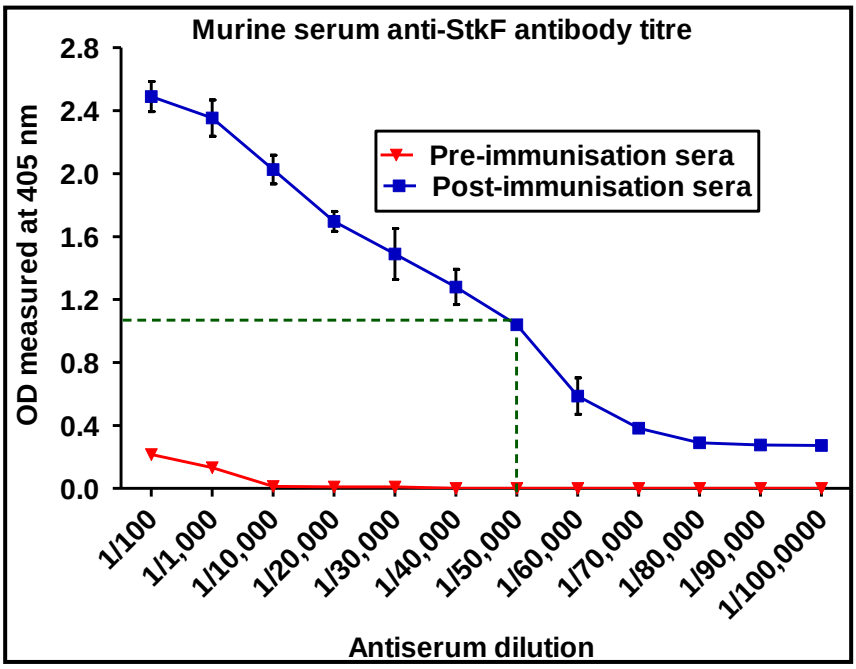


Figure 4.2.2: Anti-StkF IgG titre in mouse serum. Titration of antibody IgG (whole fraction) by indirect ELISA of pooled StkF mouse antiserum samples of one week after the 4th StkF immunisation dose against pre-immunisation serum. The result expressed as the mean of each serum dilution in three independent measurments ± SE. The titre (1:50,000) determined as the nearest dilution to the inflection point (1.029) presented on Y-axis with drawing a line down to the X-axis. There was a statistically significant difference of antibody response between pre- and post-immunisation sera using paired Student's *t*-test at $P = 0.0001$ denoted with three asterisks.

4.3 Comparing the anti-StkF IgG responses at different time points

To assess the immunogenicity of rStkF protein as determined by the time of appearance of specific antibodies in immunised mice. The OD_{405} of the pooled mice sera as well as individual sera of pre-, first- and post- immunisation bleeds were used at a dilution of 1:1,000 as this value lies in the linear part of the post immunisation standard curve against rStkF antigen. It was found that the anti-StkF IgG response significantly increased with time as indicated by three asterisks between the pre-, first- and post-immunisation serum samples.

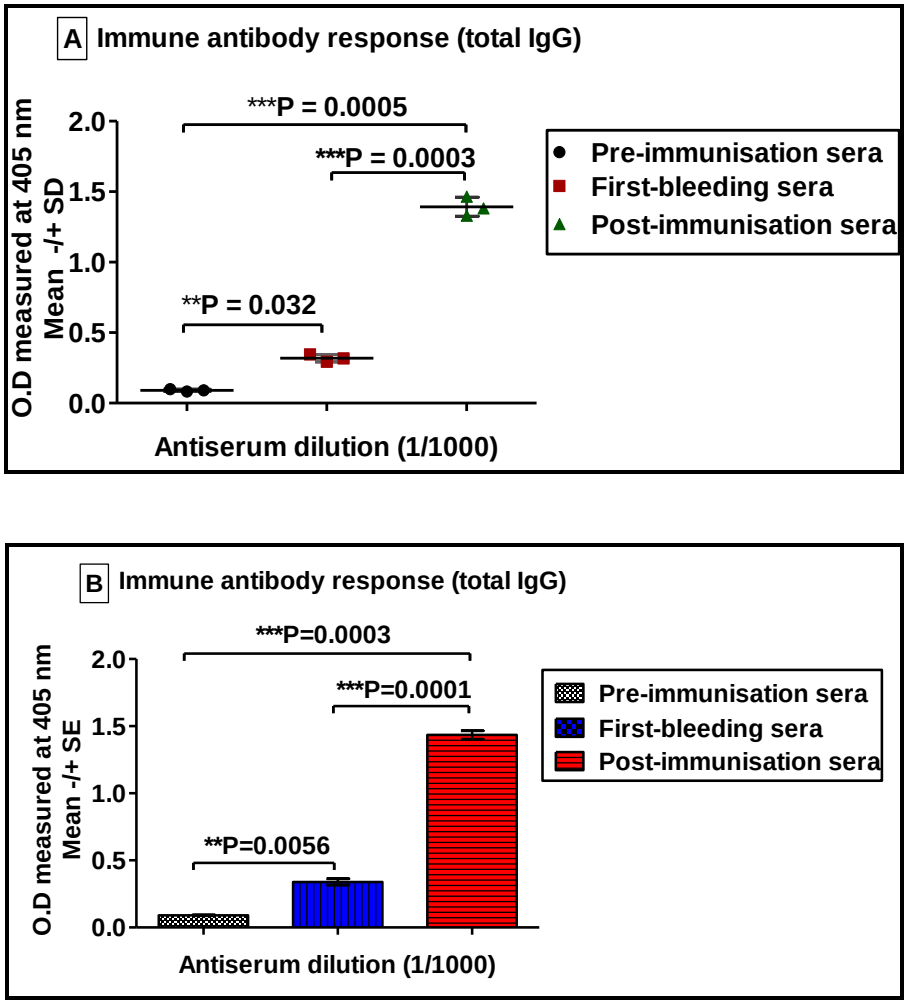


Figure 4.3.1: Immune anti-StkF IgG response at different time points after StkF immunisation in (A) individual mice sera and (B) pooled mice sera. The sera of the immunised mice were tested individually as well as pooled on ELISA plate coated with rStkF. Antibodies against rStkF were detectable three weeks after first rStkF immunisation and the antibody level increased significantly in sera taken at later time points. Results were expressed as mean ± SD of individual mice sera or mean ± SE of three independent assays of pooled sera at each time point. Using paired Student's *t*-test, there was statistically significant difference between the pre-, first- and post immunisation sera. The difference between pre-immunisation sera and first-bleed sera denoted with two asterisks at; **A**, $P = 0.032$ and **B**, $P = 0.0056$, however between post-immunisation sera and both pre- and first-immunisation sera denoted with three asterisks at **A,** $P = 0.0005$ and $P = 0.0003$, respectively**; B,** $P = 0.0003$ and $P = 0.0001$, respectively.

4.4 Type of immune response in mice

T cell immune responses can be divided into those promoting cell-mediated immunity (T helper-1 or Th1) and those promoting humoral immunity (T helper-2 or Th2). This categorization has had a profound effect on understanding immune responses to a wide range of stimuli (Lappin & Campbell, 2000). The ratio of antibodies belonging to the IgG subclasses IgG1 and IgG2a induced by immunization is routinely used as an indirect measure of the relative contribution of Th2-type cytokines versus Th1-type cytokines in the immune response. Higher IgG1/IgG2a ratios point toward a Th2-type immune response (humoral), whereas lower IgG1/IgG2a ratios are indicator of the Th1-dominant immune response (cell-mediated) (Petrushina *et al.*, 2003). To monitor the StkF antigen-specific predominant immune response produced in mice, the levels of IgG1 and IgG2a in the immunised mice sera was determined by ELISA.

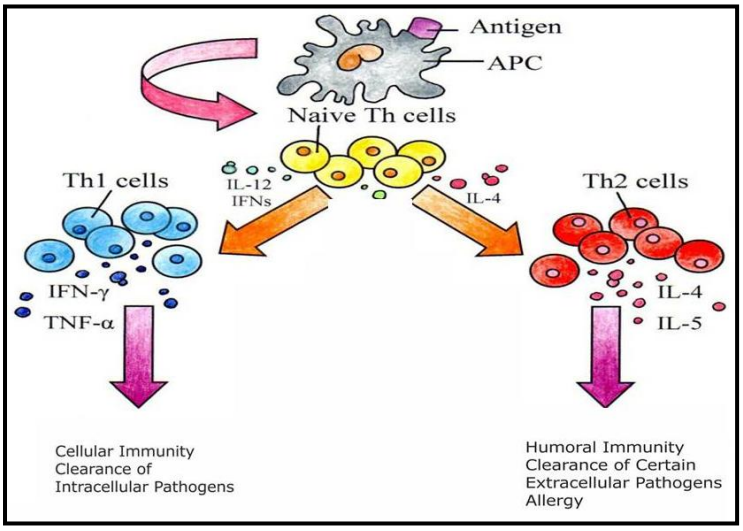


Figure 4.4.1: Schematic view of differentiation of T-helper (Th) cells into Th1 and Th2 cells in response to infectious diseases. Reproduced from D'Elios et al. (2011).

4.4.1 Levels of IgG1 and IgG2a anti-StkF antibodies

For detection of IgG1 and IgG2a anti-StkF antibodies induced after mice immunization with rStkF protein, indirect ELISA was performed using an ELISA plate coated with 1 μg / well rStkF. Pooled and individual mice sera were also assayed at 1:200 dilutions with goat anti-mouse IgG1-AP and goat anti-mouse IgG2a-AP (Southernbiotech) used as secondary antibodies. Using paired *t*-test, the immunised mice sera showed a highly significant increase in IgG1 level in comparison to the pre-immunisation sera; however the pre-and post-immunisation IgG2a levels showed a little difference. In addition, ELISA results showed an extremely significant difference between IgG1 and IgG2a levels in post-immunisation sera (IgG1 > IgG2a) denoted with three asterisks at $P < 0.0001$ using unpaired *t*-test. Thus, IgG1 was the predominant anti-StkF IgG subclass in immunised mice sera indicating the dominance of humoral immune response (Th2) following StkF mice vaccination. These results were further confirmed by measuring the levels of three cytokines (IL-2, IL-4 and INF-γ) in culture supernatant of spleen cells from StkF immunised mice.

4.4.2 IgG1/IgG2a subclass ratio in post immunisation sera

The IgG subclass profile of both individual and pooled sera of the three rStkF vaccinated mice revealed high IgG1/IgG2a ratio (1.835 / 0.305) and (1.706 / 0.2573), respectively (Fig. 4.4.2). Thus, Th2 or humoral immunity is the main immune response in mice vaccinated with rStkF protein.

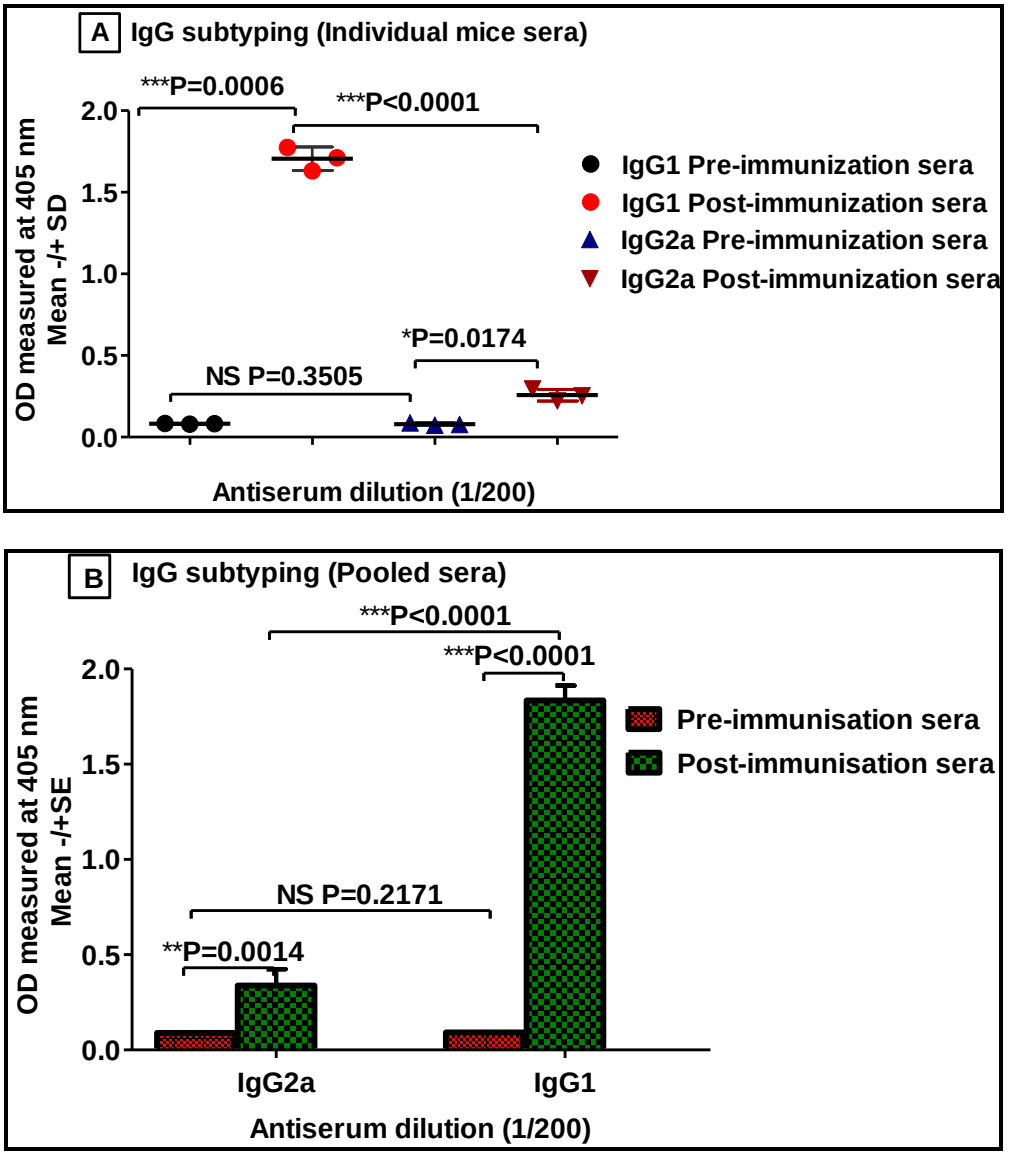


Figure 4.4.2: Effect of StkF vaccination on IgG1 and IgG2a levels in: A, Three individual mice sera; B, Pooled mice sera. The sera of the immunised mice were tested individually as well as pooled on ELISA plate coated with rStkF. ELISA results were expressed as mean ± SD of three individual mice sera or as mean ± SE of three independent mesurments of pooled sera. Using Student's paired *t*-test, the pre-and post-immunisation IgG1 levels showed a highly significant difference indicated by three asterisk at $P = 0.006$ (**A**) or at $P < 0.0001$ **(B)**. The pre- and post-immunisation IgG2a levels showed a little difference indicated by one asterisk at $P = 0.0174$ **(A)** or at $P = 0.0014$ **(B).** There was an extremely significant difference between IgG1 and IgG2a levels in post immunisation sera represented by three asterisks at $P < 0.0001$ indicating the predominance of Th2 immune response to StkF vaccination of mice.

4.4.3 Cytokine production and estimation

For further investigation into the phenotype (Th1 and Th2) of the immune response elicited by immunization with rStkF, the level of three cytokines IL-12 p70, IFN-γ (Th1 cytokines) and IL-4 (Th2 cytokine) was estimated in culture supernatant of spleen cells from immunised mice. Suspensions of splenocytes from non-immunized (control) and rStkF immunized mice were grown *in vitro* in RPMI 1640 medium supplemented with 10 % FCS and incubated at 37°C in 5 % CO_2 incubator for 72 h. The spleen cells were cultured in duplicate in the presence of rStkF protein (5 µg/well), ConA (T cell inducer) and mitomycin C (T cell inhibitor) as controls in 24-well microtitre plate. Following the incubation period, the cultures supernatants were collected and the supernatants for each duplicate were pooled. The levels of IL-12 p70, IFN-γ and IL-4 were estimated in the pooled splenocytes culture supernatants using mouse IL-4, IFN-γ and IL-12-p70 ELISA kits (ebiosciences) as per the manufacturer's instructions. There was a significant increase in IL-4 levels in the culture supernatant of splenocytes isolated from immunised mice stimulated *in vitro* with rStkF in comparison to control mice. In addition, there was a significant increase in IL-4 levels in culture supernatant from rStkF-induced immunised cells as compared to un-induced immunised cells. However, there was little increase in levels of both IL-12-p70 and IFN-γ in culture supernatants of spleen cells from immunized mice in comparison to control cells (Fig. 4.4.4).

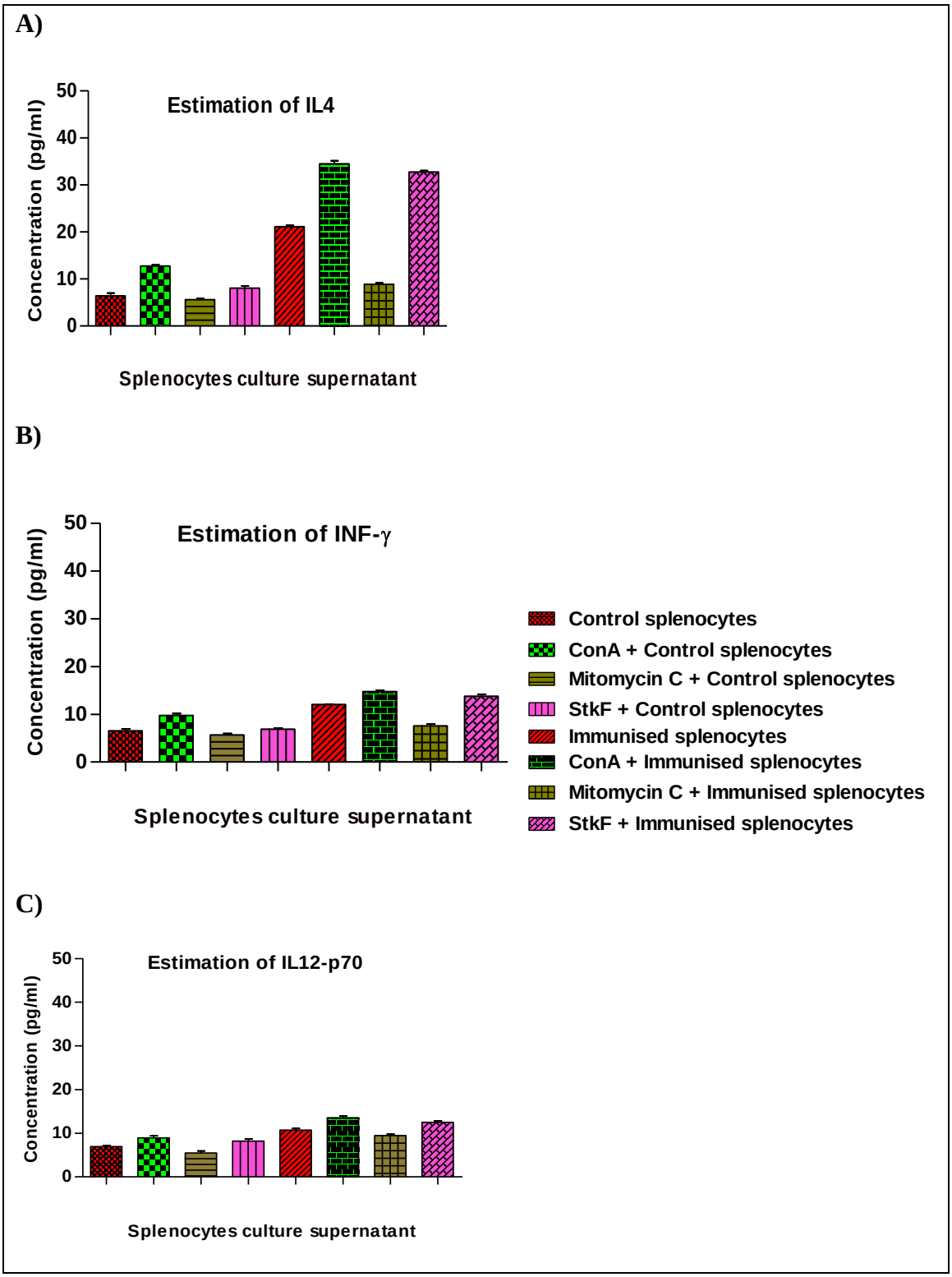


Figure 4.4.3: Estimation of cytokines in StkF immunised and control splenocytes culture supernatant. The productions of cytokines were comparable to that produced by ConA, used as positive control. Data presented as mean ± SE of triplicate. Key is the same for the three graphs.

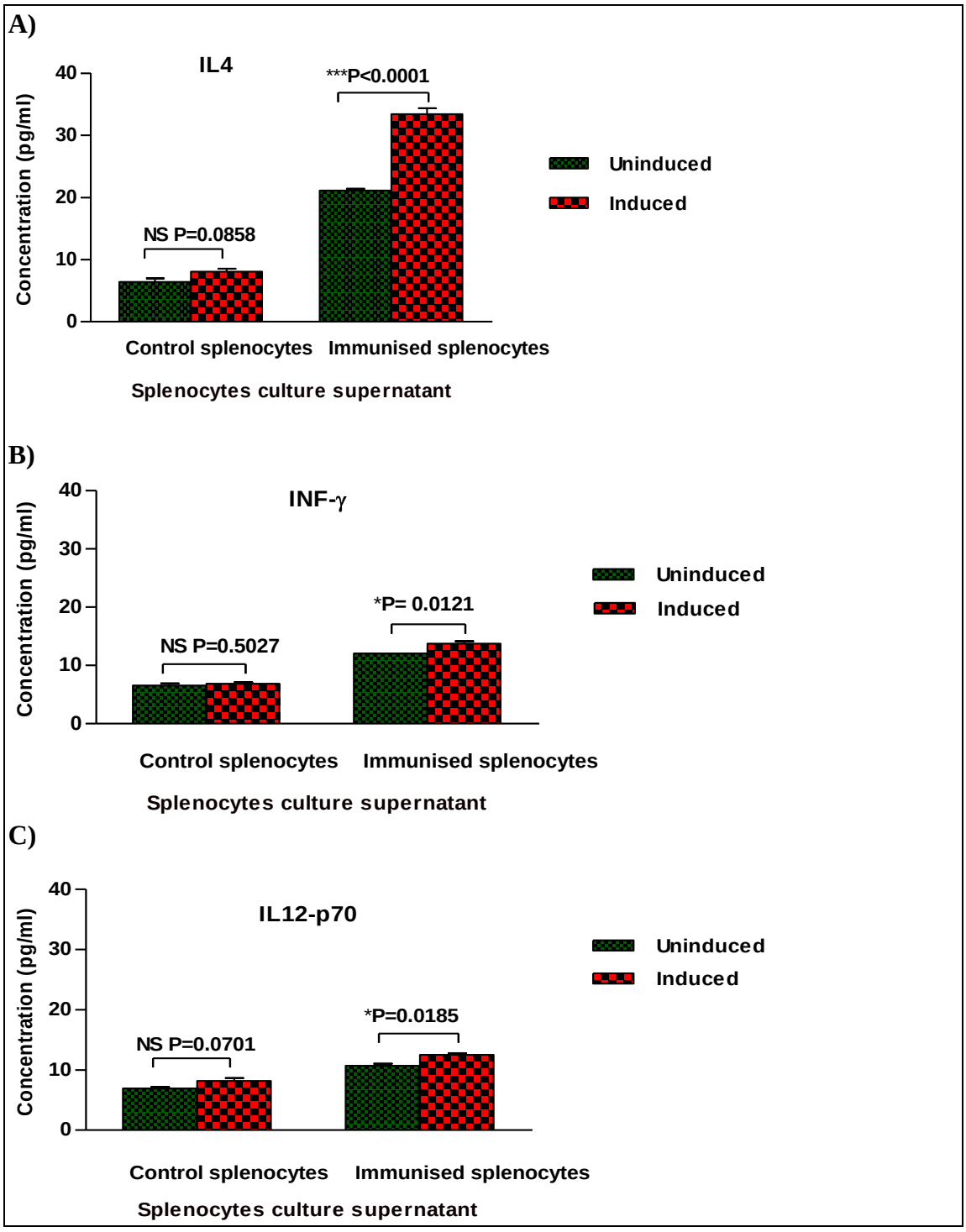


Figure 4.4.4: Effect of StkF vaccination on production of cytokines by splenic lymphocytes in mice. The cultured supernatants, in the absence (uninduced) or presence of 5 µg of rStkF (induced), of mice splenocytes (1×10^5 cells / well) collected 7 days after last rStkF immunisation, were assayed for: **A,** IL-4; **B,** IFN-γ; **C,** IL-2 by ELISA. There was increase in IL-4 level in induced StkF immunised spleen cells compared to control and un-induced immunised cells; however there was a little increase in IL-12 and IFN-γ indicating predominant humoral immune response (Th2).

4.5 Evaluation of protective efficacy of murine anti-StkF antibodies against *Salmonella* infection

Due to the lack of mouse model which replicates the systemic infection of *S.* Paratyphi A .The functional activity of the murine StkF-specific antiserum was investigated by an *in vitro* OPA using human neutrophils and an *in vitro* SBA against the non-typhoidal *Salmonella* strains HP0204 (StkF-positive) and HP0174 (StkF-negative) belonging to the serovars Kentucky and Enteritidis, respectively. The StkF status of these strains had been verified by Western blot. Human neutrophils effector cells were used, as these cells are the most abundant phagocytic cells in the peripheral blood and play an important role in controlling infection in the early stage. It was hypothesized that these assays would mirror a potential direct contribution of StkF antibodies to *in vivo* killing and clearance of the StkF-positive bacteria by host during systemic salmonellosis.

4.5.1 *In vitro* opsonophagocytosis assay using StkF-specific murine antiserum and human neutrophils

An *in vitro* opsonophagocytosis assay was used to measure the ability of serum antibodies to opsonise *Salmonella* bacteria for phagocytosis and killing by effector cells human PMN leukocytes. The *Salmonella* bacteria were pre-opsonised by incubation with pooled anti-StkF mouse immune sera. Pooled pre-immunisation serum was used as a negative control. The specificity of the assay was assessed using the StkF-negative *S.* Enteritidis strain HP0174. Killing of *Salmonella* by human neutrophils was estimated by measuring the decrease in numbers of viable bacteria. This opsonophagocytic killing assay showed the ability of mouse StkF immune serum to mediate the killing of a StkF-positive *Salmonella* strain as demonstrated by ~2.5 logs reduction in numbers of viable *S.* Kentucky strain HP0204 after 2 h of incubation with PMN leukocytes. Given the

assay design, this reduction in bacterial numbers was almost certainly due to enhanced opsonophagocytosis-mediated uptake and clearance of bacteria by human neutrophils. The finding that there was minimal to no killing of *S.* Enteritidis HP0174, a strain that does not express the StkF antigen, indicated that anti-StkF antibody mediated-killing was specific to *S.* Kentucky HP0204. The results presented as the mean log cfu count ± SE of two independent experiments (Figs. 4.5.1 and 4.5.2).

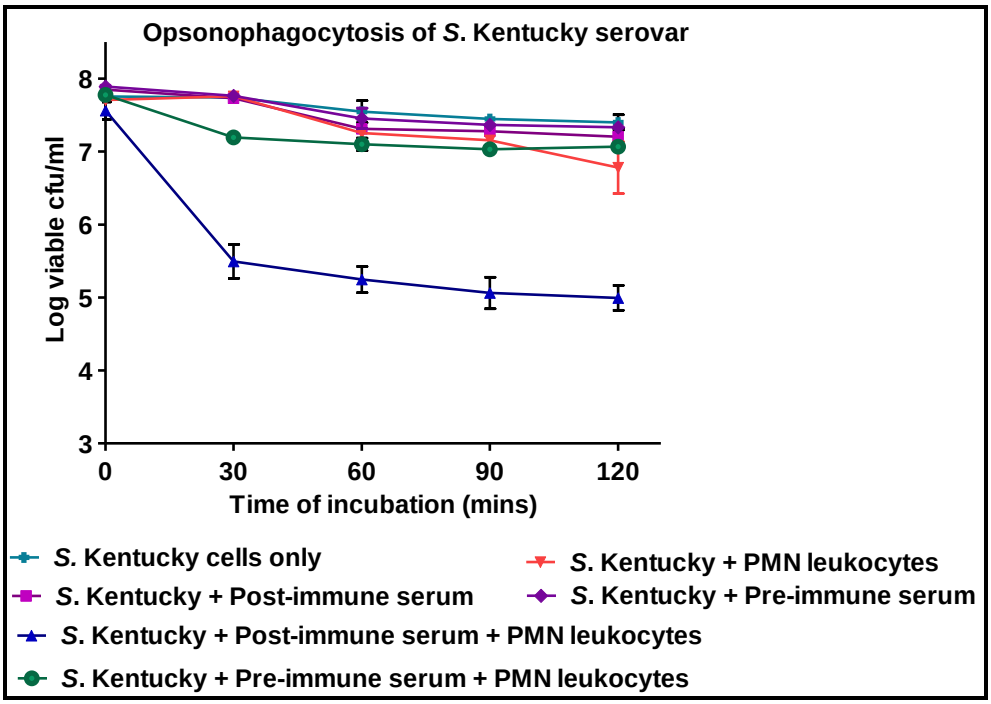


Figure 4.5.1: *In vitro* opsonophagocytosis of *S.* Kentucky strain HP0204. Determination of viable counts of *S.* Kentucky strain HP0204 at different time points following phagocytosis by human neutrophils in medium comprising 20 % (v/v) anti-StkF mouse pre- or post-immune sera. There was a significant reduction in viable counts of ~2.5 logs after 2 h incubation with PMN leukocytes following pre-opsonisation with anti-StkF post-immune sera; however, there were no reductions following pre-opsonisation with pre-immune sera or post-immune sera without addition of PMN leukocytes. *S.* Kentucky opsonised with 20 % pre-immune mouse serum was used as a negative control. The results are expressed as means ± SE of two independent experiments.

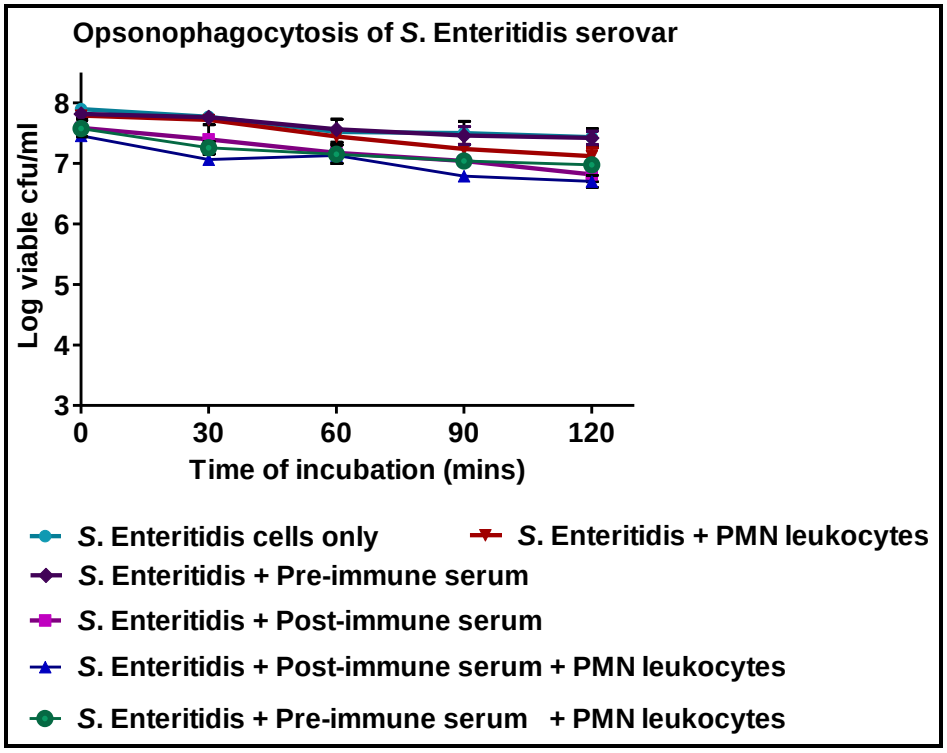


Figure 4.5.2: *In vitro* opsonophagocytosis of *S.* Enteritidis strain HP0174. Viable counts assessment of *S.* Enteritidis strain HP0174 following phagocytosis by human neutrophils using in a medium comprising 20 % (v/v) pooled anti-StkF mouse pre- or post-immune sera at different time points. There was no a significant reduction in viable counts following pre-opsonisation with 20 % of anti-StkF post-immune sera; also, there were no significant reductions following pre-opsonisation with pre-immune sera or post-immune sera without adding PMN leukocytes indicating specificity of antibodies. *S.* Enteritidis opsonised with 20 % pre-immune mouse serum was used as a negative control. The results are expressed as means ± SE of two independent experiments.

4.5.2 Examination of anti-StkF polyclonal antibody using an *in vitro* complement-mediated serum bactericidal assay (SBA)

Bactericidal antibody and terminal complement system proteins are essential factors for protection against diseases caused by Gram-negative bacteria. The complement system, which exists in the blood, both strengthens and directly enables aspects of the humoral immune response. It has been reported that complement protein is essential for killing susceptible Gram-negative bacteria. In addition to complement, lysozyme, calcium (Ca^{2+}) and magnesium (Mg^{2+}) ions are important components of extracellular fluids that are required for killing Gram-negative bacteria as they are essential for initiation of the classical complement cascade. It has also been established that IgG is able to promote killing of some Gram-negative bacteria *in vitro* in the presence of complement (Igumbor & Osayande, 2000).

According to the analysis of the immune response induced by rStkF mice vaccination, humoral immune response was the predominant immune response (section 4.4). Thus, *in vitro* complement-mediated SBA that would evaluate the ability of murine StkF-specific antiserum to promote killing of *Salmonella* bacteria by blood complement components following opsonisation of bacteria was performed using a human complement source. In this assay, the bactericidal efficiency of anti-StkF mouse immune sera was examined against *S.* Kentucky strain HP0204 (StkF-positive) and *S.* Enteritidis strain HP0174 (StkF-negative) according to the protocol and reaction mixtures mentioned in Chapter 2 (section 2.30). Immune serum induced by StkF was able to mediate serum bactericidal killing of StkF-positive *S.* Kentucky HP0204, however, minimal if any killing effect was observed with the StkF-negative *S.* Enteritidis HP0174, confirming the specificity of this assay. The results of this assay

showed that the anti-StkF complement mediated-killing is specific for the StkF-expressing strain *S.* Kentucky strain HP0204. The results are presented as the mean cfu/ml ± SE of two independent experiments (Figs. 4.5.3, 4.5.4). Bactericidal titre was 1:128 calculated as the highest serum dilution at which ≥ 50% killing of bacteria was observed.

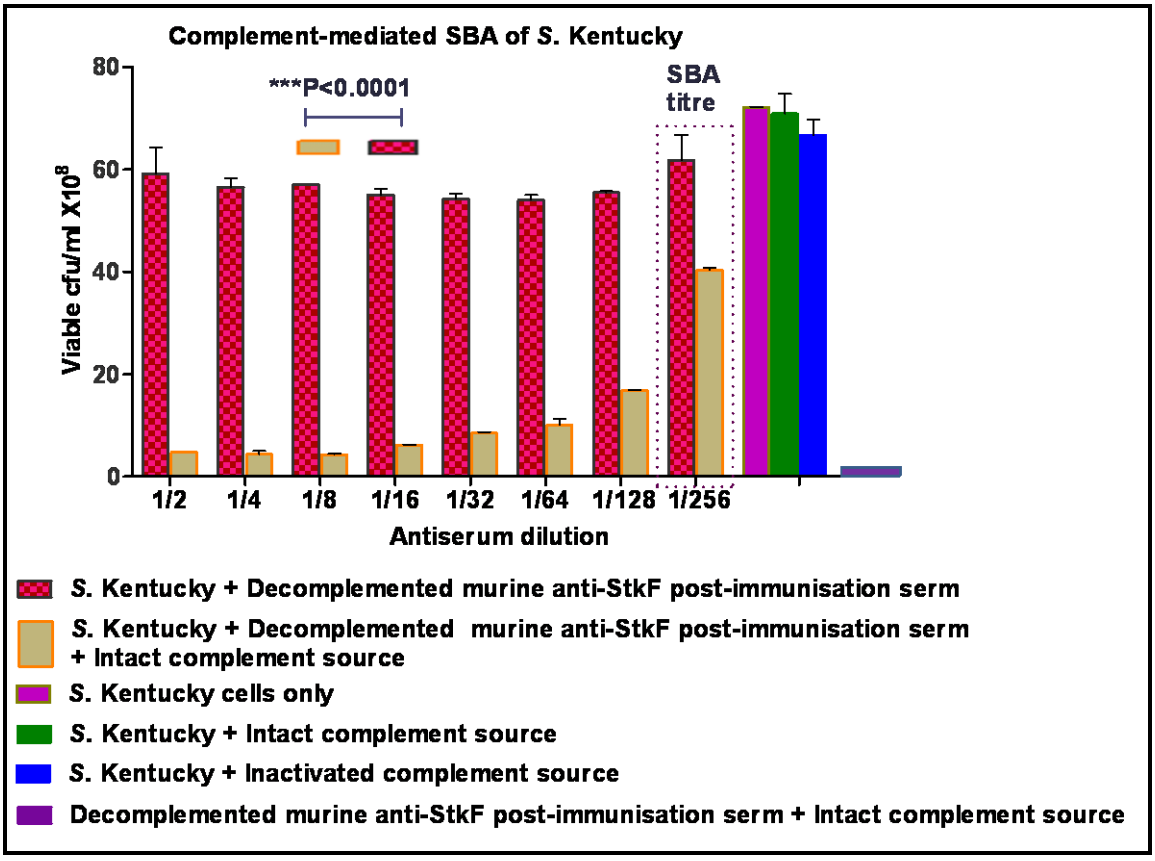


Figure 4.5.3: *In vitro* complement-mediated SBA on *S.* Kentucky strain HP0204 using polyclonal murine anti-StkF antibodies. Viable counts assessment of *S.* Kentucky strain HP0204 following incubation with different StkF mouse antiserum dilutions and human complement source (25 % of total reaction). There was a significant reduction in viable counts of the bacterium when incubated with the post-immunisation sera and intact complement source but not with antiserum alone ($P < 0.0001$). The results are expressed as mean ± SE of two independent experiments and the SBA titre (lowest serum dilution at which ≥ 50 % killing of bacteria was observed) was the dilution 1/128.

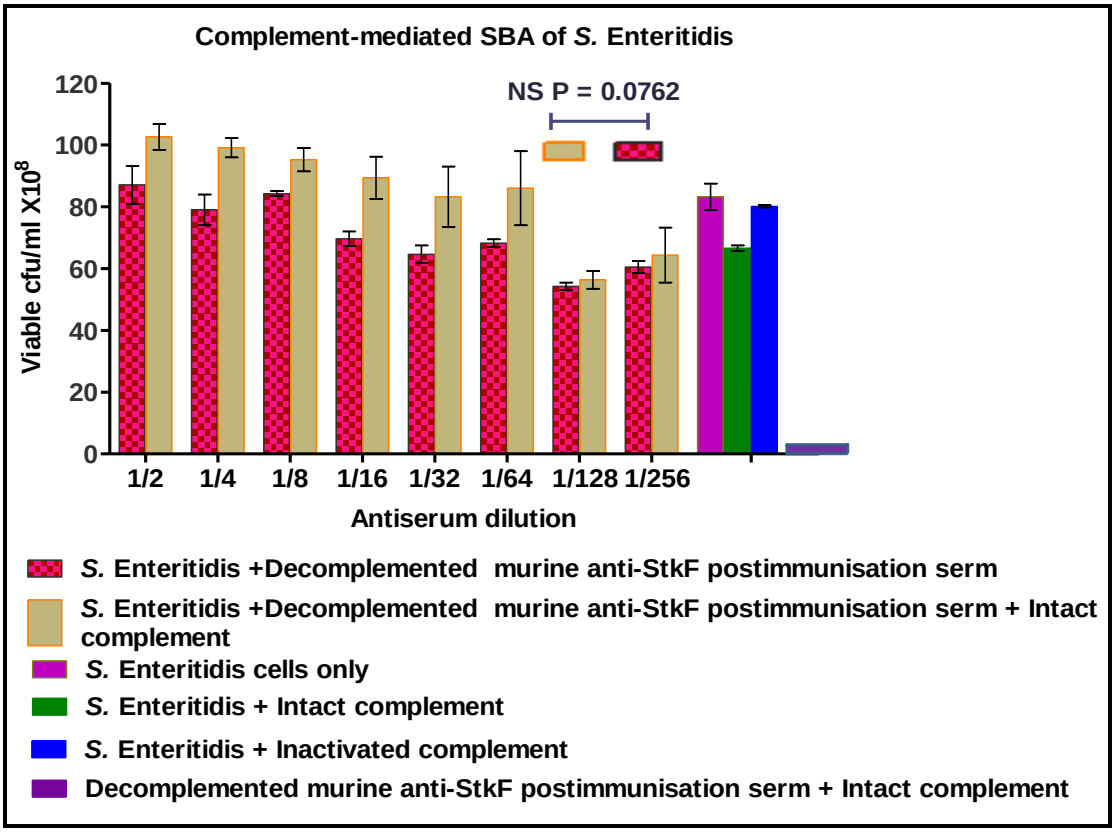


Figure 4.5.4: *In vitro* complement-mediated SBA on *S.* Enteritidis strain HP0174 using polyclonal murine anti-StkF antibodies Determination of viable counts of *S.* Enteritidis strain HP0174 strain, following incubation with different StkF mouse antiserum dilutions and human complement source (25 % of total reaction). There was no significant difference in viable counts of the bacterium when incubated with the post-immunisation sera and intact complement and incubated with antiserum alone (NS, $P = 0.076$). The results are expressed as means ± SE of two independent experiments.

4.6 Discussion

This project aimed mainly to develop a recombinant adhesin based vaccine against *S.* Paratyphi A infections. Accordingly, in this chapter, the immunogenicity of StkF protein which had been identified by bioinformatic analysis as a *Salmonella*-specific putative adhesin subunit of Stk fimbriae, carried by *S.* Paratyphi A and approximately 30 % of other *Salmonella* serovars investigated, was evaluated in BALB/c mice. In addition, the efficacy of StkF as a protective antigen against infections by an *S. enterica* strain carrying this antigen was assessed by different *in vitro* assays.

StkF appears to provide a potentially effective anti-*Salmonella* vaccine antigen due to a number of reasons. i) Surface structures, such as fimbrial adhesins, are frequently found not only to affect the virulence of bacteria but also to serve as key targets of the host immune system because these are present in extremely high numbers at the cell surface. ii) Fimbrial proteins are strong immunogens and a number of bacterial adhesins such as FimH fimbrial adhesin subunit of *E. coli* have been shown to induce strong protective immunity (Klemm & Schembri, 2000). iii) These proteins typically possess natural adhesive properties and often play critical roles in the initiation of pathogenesis (Zhang & Zhang, 2010). iv). The observation that StkF may be subject to ON–OFF phase variation by slipped-strand mispairing or recombination within a signature *stkF*-borne tandem repeat motif as discussed in Chapter 3, and the potential that this may be linked to host immune selection pressure by StkF-specific antibodies, leading to allelic OFF forms further raises the likelihood that StkF may serve as a protective antigen (Brunham *et al.*, 1993). v) The finding that rStkF protein was strongly immunogenic as revealed by the presence of very high antibody titre in the serum of immunized mice. In addition, *in vitro* assays performed with StkF-specific

murine antiserum demonstrated markedly enhanced opsonophagocytosis-mediated killing by human neutrophils, the most abundunat phagocytic cells in the blood, of *Salmonella* bacteria carrying this antigen. Augmented anti-StkF antibody-specific complement-mediated lysis of *S.* Kentucky strain HP0174 expressing this antigen was also shown. These data suggest a likely direct contribution of StkF-specific antibodies to *in vivo* killing and clearance of *Salmonella* bacteria expressing the StkF antigen by these two mechanisms.

The immunisation experiment that was performed to evaluate the immunogenicity of the His-tagged rStkF in three BALB/c mice, showed that the immunised mice produced specific anti-StkF antibodies as early as three weeks after immunisation with the recombinant protein as assessed by ELISA. In addition, there was a remarkable increase in antibody response level after further boosting doses (Fig. 4.2.1 and 4.3.1). Sera from the immunised mice obtained at six weeks after the first rStkF immunisation and following completion of the 4-dose immunization schedule reacted with rStkF at a high titre of 1:50,000 (Fig. 4.2.2). This implies that four doses of s.c. immunization with rStkF protein produced a very strong immune response indicating the high immunogenicity of StkF in the BALB/c mouse strain.

The nature of the humoral immune response produced in StkF-immunised mice was assessed by measuring IgG sub-classes, IgG1 and IgG2a, in both the pre- and post-immunisation sera collected one week after the fourth immunization dose. The production of IgG1 is primarily induced by Th2 type cytokines produced by antigen specific T cells, whereas production of IgG2a reflects the involvement of Th1 type cytokines (Petrushina *et al.*, 2003). It was found that, in comparison to the pre-immunisation sera, the post-immunisation sera had higher antibody levels of both IgG1

and IgG2a antibodies (Fig. 4.4.2)**.** However, the IgG1 level was much significantly higher than that of IgG2a in the post-immunisation sera denoted by three asterisks at $P < 0.0001$. Thus, the high IgG1/IgG2a ratio in the mice post-immunisation sera of 6.6 as measured by dividing the OD readings for the IgG1 and IgG2a ELISA assays, suggested a dominant Th2 response. To further characterize the Th1 vs. Th2 immune response phenotype generated by s.c. immunization with rStkF, splenocytes recovered from StkF-immunised and non-immunised mice (control) were cultured in *in vitro*, and the levels of the Th1 cytokines IFN-γ and IL-12, and the Th2 cytokine IL-4 were measured in cultures supernatants using cytokine-specific sandwich ELISAs. Culture supernatants of splenocytes from StkF-immunised mice showed higher IFN-γ, IL-12 and IL-4 levels than those from non-immunised mice splenocytes. However, the extent of up-regulation of IL-4 production was found to be the greatest when compared to that of IFN-γ or IL-12-p70, confirming a Th2-dominant immune response (Fig. 4.4.3, 4). These observations revealed that the mouse immune response generated with rStkF immunisation was a mixed Th1/Th2 immune response profile. However, the predominant immune response was Th2 (humoral) immune response as indicated by the high IgG1/IgG2a ratio and Th2-type cytokine profile triggered by immunisation of BALB/c mice. This is consistent with the very high anti-StkF titre measured in the pooled post-immunization serum as production of antibodies by plasma cells is mediated mainly by type 2 helper T (Th2) cells which secrete IL-4 (Okamoto *et al.*, 2005).

Complete or incomplete Freund's adjuvant (FCA and FIA) is often administered with antigens in experimental murine and rabbit immunization studies to enhance immunity. The use of FCA and FIA together with rStkF in the initial and booster doses,

respectively, may be partly responsible for this Th2 immune response profile. Indeed, Avramidis et al. (2002) have suggested that use of FCA as an adjuvant may shift the immune response predominantly towards Th2 immune response profile, as the use of other adjuvants in their study switched the response towards a Th1 profile. However, the reverse was observed in a second BALB/c mouse vaccination study. In the latter, Th2 type immune response was predominant when the antigen was mixed with alum as an adjuvant, while immunization with the same antigen mixed with CFA shifted the immune response towards a Th1 phenotype (Bansal *et al.*, 2010).

The mucosal local immune response which is potentially important in evaluating an adhesin-based vaccine that may act by inhibiting the bacterial colonisation of the mucosa by the pathogen could not be assessed in this study. Local immune response can be assayed by measuring intestinal and stool sIgA (Ghosh *et al.*, 2011). Mice were immunised in this study via the s.c route. However, an oral (mucosal) route of immunization may be more appropriate to study the action of adhesin-induced protective antibodies, as unlike the s.c. route of immunization, it may induce a strong local immune response (Holmgren & Czerkinsky, 2005). In such cases, sIgA plus a locally induced adhesin-specific IgG antibody response at the mucosal surface could prevent attachment and block the colonization. However, some reports also suggest that serum anti-adhesin IgG may transudate into mucosal secretions after parenteral vaccination and may be sufficient to block colonisation (Wizemann *et al.*, 1999 and Holmgren & Czerkinsky, 2005). Future studies to compare the local immune response and protective efficacy associated with both routes of immunization are clearly warranted.

Due to the lack of mouse model which replicates the systemic infection of *S.* Paratyphi A, the potential function of StkF-specific antibodies in protection against *Salmonella* infection was evaluated by using *in vitro* serum bactericidal and opsonophagocytosis assays. A murine oral *S.* Typhimurium infection model which mimics enteric fever has been widely used to study the human disease caused by *S.* Typhi (Santos *et al.*, 2001). However, this model is not applicable in our study because *S.* Typhimurim does not possess the StkF protein based on bioinformatics analysis and PCR investigation. The murine anti-StkF antibodies markedly enhanced *in vitro* complement-mediated killing of *S.* Kentucky strain HP0204 which possessed the *stkF* gene and was shown to express StkF by Western blot analysis. Importantly, there was no killing effect noted with the StkF-negative *S.* Enteritidis strain HP0174 indicating the specificity of the StkF murine antiserum complement mediated-killing. As there was a statistically significant difference between the numbers of viable *S.* Kentucky bacteria following incubation with different dilutions of de-complemented anti-StkF post-immunisation serum in the presence of a defined complement human source in comparison to the numbers of viable bacteria following incubation with de-complemented anti-StkF post-immunisation serum only indicated by three asterisks at $P < 0.0001$; the observed killing could clearly be linked to complement activity. Anti-StkF antibody alone exerted only a minor killing effect on bacteria in this assay. However, in the case of *S.* Enteritidis strain HP0174 there was no significant difference in viable bacterial numbers between anti-StkF-bearing samples with and without added complement ($P = 0.0762$). The bactericidal titre which is highest serum dilution at which ≥ 50 % killing of *S.* Kentucky strain HP0204 bacteria was 1:128 (Fig. 4.5.3, 4). Moreover, murine StkF antibodies enhanced the killing of *S.* Kentucky strain HP0204

by human PMN leukocytes using *in vitro* opsonophagocytosis assay, while, there was no significant effect for *S.* Enteritidis strain HP0204 The viable count of the pre-opsonised *S.* Kentucky bacteria following incubation with human PMN leukocytes for 2 hours showed a statistically significant reduction of about 2.5 logs presented by two asterisks at $P = 0.0068$, while there was no significant reduction of pre-opsonised *S.* Enteritidis at $P = 0.3831$ indicating that the StkF antibodies complement mediated-killing is serotype-specific (Fig. 4.5.1, 2). Together these findings suggest that the circulating StkF antibodies may be involved in the protection against systemic salmonellosis and a StkF-based vaccine may protect an endemic population against *Salmonella* serovars expressing this antigen.

The overall immune response observed with StkF in this study is comparable to that of the T-cell-independent Vi polysaccharide-based typhoid vaccine which has been licensed for use. The utility and validated efficacy of the latter against *S.* Typhi infections suggests that serum antibodies may confer high levels of protection against systemic salmonellosis (Hale *et al.*, 2006). Cell-mediated immune response is considered important for long-term protection against *Salmonella* infections (Holmgren & Czerkinsky, 2005). Notably, a low but significant IgG2a response, a surrogate marker of cell-mediated immunity, was also found in StkF-immunised mice. However, similar observations were reported with the pneumococcal surface adhesin PsaA (Lin *et al.*, 2010) and the outer membrane adhesin protein T2544 of *S.* Typhi (Ghosh *et al.*, 2011), which provided a protection against *Streptococcus pneumoniae* and *S.* Typhi infections in mice, respectively. In addition, StkF-specific antibody showed the same functionality as T2544-specific antibody, which enhanced *in vitro* uptake and clearance of *Salmonella* by opsonophagocytosis and augmented *in vitro* complement-mediated lysis,

indicating a contribution of specific antibodies to the *Salmonella* killing process (Ghosh *et al.*, 2011). These observations were also similar to the study characterising the functionality of *S.* Typhi-specific antibody in clearance of *Salmonella* following vaccination with a new vaccine, M01ZH09 (Ty2 Δ*aroC*Δ*ssaV*). The antibodies produced have been demonstrated by *in vitro* opsonophagocytosis and serum bactericidal assays to facilitate killing of *S.* Typhi (Lindow *et al.*, 2011). Similarly, Siggins et al. (2011) has proved the importance of the role of bactericidal and opsonic antibody in protection against invasive NTS serovars. Their experiments showed the killing of *S.* Typhimurium *in vitro* by opsonophagocytosis and serum bactericidal assay using the mouse antiserum supplemented with human complement (Siggins *et al.*, 2011). Gondwe et al. (2010) study also showed that opsonic activity of anti-*Salmonella* antibody and/or complement is required for oxidative burst and phagocyte killing of NTS by peripheral blood neutrophils, which supporting the use of murine anti-StkF serum without inactivation of complement in this study (Gondwe *et al.*, 2010). In addition, investigation of the bactericidal activity of human sera to *S.* Typhi, *S.* Paratyphi A, *S.* Paratyphi B and *S.* Paratyphi C was performed by Igumbor and Osayande (2000) revealed that antibody alone did not reduce viable count of the organisms, which implied that *in vivo* killing of S*almonella* bacteria by human serum is complement-dependent (Igumbor & Osayande, 2000).

Since animal studies have concerned cellular mechanisms as being of key importance for immunity to *Salmonella*, which partly explained by *Salmonellae* being facultative intracellular organisms (Gondwe *et al.*, 2010). Enhanced cell-mediated immune responses against StkF could potentially be induced by a different route of immunisation, use of another mouse strain and/or formulation with a distinct adjuvant

agent (Ramakrishna *et al.*, 2003). In addition, conjugating the vaccine with a protein carrier, which enhances cellular immunity as in case of PsA conjugated with capsular polysaccharide (CPS) vaccine (CPS–rPsaA conjugate) against *S. pneumoniae* (Lin *et al.*, 2010) and Vi-conjugate vaccine against *S.* Typhi (Cui *et al.*, 2010). These factors could be investigated in future StkF immunisation studies. Though, the results of StkF-specific antibody opsonophagocytosis and complement-mediated killing activity serve to link antibody and cell-mediated immunity in protection against *Salmonella*, which raises the likelihood that an antibody-inducing vaccine will protect against invasive *Salmonella* disease (Gondwe *et al.*, 2010).

Vaccine design may encounter challenges when vaccines are developed that target proteins or moieties that undergo antigenic or phase variation, but phase-variable expression of a target protein does not necessarily preclude its use as a vaccine target. Expression of type 1 fimbriae in *E. coli* is under the control of phase variation. However, an experimental vaccine based on the FimH adhesin of these fimbriae is effective in preventing *E. coli* urinary tract infections in animal models. In this animal model, a bias to the "on" phase exists in the urinary tract and a decreased efficiency in colonization exists in cells in the "off" phase. Thus, the loss of expression of the vaccine target (phase "off") in this case may have coincided with a decreased ability to establish an infection, contributing to the efficacy of this vaccine (van der Woude & Baumler, 2004). In addition, a high degree of antigenic conservation is an attribute of many adhesins, which make them ideal vaccine candidates (Wizemann *et al.*, 1999).

Chapter Five

Stk fimbriae expression and characterisation

5 Stk fimbriae expression and characterisation

5.1 Introduction

Fimbriae are filamentous, non-flagellar proteinaceous bacterial surface appendages involved in adhesion (Fronzes *et al.*, 2008). These structures have been shown to be important for host–pathogen interactions in a wide range of human pathogenic bacteria, including *Salmonella* (Kline *et al.*, 2009). Ottow (1975) classified fimbriae into six groups based on their morphology, however, group I had been further separated into four subtypes described by Duguid (1968) based on morphology, ability to haemagglutinate RBCs, and mannose haemagglutination inhibition sensitivity (Ottow, 1975). Subtype 1 fimbriae, also termed type 1 fimbriae, are the most common fimbrial type found in Gram-negative bacteria (Edwards *et al.*, 2000). However, fimbriae can be also classified based on mechanism of assembly. The genus *Salmonella* harbours fimbriae belonging to the nucleation/precipitation pathway, the chaperon-usher pathway, the alternate chaperone pathway and type IV fimbriae (Fronzes *et al.*, 2008). The presence of fimbriae has been shown to correlate with several phenotypic features. These features include haemagglutination ability (Duguid *et al.*, 1966), the formation of a surface pellicle on an undisturbed, aerobic static liquid medium, properties common to type 1 fimbriae (Old *et al.*, 1989), and contribution to the bacterial hydrophobicity which may facilitate the non-specific adherence of bacteria (Doyle, 2000).

In this chapter, bioinformatics and phenotypic characterisation of the *Salmonella stk* putative fimbrial operon present in ~30 % of *Salmonella* serovars examined by PCR in this study (Chapter 1, Table 3.3-1) and the associated Stk fimbrial structure will be discussed.

5.2 *stk* operon prediction

The prediction of the *stk* operon within the *Salmonella enterica* Paratyphi A strain ATCC 9150 genome was performed using Microbes Online Operon Predictions server available at http://www.microbesonline.org/operons/ (Price *et al.*, 2005).

A

B

Gene1	Gene2	Name1	Name2	bOp	pOp	Sep	MOGScore	GOScore	COGSim
SPA0177	SPA0178	*yadI*	*yadE*	TRUE	0.869	68.000	0.222	1.000	Y
SPA0179	SPA0180	*stkG*	*stkF*	TRUE	0.997	12.000	0.571	0.010	Y
SPA0180	SPA0181	*stkF*	*stkE*	TRUE	0.981	19.000	0.571	0.010	
SPA0181	SPA0182	*stkE*	*stkD*	TRUE	0.970	27.000	0.571	0.010	
SPA0182	SPA0183	*stkD*	*stkC*	TRUE	0.971	35.000	0.571	1.000	Y
SPA0183	SPA0184	*stkC*	*stkB*	TRUE	0.962	68.000	1.000	1.000	Y
SPA0184	SPA0185	*stkB*	*stkA*	TRUE	0.942	87.000	0.750	1.000	Y
SPA0185	SPA0186	*stkA*	*panD*	FALSE	0.010	277.00	0.000	1.000	N
SPA0186	SPA0187	*panD*	*panC*	TRUE	0.877	95.000	0.342	1.000	Y

Figure 5.2.1: Prediction of *stk* fimbrial operon within *S.* Paratyphi A ATCC 9150 genome; A, result output obtained from microbes online operon predictions server of confirmed *stk* operon (numbers below indicate intergenic distances between adjacent genes); **B,** The 'bOp' is whether each pair of adjacent genes is predicted to lie in the same operon or not. The 'pOp' value, the estimated probability that the pair is in the same operon (values near 1 or 0 are confident predictions or not, respectively, while values near 0.5 are low-confidence predictions). 'Sep' value, the distance between two genes in the base pair. MOGScore or microbes online ortholog groups of genes, is conservation ranging from 0 (not conserved) to 1 (100 % conserved), GOScore (gene ontology), describes gene products in terms of their associated biological processes, cellular components and molecular functions, smallest value means shared GO category, as a fraction of the genome, or missing if one of the genes is not characterized. COGSim, whether the genes share a COG (clusters of orthologous groups of proteins or conserved domains) category or not (Price *et al.*, 2005).

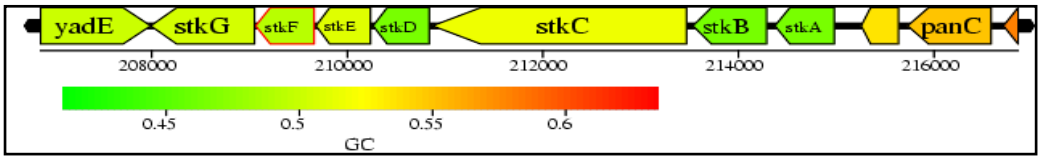


Figure 5.2.2: *stk* operon and the GC content percentage for each gene obtained from website, http://www.xbase.ac.uk/colibase/.

5.3 BLAST function of *stk* fimbrial operon nucleotide sequence

The DNA sequence of *stk* gene cluster (6992 bp) as well as the sequence with extra upstream and downstream 250 bp (7492 bp) from *S.* Paratyphi A ATCC 9150 were subjected to BLAST search against the non-redundant database of GenBank using NCBI blastn function. Both sequences showed high-level alignments with several *Salmonella enterica* genomes with all hits being annotated as encoding putative fimbrial proteins.

Table 5.3-1: DNA sequences producing significant alignments to the *stk* fimbrial gene cluster from *S.* Paratyphi A ATCC 9150

Accession	Strain	Query coverage	Alignment percentage[1]
NC_011147.1	*S.* Paratyphi A str.AKU_12601	100 %	100 %
NC_006511.1	*S.* Paratyphi A str. ATCC 9150	100 %	100 %
ABFH02000001.1	*S.* Virchow str. SL491	99 %	99 %
ABFG01000012.1	*S.* Hadar str. RI_05P066	99 %	99 %
ABEL01000008.1	*S.* Heidelberg str. SL486	99 %	99 %
NC_011083.1	*S.* Heidelberg str. SL476	99 %	99 %
ABAK02000001.1	*S.* Kentucky str. CVM29188	99 %	98 %
ABEI01000003.1	*S.* Kentucky str. CDC 191	99 %	98 %
ACBF01000005.1	*S.* Tennessee str. CDC07-0191	99 %	98 %

[1]Using blastn function, both nucleotide sequence of full-length of *stk* operon (6992 bp) and *stk* operon with extra 250 bp upstream and downstream (7492 bp) showed significant alignments with several *Salmonella enterica* genome sequences.

5.4 Prediction of sub-cellular localization of Stk fimbrial proteins

Predictions of sub-cellular localizations of *stk* encoded proteins were performed using two localisation prediction servers: PSORTb v 3.0.2 (http://www.psort.org/psortb/) (Yu *et al.*, 2010) and Gneg-mPLoc v 2.0 (http://www.csbio.sjtu.edu.cn/bioinf/Gneg-multi/) (Shen & Chou, 2010). These tools predict the five major sub-cellular localizations (cytoplasm, cytoplasmic membrane, outer-membrane, extracellular and periplasm) of proteins in Gram-negative bacteria.

Table 5.4-1: Predictions of sub-cellular localizations of *stk*-encoded proteins

Protein	Localisation prediction[1]
StkA	Extracellular (fimbrial) protein (1),(2)
StkB	Periplasmic protein (1),(2)
StkC	Outer membrane protein (1),(2)
StkD	Extracellular (fimbrial) protein (2)
StkE	Extracellular (fimbrial) protein (2)
StkF	Extracellular (fimbrial) protein (2)
StkG	Extracellular (fimbrial) protein (2)

[1]Two prediction servers were used for predicting the sub-cellular localisation of each protein encoded by *stk* operon, PSORTb v 3.0.2 (1) and Gneg-mPLoc v 2.0 (2). The numbers in brackets indicate the tool that yielded a particular prediction.

5.5 Lengths and putative functions of proteins encoded by *stk* fimbrial gene cluster

The putative functions of the proteins encoded by *stk* fimbrial operon were predicted by bioinformatics analyses and identification of conserved protein domains using the NCBI conserved domain database (CDD). CDD is a protein annotation resource that consists of a collection of well annotated multiple sequence alignment models for domains and full-length proteins to facilitate identification of conserved domains in protein sequences via RPS-BLAST. CDD includes NCBI-curated domains which use where

available 3D-structure information to define domain boundaries and provide insights into sequence/structure/function relationships. In addition to domain models imported from a number of external source databases (Pfam, **P**rotein **fam**ilies; SMART, simple modular architecture research tool; COG, clusters of orthologous groups of proteins; PRK, **PR**otein (**K**)clusters; **cl**, superfamily cluster records) (Marchler-Bauer *et al.*, 2011).

Table 5.5-1: Predicted lengths and putative functions of proteins encoded by the *stk* gene cluster

Protein	Predicted Length	Putative function
StkA (SPA0185)	201 aa	Major fimbrial subunit (pfam00419, PRK15307)[1]
StkB (SPA0184)	250 aa	Fimbrial preplasmic chaperon protein (pfam 00345,pfam02753, PRK15246, COG3121)
StkC (SPA0183)	855 aa	Fimbrial outer membrane usher protein (PRK15223, pfam 00577, COG3188)
StkD (SPA0182)	190 aa	Minor fimbrial subunit (PRK15306)
StkE (SPA0181)	185 aa	Minor fimbrial subunit (PRK15263, pfam 00419)
StkF (SPA0180)	199 aa	Minor fimbrial subunit (Potential tip adhesin), (PRK15199, pfam09160, PRK09934)
StkG (SPA0179)	353 aa	Minor fimbrial subunit (pfam00419, PRK09733)

[1]Bioinformatic predictions were achieved by identification of conserved protein domains using NCBI conserved domain database (CDD) and associated other domain models imported from a number of external source databases. Identified conserved domains super families are as indicated in brackets. Pfam, Protein families; COG, clusters of orthologous groups of proteins; PRK, PRotein (K).

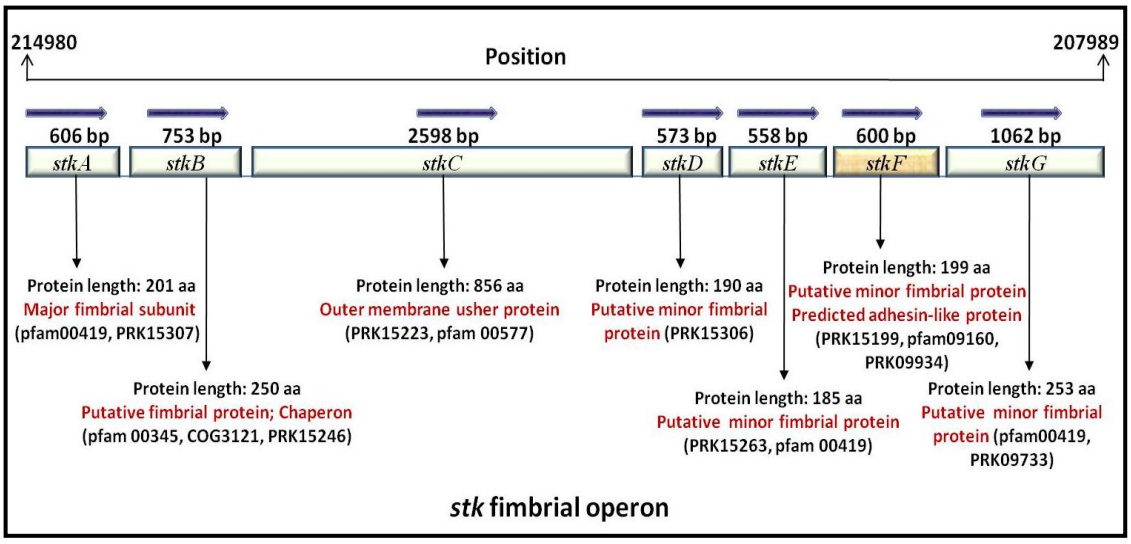


Figure 5.5.1: Genetic map *stk* fimbrial gene cluster of *S.* Paratyphi A ATCC9150. Schematic diagram showing the *stk* fimbrial operon organisation with corresponding proteins lengths and associated putative functions (conserved domains) indicated. The arrows above genes indicate the direction of transcription.

5.6 Orthologues of the *stk* fimbrial operon

The *stkABCDEFG* operon is located downstream of the *panD* gene and is an orthologue of the *yad* fimbrial operon present in *E. coli* K-12 which is formed by the genes *yadCKLM*, *htrE*, *ecpD* and *yadN*. Although the predicted amino acid sequences of matching *S.* Paratyphi A and *E. coli* fimbrial proteins only exhibit between 33.9 % and 60.3 % identity, the gene order was strictly conserved (Fig. 5.6.1). StkF is an orthologue of YadK, a predicted adhesin-like protein (Ou *et al.*, 2007).

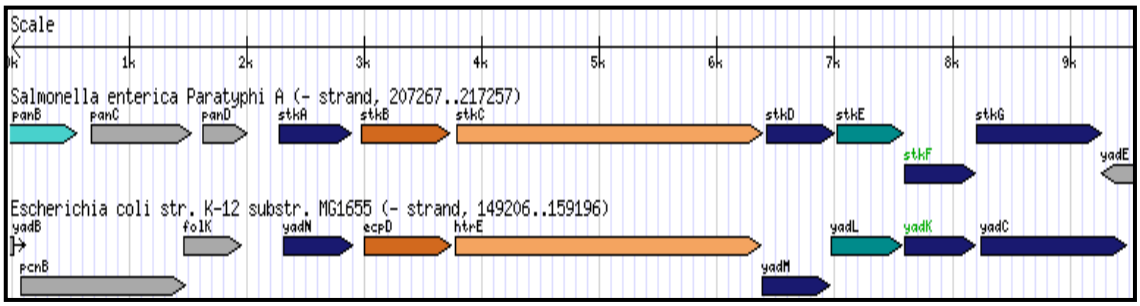


Figure 5.6.1: Schematic view of *Salmonella* Paratyphi A *stk* operon and its orthologue in *E. coli* K-12 as identified by the MicrobesOnline operon prediction server available at (http://www.microbesonline.org/operons/).

5.7 Classification of the *stk* fimbrial operon

Based on the NCBI conserved domain database search, the *stk* operon could confidently be assigned to the chaperon and usher fimbrial clades as classified by Nuccio and Bäumler scheme (2007). This assignment was further confirmed by fimbrial usher amino acid sequence comparisons against members of each sub-clade (Nuccio & Baumler, 2007). Using blastp at the protein knowledge base website (UniProt KB, http://www.uniprot.org/), the StkB chaperon protein showed a strong alignment/highest similarity with the EcpD chaperon protein. In addition, the StkC usher protein showed strong alignment/highest similarity with the HtrE usher protein. EcpD and HtrE proteins are the chaperon and usher subunits of the γ4 C/U Yad fimbriae of *E. coli* K-12. Based on this bioinformatics analysis, the *stk* fimbrial operon could be confidently allocated to the γ4 C/U sub-clade. Thus, the *stk* fimbrial operon could be considered one of the putative *S.* Paratyphi A ATCC 9150 fimbrial operons belonging to the chaperone-usher-dependent assembly class (Fig. 5.7.1).

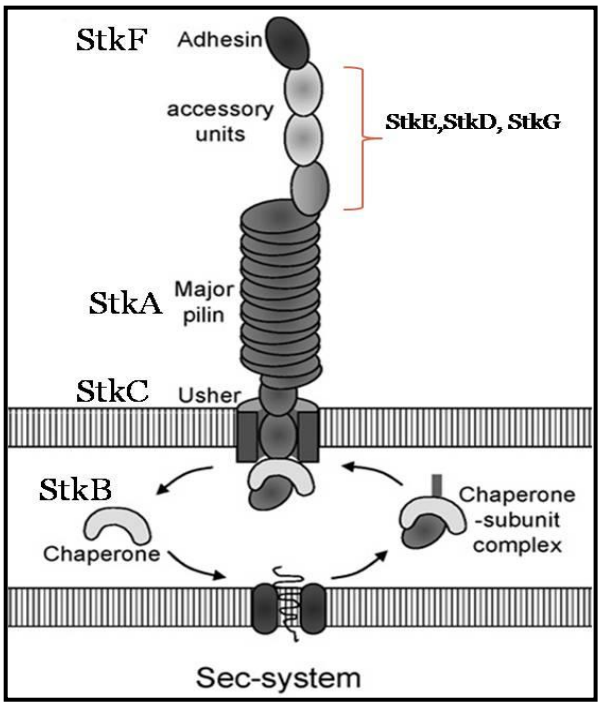


Figure 5.7.1. Model of the assembly of Stk fimbrial structure (C/U clade) based on bioinformatics analysis and sequence homology between *stk* fimbrial subunits and other fimbrial systems. Based on the classification scheme of Nuccio and Bäumler (2007), it is proposed that the *stk* operon is γ4 C/U clade and *stkF* encodes a potential adhesin subunit of Stk fimbriae, which is probably located at the tip of the fimbrial shaft. Adapted from Hori & Matsumoto (2010).

5.8 Cloning and expression of the *stk* fimbrial operons carrying the *stkF*-ON and *stkF*-OFF genes in afimbriated *E. coli* HB101

Briefly, the full-lengths of the *stk* fimbrial operons with an extra ~100 bp (7210 bp) either side carrying the *stkF*^{159bp}-ON and *stkF*^{211bp}-OFF genes from *S.* Paratyphi A strains SARB 42 and PA42, respectively, were cloned intermediately into pGEM-T easy vector and finally into the HCN pUCP20 plasmid and then transformed into afimbriated *E. coli* HB101 for Stk fimbrial expression and characterisation.

5.8.1 PCR amplifications of the *stk* operons

The full-lengths of *stk* operons with extra ~100 bp upstream and downstream was amplified by PCR from *S.* Paratyphi A SARB 42, which carries an *stkF*^{159bp}-ON form (199 aa StkF); and from *S.* Paratyphi A PA42, which carries an *stkF*^{211bp}-OFF form (60 aa, StkFv2) using the primers stk-F (5'AAGCGCGAATTCAATGGATGGCTTCATTCC3' *Eco*RI restriction site) and stk-R (5'AAGCGCAAGCTTTAATCCGATGCTGCTCAA3' *Hin*dIII restriction site). These primers were designed using Primer3 plus software by using whole length of *stk* operon with an extra 250 bp either side. Based on *insilico* PCR amplification, these primers should produce an amplicon of 7210 bp from *S.* Paratyphi A str. ATCC 9150. Gradient PCR showed that 58°C is the optimum Ta for these olignucleotides. The PCR reactions were performed using high fidelity KOD Xtreme™ Hot Start DNA Polymerase kit (Novagen, 71975) in 50 µl reactions.

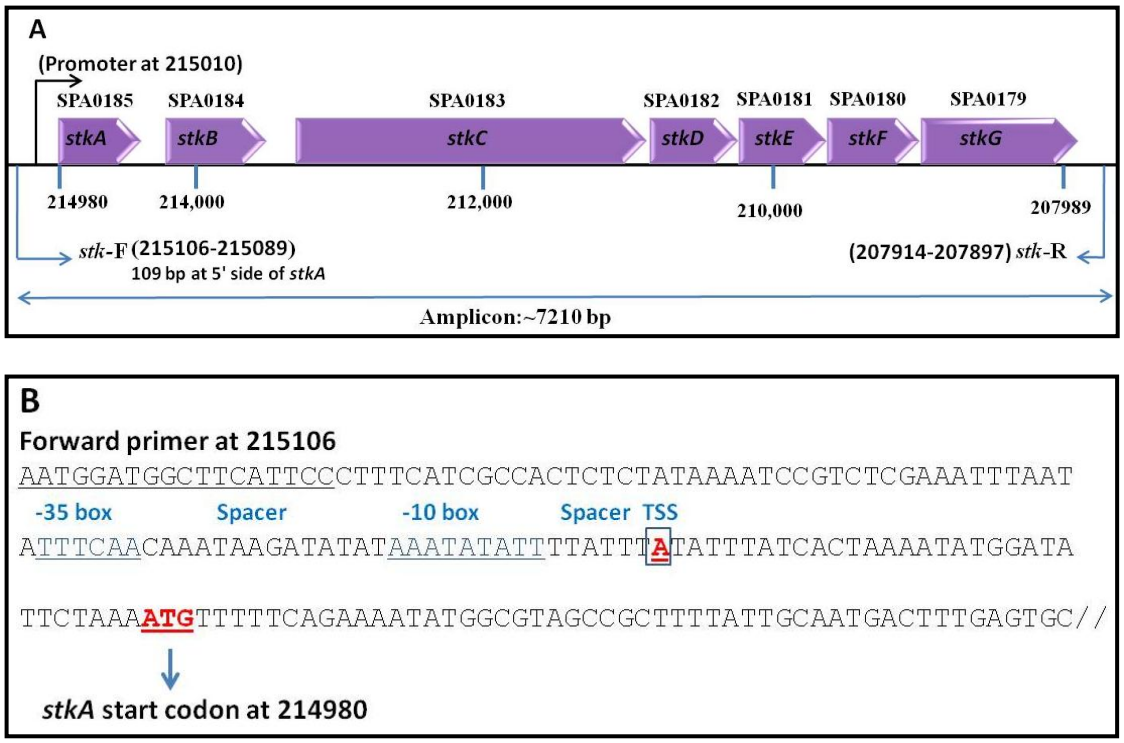


Figure 5.8.1: Schematic diagrams of the positions of *stk* gene cluster amplification primers and predicted promoter site. A, Schematic diagram of the genetic organisation of *stk* gene cluster in *S.* Paratyphi A ATCC 9150 showing the positions of stk-F (forward primer) and stk-R (reverse primer) used in *stk* operon PCR amplifications. The top most angled arrow indicates the position of the predicted promoter. **B,** Predicted promoter or transcription start position (TSS) at 215010 in the upstream sequence to *stkA* gene coding region in the chromosome of *S.* Paratyphi A ATCC 9150. The sequence from stk-F (forward primer) position at 215106 (underlined nucleotides) to starting position of *stkA* gene at 214980 (ATG in red bold font) used in prediction using BPROM (bacterial promoter prediction program) server available at (http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb). The coordinate shown corresponds to the 5'-most nucleotide for primer binding sites, promoters and start codons.

KOD Xtreme reaction setup

Component	Volume	Final conc.
DNA template	1.0 µl	1 µg
dNTPs (2 mM each)	10 µl	0.4 mM (each)
2× Xtreme Buffer	25 µl	1×
KOD Xtreme™ Hot Start polymerase	1.0 µl	0.02 U/ µl
Forward primer (10 µM)	1.5 µl	0.3 µM
Reverse primer (10 µM)	1.5 µl	0.3 µM
Sterile nH_2O	10 µl	
Total reaction	50 µl	

The PCR program for amplificationof whole *stk* operon was carried out using the following conditions:

Step 1: Polymerase activation at 94°C for 2 min

Step 2: Denaturation at 98°C for 10 sec

Step 3: Annealing at 58°C for 30 sec

Step 4: Extension at 68°C for 1 min/kb (7.5 min)

Step 5: Final extension at 72°C for 7 min

Steps 2-4 were repeated for 30 cycles.

Step 6: Cooling down at 4°C for ∞

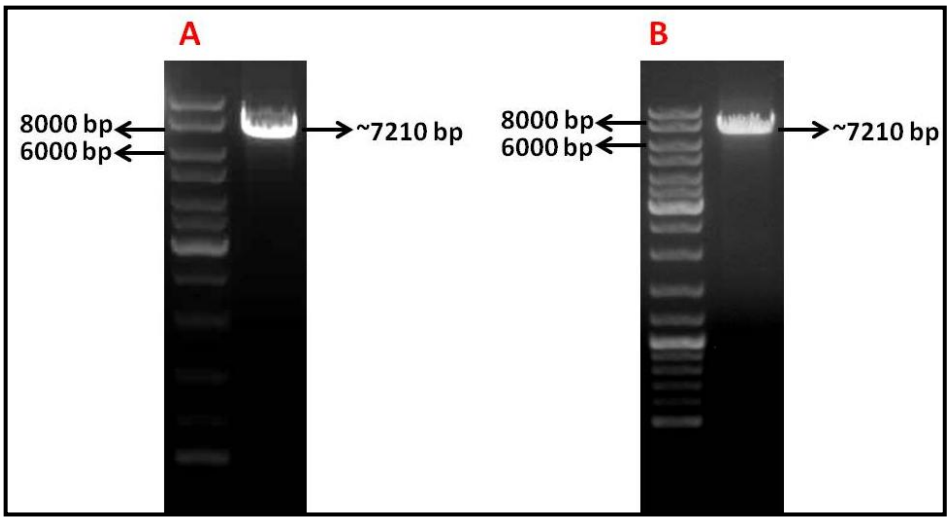


Figure 5.8.2: Agarose gel electrophoresis (0.8 %) of PCR amplified *stk* operons. A, PCR product of *stk* operon carrying the *stkF*^{159}-ON form; **B,** PCR product of *stk* operon carrying the *stkF*^{211}-OFF form; the amplicons are ~7210 bp amplified using stk-F and stk-R primers. GeneRuler™ DNA molecular weight marker (100 − 10,000 bp).

5.8.1.1 Cloning of *stk* operons into pGEM®-T Easy vector

The PCR-amplified fragments encoding Stk fimbriae were recovered from the agarose gel and subjected to A-tailing by the Promega protocol before being cloned into the pGEM®-T easy vector as the high fidelity KOD Xtreme™ Hot Start DNA Polymerase PCR amplified products have blunt ends. The A-tailed PCR amplified *stk* operon-bearing fragment was cloned into pGEM-T easy vector by ligation reaction using T4 DNA Ligase enzyme. Chemically competent *E. coli* DH5α cells were then transformed with the ligation mix to isolate recombinant pGEMstk and pGEMstkOff represent *stk* operons carrying *stkF*^{159bp}-ON form and *stkF*^{211bp}-OFF from, respectively. The positive clones were selected by white/blue screening on LA plates containing amp 100 µg/ml + 40 µg/ml X-gal, checked by colony PCR and confirmed by *Eco*RI restriction analysis of purified plasmids.

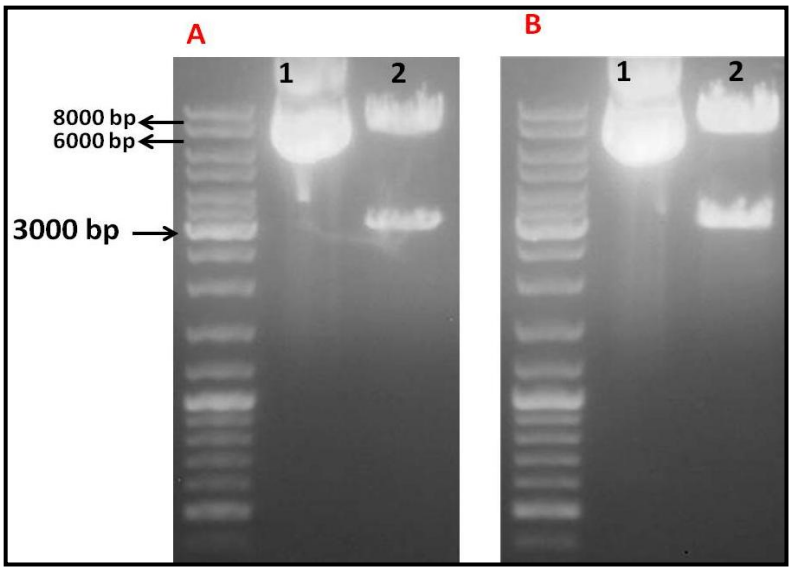


Figure 5.8.3: Agarose gel electrophoresis (0.8 % agarose) of *Eco*RI restriction analysis of the recombinant plasmids: A, pGEMstk; **B**, pGEMstkOff. The expected ~3.0 kb and ~ 7.2 kb bands were noted following digestion. Lane 1, undigested plasmid; lane 2, digested plasmid; DNA ladder is GeneRuler™ DNA molecular weight marker (100 − 10,000 bp).

5.9 Construction of expression plasmids

The vector pUCP20, which is a HCN pLAC *lacZα*, general cloning vector was used for expression of Stk fimbriae. The *stk* protein coding sequences amplified from *S.* Paratyphi A SARB 42 and Paratyphi A PA 42 were sub-cloned from pGEMstk and pGEMstkOff clones into pUCP20 vector between *Eco*RI and *Hin*dIII restriction sites to produce pUCstk and pUCstkOff, respectively.

5.9.1 Preparation of inserts and vector

The DNA inserts (*stk* operons) were prepared by restriction double digestion using *Eco*RI and *Hin*dIII enzymes of pGEMstk and pGEMstkOff to release *stk* fragments as well as digestion of pUCP20 vector by the same enzymes (Fig. 5.9.1).

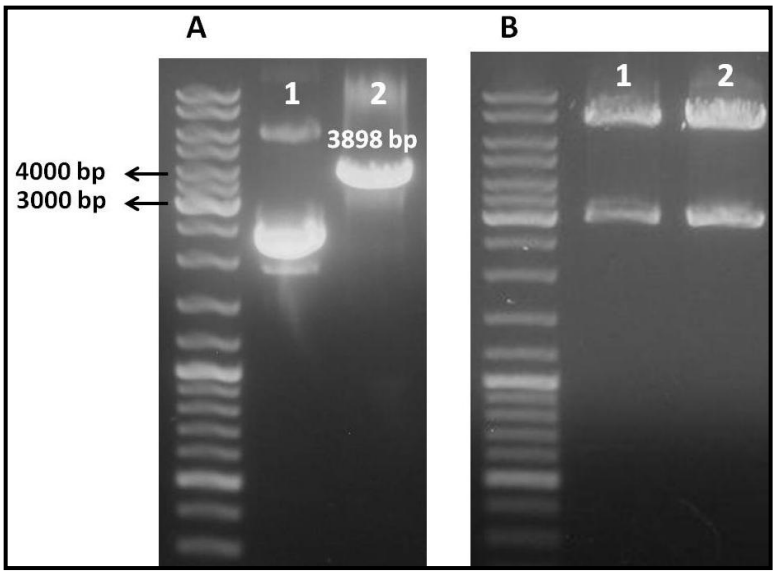


Figure 5.9.1: Preparation of *stk* operons (inserts) and pUCP20 vector for sub-cloning. Agarose gel electrophoresis (0.8 % agarose) of *Eco*RI *and Hin*dIII restriction digestion of: **A,** pUCP20 vector; the digestion was confirmed as appeared in one band at the accurate size of vector (3898 bp) represent the linearised plasmid. Lane 1, undigested plasmid; lane2, digested plasmid; **B**, Lane 1, pGEMstk; lane 2, pGEMstkOff recombinant plasmids (the inserts appeared at ~7210 bp). DNA ladder is GeneRuler™ DNA molecular weight marker (100 − 10,000 bp).

5.9.2 Sub-cloning of *stk* operons fragments into pUCP20 vector

The ~7210 bp fragments recovered from the agarose gel after restriction double digestions of pGEMstk and PGEMstkOff represent *stk* operons carrying $stkF^{159bp}$-ON form and $stkF^{211bp}$ -OFF from, respectively, were ligated into the corresponding cloning sites in linearised pUCP20 vector using T4 DNA ligase enzyme. Electrocompetent *E. coli* HB101 cells were then transformed with the ligation mix to isolate recombinant pUCstk and pUCstkOff. Transformed bacteria were grown on LA plates containing amp 100 µg/ml at 37°C. The positive clones were selected by antibiotic selection, checked by colony PCR, and confirmed by restriction double digestion using *Eco*RI *and Hind*III enzymes and PCR using insert primers (stk-F & stk-R) of purified plasmids.

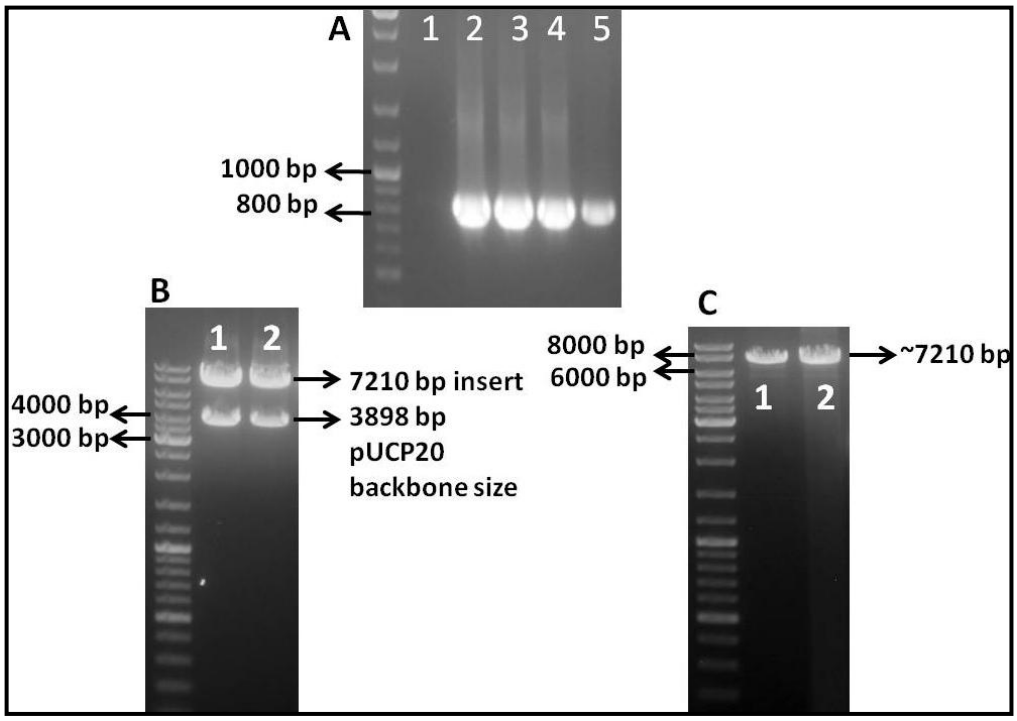


Figure 5.9.2: Analysis of pUCstk and pUCstkOff *E. coli* HB101 transformants. A, colony PCR of two colonies of pUCstk clone (lanes 2 and 3) and two colonies pUCstkOff clone (lanes 4 and 5) using insert primers stkF-F and stkF-R producing an amplicon of 849 bp; **B**, restriction analysis using *Eco*RI and *Hind*III enzymes of purified plasmid of each clone; lane 1: pUCstk, lane 2: pUCstkOff. **C,** PCR amplifications of *stk* fragments from purified plasmids using insert primers *stk*-F and *stk*-R producing the 7210 amplicon; lane 1: pUCstk, lane 2: pUCstkOff. GeneRuler™ DNA molecular weight marker (100 − 10,000 bp).

5.10 Expression of Stk fimbriae

Different growth conditions were tested for expression of *stk* fimbrial structure in *E. coli* HB101. *E. coli* HB101 transformed with pUCstk, pUCstkOff, pUCP20 vector alone and non-transformed *E. coli* HB101 (as negative control) were grown in LB broth supplemented with amp 100 µg/ml. Bacterial cultures were incubated aerobically at 37°C for 48 to 72 h with shaking at 200 rpm as well as under static growth conditions, which usually favour fimbrial expression. The static incubation was performed in

Nunclon surface bottles (Nunc-Denmark) and inoculated with overnight grown culture at 37°C with shaking at 200 rpm. Fimbrial expression was detected only in *E. coli* HB101 transformed with pUCstk after 48 h incubation in standing growth conditions but not in other recombinant strains (Table 5.11-1).

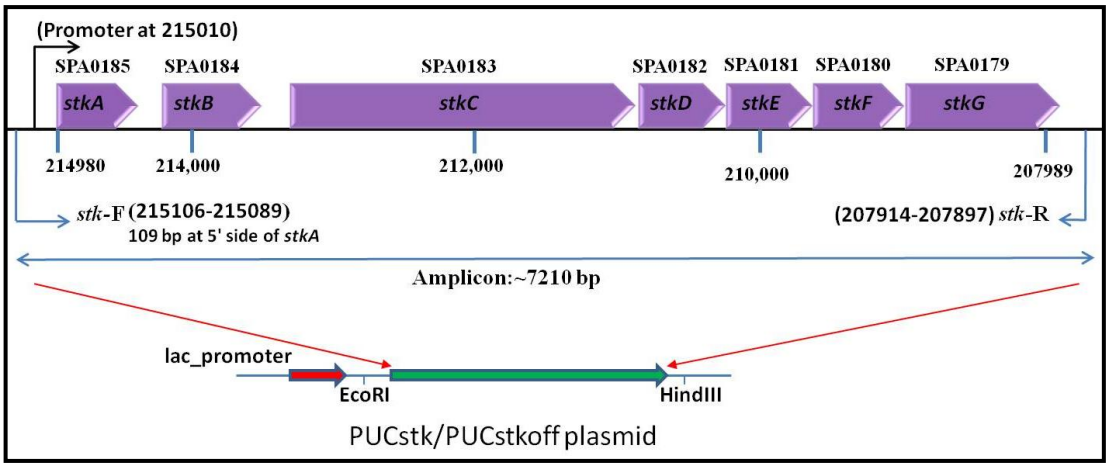


Figure 5.10.1: Construction of pUCstk and pUCstkOff plasmids transformed into *E. coli* HB101. The recombinant pUCstk and pUCstkOff plasmids harbour the entire *stk* gene cluster with extra ~100 bp on both sides carrying *stkF*-ON form and *stkF*-OFF form, respectively amplified by stk-F and stk-R primers were cloned into pUCP20 vector downstream to pLAC promoter.

5.11 Detection of Stk fimbrial structure expression

The detection of fimbrial expression in *E. coli* HB101 transformants and non-transformed *E. coli* HB101 was performed by different methods including transmission electron microscopy (TEM), haemagglutination of RBCs, cell surface hydrophobicity assay and dot blot assay using murine StkF antiserum.

5.11.1 Haemagglutination (HA) and haemagglutination inhibition (HAI)

Haemagglutination (HA) assays were carried out using horse, guinea pig and human (blood group A) erythrocytes by slide test at room temperature to detect the expression of fimbriae on the bacterial cell surface, which is likely to be attributed to the presence of type 1 fimbriae. Harvested bacterial cells grown in LB broth under different growth conditions as well as cells grown on LA agar plates were suspended in PBS (pH 7.2). HA tests were performed as described by Hagberg et al. (1981). Bacterial suspensions (50 μl) in PBS was mixed with equal volumes of a 3 % (v/v) suspension of human, guinea pig, and horse erythrocytes in PBS on a glass slide (Hagberg *et al.*, 1981). Agglutination was observed visually within 10 min and graded ++ (agglutination within 5 min), or ++++ (agglutination within 2 min). The strength of the agglutination was determined further by serial twofold dilutions in PBS of the bacterial suspensions. Mannose HAI was tested by adding 3 % (w/v) D-mannose to HA reaction. Agglutination completely reversed by D-mannose was designed as MSHA (mannose-sensitive hemagglutination).

5.11.1.1 HA patterns

HA of human, guinea pig, and horse erythrocytes by *E. coli* HB101/pUCstk were detected with bacteria grown in standing broth at 37°C for 48 h. However, *E. coli* HB101/pUCstk grown under shaking conditions at 37°C did not show HA. HA reaction with these erythrocytes was always mannose sensitive (MS), indicating the presence of MS haemagglutinating fimbrial structure. In addition, with *E. coli* HB101/pUCstk formation of a pellicle on the surface of the standing culture with the culture itself remaining uniformly turbid during incubation was observed. However, the bacteria grown on LA plates did not show any HA. *E. coli* HB101 cells, *E. coli* HB101/pPUCstkoff and HB101/pUCP20 did not show any of these phenotypes. HA titer as indicated by the highest bacterial dilution giving haemagglutination was 1:8.

Table 5.11-1: HA pattern of *E. coli* HB101/pUCstk transformant with RBCs

Source of RBCs	HA reaction[1]
Horse RBCs	++++ (strong)
Human RBCs (blood A group)	++ (weak)
Guinea pig RBCs	++++ (strong)

[1]HA reaction was performed according to Hagberg et al. (1981) using 3 % (v/v) suspension of human, guinea pig, or horse erythrocytes in PBS on a glass slide. The reaction was designated strong and weak due to time of agglutination and size of clumps, ++ (agglutination within 5 min), or ++++ (agglutination within 2 min).

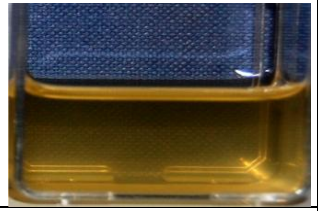
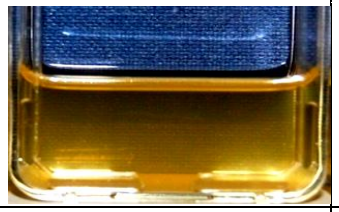
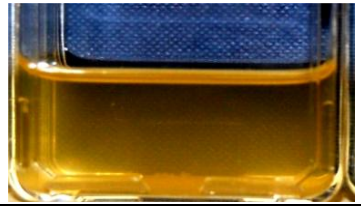
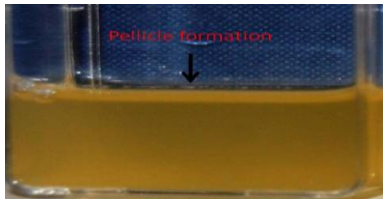
Growth conditions	Time of incubation (h)	*E. coli* HB101			*E. coli* HB101/ pUCP20[1]			*E. coli* HB101 /pUCstkOFF[2]			*E. coli* HB101/ pUCstk[3]			
		OD[4]	Pellicle	HA[5]	OD	Pellicle	HA	OD	Pellicle	HA	OD	Pellicle	HA	MHI[6]
Aerobic static broth	0	0.1	-	-	0.1	-	-	0.1	-	-	0.1	-	-	NA
	24	0.205	-	-	0.215	-	-	0.231	-	-	0.315	-	-	NA
	48	0.202	-	-	0.220	-	-	0.224	-	-	0.399	+	+	S
	72	0.170	-	-	0.171	-	-	0.171	-	-	0.775	+	+	S
														
Aerobic shaken broth	0	0.1	-	-	0.1	-	-	0.1	-	-	0.1	-	-	NA
	24	>2	-	-	>2	-	-	>2	-	-	>2	-	-	NA
	48	>2	-	-	>2	-	-	>2	-	-	>2	-	-	NA
	72	>2	-	-	>2	-	-	>2	-	-	>2	-	-	NA
LA agar plates	0 24 48 72	NA	NA	-	NA	NA	-	NA	NA	-	NA	NA	-	NA

Table 5.11-2: Phenotypic characteristics of *E. coli* HB101 expressing *stkF*-ON and *stkF*-OFF forms of the *stk* fimbrial operon.1, *E. coli* HB101 transformed by pUCP20 vector; **2**, *E. coli* HB101 transformed by pUCstkoff plasmid (pUCP20 harbouring entire *stk* operon carrying *stkF*-OFF form); **3**, *E. coli* HB101 transformed by pUCstk plasmid (pUCP20 harbouring entire *stk* operon carries *stkF*-ON form); **4**, measuring the ODs of cultures (turbidity) as an indication of growth; **5**, haemagglutination ability; **6**, mannose-haemagglutination inhibition. S, sensitive to mannose; NA, not applicable.

5.11.2 Transmission electron microscopy (TEM)

Fimbral expression was investigated by TEM performed by Stefan Hyman at Electron Microscope Unit, University of Leicester. Fimbriae were detected on the surface of *E. coli* HB101/pUCstk only.

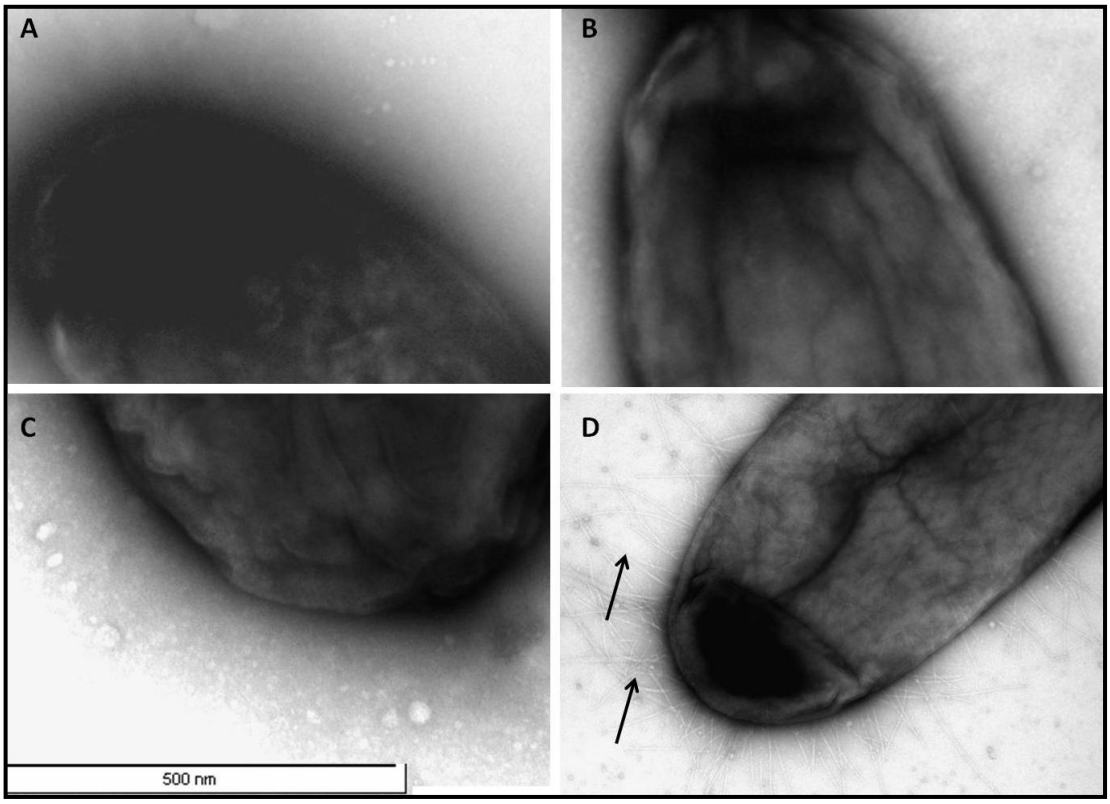


Figure 5.11.1: Transmission electron microscopy studies of fimbriae expression by the *E. coli* HB101 transformants. A, *E. coli* HB101; **B**, *E. coli* HB101/pUCP20; **C**, *E. coli* HB101/pUCstkoff; **D**, *E. coli* HB101/pUCstk. Only *E. coli* HB101/pUCstk grown in LB media at 37°C under aerobic standing conditions for 48 − 72 h displayed observable pili (arrows), which were found to be fine, abundant and peritrichously distributed.

5.11.3 Cell-surface hydrophobicity assay

The cell-surfaces hydrophobicity of *E. coli* HB101 transformants and non-transformed *E. coli* HB101 was assessed by the salt aggregation test (SAT). SAT was performed as described in Drumm *et al.* (1989) by slide agglutination of bacteria which were salted out" (aggregated) with varying concentrations of ammonium sulphate $(NH_4)_2SO_4$. In this assay, surface hydrophobicity is inversely correlated with the salt concentration required to mediate agglutination of bacteria; more hydrophobic bacteria aggregate at relatively low concentrations of salt. Bacteria agglutinated by concentrations of ammonium sulphate of 1 M or less are defined as hydrophobic (Drumm *et al.*, 1989). Bacteria were suspended in PBS at a concentration of 5×10^8 / ml. A 25 µl aliquot of each bacterial suspension was mixed on glass microscope slides with an equal volume of $(NH4)_2SO_4$ at concentrations varying between 0.0625 M and 4 M. The slides were gently rotated by hand and observed visually after 2 min for the presence of bacterial aggregation. The lowest final concentration of $(NH4)_2SO_4$ that caused aggregation was recorded as the SAT value. The SAT assay was performed in different pH values (pH 3.6, 7 and 9) and at different temperatures (4°C, 20°C and 37°C) but the change in the SAT results was not significant.

Table 5.11-3: Hydrophobicity assay of *E. coli* HB101 transformants

Strain	Lower salt concentration[1]
Non-piliated *E. coli* HB101	≥ 4.0 M
Non-piliated *E. coli* HB101/pUCP20	≥ 4.0 M
Non-piliated *E. coli* HB101/pUCstkoff	≥ 4.0 M
Piliated *E. coli* HB101/pUCstk	≥ 0.6 M

[1]SAT using $(NH4)_2SO_4$ showed a significant difference in surface hydrophobicity ($P < 0.0001$) between the piliated strain (0.6000 ± 0.12) and non-piliated strain (4.000 ± 0.12), mean ± SE of three independent experiments.

5.11.4 Dot Blot for detection of expression of Stk fimbriae

A dot blot assay using murine StkF antiserum (1:1,000) as primary antibody was used to detect expression of Stk fimbriae on the surface of *E. coli* HB101/pUCstk. *E. coli* HB101 and PBS were used as controls. A signal was only detected with *E. coli* HB101/pUCstk.

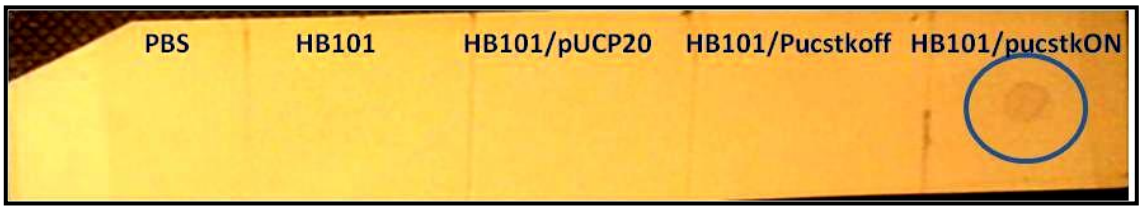


Figure 5.11.2: Dot blot immunoassay for detection of Stk fimbrial structure expression on the surface of *E. coli* HB101 clones. *E. coli* HB101/pUCP20, *E. coli* HB101/pUCstkOff, *E. coli* HB101/pUCstk as well as non-transformed *E. coli* HB101 cells and PBS as controls were applied to a nitrocellulose membrane in 3 ul dots. The membrane was incubated with murine StkF antiserum (1:1,000) as primary antibody and the goat anti-mouse IgG-AP (1:30,000) used as secondary antibody. After adding the alkaline phosphatase BCIP/NBT substrate solution (Sigma, UK-B5655), a detectable dot was found only in the section where *E. coli* HB101/pUCstk plasmid was applied indicating the expression of of Stk fimbrial structure, which includes StkF as a subunit.

5.12 Discussion

Salmonella enterica genomes include a wide variety of putative fimbrial gene clusters, however, only a few *Salmonella* fimbriae have been characterized until now. These putative fimbriae may provide different binding specificities required at different stages of the infection process and may be involved in host adaptation by conferring the ability to bind to targets in specific hosts (Forest *et al.*, 2007). The presence of a large number of fimbrial gene clusters appears to be a common feature of the genomes of *Salmonella enterica* belonging to all serovars represented by the current genome set (Humphries *et al.*, 2001).

The work in this chapter aimed to further investigate and characterise the putative *stk* fimbrial gene cluster and the Stk fimbrial structure encoded by this operon. The *Salmonella stk* putative fimbrial gene cluster was reported to be conserved and specific to *S.* Paratyphi A (Porwollik & McClelland, 2003). However, Ou et al. (2007) had shown that *stkF* homologues were found in about 30 % of *Salmonella* serovars studied. In addition, Bronowski and Winstanley (2009) showed that *S.* Kentucky and *S.* Heidelberg carry this operon. Though, reverse transcription PCR data showed variable carriage and expression of this operon by *S.* Heidelberg isolates. Given the proven role of fimbriae in pathogenicity of *Salmonella*, the variable carriage of this fimbrial operon, and the variable expression even among those strains carrying its genes, the *stk* fimbrial operon clearly deserved further investigation and characterisation of the predicted Stk fimbrium and its subunits (Ou *et al.*, 2007, Bronowski & Winstanley, 2009).

The serovar Paratyphi A *stk* fimbrial gene cluster contains seven *orfs* designated *stkABCCDEF*, predicted by Microbes Online Operon Predictions server (Fig. 5.2.1). It

was predicted to encode the putative Stk fimbriae of *S.* Paratyphi A. Intact versions of this operon were also identified by blastn analysis against available *Salmonella* genome sequences in other serovars including Virchow, Heidelberg, Kentucky and Tennessee (Table 5.3-1). In addition, an *stkF* PCR survey performed in this study demonstrated the presence of this operon in about 30 % of serovars tested (Chapter 3, Table 3.3-1).

Analysis of the biochemistry and genetics of fimbrial biosynthesis has resulted in classification of fimbriae based on assembly mechanisms. The main assembly classes found in Gram-negative bacteria include type IV fimbriae, fimbriae assembled by the extracellular nucleation/ precipitation pathway (curli), fimbriae assembled by the chaperon/usher-dependent pathway (C/U) and those put together by the alternate C/U-dependent pathway. Thus, putative fimbrial gene clusters identified by whole-genome sequencing can be identified by sequence homology of fimbrial biosynthesis genes to members of an assembly class (Nuccio & Baumler, 2007). In this study, bioinformatics characterisation revealed that the *stk* fimbrial cluster has a G + C content of 49.3 % and is a member of the C/U-dependent class. C/U fimbrial operons encode, at minimum, three different proteins: structural proteins including a major fimbrial subunit and minor fimbrial subunits, a chaperone, and an usher protein, the latter two being the assembly proteins (Nuccio & Baumler, 2007). The putative functions of Stk fimbrial subunits were predicted using NCBI conserved domain database and orthologe detection. StkA protein was annotated as the extracelluar major fimbrial subunit, and StkB and StkC proteins were annotated as the periplasmic chaperon and outer membrane usher subunits, respectively, while the other subunits were classified as extracellular fimbrial minor subunits. Additionally, StkF was annotated as a potential tip adhesin subunit

(Tables 5.4-1, 5.5-1). Importantly, none of these assignments has been experimentally verified.

The chaperones and usher assembly proteins show better sequence conservation than structural subunits and hence have been used to classify fimbrial operons belonging to the C/U class. All these fimbrial operons contain only a single usher gene, while multiple genes encoding subunits or chaperones may be present. The study of Nuccio & Baumler (2007) defined six major phylogenetic clades of the C/U fimbrial operons (α-, β-, γ-, κ-, π -, and σ-) based on the fimbrial usher protein (FUP) family. The γ-fimbrial clade was further subdivided into four sub-clades, termed γ_1-, γ_2-, γ_3-, and γ_4-fimbriae. These authors identified two subfamilies of chaperones proteins: the FGL chaperone subfamily, which assembles thin fibrillae or nonfimbrial surface structures, and the FGS chaperone subfamily, which assembles fimbrial filaments. The FGL chaperone subfamily forms a monophyletic group (γ_3-fimbriae) within the classical chaperone/usher family. However, the FGS chaperone subfamily is composed of several clades (β-, γ_1-, γ_2-, γ_4-, κ-, and π- fimbriae) (Nuccio & Baumler, 2007). The blast searches using protein knowledge base (UniProt KB) server available at (http://www.uniprot.org/) identified the HtrE usher protein and EcpD chaperone protein of the *yad* fimbrial operon present in *E. coli* K-12 as showing the highest alignments (orthogues) to StkC and StkB, respectively. The *yad* fimbrial operon is identified C/U-γ4-fimbrial clade, thus, the putative *stk* fimbrial operon was identified as a member of the γ4 fimbrial clade of the C/U class, which is known to be found in Betaproteobacteria and Gammaproteobacteria (Nuccio & Baumler, 2007).

As an experimental approach, fimbriae can be characterised phenotypically after heterologous expression in *E. coli* strains using electron microscopy as has been shown

for the *Salmonella stc* fimbrial operon (Humphries *et al.*, 2003). In this study, the *stk* fimbrial operon was cloned and expressed in the nonfimbriate *E. coli* K-12 strain HB101. Fimbriae are too narrow to be seen with the light microscope but these structures can frequently be seen by electron microscopy (Duguid *et al.*, 1966). Thus, fimbral expression was investigated by TEM. There was marked expression of surface fimbrial structures on *E. coli* HB101/pUCstk but no expression was detected on *E. coli* HB101/ pUCstkoff or *E. coli* HB101/ pUCP20 or *E. coli* HB101 cells only. In addition, the fimbriated *E. coli* showed haemagglutination of horse, human, and of guinea pig erythrocytes. Most *Salmonella* isolates produce type 1 fimbriae and consequently exhibit the MS haemagglutinating phenotype (Dwyer *et al.*, 2011). Similarly, *stk* operon appeared to encode an equivalent appendage as mannose was found to inhibit the HA caused by *E. coli*/pUCstk. In addition, a pellicle was formed at the surface of the standing growth culture of *E. coli*/pUCstk and the culture remained uniformly turbid during the whole period of incubation, both traits that are typical of type 1 fimbriae. Aerobic, static, broth culture favours optimal expression of fimbriae, which in turn allow fimbriated bacteria to establish a pellicle on the surface of the broth where growth is promoted by the free supply of oxygen (Old & Duguid, 1970). It was found from the literature that the presence of type-1 fimbriae in *Salmonella* is associated not only with mannose-sensitive adhesive properties but also with the ability to form a type-1 fimbrial pellicle at the surface of liquid media incubated statically under aerobic conditions (Old *et al.*, 1989). These observations support classification of the Stk fimbriae as members of the phenotypically-defined type 1 fimbrial group, in which fimbrial phase variation is common (Clegg & Gerlach, 1987). In addition, Stk fimbrial expression causes the *E. coli* bacterial surface to become more hydrophobic consistent with the observed phenotype of HB101/pUCstk. Importantly, as hydrophobicity of bacterial/*Salmonella*

strains has been shown to correlate positively with virulence and initial binding to host tissues (Doyle, 2000), this finding may support a role for Stk fimbriae in the pathogenesis process and/or the adhesion to inanimatye surfaces.

The non-expression (or non-production) of fimbriae in case of *E. coli* HB101/ pUCstkoff, which harbours an *stk* operon which carries the *stkF*^{211bp}-OFF allele supports the idea of Stk fimbrial phase variation mediated by repeat sequence changes in *stkF* as discussed in Chapter 3. In *E. coli* and other Enterobacteriaceae such as *S.* Typhimurium, phase switching of the *fim* operon is controlled by an oscillation between phase ON and phase OFF expression states governed by an invertible promoter-bearing fragment lying upstream of *fimA* that code for the major fimbrial subunit (Clegg *et al.*, 1996). In addition, the transcription of the *lpf* and *pef Salmonella* fimbrial operons is regulated by phase variation mediated by a random heritable switching mechanism. Whether, expression of other *Salmonella* fimbrial operons is regulated by phase variation needs to be investigated (Humphries *et al.*, 2001). Indeed, complex multigene, transcriptional and translational mechanisms that vary from fimbrial operon to fimbrial operon have been suggested or shown to contribute to such variation in *Salmonella* and other bacteria (Ou *et al.*, 2007). The expression of *H. influenza* LKP long thick pili is subjected to reversible phase variation controlled by SSM at the transcriptional level of two divergently orientated genes, *hifA* and *hifB*, encoding respectively the major fimbrial subunit and the fimbrial chaperone (van Ham *et al.*, 1993). However, not all-fimbrial phase variation occurs at the transcriptional level. The phase-variable expression of *Neisseria gonorrhoeae* type IV fimbrial adhesin PilC, caused by frame shift rely on SSM during DNA replication of regions containing highly repetitive Gs at the beginning of the coding region (Seifert, 1996).

Mutations in genes encoding the major structural fimbrial subunit as well as assembly proteins necessary for fimbrial production, such as the PapC usher and PapD chaperon proteins, usually result in the absence of fimbriae from the bacterial surface but this is not likely with mutations in minor fimbrial subunit genes (Kuehn *et al.*, 1994). However, Dwyer et al. (2011) demonstrated that the allelic variation in the tip adhesin gene of *Salmonella* strains coding for FimH not only affected adherence and biofilm formation abilities but could also influence the ability of bacteria to assemble type 1 fimbriae. The latter inference stems from the non-fimbriated and fimbriated derivatives observed amongst otherwise isogenic strains suggesting an attribution of this trait to the differences in the primary amino acid sequence of FimH adhesins (Dwyer *et al.*, 2011). This correlation between the allelic variation of minor fimbrial subunits FimH and the inability to the assemble type 1 fimbriae could explain why *E. coli* HB101/pUCstkoff fails to express any phenotypically or EM-visualizable fimbriae.

Chapter Six

Salmonella serodiagnosis

6 *Salmonella* serodiagnosis

6.1 Introduction

The 'gold standard' to confirm *S. enterica* infections is the isolation and identification of the causative agent from a suitable clinical specimen. *S. enterica* organisms, especially typhoidal *Salmonella* serovars, are usually isolated from faeces, bone marrow and/or blood specimens of patients. However, in the absence of culturable organisms, detection of specific antibodies in the sera of patients might give evidence of invasive *Salmonella* infections. Therefore, the potential value of immunoassays based on well-characterized antigens (Chart *et al.*, 2007, Nakhla *et al.*, 2011). Serodiagnosis of enteric fever has been attempted since the late 19th century by the Widal agglutination test that formed the basis of serodiagnostic tests for typhoid for over 100 years (Olsen *et al.*, 2004). Despite newer serological tests performing better than the Widal test, these have also offered unacceptable sensitivity and specificity. Therefore, none has been successfully validated or proven to be of practical value in endemic areas (Olsen *et al.*, 2004). Consequently, there is an urgent need for the development, evaluation and validation of new and improved diagnostic methods for enteric fever, which must cover the emerging threat of *S.* Paratyphi A infection (Wain & Hosoglu, 2008). Improved serological diagnosis by using new ELISAs remains a key focus (Meltzer & Schwartz, 2010).

This chapter describes the development of an ELISA based serodiagnosis of invasive *Salmonella* infections using the recombinant antigens, rStkF (*S.* Paratyphi A-specific), rStaF (*S.* Typhi-specific) and rSipA (pan-*Salmonella*).

6.2 StaF and SipA protein sequences

The gene *staF* (597 bp), a part of *S.* Typhi *sta* fimbrial gene cluster, which encodes the 198 aa StaF (a putative fimbrial protein) containing the conserved Pfam 00419 fimbrial family domain was selected as it was found to be specific to the Typhi serovar by GenomeSubstrator analysis performed by Dr Ewan Harrison, Lab 212. StaF is thought to be involved in cellular adhesion as it was found to be expressed *in vivo* in patients with *S.* Typhi bacteremia (Sheikh *et al.*, 2011). The *sipA* gene (2058 bp) encodes the 685 aa *Salmonella* pathogenicity island 1 (SPI1) effector protein SipA (*Salmonella* invasion protein A), which is secreted and/or translocated into the host cell. *sipA* gene is a member of a group of genes encoding SPI-1-dependent T3SS effector proteins, which are present in all *Salmonella* lineages and located in the core region of SPI1 (Mirold *et al.*, 2001). SipA bind to the actin of the cytoskeleton and rearranges this architectural framework, thus enhancing entry of bacteria into host cells (Yan *et al.*, 2008) (Fig. 6.2.1).

A) StaF

```
                  M  V  I  P  M  R  R  L  R  E  P  T  L
                ATGGTGATACCAATGAGACGTTTGCGCGAACCCACGCTCG
A  T  L  F  S  G  L  A  T  A  L  F  S  A  T  L  Y  A  D  T
CCACGTTATTTTCAGGGCTGGCAACAGCCCTGTTTAGCGCGACGCTGTACGCCGATACCA
N  V  N  F  T  A  S  V  Q  K  D  T  C  Q  I  K  I  D  G  N
ATGTGAATTTCACCGCCAGCGTGCAGAAGGACACCTGCCAGATAAAGATAGATGGCAATG
G  T  V  N  F  A  T  I  A  P  A  Y  F  A  D  G  I  T  A  E
GAACGGTAAACTTCGCCACCATAGCGCCCGCCTATTTTGCCGATGGCATTACCGCGGAAA
T  D  Y  E  G  G  K  E  F  T  I  K  L  I  S  C  P  I  S  D
CCGATTATGAGGGGGGGAAAGAGTTCACCATTAAGCTTATTTCTTGCCCGATCAGTGATG
G  K  I  T  N  V  T  F  N  F  A  P  L  N  G  Q  F  S  P  E
GTAAAATAACCAATGTCACCTTTAATTTCGCACCACTAAACGGGCAGTTCTCACCTGAGA
N  Q  Q  V  F  P  N  D  I  A  T  D  A  G  G  V  D  N  V  G
ATCAACAAGTTTTTCCTAATGACATCGCTACCGATGCTGGTGGCGTAGACAATGTCGGCG
V  V  I  F  T  T  D  S  P  R  T  N  V  L  N  T  D  G  S  S
TGGTGATTTTCACCACCGATTCACCACGCACCAACGTGCTTAATACGGATGGTTCATCGC
R  A  T  F  A  A  S  T  Y  S  D  T  V  W  T  F  Y  S  R  M
GGGCGACTTTTGCTGCCAGCACCTACAGCGACACCGTCTGGACCTTTTACTCCAGAATGC
Q  K  I  R  S  A  E  K  V  T  T  G  E  L  S  S  R  V  L  V
AAAAAATACGCAGTGCCGAAAAAGTGACGACCGGTGAACTTAGCAGCCGTGTACTGGTGA
N  V  S  Y  E  *
ATGTCTCCTATGAATAA
```

(B) SipA

```
                        M  V  T  S  V  R  T  Q  P  P  V  I  M
                     ATGGTTACAAGTGTAAGAACTCAGCCCCCCGTCATAATGC
P  G  M  Q  T  E  I  K  T  Q  A  T  N  L  A  A  N  L  S  A
CAGGTATGCAGACCGAGATCAAAACGCAGGCCACGAATCTTGCGGCGAATCTTTCCGCGG
V  R  E  S  A  T  A  T  L  S  G  E  I  K  G  Q  Q  L  E  D
TCAGAGAAAGTGCCACAGCAACGCTGTCAGGGGAAATTAAAGGCCAGCAACTGGAAGATT
F  P  A  L  I  K  Q  A  S  L  D  A  L  F  K  C  G  K  D  A
TTCCCGCGCTGATCAAACAGGCGAGTCTGGATGCGTTGTTTAAATGCGGGAAAGACGCCG
E  A  L  K  E  V  F  T  N  S  N  N  V  A  G  K  K  A  I  M
AGGCGTTAAAAGAAGTTTTTACCAATTCAAATAATGTCGCCGGTAAGAAAGCGATAATGG
E  F  A  G  L  F  R  S  A  L  N  A  A  S  D  S  P  E  A  K
AGTTTGCCGGCCTCTTTCGTTCAGCGCTCAACGCCGCCAGTGATTCTCCTGAGGCGAAGA
T  L  L  M  K  V  G  A  E  Y  T  A  Q  I  I  K  D  G  L  K
CGCTACTGATGAAGGTGGGGGCAGAGTATACCGCGCAAATCATAAAAGATGGCCTGAAAG
E  K  S  A  F  G  P  W  L  P  E  T  K  K  A  E  A  K  L  E
AAAAGTCAGCTTTTGGGCCATGGCTGCCAGAAACAAAGAAAGCGGAAGCGAAGCTGGAAA
N  L  E  K  Q  L  L  D  I  I  K  N  N  T  G  G  E  L  S  K
ACCTGGAAAAGCAGCTGTTAGATATCATCAAAAATAACACTGGCGGTGAATTAAGTAAAT
L  S  T  N  L  V  M  Q  E  V  M  P  Y  I  A  S  C  I  E  H
TATCGACGAATCTTGTTATGCAGGAGGTGATGCCCTATATTGCCAGCTGCATTGAACATA
N  F  G  C  T  L  D  P  L  T  R  S  S  L  T  Q  L  V  D  K
ACTTTGGCTGTACGTTAGATCCGTTAACCCGCAGCAGTCTTACGCAGCTTGTTGACAAAG
A  A  A  K  A  V  E  A  L  D  M  C  H  Q  K  L  T  Q  E  Q
CGGCGGCGAAGGCGGTTGAGGCGCTTGATATGTGCCACCAAAAATTAACGCAAGAGCAGG
G  T  S  V  G  R  E  A  R  H  L  E  M  Q  T  L  I  P  L  L
GTACCAGCGTAGGACGGGAAGCCCGGCACCTTGAAATGCAAACGTTGATACCCCTGCTGC
L  R  N  V  F  A  Q  I  P  A  D  K  L  P  D  P  K  I  P  E
TACGTAATGTTTTTGCACAAATTCCTGCAGATAAACTGCCTGACCCTAAAATTCCGGAAC
P  A  A  G  P  V  P  D  G  G  K  K  A  E  P  T  G  I  N  I
CTGCGGCTGGACCAGTACCTGATGGTGGGAAAAAAGCAGAACCTACGGGTATTAATATCA
N  I  N  I  D  S  S  N  H  S  V  D  N  S  K  H  I  N  N  S
ATATTAATATTGATAGCAGTAACCATAGCGTGGATAACAGTAAGCATATTAACAATAGCC
R  S  H  V  D  N  S  Q  R  H  I  D  N  S  N  H  D  N  S  R
GAAGCCATGTCGATAATAGCCAGCGCCATATCGATAATAGCAACCATGATAATAGCCGGA
K  T  I  D  N  S  R  T  F  I  D  N  S  Q  R  H  G  E  S  H
AGACGATTGATAATAGCCGAACATTTATTGATAACAGCCAACGCCATGGCGAGTCACACC
H  S  T  N  S  S  N  V  S  H  S  H  S  R  V  D  S  T  T  H
ATTCGACTAACAGCAGCAACGTAAGTCATAGTCATTCGCGTGTGGATTCGACTACGCATC
Q  T  E  T  A  H  S  A  S  T  G  T  I  D  H  G  I  A  G  K
AAACGGAGACCGCACACAGCGCCAGCACAGGGACAATTGACCATGGCATCGCGGGTAAAA
I  D  V  T  A  H  A  T  A  E  A  V  T  N  S  S  S  E  S  K
TTGACGTCACGGCTCATGCGACGGCAGAGGCTGTGACCAACTCTTCATCCGAAAGTAAAG
D  G  K  V  V  T  S  E  K  G  T  T  G  E  T  T  S  F  D  E
ATGGAAAGGTGGTCACGTCAGAAAAGGGCACTACGGGTGAAACAACCTCTTTTGATGAAG
V  D  G  V  T  S  K  S  I  I  G  K  P  L  Q  A  T  V  H  G
TCGATGGCGTAACCAGCAAGAGCATTATCGGCAAGCCGTTACAGGCCACGGTGCACGGCG
V  D  D  N  K  Q  Q  S  Q  T  A  E  I  V  N  V  K  P  L  A
TTGATGATAATAAACAGCAAAGCCAGACGGCAGAGATTGTGAATGTGAAGCCGCTTGCCA
S  Q  L  A  G  V  E  N  V  K  I  D  T  L  Q  S  D  S  T  V
GTCAACTGGCTGGCGTCGAGAATGTTAAAATCGATACCTTACAATCAGACTCGACGGTAA
I  T  G  N  K  A  G  T  T  D  N  D  N  S  Q  T  D  K  T  G
TTACAGGTAATAAAGCCGGTACGACCGATAATGATAATAGCCAGACAGATAAGACTGGCC
P  F  S  G  L  K  F  K  Q  N  S  F  L  S  T  V  P  S  V  T
CATTTTCTGGTTTGAAGTTTAAGCAAAATAGTTTCCTCTCAACAGTACCGAGCGTTACGA
N  M  H  S  I  H  F  N  A  R  E  A  F  L  G  V  I  R  K  A
ATATGCATTCAATACATTTCAACGCCCGTGAGGCGTTTTTGGGTGTGATACGTAAAGCCC
L  E  P  D  A  S  T  P  F  P  V  R  R  A  F  D  G  L  R  G
TGGAGCCGGATGCCTCTACACCGTTTCCTGTACGCAGAGCGTTTGACGGCTTGCGTGGGG
E  I  L  P  N  D  T  I  K  S  A  A  L  K  A  Q  C  S  D  I
```

```
AAATATTACCCAATGACACGATAAAGAGTGCAGCGTTGAAAGCGCAATGTAGCGATATTG
D  K  H  P  E  L  K  A  K  M  E  T  L  K  E  V  I  T  H  H
ACAAACACCCAGAATTGAAAGCGAAAATGGAGACGCTCAAAGAGGTCATTACTCATCATC
P  Q  K  E  K  L  A  E  I  A  L  Q  F  A  R  E  A  G  L  T
CACAAAAAGAAAAACTGGCTGAGATTGCATTGCAGTTTGCCAGAGAGGCGGGGTTGACCA
R  Q  K  G  E  T  D  Y  V  L  S  N  V  L  D  G  L  I  G  D
GGCAAAAAGGGGAAACTGACTATGTGTTAAGTAATGTGCTGGACGGCCTTATCGGAGATG
G  S  W  R  A  G  P  A  Y  E  S  Y  L  N  K  P  G  V  D  R
GTAGCTGGCGAGCTGGCCCGGCTTACGAGTCATACCTGAATAAACCTGGTGTGGATCGGG
V  I  T  T  V  D  G  L  H  M  Q  R  *
TTATTACTACCGTTGATGGCTTGCACATGCAGCGTTAA
```

Figure 6.2.1: (A) Nucleotide sequence of *staF* gene (*orf*) and the translated amino acid sequence of StaF protein (NP_804068.1) from *S.* Typhi Ty2; (B) Nucleotide sequence of *sipA* gene (*orf*) and the translated amino acid sequence of SipA protein (YP_151909.1) from *S.* Paratyphi A ATCC 9150. Sequences were obtained from NCBI. Start and stop codons are shown in green and the sequence from 5' to 3'.

6.3 BLAST functions of StkF and SipA protein sequences

Both amino acid sequences of StaF and SipA proteins were subjected to Blastp search against the non-redundant database of GenBank using NCBI. StaF protein amino acid sequence showed perfect to near-perfect hits with cognate StaF protein within the sequenced genomes of *S.* Typhi strains but did not identify any other high matching hits. Thus, StaF protein was considered as a specific diagnostic antigen for serovar Typhi. By contrast, SipA amino acid sequence matched a homologue within all sequenced *Salmonella enterica* genomes representative of diverse serovars further suggesting its suitability as a pan-*Salmonella*–specific diagnostic antigen.

Table 6.3-1: Amino acid sequences producing significant alignments to StaF protein

Accession	Strain[1]	Query coverage[2]	Alignment percentage[3]
NP_454793.1	*S.* Typhi str. CT18	100 %	198/198 (100 %)
NP_804068.1	*S.* Typhi str. Ty2		
ZP_03346717.1	*S.* Typhi str. E00-7866	97 %	194/194 (100 %)
ZP_03384612.1	*S.* Typhi str. M223		

[1]Using blastp function, StaF protein amino acid sequence showed highly matched homologues within the genomes of *S.* Typhi strains, which annotated as putative fimbrial proteins.[2]the length coverage of the input query sequence by different high scoring pairs in database. [3]the highest percent identity of the high scoring pairs in database.

Table 6.3-2: Amino acid sequences producing significant alignments to SipA protein

Accession	Strain[1]	Query coverage[2]	Alignment percentage[3]
YP_151909.1 YP_002143399.1	*S.* Paratyphi A str. ATCC 9150 *S.* Paratyphi A str.AKU_12601	100 %	685/685 (100 %)
ZP_02655532.1 NP_806485.1	*S.*Typhi str. CT18 *S.*Typhi str. Ty2	100 %	684/685 (99 %)
YP_217801.1 EFZ07439.1	*S.* Choleraesuis str. SC-B67 *S.* Choleraesuis str. SCSA50	100 %	668/685 (97 %)
ZP_03349227.1	*S.* Typhi str. E00-7866	97 %	669/670 (99 %)
NP_461803.1	*S.* Typhimurium str. LT2	100 %	696/685 (97 %)
YP_002244795.1	*S.* Enteritidis str. P125109	100 %	668/685 (97 %)
EGE35277.1	*S.* Gallinarum str. SG9	100 %	664/685 (96 %)
YP_002227626.1	*S.* Gallinarum str. 287/91	100 %	663/685 (96 %)
ZP_03219228.1	*S.* Javiana str.GA_MM04042433	98 %	658/674 (97 %)
ZP_04653486.1	*S.* Tennessee str. CDC07-0191	98 %	653/673 (97 %)
YP_002638458.1	*S.* Paratyphi C strain RKS4594	97 %	654/670 (97 %)
ZP_02683969.2	*S.* Hadar str. RI_05P066	97 %	654/670 (97 %)
YP_002042125.1 YP_002046844.1 ZP_02697901.2 YP_002216853.1 ZP_03215045.1 ZP_02667550.2 ZP_03165447.1 ZP_02832422.2 ZP_02344027.2	*S.* Newport str. SL254 *S.* Heidelberg str. SL476 *S.* Newport str. SL317 *S.* Dublin str. CT_02021853 *S.* Virchow str. SL491 *S.* Heidelberg str. SL486 *S.* Saintpaul str. SARA23 *S.* Weltevreden str. HI_N05-537 *S.* Saintpaul str. SARA29	97 %	653/670 (97 %)
EFY11231.1	*S.* Montevideo str. 315996572	97 %	655/670 (97 %)
ZP_03077844.1 ZP_02656009.2	*S.* Kentucky str. CVM29188 *S.* Kentucky str. CDC 191	97 %	648/670 (96 %)
YP_002147780.1	*S.* Agona str. SL483	97 %	648/670 (96 %)
YP_002115835.1 ZP_02660979.2	*S.* Schwarzengrund str. CVM19633 *S.* Schwarzengrund str. SL480	97 %	654/670 (97 %)

[1]Using blastp function, SipA amino acid sequence showed high level homologues within the sequenced genomes of diverse *Salmonella* serovars annotated as SPI1 effector protein.[2] and [3] as in Table 6.3-1.

6.4 PCR amplifications of *staF and sipA* genes

The full-length nucleotide sequences of the *orf* of both *staF* and *sipA* were amplified by PCR using the extensor high-fidelity PCR enzyme mix. *staF* was amplified from *S.* Typhi Ty2 genomic DNA using staF-F and staF-R primers. *sipA* was amplified from *S.* Paratyphi A SARB 42 genomic DNA using sipA-F and sipA-R primers. The primers used in the amplification of these two genes were generated by adding *Nde*I and *Xho*I restriction sites at the 5' ends of the forward and reveres primers to ensure unidirectional cloning into the expression vector pET28a. The PCR was setup in 50 µl reactions using conditions as follows.

Master Mix 1 (25 µl):	
10× Extensor Buffer 1	5.0 µl
Extensor high-fidelity PCR enzyme mix	0.25 µl
nH_2O	19.75 µl
Master Mix 2 (25 µl):	
20 mM dNTPs (10 mM)	1.5 µl
Forward primer (10 pmol/µl)	1.0 µl
Reverse primer (10 pmol/µl)	1.0 µl
DNA template	1.5 µl
nH_2O	20 µl
Both master mixes were just mixed prior to reaction cycling	

PCR conditions for cycling comprised a 2 min at 94°C of initial denaturation, followed by 30 cycles of 94°C for 15 sec (denaturation), 60°C (*sipA*) and 58.4°C (*staF*) for 30 sec (annealing), and 68.0°C for 1.5 min (extension), and a final extension at 68°C for 7 min. Reaction aliquot was resolved through 0.8 % agarose gel electrophoresis and DNA bands of PCR amplicons visualized with ethedium bromide staining under UV light (Fig. 6.4.1).

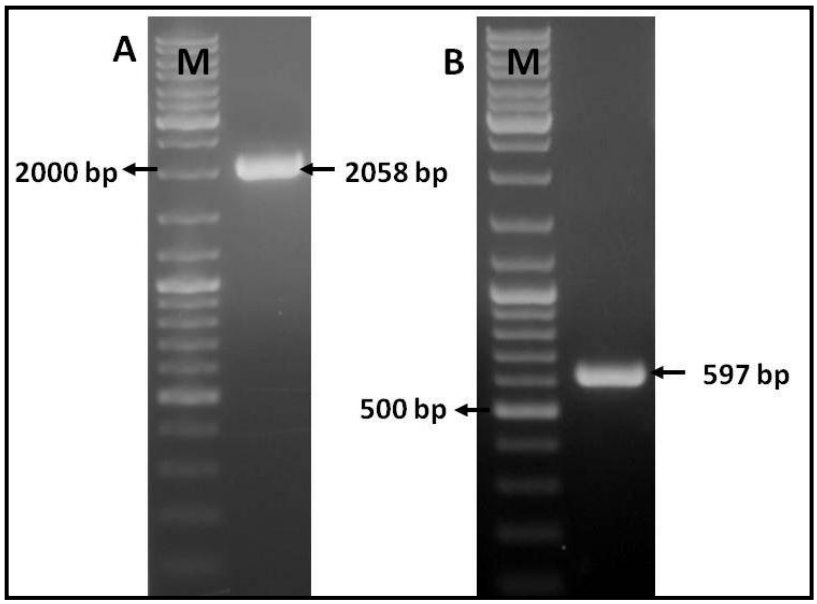


Figure 6.4.1: PCR amplifications of *staF* and sipA genes. Agarose gel (0.8 %) electrophoresis of PCR amplicons of full-length of: **A**, *sipA* gene (~2058 bp) amplified using sipA-F and sipA-R primers; **B**, *staF* gene (~597 bp) amplified using staF-F and staF-R primers; M, GeneRuler™ DNA molecular weight marker (100 − 10,000 bp).

6.4.1 Cloning of *staF and sipA* genes into pGEM®-T Easy Vector

The A-tailed PCR amplified *staF* and *sipA* fragments were cloned into pGEM-T easy vector. Electrocomptent *E. coli* DH5α cells were then transformed with the ligation mix using electroporation transformation procedures. Positive clones were selected by white / blue screening on LA plates containing Amp 100 μg/ml + X-gal 40 μg/ml, then performing colony PCR for white colonies. The success of ligation was then confirmed by *Eco*RI restriction analysis of purified plasmids (Fig. 6.4.2). The recombinant plasmids pGEMstaF and pGEMsipA were then sent for automatic DNA sequencing using T7 primer and the sequence obtained was 100 % matching with *staF* nucleotide sequence of *S.* Typhi Ty2 and *sipA* nucleotide sequence of *S.* Paratyphi A ATCC 9150 published in GenBank database.

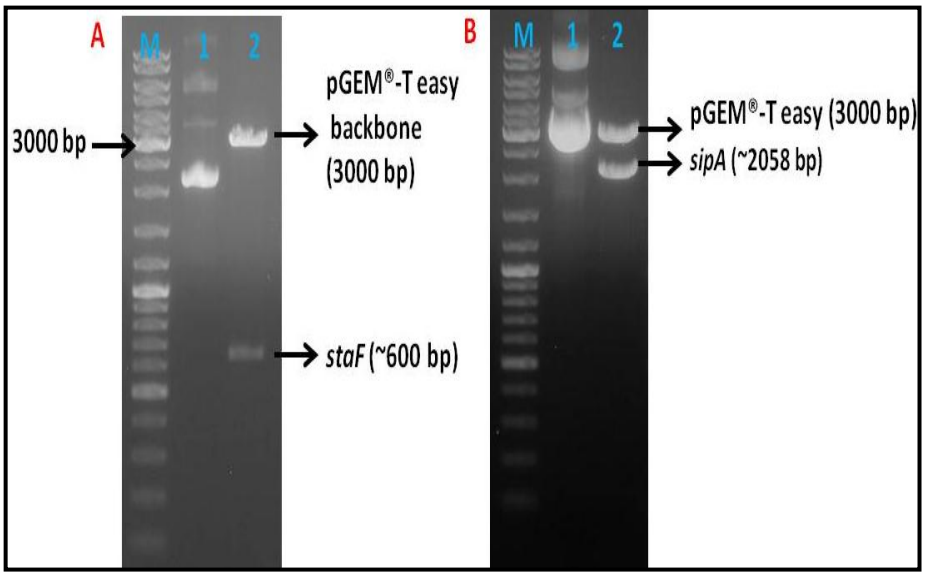


Figure 6.4.2: Agarose gel (0.8 %) electrophoresis of *Eco*RI restriction analysis of pGEMstaF and pGEMsipA recombinant plasmids. The restriction digestion confirmed the cloning as the vector contains the insert appeared in two bands after digestion, the vector at 3015 bp and the insert at: **A**, ~600 bp of *staF*; **B**, ~2058 bp of *sipA*. 1, undigested plasmid; 2, digested plasmid; M, GeneRuler™ DNA molecular weight marker (100 − 10,000 bp).

6.4.2 Construction of expression plasmids

The pET28a is a pBR322, f1-derived expression vector that induces high protein expression under control of T7 promoter which is activated by binding to host cell T7 RNA polymerase induced by IPTG. The *orf* of *staF* and *sipA* were sub-cloned from pGEMstaF or pGEMsipA into pET28a vector between *Nde*I and *Xho*I restriction sites originating the corresponding pETStaF and pETSipA expression plasmids.

6.4.2.1 Preparation of inserts and vector

The DNA inserts and vector were prepared by restriction double digestion using *Nde*I and *Xho*I enzymes of pGEMStaF and pGEMSipA to release *staF* and *sipA* fragments, and pET28a vector, respectively (Fig. 6.4.3).

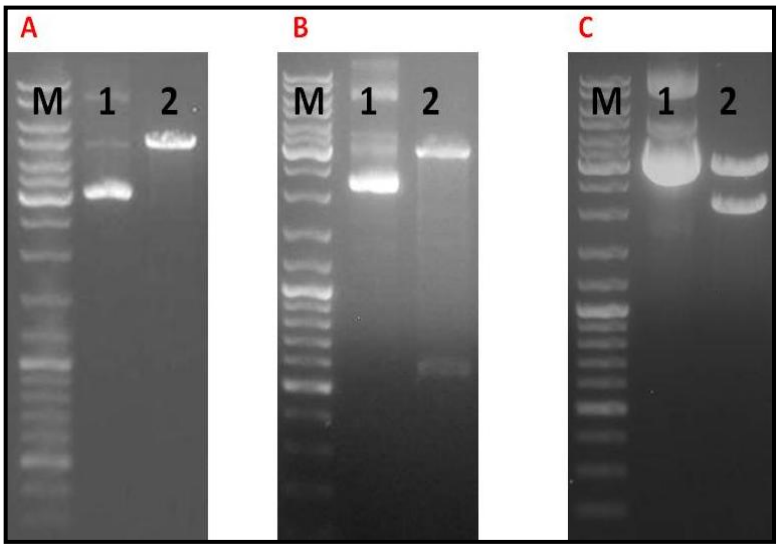


Figure 6.4.3: Preparation of *staF* and *sipA* fragments (inserts) and pET28a expression vector for ligation. Agarose gel (0.8 %) electrophoresis of *Nde*I and *Xho*I restriction digestion of: **A**, pET28a vector; the digestion was confirmed as appeared in one band at the accurate size of vector (5369 bp); **B**, pGEMstaF recombinant plasmid, the insert at ~600 bp; **C**, pGEMsipA recombinant plasmid, the insert at ~2058 bp; M, GeneRuler™ DNA molecular weight marker; 1, undigested plasmid; 2, digested plasmid. GeneRuler™ DNA molecular weight marker (100 − 10,000 bp).

6.4.2.2 Sub-cloning of *staf* and *sipA* fragments into pET28a (+) vector

The fragments recovered from the gel after restriction double digestions of pGEMstaF and pGEMsipA recombinant plasmids were ligated into the corresponding cloning sites in the linearised pET28a vector using T4 DNA ligase enzyme. From each ligation mixture, 2 µl was transformed into electrocomptent *E. coli* DH5α cells using. Positive clones were identified by growing transformed bacteria on LA plates containing Kan 50 µg/ml at 37°C. The positive clones (antibiotic selection) were identified by colony PCR, recombinant plasmid DNA isolation and restriction analysis using *Nde*I and *Xho*I (Fig. 6.4.4).

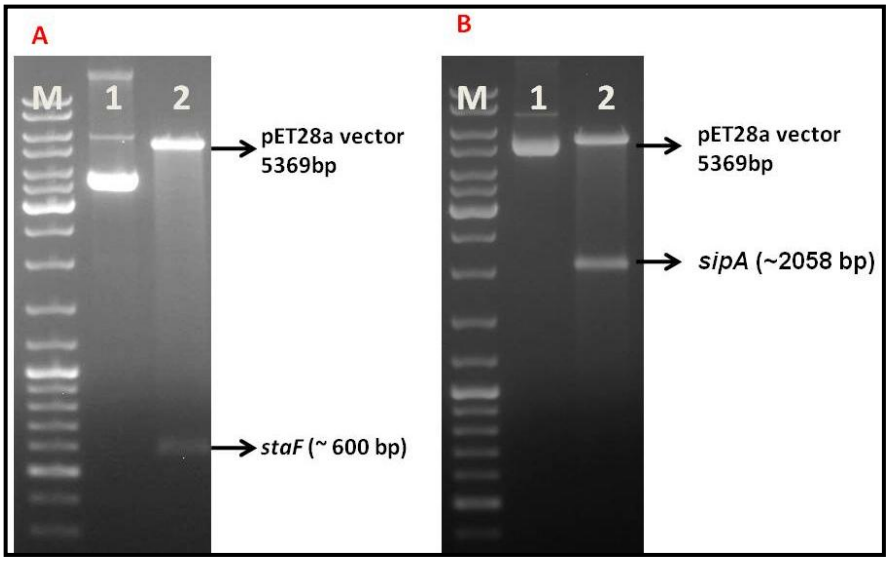


Figure 6.4.4: Analysis of pETStaF and pETSipA recombinant plasmids. Plasmid miniprep for the colony PCR positive clones and restriction analysis using *Nde*I and *Xho*I restriction enzymes of: **A**, pETStaF (pET28a plasmid harbours *staF* gene); **B**, pETSipA (pET28a plasmid harbours *sipA* gene); M, GeneRuler™ DNA molecular weight marker (100 − 10,000 bp); 1, undigested plasmid, 2, digested plasmid.

6.5 Expression of rStaF and rSipA proteins

Both pETStaF (C and N-terminal His_6-tagged *staF* in a pET-28a (+) vector, Kan resistant) and pETSipA (C and N-terminal His_6-tagged *sipA* in a pET-28a (+) vector, Kan resistant) expression plasmids were transformed into electrocompetent *E. coli* BL21, *E. coli* BL21 (DE3) and *E. coli* BL21 (DE3)pLysS. However, expression of both proteins was only observed in *E. coli* BL21 (DE3) cells. The *E. coli* BL21 (DE3) based recombinants were grown on LA plates containing Kan 50 µg/ml o/n at 37°C. Preliminary protein expression was carried out using bacteria grown in LB broth containing Kan 50 µg/ml, incubated at 37°C and 200 rpm. When the OD_{600} of the bacterial culture reached 0.5, the cells were induced by adding 1 mM IPTG. The culture was then incubated at different temperatures (15°C, 20°C, 25°C, 30°C, and 37°C) for different periods of incubation. The protein samples of the induced cell pellets were

analysed for expression by SDS–PAGE and gel staining with Coomassie blue. The highest yields of expressed rStaF and rSipA proteins were obtained at 37°C and o/n incubation (overnight expression) (Fig. 6.5.1).

6.5.1 Protein solubility examination

Examining the the solubility according to the previous used procedures showed that both rSakF and rSipA proteins were expressed as insoluble proteins (Fig. 6.5.1). Despite expression at lower incubation temperatures and/or lower IPTG concentrations both proteins remained insoluble.

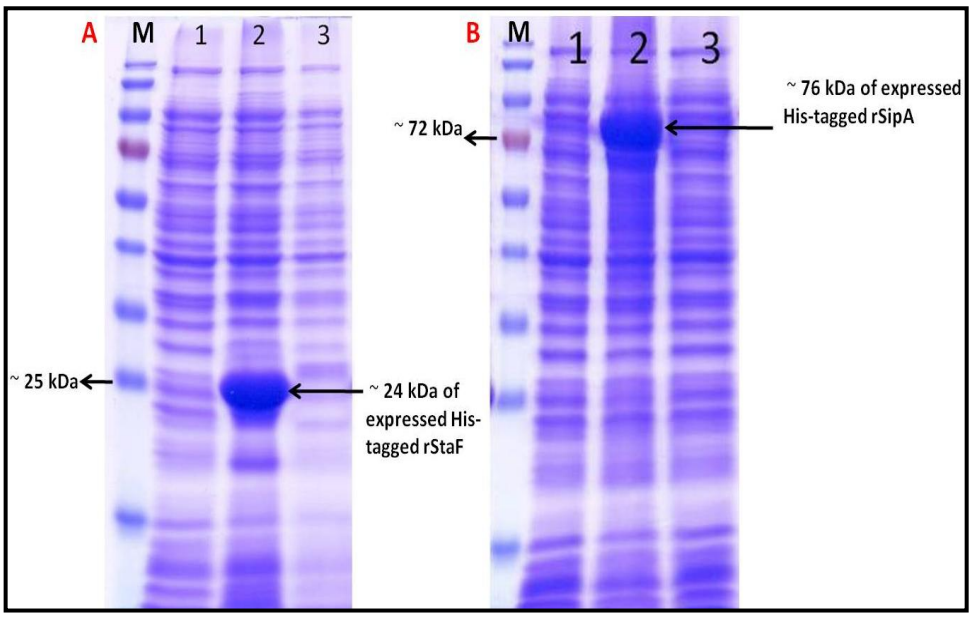


Figure 6.5.1: Pilot expression and solubility examination of (A) rStaF and (B) rSipA, His-tagged proteins. SDS–PAGE (12 %) stained with Coomassie blue loaded with pETStaF and pETSipA *E. coli* BL21 (DE3) transformants cell lysate before IPTG induction; cell debris (insoluble protein fraction) and cell lysate supernatant (soluble protein fraction) after o/n induction at 37°C. An aliquot of 20 µl of each sample was mixed with 5 µl of 5× SDS–PAGE loading dye and boiled for 5 min at 100°C, and then 20 µl of each protein cell pellet sample was loaded onto gel wells for. Lane M, PageRuler™ prestained protein M.W. marker (10 − 170 kDa); Lane 1, Pre-induction; lane 2, Expressed recombinant protein in re-suspended cell debris sample; 3, cell lysate supernatant sample.

6.6 Purification of His-tagged rStaF and rSipA proteins

The rStaF and rSipA His-tagged proteins purification was performed by immobilised metal affinity chromatography (IMAC) technology using Ni-NTA column from the cell debris fraction prepared under denaturing conditions. The clarified cell lysate supernant was loaded onto a Ni-NTA affinity column equilibrated with binding buffer (20 mM Na_2HPO_4, 500 mM NaCl and 0.5 mM imidazoles 8 M urea pH 7.4). The resin was washed using washing buffer (20 mM Na_2HPO_4, 500 mM NaCl and 0.5 mM imidazole, 2 % Tween 20, 8 M urea pH 7.4). For elution of the protein, buffers with concentration range of imidazole (20 mM - 500 mM) were used. The highly purified rStaF-6His and rSipA-6His proteins were found in the 250 mM and 500 mM imidazole elution fractions. A single band at approximately 24 kDa and 76 kDa corresponding to the predicted molecular weight of the rStaF and rSipA, respectively were detected by SDS–PAGE stained with Coomassie blue (Figs. 6.6.1 and 6.6.2). The purified protein was further confirmed by Western blot analysis using monoclonal anti-His-tag antibody.

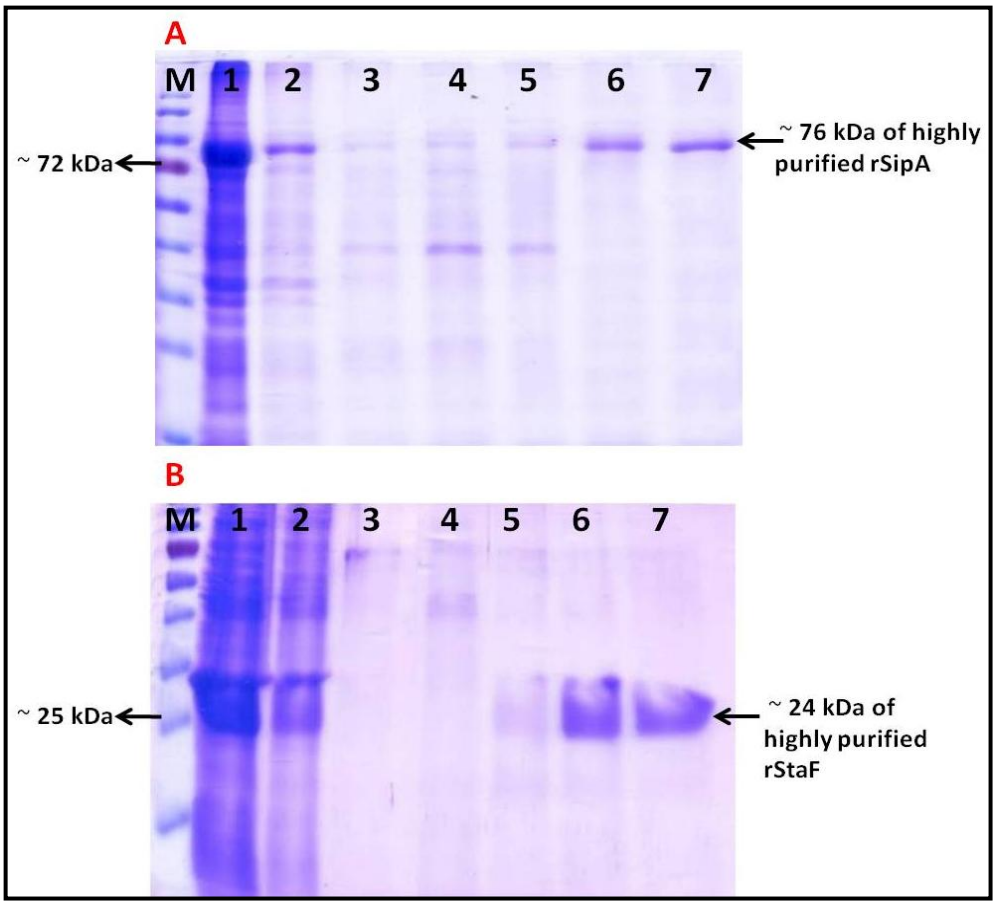


Figure 6.6.1: SDS–PAGE (12 %) of rSipA (A) and rStaF (B) His-tagged proteins purification process using IMAC under denaturing conditions. SDS–PAGE gel (12 %) stained with Coomassie blue loaded with 20 µl of the eluted fractions from the purification steps. Lane M, pageRuler™ prestained protein M.W. marker (10 − 170 kDa); lane 1, BL21 clarified cell lysate before purification steps; lane 2, cell lysate after passing through the column; lane 3, column wash with washing buffer; lane 4, column wash with elution buffer (50 mM imidazole); lane 5, column wash with elution buffer (125 mM imidazole); lane 6 and 7, highly purified eluted proteins with 250 mM and 500 mM imidazole containing containing buffers.

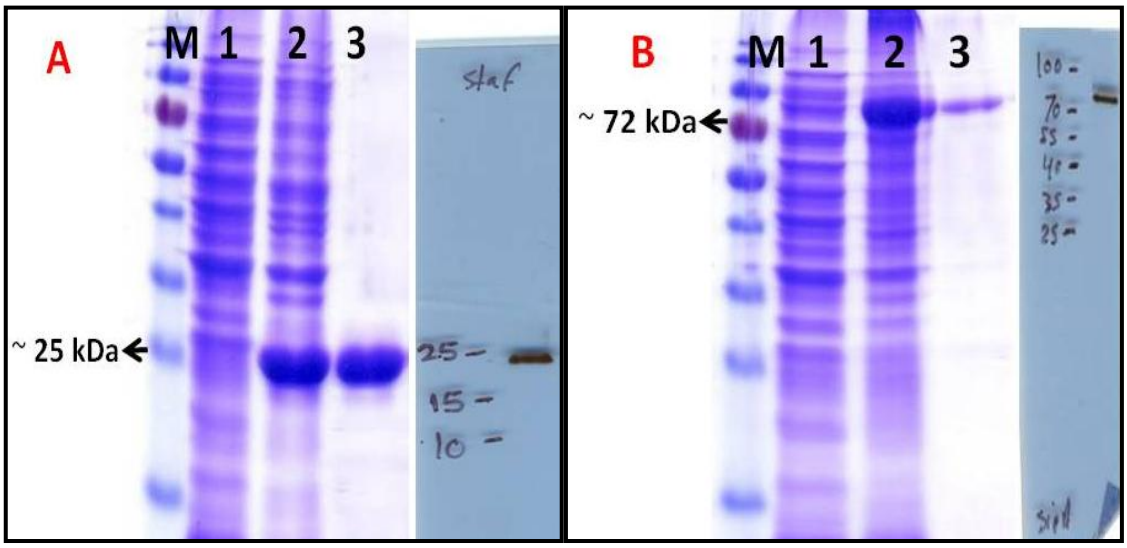


Figure 6.6.2: SDS–PAGE (12 %) analysis of (A) rStaF and (B) rSipA proteins obtained following expression and purification (optimum conditions), and matching Western blot analysis using monoclonal anti-His-tag antibody. Lane M, PageRuler™ prestained protein M.W. marker (10 − 170 kDa); lane 1, BL21 (DE3) bacterial cell lysate before induction; lane 2, BL21 (DE3) bacterial cell lysate o/n after IPTG induction; lane 3, highly purified recombinant proteins. Western blot analysis of highly purified recombinant His-tagged proteins with monoclonal Anti-His-tag® HRP conjugate antibody.

6.6.1 Purified denatured protein refolding, dialysis and concentration

Since the purification of the insoluble proteins was conducted under denaturing conditions using 8 M urea containing buffers. Denatured rStaF and rSipA antigens were refolded using particular refolding buffer by the drop dilution method. The refolded soluble protein was then assessed by SDS–PAGE followed by Coomassie blue staining and the successful refolding was evidenced by the presence of the target protein in the liquid fraction. Then, both the refolded rStaF and rSipA protein solutions were dialysed against the carbonated buffer used in ELISAs and concentrated using Amicon® Ultra-15 centrifugal filter devices with cut offs of 10 kDa for rStaF and 50 kDa for rSipA.

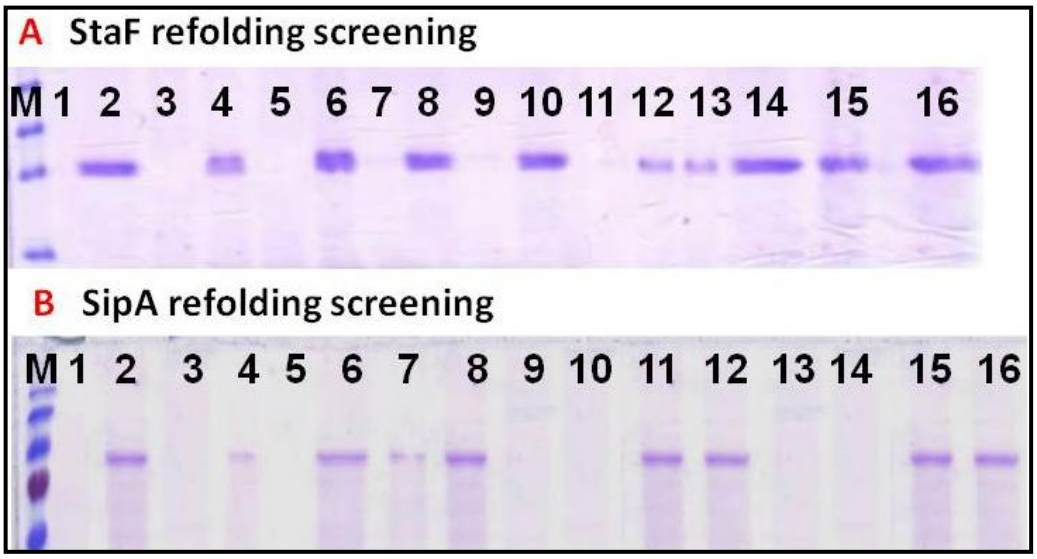


Figure 6.6.3: Coomassie blue stained SDS–PAGE (12 %) of refolding buffer composition determination of: (A) rStaF and (B) rSipA proteins. Lane M, pageRuler™ prestained protein M.W. marker; lane 1 - 15, the soluble fractions of 15 different refolding buffer compositions after dilution of the recombinant proteins for detection of the optimum conditions for refolding using the QuickFold™ Protein Refolding Kit (AthenaES, USA-0600). 40 µl of the soluble fraction was mixed with 10 µl 5× SDS–PAGE loading dye and heated at 100°C for 5 min, and then 20 µl was used in loading per well of the gel. 16) The protein in denaturing buffer sample. Successful refolding was evidenced by the presence of the target protein in the liquid fraction.

6.7 Serodiagnosis of *S. enterica* infections in humans

The rStkF common to all *S.* Paratyphi A strains and approximately 30 % of other *S. enterica* serovars, Typhi-specific rStaF and pan-*Salmonella* rSipA proteins were used in ELISAs to evaluate utility as sero-diagnostic markers of *Salmonella* infections.

6.7.1 Human serum samples

Three groups of human serum samples were tested in this study: UK, Nepalese and Egyptian groups. Group 1: UK group (n = 44) included 11 serum samples from *Salmonella* infected patients, 13 from patients with bloodstream infections caused by other Gram-negative bacteria (kindly provided by Dr. Andrew Rosser, University Hospitals of Leicester), and 20 sera from healthy UK volunteers none of whom had previously visited endemic areas as negative controls (kindly provided by Dr. Bahaa El-Aswad, Lab 231). The 11 serum samples from *Salmonella* infected patients included two samples obtained 11-12 days apart from a single patient with an invasive *S.* Virchow infection, paired serum samples taken over about 3 - 4 day intervals from two Paratyphi A-infected patients, and two Typhi-associated serum samples taken from a single patient over a one day interval (Fig. 6.7.4A).

Group 2: Nepalese group (n = 45) of sera (kindly provided by Dr. Stephen Baker, Clinical Research Unit, Vietnam; Centre of Tropical Medicine, Oxford University) included 15 serum samples from Typhi-infected and 15 from Paratyphi A-infected patients confirmed by bacterial culture. In addition, 15 serum samples from healthy Nepalese volunteers were included as negative controls (Fig. 6.7.4B).

Group 3: The Egyptian group (n = 120) of sera was kindly collected in the Northern Nile Delta by Dr. Emad Eed (Microbiolgy Dept., Faculty of Medicine,

Menoufia University). The group included 80 sera from patients infected with serovars Typhi, Paratyphi A, Paratyphi B and other non-typhoidal serovars with clinical manifestations of enteric fever and/or enteric salmonellosis identified by positive bacterial blood and/or stool cultures culture and serodiagnosis. Suspected *Salmonella* colonies were subjected to biochemical identification. Serotyping was performed using reference *Salmonella* antisera. A further 20 sera samples were obtained from food handlers as part of routine public health screening. In addition, 20 serum samples were collected from healthy volunteers from the same region (Fig. 6.7.4C).

6.7.2 ELISAs of the test antigens

After a number of standard checkerboard titrations (Crowther, 2001) ELISA experiments were performed to optimize the concentrations of antigens and the human serum sample dilutions to be used, the appropriate conditions were determined. Antigens were diluted in carbonate-bicarbonate coating buffer at 10 μg/ml for both rStkF and rStaF, and 5 μg/ml for rSip for coating the ELISA plates (Nunc Maxi-Sorp Immuno-469264). ELISAs were performed following the protocol mentioned in Chapter 2 (section 2.24) using human sera at a 1/200 dilution. The first two columns (1 and 2) of each coated plate were incubated with a number of standard reference positive control sera, which gave the highest responses to each antigen and the last two wells of the first two columns (H1 and H2) received all the reagents except sera as blank. Each plate also contained a number of sera from the healthy UK, Egyptian or Nepalese donors as negative controls. The rest of the plate received the individual test sera. The readings were recorded when the OD of the standard positive control sera attained a value of 1.5 ± 5 %. Antibody binding to BSA alone was not detected in the test sera.

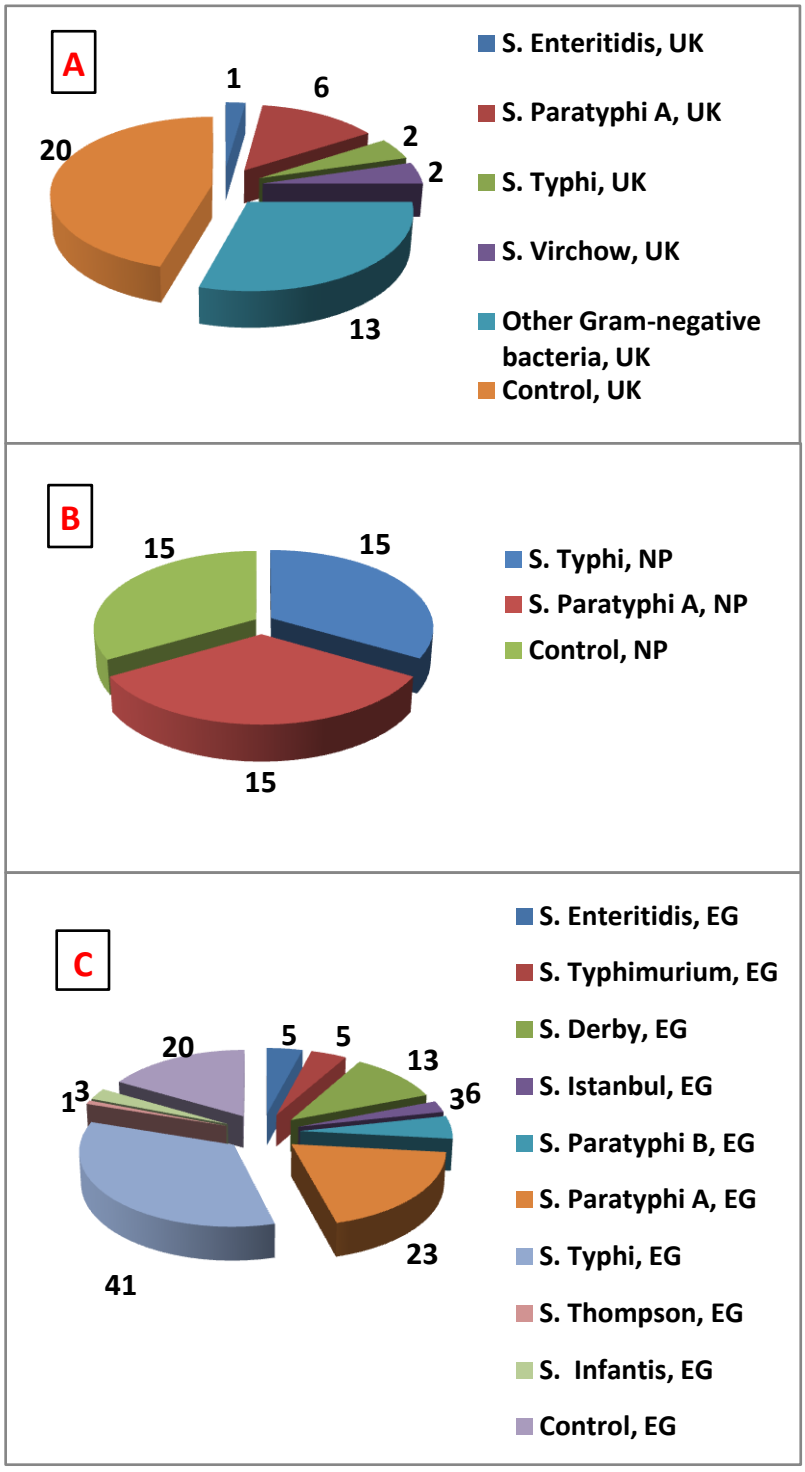


Figure 6.7.1: Human serum samples used in ELISAs in this study: A, UK sera group; **B**, Nepalese sera group; **C**, Egyptian sera group. NP = Nepalese, EG = Egyptian.

6.7.3 ELISA cut-off points of the test antigens

For evaluating the diagnostic sensitivity and specificity of rStkF, rStaF and rSipA antigens, ELISA cut-off point was applied that would serve as the threshold between positive and negative serum samples. The cut-off point to identify sera considered to have antibodies to the test antigen was calculated as two standard deviation above the mean (mean + 2SD) of the ODs values obtained with the serum samples from healthy UK individuals (20 sera), healthy Egyptian individuals (12 sera) and healthy Nepalese individuals (15 sera). The cut off points identified based on the Egyptian and Nepalese controls were significantly higher compared to these based on UK non-infected controls (Table 6.7-1). A positive test result was determined as being greater than the defined region-specific cut-offs. In addition, two other cut-off criteria were also tested (mean + 1SD) and (mean + 3SD) (see Appendix 2).

Table 6.7-1: Cut off values of the different test antigens used in ELISAs

Antigen	StkF			StaF			SipA		
Samples group	UK	NP[2]	EG[2]	UK	NP	EG	UK	NP	EG
UK cut off[1]	0.59	0.59	0.59	1.04	1.04	1.04	0.95	0.95	0.95
NP cut off[1]	-	1.26	-	-	1.34	-	-	1.26	-
EG cut off[1]	-	-	1.37	-	-	1.19	-	-	1.15

[1]The cut off point of the three test ELISAs antigens calculated as mean + 2SD of the ODs values obtained with healthy UK controls (n = 20 sera) as well as healthy Egyptian controls (n = 12 sera) and Nepalese controls (n = 15 sera).[2]NP = Nepalese and EG = Egyptian.

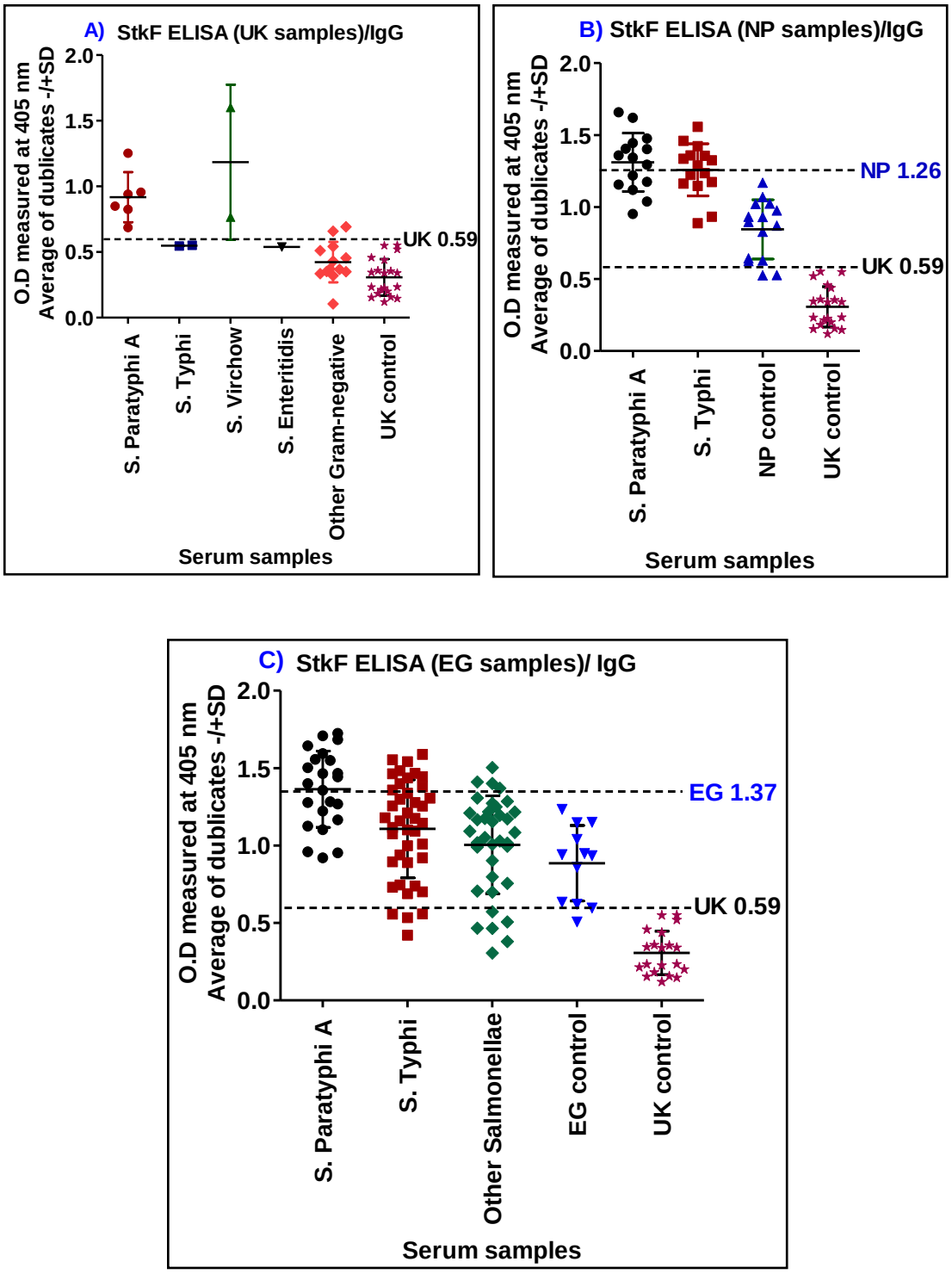


Figure 6.7.2: Serodiagnosis of *S.* Paratyphi A and other StkF-positive *Salmonella* **serovars infections using StkF antigen.** ELISAs results using StkF as an antigen in patient groups and controls of: **A**, UK; **B**, Nepalese; **C**, Egyptian, patient groups and controls. Horizontal lines indicate the cut-off limits for negative calls (mean ± 2SD of healthy controls) based on UK control samples and region-specific control samples.

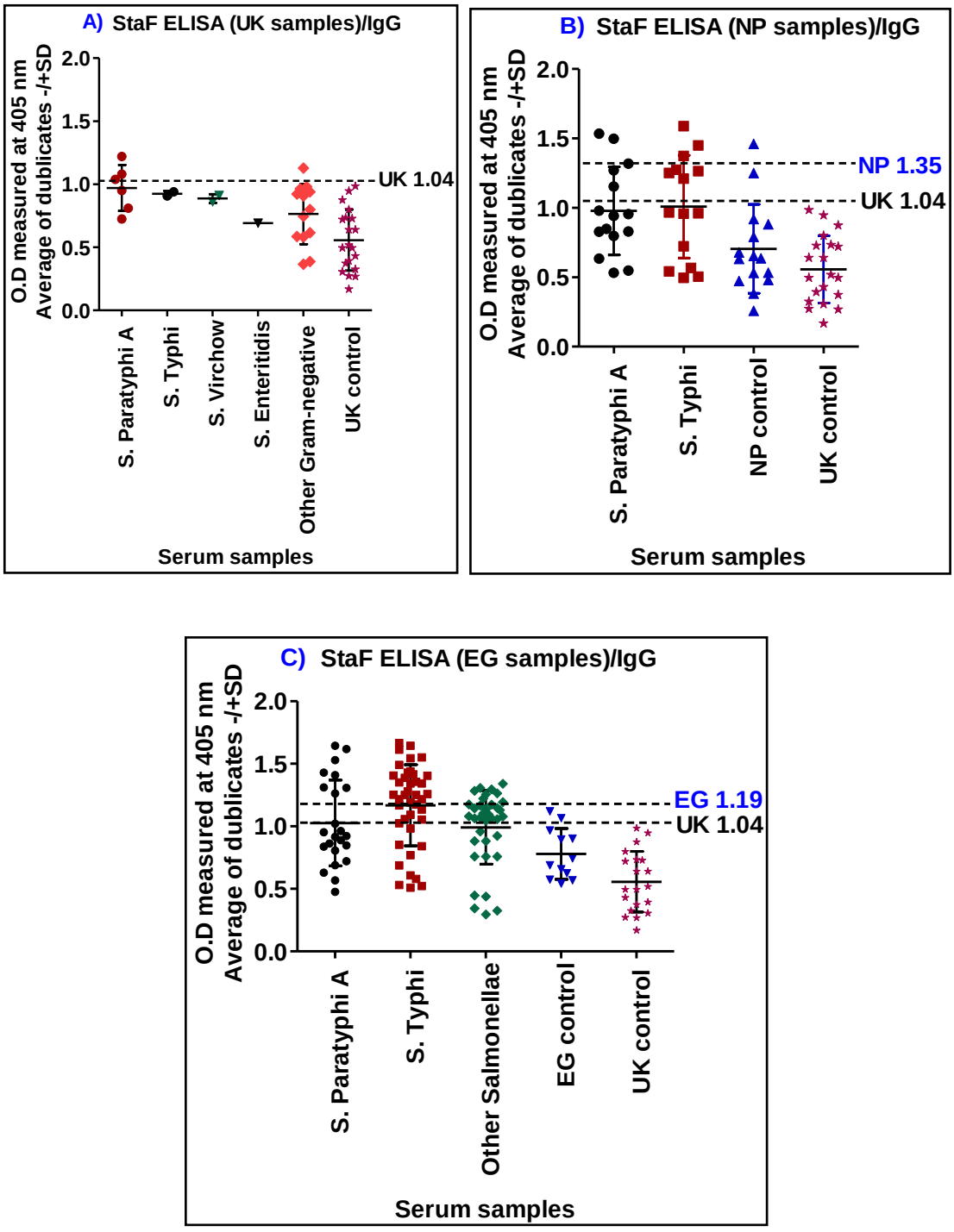


Figure 6.7.3: Serodiagnosis of *S.* Typhi infection using StaF antigen. Results of ELISAs using StaF as an antigen in patient groups and controls of: **A**, UK; **B**, NP; **C**, EG patient groups and controls. Horizontal lines indicate the cut off limits for negative calls (mean ± 2SD of healthy controls) based on UK and region-specific control samples.

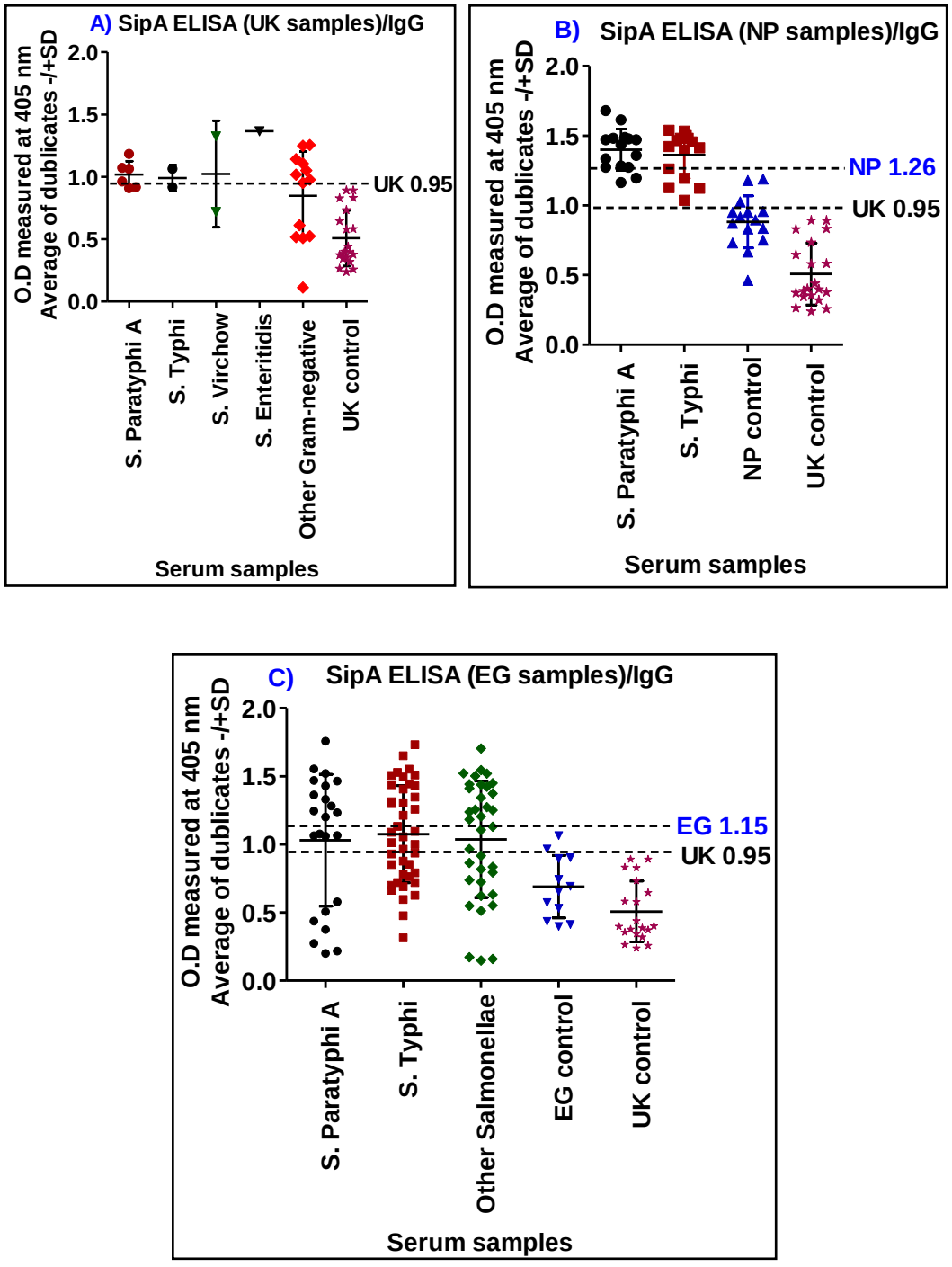


Figure 6.7.4: Serodiagnosis of invasive *Salmonella* infections using SipA antigen. ELISAs results using SipA as an antigen in patient groups and controls of: **A**, UK; **B**, Nepalese; **C**, Egyptian, patient groups and controls. Horizontal lines indicate the cut-off limits for negative calls (mean ± 2SD of healthy controls) based on UK control samples and region-specific control samples.

6.8 Evaluation of the diagnostic value of ELISA antigens

An index of the performance of a diagnostic test is calculated by measuring the sensitivity and specificity of this test. Sensitivity is defined as the percentage of individuals with a disease who are classified correctly as having the disease by the test. Specificity is defined as the percentage of individuals without the disease who are classified as not having the disease. Although, sensitivity and specificity are essential properties of a diagnostic test, positive- and negative- predictive values represent the accuracy of a diagnostic test. Negative predictive value (NPV) is the probability that a person having a negative result on a diagnostic test for a particular condition does not have the condition. Positive predictive value (PPV) is the probability that a person having a positive result on a diagnostic test for a particular condition actually has the condition (Zhou, 2003, Everitt, 2006).

The sensitivity (%) =	$\frac{\textbf{100} \times \textbf{True positives}}{\textbf{True positives + False negatives}}$

The specificity (%) =	$\frac{\textbf{100} \times \textbf{True negatives}}{\textbf{True negatives + False positives}}$

NPV (%) =	$\frac{\textbf{100} \times \textbf{True negatives}}{\textbf{True negatives + False negatives}}$
PPV (%) =	$\frac{\textbf{100} \times \textbf{True positives}}{\textbf{True positives + False positives}}$

In this study, a true positive serum is from a patient infected with the target organism that yields an OD above the cut off for this antigen. While a false negative serum from a similarly infected patient that shows an OD below or equal to the cut off for that antigen. In addition, true negative serum is from healthy UK, Egyptian (EG) or Nepalese (NP) controls, and that from a patient infected with other than the target organism shows an OD below or equal the cut off for that antigen. While false positive

serum is that taken from healthy UK, EG or NP controls, and that from a patient infected with other than the target organism shows an OD above the cut off for that antigen. For the performance characteristics, the following values were used: true positive (TP), false positive (FP), true negative (TN), and false negative (FN) were calculated.

6.8.1 Evaluation of the specificity and sensitivity of StkF, StaF and SipA antigens

The sensitivity, specificity, PPV and NPV percentages of serodiagnosis of invasive *Salmonella* infections were calculated based on culture confirmed infections as the gold standard using mean + 2SD algorithm.

StkF was examined as a specific antigen for diagnosis of *S.* Paratyphi A patients as well as infections caused by other StkF-positive *Salmonella* serovars such as serovar Virchow. In addition, its potential as a discriminatory tool between Typhi and Paratyphi A infections was also assessed. The StkF antigen based ELISA showed 100 % sensitivity, 94 % specificity, PPV of 80 % and NPV of 100 % with UK test samples. In NP test samples, StkF based ELISA showed 60 % sensitivity, 73 % specificity, 53 % PPV and NPV was 79 %, based on NP control cut off. However based on UK control cut off, the sensitivity increased to 100 %, while the specificity decreased to 57 %, PPV was 50 % and NPV was 100 %. The StkF antigen based ELISA of the EG group of sera showed sensitivity of 45 %, specificity of 86 %, PPV of 52 % and NPV of 82 %. However, based on UK control, the sensitivity increased to 97 %, while the specificity decreased to 32 %, PPV was 31 % and NPV was 97 % (Table 6.8-1).

StaF was examined as a specific antigen for diagnosis of *S.* Typhi patients, in addition to if it is able to discriminate Typhi and Paratyphi A patients. The StaF antigen

based ELISA showed 0 % sensitivity, 93 % specificity, 0 % PPV and 95 % NPV with UK test samples. The insensitivity of StaF may be due to low number of samples (two of the same patient) or anti-StaF antibody was not developed in these paired samples of one day interval. In NP group of sera, the diagnostic sensitivity was 20 %, specificity was 90 %, PPV was 50 % and NPV was 69 %, based on NP control cut off. However based on UK control group, the sensitivity increased to 47 %, while the specificity decreased to 86 %, PPV was 58 % and NPV was 79 %. The StaF antigen based ELISA of the EG group of sera showed sensitivity of 59 %, specificity of 77 %, PPV of 60 % and NPV of 76 % in UK sera group. However, based on UK control, the sensitivity increased to 73 %, while the specificity decreased to 59 %, PPV was 48 % and NPV was 81 % (Table 6.8-2).

SipA was examined as an antigen for diagnosis of any invasive *Salmonella* infections regardless of serovar. The SipA antigen based ELISA showed sensitivity of 37 %, specificity of 79 %, PPV of 50 % and NPV of 69 % in UK sera group. In NP group of sera, the diagnostic sensitivity was 80 %, specificity was 100 %, PPV was 100 % and NPV was 71 %, based on NP h control cut off. However based on UK control cut off, the sensitivity increased to 100 %, while the specificity was the same as 100 %, PPV was 100 % and NPV was 100 %. The SipA antigen based ELISA of the EG group of sera showed sensitivity of 47 %, specificity of 100 %, PPV of 100 % and NPV of 18 % based on EG control cut off. However, based on UK control cut off, the sensitivity increased to 61 %, while the specificity was the same as 100 %, PPV was 100 % and NPV was 34 % (Table 6.8-3).

Table 6.8-1: Evaluation of the sensitivity, specificity, PPV and NPV percentages of StkF ELIA-based diagnosis of of *S*. Partyphi A and other *stkF*$^{+}$*Salmonella* serovars

StkF (UK) ELISA	**Disease present**	**Disease absent**	**Total**
Test positive (UK cut off)	True positive (TP)	False positive (FP)	TP+FP
	8	**2**	**10**
Test negative (UK cut off)	False negative (FN)	True negative (TN)	FN+TN
	0	**32**	**32**
Sensitivity (%)	**100 %**		
Specificity (%)	**94 %**		
PPV (%)	**80 %**		
NPV (%)	**100 %**		
StkF (Nepal) ELISA	**Disease present**	**Disease absent**	**Total**
Test positive (NP cut off)	True positive (TP)	False positive (FP)	TP+FP
	9	**8**	**17**
Test negative (NP cut off)	False negative (FN)	True negative (TN)	FN+TN
	6	**22**	**28**
Sensitivity (%)	**60 %**		
Specificity (%)	**73 %**		
PPV (%)	**53 %**		
NPV (%)	**79 %**		
StkF (Nepal) ELISA	**Disease present**	**Disease absent**	**Total**
Test positive (UK cut off)	True positive (TP)	False positive (FP)	TP+FP
	15	**15**	**30**
Test negative (UK cut off)	False negative (FN)	True negative (TN)	FN+TN
	0	**15**	**15**
Sensitivity (%)	**100 %**		
Specificity (%)	**57 %**		
PPV (%)	**50 %**		
NPV (%)	**100 %**		
StkF (Egypt) ELISA	**Disease present**	**Disease absent**	**Total**
Test positive (EG cut off)	True positive (TP)	False positive (FP)	TP+FP
	12	**13**	**22**
Test negative (EG cut off)	False negative (FN)	True negative (TN)	FN+TN
	11	**76**	**87**
Sensitivity (%)	**45 %**		
Specificity (%)	**86 %**		
PPV (%)	**52 %**		
NPV (%)	**82 %**		
StkF (Egypt) ELISA	**Disease present**	**Disease absent**	**Total**
Test positive (UK cut off)	True positive (TP)	False positive (FP)	TP+FP
	27	**59**	**86**
Test negative (UK cut off)	False negative (FN)	True negative (TN)	FN+TN
	1	**33**	**34**
Sensitivity (%)	**97 %**		
Specificity (%)	**32 %**		
PPV (%)	**31 %**		
NPV (%)	**97 %**		

[1]These parameters were calculated based on culture confirmed infections as the gold standard using mean + 2SD algorithm.

Table 6.8-2: Evaluation of the sensitivity, specificity, PPV and NPV percentages of StaF ELIA-based diagnosis of of *S.* Typhi infection

StaF (UK) ELISA	**Disease present**	**Disease absent**	**Total**
Test positive (UK cut off)	True positive (TP)	False positive (FP)	TP+FP
	0	**3**	**3**
Test negative (UK cut off)	False negative (FN)	True negative (TN)	FN+TN
	2	**39**	**41**
Sensitivity (%)	**0 %**		
Specificity (%)	**93 %**		
PPV (%)	**0 %**		
NPV (%)	**95 %**		
StaF (Nepal) ELISA	**Disease present**	**Disease absent**	**Total**
Test positive (NP cut off)	True positive (TP)	False positive (FP)	TP+FP
	3	**3**	**6**
Test negative (NP cut off)	False negative (FN)	True negative (TN)	FN+TN
	12	**27**	**39**
Sensitivity%[1]	**20 %**		
Specificity%[1]	**90 %**		
PPV%[1]	**50 %**		
NPV%[1]	**69 %**		
StaF (Nepal) ELISA	**Disease present**	**Disease absent**	**Total**
Test positive (UK cut off)	True positive (TP)	False positive (FP)	TP+FP
	7	**6**	**13**
Test negative (UK cut off)	False negative (FN)	True negative (TN)	FN+TN
	8	**24**	**32**
Sensitivity (%)	**47 %**		
Specificity (%)	**86 %**		
PPV (%)	**58 %**		
NPV (%)	**79 %**		
StaF (Egypt) ELISA	**Disease present**	**Disease absent**	**Total**
Test positive (EG cut off)	True positive (TP)	False positive (FP)	TP+FP
	24	**15**	**39**
Test negative (EG cut off)	False negative (FN)	True negative (TN)	FN+TN
	17	**56**	**73**
Sensitivity (%)	**59 %**		
Specificity (%)	**77 %**		
PPV (%)	**60 %**		
NPV (%)	**76 %**		
StaF (Egypt) ELISA	**Disease present**	**Disease absent**	**Total**
Test positive (UK control)	True positive (TP)	False positive (FP)	TP+FP
	30	**31**	**61**
Test negative (UK control)	False negative (FN)	True negative (TN)	FN+TN
	11	**48**	**59**
Sensitivity (%)	**73 %**		
Specificity (%)	**59 %**		
PPV (%)	**48 %**		
NPV (%)	**81 %**		

[1]These parameters were calculated based on culture confirmed infections as the gold standard using mean + 2SD algorithm.

Table 6.8-3: Evaluation of the sensitivity, specificity, PPV and NPV percentages of SipA ELIA-based diagnosis of all *Salmonella* infections

SipA (UK) ELISA	**Disease present**	**Disease absent**	**Total**
Test positive (UK cut off)	True positive (TP)	False positive (FP)	TP+FP
	7	**7**	**14**
Test negative (UK cut off)	False negative (FN)	True negative (TN)	FN+TN
	4	**26**	**30**
Sensitivity%[1] **Specificity%**[1] **PPV%**[1] **NPV%**[1]	**37 %** **79 %** **50 %** **69 %**		
SipA (Nepal) ELISA	**Disease present**	**Disease absent**	**Total**
Test positive (NP cut off)	True positive (TP)	False positive (FP)	TP+FP
	24	**0**	**24**
Test negative (NP cut off)	False negative (FN)	True negative (TN)	FN+TN
	6	**15**	**21**
Sensitivity% **Specificity%** **PPV%** **NPV%**	**80 %** **100 %** **100 %** **71 %**		
SipA (Nepal) ELISA	**Disease present**	**Disease absent**	**Total**
Test positive (UK cut off)	True positive (TP)	False positive (FP)	TP+FP
	30	**0**	**30**
Test negative (UK cut off)	False negative (FN)	True negative (TN)	FN+TN
	0	**15**	**15**
Sensitivity% **Specificity%** **PPV%** **NPV%**	**100 %** **100 %** **100 %** **100 %**		
SipA (Egypt) ELISA	**Disease present**	**Disease absent**	**Total**
Test positive (EG cut off)	True positive (TP)	False positive (FP)	TP+FP
	48	**0**	**48**
Test negative (EG cut off)	False negative (FN)	True negative (TN)	FN+TN
	52	**12**	**64**
Sensitivity% **Specificity%** **PPV%** **NPV%**	**47 %** **100 %** **100 %** **18 %**		
SipA (Egypt) ELISA	**Disease present**	**Disease absent**	**Total**
Test positive (UK cut off)	True positive (TP)	False positive (FP)	TP+FP
	62	**0**	**62**
Test negative (UK cut off)	False negative (FN)	True negative (TN)	FN+TN
	38	**20**	**58**
Sensitivity% **Specificity%** **PPV%** **NPV%**	**61 %** **100 %** **100 %** **34 %**		

[1]These parameters were calculated based on culture confirmed infections as the gold standard using mean + 2SD algorithm.

6.8.2 Cross-reactions with other diseases

The potential cross-reactivity of the three tested antigens (rStkF, rStaF and rSipA) was evaluated by testing their reactivity with the 13 sera collected from patients suffering from non-*Salmonella* bacteraemic infections caused by other Gram-negative bacteria at UHL, Leicester (Table 6.8-4). In addition, the potential cross-reactivity of rStkF with sera from patients with dengue fever was tested by Dr Beti Ernawati, University of Indonesia; Jakarta, Indonesia (Fig. 6.8.1). Moreover, the cross-reaction of rStkF with sera collected from Typhi infected patients as well as the cross-reaction of rStaF with sera collected from Paratyphi A infected patients (Table 6.8-4) as well as the cross-reaction of both antigens with sera collected from patients infected with *Salmonella* other than serovars Typhi and Paratyphi A were evaluated for the three serum groups.

The rSipA antigen but not rStkF and rStaF showed cross-reaction with sera from *K. pneumoniae* infected patients. The rStaF antigen showed no cross-reaction with serum from *E. coli*-infected patients, while rStkF antigen showed lower degree of cross-reactivity as compared to rSipA against these sera samples. rStkF was the only antigen that showed cross-reaction with sera from *C. koseri* infected patient. The three antigens showed no cross reaction with sera from *P. aeruginosa*-infected patients. However, sera from a patient with a mixed *E. cloacae* and *P. aeruginosa* infections cross reacted with both the rStaF and rSipA antigens. There was no cross-reaction of rStkF with sera from patients with dengue fever sera indicating that rStkF may have a diagnostic power to differentiate between the fevers caused by *S.* Paratyphi A and dengue virus (Fig. 6.8.1 & Table 6.8-4).

The rStkF did not show any cross-reaction with sera from *S.* Typhi-infected patients in UK serum samples. However, there were substantial cross-reactions evident for both the Nepalese and Egyptian groups, even when cut offs based on cognate Nepalese and Egyptian controls were used. The rStaF showed cross-reactions with sera from *S.* Paratyphi A-infected patients in all three tested groups of sera. Although, in Egyptian group, there was no significant difference in the degree of cross-reactions based on UK or Nepalese cut offs. In Egyptian group, there was difference in number of cross-reactive sera (Table 6.8-5).

Additionally, cross-reactions of both rStkF and rStaF with sera from patients with invasive *Salmonella* infections, other than Typhi and Paratyphi A, were examined. Cross-reactions were observed with all three antigens against corresponding Nepalese and Egyptian serum samples. However, the degree of cross-reactions was reduced or even abolished by use of region-specific cut off points as compared to UK cut off points. The serum sample from the UK *S.* Enteritidis patient did not cross-react with StaF or StkF antigens (Table 6.8-6).

Table 6.8-4: Cross-reactions of test antigens with sera from UK and Indonesian patients infected with non-*Salmonella* pathogens

Antigen / Sera	StkF		StaF		SipA	
	+ve	-ve	+ve	-ve	+ve	-ve
K. pneumonia (UK, 2)[1]	0[2]	2[2]	0	2	1	1
E. coli (UK, 8)	1	7	0	8	5	3
C. koseri (UK, 1)	1	0	0	1	0	1
P. aeruginosa (UK, 1)	0	1	0	1	0	1
E. cloacae and *P. aeruginosa* (UK, 1)	0	1	1	0	1	0
Dengue fever (Indonesia, 4)	0	4	-	-	-	-

[1]Country in which patient presented and numbers of serum samples are indicated in parenthesis. Sera were classed as exhibiting a positive or negative response to each recombinant antigen based on UK and region-specific cut off points. [2]Number of samples exhibiting cross-reactivity (+ve) or not (-ve).

Table 6.8-5: Cross-reactions of StkF and StaF test antigens with sera from patients with *S*. Typhi and *S*. Paratyphi A infections (or other StkF-positive serovars), respectively

Sera \ Antigen		StkF		StaF	
		+ve	-ve	+ve	-ve
S. Typhi (UK, 2)[1]	UK cut off	0[2]	2[2]	-	-
S. Paratyphi A (UK, 6)	UK cut off	-	-	2	4
S. Typhi (NP, 15)	UK cut off	15	0	-	-
	NP cut off	8	7	-	-
S. Paratyphi A (NP, 15)	UK cut off	-	-	5	15
	NP cut off	-	-	2	13
S. Typhi (EG, 41)	UK cut off	37	4	-	-
	EG cut off	10	31	-	-
S. Paratyphi A (EG, 23)	UK cut off	-	-	8	15
	EG cut off	-	-	8	15
S. Virchow (UK, 2)	UK cut off	-	-	0	2
S. Paratyphi B (EG, 6)	UK cut off	-	-	5	1
	EG cut off	-	-	3	3
S. Typhi (Indonesia, 5)	-	0	5	-	-

[1]Country in which patient presented and the total number of serum samples are indicated in parenthesis. Sera were classed as exhibiting a positive or negative response to each recombinant antigen based on UK and region-specific cut off points. [2]Number of samples exhibiting cross-reactivity (+ve) or not (-ve).

Table 6.8-6: Cross-reactions of StkF and StaF test antigens with sera from patients with *Salmonella* infections cause by serovars other than Typhi and Paratyphi A (or other StkF-positive serovars)

Sera \ Antigen		StkF		StaF	
		+ve	-ve	+ve	-ve
S. Enteritidis (UK, 1)[1]	UK cut off	0[2]	1[2]	0	1
S. Enteritidis (EG, 5)	UK cut off	3	2	4	1
	EG cut off	0	5	1	4
S. Derby (EG, 13)	UK cut off	2	11	4	9
	EG cut off	11	2	0	13
S. Istanbul (EG, 3)	UK cut off	3	0	3	0
	EG cut off	0	3	0	3
S. Typhimurium (EG, 6)	UK cut off	3	2	4	1
	EG cut off	0	5	2	3
S. Infantis (EG, 3)	UK cut off	3	0	3	0
	EG cut off	0	3	2	1
S. Thompson (EG, 1)	UK cut off	1	0	0	1
	EG cut off	0	1	1	0

[1]Country in which patient presented and number of serum samples are indicated in parenthesis. Sera were classed as exhibiting a positive or negative response to each recombinant antigen based on UK and region-specific cut off points. [2]Number of samples exhibiting cross-reactivity (+ve) or not (-ve).

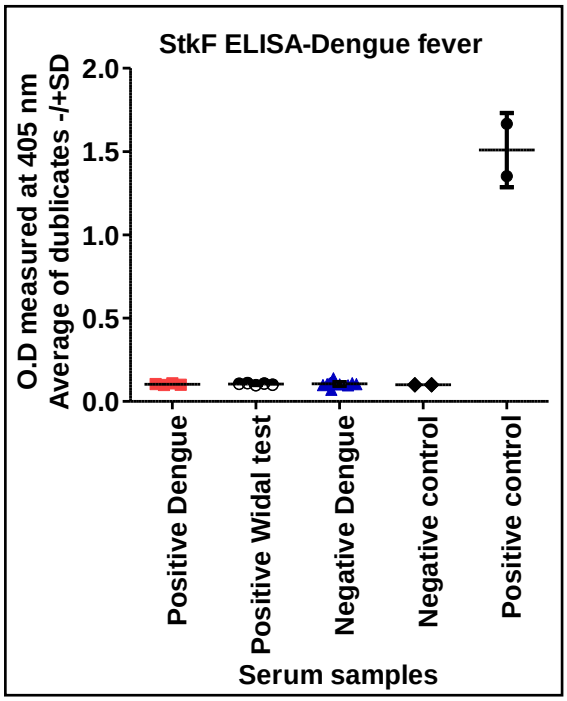


Figure 6.8.1: StkF ELISA result of dengue fever patients serum samples. Dengue-negative sera (n = 12) from healthy individuals. Dengue-positive sera (n = 4) from patients with high fever of less than 48 h from patients in Jakarta, Indonesia. Widal-positive sera (n = 5) came from patients with typhoid fever. Negative control (n = 2), ELISA test without addition of patient sera. Positive control (n = 2), dengue-antigen coated wells incubated with dengue-positive sera. There was no reaction of StkF with sera from patients suffering from dengue fever comparing to the positive controls.

6.9 Discussion

Enteric fever is a severe and potentially life-threatening worldwide disease, especially in the tropics. Given that febrile illnesses can have many causes, the accurate diagnosis of enteric typhoid and paratyphoid fevers is extremely important to facilitate the quick and appropriate treatment (Olsen *et al.*, 2004, Chart *et al*., 2007). The 'gold standard' for diagnosis of enteric fever remains the recovery of the causative organism from clinical specimens (Levine, 2009). However, serological diagnosis may be advantageous as many diagnostic laboratories in developing countries do not have facilities for blood culture. The Widal test is the basis for diagnosis of typhoid fever in many such laboratories but its drawbacks are lack specificity and sensitivity (Narayanappa *et al.*, 2010). Recent attempts, including the testing of commercially available kits, to improve serodiagnosis of enteric fever in developing countries point to superior assays as compared to the Widal test. However, none has yet found widespread application due to a number of factors that influence the sensitivity and specificity of these assays (Wain & Hosoglu, 2008, Nakhla *et al.*, 2011).

This part of my PhD project aimed to develop a practical and effective ELISA based serological diagnostic methods for invasive *Salmonella* infections, including enteric fever. This parallels recent approaches by others to improve the diagnosis of enteric fever by developing new ELISAs (Meltzer & Schwartz, 2010). This goal was achieved by examining the ability of different recombinant *Salmonella* antigens to diagnose *Salmonella* infections using ELISA through detection of specific cognate antibodies in sera from *Salmonella*-infected patients from different geographical areas. In addition, the ability of the test antigens to differentiate between infections by serovars Typhi and Paratyphi A was also examined. StaF (198 aa, 21.4 kD), the putative fimbrial

protein encoded by *staF* gene (GenBank accession, NP_804068.1), was used as a specific antigen for diagnosis of invasive *S.* Typhi infections. StaF was chosen as a *S.* Typhi-specific antigen because it was thought to be involved in cellular adhesion. Harris et al. (2006) identified StaF as one of main immunogenic bacterial proteins expressed during human infection with *S.* Typhi (Harris *et al.*, 2006, Sheikh *et al.*, 2011). Furthermore, blastp search of StaF protein amino acid sequence against the non-redundant database of GenBank showed that high matching homologues were only encoded by the genomes of *S.* Typhi, and that this protein was conserved in all four Typhi strains for which genome sequence data was available (Table 6.9-1).

SipA (685 aa, 73.9 kD), a *Salmonella* pathogenicity island 1 (SPI1) effector protein (GenBank accession, YP_151909.1), which conserved within *S. enterica* was used as a specific antigen for diagnosis of invasive *Salmonella* infections caused by all serovars. SipA or *Salmonella* invasion protein A is secreted and/or translocated into the host cell during infection (Mirold *et al.*, 2001). In addition, blastp search of SipA amino acid sequence showed the homologues proteins encoded by most available sequenced genomes of *S. enterica* serovars strains (Table 6.9-2).

The recombinant C- and N-terminal 6-His-tagged StaF and SipA proteins were expressed in *E. coli* BL21 (DE3) at 37°C and purified using Ni affinity chromatography under denaturing buffer conditions as, unlike rStkF, these proteins were expressed as completely insoluble proteins. Interestingly, there was no expression when *E. coli* BL21 or *E. coli* BL21 (DE3)pLysS was used as an expression host. It is possible that this relates to the recognized observation that efficient expressions of several recombinant proteins require particular combinations of vector, host strain, and culture conditions.

E. coli is the first choice for expression of recombinant proteins, although, over expression frequently results in insoluble aggregates or inclusion bodies. However, expression of recombinant proteins as inclusion bodies can be advantageous as large amounts of protein which are protected from proteolytic degradation are produced. In addition, in some cases production of active soluble protein can be lethal to the host cell. Many proteins that are used for commercial and/or academic applications are recovered from inclusion bodies (Clark, 1998).

Soluble rStaF and rSipA proteins were obtained by refolding of the purified proteins. Several methods may be employed, including dilution of the denatured protein solution directly into refolding buffer containing oxido-shuffling reagents comprising reduced and oxidized glutathione (GSH/GSSG) promote disulphide bond formation (oxidation). In this study the dilution process was used for refolding rStaF and rSipA. The highly purified refolded proteins were then used as antigens in ELISAs to evaluate these antigens as sero-diagnostic markers of *Salmonella* infections.

The sensitivity, specificity, NPV and PPV of ELISAs based on the three antigens was evaluated depending on calculated cut-off points for each antigen following equations shown in section 6.8. The cut off points which serve as the threshold between positive and negative serum samples; were calculated as two standard deviation above the mean (mean + 2SD) of the ODs values obtained with sera from healthy individuals in the UK, Egyptian and Nepalese groups. In addition, cut offs based on (mean + 1SD) and (mean + 3SD) were also examined (see Appendix 2). Assay performance based on both UK and region-specific cut offs were assessed (Table 6.7-1). Indeed, Egypt and Nepal cut offs were significantly higher than UK cut offs, potentially as a result of frequent exposure of individuals in the former two endemic regions to *Salmonella*

infections (Fadeel *et al.*, 2004, Kumar *et al.* 2009). This observation is also consistent with the country-to-country variable agglutination antibody titre required for diagnosis by Widal test, the current standard serological method for diagnosis of enteric fever. For example, in Iran, a Widal antibody agglutination titre cut off of > 1:40 is used (Noorbakhsh *et al.*, 2003), while in Egypt, the cut off titre is $\geq$ 1:160 for a positive result to be considered as significant (Afifi *et al.*, 2005). In Turkey, an agglutination titre of $\geq$ 1:200 is required for diagnostic significance (Willke *et al.*, 2002). This variable cut off is a key disadvantage of the Widal test (Bakr *et al.*, 2011).

In this study sensitivity, specificity, NPV and PPV of the three ELISA based serologic tests were calculated based on the gold standard of culture-confirmed *Salmonella* infections and ELISA cut off values of mean + 2SD of OD reading for serum samples from a small number of uninfected individuals from the reference population.

The sensitivity percentages of the ELISAs developed in this study are comparable to the 40 – 60 % sensitivity of the Widal test which is indicative of enteric fever (Afifi *et al.*, 2005). Indeed, depending on the recombinant protein and/or cut off examined several of the assays presently developed appear to exhibit a superior sensitivity to the Widal reaction. However, low specificity potentially due to non-specific cross-reactivity with antigens of other *Salmonella* serovars was a frequently observed problem, as is the case with the Widal test (Nakhla *et al.*, 2011). Nevertheless, selected recombinant antigen / cut off combinations resulted in a specificity of 100 % based on the small number of serum samples examined to date. Moreover, the ELISAs developed in this study showed a variable sensitivity and specificity depending on the geographic locality of the patients, which is consistent with the country-to-country variable sensitivity and

specificity of the Widal test that precludes its acceptance as a definitive diagnostic assay (Noorbakhsh *et al.*, 2003, Afifi *et al.*, 2005, Bakr *et al.*, 2011). In addition, this observation is in agreement to the presented variable ranges of sensitivities and specificities of the commercially available tests, TyphiDot, Multi-Test Dip-S-Ticks, TUBEX-TF and TUBEX-PA, when examined in different countries (Dutta *et al.*, 2006, Tam *et al.*, 2008, Wain & Hosoglu, 2008, Narayanappa *et al.*, 2010, Fadeel *et al.*, 2011, Nakhla *et al.*, 2011).

The observation that there was no apparent cross reactivity between rStkF and serum from patients with dengue fever is encouraging as it suggests diagnostic utility to differentiate between fevers caused by *S.* Paratyphi A and dengue virus (Fig. 6.9.1, Table 6.9-1). Although rStkF-based ELISA could distinguish invasive infection caused by *S.* Paratyphi A infections from those caused by *S.* Typhi, in UK group of sera, it showed a cross reactions in the NP and EG groups (Table 6.9-2). In addition, there was cross reactivity of rStkF and rStaF with sera from patients with infected caused by non-Typhi, non-Paratyphi A *Salmonella*, which may be due to similar antigens present in other *Salmonella* serovars (Nakhla *et al.*, 2011). rStkF and rStaF showed less cross reactions with sera from patients with non-*Salmonella* Gram-negative bacteria in the UK, however, SipA showed a high rate of cross reaction (62.5 %) with sera from *E. coli*-infected patients, which could potentially be explained by shared antigens among Gram-negative Enterobacteriacae (Table 6.9-2) (Minden *et al.*, 1972).

Despite the frequent use of serological diagnosis it remains fraught with specificity problems due to background levels IgG antibodies to serotype Typhi and Paratyphi A in endemic regions (Kawano *et al.*, 2007). This is a likely function of past immunisation with killed whole cell vaccines, infection in the distant past and/or non-

specific cross-reactivity with other *Salmonella* serovars (Nakhla *et al.*, 2011). Other factors that limit serological diagnosis include availability and quality of assays, routine empirical use of antibiotics, patterns of disease severity, duration, and phase of the disease at first presentation to the clinic (Nakhla *et al.*, 2011).

In conclusion, my findings on the three ELISAs are consistent with recently published data on ELISAs and other rapid serological tests, which have attempted to form the basis of an alternative to the Widal. Although these assays are simple, rapid, and cost effective; and may exhibit higher sensitivity and/or specificity than the Widal test, none are completely acceptable (Olsen *et al.*, 2004, House *et al.*, 2005, Chart *et al.*, 2007, Wain & Hosoglu, 2008, Meltzer & Schwartz, 2010). In addition, testing of these rStkF, rStaF and rSipA ELISAs in the future on much larger numbers of samples from endemic and non-endemic regions, an exploration of the utility of alternative cut offs against one or more of the three antigens examined, and/or improved interpretive algorithms that exploit result of combinations of ELISAs results may ultimately allow for improved performance characteristics.

Chapter Seven

Conclusion and Future Work

7 Conclusion and Future Work

7.1 Conclusion

Typhoid and paratyphoid fevers, which are collectively known as enteric fever, are severe systemic infections caused by the following typhoidal *Salmonella* serovars which are strictly adapted to humans: Typhi, Paratyphi A, Paratyphi B and Paratyphi C. Enteric fever continues to be a serious health risk worldwide, especially in developing countries and the tropics (Humphries *et al.*, 2001, Narayanappa *et al.*, 2010). Significantly, the incidence of enteric fever caused by *S.* Paratyphi A has shown a recent marked increase in many parts of the world, and available vaccines that protect against *S.* Typhi are not protective against *S.* Paratyphi A. Therefore, there is a growing need for an effective vaccine against *S.* Paratyphi A (Roland *et al.*, 2010). In addition, enteric fever can be clinically confused with other febrile diseases such as dengue fever, malaria and even tuberculosis, thus, early and accurate diagnosis is essential for rapid and effective management (Kawano *et al.*, 2007, Narayanappa *et al.*, 2010).

Fimbrial adhesins are believed to mediate adhesion of *S. enterica* organisms to the intestinal epithelium, a process which is considered the most important step in initial pathogenesis, preceding invasion. Genes encoding a wide variety of putative fimbriae are present in *Salmonella* serovars, although only a few *Salmonella* fimbriae have been characterized (Althouse *et al.*, 2003, Forest *et al.*, 2007). In addition, production of anti-adhesin antibody in the host to obstruct bacterial adhesion is a recent approach for developing vaccines against bacterial infections (Soto & Hultgren, 1999).

As a part of my project, the *S.* Paratyphi A *stk* putative fimbrial gene cluster and the Stk fimbrial structure encoded by this operon were further investigated and

characterised based on bioinformatics, genetic and phenotypic approaches. The serovar Paratyphi A *stk* fimbrial gene cluster contains seven *orfs* designated *stkABCCDEFG*, which were annotated in the genome sequence of strain ATCC9150 and predicted to function as an operon by Microbes Online Operon Predictions server (Chapter 5, Fig. 5.2.1). Full-length, syntenic and highly-similar clusters were also identified by blastn in sequenced genomes of several other *S. enterica* serovars: Virchow, Heidelberg, Kentucky and Tennessee (Chapter 5, Table 5.3-1). In addition, a *stkF* PCR survey revealed the presence of this gene and by extension the likely presence of the *stk* operon in 14 of 48 (~30 %) of *Salmonella enterica* serovars investigated (Chapter 3, Table 3.3-1). The remaining *stkF*-positive serovars identified are Paratyphi B, Paratyphi C, Colindale, Ohio, Braenderup, Kottbus, Saintpaul, Bareilly and Mbandaka. The data obtained from blast analysis or PCR investigation of different *Salmonella* enterica serovars indicated that the *stk* fimbrial gene cluster is also present in a number of non-typhoidal serovars, several of which exhibit broader host-ranges and/or a distinct preferred host as compared to *S.* Paratyphi A. Therefore, no simple correlation exists between the host range of the serovar or resulting disease in man and the presence of this single fimbrial operon.

In this study, bioinformatics characterisation revealed that the *stk* fimbrial cluster has a G + C content of 49.3 % and is a C/U-dependent fimbrial cluster based on the Nuccio and Baumler mechanism of assembly classification system (Nuccio & Baumler, 2007). Bioinformatics analysis of the genes of *stk* operon aimed at predicting the functions and cellular locations of the various proteins by identification of conserved domains, orthologues and other features suggested that the *stk* operon encodes three classes of proteins. These comprised the following: (1) extracellular structural proteins

StkA, the major fimbrial subunit, and the minor fimbrial subunits StkD, StkE, StkF and StkG, (2) a periplasmic chaperone StkB (assembly protein) and (3) an outer membrane usher protein StkC (assembly protein) (Chapter 5, Tables 5.4-1 and 5.5-1). Additionally, StkF was identified as a potential tip adhesin as an orthologue in *E. coli* MG1655, YadK, had also been annotated as a predicted adhesin-like protein. In addition, protein domain superfamily prediction revealed that StkF contains a domain related to the bacterial adhesin superfamily, the closest match of which was the mannose-specific binding domain of the well characterized *E. coli* type 1 fimbrial adhesin FimH. These bionformatics data strongly supported the assignment of StkF as a fimbrial adhesin subunit, suggesting that StkF could contribute to host adhesion by *S.* Paratyphi A and other *Salmonella* serovars carrying this protein through binding to mannose containing receptors. However, none of these assignments has been experimentally confirmed (Chapter 5, Fig. 5.7.1). The recognized HtrE usher and EcpD chaperone proteins encoded by the *yad* fimbrial operon present in *E. coli* K-12 showed the highest matches to StkC and StkB, respectively. The *yad* fimbrial operon, an orthologue to *stk* fimbrial operon (Chapter 5, Fig. 5.6.1), has been assigned to the C/U-γ4-fimbrial clade, supporting the same classification for the *stk* operon. Experimentally the Stk fimbrium was characterised phenotypically after heterologous expression in the afimbriate *E. coli* K-12 strain HB101. Transmission electron microscopy (TEM) analysis showed marked expression of surface fimbrial structures on *E. coli* HB101/pUCstk that coded for an intact *stk* operon but no equivalent structures on *E. coli* HB101/pUCstkoff, *E. coli* HB101/pUCP20 or *E. coli* HB101 cells only. The fimbriated *E. coli*/pUCstk demonstrated a classical MS haemagglutination phenotype with horse, human, and guinea pig erythrocytes. In addition, a pellicle was formed at the surface of standing liquid cultures of *E. coli*/pUCstk and cultures remained uniformly turbid throughout the

period of standing aerobic incubation. Both of these traits are typical of type 1 fimbriae (Chapter 5, Table 5.11-2, Fig. 5.11.1). These observations supported classification of the Stk fimbriae as member of the phenotypically-defined type 1 fimbrial group, found in several members of the Enterobacteriaceae family, in which fimbrial phase variation is common (Clegg *et al.*, 1996, Old & Duguid, 1970, Clegg & Gerlach, 1987). In addition, the surface of the *E. coli*/pUCstk bacteria was more hydrophobic than that of the comparator strains, thus linking Stk fimbriae to this trait. As surface hydrophobicity of several bacterial species including *S. enterica* has been shown to correlate positively with virulence and initial binding to host tissues (Doyle, 2000), this latter finding further supports a role for Stk fimbriae in the pathogenesis process.

Through analysis of 55 *S.* Paratyphi A strains, Ou et al. (2007) observed an *stkF* polymorphism that manifest as the *stkF*^{159bp}-ON (44/55 strains) and *stkF*^{180bp}-OFF (11/55 strains) alleles. The *stkF*^{180bp} gene, as defined by the length of the target amplicon, was predicted to code for a truncated, non-functional protein of only 46 aa (Spa0180v1) – hence its suffix 'OFF' (Chapter 3, Figs. 3.9.1 and 3.9.2). The native *stkF* form (*stkF*^{159bp}-ON) had a single 26 bp motif that comprised a 16 bp spacer and one of the two flanking 10 bp perfect direct repeats (DRs). By contrast the *stkF*^{180bp}-OFF possessed a perfect tandem repeat of this 26 bp sequence and consequently an additional a 16 bp spacer sequence and a third 10 bp DR. However, in this study, *stkF* PCR investigation of 121 *S.* Paratphi A strains failed to detect any examples of the *stkF*^{185bp}-OFF form but identified a single instance of a third form which had three perfect 26 bp tandem repeats (*stkF*^{211bp}-OFF allele), which would also constitute an OFF allele as its predicted product would be a truncated 60 aa protein (Spa0180v2) (Chapter 3, Figs 3.3.3, 3.3.4). The observed intragenic, 26 bp tandem repeat duplication or triplication

event in *stkF* would suggest slipped-strand mispairing and/or recombination as a likely mechanism for a form of low frequency phase switching at the translational level. In agreement with the above idea, whole cell lysates of *S.* Paratyphi A strain PA105 (*stkF*^{159bp}-ON) and strain PA42 (*stkF*^{211bp}-OFF) were shown via Co-IP using murine StkF antiserum to produce or not to produce intact StkF protein, respectively (Chapter 3, Fig. 3.4.5). Thus, the absence of fimbriae by EM-examination on *E. coli* HB101/pUCstkoff, carrying the *stkF*^{211bp}-OFF of PA42, and the lack of associated phenotypic traits by this strain support the idea of Stk fimbrial phase variation mediated by repeat sequence changes in *stkF*. This observation is supported by the findings of Dwyer et al. (2011) who showed that allelic variation in the gene coding for the *Salmonella* tip adhesin FimH was associated with inability to assemble type 1 fimbriae for certain variants of FimH.

To achieve the main aim of my project of developing a recombinant adhesin-based vaccine against *Salmonella* infections, especially those caused by *S.* Paratyphi A, *stkF* was cloned into the pET23a expression vector (pETStkF) and expressed under optimal conditions in the *E. coli* BL21 (DE3)pLysS expression host (Chapter 3) Then, the highly purified soluble protein was was used to immunise BALB/c mice to examine the immunogenicity of this protein. In addition, murine StkF antiserum was used in two different *in vitro* assays to assess the efficacy of rStkF as a protective antigen against infections by *S. enterica* serovars carrying this antigen. The rStkF protein appeared to serve as a potentially effective anti-*Salmonella* vaccine antigen due to a number of reasons. i) Fimbrial adhesins, present in extremely high numbers at the cell surface, are frequently found not only to affect the virulence of bacteria but also to serve as key targets of the host immune response. ii) Fimbrial proteins are strong immunogens and a

number of bacterial adhesins such as FimH of *E. coli* have been shown to induce strong protective immunity (Klemm & Schembri, 2000). iii) These proteins typically possess natural adhesive properties and often play critical roles in the initiation of pathogenesis (Zhang & Zhang, 2010). iv). The observation that *stkF* may be subject to ON–OFF phase variation by slipped-strand mispairing and/or recombination within a signature *stkF*-borne tandem repeat motif leading to allelic OFF forms, and the potential that this may be linked to host immune selection pressure by StkF-specific antibodies. v) The finding that rStkF protein was strongly immunogenic as revealed by the presence of very high antibody titre 1:50,000 in the serum of immunized mice. vi) In addition, *in vitro* assays performed with StkF-specific murine antiserum demonstrated markedly enhanced opsonophagocytosis-mediated killing by human neutrophils of *S.* Kentucky strain HP0204 which was shown to express StkF by Western blot analysis. Augmented anti-StkF antibody-specific complement-mediated lysis of *S.* Kentucky strain HP0204 was also shown. By contrast, murine StkF antiserum did not show significant killing of an *stk*-minus strain, *S.* Enteritidis strain HP0174, by either assay supporting the specificity with regards to enhanced-killing of *stkF*-positive bacteria promoted by the anti-StkF polyclonal antisera used (Chapter 4, Figs. 4.5.1, 2, 3, 4). Since the type of immune response generated may be critical to the effectiveness and safety of a potential vaccine, the ratio of IgG1 to IgG2a sub-classes in response to immunization with rStkF was determined. Given that IgG1 was the major antibody isotype induced in our studies following s.c. immunization of mice with rStkF, strong humoral (Th2-type) immune response would appear to have been promoted (Chapter 4, Fig. 4.4.2). In addition, the splenic cell-associated cytokine profile triggered by immunisation of BALB/c mice with rStkF showed that up-regulation of IL-4 production was found to be much greater than that of IFN-γ or IL-12-p70, further supporting a Th2-dominant immune response

(Chapter 4, Figs. 4.4.3 and 4.4.4). This is consistent with the very high anti-StkF titre measured in the immunised mice serum as production of antibodies by plasma cells is mediated mainly by type 2 helper T (Th2) cells which secrete IL-4 (Okamoto *et al.*, 2005). The overall immune response observed with rStkF in this study is comparable to that of the licensed T-cell-independent Vi polysaccharide-based typhoid vaccine (Hale *et al.*, 2006). The marked anti-StkF-mediated opsonophagocytosis and complement-mediated killing activity observed serve to link antibody and cell-mediated immunity in protection against *Salmonella,* raising the likelihood that an antibody-inducing vaccine will protect against invasive *Salmonella* disease (Gondwe *et al.*, 2010). Thus, the *in vitro* profile coupled with the mice immunogenicity profile stongly supports StkF a suitable recombinant *Salmonella* vaccine candidate.

The other aim of my project to develop and evaluate a simple and reliable ELISA based serological test for invasive *Salmonella* infections. rStkF, rStaF and rSipA were used in ELISAs to evaluate these antigens as sero-diagnostic markers of *Salmonella* infections. Human sera collected in UK (n = 44), Nepal (n = 45) and Egypt (n = 120) from patients with blood culture-confirmed infections and infection-free controls was examined. The sensitivity, specificity, NPV and PPV of ELISAs based on the three antigens were evaluated based on calculated UK and region-specific cut offs, the Egypt and Nepal cut-offs were significantly higher than UK cut-offs, potentially as a result of frequent exposure of individuals in the former two regions to *Salmonella* infections (Fadeel *et al.*, 2004, Kumar *et al.*, 2009). The ELISAs developed in this study showed a variable sensitivity and specificity depending on the geographic locality of the patients. This mirrors the country-to-country variable sensitivity and specificity of the Widal test which has precluded its acceptance as a definitive diagnostic assay (Noorbakhsh *et al.*,

2003, Afifi *et al.*, 2005, Bakr *et al.*, 2011). In addition, this observation is in agreement to the reported variable ranges of sensitivities and specificities of the commercially available tests, TyphiDot, Multi-Test Dip-S-Ticks, TUBEX-TF and TUBEX-PA, when examined in different countries (Dutta *et al.*, 2006, Tam *et al.*, 2008, Wain & Hosoglu, 2008, Narayanappa *et al.*, 2010, Fadeel *et al.*, 2011, Nakhla *et al.*, 2011). The observation that there was no apparent cross reactivity between rStkF and serum from patients with dengue fever is encouraging as it suggests diagnostic utility to differentiate between fevers caused by *S.* Paratyphi A and dengue virus (Chapter 6, Fig. 6.9.1 and Table 6.9-1). Although rStkF-based ELISA could reliably distinguish invasive infection caused by *S.* Paratyphi A infections from those caused by *S.* Typhi, in UK group, it showed a cross-reactions in the NP and EG groups (Chapter 6, Table 6.9-2). In addition, there was cross-reactivity of rStkF and rStaF with sera from patients infected with non-Typhi, non-Paratyphi A *Salmonella*, which may be due to similar antigens present in other *Salmonella* serovars (Nakhla *et al.*, 2011). rStkF and rStaF showed less cross reactions with sera from patients with non-*Salmonella* Gram-negative bacteria in the UK, however, SipA showed a high rate of cross reaction (62.5 %) with sera from *E. coli*-infected patients, which could potentially be explained by shared antigen epitopes among Gram-negative Enterobacteriacae (Chapter 6, Table 6.9-2) (Minden *et al.*, 1972). The performance of the three ELISAs developed in this study were consistent with recently published data on ELISAs and other rapid serological tests for enteric fever, which have attempted to form the basis of an alternative to Widal test. Despite several of these assays exhibiting higher sensitivity and/or specificity than the Widal test, none is completely satisfactory (Olsen *et al.*, 2004, House *et al.*, 2005, Chart *et al.*, 2007, Wain & Hosoglu, 2008, Meltzer & Schwartz, 2010).

7.2 Future work

The StkF immunisation work showed that rStkF is an effective and promising vaccine antigen. It should be further tested in future vaccine studies using larger numbers of mice and possibly different types of adjuvants and/or different mice strains. The *in vitro* protective immune profile induced by rStkF deserves to be investigated *in vivo* against *S.* Paratyphi A or other StkF-positive *Salmonella* serovars in an experimental mouse challenge study. Future studies to compare the local immune response and protective efficacy associated with both oral and s.c. routes of immunization are clearly warranted. The *in vivo* model used most frequently to study typhoid pathogenesis is the *S.* Typhimurium mouse oral infection model that results in a similar systemic infection to typhoid (Santos *et al.*, 2001). However, this model is not applicable in our study because *S.* Typhimurim does not possess Stk fimbriae based on bioinformatics analysis and PCR investigation. However, transformation of pUCstk plasmid into *S.* Typhimurium and expression of Stk fimbriae on its surface could solve this hurdle. In addition, production of *stk* knock out mutants of *Salmonella* should aid investigation of the role of Stk fimbriae in *Salmonella* adhesion and pathogenesis. In addition, production of monoclonal anti-StkF antibody which could then be used in protein localization studies such as immunogold EM will aid further understanding of the nature and function of these fimbriae. In addition, testing of the rStkF, rStaF and rSipA ELISAs in the future on much larger numbers of samples from endemic and non-endemic regions, an exploration of the utility of alternative cut offs against one or more of the three antigens examined, and/or improved interpretive algorithms that exploit result of combinations of ELISAs may ultimately allow for improved diagnostic performance characteristics.

Chapter Eight

References

8 References

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Appendices

Appendix 1

Chemicals, Enzymes and other Materials

Chemicals

2-Propanol (Sigma, UK)

Acrylamide/ bisacrylamide mix solution for SDS–PAGE, ProtoGel® (Geneflow, UK)

Agarose electrophoresis grade (Bioline, UK)

Ammonium persulfate (Sigma, UK)

Ampicillin (Sigma, UK)

Bovine serum albumin (Sigma, UK)

Bromphenol Blue Sigma (Sigma, UK)

Calcium chloride (Sigma, UK)

Chloramphenicol (Sigma, UK)

Coomassie Brilliant Blue R250 (Serva)

Disodium hydrogen phosphate (Sigma, UK)

Ethidium bromide (Sigma, UK)

Glucose (Sigma, UK)

Glycerol (Fisher Scientific, UK)

Glycine (Sigma, UK)

Imidazole (Fisher Scientific, UK)

IPTG (Bioline, UK)

Kanamycin (Sigma, UK)

Magnesium chloride (Sigma, UK)

Methanol (Fisher Scientific, UK)

Protein Ladder (Fermentas, UK)

SDS (Sigma, UK)

Skimmed milk powder (Oxoid)

Sodium bicarbonate (Sigma, UK)

Sodium carbonate (Sigma, UK)

Sodium Chloride (Sigma, UK)

Sodium Hydroxide (Sigma, UK)

ß-Mercaptoethanol (Sigma, UK)

TEMED (Sigma, UK)

Tris-HCl (Sigma, UK)

Triton X-100 BDH (Poole, UK)

Trizma® base (Sigma, UK)

Tween 20 (BDH, UK)

X-Gal (Bioline, UK)

Enzymes

Extensor Hi-Fidelity PCR Enzyme (ABgene, UK)

GoTaq® DNA polymerase (Promega, UK)

KOD Xtreme Hot Start DNA Polymerase (high fidelity) (Novagen, UK)

Lysozyme (Sigma, UK)

Restriction enzymes (Roche, UK and NEB, UK)

RNase (Sigma, UK)

DNaseI (Sigma, UK)

T4 DNA Ligase (Promega, UK)

Kits

ArchivePure DNA Cell/Tissue Kit for genomic DNA extraction (**5** PRIME, UK)

Wizard® *Plus* Miniprep plasmid DNA Purification System (Promega, UK)

PureYield™ Plasmid Miniprep System (Promega, UK)

TOPO TA Cloning Kit for Sequencing (Invitrogen, UK)

E.Z.N.A. ® Gel Extraction and DNA purification kit (Omega Bio-Tek, USA)

QuickFold™ Protein Refolding Kit (AthenaES, USA-0600)

Mouse IL-4 ELISA Ready-SET-Go kit (eBioscience, UK-88-7044-22)

Mouse IFN-γ ELISA Ready-SET-Go kit (eBioscience, UK-88-8314)

Mouse IL-12-p70 ELISA Ready-SET-Go kit (eBioscience, UK-88-7121)

Pierce ECL Western Blotting Substrate (PIERCE, UK)

Other materials

GeneRuler™ 0.1-10 kb DNA ladder (Fermentas, UK)

λ-*Hind*III ladder (Fermentas, UK)

dNTPs (Bioline, UK)

PageRuler™ Prestained Protein Ladder (Fermentas, UK)

Protein Assay Dye Reagent Concentrate (Bio-Rad, UK)

Whatman® Paper (Whatman, UK)

Pefabloc SC (AEBSF) (Roche, UK)

BCIP/NBT (Sigma, UK- B5655)

SIGMAFAST™ p-Nitrophenyl phosphate (Sigma, UK- N1891)

RPMI Medium 1640 (GIBCO)

Antibodies

Goat Anti-Human IgG (AP conjugated) Sigma-Aldrich A1543

Goat Anti-mouse IgG (AP conjugated) Sigma-Aldrich A3688

Mouse monoclonal anti-polyhistidine (HRP conjugated) (Sigma-Aldrich-A7058)

Goat Anti-Mouse IgG1 (AP conjugated) (Southernbiotech 1070-04)

Goat Anti-Mouse IgG2a (AP conjugated) (Southernbiotech 1080-04)

Media

Luria Bertani agar (LA)

5.0 g Bacto-tryptone (Difco)

2.5 g Bacto-yeast extract (Difco)

5.0 g NaCl

7.5g Bacto-agar (Difco)

Made up to 500 ml in dH_2O and autoclaved at 121°C at 15 psi for 15 min

Luria Bertani broth (LB)

5.0 g Bacto-tryptone

2.5 g Bacto-yeast extract

5.0 g NaCl

Made up to 500 ml in dH_2O and autoclaved at 121°C at 15 psi for 15 min

SOC broth

5.0 g Bacto-tryptone

2.5 g Bacto-yeast extract

5.0 g NaCl

Made up to 200 ml in dH_2O and autoclaved at 121°C at 15 psi for 15 min; after autoclaving, 50 µl of filter sterilised 2 M $MgCl_2$ and 200 µl of filter sterilised 1 M glucose was added to10 ml of medium.

Buffers and solutions

PBS (phosphate-buffered saline)
10× stock solution, 500 ml, pH 7.4

80 g NaCl

2.0 g KCl

11.5 g $Na_2HPO4.7H2O$

2.4 g KH_2PO_4

Made up to 500 ml in dH2O

Autoclaved at 121°C at 15 psi for 15 min

1× working solution, pH 7.4

100 ml of 10x stock buffer was completed to 1L with dH_2O, pH 7.4

TAE (Tris/acetate/EDTA) electrophoresis buffer

50× stock solution, 1 litre, pH 8.5

242 g Tris base

57.1 ml glacial acetic acid

37.2 g $Na_2EDTA.2H_2O$

Made up to 1 L in dH_2O

1× working TAE buffer, pH 8.5

100 ml of 50× stock TAE buffer was completed to 1L with dH_2O, pH 8.5

6× DNA Loading dye, pH 8.0

11.0 mM EDTA

3.30 mM Tris-HCl

2.5 % Ficoll 400 (w/v)

0.0012 % SDS

0.15 % Orange G

5× Protein sample loading buffer

10 % SDS (w/v)

5 % ß–Mercaptoethanol

20 % Glycerol (v/v)

0.2 M Tris-HCl pH 6.8

0.05 % Bromophenolblue (w/v)

Standard protein purification buffers

Lysis buffer (Native conditions), pH 7.4

20 mM Sodium Phosphate

500 mM NaCl

10 mM Imidazole, pH 7.4

Supplemented by DNaseI (1mg/ml), protease inhibitor, Pefabloc SC (10 mg/ml) and lysozyme enzyme to final concentration 0.2 mg/ml

Lysis buffer (Denaturing conditions), pH 7.4

20 mM Sodium Phosphate

500 mM NaCl

8 M Urea

10 mM Imidazole, pH 7.4

Supplemented by DNaseI (1mg/ml), protease inhibitor, Pefabloc SC (10 mg/ml) and lysozyme enzyme to final concentration 0.2 mg/ml

Binding buffer, pH 7.4

20 mM Sodium Phosphate

500 mM NaCl

10 mM Imidazole, pH 7.4

8 M Urea was added in purification under denaturing conditions

Wash buffer, pH 7.4

20 mM Sodium Phosphate

500 mM NaCl

20 mM Imidazole, pH 7.4

8 M Urea was added in purification under denaturing conditions

Elution buffer pH 7.4

20mM Sodium Phosphate

500 mM NaCl

20-500 mM Imidazole

8 M Urea was added in purification under denaturing conditions

SDS-PAGE and Western blotting buffers

10× SDS-PAGE (Tris-glycine) running buffer (1L), pH 8.3
30.3g Tris base
144.1 g Glycine
50ml 20% SDS
Completed to 1L with dH_2O

1× working solution of running buffer

100 ml of 10× stock buffer was completed to 1L with dH_2O, pH 8.3

1× Western blot transfer buffer (1L), pH 8.3

5.8 g Tris
2.9 g Glycine,
0.37 g SDS
200 ml Methanol
Completed to 1 L with d H_2O

5× Loading dye, pH 6.8

5.8 ml Tris (1 M)
0.83g SDS
2.5 ml Glycerol

1 mg Bromophenol blue

Completed to 10 ml with dH_2O

Coomassie blue stain (1L)

600 mg Coomassie blue R250 (Serva, 17525)

240 ml Methanol

100 ml Acetic acid

260 ml d H2O

De-staining Coomassie blue solution (1 L)

600 ml dH_2O

300 ml Methanol

100 ml Acetic acid

5 % skimmed milk

5 g Skimmed milk

100 ml TBS

Dialysing protein buffer, pH 7.4

20 mM Tris Hcl

145 mM NaCl

ELISA buffers

TBS, pH 7.4

10 mM Tris

140 mM NaCl

Blocking ELISA buffer

1g BSA

100 ml TBS

Coating buffer, pH 9.6

15 mM Na2CO3

35 mM Na HCO3

Washing buffer

1000 ml TBS

0.05 % Tween-20

20 % (w/v) SDS

20 g sodium dodecyl sulfate (SDS) in H_2O in a total volume of 100 ml with stirring.

Heat to dissolve, filter sterilise.

Tris buffered saline (TBS), pH 7.4

10 mM Tris-HCL

140 mM NaCl

BSA-TBS blocking buffer

TBS with 1 % (w/v) BSA, pH 7.4

Tris-glycine buffer, pH 8.3

25 mM Trizma base

192 mM glycine

0.1 % SDS

Appendix 2

Evaluation of the specificity, sensitivity, NPV and PPV of StkF, StaF and SipA antigens using mean + SD and mean + 3SD algorithms

ELISA	Mean + SD				Mean + 3SD			
	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
StkF (UK) ELISA (UK cut off)	100 %	67 %	40 %	100 %	88 %	100 %	100 %	97 %
StkF (NP) ELISA (NP cut off)	87 %	50 %	46 %	88 %	20 %	93 %	60 %	70 %
StkF (NP) ELISA (UK cut off)	100 %	46 %	44 %	100 %	100 %	57 %	50 %	100 %
StkF (EG) ELISA (EG cut off)	72 %	54 %	36 %	85 %	100 %	21 %	29 %	100 %
StkF (EG) ELISA (UK cut off)	100 %	21 %	29 %	100 %	97 %	36 %	33 %	97 %
StaF (UK) ELISA (UK cut off)	100 %	60 %	11 %	100 %	0 %	100 %	0 %	95 %
StaF (NP) ELISA (NP cut off)	47 %	77 %	50 %	74 %	0 %	100 %	0 %	67 %
StaF (NP) ELISA (UK cut off)	67 %	60 %	42 %	81 %	20 %	91 %	50 %	73 %
StaF (EG) ELISA (EG cut off)	78 %	54 %	49 %	81 %	27 %	93 %	69 %	69 %
StaF (EG) ELISA (UK cut off)	83 %	38 %	41 %	81 %	39 %	36 %	59 %	73 %
SipA (UK) ELISA (UK cut off)	91 %	61 %	44 %	95 %	27 %	100 %	100 %	79 %
SipA (NP) ELISA (NP cut off)	97 %	87 %	94 %	93 %	47 %	100 %	100 %	48 %
SipA (NP) ELISA (UK cut off)	100 %	75 %	100 %	100 %	100 %	100 %	100 %	100 %
SipA (EG) ELISA (EG cut off)	63 %	83 %	97 %	21 %	27 %	100 %	100 %	14 %
SipA (EG) ELISA (UK cut off)	75 %	75 %	94 %	74 %	47 %	100 %	100 %	27 %