DEVELOPMENT OF A PCR-BASED METHOD FOR THE DIAGNOSIS OF SALMONELLA TYPHI INFECTIONS FROM FILTER PAPER HUMAN BLOOD BLOTS

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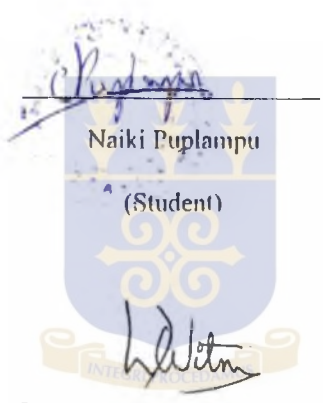
DECLARATION

This dissertation is the result of research work undertaken by Naiki Puplampu in the University of Ghana, under the supervision of Professor. M.D. Wilson and Dr Y.D. Osei.



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DEDICATION

To my parents and brothers.

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LIST OF ABBREVIATIONS

BLAST	Basic local alignment search tool
CFU	Colony forming units
CIE	Counter immunoelectrophoresis
DCA	Desoxycholate agar
EIA	Enzyme immunoassay
HLA	Human lymphocyte antigen
LPS	Lipopolysaccharide
Mab	Monoclonal antibody
MDR	Multidrug resistance
NARST	Nalidixic acid resistant *Salmonella typhi*
NCBI	National Centre for Biotechnology Information
OMP	Outer membrane protein
ONPG	o-Nitrophenyl-D-galactopyranoside
PCA	Plate counting agar
RIA	Radio immuno assay
SPI-1	*Salmonella* pathogenicity island 1
SPI-2	*Salmonella* pathogenicity island 2
SS	*Salmonella shigella*
TNTC	Too numerous to count
TSB	Tryptic Soy Broth
TSI	Triple sugar iron
TTSS	Type three secretion systems
VP	Voges proskauer

ABSTRACT

The most common diagnostic test for typhoid fever in endemic countries is the Widal test because it is fast and easy. Unfortunately, specificity is less than 75% and false positives of about 33% occur. Culture of *Salmonella typhi* on the other hand, is expensive and takes 7 days to produce significant detectable growth. Also, large volumes of blood used for diagnosis even from acute cases are not suitable for anaemic or distressed patients. A sensitive and specific PCR-based method for the detection of *S. typhi* from less than 500µl of blood has been applied successfully on field samples. The present study assessed PCR on DNA extracts from filter paper blood blots, which require much smaller volumes of blood from patients. A set of nested primers was designed for typhoid fever diagnosis by PCR. The first primer pair is *Salmonella* genus specific and amplifies a 872bp fragment while the second pair is *S. typhi* specific and amplifies a 477bp fragment. To check for specificity of the primers, they were used in a PCR reaction on DNA extracts from isolates of *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Bacillus subtilis*. The PCR results showed no amplifiction products from these bacterial cells but gave the expected product sizes from *Salmonella typhi* DNA extracts. Uninfected blood seeded with *S. typhi* was serially diluted and thereafter, aliquots plated on plate count agar (PCA). After 24 hours of incubation, bacterial colony forming units (CFUs) were counted on each plate. The serially diluted blood was blotted on filter paper to determine the threshold of detection of the PCR method. The results indicated that a minimum of 1.6CFU of bacteria in 1ml blood could be detected.

Blood samples from 185 suspected typhoid patients at University Hospital, Legon, were screened by both the Widal and the PCR methods. Out of this number, 58 (31.4%) samples were Widal positive for O and H antigens at a titre of 1/160 and seven (3.8%) were PCR positive. Blood films of 138 of the samples were screened for the malaria parasite and 12 (6.89%) were positive. A hundred and twenty-two cultured blood samples yielded 9 (7.14%) *Staphylococcus aureus* positives and 1 (0.79%) each for *Serratia marcescens* and *Yersinia enterolitica*. There was no *S. typhi* culture positive. Restriction digestion of positive PCR products with *DraI* gave the expected product sizes of 315 and 162 bps. This PCR assay can be used to detect *S. typhi* in blood blots; however, it needs to be evaluated against bacterial culture of large volumes of blood over a more extensive sample size.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Enteric or typhoid fever is a systemic, disseminated life-threatening infection of the gram-negative bacterium *Salmonella typhi*. The disease has an incubation period of 10-14 days, with an insidious onset characterized by non-specific symptoms of fever and malaise, which very often resemble those of malaria or flu (Mims *et al.*, 1998). This characteristic of typhoid fever leads to a great deal of confusion and misdiagnosis during the early stages of the disease. Without treatment, typhoid mortality rates can be as high as 10 to 15% (Ohl and Miller, 2001).

In areas of the world where safe water supply and good sewage control are not available, typhoid fever is an endemic infection (Mirza, 1995). It is estimated that there are 16 million cases of typhoid worldwide, which lead to approximately 600, 000 deaths per annum (Pang *et al.*, 1995). It is known that *Salmonella* infections, especially typhoid fever, caused by the species *S. typhi*, are a major cause of morbidity and mortality in developing countries (Cano, 1993). Case-fatality rates for typhoid fever in developing countries range between 12% and 32% as compared to less than 2% in industrialized countries (Carmeli *et al.*, 1993). Also, a study in which clinical and laboratory data were collected over 16 years (1975-1990) in the rural areas of four countries in Africa; Ghana, Zambia, Tanzania and Kenya revealed interesting results: Ghana was shown to have a national incidence rate of one in a thousand, which was the

highest among the countries under survey (Petit and Wamola, 1994). This result was based on projections from catchment areas in the various countries.

Salmonella infections have had a serious impact on the public health of Ghanaians especially in recent times, as reported on the front page of the Daily Graphic of April 6th 2001 and also by the Ghana News Agency on December 3rd 2003 when a typhoid fever outbreak at Obuasikrom and its surrounding villages in the Atwima district of the Ashanti Region of Ghana, killed two primary school pupils.

Although typhoid can be diagnosed clinically, the process is difficult and often gives uncertain results, which require a good knowledge of clinical medicine and a lot of expertise (Christie, 1974). Laboratory tests carried out in conjunction with clinical symptoms are a more effective way of diagnosing the disease because symptoms alone could be misleading. Furthermore, fever as a predominant symptom in typhoid fever is the sole manifestation in many cases and is common in most other tropical diseases an example of which is malaria (Zenilman, 1997). Thus, diagnosis cannot be done solely on the basis of symptoms, which are non-specific. Rather, definitive diagnosis requires the demonstration of the causal agent in blood, stool, urine or other clinical samples.

The most common diagnostic method used in the majority of hospitals and laboratories in the developing countries is the Widal test, an agglutination test for antibodies (Thomas, 1988). A serum sample collected on the day of the hospital visit and a second specimen collected ten to fourteen days later should show at least a fourfold rise in the

agglutinin titres to the O-antigen of *S. typhi* (Mirza, 1995). Such a rise is suggestive of a typhoid infection, although in a particular study about 15% of patients whose blood samples were *S. typhi* culture positive, did not develop significant O and H antibody titres (Brodie, 1977). Making use of the titre rise as a diagnostic method only gives a late diagnosis, which is altered by treatment because patients are usually put on antibiotic regimen as soon as the first Widal test has been performed.

Another disadvantage is that antibodies arising from previous stimuli such as vaccines, antibiotic use, sub-clinical disease, or cross-reacting antigens affect the specificity of the Widal test (Forsyth, 1990). The test is also very unreliable in endemic countries because demonstrable levels of *S. typhi* antibodies could be present in the general population anyway (Schroeder, 1968). Thus, a cut-off antibody titre level is usually determined which is used to establish a baseline level in the normal population (WHO, 2003). Any diagnostic antibody level higher than the baseline result is considered to be positive. However, this method is usually not completely reliable because the average general antibody levels in any population always vary between the different areas and with the seasons (Clegg *et al.*, 1994). The Widal test is therefore neither sufficiently sensitive nor rapid enough for the diagnosis of typhoid fever.

Laboratory culture of the organism from a clinical specimen is a very effective method for confirmatory diagnosis of typhoid fever. This is the second most common method after the Widal test for diagnosis of the disease. Blood culture especially, has a high predictive value for current enteric fever (Forsyth, 1990), although a large number of

factors such as the stage of the illness, previous infection, antibiotic use, or vaccination need to be taken into account when culture is being done (Thomas, 1988). In certain instances, suspected typhoid cases cannot be confirmed by culture probably because the levels of bacteraemia in the clinical specimens of patients are too low to be detected (Song *et al.*, 1993). *Salmonella typhi* can be isolated from more than 90% of patients with typhoid fever if blood, stool, rose spots and bone marrow aspirates are all cultured (Gilman *et al.*, 1975). A 100% positive rate of detection can be achieved if the culture of the mononuclear cell-platelet layer of blood is combined with cultures of bone marrow aspirate and rectal swab (Rubin *et al.*, 1990). In most cases, venous blood is used for culture because it is more convenient to take from patients.

The Widal diagnostic method is more rapid and cost effective than culture but as mentioned earlier, interpretation is often difficult and unreliable in endemic areas, especially with single titres (Levine *et al.*, 1986; Wicks *et al.*, 1974). The isolation of the organism from the blood of a patient by culture on the other hand, shows that the infection is active and that the patient is most likely to be suffering from an *S. typhi* infection (Mirza, 1995). However, its greatest deficiency is its inability to detect minute quantities of the organism in blood, since fewer than 10 organisms per millilitre can cause severe disease (Haque *et al.*, 1999). This implies that large quantities of blood have to be used in order for the organism to flourish in growth medium and be detected. This method is particularly inappropriate in severe cases especially in very young and febrile individuals.

Molecular methods make it possible to diagnose disease from very small quantities of clinical samples, giving them an edge over Widal and bacterial culture, methods that make use of larger volumes of diagnostic samples, with the former not being specific enough. Culture is very slow, taking between three to seven days to produce significant detectable growth (WHO, 2003). Molecular methods, especially those that are PCR based can yield results within 24 hours.

1.1.1 Rationale

The emergence of drug resistant strains of *Salmonella* has become a cause for deep concern among health professionals, especially in the developing world, because there is a rise in the occurrence of typhoid epidemics with its resultant rise in morbidity and mortality rates (WHO, 2003). In order for treatment of typhoid infections to be prompt and effective, there is a need for rapid, sensitive and specific laboratory methods for diagnosis of the disease (Guerra-Caceres *et al.*, 1979; Hoffman *et al.*, 1986; Song *et al.*, 1993). This is important when a reliable conclusion on the cause of disease is needed in a patient with recurrent fever and repeatedly negative or conflicting results from both culture and Widal tests.

Studies have been conducted to compare the efficiency of the Widal test with culture of clinical samples. One such study, in which samples from fever patients were cultured and also tested by the Widal method, revealed that Widal gave a higher infection rate than blood and stool culture. The results obtained suggested that 60 out of 200 patients (30%) were falsely diagnosed as having typhoid fever instead of malaria (Ammah *et al.*,

1999). In another study, Haque *et al.* (1999) compared the performance of blood culture, Widal and polymerase chain reaction (PCR) for the early detection of *S. typhi* in clinical samples. The authors concluded that bacterial culture gave too high an incidence rate of false negatives whilst Widal gave too high an incidence of false positive rates.

Other methods have recently been developed to overcome the low sensitivity and specificity of the conventional typhoid diagnostic methods. One of such new methods makes use of a DNA probe, specific to the Vi antigen of *S. typhi* and is able to detect the organism in the blood of patients (Rubin *et al.*, 1989). The use of a hybridisation probe requires that the bacteria be concentrated from the samples and amplification of total bacterial DNA be done before it can be detected. These requirements are often too complicated and ponderous for routine use in endemic developing countries. Some other tests have been developed as alternatives to serological and already established means of diagnosing *Salmonella* infections. Fluorescent DNA-based assays (Cano, 1993), antibody immobilization methods, enzyme immunoassays (Bailey *et al.*, 1991) and PCR have been used to identify *Salmonella* in clinical samples (Song *et al.*, 1993; Haque *et al.*, 1999). The afore-mentioned methods are quite complicated, but rapid and sensitive when compared to culture, and more specific than Widal. The PCR-based method is sensitive enough to detect very minute quantities of DNA, as low as 5 bacteria per millilitre (Haque *et al.*, 1999). It is also very specific because it targets the genomic DNA of the organism of interest.

PCR will be highly useful in determining if an infection has cleared, as opposed to Widal which detects lingering antibodies, giving the impression that an infection is still present when it has actually been cleared. The limitations of culture can also be overcome by the higher sensitivity of PCR. Although the high cost of PCR-based methods is a limitation to its use for routine diagnosis, it is being used more often in field situations for the detection of low numbers of pathogens in small samples. A unique opportunity is thus being offered by the recent slight improvements in the cost of PCR, to develop a PCR-based method that can be used routinely for typhoid diagnosis in developing countries.

The present study was designed to develop a PCR-based means of detecting *Salmonella* DNA extracted from blood stored on filter paper as blots. Filter paper blots were used as the sampling method because it is simpler to collect, and less ponderous to store, transport and process for extraction. The method is also much less painful to young and or distressed febrile patients from whom venous blood collection is difficult and from whom large volumes were unavailable. Furthermore, three new pairs of primers, which target region VI of the flagellin gene of both *S. typhi,* and *S. typhimurium* were designed. *S. typhimurium* co-existing with malaria has been found to occur at a higher rate (56.5%) than *S. typhi* and malaria co-infections (29.6%) in Cameroon (Ammah *et al.*, 1999). The new primers were designed to be used to screen severe malaria cases for dual *Salmonella*/malaria infections in a latter study. The present study was undertaken to test the *Salmonella typhi*-specific primer pair alone.

Blood samples from febrile patients, who took the Widal test, were used to establish if this PCR method would serve as a reliable diagnostic method. In addition to this, blood culture was done to detect *S. typhi*. The different sets of results were compared to ascertain if the new primers are sensitive, specific and can be used in typhoid fever diagnosis.

1.1.2 General objective

The overall goal of this study was to design a new set of oligonucleotide primers for PCR-based detection of *S. typhi* from filter paper blood blots.

1.1.2.1 Specific objectives

The specific objectives set were as follows:

i. To collect blood samples from suspected typhoid cases for culture, Widal tests, and blots on filter paper.

ii. To culture blood samples for *S. typhi* growth.

iii. To conduct Widal tests for typhoid diagnosis and prepare blood films for the detection of malaria parasites.

iv. To design oligonucleotide primers for a nested PCR based *S. typhi* diagnosis from filter paper blood blots.

v. To compare the results of the blood film, culture, Widal test, and the newly developed PCR method.

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1.2 Literature review

1.2.1 Typhoid fever, the disease

Enteric fevers, which include typhoid fever, are life-threatening infections caused by various species of bacteria belonging to the genus *Salmonella*. Typhoid fever, the most severe illness in this group of fevers, is caused by *S. typhi*. *Salmonella paratyphi* strains A, B and C on the other hand, do not cause as severe and deadly symptoms as typhoid fever, but produce the paratyphoid fevers that are generally milder (Mandal, 1996). All other Salmonellae, including *S. typhimurium* are mostly pathogenic to animals but can cause a more benign and less invasive illness of short duration in man in which intestinal symptoms such as occur in *Salmonella* food poisoning and salmonellosis are predominant (Thomas, 1988).

A typhoid fever attack is preceded by an incubation period of 10 to 20 days depending on the infecting dose (Hornick *et al*., 1970). In the first week after incubation, there is usually a slow, insidious and vague fever, with malaise and headache (Forsyth, 1990). Constipation among adults, diarrhoea, respiratory symptoms like bronchitis and a mild non-productive cough are common at this stage. During this period, the fever rises in a stepwise pattern and is usually about 0.5°C higher in the evening than in the morning. At the end of the week, the fever would have risen to its peak of 39-39.5°C. During the second week, the patient looks anorexic and apathetic with a sustained high temperature and toxaemia. Very commonly, there is a slight distension of the abdomen due to splenomegaly.

In over half of the cases, crops of pink papules (rose spots) of 2-4mm in diameter appear on the abdomen, lower chest and back. However, these are difficult to detect in dark skinned individuals (Huckstep, 1962; Wicks *et al.*, 1971). The spots are caused by bacterial embolization and rose spot cultures may be positive. During the first two weeks, bradycardia is common and the pulse may have a characteristic notch (dicrotic pulse). As the third week of the disease progresses, the patient begins to manifest the signs of severe infection, stupor worsens and the patient becomes uncomprehending, delirious and dull (typhoid state). Abdominal distension occurs with scanty bowel sounds, which are an indication of intestinal obstruction or paralysis of the bowel.

Other intestinal complications such as perforations may also occur, along with diarrhoea, which at an advanced stage of disease, is a common symptom characterised by the passing of a liquid, foul smelling yellow-green stool (pea soup diarrhoea) [Badoe, 1966]. In the event of non-treatment, death usually occurs as a result of toxaemia, myocarditis, intestinal haemorrhage and exhaustion (Mandal, 1996). If the patient survives, considerable weight loss occurs and as the disease progresses into the fourth week, the fever, mental state and abdominal distension improve slowly but steadily. Convalescence is usually prolonged (Hornick, 1985).

The clinical picture of typhoid fever varies depending on the individual, but the general picture is that of a mild illness progressing to a severe one with complications. The clinical outcome and severity of the disease are dependent on several factors, including the duration of the illness before treatment was initiated, the size of the infecting

inoculum, the antibiotic used for treatment, previous exposure to typhoid vaccines, virulence of the bacterial strain, host factors such as human lymphocyte antigen (HLA) type, concomitant infections such as HIV, immunosuppression, and the ingestion of other medications like H2 blockers and antacids that reduce gastric acid concentrations (WHO, 2003). Typhoid fever has therefore many presentations, from uncomplicated to complicated disease. Ten percent of complicated cases lead to death (GlaxoSmithKline, 2003).

1.2.2 Global distribution

Typhoid fever is a disease of public health concern in most countries of the developing world because it is associated with poor hygiene arising from overcrowding, inadequate water supply systems and lack of proper sewage disposal facilities (DuPont and Steffen, 1997). *Salmonella* infections usually occur in areas where the development of proper sanitary facilities cannot keep pace with population growth and urbanization. Social, economic and military disruptions have had the tendency to aggravate the incidence of the disease (Edelman and Levine, 1986). Three billion people worldwide do not have access to potable water and live in the high-risk areas where there is the greatest likelihood of contracting typhoid fever (WHO, 1997).

Globally, typhoid fever is far more common and widespread than paratyphoid fever (Levine *et al*., 1983). The high-risk areas are Asia and Africa with annual cases of 13.3 million and 2.7 million, respectively and annual deaths have been estimated to be as high as 440 000 in Asia and 130 000 in Africa (GlaxoSmithKline, 2003). The disease is

endemic in the Indian subcontinent, Asia, the Middle East, Africa and Central and South America (Fig. 1), with the highest incidences in Asia, the Indian sub continent and the Middle East, where almost 80% of all worldwide cases occur. In South and Central America, the estimated annual incidence is 150 cases per 100 000 inhabitants. The endemic rates in some parts of Southern and Eastern Europe are as low as 4 to 15 cases per 100 000 inhabitants per year while enteric fevers occur most exclusively as imported infections in the rest of Europe, North America and Australasia (Mandal, 1996; GlaxoSmithKline, 2003).

1.2.3 The causative agent

The genus *Salmonella* comprises non-spore bearing and non-encapsulating gram-negative flagellated bacilli. They belong to the Enterobacteriaceae family, and are characterised by the ability to ferment glucose but not lactose (Mandal, 1996).

The specific classification of *Salmonellae* remains unclear (Collin and Lyne, 1985). Members have been named as species (e.g. *S. typhi*) or serotypes (e.g. *S. enterica* ser. *typhi*), giving rise to two different schools of thought. Le Minor and Popoff (1987), who are proponents of the second school, have proposed two species within the genus *Salmonella*, i.e. *Salmonella bongori* and *Salmonella enterica*.

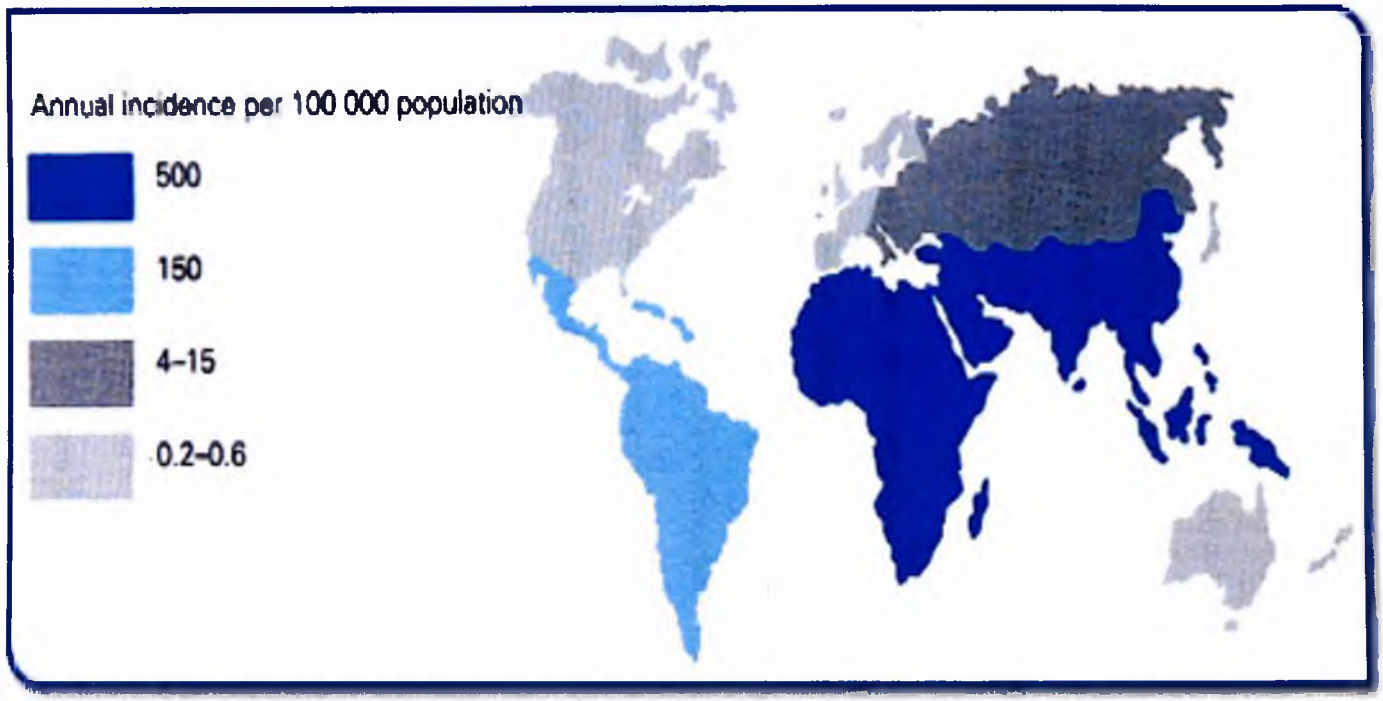

Fig. 1 The worldwide distribution of typhoid fever incidence rates (Source: GlaxoSmithKline, 2003).

All *Salmonellae* that are pathogens of warm-blooded animals belong to sub species I, which is one of six subspecies within the *enterica* species (WHO, 2003). *Salmonella typhi* was thus classified as a serotype within the subspecies I (one) of *S. enterica*. The Judicial Commission of the International Committee on Systematic Bacteriology (1990) rejected this system of nomenclature on the basis that it might confuse clinicians and be the cause of fatal outcomes. The originally accepted nomenclature, which describes *S. typhi* as a species, has therefore been retained (Ezaki *et al.*, 2000). In scientific circles, *S. typhi* is the accepted name of the causative agent of typhoid fever.

1.2.4 *S. typhi* virulence genes and pathogenesis

The genetic structure of pathogenic bacteria shows localised regions of the chromosome, which are called pathogenicity islands (Miller *et al.*, 1989; Groisman and Ochman, 1996). These islands contain virulence genes that are believed to control *S. typhi* virulence mechanisms. A representation of some virulence genes is in Figure 2, showing their roles in the virulence processes.

Typhoid fever pathogenesis in humans is not well understood because of the lack of suitable experimental models. Chimpanzees exhibit a similar disease to man only after huge infectious doses (Warren and Hornick, 1979). The information on human *Salmonella* pathogenesis comes from direct clinical and pathological observations of patients and volunteers and *in vitro* studies on cell and tissue cultures. More recently, studies using mice models infected with *S. typhimurium* have made the study of the interactions between host and pathogen easier. Although these models do not

absolutely reflect the human infection, they provide insight into the general mechanisms of microbial pathogenesis and host immunity (Ohl and Miller, 2001).

1.2.4.1 Virulence genes

Salmonella pathogenicity islands 1 and 2, (SPI-1 and SPI-2) encode virulence proteins, termed type III secretion systems (TTSSs). TTSSs are specialized systems that have evolved to modify host-cell function through the direct translocation of bacterial virulence proteins into the host cell cytoplasm (Hueck, 1998). The virulence proteins alter basic host cell functions like cytokine gene expression, membrane trafficking, signal transduction and cytoskeletal architecture. SPI-1 encodes proteins that are necessary for the invasion of intestinal epithelial cells. It also codes for proteins that induce epithelial secretory and inflammatory responses (Watson *et al.*, 1995, Galyov *et al.*, 1997). On the other hand, SPI-2 codes for proteins that are essential for intracellular replication and in the mouse enteric fever model are necessary for the establishment of systemic infection beyond the intestinal epithelium (Cirillo, *et al.*, 1998; Hensel *et. al.*, 1998).

In order to establish systemic infection, *Salmonella* serotypes that cause enteric fever must survive and replicate within the host macrophage (Fields *et al.*, 1986). Some of the genes necessary for survival in the macrophage are constituents of a two-component response regulator termed PhoP/PhoQ (Miller *et al.*, 1989). Activation of the PhoP regulator causes modifications in the protein and lipopolysaccharide components of the bacterial inner and outer membranes. These surface modifications help *Salmonella*

survival in the stressful environment of the phagosome by conferring partial resistance to the activity of antimicrobial peptides (Guo *et al.*, 1998).

Salmonella resistance to nitric oxide (NO) and related reactive nitrogen compounds produced in the macrophage is partly mediated by the synthesis of homocysteine, an antagonist of [NO] (De Groote *et al.*, 1996). *Salmonella* mutants with an alteration in their *MetL* gene are unable to synthesize homocysteine and are thus hypersensitive to S-nitrosothiol NO-donor compounds and less virulent than normal strains (Ohl and Miller, 2001). Some *Salmonella* species can also produce superoxide dismutases like SodCII that are able to inactivate reactive oxygen species (Fang *et al.*, 1999). To survive in nutrient limited environments like the phagosome, biosynthetic genes necessary for the *de novo* synthesis of essential metabolites are induced. These metabolites include aromatic amino acids and purines. However, the mutants that lack these enzymes are avirulent (Hoiseth and Stocker, 1981; McFarland and Stocker, 1987).

The flagellin gene, which is the gene of interest in this study, encodes flagellin, the monomeric protein subunit of flagellae that propel bacteria through their aqueous environments. The flagellae have been shown to aid bacteria in attaching themselves to the host cell thereby making invasion possible and contributing to bacterial virulence (Mobley *et al.*, 1996).

Flagellin proteins also carry H-antigen specificity and are inducers of pro inflammatory mediators, making them an important subject of study into bacterial virulence. The

protein is made up of carboxy and amino-end-domains coded by genes, which are highly conserved among different *Salmonella* species, as well as a number of other Gram-negative bacteria (Mimori-Kiyosue *et al.*, 1989). The middle domain on the other hand is encoded by variable nucleotide sequences within the central region of the open reading frame and this property has been exploited to type different *Salmonella* serotypes by RFLP-PCR (Hong *et al.*, 2003).

1.2.4.2. Pathogenesis

Salmonella enters the body through the mouth then passes through the stomach and the pylorus. Invasion of the epithelium of the small bowel takes place after the bacterium has crossed the intestinal mucus layer. *Salmonellae* express thin, hair-like projections called fimbriae or pili, which are made of protein sub-units and enable them to attach to the surface of the epithelial cells (Baumler *et al.*, 1996). Shortly after bacteria adhere to the apical epithelial surface, deep cytoskeletal rearrangement occurs in the host cell, the conformation of the epithelial brush border is altered and membrane ruffles are formed that reach out and enclose bacteria into large vesicles, as shown in Figure 2. This process is called bacterial mediated endocytosis and is morphologically and functionally distinct from receptor-mediated endocytosis, the mechanism by which many other pathogens enter non-phagocytic cells (Francis *et al.*, 1992, Ohl and Miller, 2001). After the bacteria have entered the cells, a fraction of the *Salmonellae* containing vesicles cross the epithelial membrane and the cells reconstitute.

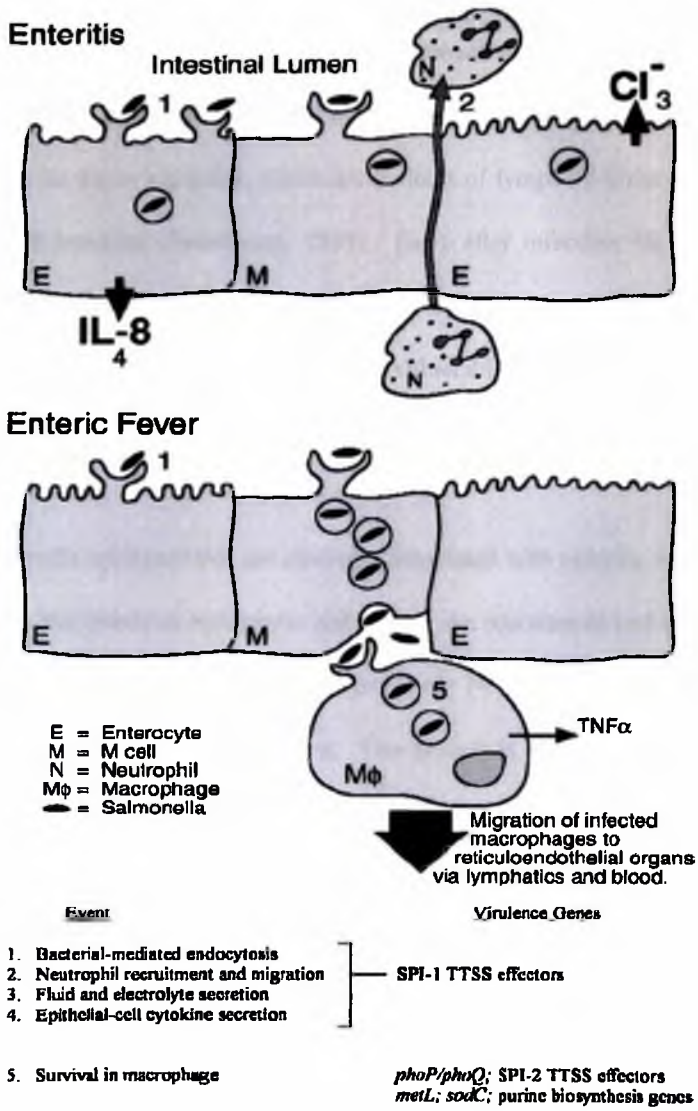

Fig 2 Schematic diagram showing the invasion processes of *S. typhi* infection and inflammatory responses. Virulence genes and their effects are also shown (Source: Ohl and Miller, 2001).

In mice, the bacteria appear to preferentially adhere to and enter the macrofold or M cells, of the intestinal epithelium. Sometimes, invasion of non-phagocytic enterocytes also occurs. M cells capture intestinal antigens through pinocytosis and transport these antigens to the Payer's patches, which are nodules of lymphoid tissue within the walls of the small intestine (Brandtzaeg, 1989). Early after infection, the Peyer's patches become hyperplastic and hyperaemic, and sometimes the overlying tissues become necrotic and ulcerated, making certain areas vulnerable to perforation. Major bleeding occurs when large blood vessels are involved in ulceration (Kearney and Kumar, 1993).

The *Salmonella* serotypes that are clinically associated with enteritis induce a secretory response in the intestinal epithelium and initiate the recruitment and transmigration of neutrophils into the intestinal lumen (Galyov *et al.*, 1997). Figure 2 depicts some of the events leading to the secretory response. This process is associated with the production of several cytokines such as interleukin-8, by the epithelial cells and is important in the recruitment of neutrophils to the submucosal space (McCormick *et al.*, 1993). Immediately after they have crossed the intestinal epithelium, *Salmonellae* encounter another obstacle of innate immunity, the submucosal macrophages.

Those serotypes that cause systemic infection are able to enter the macrophages, again by an apparently induced mechanism of macropinocytosis and subsequently activate virulence mechanisms that allow evasion of the anti-microbial functions of the phagocyte. These events permit the survival and replication of bacteria in the intracellular environment (Alpuche-Aranda *et al.*, 1994). Infected phagocytes are able to migrate and spread through the lymphatic system to the blood stream and other organs of the reticuloendothelial system, the liver, bone marrow and spleen. At this point, multiplication and proliferation of the organisms give rise to a secondary

bacteraemia, a more pronounced infection with all the signs and symptoms of disease. Headaches, constipation, high fever and prostration are common. Bacteria then flow into the bowel from the biliary tract, the patient begins to excrete the bacterium and *Salmonella* can be detected in the stool by culture (Forsyth, 1990). The spleen is also enlarged, ulceration of ileal Peyer's patches may lead to diarrhoea and finally, intestinal haemorrhage and perforation. These last two symptoms are the most important surgical complications of typhoid fever.

1.2.5 Control and prevention of typhoid fever

To prevent and control the spread of typhoid fever, it is necessary to know the mode of transmission of the causative organism. Typhoid fever is acquired by the ingestion of food or water that has been contaminated with the faeces or urine of a person who has an active disease or is an asymptomatic chronic carrier of *S. typhi* (Mirza, 1995). The carrier state arises in a patient if an individual continues to excrete the organism after he has recovered from the disease. Faecal excretion after recovery continues during convalescence and normally stops within three months, but may persist up to a year after (Bigelow and Anderson, 1933). About 3% of those recovering from typhoid fever become long-term carriers, although the carrier state sometimes arises after an asymptomatic infection (Brooks, 1996). Carriers stand a permanent risk of infecting others especially if they work in kitchens, with children or with the elderly (underdeveloped or weakened immune systems). The chance of becoming a carrier is greater among women than men (Ames and Robbins, 1943).

1.2.5.1 Improved sanitation

Good hygienic standards include measures that ensure the supply of clean water, hygienic preparation of foods, safe sewerage disposal and health education of the

general population. In most developing countries, the infrastructure for potable water supply is either defective or inadequate, making it a big challenge in the control and prevention of *Salmonella* infections.

Wastewater management techniques that prohibit the use of raw human excrement as manure and provide for adequate treatment and disposal of sewerage will ensure that in the event of an epidemic, typhoid fever is not transmitted from the irrigated crops to consumers. The proper wastewater management measures will reduce contamination of water bodies as the manure is washed into streams and lagoons. In cases where sanitary control is impossible to implement, the second alternative is to undergo vaccination, but this is expensive (Thomas, 1988).

1.2.5.2 Vaccination

Wright (1896), and Pfeiffer and Kolle (1896), independently developed typhoid vaccines in the late nineteenth century. These were suspensions of attenuated or inactivated organisms that were used to protect the military during campaigns. The effectiveness of these vaccines however, was assessed using inadequate and unquantifiable methods and protection was unreliable (Cockburn, 1955).

Two different kinds of vaccines are currently in use; the purified Vi antigen vaccine and the Ty21a strain which is a *galE* mutant (Tacket and Levine, 1995). The Vi polysaccharide vaccine can be given in a single dose subcutaneously or intra muscularly and after seven days, protection is acquired with the maximum levels of antibody developing after 28 days. Inactivated *S. typhi* vaccines are completely ineffective as oral vaccines and have been associated with high rates of systemic reactions (Parker, 1990).

The Ty21a strain was isolated after exposing *S. typhi* Ty2 strain to *N*-methyl-*N'*-nitrosoguanidine. This vaccine proved to be highly protective when three doses were given three days apart on an empty stomach in a liquid formulation. The coated capsule form of this vaccine is not as effective as the liquid formulation but both are approved for use in children aged at least 5 years (WHO, 2003). The protective ability of enteric-coated capsules 7 years after the last dose is 62%, while that of the liquid formulation is 70% after the same period of time following vaccination. The WHO recommends that antimalarial drugs be taken only three days after the end of immunisation (WHO, 2003).

1.2.6 Management and treatment of typhoid fever

In the majority of cases, infection with *Salmonella* is not lethal if effective antimicrobial therapy is administered early enough to stop the infection before it reaches a critical or deadly stage (Du Pont, 1993). More than 90% of patients can be managed at home with oral antibiotics, competent attention, and lukewarm baths to reduce temperature. These should be complemented with close medical care in the event of failure to respond to treatment, or complications (Punjabi, 2000). Generally, increased oral and intravenous fluid intake, good nutrition and the use of antipyretics are good enough measures to effectively manage typhoid infections, but a patient who develops persistent diarrhoea, vomiting, abdominal distension and pain may need to be hospitalised for professional care and treatment (WHO, 2003).

Despite the efficacy of most drugs of choice, resistance in *Salmonella typhi* exists (Mirza, *et al.*, 1996). There are two types of antibiotic resistance; the first is multiple drug resistance (MDR), which is defined as resistance to antibiotics such as co-trimoxazole (trimethoprim/sulfamethoxazole), chloramphenicol and ampicillin. The second is fluoroquinolone resistance (WHO, 2003). Figure 4 shows the global

distribution of the two kinds of antibiotic resistance. Reduced susceptibility to fluoroquinolones is indicated by *in vitro* resistance to nalidixic acid, which is a quinolone (Murdoch *et al.*, 1998). Fluoroquinolone resistance however, may either be total or partial. The so-called nalidixic-acid resistant *S. typhi* (NARST) is a marker of the levels of susceptibility of the organism to fluoroquinolones as opposed to the nalidixic acid sensitive *S. typhi*. Nalidixic acid itself is not used to treat *S. typhi* infections, but NARST gives a worse clinical response to fluoroquinolone treatment than the sensitive strains (WHO, 2003). The occurrence of multi-drug resistance to ampicillin, chloramphenicol, streptomycin, tetracycline and co-trimoxazole has been reported in Ghana. Multidrug resistance to at least three of these antibiotics was observed in thirty of the fifty-eight *S. typhi* strains isolated from clinically suspected cases of typhoid fever (Mills-Robertson *et al.*, 2002).

Chloramphenicol resistance was the first to be reported in the 1950s, two years after its introduction for typhoid treatment, although such reports were scattered and isolated (Parry *et al.*, 2002). It was not until 1972 that chloramphenicol resistant typhoid fever became a major problem. By the late 1980s, a rapid rise in the prevalence of chloramphenicol resistant strains was observed in India and Pakistan and by 1992, 70 to 80 % of strains in India, Pakistan, Bangladesh, Vietnam, the Middle East and Africa were resistant to ampicillin and trimethoprim as well as to chloramphenicol (Mizra *et al.*, 1996). Most strains are sensitive to fluorquinolones, although a recent upsurge of resistance has been observed (Rowe *et al.*, 1995). In addition, resistance to ciprofloxacin (also called nalidixic-acid resistant *S. typhi*) has been observed in Asia (Parry *et al.*, 2002). A significant number of strains from Africa and the Indian subcontinent are MDR type. A small percentage of strains from Vietnam and the Indian subcontinent are NARST strains (Miller and Pegues, 2000).

Currently, the antibiotics used for the treatment of typhoid fever are the fluoroquinolones, chloramphenicol, penicillin-based drugs, co-trimoxazole (trimethoprim/ sulfamethoxazole) and the cephalosporins.

Fluoroquinolones include drugs such as Ciprofloxacin, ofloxacin, fleroxacin, and perfloxacin. They are regarded as optimal in the treatment of typhoid fever in adults and are well tolerated, more rapidly absorbed and more effective than the former first line drugs (i.e. chloramphenicol, amoxicillin, ampicillin and co-trimoxazole) [Chinh *et al*., 2000]. Published data also suggest that the fluoroquinolones are associated with lower rates of stool carriage than the traditional first-line drugs, chloramphenicol and co-trimoxazole (White and Parry, 1996). They are active when administered orally, and penetrate well into tissues. Within the tissues, they are concentrated in the phagocytes and bile, killing *S. typhi* in its intracellular stationary phase in the macrophages and monocytes (James, 1989). The fluoroquinolones are the drug of choice in the treatment of typhoid fever all over the world and ciprofloxacin is the most commonly used in the treatment of severe enteric fever in endemic areas (DuPont, 1993). In areas where there is resistance to fluoroquinolones or they are unavailable, the other drugs like chloramphenicol, the beta-lactams and trimethoprim-sulfamethoxazole are generally used. These are inexpensive and have few or insignificant side effects (WHO, 2003).

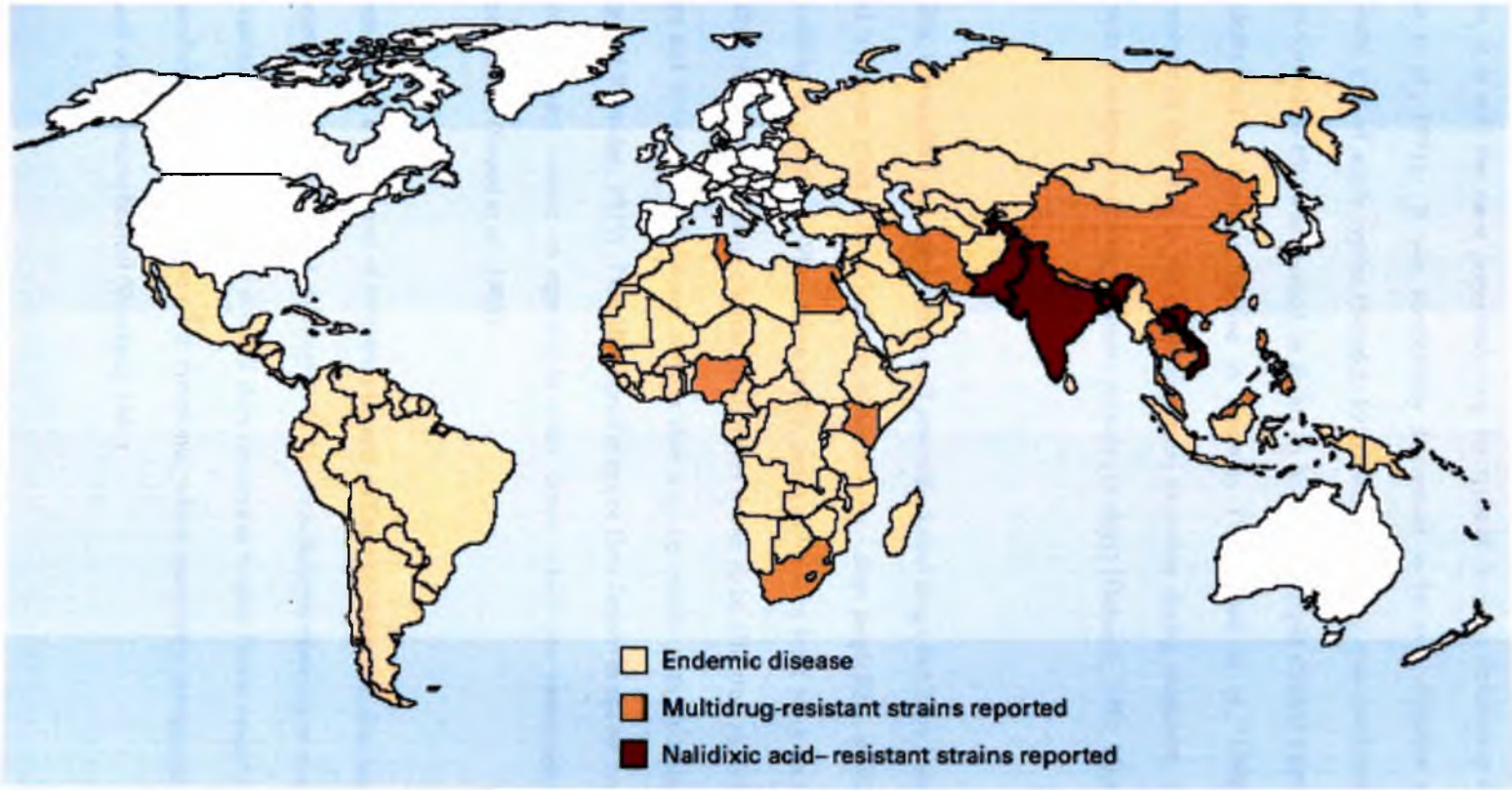

Fig 3 World map of the distribution of multidrug resistant and nalidixic acid resistant strains of *Salmonella typhi.* (Source: Parry, *et al.*, 2002)

Among the antibiotics used for typhoid treatment, chloramphenicol was previously considered the first drug of choice. Despite the risk of agranulocytosis in 1 out of 10 000 patients, it is still the most prescribed drug for typhoid fever in developing countries (Bhutta *et al.*, 1991). It was accidentally discovered to be very effective during a therapeutic trial of scrub typhus (Smadel, 1950) and its efficacy was confirmed with a placebo-controlled trial that showed its ability to bring about rapid clinical improvement of patients and a dramatic decline in mortality (Woodward *et al.*, 1948). The disadvantage of this drug is the continued faecal excretion during treatment, increased incidences of relapses and long treatment periods (14 days) [Hornick, 1985; Bhutta *et al.*, 1991].

Penicillin, amoxicillin and ampicillin are all penicillin-based drugs that have been used in typhoid treatment trials. Amoxicillin is better absorbed than ampicillin, which proved disappointing in practice. When taken orally, amoxicillin is at least twice as active as penicillin (Neu, 1974). During clinical trials, it was shown to be effective, showing fewer relapses and fewer residual chronic carriers than a group treated with chloramphenicol (Scragg and Rubridge, 1975). Penicillin based drugs or Beta-lactam drugs are considered safe for pregnant women as opposed to other drugs, which are contraindicated for pregnant women (Seoud *et al.*, 1988).

Co-trimoxazole, a compound of trimethoprim and sulfamethoxazole has also been found to be useful in treating typhoid, although only the trimethoprim component is effective. An advantage of co-trimoxazole is that it does not appear to give "toxic crisis", which is an exacerbation of toxaemia and other symptoms, which sometimes complicate typhoid treatment with chloramphenicol (Rowland, 1961).

The cephalosporins include cephamandole, cefoperazone and ceftriaxone. They have been demonstrated to be effective anti-*Salmonella* drugs (Forsyth, 1990). Among these three, ceftriaxone has especially proved useful when administered intravenously or intramuscularly over three days with some patients sometimes remaining febrile (Acharya *et al.*, 1995).

1.2.7 Diagnosis of typhoid fever infection

It is difficult to diagnose enteric fever by clinical examination, especially in the early stages, and clinical symptoms generally appear, prompting laboratory diagnosis. Diagnostic tests take three major forms:

i. Culture and identification of the bacteria.

ii. The recognition of a specific immunological response to the agent or serologic test.

iii. The direct detection of the agent of disease, its antigens, specific products or genomic elements (Forsyth, 1990).

Isolation of *S. typhi* from blood, bone marrow or a specific anatomical lesion gives a definitive diagnosis whereas clinical symptoms or the detection of a specific antibody response is suggestive of typhoid fever but cannot be used to make a definitive diagnosis (WHO, 2003).

1.2.7.1 Culture and identification of *S. typhi*

One of the most reliable diagnostic procedures is blood culture, and more than 80% of typhoid fever cases have the causative organism in their blood, although the levels of bacteria in the blood of patients are often low (Forsyth, 1990; WHO, 2003). Blood culture is often positive during the early ambulant phase and can still be detected until effective treatment is given. During relapses, it gives positive results (Forsyth, 1990).

Thus, isolation of *S. typhi* in the blood is an indication of an active infection (Mirza, 1995).

The standard primary medium for blood culture is enrichment broth such as Tryptic Soy Agar (TSA) or brain heart infusion (WHO, 2003). Fresh blood (5ml) is inoculated directly in 50ml blood culture bottles containing 45ml of broth, and incubated at 37°C in an aerobic incubator. When 10ml of blood or more have been drawn from the patient, it is usually advisable to make 5ml aliquots of the blood into liquid media in 50ml bottles immediately and as aseptically as possible, in order to have replicates of the assay.

The blood in the media is subcultured on days one, three and seven on non-selective solid media, like nutrient agar, blood agar, or selective media such as MacKonkey agar. For bile-tolerant bacteria such as *S. typhi*, MacKonkey agar is the most suitable selective solid medium and is therefore a favourite of most laboratories. This medium also inhibits the growth of Gram-positive bacteria. Subculture plates are incubated for 18-24 hr at 37°C.

Clot culture is favoured in cases where blood is available in very low quantities or has clotted. A clot is prepared or recovered from a blood sample and is either cut up with sterile scissors or loosened with streptokinase (Watson, 1955). It is then incubated for subsequent subculture as with unclotted blood. This technique usually makes use of what is left over from blood that has been used for either serological or biochemical tests. It proved useful during the Aberdeen typhoid fever outbreak in 1964 (Walker, 1965).

Stool culture is another standard diagnostic procedure, which is particularly useful for the diagnosis of typhoid carriers (Forsyth, 1990). Carriers are asymptomatic for typhoid fever and thus do not have or manifest any clinical symptoms but continue to pass *S. typhi*

in their faeces. Stool culture involves inoculating approximately 1g of stool in 10ml of Selenite F broth, which is selective for *S. typhi*, and incubating it for 19-48hr at 37°C. The inoculate is then plated on solid selective media such as MacKonkey agar, desoxycholate citrate agar (DCA), xylose-lysine desoxycholate agar, hektoen enteric agar or *Salmonella-shigella* (SS) agar and incubated at 37°C for 24hr. The process is repeated on days one, three and seven. Rectal swabs are not as reliable because of toxins in cotton wool and the drying effect of swabs, but can be used in the cases where no other specimen is available (Shaunessy *et al.*, 1948).

The specimen that has consistently shown reliability, yielding the most positive cultures has been the bone marrow aspirate (Gilman *et al.*, 1975). Bone marrow sampling is not done often because it requires skill and sterile equipment. It can also be very painful (Vallenas *et al.*, 1985). The patient is made to lie sidewise in a foetal position and a local anaesthetic is injected into the bone. A sterile syringe is inserted into the sternum from where a minimum of 1ml of bone marrow aspirate is obtained. This is done in very aseptic conditions. The sample is then cultured using the same methods as with blood. The shortcomings with this technique are the pain caused and the risk of infection and excessive bleeding (Vallenas *et al.*, 1985).

Enteric fever patients often shed *Salmonella* in their urine (Forsyth, 1990). Urine can thus be used in the diagnosis of carriers and typhoid cases although it is not a reliable method for medical diagnosis as urine cultures are positive in only 20% of all typhoid cases (Thomas, 1988). A minimum of 5ml to a maximum of 20ml of urine are mixed in an equal volume of Selenite F enrichment broth for incubation and subculture as with stool. Incubation of Selenite F cultures at 43°C sometimes may yield more *Salmonellae*.

When other methods have failed, culture of duodenal aspirate, rich in bile has been successful in detecting *S. typhi*. A kit is used to sample duodenal mucus with a nylon string (Gilman *et al*. 1979). The technique uses a string with a weighted gelatin capsule and a tape at the other end, which is attached to the cheek. The capsule is swallowed and pulled out after a meal (4 hr after), or after enough of a duodenal sample has been collected. Mucus is scraped from the string and cultured for *S. typhi*. An alkaline pH of the string confirms that it was located in the small bowel. Alternatively, a flexible endoscope is introduced through the mouth to the small intestine and a small amount of the duodenal contents is withdrawn through the endoscope. The sample is then incubated in Selenite F broth for further subculture as described for stool culture.

After isolation of bacterial colonies, subsequent identification of *S. typhi* is done in two steps. The first involves the identification of colonies by agglutination with specific antisera, followed by further confirmation by biochemical identification tests (WHO, 2003).

The physical characteristics of *S. typhi* colonies depend on the agar that is used for subculture. On blood agar, *S. typhi* usually produces non-haemolytic smooth white colonies while it normally produces smooth lactose non-fermenting colonies on MacKonkey agar. On SS and desoxycholate agar, *Salmonellae* usually produce non lactose-fermenting colonies with black centres. On Xylose-lysine-desoxycholate agar, it produces transparent red colonies with black centres and appears as transparent green colonies with black centres on Hektoen enteric agar. With Bismuth Sulphite agar, black colonies are produced (WHO, 2003).

Salmonella typhi exhibits particular biochemical properties, which are used to differentiate it from other *Salmonella* species and Enterobacteriaceae. After subculture, and when the Gram staining method has identified the organism as a gram-negative rod or bacillus, biochemical tests are performed to confirm the identity of the organism.

Salmonella typhi is positive when subjected to the motility test and negative with the urease, indole formation and citrate utilisation tests. However, the strains may be slow in reacting during the motility test. Using Triple Sugar Iron (TSI) medium as an indicator, acid in the butt of the medium (yellow colouration) in the test tube indicates fermentation of glucose, acid in the slope indicates fermentation of lactose and/or sucrose. The production of H_2S may occur in minimal quantities. Further biochemical characteristics of *Salmonellae* are as follows; *Salmonellae* produce acid from dulcitol, fail to grow in citrate and potassium cyanide media and are unable to liquefy gelatin, but bring about the decarboxylation of lysine and not ornithine. *Salmonella* is malonate, o-Nitrophenyl-D-galactopyranoside (ONPG) and Na_2HPO_4 negative (Collin and Lyne, 1985).

Paper strips, disc methods and test kits, which have the advantage of speed and savings on labour, are replacing most of the conventional biochemical tests. Kits do not give the user the choice of tests, nor require the judgement of the user; instead, numeric charts and computers are used to interpret the results. In the case of *Salmonella* where final identification depends on serology, a few biochemical screening tests are enough for one to arrive at a reliable result. Kits are the preferred testing methods when unusual organisms are being identified or epidemiological identification is being done (Collin and Lyne, 1985).

Salmonellae can also be characterised according to their somatic (O) or flagellar (H) antigens. These flagellar antigens exist in phases I and II. Phase I antigens are identified by lower-case letters and those in phase II are given Arabic numerals 1-7. Some *S. typhi* organisms and other species also have an envelope antigen for virulence (Vi). This is a surface antigen, which masks the O antigen. When present, the organisms may not agglutinate with O sera unless the suspension is boiled for ten minutes. There are over 60 somatic (O) antigens and these are in specific groups. Antigens 1-50 are distributed between Groups A-Z. Subsequent groups are 50-61 (Collin and Lyne, 1985). Table I below is an illustration of the above naming system, which was initiated by Kauffmann and White and is commonly called the Kauffmann-White scheme.

1.2.7.2 Immunological-based diagnositic methods

1.2.7.2.1 The Widal test

The Widal test is a reaction for the detection of antibodies that appear during the course of enteric fever and other *Salmonella* infections (Thomas, 1988). The principle of the test is the ability of antibodies in the patients' serum to agglutinate bacterial antigens, which are prepared from a culture suspension and are sometimes stained to aid visualisation. Agglutinins to the somatic (O) antigens develop later in the illness and their numbers reduce slowly as recovery takes place, while those to flagellar (H) antigens rise early and persist (Schroeder, 1968). The test is available commercially as a kit.

The Widal test has been used in the detection of prevalent antibodies against enteric fever agents. It has also been useful in comparing paired sera to look for specific rises in antibody levels or to test a single serum taken on admission to the hospital in the hope that significant or abnormally high antibody levels would already have been reached (Mirza,1995).

Table 1. Antigenic structure of three of the common enteric fever-causing *Salmonellae*, identified using the Kauffman-White classification scheme.

Salmonella serotype group	Name	Somatic (O) antigen	Flagellar (H) antigens	
			Phase I	*Phase II*
D	*S. typhi*	9, 12, Vi	d	
A	*S. paratyphi A*	1, 2, 12	a	
B	*S. paratyphi B*	1, 4, 5, 12	b	1, 2

[Source; Collin and Lyne, 1985].

One Widal test on a single sample on admission at the hospital only provides a late diagnosis and is sometimes modified by treatment while conducting the test on samples taken within a one week interval can be relied on in part, especially if the basal antibody levels of the general normal population are known.

The H titre levels are non-specific and variable, and they remain high for a long time, even years following infection or immunisation against typhoid fever. It is therefore of little or no value in the diagnosis of typhoid fever. A rise in titre levels of the O antibody, on two specimens taken within a two-week interval during the first two to three weeks of illness can be used for diagnosis. Unfortunately, this is sometimes impossible due to early treatment with antibiotics or the unavailability of the patient (Mirza, 1995). The results of a single serum sample therefore are considered as sufficient by some to indicate infection in the clinical setting (Shehabi, 1981). There is a limitation to the specificity of this test because of the wide distribution of somatic antigens among a variety of related organisms. *Salmonella typhi* shares O and H antigens with other *Salmonella* serotypes and has cross-reacting epitopes with other Enterobacteriaceae (WHO, 2003). Furthermore, there are patients who fail to develop the antibody levels detectable by the Widal test (Bhaskaram *et al.*, 1990) and there is also evidence that among culture proven typhoid cases, the test failed to detect antibody against the O and H antigens (Gardener, 1937).

The Widal test is also not specific because antibodies from previous stimuli confuse results. Antibodies against *Salmonella* infections have been detected among non-typhoid cases (Schroeder, 1968) and even in diseases not caused by *Salmonella*, like malaria (Sansone *et al.*, 1972). Discrepancies between results from the same laboratory have been reported especially when antigen preparations from different sources were used (Wicks *et*

al., 1971). The general conclusion is that the Widal test is unreliable even in non-endemic areas (Koeleman *et al.*, 1992). On the other hand, it has been found to be useful by some for diagnosing cases in children, because of a low prevalence of pre-existing and cross-reacting antibodies (Choo *et al.*, 1993).

1.2.7.2.2 Countercurrent-immunoelectrophoresis (CIE)

Methods developed by Tsang and Cahu (1981) and Parker (1990), have been used for typhoid diagnosis based on the principle that most bacterial antigens are negatively charged in a slightly alkaline environment, whereas antibodies are neutral. Solutions of antibody and sample fluid are analysed by electrophoresis in an agarose gel. At some point between the loading wells, a zone of equivalence occurs and the antigen-antibody complexes form a visible precipitin band. The entire procedure usually takes about an hour. Bands are often difficult to see and the agarose gel may require overnight washing in distilled water to remove non-specific precipitin residues. The use of positive and negative controls in this process is very important, since sera may contain non-specific reacting agents that form non-stable complexes in the gel. CIE is more expensive than Widal because of the initial capital outlay and the large quantities of antigen and antibody needed.

1.2.7.2.3 Haemagglutination method

This involves the use of red blood cells labelled (passively adsorbed or chemically coupled) with bacterial antigens (Coovadia *et al.*, 1986). Agglutination of the red blood cells takes place when serially diluted serum containing bacterial antibodies is reacted with the labelled red blood cells. Coovadia *et al.* (1986), used sheep red blood cells, which were sensitised with *S. typhi* lipopolysaccharide, for agglutination with serum sample. They found the process to be comparable to the Widal test but more economical,

rapid and simple. However, sensitising the red blood cells with the bacterial lipopolysaccharide is inconvenient and impractical.

1.2.7.2.4 Radio immunoassay (RIA)

With this method, radioactively labelled antibody is made to compete with the patients' unlabelled antibody for binding to a known amount of antigen (Chau *et al.*, 1981). A reduction in radioactivity of the antigen-patient antibody complex, compared with the radioactive counts measured in a control test with no antibody is used to quantify the amount of patient antibody bound to the antigen. Radioactivity is unsuitable for routine use and therefore, has not been developed for typhoid fever diagnosis. Instead enzyme and substrate systems, chemiluminescence and fluorescence are new marker methods that will make possible the development of tests as sensitive as RIA without the hazards of radioactive agents

1.2.7.2.5 Enzyme linked immunosorbent assays (ELISA)

ELISA kits are available commercially for *S. typhi* antibody detection. Testing for immunoglobulin G (IgG), IgA, and IgM anti lipopolysaccharide (LPS) of *S. typhi* antibodies by ELISA showed that the levels of all three classes of immunoglobulin anti-LPS of *S. typhi* were higher in typhoid patients than in healthy or febrile non typhoidal people (Mekara *et al.*, 1990). The ELISA assay is much more sensitive and specific than any combination of the Widal test, and hence it can be a useful tool for the serological diagnosis of typhoid fever with a single blood sample (Quiroga *et al.*, 1992). Its only drawback is that it involves several steps and is time consuming.

1.2.7.2.6 The Typhidot test

A dot enzyme immunoassay (dot EIA) has been developed to detect a 50-52kDa protein, specific to *S. typhi*, which reacts immunologically with only typhoid sera (Ismail *et al.*, 1991). In further tests in clinical settings, it proved to be simple, fast (1-3 hr), sensitive (95%), specific (75%) and to have high negative and positive predictive values. Its shortcoming is that IgG has a masking effect on IgM, the detection of which suggests acute typhoid infection. An improved version, the Typhidot-M has been developed to detect only specific IgM antibodies (WHO, 2003).

During evaluation of Typhidot and Typhidot-M in Pakistan (Bhutta and Mansurali, 1999), both tests were shown to be more sensitive at 70% and 73% respectively, compared with 65% and 55% for culture and Widal. They also have a higher negative predictive value and give quicker results, compared to Widal and culture tests (Choo *et al.*, 1998). Although Typhidot and Typhidot-M have an advantage over other serologic diagnostic tests in terms of specificity, sensitivity and positive predictive value, the higher cost of the test compared to Widal and the negative effect of regional differences, make it important that it be evaluated further to assess its suitability as a routine test in developing countries.

1.2.7.2.7 Tubex test

The Tubex test is a simple (one step), rapid (two minutes), method for the detection of typhoid specific antibodies, which takes advantage of the simplicity and user friendliness of the Widal and slide latex agglutination tests and in addition, makes use of the separation of coloured particles in solution to improve resolution and sensitivity (WHO, 2003). The antigen of interest is derived from the lipopolysaccharide (LPS) of *Salmonella*, while the tubex assay format is based on a highly sensitive and specific ELISA typhoid test, which was developed 15 years ago using the same antigen and

antibody reagents (Lim and Ho, 1983; Lim, 1986). The Tubex test is available as a modern kit, marketed under the same name, by IDL Biotech, (Sollentuna, Sweden).

The result of the test is read visually, based on the colour of the contents in the tube. The interpretation of results is done by giving arbitrarily fixed scores to the colour of the liquid in the tube. Red is given the score 0 which is the most negative result. The colour blue is the most positive result and is given the score 10. Intermediate colours based on increasing degrees of redness or blueness are given corresponding scores of 2, 4, 6, and 8. Odd numbers are given to intermediate colours where appropriate. This diagnostic method can only be used as a simple and rapid aid to the routine diagnosis of typhoid fever, either in the hospital or at the bedside.

1.2.7.2.8 IgM dipstick test

This test is used to identify *S. typhi* specific IgM antibodies in whole blood or serum samples. Any *S. typhi* specific IgM antibodies in the test sample are bound to LPS antigens along a strip on a dipstick and further stained by an anti-human IgM antibody conjugated to colloidal dye particles. A control line also on each stick is made up of anti-human IgM antibodies (WHO, 2003). The test result is read by visualisation of the intensity of the stain on the antigen strip, which is compared with the intensity of a reference strip. When no staining is observed the result is read as negative and scores of 1+, 2+, 3+ or 4+ are given to weak, moderate, moderate-strong and strong stains, respectively.

The dipstick test has been evaluated in laboratory-based studies in Egypt and Indonesia, among other countries (Ismail *et al.*, 2002; Gasem *et al.*, 2002). The studies showed 65 to 77% sensitivity and 95 to 100% specificity on culture confirmed cases at the time of first

consultation. The method has the advantage of speed and simplicity. It does not require electricity, specialised training, expertise or equipment and the reagents do not need cold storage. It holds the promise for rapid diagnosis of typhoid, especially in areas where culture facilities are not available (WHO, 2003).

1.2.7.3 Direct detection methods

1.2.7.3.1 DNA hybridisation probe technique

A DNA hybridisation probe to identify typhoid from specimens of blood has been used with some degree of success (Rubin *et al.*, 1989). The probe is specific to the Vi antigen of *S. typhi*; however, this method requires that bacteria in the blood be concentrated by lysis and centrifugation. Also, total bacterial DNA needs to be amplified by overnight incubation of the organisms on nylon filters. The concentration and amplification processes are necessary because patients with typhoid fever usually have less than 15 *S. typhi* cells per millilitre of blood and the probe cannot detect fewer than 500 bacteria. The probe technique has the disadvantage of being slow and not sensitive enough.

All the above biochemical and molecular methods have been considered as alternative serological tests to the Widal method. Quite a few of them are better than the others in terms of specificity and sensitivity (Rai *et al.*, 1989). Most of them however, have not received widespread acceptance in microbiological laboratories probably because of costs, equipment and the expertise required. The search for an ideal test is much more of an issue in developing nations where typhoid fever is still endemic and very much a public health problem. Recent developments have provided more practical methods, which are available commercially.

1.2.7.3.2 Polymerase Chain Reaction and primer design methods

Polymerase chain reaction (PCR) has been used on clinical specimens for typhoid diagnosis (Rahn *et al.*, 1992, Song, *et al.*, 1993, Hashimoto *et al.*, 1995). Different sets of primers have been designed to suit the specific need of each experiment. The DNA sequence targeted by Song *et al.* (1993) and modified by Frankel *et al.* (1994) have proved useful in this investigation because they code for the flagellin gene of *S. typhi*, in the hypervariable region VI that is unique to *S. typhi*.

PCR is an *in vitro* method of nucleic acid synthesis, by which a target DNA fragment is replicated (Erlich, 1989). It is a highly sensitive method, which is used to detect very minimal quantities of bacterial DNA in clinical samples. This property is an advantage which has the potential of setting PCR above all other diagnostic tests, knowing that patients with typhoid fever usually have less than 15 *S. typhi* cells per millilitre of blood (Song *et al.*, 1993). Thermostable *Taq* polymerase, a DNA enzyme isolated from the bacterium *Thermus aquaticus*, is used for *in vitro* synthesis of DNA. The enzyme is found in thermal hot springs and is able to withstand high temperatures without being degraded (Erlich, 1989).

The method involves three main steps, namely denaturation of duplex DNA, annealing of synthetic oligonucleotide primers to homologous regions of the DNA template and extension of the DNA primer, with dNTPs by *Taq* polymerase. At the denaturation stage, the double stranded DNA is heated to about 92-96°C and forms single strands. Then, the forward and reverse primers oriented with the 3' ends pointing towards each other, anneal to the DNA single strands, flanking the fragment of interest. Primers are short oligonucleotides usually 15 to 25 base pairs in length that match complimentary sequences adjacent to the fragment to be amplified. The annealing temperature is usually

5°C lower than the melting temperatures (Tm) of the primers and are determined using the following formula (Thein and Wallace, 1986);

Tm= 2 (A+T) + 4 (C+G) °C

(Where A = Number of Adenines, G = Number of Guanines, C = Number of Cytosines, T =Number of Thymines and Tm is the melting temperature)

At the extension step, primer elongation takes place and a new DNA strand is synthesised by *Taq* polymerase as A matches with T and C matches with G. Thus, a cycle results in two double stranded DNA fragments and with each additional cycle, the number of double stranded DNA fragments rises exponentially. The number of cycles is usually 28 to 35 and PCR is carried out in a thermal reactor into which the cycling conditions are programmed. Each reaction contains 1X PCR buffer, deoxyribonucleotide triphosphates (dNTPs), forward and reverse primers, DNA *Taq* Polymerase and the template DNA in a final volume made up of sterile double distilled water. The standard PCR amplification protocol amplifies most target DNA, but the reaction conditions are optimised to suit each application (Innis and Gelfand, 1990).

Several factors are taken into consideration when designing primers, in order to produce the required product and the desired yield. The primer length is of primary importance because specificity and annealing temperatures are partly dependent on primer length. There is a 0.25 chance of finding an A, G, C or T in any given DNA sequence, a 1/16 chance of finding any dinucleotide sequence and a 1/256 chance of finding a given 4 base sequence. Therefore, a sixteen base sequence will be present once in every 416 bases for a genome size of four billion base pairs. Thus, the association of a greater than 17 base oligonucleotide with its target sequence is a very sequence specific process (Rybicki,

1996). Oligonucleotides between 18 and 24 bases are sequence specific as long as the annealing temperature is optimal. A primer also should be complex enough so that it will not anneal to sequences other than the desired one.

The Tm of a nucleic acid duplex increases with both its length and increasing G and C content. The annealing temperature of a PCR reaction is thus set at approximately 5°C less than the lowest melting temperature of the primers, which have to be designed to have similar Tms (Innis and Gelfand, 1990). Too low a melting temperature will result in non-specific amplification, which is caused by one or both primers annealing to sequences other than the target sequence, resulting in multiple bands in an agarose gel. Optimally, the annealing temperature of a pair of primers should be between 55°C and 72°C.

Primers should also be designed to anneal to a unique sequence within the template DNA. A primer with a repetitive sequence will produce a smear after amplification. Setting up a PCR with primers containing bases that are homologous to each other or with a pair of primers that are homologous to each other will result in a partial double structure by a single primer or primer dimer, by the homologous primer pair. The former would interfere with annealing and the latter would prevent the formation of the product through competition. An efficient primer will also not have polypurine (A and G) or polypyrimidine (C and T) sequences within it; a 50% G and C content is adequate for a primer that is approximately 20 base pairs long. Mispriming is avoided if there is a G or C residue at the 3' end of primers. This "G, C Clamp" ensures correct binding at the 3' end due to the stronger hydrogen bonding of G/C residues (Dieffenbach *et al.*, 1995).

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CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials and Reagents

The oligonucleotide primers were obtained from Bioserve Biotechnologies, Laurel, USA. The PCR core kit was obtained from Roche Diagnostics Mannheim, Germany. The *Salmonella* antiserum kit was obtained from Denka Seiken, Tokyo, Japan. The 100 base pair molecular weight marker was obtained from Invitrogen Technologies, Frederick, MD, USA.

Saponin, silica gel, absolute ethanol, mineral oil, chelex-100, ficoll, molecular grade agarose, methanol, tris (hydroxymethyl) aminomethane, ethylene diamine tetraacetate disodium salt, ethydium bromide, potassium acetate, sucrose, SDS and restriction endonucleases were obtained from Sigma-Aldrich Chemie GmbH, Germany. Orange G dye was obtained from Kanto Chemical Co. Inc., Tokyo, Japan.

The Widal test kit was obtained from Omega Diagnostics Limited, Scotland, UK. The API 20E bacterial identification kit was obtained from bioMerieux SA, Marcy l'etoile, France. Bacterial culture agar and broth bases were obtained from Oxoid Limited, Hampshire, England.

2.1.1 Biological specimens

The *E. coli* strain O157:H7 was obtained courtesy Dr Aiah Gbakima, Morgan State University, USA. Isolates of *E. coli*, *Streptococcus* and *Bacillus subtilis* were obtained from the Department of Nutrition and Food Science, University of Ghana and isolates of

Staphylococcus aureus were obtained from Patholab Medical Laboratories (Adabraka, Accra).

To avoid contamination, different sets of pipettes and aerosol barrier tips were used for the cultures, DNA extraction and PCR. Where required, all the reagents were autoclaved (Eycla autoclave, Rikikakki, Tokyo) for 15min at 121lb/sq in.

2.2 Methods

2.2.1 Isolation of bacterial DNA

Bacterial isolates of *E. coli*, *Streptococcus*, *Bacillus subtilis* and *Staphylococcus aureus* were prepared for subculture and confirmatory identification and finally for DNA extraction. Each bacterial strain was streaked onto separate MacKonkey and blood agar plates and incubated for 18h or overnight at 37°C. Single isolated colonies were picked from each plate and their identity confirmed, using Gram stain and the API 20E bacterial identification kit. *S. typhi* isolates obtained from stool, blood and urine samples at the Korle-Bu Teaching Hospital and the Noguchi Memorial Institute for Medical Research were subcultured by streaking on MacKonkey, blood and Salmonella/Shigella agar. The *S. typhi* isolates were then identified by Gram stain, biochemical identification and serological methods according to Cheesbrough, (1984).

After positive confirmation of the identity of all the organisms, each isolate was subcultured on MacKonkey and blood agar plates again. A single colony was picked from each isolate and inoculated individually into separate, sterile 10ml LB medium in 100ml conical flasks. The bacterial suspensions were then incubated for 18hr at 37°C, with vigorous shaking. After overnight growth, the bacterial cells were harvested by

decanting into 15ml falcon tubes, and centrifuging at 3,000g for 10min to pellet the cells. The supernatants were then discarded.

Each pellet was resuspended in 1ml of Bender buffer (0.1M NaCl, 0.2M Sucrose, 0.1M Tris-HCl, 0.05M EDTA pH 8.0 and 0.5% SDS), mixed gently and incubated at 65°C for 30min, to lyse the bacterial cell walls. After lysis, 150µl of ice-cold 8M Potassium acetate (KAc) solution were added and incubated on ice for 5min followed by centrifugation at 21,920g for 10min. The supernatant was transferred into a fresh tube; 2X its volume of ice-cold ethanol was added and the contents mixed by rapid tube inversion and incubated for 14h at -50°C. Next, centrifugation at 21,920g for 10min was carried out and the supernatant removed completely by aspiration. The pellet was washed with 500µl of 70% ethanol in TE buffer (pH 7.5) by centrifugation for 5sec at 21,920g and the supernatant decanted. It was then further suspended in 200µl TE buffer, 400µl of phenol/chloroform added, mixed by rapid tube inversion and spun for 5min at 21,920g. The aqueous supernatant was transferred into a fresh tube, 90µl of ice-cold 8M KAc and 2X the volume of absolute ethanol were added. The tube, with its contents, was incubated at -50°C for 1hr and spun at 21,920g for 10min. Finally, the supernatant was decanted and the pellet rinsed with 70% ethanol before dissolving in 100µl of TE+RNase and stored at -50°C until ready to use.

2.2.2 Sample collection and processing

Blood samples from consenting febrile patients attending the University of Ghana Hospital were used for this study. The hospital is mainly patronised by University staff and their relatives, students and residents of the Accra metropolis and its environs. Upon clinical examination by a medical officer, patients suspected of having typhoid fever were recruited. The common clinical signs and symptoms used to identify typhoid patients

were fever, temperatures of 37°C and above, fatigue, headache, anorexia, joint and abdominal pains, vomiting, cough, diarrhoea, pallor, and abdominal tenderness. Approximately 4-5ml of venous blood were taken from each patient and used for the culture, Widal test, thick and thin blood films and filter paper blood blots. The blood films were Giemsa stained and read for malaria parasites at the University Hospital.

Approximately half (2-2.5ml) of the blood that was taken was used immediately for the Widal test and the blood film on location. One milliliter of blood was transferred into 9ml of sterile Tryptic Soy Broth (TSB) in test tubes, and the remainder put into sterile EDTA tubes for filter paper blood blotting. The blood in the EDTA tubes was transferred to the laboratory and blotted on pre-labelled filter paper. Blots were allowed to dry completely before the filter papers were placed in individual zip-lock bags containing silica gel and stored at -50°C until ready to use for DNA extraction and PCR. Sample collection was done over a period of five months, from June to October 2003.

2.2.3 Blood culture

The blood in the TSB culture tubes was incubated at 37°C in an aerobic oven and subcultured after 24 hours on plates of blood and MacKonkey agar. Sub culture plates were incubated in an aerobic oven at the same temperature. On days 3 and 7 subculture from the TSB broth was repeated. Colonies on each plate were identified by Gram stain, and biochemical tests, using the API 20E bacterial identification kit.

In the Gram stain test, a bacterial colony or part of it was picked off the agar plate with a loop and emulsified in a drop of 0.9% (w/v) NaCl/sterile double distilled water on a clean slide. This was then allowed to dry completely at room temperature before being used for the Gram stain. The slide on a rack was flooded with crystal violet and allowed to stand

for 1min before rinsing copiously for 5 sec with distilled water. Next, it was flooded with iodine solution for 1min and rinsed with distilled water for 5 sec, followed by a destaining treatment during which ethanol/acetone (50:50 v/ v) was dropped on the smear until a blue-violet colour stopped leeching out. The smear was rinsed again with distilled water for 5sec and counter stained by flooding with 0.1% basic fuchsin for 1min and rinsing copiously with distilled water for 5s. The slide was then allowed to dry at room temperature and examined under a drop of mineral oil at 100X with a compound microscope.

The isolated colonies that were identified by Gram stain to be gram-negative rods, were inoculated into a triple sugar iron (TSI) slope in a test tube, and incubated at 37°C for 18hr (overnight). *Salmonella typhi* positive isolates caused the formation of a red colouration of the agar slope indicating non-utilisation of lactose/sucrose. A yellow colouration of the agar butt in the test tube was and indication of utilisation of glucose. Also, the *S. typhi* positive isolates were not expected to produce H_2S.

The *S. typhi* isolates were then further confirmed by serology. Colonies were picked off the agar plate with an inoculation loop and emulsified on a slide with a drop (approximately 10µl) of 9% (w/v) saline solution. Drops (approximately 10µl) of antigen suspension representative of each known antigen type were mixed with an equal volume of the fresh emulsion. In *S. typhi* positive isolates, an agglutination reaction was observed with antigen compositions nine, twelve and Vi in the somatic (O) antigen group and antigen composition d in phase II of the (H) or flagellar antigen group.

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2.2.4 Widal test

The antigen kit for Widal and Weil-Felix tests was used, according to the manufacturers' instructions. A rapid slide test was performed first and the samples in which agglutination had taken place at titres of 1:160 were subjected to the tube agglutination test, for confirmation of slide results. Samples in the tubes in which agglutination was observed for both the H and O antigens at titres of 1:160 were considered as Widal positive.

2.2.5 Isolation of *S. typhi* DNA from filter paper blood blots

The Chelex-100 method described by Wooden *et al.*, (1993) was used for *S. typhi* DNA isolation from filter paper blood blots. A piece of the blot ($1.5cm^3$) was cut out using sterile scissors and placed in 1.5ml Eppendorf tubes. Then 1ml of 1X PBS (pH 7.4) and 100µl of 10% saponin were added. The contents of the tubes were mixed by inversion, incubated for 18h (overnight) at room temperature, and then centrifuged at 21,920g for 5s. The reddish PBS/saponin solution was discarded by aspiration. An aliquot of 1ml of PBS was added to the filter paper in the tube; the contents of the tube were mixed by inversion and incubated at room temperature for 15-30min. The contents of the tube were aspirated and discarded after spinning at 21,920g for 5s, then 50µl of sterile double distilled H_2O and the same volume of 20% Chelex solution were added to each filter paper in the tubes. In order to extract DNA from the filter papers the contents of each tube were incubated at 95°C for 10min, with vortexing at 2min intervals. Finally, the tubes were centrifuged at 21,920g for 5min to pellet the debris and the supernatant was transferred into fresh sterile 1.5ml Eppendorf tubes, making sure that the Chelex was not carried over. The supernatants containing the bacterial DNA were stored at -20°C until ready to use for PCR.

2.2.6 Primer design and optimisation of PCR conditions

Primers were designed by first aligning the DNA sequences of *S. typhi* and *S. typhimurium* [Appendix I]. Both sequences were obtained from the NCBI DNA sequence database, GenBank (Accession numbers L21912 and M33808 respectively) and aligned using the Align X program, which is included in the DNA and protein analysis software package, vector NTI suite 6 (InforMax, Inc.). A dot plot of the two sequences was also done using the NCBI Blast 2 software (Appendix II). The consensus sequences in the alignment map were then used to design two pairs of primers for the nested amplification of a fragment of the *S. typhi* flagellin gene. The first primer pair was designed to amplify the fragment between nucleotides 446 to 1317 (Appendix I). The pair was given the names "Sal. Gen F" (forward) and "Sal. Gen R" (reverse) and was expected to amplify an 872bp fragment of the flagellin DNA of *Salmonella typhi* and an 852bp fragment of *Salmonella typhimurium*. The second primer pair was designed to amplify the region between nucleotides 520 and 996 of the *S. typhi* flagellin gene to give a 477bp PCR product. This second pair was named "*S. typhi* flag F" for the forward primer and "*S. typhi* flag R" for the reverse primer.

After the primers had been designed (See table 2, pg 51 for details), their sequences were used to search for homologous bacterial DNA sequences in the NCBI genomic database, using the BLAST software (Altschul *et al.*, 1990) [See appendix III]. The BLAST analysis was done as a preliminary confirmatory test of the specificity of the primer pair before they were ordered from Bioserve Biotechnologies (USA) for use. The primers were then used in PCR test runs, to optimise the reaction conditions.

To optimise the reaction conditions, the annealing temperature was set at 5°C below the lowest Tm of the primer pair. Using the same PCR reagent concentrations, the reaction

was repeated, sequentially increasing the annealing temperature until a sharp, clear band of the expected size was observed upon electrophoresis. After determining the annealing temperature, the concentrations of the other reaction components namely, $MgCl_2$, primers, dNTPs and template were also individually titrated, whilst keeping the others constant. This was done until optimal conditions were obtained.

The first PCR reaction mix of 25µl contained 1X PCR buffer (10mM Tris/HCl, 1.5mM $MgCl_2$, and 50mM KCl), 100µM each of the four deoxyribonucleotide triphosphates (dNTPs) and 0.625 units of *Taq* polymerase enzyme. In addition, 1µM of each oligonucleotide primer, Sal gen F and Sal gen R and 0.5µl of the template were used. Sterile double distilled H_2O was added to make up the final volume. The reaction mix was spun down briefly at 21,920g in a microcentrifuge and overlaid with mineral oil to prevent evaporation and refluxing during the cycling reaction.

The cycling parameters for the outer PCR reaction with the *Salmonella* genus specific primers were set at 94°C for 2min (initial denaturation), followed by 35 cycles of 94°C for 1min, 59°C for 1min (annealing), 72°C for 2min (extension) and ended with a final single extension cycle at 72°C for 5min. For the second PCR reaction using the species-specific primers *S. typhi* flag F and R, the same reaction mix concentrations were used and 0.5µl of the outer PCR product used as DNA template. Cycling parameters were set at 94°C for 2min (initial denaturation), followed by 30 cycles of 94°C for 1min, 60°C for 1min (annealing), 72°C for 2min (extension) and a single extension cycle at 72°C for 5min to end the reaction. For each PCR reaction, both positive and negative controls containing DNA of *S. typhi* culture and sterile double distilled water respectively were run alongside.

An aliquot (8µl) of each PCR product was mixed with 2µl Orange G gel loading dye (5X), loaded into individual wells of a 2% agarose gel and electrophoresed in a 1X TAE buffer at 80V for 1h30min using a midi gel system. The gels were stained with 0.5µg/ml of ethidium bromide solution, visualised and photographed over a UPC dual intensity UV transilluminator (USA) at short wavelength using a Polaroid camera with a type 667 film (Polaroid, USA). The sizes of the PCR products were estimated by comparison with the mobility of a standard 100bp marker.

Table 2 DNA sequences of the synthetic oligonucleotide primers designed to amplify the flagellin gene of *Salmonella typhi*.

Name of primer	Sequence detail (5'-3')	Melting temperature (°C)	Expected size of amplified DNA (bp)
Sal gen (Forward)	GTGCCAACGACGGTGAAAC (19mer)	60	874
Sal gen (Reverse)	CGCACCCAGGTCAGAACG (18mer)	60	
S.typhi flag (Forward)	AATGTCCAAGATGCCTACACCC (22mer)	66	477
S. typhi flag (Reverse)	GCCACCATCAATAACCTTACCG (22mer)	66	

2.2.7 Determination of primer specificity

Two approaches were used to determine the specificity of the primers.

i. The primers were used in a PCR to see if they would amplify four common bacteria.

ii. An experimental restriction digest was done on the PCR product of *Salmonella typhi* DNA and the digest results were compared with those of an in-silico restriction analysis.

In the first approach, DNA was extracted from *Salmonella typhi, Staphylococcus aureus, Escherichia coli, Streptococcus,* and *Bacillus subtilis* and then used as templates in nested PCR reactions using the primers. The PCR products were then resolved by agarose gel electrophoresis for one hour and thirty minutes using a 2% gel stained in ethidium bromide, visualised under UV and photographed.

In the second approach, virtual restriction digestion was done. The complete DNA sequence of *S. typhi* flagellin gene (Accession number L21912) was retrieved from GenBank and copied into the "Jellyfish" version 1.1 software (www.biowire.com). The FIND function of this program was used to align the primer pairs with the gene sequence. The region flanked by the primers was copied, saved as a new file and then imported into DIGEST version 1.0 software (Nakisa, 1993). *In silico* analysis was then performed using the enzymes in its database WISCONSI.920. The output included the positions of the DNA sequences that each enzyme cut, the number of fragments and their sizes. It also listed all enzymes that do not restrict.

Results obtained from the *in silico* analyses, made possible the selection of an enzyme that cuts at a unique site in the middle of the fragment and another, which had no recognition site in the fragment, for restriction analysis. *Dra*I, with the recognition site; TTT↓AAA was selected to cleave the inner PCR product, giving two fragments of 315 and 162 base pairs. *Eco*RI was selected because it has no recognition site in the fragment of interest. The final reaction volume of 20µl contained 4µl of the amplified products and

was carried out following the manufacturers' recommendations at 37°C for 2 hours using the PTC thermocycler. The products were electrophoresed in ethidium bromide-stained 2% agarose gels in 1X TAE buffer and visualised under UV transillumination. A standard 100 bp ladder was used as molecular weight marker to estimate the PCR product sizes.

2.2.8 Determination of the threshold of the PCR method

Decreasing concentrations of bacterial colony forming units (CFU) in unit volumes of blood were prepared, using two different approaches.

i. *Salmonella typhi* culture suspensions were serially diluted and seeded into sterile blood to make up unit volumes of seeded blood.

ii. Sterile blood was seeded with bacteria and then serially diluted to give unit volumes of seeded blood.

The two sets of seeded blood were blotted on individually labelled filter papers and dried. The bacterial load in each dilution of either method was determined as outlined below.

In the first method, peptone (900µl) solution was pipetted into five individual sterile 1.5ml Eppendorf tubes that had been labelled one to five. Then a 2ml aliquot of *S. typhi* suspension was diluted with an equal volume of sterile LB broth. The suspension was mixed by gentle swirling and 100µl transferred into the first tube. Another fresh pipette tip was used to mix the contents of tube one and 100µl of the suspension were transferred to the next dilution blank. The contents of tube two were mixed with a fresh pipette tip and 100µl transferred to the dilution blank in tube three. Serial dilutions were carried out to give a final dilution of 10^{-5} in tube 5.

500µl of the serially diluted *S. typhi* suspensions were mixed with an equal volume of *Salmonella*-free blood in sterile 1.5ml Eppendorf tubes, and 40µl of each blood/*Salmonella* mix blotted on individually labelled filter papers, allowed to dry at room temperature and stored at –50°C. One fifth of the blots (1.5 cm^3) were later cut with sterile scissors, put in 1.5ml Eppendorf tubes and used for DNA extraction to determine the limits of detection of the PCR method.

In the second method, aliquots of 900µl of uninfected blood were pipetted into six sterile Eppendorf tubes, which were labelled one to six. The volume in the first tube was then made up to 1ml by adding 100µl of the diluted *S. typhi* bacterial culture broth then; the contents of the tube were mixed by pipetting. Starting from tube one, serial dilutions were carried out until the last tube in which a concentration of 10^{-6} of seeded blood was obtained. The content of each tube was mixed thoroughly by inversion before 20µl of the contents in each tube were blotted onto labelled filter paper. The filter papers were finally left to dry at room temperature, placed in zip-lock bags containing silica gel and stored at -50°C until ready to use for DNA extraction.

After thorough mixing, 100µl of each serial dilution (bacterial culture broth in the first method or seeded blood in the second method) were spread on plate counting agar (PCA) in a petri dish using a different sterile glass spreader for each dish. The plates were then covered and placed in an incubator at 37°C overnight for 18hr. After incubation, a felt tipped pen was used to mark the glass above each colony, which the counter automatically registered as a result of the pressure on the pen. Three replicates were prepared for each dilution. The colony or viable count per ml was calculated and the values were reported as colony forming units /ml (CFU) or viable counts per ml. The plates with more than 300 colonies were divided into either four or eight sectors.

Colonies in one sector were counted and the number of sectors factored into each calculation to determine the number of colony forming units per plate. For colonies that were too numerous, merged and indistinguishable, the plates were discarded.

In order to determine the sensitivity of the PCR reaction, the DNA extracts from the blots described above were used as templates for PCR and the products resolved by agarose gel electrophoresis. Successful amplification, producing the required fragment size of 477bps after PCR was an indication that the concentration of CFUs in that dilution was enough to be detected by nested PCR. By the above means, the threshold values for detection of *S. typhi* in blood samples were determined.

2.2.9 Data analysis

The Chi-square test was used to determine the association between the PCR and Widal results. This analysis was done using EPI INFO 6 software package (CDC, USA).

The Kappa index (K), was also calculated to determine the degree of non random agreement between the two tests, as follows:

i) $Po = \frac{a+d}{a+b+c+d}$

ii) $Pe = \frac{[(a+b)(a+c)]+[(c+d)+(b+d)]}{(a+b+c+d)^2}$

iii) $K = \frac{Po - Pe}{1 - Pe}$

Where P_o is the proportion of agreement observed between the two tests and

P_e is the proportion of agreement between both tests that occur by chance.

CHAPTER THREE

RESULTS

3.1 Clinical Studies

A total of 185 blood samples from febrile patients comprising 88 (47.57%) males and 97 (52.43%) females were used for this study. Of this number, 148 (80%) patients were 21 years and above, comprising 73 (39.46%) females and 75 (40.54%) males. The youngest recorded age was three, while the oldest was 64 years.

3.1.1 Blood culture

A hundred and twenty-two samples were cultured; nine (7.14%) were positive for *Staphylococcus aureus* and one (0.79%) each for *Serratia marcescens* and *Yersinia enterolitica*, but none for *S. typhi*.

3.2.2 Widal test

Of the total of 185 samples, 58 (31.35%) were Widal positive at a titre of 1/160 for both the O and H antigens and 127 (68.65%) samples were Widal negative. Only one Widal positive sample was culture positive for *Staphylococcus* and the other 10 culture positive samples were negative by the Widal test.

3.1.2 Malaria parasite test

Blood films were prepared and read for malaria parasites, with the following results. There were 12 (8.69%) *Plasmodium falciparum* positive blood films among 138 of the 185 samples that were screened by PCR. Among these, there were four (2.90%) Widal

positive samples and no PCR positive sample. No sample was found to be positive by all three tests.

3.1.3 PCR detection of *S. typhi* infections

As expected, the inner primer pairs amplified a DNA fragment of 477bp from extracts of the blood blots. Seven (3.78%) out of the total 185 blood blots that were screened were *S. typhi* PCR positive. However, four (2.16%) out of the 58 Widal positive samples were positive by PCR. Figure 5 is an agarose gel electrophoregram showing PCR products of the amplification of *S. typhi* from one positive control and nine patients, some Widal positive, some not.

Table 3: Grouped demographic data and diagnostic results of the study samples

Age ranges	Number	Total Males	Total Females	Widal Positives	PCR positives	Culture positives	Malaria positives
1-10	8	3	5	4	1	0	0
11-20	29	10	19	10	1	1 *St.*	3
21-30	34	15	19	8	1	5 *St*	1
31-40	17	9	8	2	1	0	0
41-50	10	5	5	1	0	1 *Y.*, 1 *St*	0
51-60	4	3	1	0	0	0	1
Above 60	1	0	1	0	0	1 *Ser.*	0
"Adults"	82	43	39	33	3	0	7
Total	**185**	**88**	97	**58**	7	**9**	**12**

Where St= *Staphylococcus*, Ser=*Serratia*, Y= *Yersinia*. See appendix V for detailed table.

3.1.4 Degree of association between the PCR and Widal methods

Table 4: Table comparing the PCR and Widal diagnostic tests.

	Widal positives	**Widal negatives**	**Total**
PCR positives	4 (a)	3 (b)	7
PCR negatives	54 (c)	124 (d)	**178**
Total	**58**	**127**	**185**

The Chi squared test revealed that there was a significant difference between the diagnosis results obtained by PCR and Widal ($\chi^2 = 48.5$, df = 1, p = 0.0000).

Also, the Kappa index (K) value was calculated to be 0.061

$$K = \frac{0.692 - 0.672}{1 - 0.672} = \frac{0.020}{0.328} = 0.061$$

According to this value, there is no association between both tests.

3.1.5 BLAST analysis

The BLAST search for the forward outer primer (Sal gen F), in the GenBank database, revealed 27 *Salmonella* sequences with the most significant alignments and these had an E or expectation value of 0.002. The search for the reverse (Sal Gen R) inner primer revealed 18 sequences with significant alignments and E values of 2e-04. A total of 17 of the same sequences were found in both outputs, including *S. typhi* and *typhimurium* flagellin genes (See Appendix III a and b).

The GenBank BLAST search of the *S. typhi* specific primer pair revealed 14 sequences that are homologous with the forward (*S. typhi*, F) primer and 15 with the reverse (*S. typhi*, R) primer. The E value of all the matches in both outputs was 2e-04. Furthermore,

12 of the same sequences, including the *S. typhi* flagellin gene were found in both searches.

Salmonella typhi flagellin gene, *Salmonella enterica* subsp. enterica, *Salmonella enterica* serovar Typhi and *Salmonella enterica* subs enterica flagellin (fliC) gene, partial cds, were found in all four BLAST search results.

3.1.6 Restriction digestion of *S. typhi* positive samples

Figure 6 is a representative 2% agarose gel electrophoregram of PCR products digested with *Dra*I. All the seven *S. typhi* PCR positive products and three *S. typhi* positive controls were digested with *Dra*I and all gave the two expected band sizes of 162bp and 314bp respectively. As expected, *Eco*RI did not restrict the amplified DNA products (Figure 7).

3.1.7 Primer specificity

DNA extracts from *E. coli*, *Streptococcus pyogenes*, *Bacillus subtilis*, *Staphylococcus aureus* and three isolates of *S. typhi* from urine, stool and blood were all analysed by PCR. All three of the *S. typhi* isolates were successfully amplified to give the expected band sizes of 872bp for the outer product and 477bp for the inner product. All the other isolates were negative (Figure 8).

3.1.8 Threshold of detection of primers

The detailed colony forming units per serial dilution of seeded blood are given in Appendix IV. Figure 9 is a picture of an agarose gel electrophoregram, of PCR products of DNA extracted from serially diluted seeded blood.

When the blood blots from the first serial dilution method were extracted and screened by PCR, the lowest detection level of the primers was 1.64 CFU/ml of blood. Also, when the blots from the second serial dilution method were extracted and screened by PCR, the lowest detection level was 1.56 CFU/ml of blood.

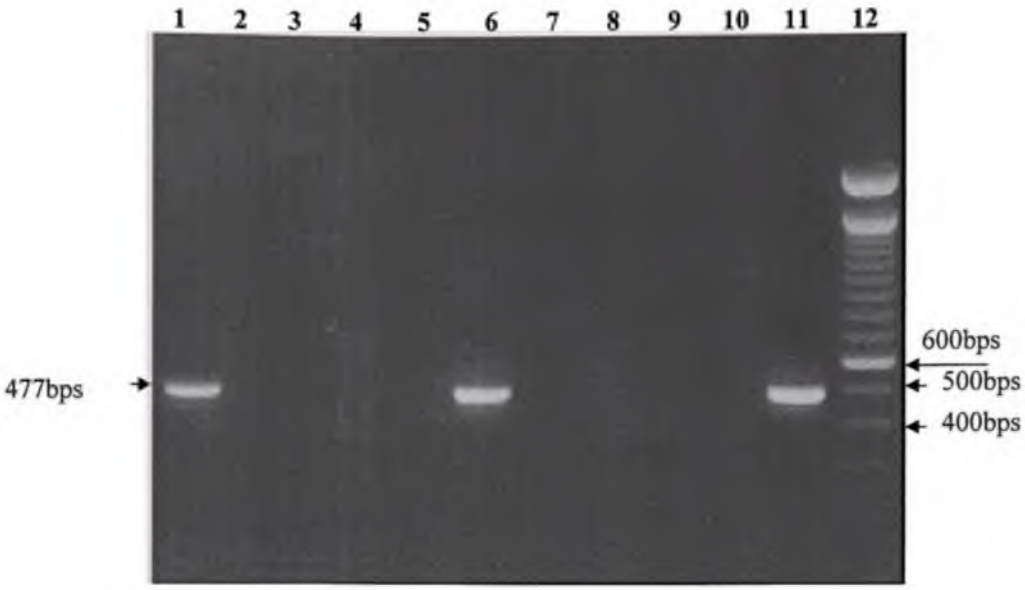

Fig 5 Agarose gel electrophoregram showing PCR products of the amplification of *S. typhi* flagellin gene.

Electrophoresis was run on agarose gel (2%) for 1h 30min at 60V using 1X TAE buffer. Gel was stained with ethidium bromide and viewed under UV transillumination to locate DNA bands

Lanes 1-5 = Widal positive samples

Lanes 6-9 = Widal negative samples

Lane 10 = Negative control

Lane 11 = *S. typhi* positive control.

Lane M = 100bp molecular weight marker.

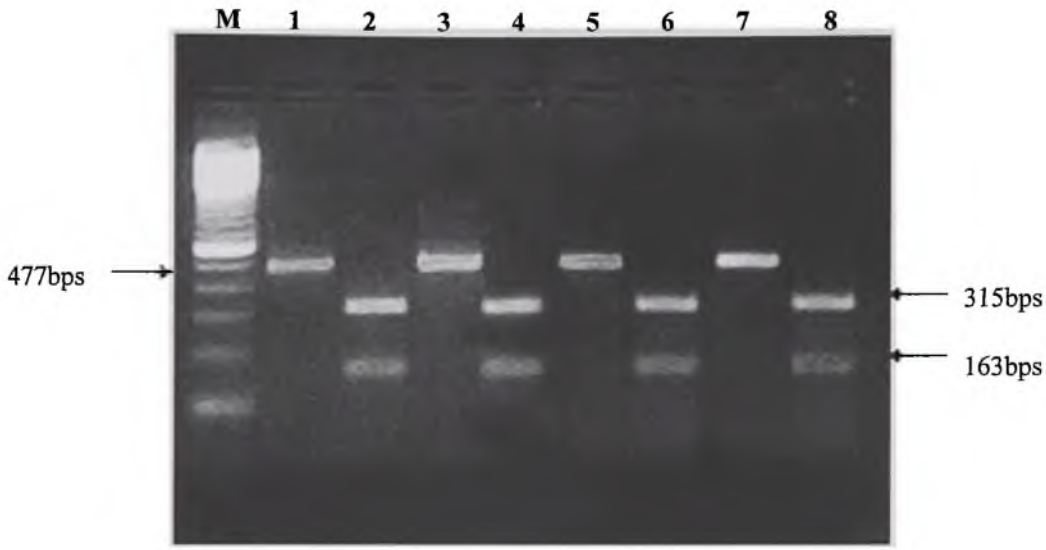

Fig 6 Agarose gel electrophoregram showing *Dra*I digested and undigested PCR products.

Electrophoresis was run on agarose gel (2%) for 1h 30min at 60V using 1X TAE buffer. Gel was stained with ethidium bromide and viewed under UV transillumination to locate DNA bands

Lane M = 100bp molecular weight marker

Lane 1 = *S. typhi* positive control undigested

Lane 2 = *S. typhi* positive control digested with *DraI*

Lanes 3, 5 and 7 =PCR products of clinical samples

Lane 4, 6, and 8 =PCR product of clinical samples digested with *DraI*

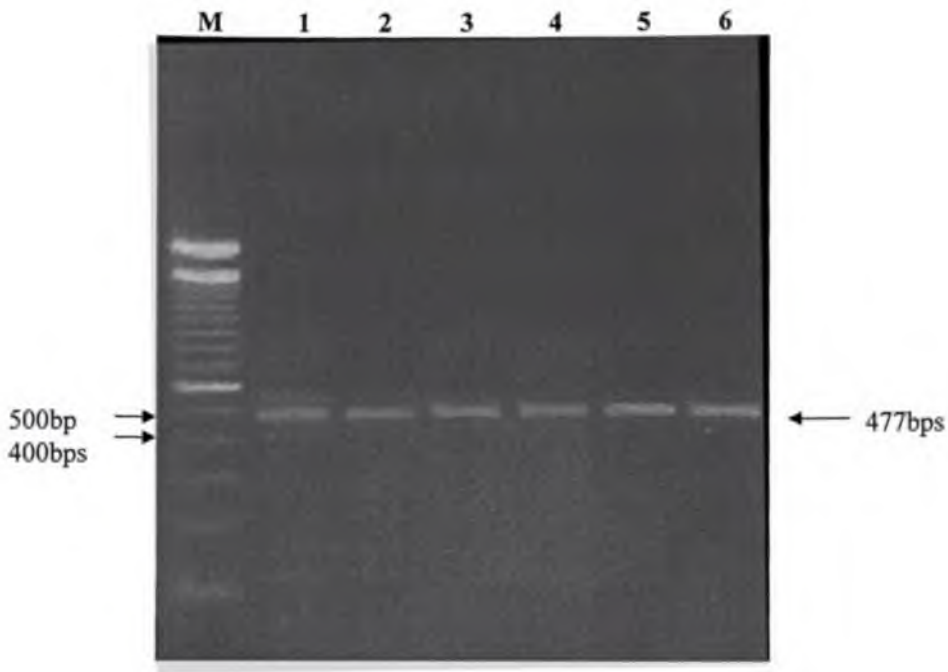

Fig 7 Agarose gel electrophoregram showing *EcoR*I digested and undigested PCR products.

Electrophoresis was run on agarose gel (2%) for 1h 30min at 60V using 1X TAE buffer. Gel was stained with ethidium bromide and viewed under UV transillumination to locate DNA bands.

Lane M: 100bp molecular weight marker.

Lane 1: PCR product of *S. typhi* positive control undigested

Lane 2: PCR product of *S. typhi* positive control digested with *Eco*RI.

Lanes 3 and 5: PCR product of clinical sample undigested

Lanes 4 and 6: PCR product of clinical sample, digested with *Eco*RI.

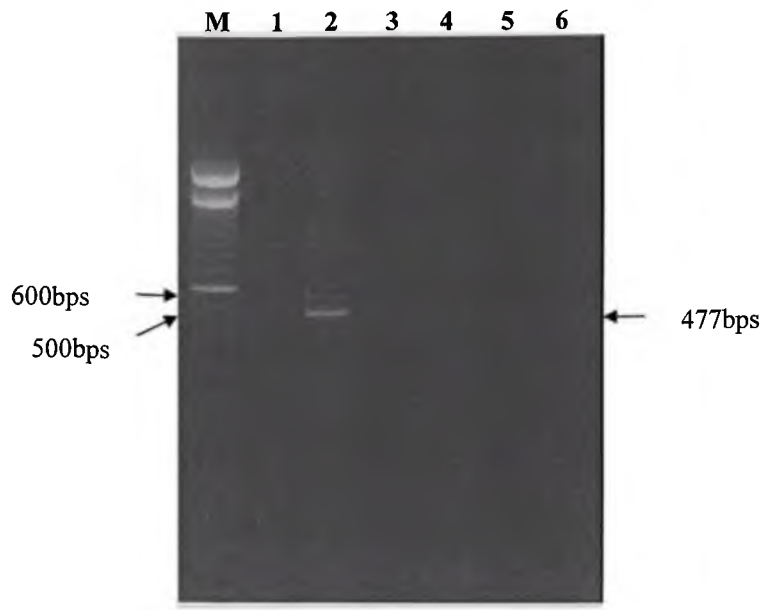

Fig 8 Agarose gel electrophoregram of PCR products of experiments testing the specificity of the developed PCR method.

Electrophoresis was run on agarose gel (2%) for 1h 30min at 60V using 1XTAE buffer. Gel was stained with ethidium bromide and viewed under UV transillumination to locate DNA bands.

Lane M = 100bp molecular weight marker.

Lane 1= Negative control (dd H_2O).

Lane 2 = *S. typhi*

Lane 3 =*B. subtilis*

Lane 4= *E. coli*

Lane 5 = *S. aureus*

Lane 6 = *S. pyogenes*

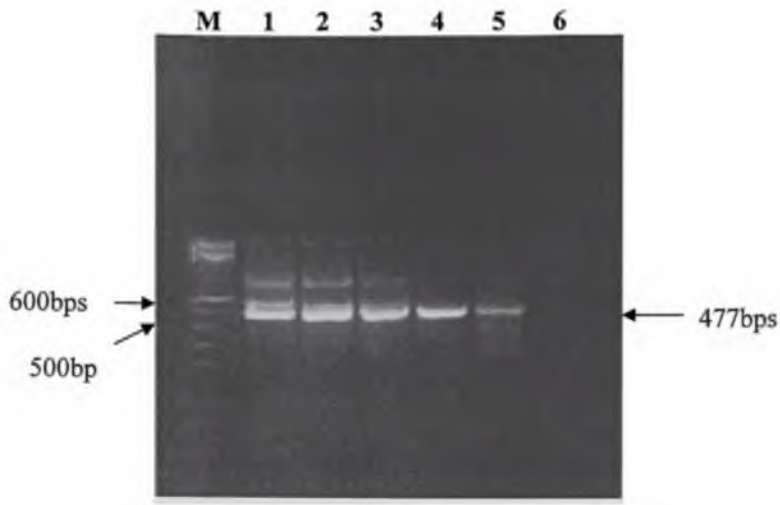

Fig 9 Agarose gel electrophoregram showing results of the PCR sensitivity experiments.

Electrophoresis was run on agarose gel (2%) for 1h 30min at 60V using 1XTAE buffer. Gel was stained with ethidium bromide and viewed under UV transillumination to locate DNA bands.

Lane M= 100bp molecular weight marker.

Lanes 1-6 show the results obtained from the serially diluted *S. typhi* infected blood at 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} fold dilutions respectively.

CHAPTER FOUR

DISCUSSION AND CONCLUSION

4.1 Discussion

This study was designed to develop a sensitive and specific PCR-based method of *S. typhi* detection from filter paper blood blots, for the rapid diagnosis of typhoid fever. The threshold of detection and specificity of the new method were also investigated. The rationale for this study is that the Widal test, which is routinely used in laboratories in Ghana, is a controversial method because it has been the cause of over diagnosis and it is known to be an indirect detection method. There is thus the need for a more specific and sensitive method. Also, the requirement for large volumes of blood from acute cases is not ideal. A better alternative would be filter paper blood blots which would require much lower volumes of blood, thus being a more comfortable sampling method.

Confirmed diagnosis of typhoid fever is reliably made on the basis of the isolation of *S. typhi* from the blood by culture. Blood culture was therefore incorporated into this study. However, *S. typhi* was not isolated from the 120 specimens of patients that were cultured, although other bacteria were isolated. These included *Staphylococcus* in six cases and 1 each of *Serratia marcescens* and *Yersinia enterolytica,* which were isolated using culture media from the same source. *Staphylococcus* in blood samples suggests septicaemia in the patient and *S. marcescens* is an agent of nosocomial infections. The latter in particular, has a peculiar hardiness that enables it to outgrow other organisms in mixed culture (Haddy *et al.*, 1996). *Yersinia enterolytica* on the other hand, is a pathogen of wild animals and is capable of infecting man through the ingestion of undercooked infected pork.

Failure to isolate *S. typhi* from blood culture may have resulted from the limitations of laboratory media, the presence of antibiotics from previous treatment, the volume of the cultured specimen, or the time of sample collection (Coleman and Buxton, 1907; Guerra-Caceres *et al.*, 1979; Wain *et al.*, 1998). However, the growth of non *Salmonella* organisms was proof that the culture media, which had already been tested for sterility and the ability to support growth, was not the reason for *S. typhi* growth failure. The history of previous antibiotic treatment of the patients was not investigated, therefore it will be impossible to determine if drug treatment affected or influenced the blood culture results. The time of sample collection cannot account for growth failure either, because the processing period achieved successful growth of other bacteria.

Some authorities have advanced that blood cultures are usually positive for *Salmonella* in the early stages of illness and that 80% of cases give positive results in the first week, with the chances of isolating the organism progressively diminishing (Thomas, 1988). The samples might have been collected and tested at too late a stage of illness to be detected by culture. Two plausible reasons for the absence of growth of *S. typhi* from the PCR positive samples are that either one or both of the volumes of blood and culture broth were not sufficient and therefore bacterial concentrations were too low for any growth to occur.

The rate of Widal positives (31.35%) describes the prevalence rate of the 1/160 titre among the febrile individuals that were recruited as presumptive typhoid patients. There might have been positives at higher titres, but 1/160 is the value that is used for diagnostic purposes in Ghana, so, this study was tailored with that in mind. If the Widal test at this titre value were used for typhoid diagnosis, the above results would indicate that the majority of the febrile individuals that were recruited for this study were suffering from

something other than typhoid fever. The conclusion here is that the Widal test at a 1/160 titer cannot be relied upon alone to diagnose typhoid fever, since the positive rates obtained here are in contradiction with the clinical symptoms, which were used as one of the criteria for the diagnosis of an active infection another confirmatory test is required in order to establish the cause of disease in the patients.

The above conclusion is a valid one, since the Widal test is known to be non-specific and sometimes downright unreliable because, it is an antibody detection procedure which does not actually demonstrate the presence of the infectious agent. The antibodies could linger after an infection has cleared, indicating a previous infection or, might have been induced by another infection altogether. Demonstration of the presence of antibodies is thus not an indication of disease and could consequently give misleading results if used exclusively for diagnosis.

Since PCR can detect the DNA of an infectious agent in tissue, the PCR method was meant to improve typhoid diagnosis and provide a reliable means of confirming a typhoid infection. However, a much lower (3.78%) positive rate than that which was achieved by the Widal test was obtained by the PCR method. These are not likely to be false positives, as great care was taken during the extraction and PCR processes to prevent contamination from occurring. Also, negative controls were used during every PCR reaction.

Would it be safe to conclude that some of the PCR negative results are false negatives? Meltzer *et al,* (1999) made the following observation in a study involving the use of PCR on cerebrospinal fluid samples that had been proven by culture to be tuberculosis meningitis positive: "The PCR has been reported to detect the equivalent of 1-10

mycobacteria in *in vitro* testing. However, lower sensitivity is found with clinical specimens. The low sensitivity may be the result of inhibitors of PCR present in the reaction, poor lysis of mycobacteria and the uneven distribution of mycobacteria in clinical specimen" A PCR negative result was obtained by Melchers *et al* (1991), when they assayed a skin biopsy sample from which a *Borrelia burdorferi* spirochete was isolated. A suggested reason for this discrepancy was that the number of spirochetes in the sample was too low.

The question of false negatives therefore arises in this study, in response to the results obtained. Although this could be a possible reason for the low number of PCR positives, one cannot make a definite conclusion, to that effect because the organism was not isolated from any of the samples.

Statistically, there was a significant difference between the PCR and Widal test results, according to the Chi square test. The conclusion is that there is no correlation between both test results. The Kappa value is a representation of the proportion of agreement between both diagnostic methods beyond that expected to occur by chance. The value from this analysis is 0.061 and indicates that there is complete disagreement between both tests. Here, a question about the reliability of the Widal test arises because the PCR is an indication of the presence of *S. typhi* DNA while, the Widal test is simply an antibody detection test. The above is further confirmation of the fact that the Widal test is an unreliable diagnostic method. Nevertheless, it is still impossible to make a firm conclusion about the reliability of PCR for *S. typhi* diagnosis, because the PCR positives have not been confirmed by any other method reliable method, i.e culture. Furthermore, the number of PCR positives is low, considering the clinical diagnosis results.

It would be interesting to further investigate if the low prevalence of *S. typhi* in the clinical samples was caused by suboptimal efficiency of PCR. A low prevalence of *Clamydia pneumoniae* was found among clinical samples with chronic cough by Mygind *et al.*, (2002) who went ahead to investigate the efficiency of the method based on real-time PCR.

As mentioned in the introduction, two pairs of primers for nested *S. typhi* flagellin gene amplification were first reported by Song *et al.*, (1993) and modified by Frankel *et al.* (1994). These are available and have been used successfully for the detection of *S. typhi* in blood samples. *S. typhi* flagellin gene is the target of these primers. The new primer set used in this study was designed to amplify *S. typhimurium* in another second round of PCR, because *S. typhimurium* is an organism of interest, which has been implicated in malaria and *Salmonella* concomitant infections in another West African country (Ammah *et al.*, 1999).

In order to increase the specificity of the amplification, a nested PCR approach was adopted. This approach significantly improved the sensitivity and specificity of malaria diagnosis (Snounou *et al.*, 1993). A BLAST Version 2, (National Centre for Biotechnology Information) search analysis of the primer pairs that were designed in this study suggested that they were specific enough to be used (See Appendices IIIa, IIIb, IIIc, and IIId).

A BLAST search of the outer primers revealed that two other *Salmonella* gene sequences, *S. rubislaw* h-1 (r) and *S. muenchen* H1-d appeared in the output with very significant E-values (0.002). However, the *S. rubislaw* gene did not match significantly with the inner primers, indicating that even if the outer pair produced an amplification product, the inner

reaction would not result in amplification and therefore diagnosis for *S. typhi* would still be possible in the presence of *S. ruhislaw* DNA.

Homolgy of the *S. muenchen* phase 1 flagellin gene fragment with the inner primer pair is less significant than it is with the outer primer pair. The E-value for the forward primers was 0.057 and is not shown in the appendix. This value is much higher than 2e-04, the E-value of the sequences producing the most significant alignments, namely, *S. typhi* flagellin gene and *S. enterica* serovar Typhi. Therefore, it is not likely that in a PCR, *S. muenchen* will be amplified over *S. typhi*, except if the former is found in superabundant numbers in the blood, which is an unlikely occurrence. This is because; *S. muenchen* is known to be an uncommon agent of gastroenteritis that produces different clinical symptoms from those of enteric fever (Song *et al*, 1993). It is unlikely that its symptoms would be confused with *S. typhi*, during clinical diagnosis.

The DNA sequences of four bacterial species were shown to have the lowest E values, indicating the most significant homology for all four primers that were used in this study. These sequences are *S. enterica* subsp. enterica serovar Typhi Ty2, *Salmonella enterica* serovar typhi strain CT18, *Salmonella typhi* flagellin gene and *Salmonella enterica* subsp. enterica flagellin (fliC) gene partial cds. The first three species are homotypic synonyms of *S. typhi*, while the last one, *S. enterica* subsp enterica is a *Salmonella* enterica serotype typhi variant that was isolated from pus in the distended gall bladder of a food handler with an *S. typhi* carrier status (Woo *et. al*. 2001). We can conclude from the BLAST results that the primers that were developed for this study are specific enough to identify *S. typhi* in clinical samples. The use of different names for *S. typhi* is due to the fact that there is an old system of nomenclature and a new system (LeMinor and Popoff, 1987),

under which all the species mentioned above are either variants of *S. typhi* or different names for the same *S. typhi* specie.

All seven of the positive PCR products were cleaved by restriction enzyme *Dra*I to give the expected band sizes predicted by the in silico analysis. This is a further confirmation that the amplified products were those of *S. typhi*.

Further proof of the specificity of the primers was demonstrated when non-*Salmonella* organisms *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli* and *Bacillus subtilis* did not produce any amplification products, and isolates of *S. typhi* from different sources (urine, stool and blood of different individuals) produced the expected band sizes of amplified DNA product. Flagellar antigen, the target for amplification, can be found in other bacterial species such as *E.coli*. Also, the d antigen can be found in other *Salmonellae* species, but the hyper variable region VI that is unique to the *S. typhi H-1d* flagellin gene, and stretches from nucleotides 969 to 1077 makes it possible to design primers that are specific for *S. typhi* DNA (Frankel *et al.*, 1989). Thus, primers with sequences that are complementary to the hyper variable region VI were designed for PCR in this study and gave satisfactory results with DNA extracts from *S. typhi* culture broths.

It was observed that DNA extracted from blood blots of clinical samples did not give recognisable bands after the first round PCR. The presence of *S. typhi* DNA was evident only after the second round PCR after which it was possible to visualise the expected bands in an agarose gel. This suggests that the concentration of *Salmonella typhi* DNA in the blood was very low, which is not surprising, as less than 10 bacteria per millilitre of blood can cause serious disease (Haque, 1999). Blood samples of healthy individuals consistently gave negative results during the study.

It is known that levels as low as 10 bacteria per millilitre can cause severe typhoid fever in patients (Pulvertaft, 1930 and Werner *et al.*, 1967). This is an indication of the importance of sensitivity in a diagnostic assay for *S. typhi* DNA in small volumes of blood. A reliable diagnostic assay should be capable of detecting DNA in the blood, irrespective of the stage of disease, as long as *S. typhi* is the cause of infection and clinical illness. The sensitivity levels achieved in this study with DNA isolated from filter paper blood blot extracts are indeed good. Haque *et al.*, (1990) made use of PCR to diagnose *S. typhi* from whole blood, and achieved sensitivities of 5 bacteria per ml of blood, a value that can be compared with 1.6± 0.4CFU per millilitre of blood, obtained in this study. The sensitivity levels achieved by Massi *et al*, 2003 was two to three genome copies of *S. typhi*, which is also comparable to those obtained in this study. The attainment of such sensitivity levels with PCR shows that the use of filter paper blood blots will not result in loss of sensitivity of the method and therefore, the filter paper blood blot method can be used to sample blood for typhoid diagnosis.

The serum antibody responses to the lipopolysaccharide (LPS) (O) and flagellum (H) antigens of serotype Typhi with Ghanaians from different backgrounds, ethnicities, and diseased states, were determined in 1955 by Hughes (Hughes, 1955). Agglutination at titres of 1/80 or less for both H and O *S. typhi* antigens were obtained in 99% of the presumably healthy subjects. Levels of 1/160 or greater were obtained in the remaining 1%, hence its use as a cut-off level for the diseased state. Lifestyles have changed since then and so have hygiene, waste management and the over population issues that have contributed to the high endemicity of typhoid in developing countries (Edelman and Levine, 1986). Therefore, the use of 1/160 antibody cut-off level might not be realistic

and may account for the high Widal positives compared to the low PCR positives that were recorded in this study.

4.2 Conclusion and Recommendation

The results of this work have shown that it is possible to use blood blots for *S. typhi* DNA extraction for the detection of *S. typhi* by PCR. It is more convenient to collect very small amounts of blood without the need for venipuncture with its painful and uncomfortable implications, especially for very sick individuals and so, this kind of PCR could become a very useful diagnostic tool. Although the study has also demonstrated that *S. typhi* can be detected at very low levels of bacteraemia, we cannot conclude that the method is suitable for use in routine diagnosis. It is suggested that the efficiency of the PCR be determined in a further study using the method used by Mygind *et al* (2002). This would help to establish if the low level of *S. typhi* positives obtained in this study was due to suboptimal PCR efficiency in clinical samples.

Also, there is a need to conduct a similar study on a bigger and more diverse sample size, comparing PCR results with Widal and culture results, making use of larger volumes of blood for the culture and higher concentrations of DNA for the PCR. A more comprehensive study will help to clear all doubts about the reliability of the primers. This must be done in order to clarify the discrepancies between the low numbers of *S. typhi* PCR positive individuals and the fact that the primers have proved to be quite sensitive.

There are disadvantages associated with the use of PCR for routine diagnosis in hospitals and laboratories. It requires among others, specialised equipment, expensive biological reagents and skilled personnel. However, it may be very suitable for epidemiology surveys, which require accurate results using large volumes of data.

Granted that Widal will remain in use for typhoid diagnosis for a long time to come, it is recommended also that studies be conducted, in which the general serum *S. typhi* antibody titre levels of a wide selection of individuals will be determined. This should be done, taking into account disparities in health, social development and in the lifestyle of Ghanaians to enable a more realistic and current cut-off antibody level to be determined.

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APPENDICES

APPENDIX I

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                1                                                    50
 TYPHIMFL     (1) --------------------------------------------------
STYFLAGLN     (1) ATGGCACAAGTCATTAATACAAACAGCCTGTCGCTGTTGACCCAGAATAA
Consensus     (1)
               51                                                   100
 TYPHIMFL     (1) --------------------------------------------------
STYFLAGLN    (51) CCTGAACAAATCCCAGTCCGCACTGGGCACTGCTATCGAGCGTTTGTCTT
Consensus    (51)
              101                                                   150
 TYPHIMFL     (1) --------------------------------------------------
STYFLAGLN   (101) CCGGTCTGCGTATCAACAGCGCGAAAGACGATGCGGCAGGACAGGCGATT
Consensus   (101)
              151                                                   200
 TYPHIMFL     (1) --------------------------------------------------
STYFLAGLN   (151) GCTAACCGTTTTACCGCGAACATCAAAGGTCTGACTCAGGCTTCCCGTAA
Consensus   (151)
              201                                                   250
 TYPHIMFL     (1) --------------------------------------------------
STYFLAGLN   (201) CGCTAACGACGGTATCTCCATTGCGCAGACCACTGAAGGCGCGCTGAACG
Consensus   (201)
              251                                                   300
 TYPHIMFL     (1) --------------------------------------------------
STYFLAGLN   (251) AAATCAACAACAACCTGCAGCGTGTGCGTGAACTGGCGGTTCAGTCTGCG
Consensus   (251)
              301                                                   350
 TYPHIMFL     (1) --------------------------------------------------
STYFLAGLN   (301) AATGGTACTAACTCCCAGTCTGACCTCGACTCCATCCAGGCTGAAATCAC
Consensus   (301)
              351                                                   400
 TYPHIMFL     (1) --------------------------------------------------
STYFLAGLN   (351) CCAGCGCCTGAACGAAATCGACCGTGTATCCGGCCAGACTCAGTTCAACG
Consensus   (351)
              401                                                   450
 TYPHIMFL     (1) --------------------------------ACCATCCAGGTTGGTGCC
STYFLAGLN   (401) GCGTGAAAGTCCTGGCGCAGGACAACACCCTGACCATCCAGGTTGGTGCC
Consensus   (401)                                 ACCATCCAGGTTGGTGCC
              451                                                   500
 TYPHIMFL    (19) AACGACGGTGAAACTATCGATATCGATCTGAAGCAGATCAACTCTCAGAC
STYFLAGLN   (451) AACGACGGTGAAACTATCGATATTGATTTAAAAGAAATCAGCTCTAAAAC
Consensus   (451) AACGACGGTGAAACTATCGATAT GAT T AA  A ATCA CTCT A AC
              501                                                   550
 TYPHIMFL    (69) CCTGGGTCTGGATACGCTGAATGTGCAACAAAAATATAAGGTCAGCGATA
STYFLAGLN   (501) ACTGGGACTTGATAAGCTTAATGTCCAAGATGCCTACACCCCGAAAGAAA
Consensus   (501)  CTGGG CT GATA GCT AATGT CAA A    TA A     A  GA A
              551                                                   600
 TYPHIMFL   (119) CGGCTGCAACTGTTACAGGATATGCCGATAC------TACGATTGCTTT-
STYFLAGLN   (551) CTGCTGTAACCGTTGATAAAACTACCTATAAAAATGGTACAGATCCTATT
Consensus   (551) C GCTG AAC GTT     A  T CC ATA       TAC   T CT T
              601                                                   650
 TYPHIMFL   (162) --AGACAATAGTACTTTTAAAGCCTCGGCTACTGGTCTTGGTGGTACTGA
STYFLAGLN   (601) ACAGCCCAGAGCAATACTGATATCCAAACTGCAATTGGCGGTGGTGCAAC
Consensus   (601)   AG C A AG A T  T A   C    CT C   T   GGTGGT C
              651                                                   700
 TYPHIMFL   (210) CCAGAAAATTGATGGCGATTTAAAATTTGATGATACGACTGGAAAATATT
STYFLAGLN   (651) GGGGGTTACTGGGGCTGATATCAAATTTAAAGATG-GTCAATACTATTTA
Consensus   (651)    G   A TG  G  GAT T AAATTT A GAT  G C   A  AT T
              701                                                   750
 TYPHIMFL   (260) -ACGCCAAAGTTACCGTTACGGGGGGAACTGGTAAAGATGGCTATTATGA
STYFLAGLN   (700) GATGTTAAAGGCGGTGCTTCTGCTGGTGTTTATAAAGCCACTTATGATGA
Consensus   (701)  A G  AAAG     G T C G  GG   T  TAAAG     TAT ATGA
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The alignment map is continued on the next page.

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                751                                                   800
 TYPHIMFL    (309) AGTTTGCGTTGATAAGACGAACGGTGAGGTGACTCTTG---CTGGCGGTG
STYFLAGLN    (750) AACTACAA---ACAAAGTTAATATTGATACGACTGATAAAACTCCGTTGG
Consensus    (751) A  T C     A AA    AA   TGA   GACT  T    CT      G
                801                                                   850
 TYPHIMFL    (356) CGACTTCCCCGCTTACAGGTGGACTACCTGCGACAGCAACTGAGGATGTG
STYFLAGLN    (797) CAACTGCCGAAGCTACAGCTATTCGGGGAACGGCCACTATAACCCACAAC
Consensus    (801) C ACT C       TACAG T   C       CG C  C A      A
                851                                                   900
 TYPHIMFL    (406) AAAAATGTACAAGTTGCAAA------TGCTGATTTGACAGAGGCTAAAGC
STYFLAGLN    (847) CAAATTGCTGAAGTAACAAAAGAGGGTGTTGATACGACCACAGTTGCGGC
Consensus    (851)  AAA TG   AAGT  CAAA      TG TGAT  GAC    G T   GC
                901                                                   950
 TYPHIMFL    (450) CGCATTGACAGCAGCAGGTGTTACCGGC----------ACAGCATCTG--
STYFLAGLN    (897) TCAACTTGCTGCAGCAGGGGTTACTGGCGCCGATAAGGACAATACTAGCC
Consensus    (901)    A T  C GCAGCAGG GTTAC GGC          ACA  A   G
                951                                                  1000
 TYPHIMFL    (488) TTGTTAAGATGTCTTATACTGATAATAACGGTAAAACTATTGATGGTGGT
STYFLAGLN    (947) TTGTAAAACTATCGTTTGAGGATAAAAACGGTAAGGTTATTGATGGTGGC
Consensus    (951) TTGT AA  T TC T T   GATAA AACGGTAA   TATTGATGGTGG
                1001                                                 1050
 TYPHIMFL    (538) TTAGCAGTTAAGGTAGGCGATGATTACTATTCTGCAACTCAAAAT---AA
STYFLAGLN    (997) TATGCAGTGAAAATGGGCGACGATTTCTATGCCGCTACATATGATGAGAA
Consensus   (1001) T  GCAGT AA  T GGCGA GATT CTAT C GC AC  A  AT   AA
                1051                                                 1100
 TYPHIMFL    (585) AGATGGTTCCATAAGTATTAATACTACGAAATACACTGCAGATGACGGTA
STYFLAGLN   (1047) AACAGGTGCAATTACTGCTAAAACCACTACTTATACAGATGGTACTGGCG
Consensus   (1051) A   GGT C AT A T  TAA AC AC A  TA AC G  G T   GG
                1101                                                 1150
 TYPHIMFL    (635) CATCCAAAACTGCACTAAACAAACTGGGTGGCGCAGACGGCAAAACCGAA
STYFLAGLN   (1097) TTGCTCAAACTGGAGCTGTGAAATTTGGTGGCGCAAATGGTAAATCTGAA
Consensus   (1101)    C  AAACTG A      AAA T GGTGGCGCA A GG AAA C GAA
                1151                                                 1200
 TYPHIMFL    (685) GTTGTTTCTATT---(K)TGGTAAAACTTACGCTGCAAGTAAAGCCGAAGG
STYFLAGLN   (1147) GTTGTTACTGCTACCGATGGTAAGACTTACTTAGCAAGCGACCTTGACAA
Consensus   (1151) GTTGTT CT  T   G TGGTAA ACTTAC   GCAAG  A    GA
                1201                                                 1250
 TYPHIMFL    (732) TCACAACTTTAAAGCACAGCCTGATCTGGCGGAAGCGGCTGCTACAACCA
STYFLAGLN   (1197) ACATAACTTCAGAACAGGCGGTGAGCTTAAAGAGGTTAATACAGATAAGA
Consensus   (1201)  CA AACTT A A CA     TGA CT    GA G    T C    A  A
                1251                                                 1300
 TYPHIMFL    (782) CCGAAAACCCGCTGCAGAAAATTGATGCTGCTTTGGCACAGGTTGACACG
STYFLAGLN   (1247) CTGAAAACCCACTGCAGAAAATTGATGCTGCCTTGGCACAGGTTGATACA
Consensus   (1251) C GAAAACCC CTGCAGAAAATTGATGCTGC TTGGCACAGGTTGA AC
                1301                                                 1350
 TYPHIMFL    (832) TTACGTTCTGACCTGGGTGCG----------------------------
STYFLAGLN   (1297) CTTCGTTCTGACCTGGGTGCGGTTCAGAACCGTTTCAACTCCGCTATCAC
Consensus   (1301)  T CGTTCTGACCTGGGTGCG
```

Align X-generated alignment map of *S. typhi* and *S. typhimurium* DNA sequences. The concensus sequence is shaded grey while the *S. typhi* and *S. typhimurium* specific primer sequences are shaded yellow.

TYPHIMFL = *S. typhimurium* (Accession number M33808).
STYFLAGLN= *S. typhi* (Accession number L21912).

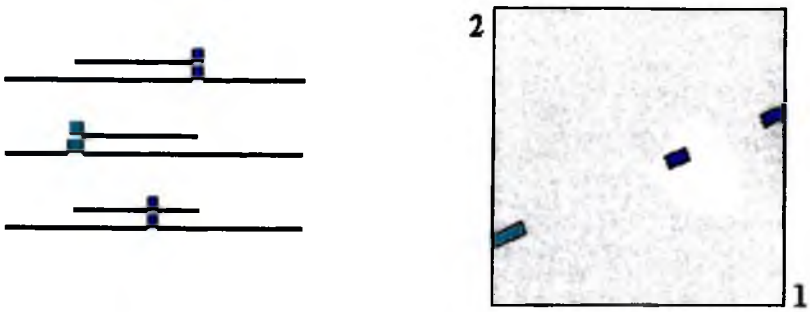
The *Salmonella* genus-specific forward primers correspond to nucleotides 446 to 464 of the *S. typhi* DNA sequence and the genus specific reverse primers correspond to nucleotides 1300 to 1317 of the DNA sequence.

The *S. typhi* species-specific forward primers correspond to nucleotides 520 to 541 of the *S. typhi* DNA sequence and the reverse primers correspond to nucleotides 975 to 996 of the DNA sequence.

APPENDIX II

Sequence 1 lcl|seq_1 **Length** 852 (1 .. 852)

Sequence 2 lcl|seq_2 **Length** 2001 (1 .. 2001)




```
Score = 98.7 bits (51), Expect = 2e-17
Identities = 63/69 (91%)
 Strand = Plus / Plus

Query: 784  gaaaacccgctgcagaaaattgatgctgctttggcacaggttgacacgttacgttctgac 843
            |||||||| |||||||||||||||||||| |||||||||||||| ||  | |||||||||
Sbjct: 1249 gaaaacccactgcagaaaattgatgctgccttggcacaggttgatacacttcgttctgac 1308

Query: 844  ctgggtgcg 852
            |||||||||
Sbjct: 1309 ctgggtgcg 1317

Score   98.7 bits (51), Expect   2e-17
Identities   81/96 (84%)
 Strand = Plus / Plus

Query: 1    accatccaggttggtgccaacgacggtgaaactatcgatatcgatctgaagcagatcaac 60
            |||||||||||||||||||||||||||||||||||||||||||||||| ||| | ||  | |||| |
Sbjct: 433  accatccaggttggtgccaacgacggtgaaactatcgatattgatttaaaagaaatcagc 492

Query: 61   tctcagaccctgggtctggatacgctgaatgtgcaa 96
            ||| | || ||||| || |||| ||| ||||| |||
Sbjct: 493  tctaaaacactgggacttgataagcttaatgtccaa 528

Score = 41.1 bits (21), Expect = 3.8
Identities = 47/60 (78%)
 Strand = Plus / Plus

Query: 508  gataataacggtaaaactattgatggtggtttagcagttaaggtaggcgatgattactat 567
            ||||| |||||||| ||||||||||||| | ||||| || | ||||| |||| ||||
Sbjct: 967  gataaaaacggtaaggttattgatggtggctatgcagtgaaaatgggcgacgatttctat 1026
```

Dot plot (above) and BLAST analysis (below) of *S. typhi* (sequence 1) and *typhimurium* (sequence 2) flagellin gene nucleotide sequences generated by the NCBI BLAST 2 analysis programme.

APPENDIX III

Sequences producing significant alignments:

GI number	Accession number	Region	Organism name	Score (bits)	E Value
gi|16420488	AE008787.1		*S. typhimurium* LT2, sect...	38	0.002
gi|29136933	AE016837.1		*S. enterica* subsp. enter...	38	0.002
gi|27448112	AF336929.1		*S. enterica* subsp. enter...	38	0.002
gi|47470	X04505.1	SRH1R	*S. rubislaw* H-1(r) ..	38	0.002
gi|16903563	AF425736.1	AF425736	*Salmonella enterica* subs...	38	0.002
gi|16226012	AF420426.1	AF420426	*Salmonella enterica* subs...	38	0.002
gi|16226008	AF420425.1	AF420425	*S. enterica* subs...	38	0.002
gi|16502975	AL627272.1		*S. enterica* serovar Typ...	38	0.002
gi|13377573	AF332601.1	AF332601	*S. enterica* subs...	38	0.002
gi|882142	U17176.1	SCU17176	*S. choleraesuis* ATCC...	38	0.002
gi|882140	U17175.1	SCU17175	*S. choleraesuis* ATCC...	38	0.002
gi|882134	U17172.1	SCU17172	*S. choleraesuis* ATCC...	38	0.002
gi|882132	U17171.1	SCU17171	*S. choleraesuis* ATCC...	38	0.002
gi|217062	D13689.1	STYFLICS	*S.typhimurium* gene ...	38	0.002
gi|217067	D13690.1	STYHIN	*S.abortus-equi* genes ...	38	0.002
[redacted]					
gi|153978	M11332.1	STYFLGH1I	*S. typhimurium* H-1-...	38	0.002
[redacted]					
gi|8895078	AF159459.1	AF159459	*S. choleraesuis* f...	38	0.002
gi|47385	X03393.1	SPH1A	*S. paratyphi*-A H-1a gen...	38	0.002
gi|47233	X03395.1	SMH1D	*S. muenchen* H1-d gene f...	38	0.002
gi|46855	X03394.1	SCH1C	*S. cholerae-suis* H-1c g...	38	0.002
gi|24306147	AF487406.1		*E. tarda* flagellin gen...	34	0.037
gi|16421311	AE008826.1		*S. typhimurium* LT2, sect...	34	0.037
gi|4001806	AF045151.1	AF045151	*Salmonella typhimurium* ph...	34	0.037
gi|882144	U17177.1	STU17177	*S. typhimurium* phase...	34	0.037
gi|882136	U17173.1	SCU17173	*S. choleraesuis* ATCC...	34	0.037

b

Sequences producing significant alignments:

GI number	Accession number	Region	Organism name	Score (bits)	E Value
gi|16420488	AE008787.1		*Salmonella typhimurium* LT2, sect.	36	0.009
gi|29136933	AE016837.1		*Salmonella enterica* subsp. enter.	36	0.009
gi|27448112	AF336929.1		*Salmonella enterica* subsp. enter..	36	0.009
gi|47470	X04505.1	SRH1R	*S. rubislaw* H-1(r) gene for pha..	36	0.009
gi|16226012	AF420426.1	AF420426	*Salmonella enterica* subs...	36	0.009
gi|16226008	AF420425.1	AF420425	*Salmonella enterica* subs...	36	0.009
gi|16502975	AL627272.1		*S. enterica* serovar Typ...	36	0.009
gi|13377573	AF332601.1	AF332601	*S. enterica* subs...	36	0.009
gi|882138	U17174.1	SCU17174	*S. choleraesuis* ATCC..	36	0.009
gi|217062	D13689.1	STYFLICS	*S.typhimurium* gene for phas...	36	0.009
gi|217067	D13690.1	STYHIN	*S.abortus-equi* genes for inac..	36	0.009
[redacted]					
gi|153978	M11332.1	STYFLGH1I	*S. typhimurium* H-1-...	36	0.009
[redacted]					
gi|8895078	AF159459.1	AF159459	*S. choleraesuis* f...	36	0.009
gi|47233	X03395.1	SMH1D	*S. muenchen* H1-d gene f...	36	0.009
gi|46855	X03394.1	SCH1C	*S. cholerae-suis* H-1c g...	36	0.009
gi|882132	U17171.1	SCU17171	*S. choleraesuis* ATCC...	30	0.58

a. Results of the BLAST analysis of forward (top) and reverse (below) *Salmonella* genus flagellin specific primers (outer primers).

Sequences producing significant alignments:

GI number	Accession number	Region	Organism name	Score (bits)	E Value
gi|29136933	AE016837.1		*Salmonella enterica* subsp. enter..	44	2e-04
gi|38050054	AY353434.1		*Salmonella enterica* strain CDC s..	44	2e-04
gi|38050052	AY353433.1		*Salmonella enterica* strain CDC s..	44	2e-04
gi|38049938	AY353376.1		*Salmonella enterica* strain CDC s..	44	2e-04
gi|38049936	AY353375.1		*Salmonella enterica* strain CDC s..	44	2e-04
gi|38049934	AY353374.1		*Salmonella enterica* strain CDC s..	44	2e-04
gi|38049928	AY353371.1		*Salmonella enterica* strain CDC s..	44	2e-04
gi|38049789	AY353302.1		*Salmonella enterica* strain CDC s..	44	2e-04
gi|16502975	AL627272.1		*Salmonella enterica* serovar Typ..	44	2e-04
gi|13377573	AF332601.1	AF332601	*Salmonella enterica* subs..	44	2e-04
[redacted]					
gi|38049932	AY353373.1		*Salmonella enterica* strain CDC s..	36	0.057
gi|38049930	AY353372.1		*Salmonella enterica* strain CDC s..	36	0.057
gi|153999	M34824.1	STYFLICDA	*S.muenchen* phase 1 flag...	36	0.057

d

Sequences producing significant alignments:

GI number	Accession number	Region	Organism name	Score (bits)	E Value
gi|29136933	AE016837.1		*Salmonella enterica* subsp. enter..	44	2e-04
gi|38050054	AY353434.1		*Salmonella enterica* strain CDC s..	44	2e-04
gi|38050052	AY353433.1		*Salmonella enterica* strain CDC s..	44	2e-04
gi|38049938	AY353376.1		*Salmonella enterica* strain CDC s..	44	2e-04
gi|38049936	AY353375.1		*Salmonella enterica* strain CDC s..	44	2e-04
gi|38049934	AY353374.1		*Salmonella enterica* strain CDC s..	44	2e-04
gi|38049932	AY353373.1		*Salmonella enterica* strain CDC s..	44	2e-04
gi|38049930	AY353372.1		*Salmonella enterica* strain CDC s..	44	2e-04
gi|38049928	AY353371.1		*Salmonella enterica* strain CDC s..	44	2e-04
gi|38049789	AY353302.1		*Salmonella enterica* strain CDC s..	44	2e-04
gi|16502975	AL627272.1		*Salmonella enterica* serovar Typ...	44	2e-04
gi|13377573	AF332601.1	AF332601	*Salmonella enterica* subs...	44	2e-04
gi|47924	X16406.1	STRVIFG	*Salmonella typhi* DNA for the .	44	2e-04
gi|153999	M34824.1	STYFLICDA	*S.muenchen* phase 1 flagelli. .	44	2e-04
[redacted]					

b. Results of the BLAST analysis of *Salmonella typhi* specific forward primer (above) and *Salmonella typhi* specific reverse primer (below), these being primers for the inner reaction.

The raw score S for an alignment is calculated by summing the scores for each aligned position and the scores for gaps.

The E (Expectation)-value is a parameter that describes the number of hits one can "expect" to see just by chance when searching a database of a particular size. The lower the E-value, or the closer it is to "0" the more "significant" the match is.

APPENDIX IV. Table showing the serial dilutions of seeded blood (extreme left column) and the PCR status of those dilutions (extreme right). The concentration of colony forming units (CFUs) per serial dilution is shown in the penultimate columns.

	Dilutions	Dil. Factor.	Blot	DNA xtrct.	Template	CFU 1	CFU/ul	
1	1	0.001	8	0.01	0.5	TNTC		PCR +ve
2	0.1	0.001	8	0.01	0.5	TNTC		PCR +ve
3	0.01	0.001	8	0.01	0.5	41	0.00164	PCR +ve
4	0.001	0.001	8	0.01	0.5	0	0	PCR -ve
5	0.0001	0.001	8	0.01	0.5	0	0	PCR -ve
	Dilutions	Dil. Factor.	Blot	DNA xtrct.	Template	CFU2	CFU/ul	
1	1	0.001	8	0.01	0.5	TNTC		PCR +ve
2	0.1	0.001	8	0.01	0.5	TNTC		PCR +ve
3	0.01	0.001	8	0.01	0.5	2378	0.09512	PCR +ve
4	0.001	0.001	8	0.01	0.5	476	0.01904	PCR +ve
5	0.0001	0.001	8	0.01	0.5	45	0.0018	PCR +ve
	Dilutions	Dil. Factor.	Blot	DNA xtrct.	Template	CFU3	CFU/ul	
1	1	0.001	8	0.01	0.5	TNTC		PCR +ve
2	0.1	0.001	8	0.01	0.5	TNTC		PCR +ve
3	0.01	0.001	8	0.01	0.5	1406	0.05624	PCR +ve
4	0.001	0.001	8	0.01	0.5	237	0.00948	PCR +ve
5	0.0001	0.001	8	0.01	0.5	39	0.00156	PCR -ve
	Dilutions	Dil. Factor.	Blot	DNA xtrct.	CFU 1	CFU/ul		
1	1	0.001	1	0.01	TNTC		PCR +ve	
2	0.1	0.001	1	0.01	TNTC		PCR +ve	
3	0.01	0.001	1	0.01	TNTC		PCR +ve	
4	0.001	0.001	1	0.01	1568	0.01568	PCR +ve	
5	0.0001	0.001	1	0.01	156.8	0.001568	PCR +ve	
6	0.00001	0.001	1	0.01	15.6	0.000156	PCR -ve	

APPENDIX V: Table showing demographic status and diagnostic test results of study samples. The shaded boxes are the positive results.

Sample ID	Age	Sex	Widal status	Parasitaemia	PCR result	Culture
UH001	0	F		Pos	Neg	
UH002	9	F		Neg	Neg	
UH003	0	M		Neg	Neg	
UH004	13	M		Neg	Neg	
UH005	0	M		Neg		
UH006	0	M	Neg	Neg	Neg	
UH007	20	F			Neg	
UH008	25	M			Neg	
UH009	0	F				
UH010	0	F		Neg	Neg	
UH011	0	M			Neg	
UH012	0	F	Neg		Neg	
UH013	0	M	Neg		Neg	
UH014	0	M	Neg			
UH015	0	M	Neg		Neg	
UH016	0	F	Neg		Neg	
UH017	0	F		Neg	Neg	
UH018	0	M		Neg	Neg	
UH019	0	M			Neg	
UH020	0	M	Neg	Neg	Neg	
UH021	0	M	Neg	Neg	Neg	
UH022	0	M	Neg	Neg	Neg	
UH023	0	F	Neg	Neg	Neg	
UH024	0	F	Neg	Neg	Neg	
UH025	0	F	Neg	Neg	Neg	
UH026	0	M	Neg	Neg	Neg	
UH027	0	M		Neg	Neg	
UH028	0	M			Neg	
UH029	0	F			Neg	
UH045	0	F	Neg		Neg	
UH046	0	M		Neg	Neg	
UH047	0	M		Neg	Neg	
UH048	5	F			Neg	
UH049	0	M			Neg	
UH050	10	F		Neg	Neg	
UH051	0	F			Neg	
UH058	0	F			Neg	
UH060	20	M			Neg	
UH064	0	F			Neg	
UH065	45	F			Neg	
UH066	4	F		Neg	Neg	
UH067	0	F		Neg	Neg	
UH068	17	M		Neg	Neg	
UH069	21	F		Neg	Neg	

UH091	24	F	[redacted]		[redacted]	
UH092	21	F	[redacted]		Neg	
UH094	26	F	[redacted]	[redacted]	Neg	
UH095	23	M	[redacted]		Neg	
UH097	0	M	[redacted]	Neg	Neg	
UH099	0	M	[redacted]	Neg	Neg	
UH101	20	M	Neg	Neg	Neg	
UH121	0	M	[redacted]	Neg	Neg	
UH124	0	M	Neg	Neg	Neg	
UH125	0	F	Neg	Neg	Neg	
UH126	0	M	Neg	Neg	Neg	
UH127	0	M	[redacted]	Neg	Neg	
UH128	0	F	Neg	Neg	Neg	
UH129	0	F	[redacted]	Neg	Neg	
UH130	0	F	[redacted]		Neg	
UH131	0	M	Neg	Neg	Neg	
UH132	0	F	Neg	Neg	Neg	
UH133	0	M	Neg	Neg	Neg	
UH134	0	F	Neg	[redacted]	Neg	
UH136	0	M	Neg	Neg	Neg	
UH137	0	F	Neg	Neg	Neg	
UH138	0	F	Neg	[redacted]	Neg	
UH139	0	M	Neg	Neg	Neg	
UH140	0	F	[redacted]	Neg	Neg	
UH141	0	F	Neg	Neg	Neg	
UH142	0	M	Neg	Neg	Neg	
UH143	0	M	Neg		Neg	
UH144	0	M	Neg	[redacted]	Neg	
UH145	0	M	Neg	Neg	Neg	
UH146	0	F	Neg		Neg	
UH147	0	F	Neg	Neg	Neg	
UH148	0	M	Neg		Neg	
UH149	0	F	Neg	Neg	Neg	
UH150	0	F	Neg		Neg	
UH151	0	F	Neg	Neg	[redacted]	
UH153	27	F	Neg		Neg	Neg
UH154	40	F	Neg		Neg	Neg
UH155	37	M	Neg		Neg	Neg
UH156	24	M	Neg		Neg	Neg
UH157	0	F	[redacted]		Neg	Neg
UH158	27	F	Neg		Neg	Neg
UH159	27	F	Neg		Neg	Neg
UH160	23	F	Neg		Neg	Neg
UH161	27	F	Neg	Neg	Neg	Neg
UH162	40	F	Neg		Neg	Neg
UH163	20	F	Neg	Neg	Neg	Neg
UH164	16	M	Neg		Neg	Neg
UH165	20	M	Neg	Neg	Neg	Neg
UH167	29	M	Neg	Neg	Neg	Neg
UH168	25	M	Neg	Neg	Neg	Neg

UH169	24	F	Neg	Neg	Neg	Neg
UH170	14	F	Neg	Neg	Neg	Neg
UH171	30	F	Neg	Neg	Neg	Neg
UH172	38	M	[redacted]	Neg	Neg	Neg
UH173	20	F	Neg	Neg	Neg	Neg
UH174	23	M	Neg		Neg	Neg
UH175	17	M	Neg	Neg	Neg	Neg
UH176	17	M	Neg	Neg	Neg	Neg
UH177	30	M	Neg		Neg	Neg
UH178	6	M	Neg		Neg	Neg
UH188	0	M	[redacted]		Neg	Neg
UH189	0	F	[redacted]	Neg	Neg	Neg
UH190	0	F	[redacted]	Neg	Neg	Neg
UH191	0	F	[redacted]	Neg	Neg	Neg
UH192	0	M	[redacted]	Neg	Neg	Neg
UH193	0	M	Neg		Neg	Neg
UH194	0	F	[redacted]	Neg	[redacted]	Neg
UH195	0	M	Neg	Neg	Neg	Neg
UH196	0	M	Neg	Neg	Neg	Neg
UH198	0	M	Neg		Neg	Neg
UH199	31	M	Neg	Neg	Neg	Neg
UH200	53	F	Neg	Neg	Neg	[redacted]
UH201	19	F	Neg	Neg	Neg	Neg
UH202	0	M	Neg	Neg	Neg	Neg
UH203	16	F	Neg	[redacted]	Neg	Neg
UH204	30	F	Neg	Neg	Neg	[redacted]
UH205	20	F	[redacted]	Neg	Neg	Neg
UH206	22	F	Neg	Neg	Neg	Neg
UH207	36	F	Neg		Neg	Neg
UH208	64	F	Neg		Neg	[redacted]
UH209	50	M	Neg	Neg	Neg	[redacted]
UH210	31	F	Neg		Neg	Neg
UH211	34	M	Neg	Neg	Neg	Neg
UH212	21	M	Neg	Neg	Neg	Neg
UH213	10	M	Neg	Neg	Neg	Neg
UH214	27	M	Neg	Neg	Neg	Neg
UH215	36	F	Neg	Neg	Neg	Neg
UH216	23	M	Neg	Neg	Neg	[redacted]
UH217	0	M	Neg		Neg	Neg
UH218	14	F	Neg	Neg	Neg	[redacted]
UH219	17	F	Neg		Neg	Neg
UH220	50	F	Neg		Neg	Neg
UH221	25	M	Neg	Neg	Neg	Neg
UH222	30	M	Neg	Neg	Neg	Neg
UH223	28	F	Neg	Neg	Neg	Neg
UH224	40	M	Neg	Neg	Neg	Neg
UH225	34	M	Neg	Neg	[redacted]	Neg
UH226	32	M	Neg	Neg	Neg	Neg
UH227	52	M	Neg	[redacted]	Neg	Neg
UH228	15	F	Neg	Neg	Neg	Neg

UH229	48	M	Neg	Neg	Neg	Neg
UH230	8	F	Neg	Neg	Neg	Neg
UH231	33	F	Neg	Neg	Neg	Neg
UH232	28	F	[redacted]	Neg	Neg	[redacted]
UH233	58	M	Neg	Neg	Neg	Neg
UH234	45	F	Neg	Neg	Neg	Neg
UH235	9	M	Neg	Neg	Neg	Neg
UH236	0	F	Neg	Neg	Neg	Neg
UH237	23	M	Neg	Neg	Neg	Neg
UH238	50	M	Neg	Neg	Neg	Neg
UH239	11	F	Neg	Neg	Neg	Neg
UH240	17	F	[redacted]	Neg	Neg	Neg
UH241	16	F	Neg	Neg	Neg	Neg
UH242	11	F	Neg	Neg	Neg	Neg
UH243	12	F	Neg	Neg	Neg	Neg
UH244	48	M	Neg		Neg	Neg
UH245	17	F	[redacted]	Neg	Neg	Neg
UH246	42	M	Neg	Neg	Neg	Neg
UH247	17	M	Neg	Neg	Neg	Neg
UH248	50	F	Neg	Neg	Neg	Neg
UH249	34	M	Neg	Neg	Neg	Neg
UH250	27	F	[redacted]	Neg	Neg	Neg
UH251	40	F	[redacted]	Neg	Neg	Neg
UH252	13	F	[redacted]	Neg	Neg	Neg
UH257	35	F	Neg	Neg	Neg	Neg
UH258	28	F	Neg	Neg	Neg	Neg
UH259	16	F	[redacted]	Neg	Neg	Neg
UH260	45	F	Neg	Neg	Neg	Neg
UH261	29	M	Neg	Neg	Neg	[redacted]
UH262	24	F	[redacted]	Neg	Neg	Neg
UH263	26	M	Neg	Neg	Neg	Neg
UH264	36	M	Neg	Neg	Neg	Neg
UH265	25	F	Neg	Neg	Neg	Neg
UH266	52	M	Neg	Neg	Neg	Neg
UH267	28	F	Neg	Neg	Neg	[redacted]
UH268	0	F	Neg	Neg	Neg	Neg
UH269	0	F	Neg	Pos	Neg	Neg
UH270	0	F	Neg	Neg	Neg	Neg
UH271	0	M	Neg	Neg	Neg	Neg
UH272	0	M	Neg	Neg	Neg	Neg
UH108	16	M	Neg	[redacted]	Neg	0