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Molecular Diagnosis of Enteric Fever: Progress and Perspectives

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1. Introduction

Enteric fever is a severe systemic Gram-negative bacterial infection caused by several serovars of *Salmonella enterica* subspecies *enterica*, including *S.* Typhi and *S.* Paratyphi serotypes A (most commonly), B and C. It is characterised by high fever and a myriad of other non-specific features, including abdominal pain and constipation, headache, myalgia and arthralgia, cough, lymphadenopathy and rash. *S.* Typhi, the human-specific causative agent of typhoid fever, is thought to account for an estimated 21 million new cases and 216,000 deaths every year (Crump et al., 2004). *S.* Typhi is generally transmitted in food and water contaminated with faeces from those excreting bacteria, either during the acute illness or during chronic asymptomatic carriage, although infection of health-care or laboratory workers through poor hygiene practices or accidental exposure is also described. Transmission in regions with adequate sanitation and sewage facilities is uncommon as, in general, a relatively high inoculum is required to survive the gastric acid environment and cause infection. Enteric fever is therefore most common in resource-poor settings where the provision of clean drinking water and sewage disposal facilities is inadequate. South and Central Asia, Africa and South and Central America are considered endemic for this disease and particularly high incidence rates are found in the Indian sub-continent and South-east Asia, with rates exceeding 100 per 100,000 population per year (Bhan et al., 2005). In other countries typhoid fever remains an important consideration for travellers both pre- and post-travel (Levine et al., 1982; Ackers et al., 2000; Bhan et al., 2005).

The accurate and rapid clinical diagnosis of enteric fever in these regions is obfuscated by the range of other common fever-causing infections including malaria, dengue fever, leptospirosis, melioidosis and the rickettsioses. Accurate diagnosis to differentiate typhoid fever from these conditions is often difficult, both in the clinic and in the laboratory, but is imperative for effective treatment selection. Even in highly-resourced western countries, physicians often start typhoid treatment empirically whilst awaiting confirmation of the diagnosis. Treatment decisions are further complicated by the increasing prevalence of antibiotic resistance amongst clinical isolates due to plasmid-mediated multidrug resistance (in particular the gyrA gene mutation, conferring variable fluoroquinolone resistance in both *S.* Typhi and *S.* Paratyphi A (Chau et al., 2007)) and the potential for extended-spectrum β-lactamase (ESBL) and carbapenemase-producing strains (Al Naiemi et al., 2008; Pokharel et al., 2006; Nordmann et al., 2008). Rates of illness caused by *S.* Parathyphi and

non–typhoidal *Salmonella* are increasing in many endemic areas further complicating accurate laboratory testing (Ochiai et al., 2005; Palit et al., 2006).

It has long been accepted that vaccines represent the most cost effective approach to control typhoid infection, especially in the era of widespread and increasing antibiotic resistance (Parry et al., 2002; Whitaker et al., 2009). However, few countries have taken up routine typhoid immunization, partially due to uncertainty on disease burden and vaccine effectiveness. The development of cheap and reliable enteric fever diagnostics would play a key role in more accurately defining the scale of the problem and thus facilitating both long-term disease control and individual patient treatment (Baker et al., 2010). A combination of accurate diagnosis, effective vaccination and directed treatment could ultimately lead to the eradication of this human-restricted infection if appropriately implemented. Here we review the current means available for enteric fever diagnosis and the progress being made in improving molecular diagnostics in particular.

2. Clinical diagnosis of enteric fever

Enteric fever may affect individuals of any age; recently it has been shown to affect a much higher proportion of children aged less than 5 years than previously thought, causing a similar range of signs and symptoms to those seen in adults (Sinha et al., 1999). Immunosuppressed individuals, those with reduced gastric acid production, biliary and urinary tract abnormalities, haemoglobinpathies and other concomitant infectious diseases (including malaria and schistosomiasis) are at higher risk of acquiring infection and at risk of developing more severe or disseminated disease (Gotuzzo et al., 1991; Khosla et al., 1993; Mathai et al., 1995; Bhan et al., 2002; Crawford et al., 2010).

The clinical presentation of typhoid fever is notoriously variable, ranging from non-specific fever symptoms to fulminant Gram-negative sepsis with multisystem disease. The incubation period is classically 10 to 14 days although can range from 5 to 21 days. Early evidence suggested that as well as asymptomatic carriers, some individuals are capable of remaining asymptomatic and afebrile despite demonstrable bacteraemia (Snyder et al., 1963). The incubation period is likely to be directly proportional to the inoculum ingested and the cell-mediated immune response of the individual infected, although precise correlates of protection have yet to be determined (Sztein, 2007).

In the early days following infection, individuals may develop diarrhoea and abdominal discomfort. Diarrhoea is thought to be more common in certain geographic areas and in individuals with HIV/AIDS and in children less than 1 year of age (Butler et al., 1991). After a variable asymptomatic duration, individuals may develop constipation (10-38%), abdominal pain (30-40%), headache (often a dull frontal aching, 62%) and fever (Stuart & Pollen, 1946; Clark et al., 2010). Various studies have shown that fever is present in from 75 to 100% of microbiologically-confirmed cases on presentation (Stuart & Pullen, 1946; Butler et al., 1991; Clark et al., 2010); it classically starts low and increases in a saw-toothed pattern, often to between 39 and 40°C by the second week (see figure 1).

The spectrum of symptoms experienced is highly varied, and therefore the diagnosis may be missed particularly in areas where other febrile illnesses, such as malaria, tuberculosis or dengue, are common. Other presentations may include a more 'food poisoning' type illness with diarrhoea and vomiting or a predominantly respiratory presentation with symptoms

including cough and audible crackles on chest auscultation. Other clinical findings of note include a relative bradycardia (Faget's sign, which occurs in less than 50% of patients), hepatosplenomegaly (20 to 50%) and Rose spots (up to 25%), which are classically described as salmon pink evanescent maculopapular spots seen towards the end of the first week of illness on the trunk, and from which *S.* Typhi may be cultured if biopsied (Parry et al., 2002; World Health Organization, 2003).

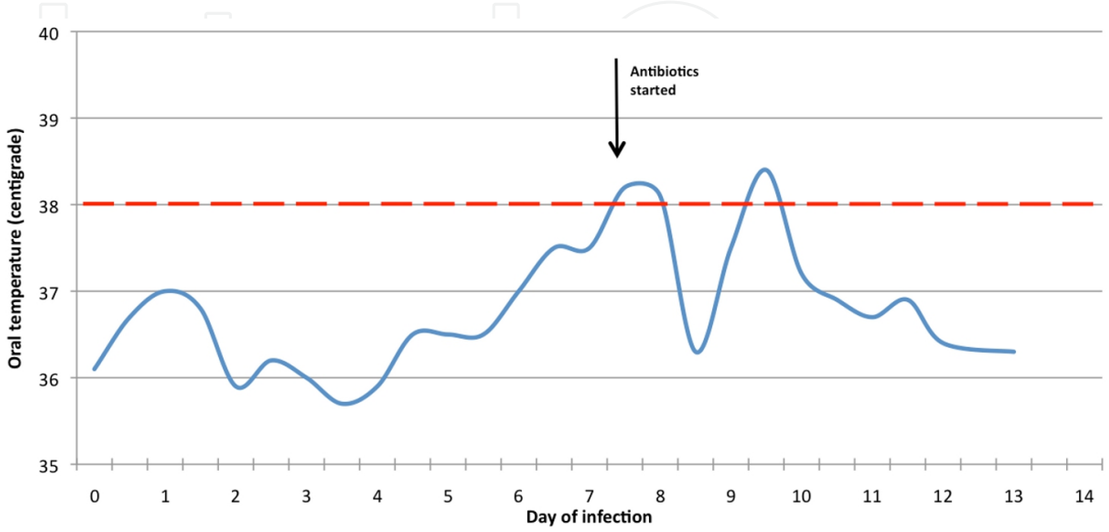

Fig. 1. The variation of oral temperature during typhoid infection

Presentation of neonatal typhoid fever resulting from vertical transmission during late pregnancy is usually within 3 days of delivery; signs including fever, vomiting, diarrhoea, and abdominal distension (Bhan et al., 2005). Significant hepatomegaly and jaundice and seizures can occur (Butler et al., 1991). Typhoid fever typically presents as a milder or atypical illness, often as a severe pneumonia, in children younger than 5 years (Mahle & Levine, 1993). The rate of severe complications is lower than in older age-groups (Mahle & Levine, 1993; Chiu & Lin, 1999; Sinha et al., 1999; Bhan et al., 2005).

Duration of illness before therapy, choice of antimicrobial therapy, strain virulence, inoculum size, previous exposure or vaccination, and other host factors such as HLA type, AIDS or other immune suppression, antacid consumption or concomittant *H. pylori* infection (Bhan et al., 2002) affect severity of the disease. Depending on the clinical resources available, approximately 10–15% of patients may develop more severe disease charaterised by the development of abdominal complications (Bhan et al., 2005). Gastrointestinal bleeding, intestinal perforation and typhoid encephalopathy are the commonest complications (Ali et al., 1997; Parry et al., 2002; World Health Organization, 2003; Bhan et al., 2005). The more details of clinical features of typhoid complications are described in the Seminar by Bhan et al. (Bhan et al., 2005).

Traditionally, the clinical features of paratyphoid fever were thought to be similar or milder than those of typhoid fever. With increasing incidence and more data now available, studies have started to demonstrate an equivalent or even increased rate of complications with paratyphoid infections (Ekdahl et al., 2005; Meltzer et al., 2005; Vollaard et al., 2005; Maskey et al., 2006; Woods et al., 2006). *S.* Paratyphi A, B or C may present with either systemic (Lee

et al., 2000; Rajagopal et al., 2002; Mohanty et al., 2003) or localised infection (Fangtham et al., 2008). A relapse rate of 8% has been reported with *S.* Paratyphi A which is increasing in incidence throughout Southeast Asia (Ochiai et al., 2005; Woods et al., 2006; Fangtham et al., 2008) and may be associated with higher rates of complicated disease and outbreaks of infection (Khan et al., 2007; Pandit et al., 2008; Patel et al., 2010). *S.* Paratyphi A and B may present with a non-specific *Salmonella* gastroenteritis with diarrhoea being a predominant symptom (Thisyakorn et al., 1987; Yang et al., 2010). Gastrointestinal symptoms are usually not present with *S.* Paratyphi C infection but there have been cases with systemic complications such as septicaemia and arthritis (Lang et al., 1992).

3. Laboratory diagnosis of enteric fever

Current widely used methods for the diagnosis of individuals with enteric fever include bacterial culture, microscopy and serological assays, specifically the Widal test, which have been recently reviewed by Bhan et al. (2005), Bhutta (2006), Kundu et al. (2006), Wain & Hosoglu (2008) and Parry et al. (2011). Molecular diagnostics of enteric fever, in particular nucleic acid amplification by polymerase chain reaction (PCR), have been growing rapidly in last decade although they are confined within the research setting.

3.1 Bacterial culture

Accurate diagnosis of enteric fever requires isolation (or detection) of the causative organism, preferably from a sterile site (World Health Organization, 2003). Even though an array of specimens including whole blood, bone marrow, stool, duodenal fluid, urine and skin (Rose spots) (Gilman et al., 1975; Vallenas et al., 1985; Hoffman et al., 1986; Rubin et al., 1990) have historically been shown to harbor cultivable bacteria, blood is the most common specimen submitted for culture of *S.* Typhi (Parry et al., 2002; Wain and Hosoglu 2008). Between 45 and 70% of patients with typhoid fever may be diagnosed by blood culture (World Health Organization, 2003; Wain et al., 2001, 2008). The sensitivity of culture from blood is dependent on a variety of factors including the volume of blood taken (and its ratio to enrichment broth), pre-treatment with antibiotics and delay in transportation of the sample to the laboratory (Wain et al., 2008). As the number of circulating bacteria may be extremely low and predominantly intracellular (over 50% in one study (Wain et al., 2001)), any of these variables may significantly affect the growth and therefore the isolation rate. Use of selective media such as ox bile broth may increase this rate, as, while selective for bile resistant organisms, it inhibits some of the bactericidal activity of blood and is capable of releasing intracellular bacteria (Coleman & Buxton, 1907; Kaye et al., 1966; Wain et al., 2008). Research performed in our laboratory has also confirmed that bile (as ox bile soy tryptone broth) causes selective lysis of mammalian cells whilst leaving bacterial cells intact and capable of unhindered growth in culture (Zhou & Pollard, 2010). Whilst useful for research settings, selective culture of blood in bile-containing media outside of highly endemic regions is unhelpful in the general microbiology laboratory although alternative additives such as saponin have also been investigated (Murray et al., 1991; Wain & Hosoglu 2008; Wain et al., 2008).

Although it is thought that a significant inoculum is required to cause typhoid fever, in those with enhanced susceptibility, ingestion of even a small number of *S.* Typhi organisms may be sufficient to cause infection. Previous studies using a typhoid challenge model in healthy adult volunteers demonstrated that as few as 10^5 organisms were capable of causing disease

following gastric acid suppression using milk (Glynn et al., 1995). In ongoing challenge studies, we have demonstrated that as few as 700 colony forming units (CFU) of non-attenuated live *S.* Typhi may cause clinical illness after gastric acid suppression using sodium bicarbonate. That very low numbers of *S.* Typhi are found circulating in the bloodstream at onset of symptoms in most typhoid cases is therefore not surprising; in 81 patients diagnosed with typhoid fever, a median level of 0.3 (IQR, 0.1-10) bacteria per millilitre of blood was found (Wain et al., 2001). Therefore, one of the key issues in typhoid diagnostics is how to detect the extremely low level bacteraemia present in a sick patient. Even using modern PCR and related diagnostics, current studies often still employ a pre-culture stage in order to try and maximise the organism detection rate (Nga et al., 2010; Zhou & Pollard, 2010).

Bone marrow harbors over 10 times as many organisms per unit volume than in the blood (Wain et al., 2008). Aside from the degree of patient discomfort involved, bone marrow aspiration and culture may therefore represent a useful addition to blood culture if appropriate facilities exist, particularly in patients who have been heavily antibiotic exposed (Wain et al., 2001) or who are being investigated for haematological conditions or pyrexia of unknown origin simultaneously (Volk et al., 1998).

Stool specimens are commonly collected during the diagnostic work-up of patients with typhoid infection, but there may be difficulty in obtaining specimens due to constipation when rectal swabs are a less good alternative. Stool should be cultured in selenite enrichment broth to maximise the culture yield (Moriñigo et al., 1993) for which standard selenite F medium appears at least as effective as selenite supplemented with mannitol (selenite M) (Wain et al., 2008). The results of a positive stool culture need to be interpreted in light of the clinical condition of the patient to exclude healthy carriers (such as `Typhoid Mary') (Soper, 1939). Stool cultures obtained from acutely ill patients may become positive before blood cultures, immediately preceeding either the primary or secondary bacteraemic phase, and their sensitivity increases with the quantity obtained (Personal observations; Wain et al., 2008). Stool cultures are therefore a useful aid to diagnosis and to guide public health prevention activities in certain settings.

Rose spot skin biopsies (Gilman et al., 1975; Wain et al., 1998) and urine samples may also be used for culturing *S.* Typhi, the latter being culture positive in approximately 7% of confirmed cases (Gilman et al., 1975). Duodenal contents obtained using a duodenal string test or aspiration may be more useful for culture identification of causative organisms, but the procedures required are often poorly tolerated, particularly by young children (Vallenas et al., 1985).

Most diagnoses of enteric fever are still made by blood culture followed by microbiological identification. However, blood culture, whilst considered "routine" in most resource-rich settings, is expensive, requiring specialist facilities and personnel, and time-consuming, taking at least 2 to 5 days for organism growth and positive identification.

3.2 Serological tests

Several serological tests have been developed in order to detect the presence of either *S.* Typhi antigens or the antibody response to it. The classic Widal test, a tube agglutination test developed by Widal F. in 1896 (Widal et al., 1896), detects the presence of agglutinating antibodies in the serum of infected/exposed patients against lipopolysaccharide (LPS; O)

and flagella (H) antigens of *S.* Typhi (Olopoenia & King, 2000; World Health Organization, 2003). These antibodies present at 6 to 8 days and 10 to 12 days respectively, following infection; a 4-fold rise in either of these antibodies between acute and convalescent sera is diagnostic (World Health Organization, 2003). The test is only moderately specific for typhoid infection; however, studies from several areas, predominantly endemic for typhoid infection, demonstrate a significant variation in assay performance particularly when using a single Widal test result to make a typhoid fever diagnosis. Reasons for false-positive test results may include previous vaccination or exposure to natural infection, cross-reactivity with epitopes from other enterobacteriaceae or concomittant infections including malaria, typhus and other causes of bacteraemia (Reynolda et al., 1970; Levine et al., 1978; Olopoenia & King, 2000; House et al., 2001; World Health Organization, 2003; Omuse et al., 2010). Likewise, false-negative tests are also seen which may be due to previous antibiotic exposure or other medical conditions capable of reducing the antibody response generated. Widal tests are relatively inexpensive however, particularly in comparison to bacterial culture methods, and are therefore still widely used (Bakr et al., 2011) and are possibly of more benefit in non-endemic settings (Levine et al., 1978; Chew et al., 1992).

Much effort has been put into improving on the classic Widal test over the last twenty years specifically in order to improve the speed and reliability of serological testing (Bhutta & Mansurali, 1999; House et al., 2001; Gasem et al., 2002; Hatta et al., 2002; Jesudason et al., 2002; Olsen et al., 2004; Tam et al., 2008; Fadeel et al., 2011). Several of these assays have subsequently become commerically available; Typhidot® (Malaysian Biodiagnostic Research SDN BHD, Malaysia) and TUBEX assays (IDL Bideh, Solletuna, Sweden) are discussed in further detail below.

Typhidot® is a dot enzyme-linked immunosorbent assay capable of detecting both IgM and IgG antibodies against a *S.* Typhi-specific 50kDa outer membrane protein (OMP) (Ismail et al., 1991; Choo et al., 1994, 1999). OMP dotted onto a nitrocellulose strip is probed with test sera and developed using peroxidase-conjugated antihuman IgM/IgG antibodies and a substrate for colour development (Choo et al., 1994; Kawano et al., 2007).

TUBEX-TF® is an inhibition binding assay that detects the presence of the O9 component of *S.* Typhi LPS. Binding of *S.* Typhi LPS (O9) antibody-coated indicator to *S.* Typhi LPS (antigen)-coated magnetic particles is inhibited by patient sera containing anti-O9 antibodies, which results in a quantitative red-blue colour change (Lim et al., 1998; Oracz et al., 2003). Elevated levels of anti-O9 IgM antibodies together with typical clinical symptoms of typhoid fever probably indicates acute infection with *S.* Typhi (Tam & Lim, 2003; Feleszko et al., 2004; Tam et al., 2008). Subsequent modification of the antigens used has resulted in a similar test for paratyphoid fever which has demonstrated early promise (Tam et al., 2008).

In clinical studies involving small cohorts of hospitalized patients, both the Typhidot and TUBEX tests have demonstrated good performance in clinically suspected typhoid fever cases in comparison to the Widal test, particularly in early infection (Bhutta & Mansurali, 1999; House et al., 2001; Jesudason et al., 2002; Olsen et al., 2004; Begum et al., 2009; Narayanappa et al., 2010). In larger studies both in Asia and Africa, the new generation serological tests have compared less favourably (Dutta et al., 2006; Ley et al., 2011). Data from a large community-based surveillance study in Calcutta from 6697 patients with fever for 3 or more days demonstrated that, using a cut-off of fever for >5 days, the Widal

test was more sensitive overall than the other two tests (Widal sensitivity 67%, specificity 85%, PPV 75%, NPV 79%; Typhidot 59%, 75%, 89% and 33%; Tubex 55%, 81%, 72% and 66%)(Dutta et al., 2006). The Widal test was also significantly cheaper but took longer to produce a result. One concern raised by the authors was that there was relatively poor standardisation of the kit reagents in the two newer tests and this may have had an effect due to the large number of tests performed.

More recently, the Dri-Dot Latex agglutination and IgM lateral flow assays have been developed by KIT Biomedical Research, Royal Tropical Institute, The Netherlands, and are simple to use for diagnosis of enteric fever. The validation study of the Dri-Dot Latex agglutination and IgM lateral flow assays for the diagnosis of typhoid fever, carried out in patients with clinically diagnosed typhoid fever in an Egyptian population, has demonstrated that the sensitivity and specificity were 71.4% and 86.3% for the Dri-Dot, and 80% and 71.4% for IgM Lateral Flow assay, respectively. A major limitation of these serologic tests is the limited sensitivity at the early stage of the disease. The sensitivity of these assays was increased to 84.3% when both tests were performed in parallel but the specificity decreased to 70.5%. Given that these assays are rapid and provide easy-to-interpret results, they may be useful for diagnosis of enteric fever in typhoid-endemic countries (Nakhla et al.,2011; Smith et al., 2011).

In summary, although several alternatives exist for diagnosing typhoid serologically, to-date the newer tests have not improved greatly on the performance of a test that is over a century old. With newer techniques for antigen discovery becoming available and an increasing amount of data being collected regarding the immune response to typhoid and paratyphoid infection, rapid and more effective diagnostic serological tests for typhoid infection are likely to become available in the near future.

3.3 Molecular diagnosis of enteric fever

Detecting the presence of *S.* Typhi in clinical samples using highly sensitive molecular techniques is not a recent development. In the 1980s, Rubin et al. designed and used a DNA probe cloned from *Citrobacter freundii* which has similar Vi antigen to *S.* Typhi for detection of *S.* Typhi and demonstrated 99% specificity and sensitivity using lactose-negative colonies or previously identified bacteria from febrile patients in Peru and in Indonesia (Rubin et al., 1988). As a direct diagnostic method however, the DNA probe method cannot detect less than 500 bacteria per ml of blood; patients with typhoid generally have fewer than 15 *S.* Typhi bacteria per ml (Watson, 1955; Wain et al., 1998). The DNA probe method was refined in a further study (Rubin et al., 1989), in which blood samples (and other specimens including bone marrow aspirates) were taken from patients presenting with febrile symptoms and concentrated by centrifugation using a DuPont Isolator tube, followed by overnight incubation of the bacteria on nylon filters. This modification allowed the detection of *S.* Typhi in 42% (13/32) of samples from patients with culture-confirmed typhoid fever using the equivalent of 2.5 ml of blood, compared with 53% (17/33) of these patients by culture of 8 ml peripheral blood. Additionally the probe detected 4 of 47 patients from whom *S.* Typhi was not isolated by culture, suggesting superior sensitivity could be achieved.

These early studies supported the introduction and development of further nucleic acid amplification tests to enable the rapid detection of very small numbers of bacterial

components, thus providing new tools for sensitive and specific detection, identification and subsequently resistance testing of microorganisms starting from non-cultured sample material. Aside from the significant time saving over standard culture methods and the ability to detect much smaller number of bacteria, as with other organisms, nucleic acid amplification overcomes the issue of non-culturable or dead material, as is often seen with previous antibiotic treatment (Darton et al., 2009; Ho et al., 2009; Rello et al., 2009). After the early studies using DNA probes and hybridization techniques attention was turned to the use of polymerase chain reaction (PCR) methods for the detection of both *S.* Typhi and *S.* Paratyphi A for diagnosis of enteric fever.

3.3.1 Gene targets of PCR based assays for diagnosis of enteric fever

Generally any genomic sequences specific for *S.* Typhi or Paratyphi can be used as the PCR targets, and are easily available from the published DNA data bases. The widely researched targets for *S.* Typhi PCR-based assays include the *S.* Typhi flagellin gene *fliC-d* (Song et al., 1993; Hague et al., 1999, 2001; Kumar et al., 2002; Prakash et al., 2005; Ambati et al., 2007; Hatta & Smits 2007; Nandagopal et al., 2010; Nath et al., 2010), the *viaB* region encoding the Vi antigen of *S.* Typhi (Hashimoto et al., 1995), the *Salmonella* invasion gene *invA* (Cocolin et al., 1998), *hilA* gene encoding a transcription factor of *S.* Typhi (Sánchez-Jiménez & Cardona-Castro, 2004), Vi polysaccharide export ATP-binding protein *vexC* gene (Farrell et al., 2005), ST5 gene (Aziah et al., 2007), an iron-regulated gene *iroB* (Bäumler et al., 1997), 5S-23S spacer region (Zhu et al., 1996), and a heat shock protein *groEL* gene (Nair et al., 2002).

Other gene targets are also used in multiplex PCR assays, including the tyvelose epimerase gene (*tyv*; previously *rfbE*), *fliC-d*, *fliC-a* and the paratose synthase gene (*prt*; previously *rfbS*) (Hirose et al., 2002; Ali et al., 2009), *invA*, *viaB*, *fliC-d* and *prt* (Kumar et al., 2006), the outer membrane protein C (*ompC*), the putative regulatory protein gene STY4220, the intergenic region (SSPAI) between SSPA1723a and SSPA1724 in serovar Paratyphi A, and *stgA* (a fimbrial subunit protein) in serovar Typhi (Ngan et al., 2010), *stkF* (a putative fimbrial protein), *spa2473*, *spa2539*, *hsdM* (DNA methyltransferase) of *S.* Paratyphi (Ou et al., 2007).

Both *S.* Typhi and *S.* Paratyphi A have extremely limited genetic diversity within their populations and between 1 and 3% of the gene content of the *S.* Typhi and *S.* Paratyphi A genomes are unique (Roumagnac et al., 2006). This may aid DNA test specificity over other Gram-negative organisms. Further genomic exploration of both *S.* Typhi and *S.* Paratyphi A will identify new and better targets and then lead to novel nucleic acid based tests.

3.3.2 Sensitivity and specificity of PCR based assays for diagnosis of enteric fever

PCR-based tests for detecting the causative pathogens of enteric fever have developed rapidly over the last decade; however questions regarding the clinical utility and standardization of tests remain. Key to these issues is the array of methodologies used and variable sensitivities and specificities found. Song et al. (1993) was the first to apply PCR for detection of *S.* Typhi in clinical samples in an attempt to overcome the need for a pre-incubation or concentration step. Two pairs of oligonucleotide primers were designed to amplify the Hd flagella gene (*fliC-d*) of *S.* Typhi by nested PCR. This nested PCR had a minimum detection limit of 10 bacteria as determined by dilutions of DNA from *S.* Typhi and proved highly sensitive and specific using both laboratory and clinical samples. *S.* Typhi DNA was detected in 11 of 12 clinical specimens

from patients with confirmed typhoid fever, whereas 10 blood specimens from patients with other febrile disease were all negative. Furthermore, this nested PCR also detected *S.* Typhi DNA from blood samples of 4 patients with suspected typhoid fever on the basis of clinical features but with negative cultures. Since then, many studies on the use of the nested PCR for detection of *S.* Typhi and diagnosis of typhoid fever have been published (Hague et al., 1999, 2001; Kumar et al., 2002; Prakash et al. 2005; Ambati et al., 2007; Hatta & Smits, 2007; Nath et al., 2010; Nandagopal et al., 2010). A nested PCR method was also developed using the *viaB* gene target, but its use in clinical diagnosis of enteric fever remains to be tested even though it demonstrated good sensitivity and specificity in tests performed on DNA samples isolated from clinical bacterial isolates (Hashimoto et al., 1995).

The nested PCR approach significantly improved the detection rate compared to that of blood culture and the Widal test; however its limitations include the longer time taken to perform and the more contaminations in comparison to a conventional PCR assay. Massi et al. utilized just one pair of primers ST1 and ST4 that Song et al. (1993) used for PCR detection of *S.* Typhi, and demonstrated that this single round PCR was also specific and could detect as little as 2-3 copies of *S.* Typhi DNA as determined by serial dilution of genomic DNA from *S.* Typhi (Massi et al., 2003). Using this conventional PCR method, genomic *S.* Typhi DNA was detected in 46 of 73 blood samples collected from patients with clinically suspected typhoid fever who had fever within 3 days of hospitalized admission, and who received no prior antibiotic treatment. PCR compared favourably (63% positivity amongst the clinically suspected cases) to blood culture (13.7%) and the Widal test (35.6%), using these 73 samples. The time taken for PCR analysis of each sample was less than 12 h, rather than 16 h for the nested PCR (Song et al., 1993) and between 3 to 5 days for blood culture.

Conventional PCR generally detects amplification using an agarose gel, which has limitations in sensitivity and speed. Cocolin et al. developed a PCR-microtitre plate hybridization technique for detection of *S.* Typhi *invA* by PCR, and demonstrated enhanced sensitivity and faster availability of results in comparison to a standard agarose gel electrophoresis approach (Cocolin et al., 1998). Other PCR assays were also researched on different gene targets in order to find a rapid and sensitive detection of *S.* Typhi in clinical specimens (Zhu et al., 1996; Bäumler et al., 1997; Nair et al., 2002; Sánchez-Jiménez & Cardona-Castro, 2004; Farrell et al., 2005; Nizami et al., 2006; Aziah et al., 2007).

Real-time PCR (RT-PCR), which is generally detected by measuring a fluorescent signal and has several advantages over conventional PCR has recently been explored, yet not exhaustively, for detection of both *S.* Typhi and *S.* Paratyphi A. Massi et al. applied TaqMan-based real-time PCR (TaqMan assay) to the quantification of *S.* Typhi in the blood of patients suspected of having typhoid fever by targeting the *S.* Typhi flagellin gene in genomic DNAs isolated from blood samples (Massi et al., 2005). Of 55 blood samples taken from suspected typhoid fever patients, eight blood samples with a positive blood culture had *S.* Typhi loads ranging from 1.01×10^3 to 4.35×10^4 copies/ml blood, and from 47 blood samples with negative blood culture, there were 40 (85.1%) TaqMan assay-positive samples with loads ranging from 3.9 to 9.9×10^2 copies/ml blood. In their study, the TaqMan assay detected more than 10^3 copies/ml blood of *S.* Typhi in all of the blood culture-positive samples, whereas less than 10^3 copies/ml blood of *S.* Typhi were detected in the blood culture-negative samples. This suggests that a TaqMan assay may be useful for assessing *S.* Typhi loads, especially in cases of suspected typhoid fever with negative results from the standard blood culture test.

Farrell et al. developed broad-range (Pan) *Salmonella* and *S.* Typhi specific real-time PCR assays using LightCycler (Roche Diagnostics, Indianapolis, IN). Using direct stool samples the pan-*Salmonella* assay was validated with 96% (53/55) sensitivity and 96% (49/51) specificity. However, the *S.* Typhi-specific PCR assay was not sufficiently validated due to the low incidence of *S.* Typhi infections in the test region (Farrel et al., 2005).

All these studies demonstrated that the sensitivity and specificity of PCR assays was significantly better compared to that of blood culture and/or the Widal test, and some selected evaluation studies of these tests are summarized in Table 1.

Test used	Target gene	Samples (n) tested		Blood culture	PCR	Widal test	Reference
nested PCR	*fliC-d*	suspected	16	12BC+ 4BC-	11/12BC+ 4/4BC-		Song et al. 1993
		control	10 febrile		0/10		
nested PCR	*fliC-d*	suspected	55	8BC+ 47BC-	8/8BC+ 24/47BC-	6/8BC+ 23/47BC-	Hague et al. 2001
		control	20 nonfebrile		0/20	9/20	
nested PCR	*fliC-d*	suspected	40	20BC+ 20BC-	20/20BC+ 12/20BC-		Kumar et al. 2002
		control	None				
nested PCR	*fliC-d*	suspected	63	17BC+ 46BC-	17/17BC+ 36/46BC-	12/17BC+ 4/46BC-	Prakash et al. 2005
		Control	25 nonfebrile		0/25	1/25	
nested PCR	*fliC-d*	suspected	119	68BC+ 51BC-	67/68BC+ 26/51BC-	34/68BC+ 11/51BC-	Hatta & Smiths 2007
		control	12 febrile		0/12	4/12	
nested PCR	*fliC-d*	suspected	42	14BC+ 38BC-	14/14BC+ 29/38BC-	7/14BC+ 19/38BC-	Ambati et al. 2007
		control	11 febrile 8 nonfebrile		0/11 0/8	2/11 0/8	
nested PCR	*fliC-d*	suspected	291	6BC+ 285BC-	6/6BC+ 8/285BC-		Nandagopal et al. 2010
		control	10 febrile		0/10		
PCR	*viaB*	suspected	203	26 BC+ 177BC-	10/26BC+ 12/177BC-		Nizami et al. 2006
		control	None				
PCR	*hilA*	suspected	37	34BC+ 3BC-	34/34BC+ 3/3BC-		Sánchez-Jiménez & Cardona-Castro 2004
		control	35 infected with other pathogens 150 healthy volunteers		0/35 0/150		

Test used	Target gene	Samples (n) tested		Blood culture	PCR	Widal test	Reference
PCR	*fliC-d*	suspected	82	28BC+	59/82		Haque et al. 1999
		control	20 nonfebrile		0/20		
PCR	*fliC-d*	suspected	73	10BC+ 63BC-	10/10BC+ 36/63BC-	10/10BC+ 16/63BC-	Massi et al. 2003
		Control	None				
PCR	ST-50	suspected	33BC+ broths		29/33		Aziah et al. 2007
		control	40BC- broths		0/40		
PCR	*fliC-d*	suspected	820	78BC+ 742BC-	73/78BC+ 95/742BC-		Chaudhry et al. 2010
		control	None				
RT-PCR	*fliC-d*	suspected	55	8BC+ 47BC-	8/8BC+ 40/47BC-		Massi et al. 2005
		control	26 nonfebrile		0/26		

BC: Blood culture; BC+: Blood culture positive; BC-: Blood culture negative

Table 1. The results of selected studies on the sensitivity and specificity of PCR, blood culture and Widal test on blood samples from patients with suspected enteric fever

3.3.3 Multiplex PCR detection for *S.* Typhi and *S.* Paratyphi

Classically *S.* Typhi has been considered as the major cause of enteric fever; however, in recent years *S.* Paratyphi and Vi-negative variants of *S.* Typhi have emerged rapidly (Wain et al., 2005; Dong et al., 2010). *S.* Paratyphi A is a causative agent of paratyphoid fever and has become a major cause of enteric fever in Asia. For example, more than 80% of enteric fever outbreaks have been caused by *S.* Paratyphi since 1998, three years after Vi polysaccharide typhoid fever vaccine was introduced in Guangxi province China (Dong et al., 2010). The largest one (495 episodes), which occurred in 2004 in Luocheng County, was caused by a contaminated water supply system. *S.* Paratyphi has been the predominant cause of enteric fever in Guangxi province China since 1999 (Dong et al., 2010). Studies from India and Nepal also suggested that paratyphoid fever caused by *S.* Paratyphi A can contribute up to half of all cases of enteric fever in some settings (Ochiai et al., 2005; Woods et al., 2006). PCR tests using *S.* Typhi specific primers appear to be sensitive to detect typhoid fever, but cannot detect paratyphoid fever. Recent developments in multiplex PCR methods have addressed the issue of paratyphoid as well as typhoid fever diagnosis.

Hirose et al. developed a complex PCR using the primers for O, H, and Vi antigen genes, *tyv* (*rfbE*), *prt* (*rfbS*), *fliC-d*, *fliC-a*, and *viaB*, for the rapid identification of *S.* Typhi and *S.* Paratyphi A. This assay was able to accurately identify and distinguish *S.* Typhi and *S.* Paratyphi A from laboratory isolates; however, its clinical use was not assessed (Hirose et al., 2002). Similarly, Levy et al. developed a multiplex PCR to identify *Salmonella* serogroups A, B and D, and Vi-positive strains. Blinded testing of 664 Malian and Chilean *Salmonella* blood isolates demonstrated 100% sensitivity and specificity; again clinical utility was not assessed (Levy et al. 2008). Kumar et al. explored another set of target genes including those

responsible for invasion (*invA*), O (*prt*), H (*fliC-d*) and Vi (*viaB*) antigen genes in a multiplex PCR, and demonstrated accurate identification of laboratory isolates and 100% detection probability when a cell suspension of 10^4 CFU/ml (500 CFU per reaction) was used. *S.* Typhi bacteria were artificially inoculated into water and food (milk and meat rinse) samples and detected by the multiplex PCR after overnight pre-enrichment in buffered peptone water. No *Salmonella* bacteria could be detected from water samples collected from the field by the multiplex PCR or standard culture method (Kumar et al., 2006).

Using the same target genes as Hirose et al. (Hirose et al., 2002), Ali et al. further optimised the primers and applied the nested multiplex PCR directly to clinical blood specimens for diagnosis. Of 42 multiplex PCR-positive blood samples, they showed that 26, 9, and 2 were Vi-positive *S.* Typhi, Vi-negative *S.* Typhi and *S.* Paratyphi A, respectively, and five patients had a mixed infection. Tests with several common pathogens confirmed that the assay was specific (Ali et al., 2009).

The analysis of the genome of *S.* Paratyphi led Ou et al. to identify four gene targets (*stkF*, *spa2473*, *spa2539* and *hsdM)* which were used to develop a highly discriminatory multiplex PCR assay (Ou et al., 2007). A valuation study using spiked blood and stool samples demonstrated that the sensitivity of the discriminatory multiplex PCR was 1 x 10^5 CFU/ml and 2 x 10^5 CFU/ml, respectively, and however, the sensitivity can be increased to 1 x 10^4 CFU/ml and 2 x 10^3 CFU/ml after 5 h culture enrichment (Teh et al., 2008). Nagarajan et al. have further improved upon the existing PCR-based diagnostic technique by using one pair of primers that is unique to *S.* Typhi and *S.* Paratyphi A, corresponding to the STY0312 gene in *S.* Typhi and its homolog SPA2476 in *S.* Paratyphi A, and another pair that amplifies the region in *S.* Typhi CT18 and *S.* Typhi Ty2 corresponding to the region between genes STY0313 to STY0316 but which is absent in *S.* Paratyphi A. The possibility of a false-negative result arising due to mutation in hypervariable genes has been reduced by targeting a gene unique to typhoidal *Salmonella* serovars as a diagnostic marker. This set of primers can also differentiate between *S.* Typhi CT18, *S.* Typhi Ty2, and *S.* Paratyphi A, which have stable deletions in this specific locus. The PCR assay designed in this study has a sensitivity of 95% compared to the Widal test which has a sensitivity of only 63% (Nagarajan et al., 2009). Ngan et al. developed another multiplex PCR format in which the outer membrane protein C (*ompC*) was used for detection of members of the *Salmonella* genus, the putative regulatory protein gene STY4220 for the presence of either *S.* Typhi or *S.* Paratyphi A, and the intergenic region (SSPAI) between SSPA1723a and SSPA1724 in serovar Paratyphi A and a fimbrial subunit protein *(stgA*) in serovar Typhi for differentiation between *S.* Typhi and *S.* Paratyphi. This multiplex PCR was evaluated using 124 clinical and reference *Salmonella* serovars and both *S.* Typhi and *S.* Paratyphi A were detected at 100% specificity and sensitivity. This multiplex PCR reaction can detect approximately 1 pg of *Salmonella* genomic DNA. When tested on 8 h enriched spiked blood samples of serovars Typhi and Paratyphi A, the sensitivity was estimated at 4.5 x 10^4 - 5.5 x 10^4 CFU/ml, with similar detection levels observed for spiked fecal samples (Ngan et al., 2010).

Recently Nga et al. used a novel multiplex three colour real-time PCR assay to detect specific target sequences in the genomes of *S.* Typhi and *S.* Paratyphi A. The assay was validated and demonstrated a high level of specificity and reproducibility under experimental conditions with the DNA extracted from blood and bone marrow samples

from culture positive and negative enteric fever patients. All bone marrow samples tested were positive for *Salmonella*; however, the sensitivity on blood samples was limited. The assay demonstrated an overall specificity of 100% (75/75) and sensitivity of 53.9% (69/128) on biological samples. The data on the PCR detection limit suggested that PCR performed directly on blood samples may be an unsuitable methodology and a potentially unachievable target for the routine diagnosis of enteric fever because the bacterial load of *S.* Typhi in peripheral blood is low, often below the limit of detection by culture and, consequently, below detection by PCR (Nga et al., 2010).

3.3.4 Novel blood culture PCR system and application in human challenge study

An alternative strategy to increase the sensitivity and specificity of PCR is PCR amplification on the blood culture after a short period of incubation. We have recently developed a fast and highly sensitive blood culture PCR method for detection of *Salmonella* serovar Typhi (Zhou & Pollard, 2010). The method uses an optimised ox bile tryptone soy broth for blood culture with subsequent PCR assay in an attempt to reduce the turn-around time for diagnosis and increase diagnostic sensitivity. By using a 5-hour incubation, 3 CFU of *S.* Typhi cells could mutliply over about 10 generations. This was assessed by a time-course experiment, the results of which were published (Zhou & Pollard, 2010) and are cited here in Table 2.

Incubation time (hour)	CFU[a]	*filC-d* amplicons[b]
0	3	---
1	4	---
2	17	----
3	105	+++
4	209	+++
5	4461	+++

*Three bacteria of *Salmonella* serovar Typhi were incubated in the tryptone soy broth containing 2.4% ox bile and 20% blood. [a] The mean of three independent experiments; [b] *Salmonella* serovar Typhi *fliC-d* amplicons resulting from PCR using the DNA templates prepared from three independent cultures.

Table 2. The growth and PCR detection of *S.* Typhi in ox bile tryptone soy broth blood culture*.

The sensitivity of this blood culture-PCR method was equivalent to 0.75 CFU per millilitre of blood which is similar to the level of clinical typhoid samples which regular PCR cannot detect. The whole blood culture PCR assay takes less than 8 hours to complete rather than several days for conventional blood culture. This novel blood culture PCR method is superior in speed and sensitivity to both conventional blood culture and PCR assays. Its use in clinical diagnosis may allow early detection of the causative organism and facilitate initiation of prompt treatment among patients with typhoid fever. The recent use of this novel culture PCR method to our ongoing human typhoid challenge studies has proved that the advantage of combining culture and PCR amplification is an increase in the speed of a positive confirmatory diagnosis, even though it is unlikely to produce a greater level of sensitivity than that of traditional culture alone. However, practical clinical use in diagnosing enteric fever of this culture PCR system remains to be proved, in particular, using blood samples with antibiotic pre-treatment.

4. Future perspectives

Blood culture has some distinct advantage over other diagnostic methods, such as the combination of bacterial identification with antibiotic susceptibility, and an unquestioned role in providing epidemiological data; however, it has many problems related to its relatively long turnaround time and low sensitivity, especially in patients receiving antibiotic treatment. Detection of bacterial DNA in whole blood by PCR assay is the methodology most able to substantially decrease the turnaround time without bias from the inhibitory effect of antibiotics, yet the published PCR assays for diagnosis of enteric fever are in limited use. Further investigation to develop rapid and reliable diagnostics for enteric fever are urgently needed.

One of the limiting factors in the use of current PCR methodology in clinical diagnosis of enteric fever is the low number of bacteria circulating in the blood of enteric fever patients. Advancement in the use of PCR would require the capture and amplification of a smaller number of bacteria (maybe even a single organism) in blood or other bodily fluids. Such a task is not insurmountable but it will be a challenge to make it cost effective (Baker et al., 2010). An alternative approach to increase the PCR assay sensitivity and specificity is to remove the interfering human genomic DNA present in the samples. To achieve this, selective lysis of human genomic DNA with external nuclease may be usful, as proven in pathogen identification in patients with sepsis (Horz et al., 2008; Handschur et al., 2009). Removal of dominant human genomic DNA causes enrichment of bacterial DNA, thus improving sensitivity and specificity of PCR assays. Using *S.* Typhi spiked blood samples, we have demonstrated that this approach can increase the sensitivity of PCR assays by more than 1,000 fold (unpublished result). However, a field trial with clinical typhoid specimens is needed to confirm the laboratory findings. Reverse transcription PCR may be another choice to detect such a low number of bacteria in typhoid patient blood as the higher number of copies of mRNA for a specific gene target could increase the PCR assay sensitivity. The *fliC* of *S.* Typhi was used as target in the reverse transcription-multiplex PCR assay for simultaneous detection of *Escherichia coli* O157:H7, *Vibrio cholerae* O1 and *S.* Typhi (Morin et al., 2004).

The study on host specific responses to enteric fever may identify signatures of host-pathogen interactions with *S.* Typhi, which will form the basis of development of new molecular diagnostics for enteric fever. Activation of host specific genes or pathways during infection could be identified using DNA microarrays; a physiological signature or metabolic product associated with typhoid could be studied with mass spectrometry or other proteomic technologies. For example, surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectroscopy has been used in identifying SARS protein biomarkers (Mazzulli et al., 2005). All these new technological approaches may add insight into proteins as biomarkers of typhoid infection, and potentially result in a new generation of novel molecular diagnostics for enteric fever.

Enteric fever is endemic in resource poor countries, and development in new technologies should focus on how these can be applied to location with limited resources. Efforts are being made to simplify typhoid PCR assays using pre-prepared and freeze-dried regents (Aziah et al., 2007). However, new PCR technologies, such as isothermal PCR, are of particularly practical use in the diagnosis of enteric fever, as these methods allow for the

possibility of developing less-complicated and less-expensive machinery than is necessary for conventional PCR. Several isothermal PCR technologies have been developed (Gill & Ghaemi, 2008), including strand displacement amplification (SDA) (Walker et al., 1992), loop-mediated amplification (LAMP) (Notomi et al., 2000), and helicase-dependent amplification (HDA) (Vincent et al., 2004). Recently, Francois et al. have examined the robustness of LAMP for bacterial diagnostic applications using *S.* Typhi as the target organism (Francois et al., 2011), and demonstrated that LAMP is more sensitive than conventional qPCR and is also a very robust, innovative and powerful molecular diagnostic method. However, SDA, HDA and/or other isothermal amplification methods could be more advantageous over LAMP in multiplex amplifications. The recent surge in paratyphoid disease makes it necessary to develop new diagnostics for detection of both S. Typhi and Paratyphi. Another advantage of isothermal PCR is its potential for use in resource poor or point-of-care settings.

In summary, advancement in genomics and proteomics will further our understanding of molecular pathogenesis of enteric fever, and eventually lead to identification of new targets which could form the basis for new molecular diagnostics. With progress in new technologies, we expect that a new generation of fast and sensitive molecular diagnostics for enteric fever will be developed in the near future.

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