Rapid diagnosis of typhoid fever by an in-house flagellin PCR

Enteric fever is an important public-health problem in developing countries. Effective control of the disease requires early detection of cases, definitive treatment and elimination of the source of infection, as well as improvement of the public-health system. Symptoms and signs of the disease are non-specific and laboratory testing is essential for diagnosis. Accurate diagnosis of cases is often not possible as empirical antibiotic therapy decreases the sensitivity of isolation procedures and serological tests lack specificity in endemic areas. Enteric fever is a common cause of morbidity in the Indian subcontinent and *Salmonella* Typhi accounts for approximately 90 % of infections. *Salmonella* Paratyphi A is less common but has shown a trend of rising incidence over the past few years, whereas *S. Paratyphi* B is exceedingly rare. Typhoid fever is an acute systemic infection caused by the bacterium *S. Typhi*. Worldwide an estimated 21.5 million people develop typhoid fever annually. The diagnosis of typhoid fever still relies on conventional serology and blood culture identification systems. The isolation procedures are very time consuming and expensive. PCR assays facilitate prompt and appropriate treatment of confirmed cases and also help in the detection of carriers thereby limiting spread.

Based on the flagellin gene sequence, we previously developed an in-house PCR assay for the early and rapid detection of *S. Typhi* in clinical samples. This assay utilizes primers specific to unique sequences in the VI–VIII region of the dH flagellin gene. We reported our results about a decade ago and our work was among the earliest of the small number of published reports available at that time (Chaudhry *et al.*, 1997; Frankel *et al.*, 1989). Over the years we have been using this assay for diagnostic purposes and here we present data compiled over the last decade. The advanced molecular assays like nested PCR, multiplex and real-time PCR have been the method of choice over the years owing to their better sensitivity.

However, in our experience, conventional PCR is equally sensitive, less time consuming and less expertise is required compared with the advanced molecular methods for detecting *S. Typhi* (Chaudhry *et al.*, 1997).

A total number of 820 patients suspected of having typhoid fever, belonging to all age groups, were included in the study. The samples were collected at the All India Institute of Medical Sciences, New Delhi, India, during the last decade. Ethical approval for conducting this study was received from the institutional human ethical committee. Blood samples were collected from the patients for use in culture and PCR. A 5 ml aliquot of blood in the case of adults and 2.5 ml in the case of the paediatric age group was inoculated in to brain heart infusion broth for culture. A 2 ml aliquot of blood was taken in citrate vials for PCR. Blood was cultured by standard techniques and the isolates were identified using Gram stain and then by standard biochemical tests.

For PCR, DNA was extracted using a QIAamp blood kit (Qiagen). A 200 µl blood sample was processed as per the kit instructions by column purification for effective removal of inhibitory substances.

A 10 µl aliquot of this extract was used for PCRs. The RK1 (5'-TGG GCG ACG ATT TCT ATG CC-3') and RK2 (5'-TTT CGC GAA CCT GGT TAG CC-3') primers used (Chaudhry *et al.*, 1997) generated a 486 bp amplicon. Amplification was carried out with 20 pmol each primer in 25 µl PCR mixture containing 0.625 units *Taq* DNA polymerase, 2.5 mM MgCl₂, 200 µM dNTP and 1 × PCR buffer (Perkin Elmer), with 10 µl DNA added to each tube, and the reactions were performed in a DNA thermal cycler (MJ Research) as follows: 40 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min 15 s, elongation at 72 °C for 3 min; and a final extension of 5 min at 72 °C. PCR products were visualized in 1.2 % agarose gel stained with ethidium bromide (0.5 µg ml⁻¹) (Fig. 1). A positive and a negative control were systematically run in parallel. This PCR is specific only for *S. Typhi*. It does not amplify *S. Paratyphi* A and B.

Out of the 820 samples tested, 168 were positive for *S. Typhi* by PCR, while only 73 of these showed growth on culture after 48–72 h of incubation. Apart from five cases, all the PCR-negative samples were sterile on blood culture following a week of incubation (Table 1). The sensitivity and specificity of this assay was assessed to be

Fig. 1. *S. Typhi*-specific 486 bp amplicon in clinical specimens. Lane 1, standard strain (486 bp); lane 6, 1 kb ladder DNA marker (the 500 bp band is indicated); lane 2, 3, 4, 5, 7, 8, 9 and 10, clinical samples.

Table 1. Detection of *S. Typhi* in blood samples: comparison of blood culture with PCR detection

	Blood culture		Total no.
	Positive	Negative	
PCR positive	73	95	168
PCR negative	5*	647	652
Total	78	742	820

*Blood sample stored for a long time in citrate.

93.58 and 87.19 %, respectively, when compared with blood culture as the gold standard, with positive and negative predictive values of 43.45 and 99.23 %, respectively.

PCR has been used to detect *Salmonella* serovars in samples from different sources of animal origin (Oliveira *et al.*, 2002; Patel & Bhagwat, 2008). A DNA-based assay based on alleles of genes encoding the three phases of flagellar H antigen to identify serovar-specific antigens has also been reported to show partial success (McQuiston *et al.*, 2000). Another PCR assay that uses the 16S and 23S ribosomal spacer region to facilitate the discrimination of serovars has been documented (Bakshi *et al.*, 2002). The applicability of multiplex PCR amplifying multiple genes in a single reaction that detects the phage type DT104 by amplifying two virulence determinants and one or more resistance genes has been described (Carlson *et al.*, 1999). A similar multiplex PCR for the specific detection of *Salmonella*, targeting multiple gene loci, was also applied to environmental samples (Way *et al.*, 1993). Multiplex PCR has also been used in many other studies on various samples from humans and animals (Lee *et al.*, 2009).

Real-time PCR has the advantages of a shorter processing time and lower cost by eliminating post-amplification gel analysis. It has increased throughput and reduces the chances of carryover contamination. It also enables quantitative analysis of gene products, predicting the actual pathogen load in the clinical sample (Heid *et al.*, 1996). The real-time PCR principle has

also been extended to multiplex reactions using many sequence-specific probes called molecular beacons (Chen *et al.*, 2000). Since this approach is highly discriminatory and specific, it enables the detection of as few as 2 c.f.u. per PCR.

Although recently there has been more focus on these more advanced molecular methods, in our experience, conventional PCR gives results that are as good, with the advantage of simplicity and a lesser requirement for expertise. This we concluded based on our earlier study where we found sensitivity with one round of PCR to be very high (about 28 pg). The sensitivity and specificity of the assay in that study was estimated to be 100 and 93.7 %, respectively, with positive and negative predictive values estimated at 84 and 100 %, respectively (Chaudhry *et al.*, 1997). Our observations have been consistent with previous studies, which inferred that various PCR methods have a better sensitivity than blood culture for the diagnosis of typhoid (Hatta & Smits, 2007). The efficacy of blood culture decreases with the duration of illness, and the use of antibiotics before collection of blood samples severely affects the isolation rate (Coleman & Buxton, 1907; Wain *et al.*, 2001). Our PCR results were ready in less than 10 h, compared with 3–5 days for blood culture confirmation. Our results suggest that PCR-based diagnosis is particularly useful for all clinically suspected cases of typhoid fever caused by *S. Typhi*, mainly those with problematic and varying clinical manifestations. Moreover, several additional cases of typhoid fever, not detected in culture, were also identified by PCR. The follow-up of PCR positive/culture negative cases revealed a good therapeutic response to specific antibiotic therapy as reported by clinicians. The technique therefore seems reliable and simple, and enables early and accurate detection of *S. Typhi* in human clinical samples. However, this PCR does not detect *S. Paratyphi* A and B for which we have earlier reported a multiplex PCR (Chaudhry *et al.*, 2005) to simultaneously detect both *S. Typhi* and *S. Paratyphi*.

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