a normal cerebrospinal fluid examination. Severe *C jejuni* infections have been reported in patients with immunodeficiency,[5] such as HIV infection.[6] Only one case of *C jejuni* septicaemia occuring in a post-splenectomy patient has been reported previously, and that patient was also an iron overloaded thalassaemic with chronic liver disease who also had diabetes mellitus.[7] There appears to be no obvious reason why *C jejuni* would show a predilection for growth in such patients, unlike *Yersinia* spp which grows more readily in iron overloaded patients.[8] Septicaemic shock is thought to occur in severe *C jejuni* infections because of the bacteria's lipopolysaccharide content,[9] and perhaps because of the secretion of an enterotoxin.[10]

Thalassaemia is a common indication for splenectomy, and these patients may be immunocompromised in other ways, for example by the effects of multiple blood transfusions, viral infection, iron overload, or diabetes mellitus. In addition, the presence of chronic liver disease and impaired cardiac function in this patient would have impaired his ability to counter the infection and withstand its effects. Our patient was given gentamicin (to which the organism was sensitive) from the time of admission, but he still died owing to a combination of septicaemic shock and hepatic encephalopathy. Although the dose of gentamicin was as recommended (5 mg/kg/day), no loading dose was given and the post-dose serum concentration was subtherapeutic. These factors may also have contributed to the poor clinical response. The possibility of a campylobacter septicaemia should be considered when a splenectomised thalassaemia patient presents with high grade fever, and appropriate high dose antibiotic coverage should be instituted.

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Standardisation of polymerase chain reaction for the detection of *Salmonella typhi* in typhoid fever

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Accepted for publication
22 January 1997

Abstract

To improve the diagnosis of *Salmonella typhi* infection, a polymerase chain reaction (PCR) assay was developed for the amplification of the dH flagellin gene of *S typhi*. Primers were designed from dH flagellin gene sequence which will give an amplification product of 486 base pairs. In tests to study the specificity of the assay, no amplification was seen in non-salmonella strains or salmonella strains with flagellar gene other than "d". Sensitivity tests determined that 28 pg of *S typhi* target DNA or 3×10^2 target bacteria could be detected by the PCR assay. Subsequently, the PCR technique was used for detection of *S typhi* in blood or clot cultures from 84 patients clinically suspected of having typhoid fever, and from 20 healthy control subjects. Twenty five of 84 samples from clinically suspected cases were positive by PCR; four of which were culture negative. No amplification was seen in samples from patients who were culture positive for organisms other than *S typhi* or from controls. The time taken for each sample for PCR analysis was less than 48 hours compared with three to five days for blood or clot culture. PCR appeared to be a promising diagnostic test for typhoid fever.
(*J Clin Pathol* 1997;**50**:437–439)

Keywords: *Salmonella typhi*; polymerase chain reaction; typhoid fever

Typhoid fever, a septicaemic disease caused by *Salmonella typhi*, is a serious health problem in developing countries.[1 2] Diagnosis of typhoid fever currently relies on blood culture and Widal's test. Blood cultures are negative in 30–65% of cases with typhoid fever because of

Table 1 Bacterial strains used to develop a PCR assay for the diagnosis of typhoid fever

Species	*Flagellar type*	*Sources*
Salmonella strains		
S typhi (6)	d	AIIMS
S livingstone	d, i, w	CRI, Kasauli
S stanlay	d, i, z	CRI, Kasauli
S schwarzangrund	d	CRI, Kasauli
S paratyphi A	a	AIIMS
S paratyphi B	b	CRI, Kasauli
S paratyphi C	c	CRI, Kasauli
S typhimurium	i	CRI, Kasauli
S cholerasuis	c	CRI, Kasauli
S enteritidis	g, m	CRI, Kasauli
S senftenberg (JT1502)	I, s, t	CRI, Kasauli
Non-salmonella strains		
Escherichia coli		AIIMS
Klebsiella pneumoniae		AIIMS
Klebsiella spp		AIIMS
Staphylococcus aureus		AIIMS
Pseudomonas aeruginosa		AIIMS
Enterobacter spp		AIIMS
Bacteriodes melaninogenicus		AIIMS
Clostridium perfringens		AIIMS
Acinetobacter spp		AIIMS
Diphtheroids		AIIMS

AIIMS, All India Institute of Medical Sciences; CRI, Central Research Institute.

prior administration of antibiotics or a low number of organisms.[3-5] Negative blood culture reports in patients with typhoid fever underestimates the actual incidence of disease. Widal's test has been found to be non-specific and difficult to interpret in areas where typhoid fever is endemic.[6 7] No non-cultural test for typhoid fever has been consistently shown to be sufficiently sensitive and specific.[1] There is, therefore, a need to develop a highly sensitive and specific method for the diagnosis of patients with negative blood cultures. We report the development and evaluation of a polymerase chain reaction (PCR) assay to detect *S typhi* from peripheral blood of patients with typhoid fever by amplification of dH flagellin gene.

Methods

Six *S typhi* strains and 10 non-salmonella strains were grown overnight in Luria broth (table 1) and tested to study the specificity of the PCR assay. S901 (motile), a standard strain of *S typhi* was used as positive control. The primers were designed based on published dH flagellin gene sequence[2] using Oligo Computer Program. To investigate the sensitivity of the system, overnight culture of S901 was titrated by counting colonies on nutrient agar plates after 10-fold serial dilution of organisms ranging from 10^6 to 10^1; DNA extracted from *S typhi* was serially diluted to determine the minimum amount of DNA detectable by PCR.

Blood samples were collected from 84 patients with clinically suspected typhoid fever attending our institution as well as from 20 normal healthy individuals to be used as negative controls. Serum was removed from 5 ml of blood collected in a sterile tube. The clot was then added to 5 ml of 10% bile broth. Clots were broken by vortexing with sterile glass beads for five minutes and incubated overnight at 37°C. The following morning DNA was extracted by boiling method.[8] For 36 samples, DNA was extracted from citrated blood by lysis method using Proteinase K and Triton X-100.[9] The target for amplification was the dH flagellin gene. A 486 base pair region was amplified with specific primers RK1 (5' TGG GCG ACG ATT TCT ATG CC 3') and RK2 (5' TTT CGC GAA CCT GGT TAG CC 3'). Amplification was carried out with 50 pmol of each primer in 25 µl of the PCR solution containing 0.625 units of Taq DNA polymerase, 2.5 mM $MgCl_2$, 200 µm each of dATP, dCTP, dTTP, dGTP, and 1 × PCR buffer (Perkin Elmer, Rotkreuz, Switzerland). The reactions were performed in a DNA thermocycler (MJ Research Inc, Massachusetts, USA) as follows: 40 cycles of denaturation at 94°C for one minute, annealing at 57°C for one minute 15 seconds, elongation at 72°C for three minutes, and final extension of five minutes at 72°C. PCR products were visualised on a 1.2% agarose gel stained with ethidium bromide.

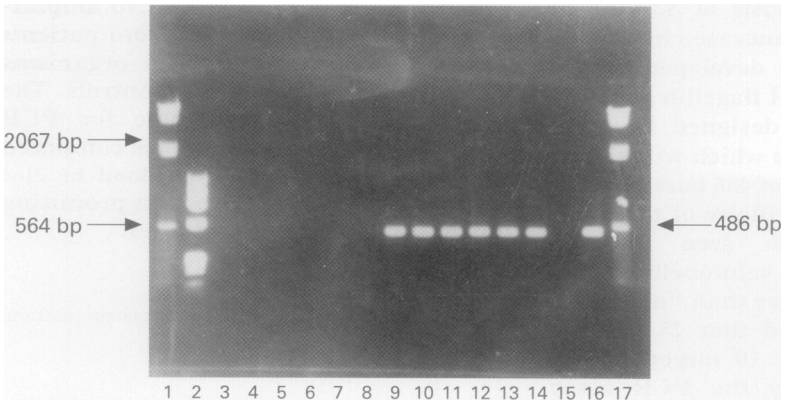

Figure 1 Specificity of the PCR assay for the detection of the flagellin gene of S typhi. *Lane 1 and 17,* Hind *III digest marker; Lane 2, X174 marker; Lane 3,* Escherichia coli*; Lane 4,* Staphylococcus aureus*; Lane 5,* Pseudomonas aeruginosa*; Lane 6,* Salmonella paratyphi *A; Lane 7; acinetobacter; Lane 8,* S paratyphi *B; Lane 9,* S typhi *I; Lane 10,* S typhi *II; Lane 11,* S typhi *III; Lane 12,* S typhi *IV; Lane 13,* S typhi *I (DNA extracted 10 days previously); Lane 14,* S typhi *II (DNA extracted 10 days previously); Lane 15, water (no DNA); Lane 16,* S typhi *S901 (mobile) reference strain.*

Results

PCR with RK1 and RK2 primers was found to be specific for dH flagellin gene, amplifying the *S typhi* specific band of 486 base pairs. No amplification was seen in any non-salmonella strain or the other seven *Salmonella* spp with flagellar gene other than "d", or in DNA extracted from humans (fig 1). We also investigated other *Salmonella* spp containing dH flagellin gene—*S stanley*, *S livingstone*, and *S Schwarzangrund*—and amplification of the 486 base pair segment was seen.

The minimum number of organisms detected by PCR was 3×10^2. The amount of DNA which could be detected after serial dilution was 28 pg (fig 2).

Of 84 patients with clinically suspected typhoid fever, 21 were both culture and PCR positive for *S typhi*. Four additional cases were. positive by PCR but culture negative after 48 hours (table 2). No amplification was seen in patients that were culture positive for organisms other than *S typhi* or in controls

Discussion

Isolation of *S typhi* from blood is the most satisfactory method of diagnosis in the early stage of typhoid fever as bacteraemia is present dur-

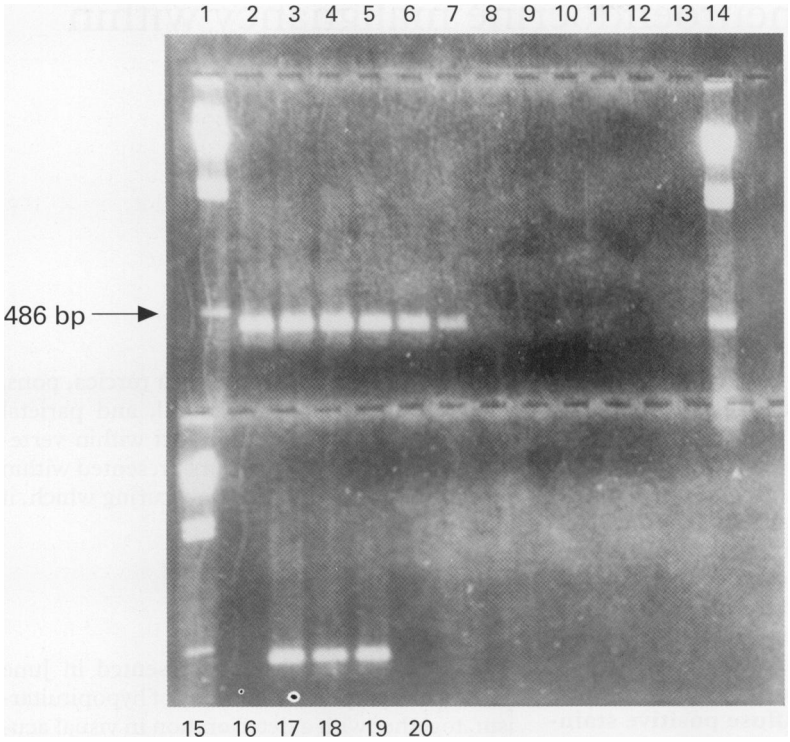

Figure 2 Sensitivity of the PCR assay with serially diluted DNA from S typhi S901. Lanes 1, 14, and 15, marker; Lane 2, 2800 ng; Lane 3, 280 ng; Lane 4, 28 ng; Lane 5, 2.8 ng; Lane 6, 280 pg; Lane 7, 28 pg; Lane 8, 2.8 pg; Lane 9, 280 fg; Lane 10, 28 fg; Lane 11, 2.8 fg; Lane 12, negative control (distilled water); Lane 13, S typhi with no enzyme; Lane 16, Human DNA; Lane 17, S typhi S901 (mobile) reference strain; Lane 18, S typhi I; Lane 19, S typhi II; Lane 20, PCR mix only.

ing the first week of illness. Negative blood culture results, because of low concentrations of bacteraemia or previous antibiotic treatment,[3 8] in suspected cases of typhoid fever may lead to misdiagnosis and improper treatment. Sensitivity can be increased if clinical specimens can be obtained for culture from bone marrow; however, this is an invasive technique and it is not amenable to routine use in patients with typhoid fever. Blood culture is widely practised because of its simplicity, safety, and non-invasiveness. PCR, a highly sensitive method to detect very low quantities of infectious organisms shows promise for typhoid fever diagnosis. Compared with the study by Song *et al*,[10] sensitivity of our PCR using RK1 and RK2 primers was very high (28 pg versus 4 ng by one round of PCR). We could detect as few as 300 bacteria by a single round of PCR compared with 10^6 bacteria by Song *et al.*

Table 2 Results of culture and PCR assay

	Blood/clot culture		
	Positive	*Negative*	*Total*
PCR positive	21	4	25
PCR negative	0	59	59
Total	21	63	84

Other *Salmonella* spp containing dH flagellin do not infect humans. In addition, these primers were designed to amplify a region of *S typhi* that is not affected by the deletion mutation reported by Song *et al.*[11] Therefore, it seems to be a more promising diagnostic approach compared with the conventional procedure of culturing and identifying *S typhi* strains with dH antisera that may miss *S typhi* strains with mutated flagellin gene, that is, jH rather than dH.

The specificity of PCR was 93.7% and sensitivity was 100%. The predictive positive value was 84% and predictive negative value was 100%. Four culture false positive cases were actually additional cases of typhoid fever detected by PCR. There were no false negative cases.

PCR detection of *S typhi* took less than 48 hours, compared with five to seven days for blood or clot culture. Therefore, PCR was a rapid, sensitive, and specific test for the diagnosis of typhoid fever, especially where blood culture was negative because of prior antibiotic treatment, low level of bacteraemia, and culture done in late stages of disease, thus enabling the clinician to use appropriate treatment and to avoid diagnostic delay.

We gratefully acknowledge the help of Dr SK Panda, Department of Pathology, with synthesis of the primers. We also acknowledge Drs B Das, L Ming, and Jaya Tyagi, Department of Biotechnology, Dr AB Dey, Department of Medicine, and Dr Sundram, Department of Biostatistics, All India Institute of Medical Sciences. The technical assistance of Mr Salek Chand, Mr Pooran Arya, and Mr Ramesh Yadav is also acknowledged. Financial assistance from the department of biotechnology, New Delhi (project no. BT/R+D/9/11/94) is acknowledged.

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