J Infect Chemother (2003) 9:233–237
DOI 10.1007/s10156-003-0256-4
© Japanese Society of Chemotherapy and The Japanese Association for Infectious Diseases 2003

ORIGINAL ARTICLE

**Muhammad Nasrum Massi · Toshiro Shirakawa
Akinobu Gotoh · Acharya Bishnu · Mochammad Hatta
Masato Kawabata**

Rapid diagnosis of typhoid fever by PCR assay using one pair of primers from flagellin gene of *Salmonella typhi*

Received: August 21, 2002 / Accepted: June, 3 2003

Abstract Using the polymerase chain reaction (PCR) assay, we developed a rapid diagnosis method for *Salmonella* typhi infection in blood specimens from patients with typhoid fever. Primers were designed from the flagellin gene sequence, which would give an amplification product of 367 base pairs. In this study, the specificity of the assay, with no amplification, was seen for the other *Salmonella* strains with the flagellin gene, and not for non-*Salmonella* bacteria. For the sensitivity test, the protocol described allowed the detection of two to three copies of the *Salmonella typhi* genome, as determined by serial dilution of genomic DNA from *Salmonella typhi*. With the PCR technique, genomic DNA of *Salmonella typhi* was detected in 46 of 73 blood samples collected from patients with clinically suspected typhoid fever who had fever within 3 days of admission to the General Hospital, Makassar, South Sulawesi, Indonesia, and who had had no prior antibiotic treatment. The PCR results (63% positive cases) were compared with those of blood culture (13.7% positive cases) and the Widal test (35.6% positive cases), using the same samples from each of the 73 patients admitted to the General Hospital in Makassar. The time taken for PCR analysis of each sample was less than 12 h, compared with 3 to 5 days for blood or clot culture. The PCR with one pair of primers can be used as a novel, rapid diagnotic method for typhoid fever, particularly when results of standard culture assays are negative.

Key words PCR · Blood culture · Widal test · *Salmonella typhi*

M.N. Massi · A. Gotoh · A. Bishnu · M. Kawabata
International Center for Medical Research, Kobe University School of Medicine, Kobe, Japan

T. Shirakawa (✉) · A. Gotoh
Division of Urology, Department of Organ Therapeutics, Faculty of Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan
Tel. +81-78-382-6155; Fax +81-78-382-6169
e-mail: toshiro@med.kobe-u.ac.jp

M. Hatta
Department of Microbiology, Faculty of Medicine, Hasanuddin University, Makassar, South Sulawesi, Indonesia

Introduction

Typhoid fever remains an important cause of morbidity and mortality in many developing countries. *Salmonella typhi* is responsible for the pathogenicity of this infectious disease. A rapid and sensitive method for the detection of *Salmonella typhi* would help both in preventing the spread of outbreaks and in clinical diagnosis.[1]

Several different techniques are used for the diagnosis of typhoid fever. The Widal test and blood culture remain the only universally practiced diagnostic procedures, because other methods are invasive, tedious, and time-consuming, or are expensive.[2] The Widal test has been found to be nonspecific and difficult to interpret in areas where typhoid fever is endemic.[3,4] Blood culture requires about 5 days before a negative result can be obtained. It takes at least 2 days for clinical laboratories to give a positive report. Although rapid detection methods have been developed, such as the use of DNA or RNA probes and immune-detection methods, these methods are deficient with regard to sensitivity and specificity.[1,5]

The development of polymerase chain reaction (PCR) technology has the potential to solve these problems. The PCR technique seems ideally suited for this rapid and sensitive detection goal. This method potentially allows amplification of the target DNA from one copy.[1]

In this study, we report the development and evaluation of a PCR assay, used in samples from patients with typhoid fever, by amplification of the flagellin gene of *Salmonella typhi*; we also compared the results of this PCR assay with those of blood culture and the Widal test. This assay could be used as a novel tool to reinforce the clinical diagnosis of typhoid fever, especially in blood culture-negative patients.

Patients, materials and methods

Clinical samples

All of the blood specimens were from patients with suspected typhoid fever who were admitted to the General Hospital, Makassar, South Sulawesi Indonesia. The blood specimens included in this study were obtained from 73 patients had had fever for 3 days or less; no anti-typhoid treatment had been given from admission until the blood samples were collected. The mean age of the patients was 20.9 years (range, 5–47 years). They had a mean temperature on admission of 38.0°C (range 37.0°C–40°C). The main clinical symptoms were fever, headache or confusion, relative bradycardia, hepatomegaly, splenomegaly, and abdominal discomfort. Approximately 10 ml of blood specimen was collected from the patients, using a 10-ml syringe; 5 ml of the specimen was directly (at the bedside) put into a bottle containing 15 ml bile broth for blood culture; 1 ml was placed in a tube for extracting DNA, and the rest of the fresh blood in the 10-ml syringe was separated by centrifuge in our laboratory on the same day, and the collected serum samples were stored at 4°C until used for the Widal test.

Healthy controls

Blood samples were collected from 20 healthy people with no recent history of fever within the previous 6 months.

Ethical considerations

Before the study was undertaken, patients were informed about the purpose of the study, and consents were obtained from all of the patients or their parents/guardians.

Blood culture

Blood culture was performed by the inoculation of 15 ml of bile broth (Merck, Rahway, NJ, USA) with 5 ml of freshly collected blood. Cultures were incubated for 24 h at 37°C. A 1-ml culture sample was then plated on *Salmonella-Shigella* agar. After incubation for 24 h at 37°C, colonies were examined by Gram staining and biochemical test to identify *Salmonella typhi*-positive cultures.

Widal test

The Widal test procedure, using O antigen, was performed according to the manufacturer's protocol (Murex Biotech, Dartford, UK). Briefly, twofold serial dilutions (1:20–1:1280) of the serum sample were prepared. One drop (about 25 µl) of O antigen suspension was added to each tube containing the diluted sample. Antigen and serum were mixed and incubated at 50°C. Tubes were checked for agglutination after 4 h. According to routine diagnostic criteria, a titer of 1:320 or more was considered positive.

Bacterial strains

Strains of 14 species of Salmonella and non-Salmonella organisms, including *Salmonella paratyphi* A, B, C; *Salmonella typhimurium*; *Salmonella cholerasius*; *Salmonella enteritidis*; *Escherichia coli*; *Citrobacter freundii*; *Klebsiella pneumoniae*; *Enterobacter hapniae*; *Alkaligenes faecalis*; *Proteus mirabilis*; *Pseudomonas aeroginosa*; and *Providentia alkalifacieus*, were obtained from the Department of Microbiology, University of Hasanuddin, Indonesia, and from the Japan Collection of Microorganisms (JCM). These strains were used for testing the specificity and sensitivity of the PCR assay, and two pure strains of *Salmonella typhi* were studied for the standardization of PCR conditions and as reference strains. All of the bacterial strains that we used in this study were grown overnight in Brain Heart Infusion agar (Oxoid, Basingstoke, UK) and then subjected to DNA extraction.

Extraction of DNA

Genomic DNAs from the cultured bacteria and the blood specimens for PCR were extracted according to the protocol's instructions, using a QIAamp blood mini kit (Qiagen, Hilden, Germany). Just 200 µl of sample, or bacterial strains in 200 µl of Tris-EDTA (TE) buffer or whole blood are recommended for this kit for DNA extraction. The purity of the extracted DNA was checked by measurement of A_{260} and A_{280} unit of double-stranded DNA and agarose gel electrophoresis.

PCR primers

One pair of oligonucleotide primers was designed from the complete sequence of the flagellin gene of *Salmonella typhi*, which was first reported by Song et al.[3] The sequence of the forward primer (ST1) was 5′-ACT GCT AAA ACC ACT ACT′3 (nucleotides 1060 to 1077 in the sequence with GenBank accession number L21912), and that of the reverse primer (ST4) was 5′-TGG AGA CTT CGG TCG CGT AG-′3 (nucleotides 1407 to 1426 in the sequence with GenBank accession number L21912). These primers could amplify a 367-bp fragment of the flagellin gene.

PCR conditions

PCR amplification of the flagellin gene was done according to a published protocol with a little modification.[6] The DNA extracts were amplified with 0.5 µM primers. The PCR mixture (50 µl) contained bacterial DNA, PCR buffer (10 mM Tris/HCl (pH 8.3), 50 mM KCl, 3 mM MgCl, and 0.01% gelatin), 200 µM of each dNTP, and 1.0 U AmpliTaq Gold enzyme (Roche Molecular Systems, Branchburg, NJ, USA). The mixtures were amplified for 40 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, with a final exten-

sion at 72°C for 10 min, in an automated thermal cycler (Control system PC-710; Astec; Tokyo, Japan). An aliquot of 10 μl of each amplified product was submitted to electrophoresis in 2% (wt/vol) agarose gel, with a DNA Molecular Weight Marker VI (Roche Diagnostics, Mannheim, Germany) in parallel. Electrophoresis in Tris-Acid-ethylenediaminetetraacetic acid (EDTA) buffer was performed at 100 V for 45 min. The gel was stained with ethidium bromide (0.5 μg/ml) for 15 min, rinsed, and photographed under ultraviolet light illumination for the presence of a 367-bp band.

Results

Specificity of the PCR assay

To test the specificity of the primers for *Salmonella typhi*, PCR was carried out with extracted DNA from six *Salmonella* strains and eight non-*Salmonella* bacteria, using the same PCR conditions. With this one pair of primers, an expected 367-bp PCR amplified product was obtained only with *Salmonella typhi* strains. None of the non- *Salmonella typhi* strains, which are closely related to *Salmonella typhi*, showed this 367-bp PCR product. Bacterial strains from eight other genera tested in this study showed no amplification products at all using this specific pair of primers (Fig. 1).

Sensitivity of the PCR assay

Assuming that one *Salmonella typhi* bacterium contains approximately 3.48 fg genomic DNA,[1,7] the system is able to detect two to three genome copies of *Salmonella typhi*. The amount of DNA that could be detected after serial dilution was 8.91 fg of genomic DNA (Fig. 2). These results were obtained with purified genomic DNA isolated from cultured *Salmonella typhi* as the template in the PCR, using a QIAquick PCR purification kit (Qiagen).

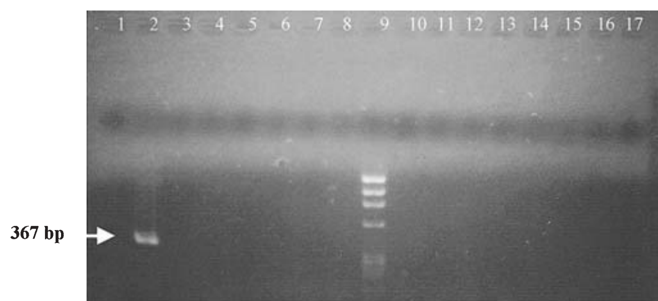

Fig. 1. Specificity of the polymerase chain reaction (PCR) for detection of the flagellin gene of *Salmonella typhi*. *Lane 1*, negative control (DNA mononuclear cell); *lane 2*, *S. typhi*; *lane 3*, *S. typhimurium*; *lane 4*, *S. cholerasius*; *lane 5*, *S. enteritidis*, *lanes 6 to 8*; *S. paratyphi* A, B, and C; *lane 9*, molecular weight marker; *lane 10*, *Escherichia coli*; *lane 11*, *Citrobacter freundii*; *lane 12*, *Klebsiella pneumoniae*; *lane 13*, *Enterobacter hapniae*; *lane 14*, *Alkaligenes faecalis*; *lane 15*, *Proteus mirabilis*; *lane 16*, *Pseudomonas aeroginosa*; and *lane 17*, *Providentia alkalifacieus*

PCR, blood culture, and Widal test in patients with suspected typhoid fever and in healthy persons

We collected samples from people who had no history of fever within the previous 6 months. We used these samples as a negative control. PCR and blood culture were negative in all 20 of the healthy people; however, we found 5 (25%) positive cases on the Widal test, with a titer of 1:320 or more for *Salmonella typhi* O antigen (Fig. 3). Of the 73 patients with suspected typhoid fever, 46 (63%) patients were positive for the PCR assay, 10 (13.7%) patients were positive for blood culture, and 26 (35.6%) patients were positive for the Widal test (Fig. 3).

PCR and Widal test in blood-culture-positive and -negative patients with suspected typhoid fever

Of the 73 patients with suspected typhoid fever, only 10 (13.70%) patients were positive for blood culture, and the

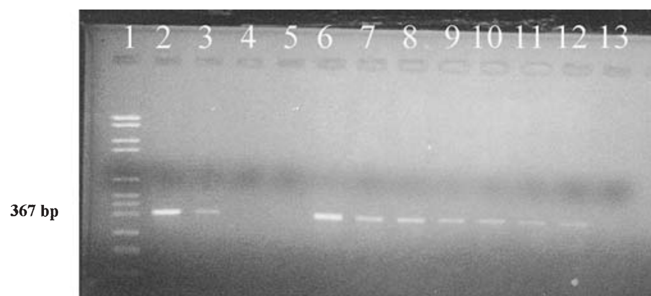

Fig. 2. Sensitivity with serial diluted DNAs from *S. typhi*. *Lane 1*, molecular weight marker; *lanes 2 and 3*, positive controls (*S. typhi* DNA); *lanes 4 and 5*, negative controls (TE buffer and mononuclear cell DNA only); *lane 6*, *S. typhi* DNA, 8.91 ng; *lane 7*, *S. typhi* DNA, 891 pg; *lane 8*, *S. typhi* DNA, 89.1 pg; *lane 9*, *S. typhi* DNA, 8.91 pg; *lane 10*, *S. typhi* DNA, 891 fg; *lane 11*, *S. typhi* DNA, 89.1 fg; *lane 12*, *S. typhi* DNA, 8.91 fg; *lane 13*, *S. typhi* DNA, 0.891 fg. *S. typhi* DNA in lanes 6 to 13 was mixed with 2 μg of mononuclear cell DNA

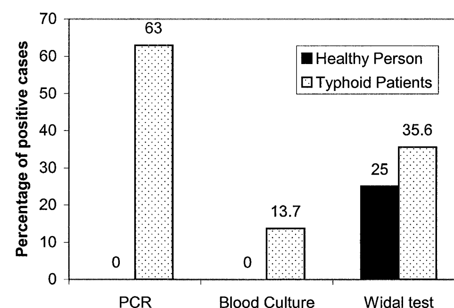

Fig. 3. Comparison of PCR, blood culture, and Widal test findings in patients with suspected typhoid fever and in healthy persons. In the healthy persons, PCR and blood culture were negative in all 20 subjects. The Widal test (with a titer of 1:320 or more for *Salmonella typhi* O antigen) was positive in 5 (25%) of these subjects. Of the 73 patients with suspected typhoid fever, 46 (63.01%) were positive for the PCR assay, 10 (13.7%) were positive for blood culture, and 26 (35.6%) were positive for the Widal test

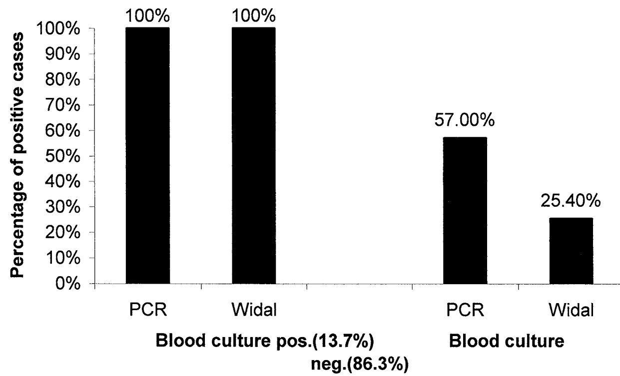

Fig. 4. Comparison of PCR and Widal test findings in blood culture-positive and -negative patients. Of 73 patients with suspected typhoid fever, 10 (13.7%) were positive for blood culture, and all 10 blood-culture-positive patients were positive for PCR and the Widal test. Of the 63 (86.3%) patients who were negative for blood culture, the PCR assay was positive in 36 patients (57%) and the Widal test was positive in 16 patients (25.4%)

other 63 (86.3%) patients were negative for blood culture. All of the patients positive for blood culture were also positive for PCR and the Widal test (Fig. 4). Of the 63 patients negative for blood culture, 36 (57%) patients were positive for PCR, and 16 (25.4%) patients were positive for the Widal test (Fig. 4).

Discussion

A rapid and sensitive method for the detection of *Salmonella typhi* would help in relieving patients' suffering, and would protect from the fatal complication of typhoid-like perforation of the intestines and typhoid meningitis. The early and definitive diagnosis of typhoid fever can make possible specific treatment at an early stage of the disease, leading to rapid elimination of the pathogen and helping in epidemiological investigation during foodborne outbreaks.[2]

The most favored methods for the diagnosis of typhoid fever currently rely on blood culture and the Widal test. Even though the Widal test is easy and is a little bit cheaper than other methods, it cannot detect the disease in the early stage, because specific antibodies take at least 1 week to reach detectable levels. Also, it becomes nonspecific and is particularly unreliable with single titers.[2,3] In the present study, we found the Widal test was positive in 25% of healthy persons without clinical signs, because these healthy persons live in Makassar city, an area endemic for typhoid in Indonesia. This finding suggested that the Widal test could give an unacceptable level of false-positive results.

Salmonella typhi can be isolated from more than 90% of patients with typhoid fever if bone marrow, blood, stool and intestinal secretions are all cultured. We found only 13.7% positivity for blood culture in this study, maybe because of low bacteremia in most of our blood samples, or maybe because of other factors, such as the bacteriostatic effect of antibiotics already administered to some patients before blood culture was done, and maybe depending the total amount of blood used in this culture, the time of blood collection, the type of culture medium employed, the host's immune response system, and the intracellular characteristics of *Salmonella typhi*.[2,3,7,8,9] In this study, in the blood-culture-negative patients with clinically suspected typhoid fever the PCR assay detected positive results in 57% of these patients. This finding indicated that the PCR assay could detect the *Salmonella typhi* infection from patients showing negative results for blood culture, these negative results possibly being due to the low level of bacteremia in the blood of patients with suspected typhoid fever in this study.

DNA probe hybridization methods can detect *Salmonella typhi*, but their sensitivity is poor because they cannot detect fewer than 500 bacteria/ml.[10,11,12,13] This problem of sensitivity could be circumvented by using the PCR, which can detect very small amounts of DNA by enzymatic amplification with absolute specificity within several hours. For PCR, we preferred to target the flagellin gene, because the flagellin gene of *Salmonella typhi* has unique nucleotide sequences in the hypervariable region of the gene,[3,14,15,16,17] which are different from those sequences in other strains of *Salmonella*. Our PCR assay using one pair of primers from the flagellin gene was specific for the *Salmonella typhi* strain. This PCR assay showed a positive result only in the *Salmonella typhi* strain, but not in six other *Salmonella* strains or eight non-*Salmonella* bacteria. Other strains of *Salmonella* have no significant sequencing homology with the primers that we used in this study. The sensitivity of this PCR assay was also very high; this PCR could detect a positive result from 8.91 fg of genomic DNA of *Salmonella typhi*, which corresponds to two to three bacteria of *Salmonella typhi*. This PCR assay was also very rapid, with a single-round PCR procedure taking less than 12 h because there was no need to run the procedure again for nested PCR.

In conclusion, our results suggest that the PCR assay be used as a rapid diagnostic method for the detection of typhoid fever, especially in endemic areas, where the Widal test shows nonsignificant differences between patients with suspected typhoid fever and healthy individuals, and where blood culture is negative because of prior antibiotic treatment, a low level of bacteremia, and culture done in the late stages of the disease. Therefore, the PCR assay can be of singular importance for the detection of early cases of typhoid, which is important not only for the treatment of patients but is also necessary for control of the disease.

Acknowledgments We thank Irfan Idris, M.D., and Marcus, Lembong, Department of Microbiology, Hasanuddin University, Indonesia, for their kind gift of various *Salmonella* strains and non-*Salmonella* strains. We also thank Mardiah Suci, M.D., Department of Hematology, ICMR, Kobe University, Japan for kindly providing the human DNA mononuclear cells.

References

1. Zhu Q, Lim CK, Chan YN. Detection of *Salmonella typhi* by polymerase chain reaction. J Appl Microbiol 1996;80:244–51.
2. Haque A, Ahmed J, Qureshi JA. Early detection of typhoid fever by polymerase chain reaction. Ann Saudi Med 1999;19:337–40.
3. Song JH, Cho H, Park MY, Na DS, Moon HB, Pai CH. Detection of *Salmonella typhi* in the blood of patients with typhoid fever by polymerase chain reaction. J Clin Microbiol 1993;31:1439–43.
4. Chaudhry R, Laxmi BV, Nisar N, Ray K, Kumar D. Standardization of polymerase chain reaction for the detection of *Salmonella typhi* in typhoid fever. J Clin Pathol 1997;50:437–9.
5. Hashimoto Y, Itho Y, Fujinaga Y, Khan AQ, Sultana F, Miyake M, et al. Development of nested PCR based on the ViaB sequence to detect *Salmonella typhi*. J Clin Microbiol 1994;33:775–7.
6. Widjojoatmodjo MN, Fluit AC, Verhoef J. Rapid identification of bacteria by PCR-single-strand confirmation polymorphism. J Clin Microbiol 1994;32:3002–7.
7. Cocolin L, Manzano M, Astori G, Botta GA, Cantoni C, Comi G. A highly sensitive and fast non-radioactive method for the detection of polymerase chain reaction products from *Salmonella* serovars, such as *Salmonella typhi*, in blood specimens. FEMS Microbiol Immunol 1998;22:233–9.
8. Gilman RH, Terminel M, Levine MM, Hernandez-Mendoza P, Hornick RB. Relative efficacy of blood, urine, rectal swab, bone marrow, and rose spot cultures for recovery of *Salmonella typhi* in typhoid fever. Lancet 1975;1:1211–3.
9. Gaviria-Ruiz MM, Caardona-Castro NM. Evaluation and comparison of different blood culture techniques for bacteriological isolation of *Salmonella typhi* and *Brucella abortus*. J Clin Microbiol 1995;33:868–71.
10. Rubin FA, McWhirter PD, Burr D, Punjabi DH, Lane E, Kumala S, et al. Rapid diagnosis of typhoid fever through identification of *Salmonella typhi* within 18 hours of specimen acquisition by culture of the mononuclear cell-platelet fraction of blood. J Clin Microbiol 1990;28:825–7.
11. Rubin FA, McWhirter PD, Punjabi NH, Lane E, Sudarmono P, Pulungsih SP, et al. Use of DNA probe to detect *Salmonella typhi* in the blood of patients with typhoid fever. J Clin Microbiol 1989;27:1112–4.
12. Rubin FA, Kopecko DJ, Sack RB, Sudarmono P, Yi A, Maurta D, et al. Evaluation of a DNA probe for identifying *Salmonella typhi* in Peruvian and Indonesian bacterial isolates. J Infect Dis 1988;157:1051–3.
13. Rubin FA, Kopecko DJ, Noon KF, Baron LS. Development of a DNA probe to detect *Salmonella typhi*. J Clin Microbiol 1985;22:600–5.
14. Frankel G. Detection of *Salmonella typhi* by PCR. J Clin Microbiol 1994;32:1415.
15. Frankel G, Newton SMC, Schoolnik GK, Stocker BAD. Unique sequences in region VI of the flagellin gene of *Salmonella typhi*. Mol Microbiol 1989;3:1379–83.
16. Frankel G, Newton SMC, Schoolnik GK, Stocker BAD. Intragenic recombination in a flagellin gene: characterization of the H1-j gene of *Salmonella typhi*. EMBO J 1989;8:3149–52.
17. Edwards PR, Ewing WH. Identification of *Enterobacteriaceae*. 3rd Ed. Minneapolis: Burgess; 1972. p. 7–20; p. 146–258.