# IDENTIFICATION AND SEQUENCING OF *SALMONELLA ENTERICA* SEROTYPE TYPHI ISOLATES OBTAINED FROM PATIENTS WITH PERFORATION AND NON-PERFORATION TYPHOID FEVER

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Abstract. We describe the characterization of *Salmonella enterica* serovar Typhi, isolated from the blood of patients with perforation and non-perforation typhoid fever, by a combination of conventional microbiological tests, *16S rRNA* gene sequencing, and *flagellin* gene and *CDP-tyvelose epimerase (rfbE)* gene sequencing. The *16S rRNA* gene sequencing showed that there were four base mutations from perforation samples and only three from non-perforation samples. These findings indicated that the isolates were a strain of *Salmonella enterica.* The *flagellin* gene sequences from the two groups were 100% identical to that of the *H1-d flagellin* gene of serovar Typhi. Sequences of the *rfbE* from both groups were also 100% identical.

## INTRODUCTION

*Salmonella enterica* serovar Typhi, the cause of typhoid fever, is a pathogen that has a major impact on public health in many developing countries, with an annual incidence of 16 to 17 million cases and which leads to approximately 600,000 to 700,000 deaths (Thong *et al,* 1996).

*Salmonella enterica* serovar Typhi continues to cause severe disease in many parts of the world, its most feared complication being perforation of ulcerated Peyer's patches within the small intestine, leading to peritonitis with associated mortality. The disease is characterized by prolonged fever, bacterial replication in the reticulo-endothelial system (RES) and significant inflammation of the lymphoid organs of the small intestine (Everest *et al,* 2001).

In developing countries, typhoid fever causes at least 5% of all deaths, with markedly different rates where typhoid fever is endemic. The reasons for these differences in disease severity are not known but may be related to differences in health care facilities, host immune responses, genetic factors, and perhaps in the strains of *Salmonella enterica* serovar Typhi circulating in areas of endemicity (Zhu *et al,* 1996; Everest *et al,* 2001).

In the present study, we characterized *Salmonella enterica* serovar Typhi isolated from patients with perforation and non-perforation typhoid fever by a combination of conventional microbiological tests, *16S rRNA* gene sequencing, *flagellin* gene sequencing, and *CDP-tyvelose epimerase (rfbE)* gene sequencing. We sought to re-investigate a possible correlation between disease severity and strain differences.

## MATERIALS AND METHODS

### Clinical isolates

Human isolates of *Salmonella enterica* serovar Typhi recovered from blood specimens were used in the present study. A total of 11 isolates were obtained from 11 blood samples of patients with perforation (6 patients) and non-

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perforation (5 patients) typhoid fever in the South Sulawesi Province of Indonesia between February 2000 and October 2001. Of the 11 patients, 6 were males and 5 were females; they ranged in age from 13 to 54 years. Before the study was undertaken, the patients were informed about the purpose of the study, and their consents were obtained directly or from their parents/guardians. The main clinical symptoms for non-perforation samples were fever, headache or confusion, relative bradycardia, hepatomegaly, splenomegaly, and abdominal discomfort; those for perforation samples included hematemesis and / or melena, altered consciousness, meningeal signs, and intestinal perforation. All blood specimens were collected and handled according to standard protocols, and all suspect colonies from a positive blood culture were identified by standard conventional biochemical methods (Murray *et al,* 1999).

### Blood culture

A 5 ml sample of peripheral blood from each patient was collected in 15 ml of bile-broth (Merck, Rahway, NJ). Cultures were incubated for 24 hours at 37ºC. A 1 ml culture sample was then plated on Salmonella-Shigella agar. After incubation for 24 hours at 37ºC, the colonies were examined by Gram staining and conventional biochemical tests with positive results (via nitrate reduction, lysine decarboxylase, $H_2S$, glucose oxidation, and fermentation or oxidation of mannitol and sorbitol) used to identify *Salmonella enterica* serovar Typhi - positive cultures.

### Widal test

The Widal test procedure using O antigen was performed according to the manufacturer's protocol (Murex Biotech Ltd, Temple Hill, Dartford, United Kingdom) with all the samples being checked for agglutination with a titer of more than 1: 320 being considered positive.

### Dipstick test

Serum samples were diluted (1/50) in the detecting reagent (containing dye-labeled, anti-human IgM antibodies). Nitrocellulose dipsticks coated with heat-inactivated serovar *Salmonella* typhi were immersed in the diluted serum and incubated at room temperature for 3 hours. The strips were then washed and dried at room temperature. The sera were graded (0 to 4) according to the staining intensity of the colored band corresponding to the antigen (Hatta *et al*, 2001).

### Isolation of genomic DNA

Genomic DNA from the cultured bacteria in 200 μl of Tris-EDTA (TE) buffer for PCR was extracted according to the protocol's instructions using a QIAamp blood mini Kit (Qiagen, Hilden, Germany). The purity of the extracted DNA was checked by measurement of $A_{260}$ and $A_{280}$ and by agarose gel electrophoresis.

### PCR amplification

The bacterial DNA extract and control were amplified using 0.5 μM primers (Table 1). 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, Inc, Brachburg, New Jersey, USA). The mixtures were amplified for 40 cycles at 94ºC for 1 minute, 55ºC for 1 minute, and 72ºC for 2 minutes, with a final extension at 72ºC for 10 minutes in an automated thermal cycler (Control system PC-710; Astec; Tokyo, Japan). An aliquot of 10 μl of each amplified product was electrophorese in 2% (wt/vol) agarose gel, with a DNA Molecular Weight Marker VI (Roche Diagnostics GmbH, Mannheim, Germany) in parallel (Massi *et al*, 2003).

### DNA sequencing

The PCR product was gel purified using the QIAquick PCR purification kit (QIAgen, Hilden, Germany). Both strands of the PCR product were sequenced twice with an ABI 310 automated sequencer according to the manufacturer's instructions (Perkin-Elmer, Foster City, Calif.) using the PCR primers (Table 1). The sequences of the PCR products were compared with known *16S rRNA, flagellin,* and *rfbE* gene sequences in the GeneBank database, which was performed at NCBI using the BLAST network services (Altschul *et al*, 1990).

## RESULTS

### Standard conventional microbiological tests

based on blood culture showed that the profile of all the strains were compatible with serovar Typhi. Serological tests on the basis of standard criteria such as the Widal test and dipstick test also indicated that all of the strains in the serum samples of the patients with perforation and non-perforation were *Salmonella enterica* serovar Typhi (Table 2).

PCR amplification using different pairs of primers for the *16S rRNA* gene showed bands of 428 bp, 484 bp, and 483 bp. There were four base differences [+565 (G→A), +602 (G→A),

Table 1
Details of the primers used for PCR amplification for detection and sequencing of *Salmonella enterica* serovar Typhi from perforation samples and non-perforation samples.

| Serial no. | Gene | Primer | Sequence | Nucleotide position |
|---|---|---|---|---|
| 1 | *16S rRNA* | SR1 | 5′ agtttgatcctggctcag 3′ | 3-20 (AC: Z47544) |
| 2. | *16S rRNA* | SR2 | 5′ agtactttacaacccgaagg 3′ | 411-430 (AC: Z47544) |
| 3. | *16S rRNA* | SR3 | 5′ aagtactttcagcgggga 3′ | 424-441 (AC: Z47544) |
| 4. | *16S rRNA* | SR4 | 5′ ttgagttttaaccttgcgg 3′ | 898-916 (AC: Z47544) |
| 5. | *16S rRNA* | SR5 | 5′ aactcaaatgaattgacgg 3′ | 901-919 (AC: Z47544) |
| 6. | *16S rRNA* | SR6 | 5′ aggcccgggaacgtattcac 3′ | 1364-1383 (AC: Z47544) |
| 7. | *Flagellin* | ST1 | 5′ caagtcattaatacaaacagcc 3′ | 7-28 (AC: L21912) |
| 8. | *Flagellin* | ST2 | 5′ aagtcccagtgttttagagc 3′ | 491-510 (AC: L21912) |
| 9. | *Flagellin* | ST3 | 5′ actgggacttgataagct 3′ | 501-518 (AC: L21912) |
| 10. | *Flagellin* | ST4 | 5′ aattgcacctgttttctc 3′ | 1042-1059 (AC: L21912) |
| 11. | *Flagellin* | ST5 | 5′ actgctaaaaccactact 3′ | 1060-1077 (AC: L21912) |
| 12. | *Flagellin* | ST6 | 5′ ttaacgcagtaaagagag 3′ | 1504-1521 (AC: L21912) |
| 13. | *rfbE* | SF1 | 5′ tgaagttggtagtggagagg 3′ | 1-20 (AC: AF332602) |
| 14 | *rfbE* | SF2 | 5′ ccatacaaggattgtcaatag 3′ | 482-502 (AC: AF332602) |
| 15. | *rfbE* | SF3 | 5′ ctattgacaatccttgtatgg 3′ | 482-502 (AC: AF332602) |
| 16. | *rfbE* | SF4 | 5′ gcatgcaaaacatccctaact 3′ | 921-941 (AC: AF332602) |
| 17. | *rfbE* | SF5 | 5′ agttagggatgttttgcatgc 3′ | 921-941 (AC: AF332602) |
| 18. | *rfbE* | SF6 | 5′ tcctcacgtcagcttcatct 3′ | 1294-1313 (AC: AF332602) |

AC: GenBank accession no.

Table 2
The results of blood culture assay, Widal test, Dipstick test from patients with perforation and non-perforation typhoid fever.

| Typhoid fever patients with | Blood culture test | Widal test (O antigen) | Dipstick test |
|---|---|---|---|
| Perforation | | | |
| No 1 | Positive | 1/320 | +2 |
| No 2 | Positive | 1/320 | +3 |
| No 3 | Positive | 1/640 | +4 |
| No 4 | Positive | 1/320 | +2 |
| No 5 | Positive | 1/640 | +1 |
| No 6 | Positive | 1/320 | +3 |
| Non-perforation | | | |
| No 1 | Positive | 1/320 | +2 |
| No 2 | Positive | 1/320 | +1 |
| No 3 | Positive | 1/320 | +1 |
| No 4 | Positive | 1/320 | +1 |
| No 5 | Positive | 1/320 | +2 |

+1079 (C→T) and +1260 (T→C)] between all of the isolates from the perforation samples and *Salmonella enterica* serovar Typhi (Genbank accession no. Z47544) but only three base differences [+565 (G→A), +602 (G→A), and +1079 (C→T)] between all of the isolates from the non-perforation samples and *Salmonella enterica* serovar Typhi (GenBank accession no. Z47544). These sequencing results showed 99% homology between the isolates and *Salmonella enterica* serovar Montevideo with identities 1371/1376 (GenBank accession no. AF227867), *Salmonella enterica* serovar Typhi with identities 1377/1381 (Gen Bank accession no. Z47544), *Salmonella enterica* serovar Typhimurium with identities 1374/1379 (GenBank accession no. AF227869), and *Salmonella* Dublin with identities 1373/1380 (GenBank accession no. AF227868); there was 98% homology between the isolates and *Salmonella* species with identities 1366/1381 (GenBank accession no. X80676), *Salmonella enterica* serovar Paratyphi *A* with identities 1354/1381 (GenBank accession no. U88546), and *Salmonella enterica* serovar Paratyphi C with identities 1355/1381 (GenBank accession no. U88548); and there was 97% homology between the isolate and *Salmonella enterica* serovar Enteritidis with identities 1351/1381 (GenBank accession no. U90318).

PCR amplification of the *flagellin* gene of the bacteria showed bands of 504 bp, 559 bp, and 462 bp. There were no base differences between the *flagellin* gene sequences of any of the 11 isolates and that of serovar Typhi (GenBank accession no. L21912). There was 97% homology between the isolates and *Salmonella enterica* serovar Munchen with identities 1480/1515 (GenBank accession no. X03395), 84% homology between the isolates and *Salmonella enterica* serovar Enteritidis with identities 388/458 (GenBank accession no. M84974), *Salmonella* Dublin with identities 387/458 (GenBank accession no. Z15067). These findings show that the *flagellin* gene of all the isolates is the same as the *H1-d flagellin* gene of serovar Typhi.

PCR amplification of the *rfbE* gene of the bacteria showed bands of 502 bp, 460 bp, and 393 bp. There were no base differences between the *rfbE* gene sequences of any of the 11 isolates and that of serovar Typhi (GenBank accession no. M29682).

## DISCUSSION

The present study sought to identify an association between strain characteristics of the bacteria that cause typhoid fever and disease severity, since the observed differences in disease severity have been unexplained. This condition may be related to differences in health care facilities, host immune responses, genetic factors, and perhaps to differences in the strains of *Salmonella enterica* serovar Typhi circulating in areas of endemicity. Previous findings showed an association between the genotype of the *Salmonella enterica* serovar Typhi, as assessed by the PFGE pattern, and its ability to cause fatal illness (Thong *et al,* 1996). A previous study identified an isolate that was a variant of *Salmonella enterica* serovar Typhi rather than a new serovar of *Salmonella enterica* from a patient with acute cholecystitis and cholangitis using several methods (Woo *et al,* 2001). A more recent study, however, reported an association between flagellar serovar and decreased severity of illness and invasiveness (Grossman *et al,* 1995).

The present study found that the same strains of *Salmonella enterica* serovar Typhi caused perforation and non-perforation typhoid fever, as determined by blood culture assay with conventional biochemical tests, Widal test, dipstick test, and sequencing of the *flagellin*, *rfbE*, and *16S rRNA* genes. However, these was one base difference between the *18S rRNA* gene sequence of perforation and non-perforation samples. Classification by *16S rRNA* sequences has been of limited usefulness below the species level (Woo *et al,* 2001).

Our results are consistent with those from a study performed by Heneine *et al* (1991) who characterized four *Salmonella enterica* serovar Typhi isolates from patients with severe and mild typhoid fever by using several methods, including multilocus enzyme electrophoresis, protein profiles, ribotyping, plasmid analysis, and phage typing. No association between the character-

istics of these clones and disease severity was found. Franco *et al* (1992) used envelope protein profiles, restriction fragment length polymorphism of chromosomal DNA, and immune response to envelope protein and also found no clear correlation between strain characteristics and disease severity.

Based on a combination of results from these phenotypic and genotypic tests, we conclude that the isolates from blood culture samples of patients with perforation typhoid fever are the same as those from patients with non-perforation typhoid fever. It would be valuable to precisely identify the organisms responsible for perforation and non- perforation typhoid if their different genotypes led to symptoms requiring markedly different treatment and follow-up.

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