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# Screening, phylogenetic analysis and antibiotic sensitivity pattern of *Salmonella enterica* serovar Typhi isolates from typhoid asymptomatic carriers

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ABSTRACT

**Objective:** To isolate the *Salmonella enterica* serovar Typhi (*S. typhi*) from asymptomatic typhoid carriers in the local population. To assess the antibiotic sensitivity and resistant pattern of *S. typhi* isolates against viable antibiotics and phylogenetic analysis of *S. typhi* isolates on the basis of 16S rDNA gene. **Methods:** *S. typhi* was isolated and identified based on the cultural characteristics on BSA (Bismuth Sulphite Agar), MacConkey agar, agglutination test with specific antiserum and phylogenetic analysis. *S. typhi* isolates were tested for sensitivity and resistant pattern with a number of viable antibiotics by disc diffusion method. **Result:** A total of 15 bile samples were collected from the food handlers to screen the typhoid asymptomatic carriers. Positive result was yielded for 3 out of 15 samples. *S. typhi* isolates showed resistant to amphicillin (100%), tetracyclin (100%), rifampicin (66.5%), ofloxacin (33.5%), cloxacillin (33.5%) and susceptibility to gentamycin (100%), amikacin (100%), chloramphenicol (100%), streptomycin (100%), kanamycin (100%), cprofloxacin (100%), amoxycillin (66.5%) and ofloxacin (66.5%). **Conclusions:** This study demonstrates the outbreak of typhoid fever occurs through asymptomatic carrier. In addition, this study also reveals the occurrence of considerable drug resistant among the *S. typhi* isolates.

## 1. Introduction

Typhoid fever is a generalized infection of the reticuloendothelial system (spleen, liver, bone marrow), gut-associated lymphoid tissue and gall bladder caused by the highly human host restricted pathogen *Salmonella enterica* serovar Typhi (*S. typhi)*[1]. The annual global estimates for typhoid fever are 21 million episodes with 216 000 deaths in 2000[2]. It is characterized by the ingestion of food and water contaminated with human faeces. Individuals recovering from typhoid fever act as a chronic carriers and shed bacteria for months. So, it is important that food handlers abstain from working during and immediately following *S. typhi* infection. Typhoid is one of the most wide spread of all bacterial diseases in India. The main source of typhoid is asymptomatic carriers. An individual can symptomatically carry the typhoid germ days to years without showing any symptoms of typhoid fever. The rate of resistance development in bacteria has been found to be increasing. It is necessary to maintain proper hygienic conditions in order to prevent the spread of the typhoid fever[3].

Generally typhoid can be treated with antibiotics such as ampicillin, amoxicillin, ciprofloxacin, gentamycin, amikacin, chloramphenicol, co-trimaxazole, nalidixic acid, oflaxacin, tetracycline *etc.* The population of multi-drug resistant (MDR) *S. typhi* is steeply increasing in Indian subcontinent, Southeast Asia and other geographical regions. Now days, resistance of pathogens to antibiotics is a problem that makes each of us to worry. The rate of resistance development in bacteria has been found to be increasing. Several diseases causing bacteria including typhoid-causing *Salmonella* species have now become resistant to one or more antibiotics[4].

Thereby, the present investigation aimed at screening, identification, antibiotic sensitivity and resistant pattern of *S. typhi* isolates from typhoid asymptomatic carriers.

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## 2. Materials and methods

### 2.1. Sample collection

On the historical background of suspected individuals who previously suffered from typhoid were chosen for this study. A total of 15 bile samples were collected from the people of different age group working as cooks in the hotels in Salem District, Tamil Nadu, India for the isolation of typhoid bacilli. The collected samples were transported to the laboratory using screw-capped tubes with Cary-Blair medium[5].

### 2.2. Identification S. typhi isolates

#### 2.2.1 Cultural characteristics

The samples were transferred to the Selenite-F (Hi-media) broth to enhance the growth of the organism and it was incubated at 37 ℃ for 24 h. After the growth was observed in the enrichment media, they were inoculated into the MacConkey agar media (Hi-media) Bismuth Sulfite Agar (BSA) (Hi-media), *Salmonella* Differential Agar (SDA) (Hi-media) respectively and incubated at 37 ℃ for 24 h.

#### 2.2.2. Preliminary tests and biochemical tests

Gram′s staining, Motility test, Catalase test, Oxidase test, Sugar fermentation, IMVIC test (Indole production, Methyl Red test, Vogues-Proskauer test, Citrate test), Urease test and Triple Sugar Iron test were done according to the procedure of Cappuccino and Sherman[6].

#### 2.2.3. Confirmatory test for identification of S. typhi

The bacterial agglutination test (high titre serum agglutination test) was adopted to identify *S. typhi*. This procedure was carried out on an ordinary glass slide. A drop of saline was placed in the three circles and a small amount of culture of *S. typhi* from the solid medium was emulsified using inoculation loop. A drop of antiserum of *S. typhi* was added respectively. Then, both were mixed and observed for agglutination. A drop of saline with culture was kept as control.

### 2.3. Antibiotic sensitivity test

Antibiotic sensitivity test was done for the isolates using Muller-Hinton agar (MHA) medium (Hi-media) by disc diffusion method[7]. Culture tubes containing sterilized nutrient broth were inoculated with few colonies of isolates. A bacterial suspension was produced by incubating the tubes for 24 h. For the sensitivity test the MHA plates were prepared and the plates were dried for 30 min before inoculation. The bacterial suspension was swabbed evenly on the whole surface of the medium with sterile cotton swab. After the inoculum had dried, the antibiotic discs were placed on the MHA with sterilized forceps. Then, the plates were incubated at 37 ℃ for 24 h. After the incubation period, the zones of inhibition were observed and compared with standard antibiotic sensitivity chart (Hi-media).

### 2.4. Amplification of 16S rDNA gene and sequencing

Genomic DNA of *S. typhi* isolates were prepared by using CTAB/NaCl method[8]. The genomic DNA were detected by electrophoresis in 1% agarose gel (Hi-media) stained with ethidium bromide. PCR primers were used [Forward: AGA GTT TAG TCC TGG CTG AG; Reverse: ACG GCT ACC TTG TTA CGA CTT] to amplify the genomic DNA. The reaction mixture for PCR amplification was prepared in a total volume of 50 μL with the template DNA, Taq DNA polymerase (0.5 μL) dNTPs (1 μL), and forward and reverse primers (2 μL). The PCR reaction details were as follows: initial denaturation at 94 ℃ for 2 min, cycle denaturation at 94 ℃ for 30 s, annealing at 49 ℃ for 1 min, extension at 72 ℃ for 1 min, 34 cycles in total and final extension at 72 ℃ for 7 min. 16S rDNA sequencing were done at Helini Biomolecules, Chennai, Tamil Nadu, India.

### 2.5. Phylogenetic analysis

MEGA 4 software was used to align the DNA sequences and using Clustal W for the formation of phylogenetic tree.

## 3. Results

### 3.1. Screening and identification of S. typhi isolates

A total of 15 bile samples were used to screen typhoid asymptomatic carrier, out of which three samples only yield positive result. The isolates were identified based on the cultural characteristics on enriched, selective and differential medium. The production of red colour deposits in the selective broth, jet black colonies on BSA, and colour less colonies on MacConkey agar by the isolates indicates the presence of *S. typhi*. The preliminary and biochemical tests showed for the isolates *viz*. Gram negative; motile rods; catalase positive; oxidase negative; gulucose-maltose-mannital fermented; lactose-not fermented; indole-negative; methyl red positive; vogues proskauer test-negative; citrate-positive; triple sugar iron test-alkaline slant/acid butt with $H_2S$ production and urease test-negative. From these results, the isolates were identified as *S. typhi*. They were confirmed by bacterial agglutination test.

### 3.2. Antibiotic sensitivity and resistant pattern of S. typhi isolates

*S. typhi* isolate-1 from the asymptomatic typhoid carriers showed sensitivity against gentamycin, amikacin, chloramphenical, streptomycin, kanamycin, cloxacillin, ciprofloxacin, amoxycillin and resistant to amphicillin, tetracyclin, ofloxacin, rifampicin. *S. typhi* isolate-2 from asymptomatic typhoid carriers showed sensitivity against gentamycin, amikacin, ofloxacin, chloramphenicol, streptomycin, cloxacillin, kanamycin, ciprofloxacin, amoxycillin and it is resistant to tetracyclin, amphicillin, rifampicin. *S. typhi* isolate-3 showed sensitivity against gentamycin, amikacin, chloramphenicol, streptomycin,

kanamycin, ciprofloxacin and resistant to amphicillin, tetracyclin, cloxacillin, ofloxacin (Table 1).

**Table 1**
Antibiotic sensitivity and resistant pattern of *S. typhi* isolates (zone of inhibition in mm).

| Antibiotics | Isolate 1 | Isolate 2 | Isolate 3 |
|---|---|---|---|
| Amphicillin (25 μg) | R | R | R |
| Amoxicillin (25 μg) | 21(S) | 21(S) | 14(I) |
| Amikacin (30 μg) | 18(S) | 17(S) | 23(S) |
| Chloramphenicol (25 μg) | 21(S) | 20(S) | 21(S) |
| Ciprofloxacin (30 μg) | 22(S) | 23(S) | 24(S) |
| Cloxacillin (10 μg) | 15(S) | 20(S) | R |
| Gentamycin (30 μg) | 23(S) | 16(S) | 23(S) |
| Kanamycin (30 μg) | 19(S) | 19(S) | 19(S) |
| Nalidixicacid (30 μg) | 16(I) | 20(I) | 17(I) |
| Ofloxacin (30 μg) | 10(R) | 16(S) | 18(R) |
| Rifampicin (30 μg) | 15(R) | 15(R) | 15(R) |
| Streptomycin (25 μg) | 16(S) | 18(S) | 18(S) |
| Tetracyclin (10 μg) | 12(R) | 12(S) | R |

S-Sensitive; R-Resistant; I-Intermediate.

### 3.3. PCR amplification of 16S rDNA gene and sequencing

The amplified 16S rDNA product was examined by agarose gel electrophoresis, revealed 1 500 bp amplified product (Figure 1). The amplified product was purified and sequenced.

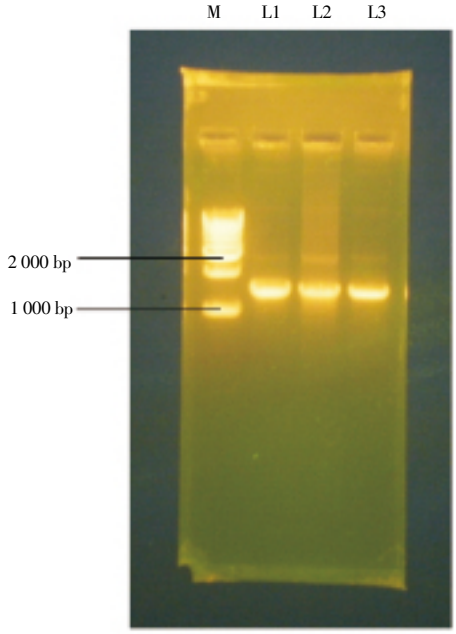


**Figure 1.** Amplified 16S rDNA of *S. typhi* isolates. Lane 1-Marker; Lane 2-*S. typhi* isolate 1; Lane 3-*S. typhi* isolate 2-*S. typhi*; Lane 3 *S. typhi* isolate 3.

### 3.4. Phylogenetic analysis of S. typhi isolates on the basis of 16S rDNA sequence

A set of three *S. typhi* isolates sequences representing major evolutionary lineages within bacteria kingdom was used in our analysis. They were selected in order to create a representative set that covers the diversity of bacteria as much as possible. Therefore, within the defined orders of three sample sequence, the preference was given to the phylogenetically most distantly related species. The phylogenetic data were obtained by alignment and phylogenetic analysis of the nucleotide sequence. The sequence were first aligned by using CLUSTAL W then the neighbor joining analysis was used to reconstruct phylogenetic trees with the MEGA 4 computer software program. By using MEGA 4 package, neighbour-joining evolution, maximum parsimony and boots trapping methods were found out. *S. typhi* gene from isolate 1, *S. typhi* strain T4, T7 and *S. typhimurium* from isolate 2, *S. typhi* and *S. paratyphi* strain A5 from isolate 3 were found to be distantly related to each other (Figure 2, 3 & 4).

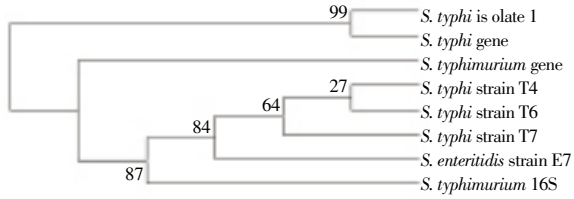


**Figure 2.** *S. typhi* isolate-1 evolutionary relationships of 8 taxa.

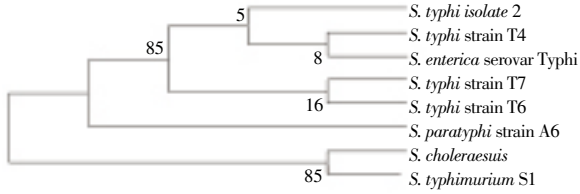


**Figure 3.** *S. typhi* isolate-2 evolutionary relationships of 8 taxa.

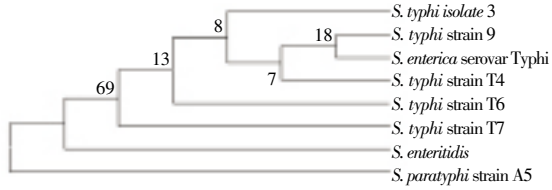


**Figure 4.** *S. typhi* isolate-3 evolutionary relationships of 8 taxa.

## 4. Discussion

*S. typhi* is the major known biliary pathogen[9]. In this study three *S. typhi* isolates were screened from bile of asymptomatic typhoid carriers. *S. typhi* was isolated and identified based on the cultural characteristics on Bismuth Sulphite Agar, MacConkey agar[10]. The food handlers prominently play a role in disseminating typhoid bacilli through different food products and water[3]. People affected by *Salmonella* species known to excrete *Salmonella* sp even after disapperence of symptoms and control of disease. Such people are known as asymptomatic carriers[11]. Antibiotic-

resistant *Salmonella enterica* strains are spreading throughout the world[12-18]. In this study, we have reported that Amphicillin and Tetracyclin are (100%) resistant to *S. typhi* isolates 1, 2, and 3. *S. typhi* isolated from the stool and urine samples in America 1999-2006 among two hundred seventy two (13%) isolates resistant to Amphiclin, Chloramphenicaol and Trimethoprim/Sulfamethoxazole[19]. In 1990, ciprofloxacin is an alternative to Chloramphenicol for the treatment of typhoid fever patients. In 1995, four (1.7%) of 237 isolates of *S. typhi* in the UK showed reduced sensitivity to ciprofloxacin[20]. This present study revealed *S. typhi* isolates were sensitive to the viable antibiotics *viz.* 100% gentamycin, amikacin, chloramphenicol, streptomycin, kanamycin, ciprofloxacin; 66.5% amoxycillin and ofloxacin. However, *S. typhi* isolates showed resistant to the viable antibiotics *viz.* 66.5% rifampicin; 33.5% ofloxacin and cloxacillin.

The 16S rDNA gene sequence of *S. typhi* isolates were amplified and sequenced. These sequences were analysed by comparison with reference organisms from the family *Enterobacteriaceae* and belong to the gamma subdivision of the class of *Proteobacteria*[21]. DNA sequence is used to find out the phylogenetic relationships between closely related species of *S. typhi* gene and *S. typhimurium* gene from sample 1 having 99% similarity. *Salmonella typhi* strain T7 and *S. typhimurium* strain S1 comes from the same root of taxan having 85% similarity, sample-3 is distantly related to *S. enteritidis* and *S. paratyphi* strain A5 having 69% similarity with in all constructed three trees sample one having the highest are present in the similarity and also they clade. From this, sample-1 suggests an evolutionary pathway for the novel form.

## Conflict of interest statement

We declare that we have no conflict of interest.

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