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RESEARCH ARTICLE

PREVALENCE AND ANTIBIOTIC SUSCEPTIBILITY PROFILING OF *SALMONELLA TYPHI* FROM TYPHOID PATIENTS IN AMRAVATI CITY (MAHARASHTRA) INDIA

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ABSTRACT

This study was conducted in order to known the occurence of MDR *Salmonella typhi* in Amravati city. Widal positive blood samples were collected and analysed for typhoid causative agent *Salmonella typhi*. The *Salmonella typhi* isolates were obtained and confirmed by various morphological, biochemical, cultural and molecular methods. Antibiotic susceptibility testing of *Salmonella typhi* isolates was carried out using disk diffusion method. Out of 400 blood samples, 230 samples showed the presence of *Salmonella typhi.* The isolates were obtained from blood specimen includes 93 (40.43%) *Salmonella typhi* isolates from male and 137 (59.57%) from female patients. The prevalence of *Salmonella typhi* isolates was found to be 57.5% in Widal positive blood samples with the 1:160 dilution. Out of 230 isolates of *Salmonella typhi* 124 isolates showed the resistance to more than five antibiotics among the 10 antibiotics tested and the prevalence rate of MDR *Salmonella typhi* was found to be 53.91%. The widespread resistance patterns were observed with amoxycillin, cefpodoxime, chloramphenicol, kanamycin, tetracycline and trimethoprime. Among all the tested antibiotics ciprofloxacin and ofloxacin were found to be the most active antibiotics against (94.35%) *Salmonella typhi* isolates followed by co-trimoxazole against (83.48%) and nalidixic acid against (69.57%) *Salmonella typhi* isolates. From the present study it is clear that occurrence of MDR *Salmonella typhi* strains are high in the Amravati city and suggested to use the antibiotics ciprofloxacin and ofloxacin as a first line treatment for typhoid patients in the city.

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INTRODUCTION

Enteric fever is a universal public health problem. Almost 80% of the cases and deaths are reported in Asia and the rest occur mostly in Africa and Latin America (WHO, 1996). Typhoid fever is estimated to cause 16-33 million illnesses and 500,000 to 600,000 deaths annually in endemic areas. The World Health Organization identified typhoid as a serious public health problem. Its incidence reported to be highest in children and young adults between 5 and 19 year old (WHO, 2007). Typhoid fever is evolving as a major threat with high morbidity and mortality rate in developing countries (Crump *et al*., 2004). Typhoid fever is a severe bacterial disease caused by *Salmonella enterica* serotype Typhi and emerging as one of the most wide spread of all bacterial diseases in India (Chandrasekaran and Balakrishnan, 2011).

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Salmonella enterica serovar Typhi and Paratyphi A are the most common types of infective agents responsible for enteric fever in India, especially in summer season (Jesudason and John, 1992). The emergence of antimicrobial resistance, particularly the multidrug resistance to ampicillin, chloramphenicol and co-trimoxazole, has further complicated the treatment and management of enteric fever (Mourad *et al*., 1993). Since, 1960 antibiotic resistance among *Salmonella typhi* has been reported in India and first outbreak of multidrug resistance *S. typhi* was reported in Calicut (Agarwal, 1962). Recently, the outbreak of *Salmonella typhi* (enteric fever) was reported in sub-urban area of North India and maximum cases were in the age group of 5-14 years. Most importantly, all the strains were resistant to ampicillin and nalidixic acid (Singla *et al*., 2013). Effective treatment for typhoid fever was been a great challenge in scientific community. Over a time, vaccines have been developed against strains of *Salmonella* (Myron *et al*., 1976). On the other hand, drawbacks in the take up of vaccines to manage typhoid fever, antibiotics therapy remains a feasible option in combating the infection.

Several antibiotics used for typhoid treatment are ampicillin, chloramphenicol, β-lactams, sulphademidine, gentamicin, aminoglycosides, nalidixic acid, ciprofloxacin and the fluoroquinolones (norfloxacin and ofloxacin) (Girgis *et al*., 1999; Kadhiravan *et al*., 2005). Continuous use of these antibiotics leads to the development resistance among organisms that creates a major threat in use of antibiotic therapy policies. Severity of infections increasing day by day due to the antibiotic resistance, which is mainly responsible for high mortality rate (Davies and Davies, 2010). Early and proper antibiotic administration is important in order to reduce mortality in typhoid infection cases especially caused by *Salmonella typhi* (Cooke and Wain, 2004). The aim of the present study was to determine the prevalence of MDR *Salmonella typhi* to the most widely used antibiotics *viz.* amoxicillin, cefpodoxime, chloramphenicol, ciprofloxacin, kanamycin, ofloxacin, nalidixic acid, co-trimoxazol, tetracycline and trimethoprime.

MATERIALS AND METHODS

Collection of Blood Samples

A total of 400 Widal positive blood samples with titre of 1:160 were collected from Irwin hospital of Amravati city, Maharashtra (India). The blood samples were packed well on ice pack and brought to laboratory. All samples were processed within 6 hrs of span.

Isolation and Characterization of *Salmonella typhi* from Blood Specimen

A 1 ml of blood sample was inoculated into a tube containing 9 ml of brain heart infusion broth and incubated at 37°C for 72 hrs. The growth from blood cultures was processed for isolation and identification of *Salmonella typhi* isolates (Shetty *et al*., 2012). The resulting inoculums was streaked on selective media bismuth sulphide agar (BSA) and xylose lysine deoxycholate (XLD) agar and incubated at 37°C for 72 hrs. The colonies with characteristics feature of jet black and pink with black centre colonies were stocked on to nutrient agar slants and kept at 4°C until use. Besides selective isolation of *Salmonella typhi,* cultures were further confirmed on the basis of morphological characteristics and biochemical tests (Collee *et al*., 1996).

Molecular characterization using 16S rRNA gene sequencing was only done with the ST-1 isolate, because of high cost of analysis. Bacterial Genomic DNA was isolated using the InstaGene™ Matrix Genomic DNA isolation kit. The procedure was followed according to the instructions given in kit. Using 16S rRNA Universal primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (ACGGYTACCTTGTTACGACTT), gene fragment was amplified using MJ Research Peltier Thermal Cycler. The PCR product was sequenced using the 785F/907R primers. Sequencing reactions were performed using a ABI PRISM® BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems). Sequence data was aligned and analyzed for identifying the sample.

Bioinformatics protocol

The 16s rRNA sequence was blast using NCBI blast similarity search tool. The sequences of these 16S rRNA genes were compared against the sequences available particularly for *Salmonella typhi* from GenBank using the BLASTN program (Altschul *et al*., 1990) and were aligned using CLUSTAL W software (Thompson *et al*., 1994). Distances were calculated according to Kimura's two-parameter correction (Kimura, 1980). The phylogeny analysis of our sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment. The neighbour-joining method was used to construct phylogenetic tree. Bootstrap analysis was done based on 1000 replications. The Phylogenetic analysis was conducted using MEGA 4 package (Tamura *et al*., 2007).

Antibiotic Sensitivity Testing

All the *Salmonella typhi* isolates were tested against 10 standard antibiotics *viz.* Amoxicillin (10mcg), Cefpodoxime (10mcg), Chloramphenicol (10mcg), Ciprofloxacin (10mcg), Kanamycin (5mcg), Ofloxacin (5mcg), Nalidixic acid (30mcg), Co-Trimoxazol (25mcg), Tetracycline (10mcg) and Trimethoprime (10mcg) using disk diffusion method (Bauer *et al*., 1966). The growth medium Mueller Hinton agar was prepared and poured in 90 mm Petri plates. The discs of the standard antibiotics (Hi-media Pvt. Ltd. Mumbai) were placed onto the surface of the Mueller Hinton agar plate, which was prelawned with the appropriate inoculum size by streaking the cotton swab uniformly by turning the plate 60° between each streaking aseptically. The plates were left undisturbed for 30 minutes to allow appropriate diffusion (Dorman and Deans, 2000) into the agar. After diffusion the plates were incubated aerobically at 37°C for 24 hrs. This was followed by measurement of zone of inhibition (ZOI) exhibited by the isolates to the standard antibiotic discs (NCCLS, 1999). The results of antibiotic susceptibility testing of *Salmonella typhi* isolates was interpretated and categorized as ZOI of ≥ 18 mm (Sensitive), 13-17 mm (Intermediate sensitive) and ≤ 12 mm (Resistant) as described by Nkang *et al*., 2009.

RESULTS

Isolation and Identification of *S. typhi*

Out of 400 blood samples collected, 230 samples showed the presence of *Salmonella typhi.* The isolates were obtained from blood specimen includes 93 (40.43%) *Salmonella typhi* isolates from male and 137 (59.57%) from female patients. The prevalence of *Salmonella typhi* isolates was found to be 57.5% in Widal positive blood samples with the 1:160 dilution. The black colonies with sheen on bismuth sulphite agar and red colonies with black centres on XLD medium subjected to the morphological and biochemical tests showed that the isolates were Gram negative, motile rods, indole negative, methyl red positive, Voges Proskauer negative, Simmons citrate utilization negative and positive triple sugar iron agar test. From these results, the isolates were identified as *Salmonella typhi* and as a representative isolate ST-1 was confirmed by 16S rRNA sequencing method.

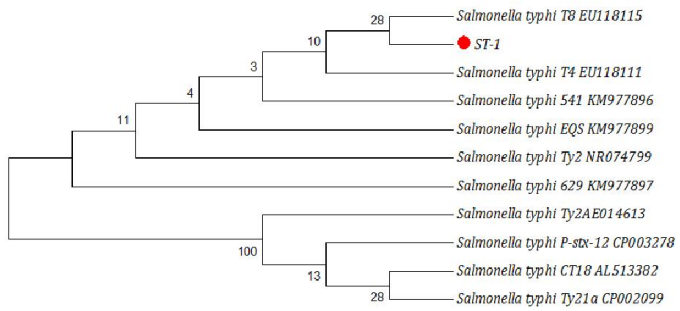

Figure 1. Phylogenetic tree created on the basis of comparison of the 16S ribosomal DNA sequence of *Salmonella typhi* isolate ST-1 to the closest phylogenetic relatives(The tree was created by the neighbor-joining method and numbers on the tree indicates the percentages of bootstrap sampling)

According to 16S rRNA gene sequences, isolate ST-1 shown a high level of similarity with the type strain of *Salmonella typhi* and showed a substantial degree of relatedness to reference 16S rRNA sequence of *Salmonella enterica* serovar Typhi in the database. The 16S r RNA sequence of ST-1 isolate, when compared with complete genome of *Salmonella enterica* serovar Typhi available in database showed high similarity value to *Salmonella enterica* serovar Typhi AL513382 (99%), CP002099 (99%) and CP003278 (99%) with maximum alignment score of 2689, while in case of partial sequence maximum alignment score of 2684 was observed with *Salmonella enterica* serovar Typhi EU118115 and KM977896. The phylogenetic data were obtained by alignment and phylogenetic analysis of the nucleotide sequence. Phylogenetic tree including the isolate and related phylogenetic neighbors were constructed using the MEGA 4.0 software program (Figure 1). The isolated bacterial nucleotide and phylogeny relationship with NCBI nucleotide database has clearly revealed that, the given sample is belongs to the taxa of *Salmonella enterica* serovar Typhi.

Antibiotic Profiling of *Salmonella typhi* Isolates

In vitro antimicrobial susceptibility test has an important role in suggesting proper antimicrobial chemotherapy in cases of life threatening infections, where patient not respond to medication. In the present study antibiotic susceptibility was studied for 230 isolates of *Salmonella typhi* and examined towards 10 antibiotics using Kirby-Bauer disk diffusion method on Muller Hinton agar (Table 1) Antibiotics with known concentrations were included in the study are amoxicillin (10mcg), cefpodoxime (10mcg), chloramphenicol (10mcg), ciprofloxacin (10mcg), kanamycin (5mcg), ofloxacin (5mcg), nalidixic acid (30mcg), co-trimoxazol (25mcg), tetracycline (10mcg) and trimethoprime (10mcg). Out of 230 isolates of *Salmonella typhi* 124 isolates showed the resistance to greater than five antibiotics among the 10 antibiotics tested and the prevalence rate of MDR *Salmonella typhi* was found to be 53.91%. Among 230 isolates of *Salmonella typhi,* 228 (99.13%) isolates showed the highest resistance to amoxicillin. The antibiotics ciprofloxacin and ofloxacin were found to be the most active antibiotics against (94.35%) *Salmonella typhi* isolates followed by co-trimoxazole against (83.48%) and nalidixic acid against (69.57%) *Salmonella typhi* isolates. However, highest intermediate sensitivity was observed in tetracycline against 31.74% *Salmonella typhi* isolates followed by trimethoprime against (24.78%), chloramphenicol against (20.87%) and cefpodoxime against (19.57%) *Salmonella typhi* isolates. In case of amoxicillin 99.13% isolates showed resistance, while one isolates was intermediately sensitive and one was sensitive isolate. Overall, amoxicillin was found to be least effective antibiotic as compared to all the tested antibiotics. For cefpodoxime antibiotic 123 (53.48%) *Salmonella typhi* isolates were resistant, 45 (19.57%) intermediately sensitive and 62 (26.96%) were sensitive isolates.

Table 1. Antimicrobial susceptibility pattern of *Salmonella typhi* isolates

Antibiotic	*Salmonella typhi* isolate (%) (n=230)		
	Resistant	Intermediate sensitive	Sensitive
Amoxycillin	228 (99.13)	1(0.43)	1(0.43)
Cefpodoxime	123 (53.48)	45(19.57)	62(26.96)
Chloramphenicol	182 (79.13)	48(20.87)	0(0)
Ciprofloxacin	12 (5.22)	1(0.43)	217(94.35)
Kanamycin	195 (84.78)	11(4.78)	24(10.43)
Ofloxacin	13 (5.65)	0 (0)	217(94.35)
Nalidixic acid	48 (20.87)	22(9.57)	160(69.57)
Co-trimoxazole	22 (9.57)	16(6.96)	192(83.48)
Tetracycline	149 (64.78)	73(31.74)	8(3.48)
Trimethoprime	166 (72.17)	57(24.78)	7(3.04)

In case of co-trimoxazole 83.48% isolates were effectively sensitive, 6.96% isolates were intermediately sensitive and 9.57% isolates were resistant. Whereas tetracycline and trimethoprime antibiotics were found to be least sensitive after amoxycillin by showing sensitivity only against 3.48 and 3.04% isolates, respectively. Out of 230 isolates, 149 and 166 isolates displayed resistance, 73 and 57 isolates showed intermediate sensitive to tetracycline and trimethoprime antibiotics, correspondingly. All the tested isolates exhibited multiple antibiotics resistance (MAR) index in the range of 0.2 to 0.9. The highest MAR index of >0.5 was recorded against 121 (53%) isolates. In the study area inspection of antibiotic resistance of *Salmonella typhi* isolates is useful in establishing new criteria of antimicrobial chemotherapy in the typhoid treatment. The individual highest MAR index value of 0.9 was observed against TYD-16, TYD-36, TYD-81, TYD-106, TYD-126, TYD-202 and TYD-228 isolates. However, MAR index of 0.8 was recorded against TYD-15, TYD-64, TYD-65, TYD-125, TYD-155, TYD-166, TYD-171, TYD-193, TYD-201 and TYD-222 isolates. Overall, the MDR isolates of *Salmonella typhi* exhibited poor susceptibility to antibiotics including amoxycillin, cefpodoxime, chloramphenicol, kanamycin, tetracycline and trimethoprime. All the tested isolates were more or less consistently susceptible to ciprofloxacin, ofloxacin, co-trimoxazole and nalidixic acid.

DISCUSSION

The outbreaks of typhoid have been documented from different parts of India (Choudhary *et al*., 2013; Nagshetty *et al*., 2010; Madhulika *et al*., 2004). In the current study out of 400 blood samples, 230 showed the presence of *Salmonella typhi* with the isolation rate of 57.5%. Alike, Hasan *et al*., (2011) reported 16% isolation rate of *Salmonella typhi* in blood cultures, which is much lesser than present findings.

However, Akter *et al*., (2012) isolated the *Salmonella typhi* from blood samples as a major species of typhoid infection with an isolation rate of 73.81%. In the present study, out of 230 isolates of *Salmonella typhi,* 124 isolates were multi-drug resistant (MDR) showing the resistance to more than five antibiotics with the their greater prevalence rate of 53.91%, than sensitive *Salmonella typhi* (46.09%). The prevalence rate of MDR *Salmonella typhi* was in agreement with the findings of Misra *et al*., (2005) who reported the outbreak of MDR *Salmonella typhi* with the prevalence rate of 63% in Mumbai Garrison. Similarly, an outbreak of MDR *Salmonella typhi* was reported previously in different parts of India (Madhulika *et al*., 2004; Joshi and Amarnath, 2007; Sathiamoorthi *et al*., 2011; Menezes *et al*., 2012) and in other countries (Onyango *et al*., 2008; Muyembe-Tamfum *et al*., 2009). The antimicrobial susceptibility pattern of *Salmonella typhi* isolates from blood specimen in India have been described by Poudel *et al*., (2014) and reported the higher prevalence rate (78.4%) of MDR *Salmonella typhi*.

In the present study ciprofloxacin and ofloxacin were found to be the most active antibiotics against 94.35% isolates of *Salmonella typhi,* followed by Co-trimoxazole against 83.48% isolates and nalidixic acid against 69.57% *Salmonella typhi* isolates. Effectiveness of ciprofloxacin was previously verified by Li *et al*., (2013) in the treatment of Salmonella induced systematic infections. However, the decreased susceptibility of ciprofloxacin was reported by Gaborieau *et al*., (2010). Menezes *et al*., (2012) reported 78% *Salmonella typhi* isolates as nalidixic acid resistant (NAR) while in our study 48% isolates found to be nalidixic acid resistant. In our study, the decreased rate of resistance was observed with ciprofloxacin (5.22%), ofloxacin (5.64%) and co-trimoxazole (9.57%). Drug resistance is fast becoming a major problem in the management of this infection. Chloramphenicol resistance became established globally in the *S. typhi* population. In a different study from Canada resistance of *Salmonella typhi* isolates to nalidixic acid was observed 80% while resistance to β-lactams remained steady during 2002-2007 (Morris *et al*., 2009).

Comparatively in our study nalidixic acid resistance (20.87%) was observed against 48 *Salmonella typhi* isolates. A study of 41 cases of typhoid fever from Cambodia demonstrated MDR rates of 56% against ampicillin, trimethoprim and chloramphenicol (Kasper *et al*., 2010), while in our study the resistance rate was recorded 79% for chloramphenicol and 72% towards trimethoprime. In contrast to trimethoprime resistance in *Salmonella typhi,* the susceptibility has been reported between 43% and 100% for trimethoprim which was recorded between 2006-2013 in Lebanan (Kanj *et al*., 2015). From the above studies it is clear that resistance pattern among *Salmonella typhi* isolates diverge with the geographical region. Differences in the resistance pattern created the need to examine typhoid patients and implement new chemotherapeutic policies.

Conclusion

Accurate diagnosis and regular susceptibility testing has become increasingly important in treating typhoid patients. In present investigation, the prevalence of *Salmonella typhi* isolates was found to be 57.5% in Widal positive blood samples with the 1:160 dilution. Antibiotic susceptibility pattern of ciprofloxacin against *Salmonella typhi* isolates showed the highest effectiveness by showing sensitivity against 94.35% isolates and one isolates was found to be intermediately sensitive. The highest MAR index of >0.5 was recorded against 121 (53%) isolates. In the study area inspection of antibiotic resistance of *Salmonella typhi* isolates is useful in establishing new criteria of antimicrobial chemotherapy in the typhoid treatment.

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