Original article

Phenotypic characterization and antibiogram of Salmonella isolated from enteric fever patients at a tertiary care hospital

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Abstract:

Background: Enteric fever (EF) is a major problem especially in developing countries. Trend of antibiogram in *Salmonella* is changing continuously. Hence, monitoring of the prevailing serotypes and their antibiogram is imperative.

Aims: 1.To characterize the prevalent serotypes of *Salmonella* isolated from blood culture of EF patients. 2. To study the antimicrobial resistance pattern of these isolates 3.To determine the Minimum Inhibitory Concentration (MIC) of ciprofloxacin against the isolates.

Methods: Fifty isolates of *Salmonella* recovered from blood culture/clot culture of suspected cases of EF, identified by standard biochemical methods and serotyping were studied. Antimicrobial susceptibility was tested by Kirby-Bauer disc diffusion method. The MIC of ciprofloxacin against these isolates was determined by E test and compared with their corresponding disc diffusion results.

Results: Of the 50 isolates, 32;64% were *Salmonella* Typhi, 12;24% *S.* Paratyphi A and 6;12% *S.* Paratyphi B. Among these, 32;64% isolates were from paediatric patients and 18;36% from adults. Isolates recovered from Indoor, Outpatients' department and Intensive care units patients were 35;70%, 9;18% and 6;12% respectively. More than 90% of the isolates were susceptible to ampicillin, chloramphenicol and co-trimoxazole and all were sensitive to ceftriaxone and azithromycin. Isolates showed mixed antibiogram for fluoroquinolones, with 17;34% being ciprofloxacin susceptible and 37 (74%) ofloxacin susceptible while only 4 (8%) were susceptible to nalidixic acid. Results of zone diameter and MIC were in total agreement.

Conclusion: The widespread emergence of resistance to fluoroquinolones and reappearance of sensitivity to first line drugs has reinforced the need for antibiotic recycling for uncomplicated enteric fever.

Key words: Salmonella, serotypes, minimum inhibitory concentration, antibiogram, ciprofloxacin, ceftriaxone

Introduction:

Enteric fever (EF) is endemic in many developing countries with high incidence in Asia and medium incidence in Africa and Latin America/Caribbean regions.[1] It is caused by *Salmonella enterica* serovar Typhi (*S.* Typhi) and less commonly by *Salmonella enterica* serovar Paratyphi (*S.* Paratyphi A, B and C).[2]

The continually changing antibiogram and emergence of drug resistance in *Salmonella* have resulted in treatment failure.[3] This not only prolongs the illness but also increases risk of complications as well as transmission. The era of multidrug resistant (MDR)

Salmonella has dawned. Multidrug resistance (MDR) is defined as resistance to all first line antimicrobials viz. ampicillin, chloramphenicol and cotrimoxazole (ACCo). MDR paved the way for the discovery of quinolones and flouroquinolones (ciprofloxacin, ofloxacin, etc). However, low level resistance to ciprofloxacin soon developed.[3] There were ever increasing reports of treatment failure with ciprofloxacin in patients with EF.[4] This led to calls for modifications in ciprofloxacin breakpoints for better detection of ciprofloxacin resistance in order to improve therapeutic outcome.[5] In 2012, Clinical and Laboratory Standards Institute (CLSI) published evidence-based revision of the minimum inhibitory concentration (MIC) of ciprofloxacin and the disc diffusion interpretative criteria.[6] The changing antibiogram patterns and serotypes necessitate their continuous monitoring.

Aims and Objectives: 1.To characterize the prevalent phenotypes (serotypes) of *Salmonella* isolated from blood culture/clot culture of Enteric fever patients

2. To study the antimicrobial resistance pattern of these isolates

3. To determine the Minimum Inhibitory Concentration (MIC) of ciprofloxacin against the isolates.

Materials and methods :

The study was conducted in the Department of Microbiology, at a tertiary care teaching hospital, after approval of the study protocol by the Institutional Ethics committee. Fifty isolates of *Salmonella* recovered from Blood specimens (blood culture/clot culture) from suspected cases of Enteric fever, in 2012-2014 were studied.

Sample collection and processing

Peripheral venous blood (5ml from children and 8-10ml from adults) was collected under aseptic precautions in Brain heart infusion broth (BHIB) or BACTEC Peds Plus/F or BACTEC Aerobic Plus/F broth medium) for conventional or automated blood culture (BACTEC 9120 System) respectively. For clot culture, 5ml venous blood was collected in plain vacutainer. For conventional blood culture, the BHIB was incubated at 37^0C for 18- 24 h. Blind subcultures were done on blood agar (BA) and MacConkey agar (MA) on 2^{nd}, 4^{th} and 7^{th} day of incubation. In case of automated culture, inoculated BACTEC bottles were incubated in BACTEC 9120 system. If a bottle flashed positive, smear was prepared for Gram staining and the sample was inoculated on BA and MA and the plates incubated as above. For clot culture, 5 ml of bile broth was added to the clot and incubated for 24 hours. Subcultures were done on 2^{nd}, 4^{th} and 7^{th} day of incubation, on MA. Colonies suggestive of *Salmonella* species were further processed for identification and characterization. All the clinical isolates of *Salmonella* were identified using standard microbiological techniques and were further confirmed by serotyping with specific antisera (Denka seiken, Japan).[7]

Antimicrobial susceptibility testing:

The antimicrobial susceptibility testing of the isolates was done by Kirby-Bauer disc diffusion method using Mueller-Hinton agar medium and commercially available antibiotic discs (Hi-Media Laboratories Ltd, Mumbai), in accordance with the CLSI 2012 guidelines and interpretative criteria. The antimicrobial agents tested were ampicillin (10 µg), chloramphenicol (30 µg), trimethoprim/sulfamethoxazole (co-trimoxazole) (1.25/23.75 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), ofloxacin (5 µg), ceftriaxone (30µg),

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cefotaxime (30 µg), cefixime (5 µg) and azithromycin (15 µg). The diameter of the zones of inhibition were measured and interpreted according to CLSI 2012 guidelines .[6] For ciprofloxacin, the zone diameters were; susceptible (S)>=31, intermediate susceptible (I) 21-30, resistant (R)<= 20 mm. Isolates with intermediate levels of susceptibility were included in the percentage of resistant organisms for final analysis. For azithromycin, zone diameter of ≥19 mm was susceptible and ≤18 mm was resistant based on the British Society for Antimicrobial Chemotherapy (BSAC's) 2012 document.[8]

Minimum inhibitory concentrations of ciprofloxacin:

Minimum inhibitory concentrations of ciprofloxacin were determined by Epsilometer (E test) according to the manufacturer's instructions (Hi-Media Laboratories Ltd, Mumbai). The E test strip contained ciprofloxacin in the concentrations ranging from 0.032 - 32 µg/ml. The zone of inhibition was observed in the form of an ellipse. The MIC value was taken as the reading on the E test strip where the zone of inhibition intersected with E test strip. It was then interpreted as susceptible, intermediate or resistant as per CLSI 2012 guidelines (S <=0.06 ,I 0.12-0.5, R>= 1 (µg/ml).

Escherichia coli ATCC 25922 was used as control strain for antimicrobial susceptibility testing and MIC determination.

Sensitivity Index (SI) was calculated as the ratio of Sensitive (S) percentage / Resistant (R) percentage.[9]

Statistical analysis: The data was entered in Microsoft Excel. Percentages were obtained for qualitative data.

Results:

Out of 50 isolates of *Salmonella, S.*Typhi (32; 64%) predominated followed by *S.* Paratyphi A (12; 25.5%) and *S*. Paratyphi B (6; 12.8%). A total of 32; 64% isolates were obtained from pediatric patients while 18; 36% were from adults. Maximum isolates (41; 82%) were recovered from indoor patients and (9;18%) were from patients who were treated through the Outpatients' department (Table 1).

Majority of the isolates, were susceptible to ampicillin, chloramphenicol, and cotrimoxazole i.e. 49 (98%), 50 (100%), and 47 (94%) respectively while only 4 (8%) were susceptible to nalidixic acid. Isolates showed mixed antibiogram for fluoroquinolones, with 17 (34%) being ciprofloxacin susceptible and 37 (74%) ofloxacin susceptible. All 50 isolates in the series were susceptible to third generation cephalosporins and azithromycin (Table 2). None of the isolates was MDR.

Sensitivity index of 50 isolates was 100 for cephalosporins, azithromycin, and chloramphenicol; 49 for ampicillin, 15.7 for cotrimoxazole, 3.7 for ofloxacin, 1.54 for ciprofloxacin and 0.08 for nalidixic acid (Table 2).

Results of zone diameter and MIC for ciprofloxacin were in total agreement as shown in Table 3.

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Table 1: Demographic distribution of Patients from whom Salmonella was isolated (n=50)

***Salmonella* species**	Age Group		Gender		Location		
Salmonella species	Pediatric	Adult	Male	Female	Ward	OPD	ICU
S. Typhi (n=32; 64%)	21 (42%)	11 (22%)	20 (40%)	12 (24%)	22 (44%)	7 (14%)	3 (6%)
S. Paratyphi A (n=12;24%)	6 (12%)	6 (12%)	5 (10%)	7 (14%)	9 (18%)	2 (4%)	1 (2%)
S. Paratyphi B (n=6;12%)	5 (10%)	1 (2%)	3 (6%)	3 (6%)	4 (8%)	0	2 (4%)
Total (n=50)	32 (64%)	18 (36%)	28 (56%)	22 (44%)	35 (70%)	9 (18%)	6 (12%)

Table 2: Antibiogram of *Salmonella* from Blood specimen (n = 50)

Antimicrobial agent	***Salmonella* Typhi**	***Salmonella* Paratyphi A**	***Salmonella* Paratyphi B**	***Salmonella enteric***
	Sensitive	**Sensitive**	**Sensitive**	**Sensitive**
	n %	**n %**	**n %**	**n %**
Ampicillin	32 (100)	11 (91.67)	6 (100)	49 (98)
Chloramphenicol	32 (100)	12 (100)	6 (100)	50 (100)
Cotrimoxazole	30 (93.7)	11 (91.7)	6 (100)	47 (94)
Nalidixic acid	2 (6.2)	0 (0)	2 (33.3)	4 (8)
Ciprofloxacin	11 (34.3)	3 (25)	3 (50)	17 (34)
Ofloxacin	28 (87.5)	5 (41.7)	4 (66.7)	37 (74)
Ceftriaxone	32 (100)	12 (100)	6 (100)	50 (100)
Cefotaxime	32 (100)	12 (100)	6 (100)	50 (100)
Cefixime	32 (100)	12 (100)	6 (100)	50 (100)
Azithromycin	32 (100)	12 100)	6 (100)	50 (100)

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Table 3: Minimum Inhibitory Concentration (MIC) and Zone diameters of Ciprofloxacin against Salmonella isolates. (n=50)

MIC values (µg/ml)	Zone diameter (mm)	*S.* Typhi (n=32)	*S.*Paratyphi A (n=12)	*S.*Paratyphi B (n=6)
0.032	32,33,33,33	3	0	1
0.047	31,31,32,32,32,32,32	5	2	0
0.064	31,31,31,31,31,32	3	1	2
0.125	29,29,29,30	3	1	0
0.25	28,30,30	1	1	1
0.38	26,27,28,28	4	0	0
0.50	25,27,27,27	4	0	0
0.75	24,25,25,26,26,26,28	6	1	0
1	19	0	0	1
1.5	18	0	1	0
2	17,19,19,19	2	1	1
8	15,16	0	2	0
12	16	0	1	0
32	12,12	1	1	0

Discussion

Enteric fever is endemic in India and is still a major cause of morbidity and mortality across age groups. In the present study, maximum isolates (32;64%) were from the patients of paediatric age group resulting in paediatric to adult ratio of 1.6:1. It is evident from numerous reports that the paediatric population is more susceptible to enteric fever.[10,11,12] Gupta *et al.* from Chandigarh, in their three-year study (2008 to 2010), noted that 54.6% of their 302 isolates were recovered from children, the ratio of paediatric to adult being 1.2:1.[13] Children are more likely to suffer from clinical infection than adults who usually develop immunity from recurrent subclinical /clinical infection. In our series, majority of isolates were recovered from hospitalized patients

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with the ratio of patients hospitalized to those treated through the Outpatients' department being 4.22:1; a high rate of hospitalization was also observed by Capoor *et al.* in their 5 year study (2001 to 2006) with 3.8% increase in hospitalization noted over the years.[14] This could be due to a commensurate increase in the numbers of *Salmonella* strains with reduced susceptibility to ciprofloxacin which have been shown to be associated with treatment failures and morbidity.[3]

Salmonella enterica serovar Typhi (*S.* Typhi) classically causes enteric fever in India and is considered as the commonest serovar (serotype). Infections due to Paratyphoidal *Salmonella* were rare till 1996. An increase in *Salmonella enterica* serovar Paratyphi A (*S.* Paratyphi A) infections was first reported from north India.[15] Soon *S.* Paratyphi A gained importance as a rapidly emerging pathogen causing enteric fever in India.[16,17,18] In the present study, although *S.*Typhi (32;64%) was predominant, 36% of the cases were due to paratyphoidal salmonella (*S.* Paratyphi A in12;24% and *S.* Paratyphi B in 6;12%). Muhammad *et al* reporting from a teaching hospital in Pakistan (2013) detected *S.*Typhi, *S.*Paratyphi A and *S.*paratyphi B in 54.7%, 32.1% and 13.2% cases respectively.[19] Similar findings were noted by Lakshmi et al (2006, Hyderabad) and Choudhary *et al*. (2009, Chennai)'.[20,21]

The antibiotic susceptibility pattern of *Salmonella* has been changing with time and geographical location. In our study, 94% of the isolates were susceptible to the first line drugs ampicillin, chloramphenicol and cotrimoxazole (A C Co) and none of the isolates was MDR. Garg *et al*. from north India also observed high sensitivity to first-line agents with none of their isolates being MDR.[22] While Jog et al noted a return of sensitivity to the first line agents, Shastri et al also from Mumbai detected only one MDR isolate in their study.[23, 24] Srirangaraj *et al* [25] from Pondicherry (2014) also reported high susceptibility to the first line drugs, with one MDR *Salmonella* isolate, although an earlier study also from Pondicherry (2011) had shown only 66% of the *S.* Typhi isolates to be susceptible to first-line antimicrobials, with 22% being MDR.[26] A 12-year study (2001-2012, Chandigarh) by Singhal *et al* observed increase in susceptibility to first line antibiotics and decrease in MDR strains over the years. [12] The remarkable reversal in antibiogram, especially to ampicillin and chloramphenicol may be due to a decrease in use of these first line antibiotics for enteric fever, thus resulting in reduced selection pressure. Due to the lack of exposure, *Salmonella* isolates might have lost molecular mechanisms responsible for resistance. Emergence of de novo susceptible strains or the loss of high molecular weight self-transmissible plasmids might be other explanations for re-emergence of susceptibility.[27,28] The high rate of NA resistance seen in our study has also been noted in other studies from various parts of India including Mumbai.[29,23,24] In our study, 17(34%) isolates were susceptible to ciprofloxacin while the majority (44%) fell in the intermediate susceptibility range with respect to zone diameter as well as MIC. Similarly, many studies have shown an increase in isolates with intermediate susceptibility compared to susceptible strains. Increase in resistance to ciprofloxacin is indicative of irrational use and overuse of the drug in the treatment of typhoid fever as well as several other infections, both in the community and in the hospital. Incomplete treatment in developing countries may also be a contributing factor. We noted discrepancies

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in susceptibility of ciprofloxacin with ofloxacin. While 17 isolates showed susceptibility to both ciprofloxacin and ofloxacin, 20 isolates showed susceptibility to ofloxacin and intermediate susceptibility to ciprofloxacin. These observations were concurrent with several other studies like Kawser *et al.* and Capoor *et al.*[30,31] Conversely, Mandal *et al* (2003) observed that ofloxacin resistance in *S*. enterica serovar Typhi was not detected by either the MIC breakpoints or the equivalent zone diameters suggested by NCCLS (now CLSI) and suggested a re-evaluation of these breakpoints for ofloxacin.[32] Subsequently, in 2013, ofloxacin disc diffusion zone diameter interpretative criteria for *S*. Typhi has been removed and MIC interpretative criteria has been revised.[33]

All our isolates were susceptible to third generation cephalosporins. Majority of the studies have shown 100% susceptibility [34,23,24] with a few unfortunate reports of resistant strains in Indian subcontinent.[11,19,35,36] Among third generation cephalosporins, cefixime being orally active can be utilised for the treatment of ambulatory patients and children. Use of ceftriaxone is restricted by the parenteral route of administration besides its cost. With concerns of resistance developing to ceftriaxone, it should be used cautiously and only in proven cases of enteric fever.

Prior to modifications in CLSI 2012 guidelines, it was noted that decreased susceptibility to ciprofloxacin (DSC) was associated with therapeutic failure.[3] However, these cases could not be detected by the CLSI breakpoints for ciprofloxacin, existing at that time. In view of this, CLSI 2012 guidelines made recommendations to use separate ciprofloxacin interpretative criteria for all *S.* Typhi strains and extraintestinal *Salmonella spp*. for disc diffusion method as well as MIC. The results of disc diffusion testing and MIC were in total agreement in our study. These modified breakpoints will thus be useful wherever MIC determination is not feasible. The isolates showing intermediate susceptibility to ciprofloxacin as per revised guidelines may either be due to first step QRDR mutations or plasmid mediated mechanisms of resistance.[37] In cases infected with such isolates, clinicians now have the option of either adjusting the dosage and duration of treatment with a fluoroquinolone or prescribing another drug. However, to maintain the efficacy of flouroquinolones in both developing and developed countries, this class of antimicrobial agents must be reserved for treatment of invasive disease and not for prophylaxis.[38]

Conclusion

Re-emergence of *Salmonella* strains susceptible to ampicillin, chloramphenicol and cotrimoxazole calls for the use of these drugs in uncomplicated enteric fever. With the revision in CLSI (2012) breakpoints for ciprofloxacin with respect to extraintestinal *Salmonella,* the disc diffusion test results of ciprofloxacin may now be utilised for guiding treatment whenever MIC determination is not routinely feasible. Although, continued susceptibility to the third generation cephalosporins is encouraging, drugs like ceftriaxone should be used with caution.

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