Original Article

Antibiotic susceptibility pattern of *Salmonella enterica* serovar typhi and *Salmonella enterica* serovar paratyphi A with special reference to quinolone resistance

Abstract

Background and Objectives: Typhoid fever is endemic in India. Extensive use of first-line antibiotics has led to the emergence of multi-drug resistant (MDR) *Salmonella typhi*. Ciprofloxacin has become empirical therapy of choice against MDR salmonellae. Recent year's emergence of low-level ciprofloxacin resistance in salmonellae resulted in delayed response and serious complications. Nalidixic acid (NA) screen test is used as surrogate marker for detection low-level ciprofloxacin resistance. In this study, we evaluated prevalence of MDR and low-level ciprofloxacin resistant *S. typhi* and *Salmonella paratyphi* A. **Materials and Methods:** A total of 50 blood culture isolates of *S. typhi* and *S. paratyphi A* were tested for antibiotic susceptibility according to Clinical Laboratory Standards Institute (CLSI) method. Minimal inhibitory concentration (MIC) to ciprofloxacin was carried out by E-test and agar dilution method. **Results:** Among the 50 salmonella isolates, 80% were *S. typhi* and 20% were *S. paratyphi A*. MDR was found in 2% *S. typhi*. NA resistant salmonellae showed ciprofloxacin MIC ranging from 0.25 to 0.75 μg/ml. One isolate of *S. typhi* showed ciprofloxacin MIC of 32 μg/ml and was also resistant to ceftriaxone. NA screen test for low-level ciprofloxacin resistance was 100% sensitive and 97.9% specific. **Interpretation and Conclusion:** NA resistant isolates should be tested for ciprofloxacin MIC to decide therapeutic options. The current CLSI breakpoints may have to be re-evaluated for salmonellae.

Key words:
Ceftriaxone resistance, ciprofloxacin resistance, fluoroquinolone resistance, nalidixic acid resistance, Salmonella typhi, typhoid fever

Introduction

Typhoid fever is a major cause of morbidity and mortality with an estimated global incidence of 21.6 million typhoid fever cases and approximately 220,000 annual deaths.[1] The disease is endemic in India and other Southeast Asian countries, where nearly 80% of the world's typhoid fever cases occur. *Salmonella typhi* and *Salmonella paratyphi A* are the predominant species responsible for the enteric fever in India.

Untreated enteric fever patients have a mortality rate of 10–30%[1,2] and appropriate treatment reduces the mortality to 0.5%.[2] Multi-drug resistance (MDR) in *Salmonella* is defined as resistance to ampicillin, chloramphenicol, and trimethoprim – sulfamethoxazole (ACCo).[2,3] It was first

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reported in 1987 from China, South Asia, and Southeast Asia and in 1992 from Calicut, India.[4]

The emergence of MDR *S. typhi* (MDRST) led to the use of ciprofloxacin as the first-line drug in therapy.[5] Because of widespread use of ciprofloxacin, in 1993, *S. typhi* strains showing decreased susceptibility to fluoroquinolones (minimal inhibitory concentration [MIC] of 0.25–1 μg/ml) appeared.[6-8] Low-level ciprofloxacin resistance cannot be detected *in vitro* by a standard disk diffusion method using 5 μg ciprofloxacin disk, as these strains are interpreted as susceptible according to Clinical Laboratory Standards Institute (CLSI) recommendations.[7,8] However, such strains show *in vitro* resistance to 30 μg nalidixic acid (NA) disk and are called NA resistant *S. typhi* (NARST).[8] Therefore, NA can be used as a screening agent for detection of low-level ciprofloxacin resistance.[6,9] Treatment of enteric fever caused by NARST strains with ciprofloxacin has led to therapeutic failure.[8] In recent studies, high-level ciprofloxacin resistance with MIC of ≥4 μg/ml[2,10-12] and ceftriaxone resistant[13] *S. typhi* has been reported.

The purpose of this study was to screen for MDR *Salmonella* and to detect fluoroquinolone resistance among *S. typhi* and *S. paratyphi A* isolates obtained from blood samples of enteric fever patients.

Materials and Methods

Children and adults with a clinical suspicion of enteric fever attending on an outpatient or inpatient basis were included in the study. Patients having a fever with obvious foci of infection or fever due to other causes were excluded from the study.

Sample collection

Under aseptic precautions, 5 ml and 10 ml of venous blood was collected from children and adults respectively. Blood samples collected was inoculated aseptically into biphasic medium containing brain heart infusion (BHI) agar slant and BHI broth and was incubated at 37°C. Subcultures were done twice daily by tilting the bottle so that the broth runs over the slant and incubated at 37°C aerobically. If no growth was observed on agar slant, repeated subcultures were done for 7 consecutive days and bottles were discarded as sterile. If any growth on the agar slant was observed, the colonies were subcultured onto blood agar and MacConkey agar. The growth was identified by standard biochemical and agglutination tests.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was carried out by Kirby–Bauer disk diffusion method according to CLSI recommendations. Briefly 3–5 well isolated morphologically similar colonies were inoculated into the nutrient broth and incubated at 37°C for 2 h. Turbidity was adjusted to 0.5 McFarland standard and lawn culture was made on Mueller-Hinton agar and antibiotic disks were applied, plates were incubated at 37°C for 18–24 h. The diameter of the zone of inhibition was measured and interpreted according to CLSI recommendations. Quality control was performed by testing *Escherichia coli* ATCC 25922.

Nalidixic acid screen test

Isolates showing the NA (30 μg) disk zone size of ≤18 mm on disk diffusion testing were considered as NA resistant salmonellae (NARS) strains.

Ciprofloxacin minimal inhibitory concentration detection

- Agar dilution method: Agar dilution was done according to CLSI guidelines. Bacterial suspensions equivalent to 0.5 McFarland standard were inoculated onto Mueller-Hinton agar containing serial dilutions of ciprofloxacin (0.064–64 μg/ml). Plates were incubated at 37°C for 24 h. MIC were recorded as the lowest concentration of antimicrobial agent that completely inhibits growth, disregarding a single colony, or a faint haze caused by the inoculum. The quality control of the procedure was achieved by *E. coli* ATCC 25922
- E-test (AB Biodisk, Solna, Sweden): Ciprofloxacin MIC detection using E-test was performed according to manufacturer's instructions. A lawn culture of the 0.5 McFarland suspension of test organism was made on Mueller-Hinton agar. A ciprofloxacin E-strip was applied on the agar surface with the MIC scale facing upwards. The plates were incubated at 37°C for 18–24 h. The MIC values were read where the edge of the inhibition ellipse intersects the strip. The quality control of the procedure was achieved by *E. coli* ATCC 25922.

Results

A total of 2500 blood cultures were done of which 500 (20%) yielded bacterial growth and among them 50 (2%) were salmonellae. The blood culture positivity for salmonellae was 20 per 1000 febrile episodes. Among the 50 salmonellae isolates, the predominant serotype was *S. typhi* 40 (80%) followed by *S. paratyphi A* 10 (20%).

Of the 50 salmonellae, 32 were isolated from males and 18 from females (male: female ratio 1.7:1). The highest number of isolates (36%) was obtained from patients in 11 to 20 years age group. Median age group of patients was found to be 16 years (range 2–57). The maximum number of isolates was obtained from the patients attending hospital during the month of July.

Antibiogram of salmonellae is shown in Table 1. *S. typhi* showed 87.5%, 95% and 97.5% sensitivity to ampicillin,

Table 1: Antimicrobial susceptibility pattern of *S. typhi* and *S. paratyphi A*

Isolate	A *n* (%)	C *n* (%)	CO *n* (%)	CI *n* (%)	NA *n* (%)	CF *n* (%)
S. typhi (40)	35 (87.5)	38 (95)	39 (97.5)	39 (97.5)	1 (2.5)	39 (97.5)
S. paratyphi A (10)	10 (100)	10 (100)	10 (100)	10 (100)	0 (00)	10 (100)
Total (50)	45 (90)	48 (96)	49 (98)	49 (98)	1 (2)	49 (98)

A – Ampicillin (10 μg/disk); C – Chloramphinicol (30 μg/disk); CO – Co-trimoxazole (25 μg/disk); CI – Ceftriaxone (30 μg/disk); NA – Nalidixic acid (30 μg/disk); CF – Ciprofloxacin (5 μg/disk) (HiMedia lab ltd, Mumbai); *S. typhi – Salmonella typhi; S. paratyphi – Salmonella paratyphi*

chloramphenicol, and co-trimoxazole, respectively. All *S. paratyphi* isolates were 100% sensitive to ACCo drugs. MDR was found in 2% (*n* = 1) of *S. typhi*. In disk diffusion testing, NA resistance was found in 98% (*n* = 49) isolates. MIC values obtained in agar dilution method were comparable to E-test method. ATCC *E. coli* showed a ciprofloxacin MIC of 0.064 μg/ml. The isolates which were NA resistant on disk diffusion testing had ciprofloxacin MIC's between 0.25 μg/ml and 0.75 μg/ml in E-test. Median ciprofloxacin MIC for NARS strains was 0.38 μg/ml (range 0.25–0.75). One isolate of *S. typhi* which was NA sensitive had ciprofloxacin MIC of 0.064 μg/ml. The NA screen test was 100% sensitive and 97.9% specific and had 100% negative predictive values and 98% positive predictive value for detection of low-level ciprofloxacin resistance among the salmonellae causing enteric fever.

High-level ciprofloxacin resistance (>32 μg/ml) was found in 1 (2%) strain of *S. typhi*. It was also resistant to ceftriaxone in disc diffusion testing. On further testing, the isolate was confirmed as AmpC β-lactamase producer.

Discussion

The study showed typhoid prevalence in and around Bengaluru to be 20/1000 febrile episodes with blood cultures taken into consideration. In India, typhoid prevalence was reported to be 28.1/1000 febrile episodes.[14]

We noted a significant increase in sensitivity of typhoidal salmonellae to chloramphenicol (95%), ampicillin (87.5%), and co-trimoxazole (97.5%) when compared to previous findings.[15-17] The re-emergence of sensitivity to ACCo drugs is due to the removal of the selective pressures and extensive use of quinolones as first-line drugs, resulting in loss of the MDR phenotype. Currently in India, the incidence of MDRST varies from 7% to 55% and our study showed 2%.[2,15-17]

Many studies have reported cases of enteric fever treated with fluoroquinolones had a prolonged defervescence time or treatment failure.[9,18] The ciprofloxacin MIC of such strains is steadily increasing, although the MIC values were still below CLSI recommended breakpoint (≤1 and ≥4 μg/ml).[7,18] Such strains were found NARS. Resistance to NA in *S. typhi* has been reported to be mediated by a single point mutation at the quinolone resistance determining region of the *gyrA* gene.[2] Complete resistance to fluoroquinolones is usually associated with a double mutation in *gyrA* gene. In India, prevalence of NARST varies from 57% to 97%. In the present study, NA resistance was noticed in 97.5% of *S. typhi* and 100% of *S. paratyphi A* isolates. Among the 49 NARS strains, one *S. typhi* sereotype was resistant to ciprofloxacin and 48 were interpreted as ciprofloxacin sensitive according to current CLSI recommended zone sizes. NARS strains had mean ciprofloxacin zone of 24.71 ± 1.38 mm (range 21–28) while NA sensitive isolates showed a zone of 36 mm. The finding of the present study was similar to that published elsewhere.[6,19,20]

NARS had a median ciprofloxacin MIC of 0.38 μg/ml (range 0.25–0.75 μg/ml). Rupali *et al*.[21] found median ciprofloxacin MIC of 0.5 μg/ml (range 0.25–1 μg/ml) among NARS strains. NA screen test for low-level ciprofloxacin resistance was 100% sensitive and 97.9% specific when ciprofloxacin MIC of ≥0. 25 μg/ml was taken as the breakpoint and the zone of inhibition as ≤28 mm by disk diffusion method. One strain of *S. typhi* which was high-level ciprofloxacin-resistant was also NA resistant, hence decreases specificity. The result of NA screen test was comparable to the reports of previous studies.[6,12,19]

The scatter diagram [Figure 1] of ciprofloxacin MIC values against ciprofloxacin zone showed clustering of low ciprofloxacin-resistant strains in an area between 21 and 28 mm of ciprofloxacin zone. Hence, ciprofloxacin susceptible salmonellae on disk diffusion testing with a zone of >21–≤28 mm can be presumptively considered as strains with decreased susceptibility to ciprofloxacin.

One strain of *S. typhi* showed high-level resistance to ciprofloxacin (>32 μg/ml). The isolate was also an AmpC β-lactamase producer. Recently, *S. typhi* producing the ACC-1 type of AmpC β-lactamase has been reported.[13] There have been reports of *S. paratyphi A* and *S. typhi* showing high-level resistance to ciprofloxacin (>4 μg/ml).[10-12] To our knowledge, there have been no reports of the occurrence of high-level ciprofloxacin resistance and AmpC β-lactamase production in a single isolate of *S. typhi*. The occurrence of high-level ciprofloxacin resistance and third-generation cephalosporin resistance in a single isolate is an alarming situation, and it is recommended that their use should be restricted to empirical therapy of typhoid fever only. There

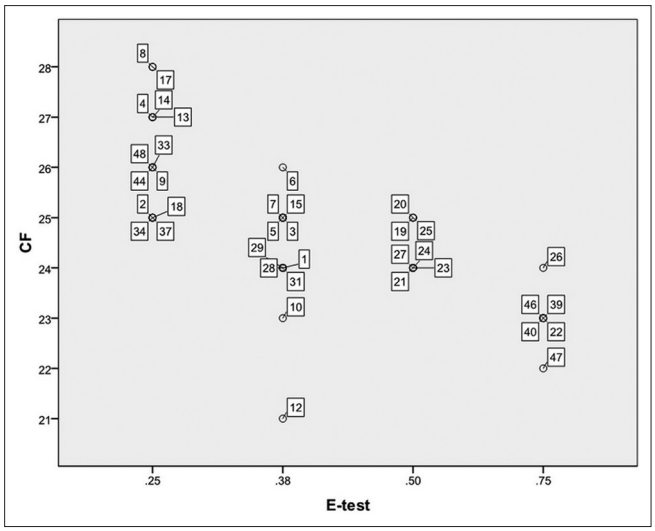

Figure 1: Scatter diagram of ciprofloxacin minimal inhibitory concentration (μg/ml) against ciprofloxacin zone (mm)

is an urgent need of national guidelines on the proper usage of antibiotics, and it has to be implemented at earliest.

Low-level ciprofloxacin resistant *Salmonella* infection need prolonged or increased dose of ciprofloxacin therapy. A study done by NK Pal *et al*.[3] showed 70% of patients with NARST infection treated with ciprofloxacin for the prolonged period showed therapeutic failure. Ceftriaxone, cefixime, and azithromycin has good activity against NARST isolates, but they are expensive.[9,16] Shifting back to ACCo drugs is good alternative, but their clinical outcome is questionable, and re-emergence of MDR isolates has to be kept in mind.

Conclusion

It is important to identify NA resistance in *Salmonella* as a predictor for decreased fluoroquinolone susceptibility. The current CLSI breakpoints may have to be re-evaluated for *Salmonella* and clinicians may have to reconsider the use of quinolones as the drug of choice for enteric fever cases!

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Conflicts of interest
There are no conflicts of interest.

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