

REDUCED SUSCEPTIBILITY TO FLUROQUINOLONE AND MOLECULAR ANALYSIS OF CIPROFLOXACINE RESISTANCE IN *SALMONELLA TYPHI* IN BANGLADESH

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Abstract

The study of the trend of infection, susceptibility to antibiotics and molecular level analysis of the cause of reduced susceptibility of *Salmonella typhi* isolates from the patients in Bangladesh were studied. Out of 9040 blood cultures obtained during the study period, 1266 (14.0%) showed significant growth. Nearly three-fourths of the positive blood cultures were due to *S. typhi* and rests were mostly of *S. paratyphi* A. The prevalence was highest between the age group 25 and 60 months. Male showed slightly higher rate of infection than female. Among all 943 *S. typhi* isolates, 42.6, 42 and 41.4% were sensitive to ampicillin, cotrimoxazol and chloramphenicol, respectively. All isolates were sensitive to ceftriaxon and ceftazidim; 9 isolates were ciprofloxacin resistant, others were moderate to highly sensitive; whereas, only 2.2% isolates were sensitive and almost all (97.8%) were found resistant to nalidixic acid. The E-strip test among 411 isolates showed the MIC value of 53 isolates nearer to the very sensitive (< 0.125 µg/ml), 252 isolates between 0.125 and 0.5 µg/ml, 95 isolates between 0.5 and 2.0 µg/ml and rest other 11 isolates showed from > 2.0 µg/ml to very highly resistant (512 µg/ml). VNTR pattern of all ciprofloxacin resistant *S. typhi* was also same. Restriction fragment analysis of *gyrase-A* gene indicated point mutations in different loci that bear the cause of being resistant to ciprofloxacin.

Introduction

Typhoid fever is endemic in Bangladesh as so in the Indian subcontinent with high prevalence among under-five children.⁽¹⁾ Prevalence of multi-drug resistant typhoid strains lead to use the ciprofloxacin as the drug of choice for routine outpatient treatment. This resulted in rapid emergence of *Salmonella enterica* serovar *typhi* isolates with reduced susceptibility to ciprofloxacin and associated delay in response or treatment failure to ciprofloxacin therapy. These strains are still considered 'susceptible' to ciprofloxacin based on the National Committee for Clinical Laboratory Standards (NCCLS) cut-off values [i.e., zone ≤ 20 mm; minimum inhibitory concentration (MIC) ≥ 2.0 µg/ml], and are termed as nalidixic acid-resistant *S. typhi* (NARST) on the basis of

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their resistance to nalidixic acid. At present, based on NCCLS guidelines, *S. typhi* strains with true resistance to ciprofloxacin, (MIC \geq 2.0 $\mu\text{g/ml}$) have been identified; the highest MIC level reported from clinical isolate of *S. typhi* is $> 32 \mu\text{g/ml}$. The decreased level of susceptibility of NARST strains to ciprofloxacin arisen from point mutations in the QRDR region of *gyrA* gene, either at ser-83 or asp-87 or both.⁽²⁾ Present work was undertaken to study the prevalence of reduced susceptibility to fluoroquinolone and then molecular analysis of ciprofloxacin resistant *Salmonella* serovar *typhi* in Bangladesh.

Materials and Methods

The blood samples were collected from unvaccinated patients who were clinically diagnosed with typhoid fever and admitted to Dhaka Shishu hospital, Popular Diagnostic Center and Kumudiny Hospital. Although, sample collection was started from 1997, samples collected from June, 2005 to August, 2006 were used. Patients' age, sex, duration of typhoid fever, name of the *Salmonella typhi* isolates, antibiotic sensitivity and minimum inhibitory concentration (MIC) for fluoroquinolone (ciprofloxacin) were recorded. Blood cultures were made using standard methodology on Blood-Agar, Chocolate-Agar and MacConkey-Agar medium. The isolates of *S. typhi* were confirmed by standard biochemical (API-20E system, BioMeriux, France) and serologic (Murex Diagnostics, Dartford, England) means. The antibiotic susceptibility test was done by Disc Diffusion method, using Muller-Hinton agar plate⁽³⁾ according to the NCCLS guidelines.⁽⁴⁾ All statistical analyses were done using EpiData software. DNA was extracted from pure culture of *Salmonella typhi* on MacConkey-agar plate by heat-lysis method. Molecular typing was done by multiplex PCR for Variable-Number Tandem Repeats (VNTR) using primers flanking three VNTR loci (TR1, TR2 and TR3) (Table 1) as described by Liu *et al.* The PCR amplified product of *gyraseA* gene was confirmed by running it through 1% polyacrylamide gel and after cutting into fragments by Hinf-1 restriction enzyme. The probability of point mutation was confirmed by running it through 2% polyacrylamide gel.

Results and Discussion

Out of the 9040 blood cultures obtained during the study period, 1266 (14.0%) yielded significant growth. Nearly three-fourths of the positive blood cultures (74.5%, 943 of 1266) were *S. typhi* and others were mostly *S. paratyphi* A. The isolation rate from boys and girls was apparently similar (Fig. 1).

The present laboratory-based study, showed the prevalence of *S. typhi* among the age group between 2 and 10 years.

Moreover, the high rate of isolation and degree of bacteremia in younger children suggest that they are more vulnerable to the infection. This argues against the view that

typhoid fever typically is mild and-or sub-clinical in younger patients and mandates a reassessment of the epidemiology and severity of typhoid fever in the community in endemic areas. The passive nature of case detection in the present study however may have biased the data toward more severe disease because only very ill patients typically are taken to physician and furthermore only a portion of them considered being able to afford the investigation cost may have been sent to the referral Centre. Despite this limitation the large number of patients evaluated in this study that were confirmed to

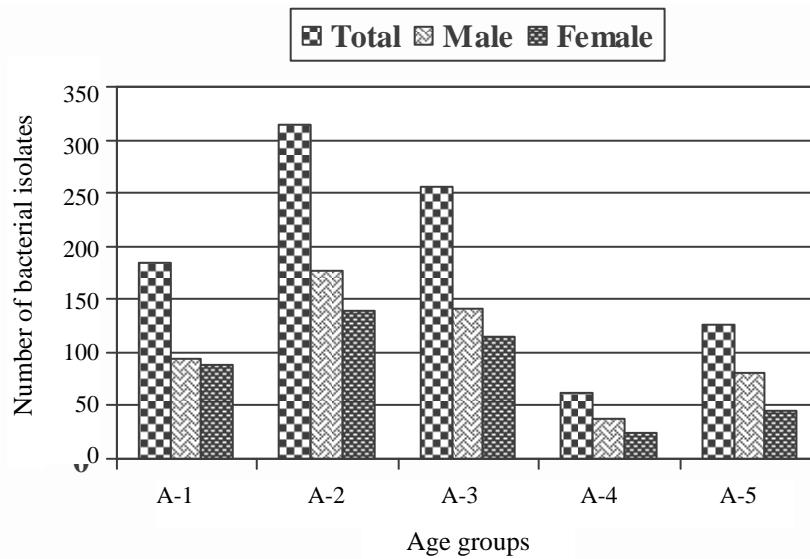


Fig. 1. Distribution of bacterial isolates with respect to different age groups of the patients (A1 is for 0 to 24 months; A2 for 25 to 60 months; A3 for 61 to 120 months; A4 for 121 to 180 months and A5 for 181 months to maximum age of our sample collections.)

have typhoid fever proved the prevalence of the disease. Moreover, the patients undertaken to this study were largely from the middle and upper income population of Dhaka city who have access to pipe water. Thus the incidence of typhoid fever in urban slum and rural areas of Bangladesh, which do not have safe water supplies, might be even higher than present data would indicate. The isolation rate was highest between the age group 25 and 60 months and male showed slightly higher rate of infection than female. These results will help in taking decision for efficacious trial of typhoid vaccine within this age group, particularly pre-school children.

In addition, the isolation rate was highest in the summer and monsoon season (May to October), with peaks in September (9.7% in 2005 and 12.3% in 2006) and was relatively low from November to March (Fig. 2). This trend was also observed in the year preceding to the study period. Among all 943 *Salmonella typhi* isolates studied, 42.6% of the total isolates were sensitive to ampicillin, whereas 57.4% resistant; 42% of the total

isolates were sensitive to cotrimoxazol, whereas 57.4% are resistant. On the other hand, all isolates were sensitive to ceftriaxon and ceftazidim; 41.4% of the total isolates were sensitive to chloramphenicol and 58.6% are resistant; 9 isolates collected were ciprofloxacin resistant, others were moderate to highly sensitive; however, only 2.2% isolates were nalidixic acid sensitive and almost all (97.8%) were found resistant (Fig. 3).

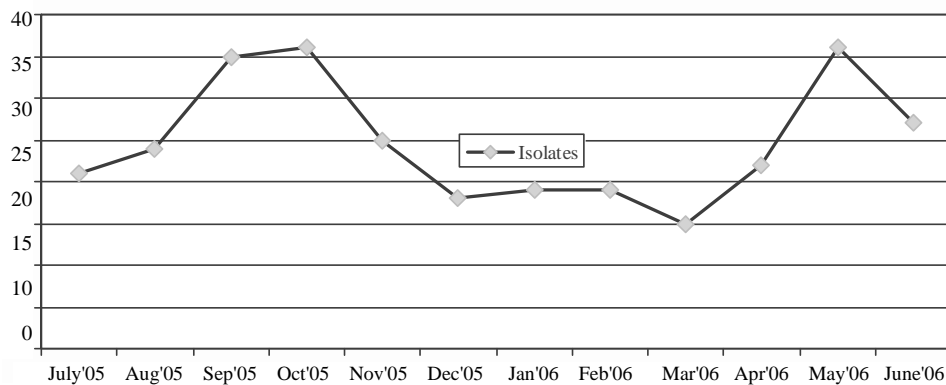


Fig. 2. Incidence of *S. typhi* isolates with patients during a year.

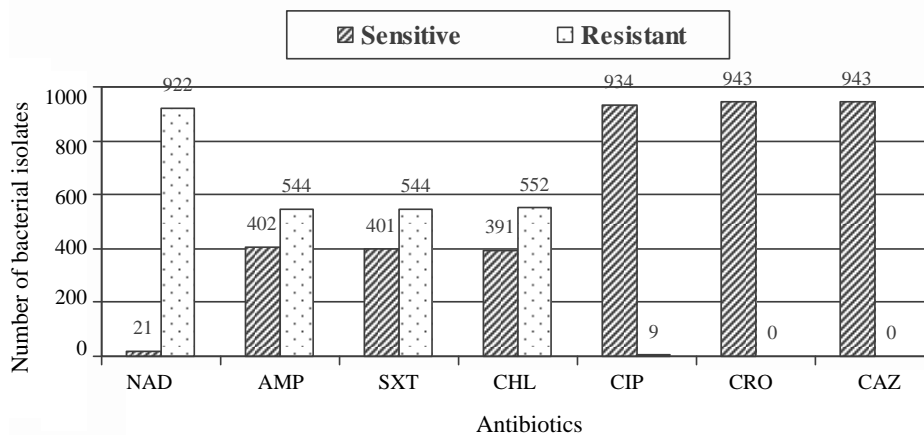


Fig. 3. Antibiogram of *Salmonella typhi* to different antibiotics.

Present study shows a drastic increase in the annual proportion of reduced fluoroquinolone susceptibility from 3.9 to 23.5% among all the *Salmonella* serover *typhi* in Bangladesh between 1999 and January 2007. Among all 943 *S. typhi* isolates studied, E-strip test was performed for 411 isolates of which 53 showed the MIC value nearer to the very sensitive ($< 0.125 \mu\text{g/ml}$), 252 showed between 0.125 to $0.5 \mu\text{g/ml}$, 95 showed between 0.5 to $2.0 \mu\text{g/ml}$ and rest other 11 isolates showed from $> 2.0 \mu\text{g/ml}$ to vary highly resistant e.g. $512 \mu\text{g/ml}$ (Fig. 4).

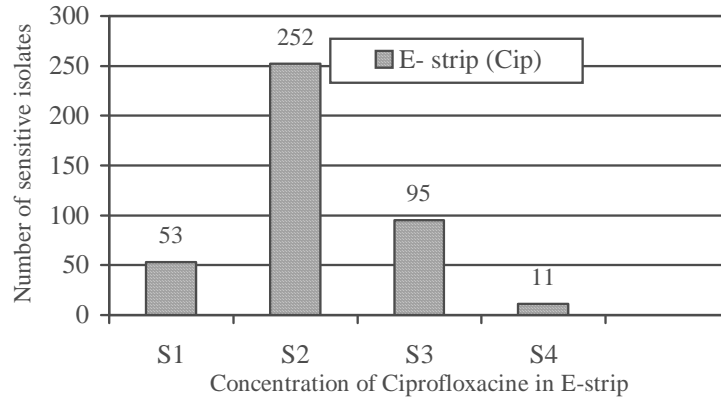


Fig. 4. Status of susceptibility of *S. typhi* isolates to Fluoroquinolone. (S1 is < 0.125, S2 is in between 0.125 and 0.5, S3 is in between 0.5 and 2.0 and S4 is > 2.0 µg/ml, respectively.)

All the ciprofloxacin resistant isolates were also highly resistant to ampicillin (> 256 µg/ml), cotrimoxazole (> 32 µg/ml), chloramphenicol (> 256 µg/ml), ciprofloxacin (> 32 µg/ml) and nalidixic acid (> 256 µg/ml), and were susceptible to ceftriaxone (0.094 µg/ml) according to their MIC of respective antibiotics. All three isolates were found to be identical by Api 20E score (4404552) and VNTR type (Fig. 5).

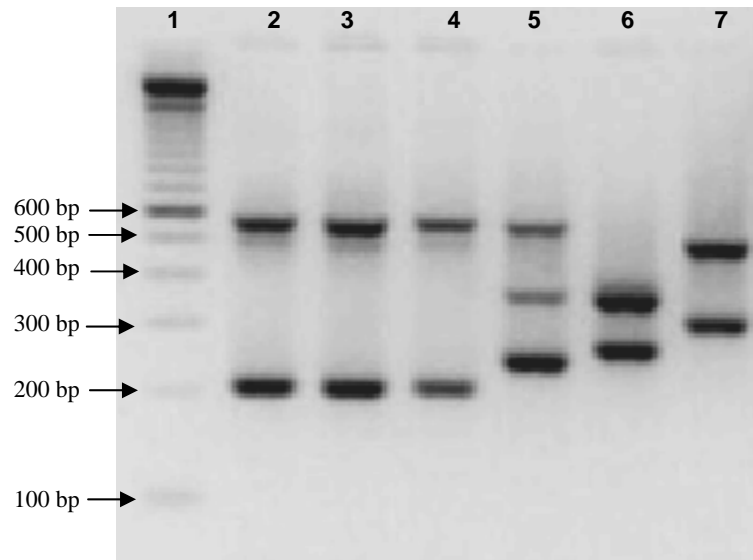


Fig. 5. VNTR pattern of ciprofloxacin resistant *S. typhi* isolates with that of comparative sensitive isolates (Lane 1: 100 bp marker; Lane 2-4: Ciprofloxacin resistant isolates (MIC 1024 µg/ml), Lane 5-7: Ciprofloxacin sensitive isolates, MIC 0.032, 0.064, 0.25 µg/ml, respectively).

When looking for reasons for the rapidly increased quinolone resistance in *Salmonella typhi* isolates, three issues must be considered: transferable resistance, mutational resistance, and clonal spread. Until now, transferable resistance to the quinolone

antimicrobial group has been described in one preliminary report.⁽⁵⁾ As far as we know, however, transferable fluoroquinolone resistance appears to be rare in bacteria *in vivo*. Thus, either clonal spread or resistance due to mutations in chromosomal genes remains the potential mechanism accounting for the high level of reduced fluoroquinolone susceptibility to fluoroquinolone in Bangladesh as well as in Southeast Asia. In Bangladesh, the possibility of clonal spread as a major contributing factor was excluded by identification of 8 serotypes among the quinolone-resistant isolates. In addition, some of these serotypes contained different antimicrobial resistance patterns along with their different VNTR patterns. Based on these data, we can conclude that the reduced fluoroquinolone susceptibility of *Salmonella typhi* in Bangladesh primarily involves mutations in the chromosomal genes. This concept is consistent with our VNTR experiment and finally proved by sequencing data where all 8 *Salmonella* isolates with reduced fluoroquinolone susceptibility were analyzed in ICDDR,B have shown point mutation leading to nucleotides change in their QRDR of the *gyraseA* gene.

Taq-DNA polymerase successfully amplified the 195 bps *gyraseA* gene from the genomic DNA of ciprofloxacin sensitive and ciprofloxacin resistant *Salmonella* serover *typhi* using primer of *gyraseA* (Fig. 6)



Fig. 6. Amplified *gyraseA* gene using PCR.

These PCR amplified products then showed different multiple fragments upon the treatment of *Hinf-1* which showed a clarifying image indicating point mutation at different loci of QRDR region of the *gyraseA* gene (Fig. 7).

It is noteworthy that the less susceptible subpopulation has already undergone one point mutation and thus is potentially inclined to a second mutation, which leads to high level fluoroquinolone resistance. Admittedly, fluoroquinolone-resistant *Salmonella* strains are still extremely rare, and they are usually counter selected in field conditions.⁽⁶⁾ Even so, one can think of highly fluoroquinolone-resistant *Salmonella* strains will inevitably emerge, if the less susceptible strains and quinolone pressure coexist. On this account,

global surveillance on reduced fluoroquinolone susceptibility of *Salmonella* also is cared for epidemiologic reasons.

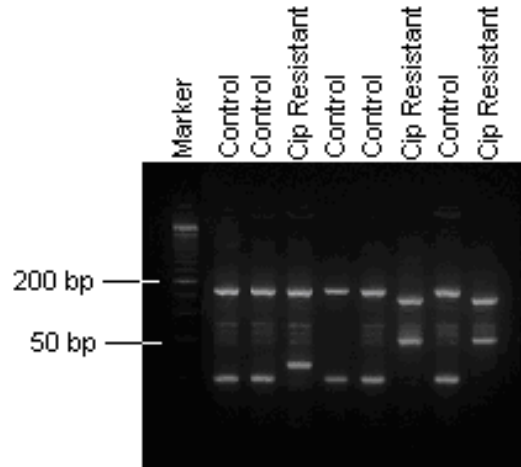


Fig. 7. *Hinf*-I treated fragments of *gyrase-A* PCR products.

The orchestrated increase in the incidence of fluoroquinolone resistance in *Salmonella* and other enteric bacteria, especially *Campylobacter* sp. and *E. coli*⁽⁷⁻¹⁰⁾, drives a situation that impedes the effectiveness of this antimicrobial group. The significantly common multi drug resistance observed here among the fluoroquinolone-resistant *Salmonella* compared with the susceptible population (47.4% vs. 11.5%) is also a matter of concern. This finding suggests that the abrupt use of fluoroquinolone for multi drug resistant *Salmonella* provoke a question of whether the same could happen to other bacterial species. In that case, the likelihood of the emergence of notable pathogens resistant to fluoroquinolones as well as to other commonly used drugs will certainly increase if the consumption of fluoroquinolones continues to grow. Collectively, these data indicate that prudent use of the fluoroquinolone antimicrobial group is warranted to prevent further development of resistance and to preserve the usefulness of this valuable drug.

In conclusion, it has been shown that reduced susceptibility of *Salmonella* to the fluoroquinolone group was significantly associated with multi drug resistance. Moreover, all quinolone-resistant *Salmonella* isolates had undergone a point mutation in the QRDR of the *gyrA* gene. In contrast to previous reports on quinolone resistance in a specific clone or in a few *Salmonella* serotypes, the reduced fluoroquinolone susceptibility of our isolates was nonclonal. These data provide further evidence of the rapid spread of multidrug-resistant pathogens from one continent to another. The emergence of antimicrobial resistant pathogen in any part of the world may have global implication and is therefore a universal concern.

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