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Original Research Article

Increased resistance to Nalidixic acid and Ciprofloxacin in *Salmonella* isolates from the Sub Himalayan region

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ABSTRACT

Background: During the last two decades, increased resistance to nalidixic acid and ciprofloxacin has become a cause of global concern. The present study was undertaken to ascertain nalidixic acid and ciprofloxacin resistance in *Salmonella* isolates from our region. To know the true pattern of ciprofloxacin resistance by determining the minimum inhibitory concentration (MIC) through E-test.
Methods: All the *Salmonella* isolates recovered from blood cultures were screened for nalidixic acid resistance using 30µg disc by the Kirby Bauer disc diffusion method. Ciprofloxacin susceptibility was done both by disc diffusion and MIC using CLSI breakpoints.
Results: We analysed a total of 80 Salmonella isolates during the last three years. *Salmonella enterica serovar Typhi* was the predominant serovar in 51 (64.8%) isolates, followed by *Salmonella enterica serovar Paratyphi* A comprising 28 (36.2%) isolates. Amongst the total isolates 78 (97.5%) were nalidixic acid resistant. Of these 54 (67.5%) showed intermediate susceptibility and 9 (11.2%) were ciprofloxacin resistant by the disc diffusion technique. On the contrary 29 (36.2%) had decreased susceptibility to ciprofloxacin; while a larger number 38 (47.5%) were detected resistant to ciprofloxacin on determination of MIC by the E-test.
Conclusions: Screening for nalidixic acid acts as a surrogate marker to detect ciprofloxacin resistance. However, the true pattern of ciprofloxacin resistance can be determined by calculating the MIC by the E-test.

Keywords: Ciprofloxacin minimum inhibitory concentration (MIC), Nalidixic acid resistance, *Salmonella enterica*

INTRODUCTION

Enteric fever caused by *Salmonella enterica* serotype Typhi and Paratyphi A, B, C is a major public health problem in the developing countries. There is a potential threat to developed nations due to increasing immigration. The estimated incidence of typhoid fever is approximately 27 million cases each year.[1] Enteric fever if not treated, carries a mortality rate of 10% to 30% but with appropriate treatment there is a drastic reduction in the rate to 0.5%.[2] The emergence of multidrug resistant (MDR) strains of *Salmonella enterica* which are resistant to chloramphenicol, ampicillin and cotrimoxazole, since the last two decades pose a grave concern. Ciprofloxacin, a fluoroquinolone was introduced in 1990s as the first line therapy against MDR *Salmonella typhi*.[3] Fluoroquinolones are characterized by excellent intracellular penetration and achieve a high concentration in all body fluids and tissues. Ciprofloxacin is the drug of choice in developing countries due to low cost, oral route of administration and easy availability. However, unauthorized dispensing and irrational use of this drug in

human and animal therapeutics is responsible for emergence of ciprofloxacin resistance. Since the last decade there are reports of ciprofloxacin resistance in S*almonella* and increased minimum inhibitory concentration (MIC) of fluoroquinolones.[4] Outbreaks of enteric fever due to nalidixic acid resistant strains (NAR) with reduced susceptibility to ciprofloxacin have been reported from India and neighbouring countries.[5-7]

The routinely used ciprofloxacin disc diffusion method fails to detect the low level of resistance. Treatment failures with ciprofloxacin have been reported where the isolates had higher MIC of ciprofloxacin, though they were detected susceptible by the in vitro disc diffusion testing and recommended MIC breakpoints. Nalidixic acid susceptibility is considered as a surrogate marker to predict decreased susceptibility to ciprofloxacin.

Thus, all isolates of *Salmonella typhi* and *Salmonella Paratyphi A* should be screened for nalidixic acid resistance to avoid false reporting of ciprofloxacin susceptibility. Further determination of MIC of ciprofloxacin is essential to assess the true susceptibility pattern. The present study was undertaken to detect the prevalence of NAR strains and ciprofloxacin resistance by the disc diffusion and MIC testing, which would assist in formulating treatment guidelines.

METHODS

Eighty *Salmonella* species isolated from blood culture of patients suspected to be suffering from enteric fever, attending the outpatient department or admitted in Indira Gandhi medical college Shimla, Himachal Pradesh, India, during March 2010 to March 2013 were included in the present study. Blood culture samples collected with sterile precautions in brain heart infusion broth were incubated aerobically at 37°C followed by subculture on blood agar and Mac Conkey agar after 24 hours. The growth of non-lactose fermenting colonies was further identified by conventional biochemical reactions and confirmed by serotyping using antisera for *Salmonella* (polyvalent O, O2, O9 from CRI Kasauli). Susceptibility testing to nalidixic acid (30µg) and ciprofloxacin (5 µg) was performed by the standard Kirby Bauer disc diffusion method and interpreted as per the clinical laboratory standards institute (CLSI) guidelines.[8]

Nalidixic acid is a prototype quinolone which is used for in vitro screening tests for determining ciprofloxacin resistance. The zone diameter obtained around the 30µg nalidixic acid disc obtained from (Hi Media Laboratories, Mumbai, India) was measured for all isolates. These were further classified as being nalidixic acid resistant (NAR) (ZDI≤13mm), nalidixic acid susceptible (NAS) (ZDI≥19mm), according to the current CLSI guidelines. Further ciprofloxacin susceptibility was determined by measuring the zone of inhibition around 5 µg ciprofloxacin disc obtained from (Hi Media Laboratories, Mumbai, India). The isolates were labeled as ciprofloxacin susceptible (ZDI ≥31mm), intermediate (ZDI=21-30mm) and resistant (ZDI≤20mm), according to the current CLSI guidelines. The Minimum inhibitory concentration (MIC) to ciprofloxacin was determined for all the isolates using E test strips obtained from (Hi Media Laboratories, Mumbai, India), and interpreted according to the current CLSI guidelines.[8] Ciprofloxacin MIC (µg/ml) was interpreted as (susceptible ≤0.06, intermediate 0.12-0.5, resistant ≥1).8 *Escherichia coli* ATCC 25922 strains was used for quality control.

Statistical analysis

The data analysis was done using SPSS version 16.0 (IBM SPSS, Chicago, USA). We calculated the p value by the bivariate correlation and Student's t test was applied to determine the association between the parameters a) nalidixic acid and ciprofloxacin resistance based on disc zone diameter. b) nalidixic acid disc zone diameter and ciprofloxacin MIC c) ciprofloxacin sensitivity pattern by measurement of disc zone diameter and ciprofloxacin MIC by E test. A p value of ≤0.05 was considered as significant.

Institutional ethics committee of Indira Gandhi medical college and hospital, Shimla, Himachal Pradesh, India, reviewed and approved the study.

RESULTS

A total of 80 isolates of *Salmonella* species were recovered from blood culture samples. The mean age was 24.22 ±11.28 years. The isolation rate was higher in male patients, 56 (70%) as compared to 24 (30%) females. Of the total, 42 (52.5%) isolates were obtained from admitted patients whereas 38 (47.5%) from outpatients. Overall Salmonella typhi was the predominant serotype, comprising 51 (63.8%) isolates, followed by 29 (36.2%) isolates of *Salmonella paratyphi* A. However, in year 2012 *Salmonella paratyphi* A was the predominant species in 25 out of 47 (53.19%) positive blood cultures.

There were 78 (97.5 %) nalidixic acid resistant isolates and 2 (2.5 %) were nalidixic acid susceptible based on measuring the zone diameter around 30µg nalidixic acid disc and interpreted according to the current CLSI guidelines. Ciprofloxacin susceptibility was determined both by the Kirby Bauer disc diffusion method and calculation of MIC of ciprofloxacin by the E-test. According to the disc diffusion method there were 17 (21.2%) ciprofloxacin susceptible isolates.

The number of isolates showing intermediate susceptibility and resistance were 54 (67.5%), and 9 (11.2%) respectively. In comparison determination of MIC of ciprofloxacin by the E-test detected only 13 (16.2%) isolates with MIC of ciprofloxacin≤0.06ug/ml as susceptible. On the contrary 38 (47.5%) isolates had MIC ≥1 µg/ml were detected resistant and 29 (36.2%) isolates

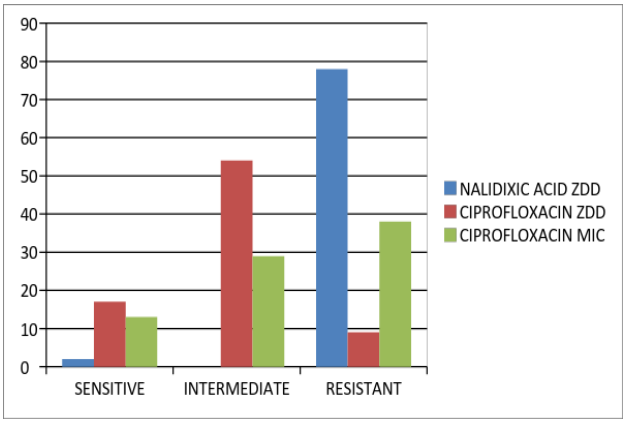
had MIC of ciprofloxacin between (0.12-0.5 µg/ml) showings decreased susceptibility according to the current CLSI guidelines (Table 1).

Table 1: Depicts the susceptibility pattern to nalidixic acid and ciprofloxacin disc diffusion method and ciprofloxacin MIC.

Antibiotic	Sensitive	Intermediate	Resistant
Nalidixic acid disc(30ug) zone of inhibition in mm	2 (2.5%)	0	98 (78.5%)
Ciprofloxacin disc (5ug) zone of inhibition in mm	17 (21.2%)	54 (67.5%)	9 (11.2%)
Ciprofloxacin MIC(ug/ml)	13 (16.2%)	29 (36.2%)	38 (47.5%)

This highlights the fact that a larger proportion of the isolates were detected resistant by the E-test which either appeared to be falsely susceptible or had intermediate susceptibility (Figure 1).

Figure 1: Clustered cylinder graph showing the various isolates of *Salmonella enterica* AND THEIR sensitivity pattern.



DISCUSSION

Enteric fever is an important cause of morbidity and mortality, being more prevalent in the tropics, and afflicting the younger age group more frequently. This preponderance maybe explained on the basis that the youth are more socially mobile and likely to consume contaminated food and water outdoors.[9]Similar findings were observed in present study, the mean age being 24.22 years. In India, enteric fever occurs predominantly due to *Salmonella enterica* serovar typhi followed by *Salmonella enterica* serovar *paratyphi* A and rarely due to *Salmonella enterica serovar paratyphi* B.[11-13] This corroborates with our finding where *Salmonella typhi* had higher isolation rate of 63.8% followed by *Salmonella paratyphi* A 36.2% and no isolate of *Salmonella enterica* serovar paratyphi B.

Over the years high isolation rates of *Salmonella paratyphi A* have been reported from several parts of India including our own region in the year 2004.[13-15] *Salmonella* species which exhibit in vitro resistance to nalidixic acid, a quinolone antibiotic has decreased susceptibility to ciprofloxacin and consequently therapeutic failure during therapy.[3,16] Nalidixic acid resistance is a surrogate marker to detect decreased ciprofloxacin susceptibility. Variable isolation rate of NAR strains from 2.3% to 96% has been documented.[3,17] Similar findings have been reported from neighbouring regions as Pakistan, Bangladesh, Nepal and other countries.[9,18-20]

In the present study high isolation rate of NARST 97.5% in comparison to earlier studies was seen.[7,21] Previously in year 2001-2006 the frequency of NARST in our region was 66.47%.[15] Emergence of nalidixic acid resistant *Salmonella* and reports of infection with these strains showing increased resistance to ciprofloxacin from typhoid endemic areas have generated great concern.[5] In our region an increase in resistance to nalidixic acid from13.38% in 1993 to 66.47% in 2000 to 2006 and 97.5% presently, and for ciprofloxacin being 3.67% in 1993 to 9.41% in 2000 to 2006 and presently 47.5%.[15,21] This pattern probably reflects the indiscriminate and irrational use of this drug in treatment of typhoid and other unrelated infections. Incomplete treatment may be another factor which contributes the development of resistance.

However, there is no data to study the association between nalidixic acid resistance and ciprofloxacin resistance either by the disc diffusion method or determination of MIC from our region. In present study, nalidixic acid resistant isolates 36.2% had decreased ciprofloxacin susceptibility (DCS, MIC 0.12-0.5ug/ml) on performing the E test. This was more in comparison to findings of Anjum et al (11%) and Threfall et al (23%).[18,20]

Student's t test was applied to find out the association between nalidixic acid sensitivity pattern by measuring the zone of inhibition and ciprofloxacin MIC by the E test. The mean (SD) was 0.662 +/-(0.711) with p value of 0.000 at 95% confidence interval (CI): -0.821 to -0.504, which was highly significant. Similarly, bivariate correlation between nalidixic acid and ciprofloxacin MIC (p value=0.01) is significant. This concurs with findings of Mandal et al who observed the association between

nalidixic acid resistance and reduced ciprofloxacin susceptibility to be significant ($p \leq 0.001$).[22] This highlights the fact that there is a correlation between resistance to nalidixic acid and reduced susceptibility to ciprofloxacin.[5] Isolates with DCS (MIC 0.125-0.5ug/ml are more likely to have prolonged fever clearance times, higher rates of treatment failure and require higher dose of ciprofloxacin (10mg/kg bd for 10 days).[1]

In the present study, there were 11.2% isolates which were resistant to ciprofloxacin and nalidixic acid by the disc diffusion method. Student's t test showed a mean (SD) 1.075 +/- (0.546) with p value of 0.000 at 95% confidence interval (CI): -1.197 to -0.953, which was highly significant. Our finding coincides with finding of Hanken et al and Chandel et al which support the use of nalidixic acid 30µg disc as a screening method to detect low level of resistance.[23,24] Nalidixic acid disc diffusion assay should be used as an indicator to detect salmonella isolates with decreased ciprofloxacin susceptibility. With the adoption of CLSI 2012 guidelines where isolates with MIC ≥1 µg/ml are resistant to ciprofloxacin, more number of isolates 47.5% were detected as resistant.

In contrast only 11.2% were resistant by the ciprofloxacin disc diffusion method. This suggests that strains with higher MIC to ciprofloxacin may not be detectable by the disc diffusion test. It is advocated that MIC for ciprofloxacin should be determined in the diagnostic laboratories routinely for all the nalidixic acid strains.[12] Similar findings were seen in this study where Student's *t* test was used to find out the association between ciprofloxacin sensitivity pattern by measuring zone of inhibition and ciprofloxacin MIC.

The paired mean (SD) difference was 0.413 (0.724) with p value of 0.000 at 95% confidence interval (CI): 0.251 to 0.574. The maximum MIC observed in current study was ≥32 µg/ml in 9 (11.25%) isolates. Several studies from North and South India have reported elevated level ciprofloxacin resistance with MIC ranging from 8 to 64µg/ml.[1] Also elevated levels ciprofloxacin resistant strains with MIC 512, 128 µg /ml have been isolated from Bangladesh and Japan respectively.[19,25]

The exact mechanism of fluoroquinolone resistance in *Salmonella enterica* serotype typhi and *Salmonella enterica* serotype paratyphi A is not fully understood. Quinolone resistance in *Salmonella* occurs due to a single point mutation in the quinolone resistance determining region (QRDR) of gyr A gene that leads to simultaneous resistance to nalidixic acid and reduced susceptibility to ciprofloxacin.[26] In contrast high level ciprofloxacin resistance (MIC range 8 to >32 µg/ml) above the established breakpoints may be due to either a) cumulative impact of mutations in many genes b) decreased membrane permeability c) active efflux pump d) presence of plasmid encoded qnr genes that encode a protein that protects DNA gyrase from ciprofloxacin. This is of great concern since horizontal transfer of quinolone resistance would facilitate rapid dissemination of quinolone resistance genes so further restricting the use of these antimicrobial agents.

Moreover, the varying levels of the ciprofloxacin MIC in strains with the same type of *gyrA* mutation suggests that some other mechanisms may also be involved in high-level fluoroquinolone resistance in *S. Typhi* and *S. Paratyphi* A. Further sequencing of nalidixic acid resistant isolates is essential to study the exact mechanism of resistance.

CONCLUSION

Current study shows a high prevalence of NAR and ciprofloxacin resistance in Salmonella species circulating in our region. Nalidixic acid disc diffusion method can be used to predict decreased ciprofloxacin susceptibility in resource poor settings and has important application in the clinical laboratories. Resistance to nalidixic acid is associated with high MIC to ciprofloxacin in Salmonella isolates. Detection of ciprofloxacin resistance by the Kirby Bauer disc diffusion method is less efficient, so MIC of ciprofloxacin should be determined by the E test. Optimal interpretation of ciprofloxacin susceptibility by MIC method should be established in cases of enteric fever where nalidixic acid resistance is reported. The findings of the current study suggest that in this era of ciprofloxacin resistance, ciprofloxacin should not be considered the drug of choice. It is the need of the hour that the health care authorities should formulate alternative treatment guidelines.

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