Fluroquinolone Resistance in Typhoidal Salmonella and its Detection by Nalidixic Acid Disc Diffusion

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Introduction

Typhoid fever is a major cause of morbidity and mortality in the third world countries. Since the last decade the emergence of the multidrug resistant strains of Typhoidal salmonellae dictated the use of fluoroquinolones as the first line drugs for empirical therapy in typhoid fever.1,2 Fluoroquinolones not only have an excellent intracellular penetration and achieve high levels in all body fluids and tissues but also are very effective in vivo and vitro as far as microbial killing is concerned.3 These properties make them an excellent choice in this disease. 4.5

The availability of these drugs across the counter and its indiscriminate use in the third world counties including Pakistan, has started to compromise the efficacy of these wonder drugs.6 There are several reports of treatment failure after administration of ciprofloxacin to patients with enteric fever.7,8 The Salmonella enterica serovar Typhi (ST) isolates, from patients not responding to ciprofloxacin therapy (less susceptible isolates), showed increased MIC to ciprofloxacin.9,10 Although the MIC of ciprofloxacin of these isolates was within the recommended susceptible range, but was in higher range than those of with strains showing successful treatment of enteric fever. This resistance is however, not detected by the ciprofloxacin disc using NCCLS breakpoints in clinical diagnostic laboratories, resulting in ciprofloxacin being administered as the drug of choice to treat enteric fever. This delays the decision to switch over to third generation cephalosporins or other appropriate antibiotics. There was a need to improve the recommended MIC of quinolones against typhoid salmonellae. Some researchers have suggested the new recommendations for susceptible MIC of quinolones against typhoid salmonellae.11,12 It has been suggested that the isolates revealing MIC of <0.125µg/ml are susceptible, MIC between 0.125 and 1µg/ml reduced susceptible and MIC of >1µg/ml are resistant.13 Studies also revealed that ST isolates from patients not responding to ciproflxacin therapy showed resistance to nalidixic acid.14,15 This is due to the fact that mutation in DNA gyrase, a mechanism responsible for ciprofloxacin resistance, also correlates with resistance to nalidixic acid, the first generation of quinolones. 16 Taking advantage of this fact Hakanen A et al and Kapil A et al, used nalidixic acid susceptibility test and proved it useful as a tool to screen salmonella isolates with decreased ciprofloxacin susceptibility.17,18 The present study was designed at pathology department Army Medical college Rawalpindi to explore the possibility of detection of resistance to quinolones in typhoid salmonella using 30mg nalidixic acid disc, instead of 5mg disc of ciprofloxacin used presently.

Materials and Methods

The study was carried out on isolates of Typhoidal salmonellae, (n = 100), isolated from routine clinical blood culture samples from patients having fever, at Army Medical College Laboratory, Rawalpindi during the year 2001-2002. The blood samples were collected in brain heart infusion broth, incubated at 37°C. Sub cultures were done on day 1,2,4,7 on blood agar and MacConkey agar. Non-lactose fermenting colonies of Gram-negative bacilli were

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serovar Typhi (ST) and Salmonella enterica serovar Paratyphi A (SPA) were confirmed serologically using standard antisera (Welcome reagents Ltd. UK). The isolates revealing serotyping as 09, 12, (Vi), Hd were labelled as ST and sertotyping as 01, 2,12, Ha, were labelled as SPA. Antimicrobial sensitivity testing of the isolates was done on Muller Hinton agar (Mast Diagnostic Group Ltd., Mersyside,U.K). The disc diffusion antimicrobial

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sensitivity was performed using discs of nalidixic acid 30µg and ciprofloxacin 5µg (Oxoid Ltd., Sollentuna, Sweden). Modified Kirby-Bauer disc diffusion method was employed for susceptibility testing. Diameter of zones of inhibition of bacterial growth was measured and sensitivity / resistance was interpreted according to the National Committee for Control Laboratory Standard (NCCLS). Minimum inhibitory concentration (MIC) of these drugs against the same isolates was also determined in parallel using the 'E' test (AB-Biodisc, Sweden). The results were interpreted according to NCCLS recommendations.

Escherichia coli ATCC 25922 was used as a control strain. The results of E-Test were used for final analysis in the study. Statistical analysis was done by SPSS 10.0 for Windows (Statistical package for social sciences).

Results

A total of n = 100 typhoid salmonellae (ST, n = 41; SPA, n = 59) were analysed. When considering the NCCLS criteria, there were 17 isolates showing resistance to nalidixic acid (MIC, > 32µg/ml) and remaining 83 isolates were susceptible to nalidixic acid (MIC < 16µg/ml). Where

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as all isolates had ciprofloxacin MIC <1µg/ml, which according to the NCCLS criteria are susceptible to ciprofloxacin. Similarly when considering the inhibition zone diameter of NAL disc, it revealed 17 isolates resistant to nalidixic acid (zone <13mm) and 83 susceptible to nalidixic acid (zone > 19mm). All the isolates however, revealed inhibition zone of >21mm around the disc of ciprofloxacin, indicating all the isolates to be susceptible to Cip. The mean inhibition zone diameters around NAL and Cip discs are shown in the table. MIC of ciprofloxacin and NAL against typhoid salmonellae showed a linear correlation (r = 0.899) (Figures 1a,1b). Similarly the zone of inhibition with NAL disc (30µg) and with ciprofloxacin disc (5µg) showed linear correlation with

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their corresponding MICs (r = 0.79 and r = 0.7 respectively) (Figures 2a, 2b and 3a, 3b). When the zone of inhibition around NAL disc is evaluated against Cip MIC, it also revealed a linear correlation (r = 0.77) (Figures 4a, 4b). If we consider the new proposed criteria for MIC of Cip against typhoid salmonella, 89 isolates revealed MIC of Cip <0.125µg/ml and hence susceptible to Cip, 11 isolates revealed MIC of Cip >0.125µg/ml, and hence having reduced susceptibility to Cip. None of the isolates revealed Cip MIC, 1 or more than 1µg/ml. This result corresponds with the result interpreted through nalidixic acid. The relevance of using resistance to nalidixic acid as a marker for decreased ciprofloxacin susceptibility in salmonella was evaluated by comparing the MICs of ciprofloxacin and nalidixic acid for the 100 Salmonella isolates (Figure 1a). When MIC of Ciprofloxacin >0.125µg/ml was adopted as a break point, screening for nalidixic acid resistance (MIC >32µg/ml) led to the detection of all 11 isolates with decreased ciprofloxacin susceptibility (MIC > 0.125µg/ml) and, in addition, 6 of the 89 susceptible isolates. Thus the sensitivity of the approach was 100% and specificity was 93.6%. Based on the MIC of ciprofloxacin and Zone diameters around 30µg nalidixic acid discs for the 100 salmonella isolates tested (Figure 4a), screening for nalidixic acid resistance (inhibition zone diameter, <13mm) led to detection of all isolates for which the MICs were >0.125µg/ml. When this MIC was used as a break point of decreased ciprofloxacin susceptibility, the sensitivity of the nalidixic acid disc screening was 100% and the specificity was 92.7%. The applicability of the 5µg ciprofloxacin disc diffusion test in detecting decreased ciprofloxacin susceptibility was assessed (Figure 3a). The MICs for 11 of the 48 isolates with an inhibition zone diameter of < 36mm, were >0.125µg/ml, where as for all of the isolates with a zone diameter of >36mm the MICs were < 0.064µg/ml.

Table. The meaninhibitionzone diameters for nalidixic acid and ciprofloxation disc for NARST and NASST isolates.

Agent (disk content)	Nalidixic acid-resistance isolates MIC > 32 ug/ml	Nalidixic Acid-susceptible isolates MIC < 8ug/ml
	Mean + SD Range (mm) (mm)	mean + SD Range (mm) (mm)
Nalidixic Acid (30ug) a	6.0 b + 00 –	29.66 + 6.35 22.50
Ciprofloxacin (5ug) a	24 + 3.92 20-36	39.79 + 6.14 22.58

a Disk content as recommended by the NCCLS

b Standard Deviation cannot be calculated.

Thus, when an MIC of >0.125µg/ml was adopted as a break point, the ciprofloxacin inhibition zone diameter of <36mm yielded a 100% sensitivity and a 89.6 % specificity in screening for decreased ciprofloxacin susceptibility.

Discussion

Out of 100 isolates 41% were Salmonella enterica serovar Typhi (ST) and 59% Salmonella enterica serovar Paratyphi A. Seventeen isolates (ST) were found resistant (NARST) and 83 sensitive to nalidixic acid (NASST) on disc diffusion testing. MIC determination against nalidixic acid (NAL) showed similar results. Thus there was no ambiguity in both the methods, which correlated well. All isolates were sensitive to ciprofloxacin on disc diffusion testing as per the existing NCCLS criteria. Similar results were seen on MIC determination but those isolates which were nalidixic acid sensitive had MIC values <0.023µg/ml and those isolates which were resistant to nalidixic acid had MIC values 0.064 - 0.19 µg/ml, clearly indicating that although the MIC values of ciprofloxacin against all the isolates are less than the breakpoint criteria for resistance but the isolates resistant to nalidixic acid had higher MIC as

compared to those which are nalidixic acid sensitive. Thus an inference can be drawn that: 1. Disc diffusion test to detect resistance against nalidixic acid is reliable as it correlates well with the MIC values. 2. Ciprofloxacin disc diffusion test cannot pick up the isolates that show a higher MIC values against ciprofloxacin. 3. The isolates, which have higher MIC values against ciprofloxacin, are the same, which were resistant to nalidixic acid on disc diffusion as well as MIC test.

Thus disc diffusion testing against nalidixic acid can pick up the isolates for which MIC of ciprofloxacin are >0.064µg/ml. These isolates, which have relatively higherMICs against ciprofloxacin, are the ones responsible for treatment failures. Fluoroquinolones have become the treatment of choice for multi drug resistant typhoid fever.19,20 Indeed these are the only effective oral drugs in this clinical situation.4,5,9 Most of the medical practitioners have almost stopped using conventional antityphoid drugs. There are reports of treatment failure to quinolones due to reduced fluoroquinolones susceptibility.19,21 Studies conducted in Finland during 1995 to 1999 on Salmonellae isolates from Finnish travelers returning from abroad, showed that annual proportions of reduced ciprofloxacin susceptibility (MIC >0.125 mg/ml) among travelers isolates increased from 3.9% to 23.5%. The increasing trend was outstanding among the isolates from South East Asia. 19 Treatment failures with quinolones were significantly more common in patients infected with nalidixic acid resistant Salmonella typhi (NARST), than in those infected with nalidixic acid sensitive Salmonella typhi (NASST). Viet Nam study showed 2.1% isolates resistant to nalidixic acid with raised MICs to ciprofloxacin.8 Similar results were observed in our study. In recent years studies have been focused on fluoroquinolone resistance in Salmonella isolates with special reference to strains for which ciprofloxacin MICs were >0.125 mg/ml.17,19,20 In England and Wales low level resistance (Cip. MICs values >0.125 mg/ml) was identified in 1996 in 7% of Salmonella typhi and 4% of Salmonella paratyphi A.14 While in our study it was 11%. This result shows that low-level resistance is more in our set up. This finding supports the out come of the studies carried out in Finland that the increased prevalence of such isolates is more in South East Asia. 19 Ciprofloxacin 5µg disc failed to detect these less susceptible ST. Earlier workers recommended the use of 30µg nalidixic acid disc to detect these less susceptible Salmonella isolates in routine susceptibility testing, based on solitary cases of fluoroquinolone treatment failure. 14,19 In our collection of Salmonella enterica serovar Typhi (ST) isolates, nalidixic acid susceptibility testing proved both sensitive and specific in screening for isolates with decreased ciprofloxacin susceptibility. Identification of nalidixic acid resistance by the disc diffusion method led to the detection of all isolates for which the MICs of ciprofloxacin were >0.125µg/ml. Our findings coincide with the findings of Haknan et. al and Chandel Das et. Al.17,18 Thus use of 30µg nalidixic acid disc is a more direct and reliable method for detecting low level resistance. Though the number of isolates analyzed in our study was limited, nevertheless it needs to be followed by in vivo clinical trials in conjunction to molecular detection of the mutations in the gyrA gene.

Conclusion

To conclude, all the Salmonella enterica serovar Typhi strains manifesting decreased ciprofloxacin susceptibility, were NARST. These strains however are interpreted as ciprofloxacin sensitive by the disc diffusion test using NCCLS guidelines. This indicates that there is a need to redefine the zone diameter interpretive standards for Salmonella enterica serovar Typhi, as the previous studies suggested.8,11 Till the NCCLS break point of ciprofloxacin for the interpretation of resistance in typhoid salmonellae is revised, detection of nalidixic acid resistance by disc diffusion method can be used to screen for decreased ciprofloxacin susceptibility which can help the treating physician to decide on an alternative antibiotic for patients infected with these strains. Further studies are required to: a. Correlate the treatment failures in patients with the ciprofloxacin MICs to establish a new ciprofloxacin MIC breakpoint. b. Establish new criteria of zone diameters for ciprofloxacin disc diffusion testing, lowering the disc contents from 5 mg may lead to the establish a new criteria of sensitivity and resistance against Salmonellae which may be able to detect the isolates having raised MICs. c. Nalidixic acid resistant isolates should be subjected to molecular analysis for detection of mutations in gyr A gene

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Abstract

Objective:To determine the presence of fluoroquinolone resistance in Typhoidal salmonellae at Rawalpindi and the role of nalidixic acid in predicting the resistance against fluoroquinolones in Microbiology Laboratory, Department of Pathology, Army Medical College, Rawalpindi.

Methods:

One hundred consecutive clinical isolates of Typhoidal salmonellae isolated from blood culture samples were studied. The organisms were identified biochemically and serologically by standard technique. Sensitivity testing was carried out against nalidixic acid and ciprofloxacin by modified Kirby bauer disc diffusion method and minimum inhibitory concentration (MICs) were determined by E-test.

Results:

Seventeen percent of the isolates were resistant to nalidixic acid. Nalidixic acid disc diffusion and MIC estimation by E-test were 100% comparable. All isolates were sensitive to ciprofloxacin but the isolates which were resistant to nalidixic acid had raised MIC values against ciprofloxacin.

Conclusion:

Typhoidal salmonellae have not shown an overt in vitro resistance against fluoroquinolones in our set up but a significant population has emerged with raised MIC values. Nalidixic acid susceptibility test can be used an indirect evidence of resistance to quinolones (jpma 54:295;2004).