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Nalidixic acid screening test in detection of decreased fluoroquinolone susceptibility in salmonella typhi isolated from blood

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NALIDIXIC ACID SCREENING TEST IN DETECTION OF DECREASED FLUOROQUINOLONE SUSCEPTIBILITY IN *SALMONELLA TYPHI* ISOLATED FROM BLOOD

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ABSTRACT

Objective: To determine the validity of nalidixic acid screening test in the detection of high MICs of fluoroquinolone against *Salmonella(S.) typhi* isolated from blood and correlate zone diameters of ofloxacin with that of MIC value for nalidixic acid sensitive and resistant strains.

Design: Cross-sectional analytical study.

Place and Duration of Study: Clinical Microbiology Laboratory of the Aga Khan Hospital, Karachi from January 2002 to December 2003.

Patients and Methods: Two hundred *S. typhi* isolates from blood were included for nalidixic acid screening and ofloxacin susceptibility. Antibiotic susceptibilities for both the antibiotics were obtained by disc diffusion method whereas MICs were determined by standard agar dilution method as recommended by NCCLS guidelines. Sensitivity, specificity and correlation between both antimicrobial susceptibility methods were calculated and results expressed as scattergrams.

Results: The results broadly classify *S. typhi* isolates into nalidixic acid resistant strains with no zone of inhibition around 30 μg nalidixic acid disc and nalidixic acid sensitive strains with mean zone of inhibition of 24.9mm. All *S. typhi* isolates with ofloxacin MIC of ≥0.125 μg/ml were found to be nalidixic acid resistant (MIC ≥32 μg/ml) whereas the isolates with ofloxacin MIC ≤0.06 μg/ml were nalidixic acid sensitive (MIC ≤8 μg/ml). Screening for nalidixic acid resistance was found to be 100% sensitive and 97% specific in identifying *S. typhi* strains with reduced susceptibility to fluoroquinolone (MIC ≥0.125 μg/ml).

Conclusion: Nalidixic acid resistance as a screening method is proved to be significant in identifying *S. typhi* isolates with reduced susceptibility to fluoroquinolones. It is also suggested that inhibition zone of ≤25mm around 5μg ofloxacin disc is appropriate as a selection criterion to detect *S. typhi* isolates with reduced susceptibility to fluoroquinolones.

KEY WORDS: *Salmonella typhi. Fluoroquinolone resistance. Nalidixic acid screening blood isolates.*

INTRODUCTION

Typhoid fever has long been endemic within South Asia. Multidrug-resistant *Salmonella typhi* (MDRST), defined as strains exhibiting en-bloc resistance to chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole (TMP-SMX), was first reported from South Asia in late 80s.[1,2] Multidrug resistant *S. typhi* is now endemic in our region[3] along with overall increase in isolation rate and various reported outbreaks.[4-7]

Until recently, these multidrug-resistant strains have remained uniformly susceptible to fluoroquinolones and third generation cephalosporins. The two fluoroquinolones ofloxacin and ciprofloxacin are successful, safe, simple and cost effective mode of therapy with excellent cure rates as compared to intravenous ceftriaxone.[8-14] Isolation rate of multidrug resistant *S. typhi* from Pakistan is reported to be around 40% to 67.9%[14,15] which are uniformly susceptible to fluoroquinolones.

A steady rise has, however, been observed in the incidence of fluoroquinolone resistant typhoid fever. These isolates have ofloxacin MIC_{90} of 0.5 mg/L compared with 0.06 mg/L for fluoroquinolone susceptible isolates. Routine susceptibility methods as recommended by National Committee for Clinical Laboratory Standards (NCCLS)[16] fail to detect higher MICs of fluoroquinolones giving false susceptibility result, which appears to be responsible for growing number of treatment failures.[17]

Results showed that nalidixic acid resistant strains exhibited higher MIC values for ofloxacin and ciprofloxacin as compared to nalidixic acid sensitive strains despite the fact that these isolates were reported to be susceptible using recommended zone diameter from NCCLS.[16-20] It is recommended that screening of all isolated *S. typhi* for nalidixic acid resistance should be conducted to alert physicians about

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the possibility of treatment failure with fluoroquinolones.[17,20] This study was conducted to explore the validity of nalidixic acid screening test in detection of high MICs of fluoroquinolone against *Salmonella typhi* isolated from blood in a tertiary care referral laboratory in a typhoid endemic area. Our second objective was to correlate the zone diameters of ofloxacin with that of MIC values for both nalidixic acid sensitive and resistant strains.

Patients and Methods

Two hundred *Salmonella typhi* isolates from blood culture samples submitted to clinical microbiology laboratory between January 2002 to December 2003 were included in this study for nalidixic acid screening and fluoroquinolone susceptibility. Isolates were identified by API20E (Bio Murex, France). Serological confirmation was done by slide and tube agglutination method using type specific antisera (Difco Laboratries).

Antibiotic susceptibility of these isolates was determined by Kirby Bauer method using nalidixic acid (30μg) and ofloxacin (5μg) disks according to NCCLS (National Committee for Clinical Laboratory Standards) guidelines.[16] Minimum inhibitory concentration (MIC) of ofloxacin was determined by standard agar dilution method using Mueller Hinton agar as described in NCCLS guidelines.[21] *E. coli* ATCC strain 25922 was used as control in susceptibility testing by agar dilution method.

As per NCCLS recommendations[16,21] *Salmonella typhi* isolates with inhibition zone diameter of ≥ 19mm around 30μg nalidixic acid disk and minimum inhibitory concentration (MIC) of ≤16μg/ml by standard agar dilution method are classified as nalidixic acid sensitive *Salmonella typhi* , whereas, isolates with inhibition zone diameter of ≤13mm around 30μg nalidixic acid disk and minimum inhibitory concentration of ≥32μg/ml by standard agar dilution method are categorized as nalidixic acid resistant *Salmonella typhi*. Isolates with reduced susceptibility to fluoroquinolones is defined as *S. typhi* strains with the MIC of ≥0.125μg/ml for ofloxacin by standard agar dilution method.

Susceptibility data of all *Salmonella typhi* isolates for the above mentioned antibiotics was analyzed by using descriptive statistics of SPSS software version 13.0. Mean inhibition zone diameter of ofloxacin (± standard deviation) has been calculated for both nalidixic acid sensitive and resistant strains. Software EpiCal 2000 was used for determination of p-value which was considered to be significant if ≤0.01. Pearson correlation was calculated to demonstrate association between both the antimicrobial susceptibility methods against ofloxacin and nalidixic acid. Correlation was considered to be significant at 0.01 level (2-tailed). Sensitivity and specificity were calculated and results were expressed in the form of scattergrams.

Results

All *S. typhi* isolates included in the study (n=200) were tested for ofloxacin susceptibility by disc diffusion method and considered sensitive as per NCCLS recommendation (inhibition zone diameter ≥ 16). Mean inhibitory zone diameter around nalidixic acid and ofloxacin disks are shown in Table I along with p-values. These results broadly classify these isolates into two distinct subgroups; nalidixic acid resistant *S. typhi* with no zone of inhibition and nalidixic acid sensitive *S. typhi* with mean zone diameter of 24.9mm around 30μg nalidixic acids disk (Table I). This difference between inhibition zone diameter around nalidixic acid disk has been significant (p-value ≤ 0.01). Significant difference in zone diameter for ofloxacin by disc diffusion method was also observed for the above mentioned strains showing a definite association between nalidixic acid resistance and decreased fluoroquinolone susceptibility.

Table I: Mean zone diameter around nalidixic acid and ofloxacin for *S. typhi* strains isolated from blood.

Agent (disk content)	Nalidixic acid resistant isolates (MIC ≥32μg/ml)		Nalidixic acid sensitive isolates (MIC ≤6μg/ml)		p-value
	Mean ± SD	Range (mm)	Mean ± SD	Range (mm)	
Nalidixic acid (30μg)	0 ± 0	0	24.9 ± 2.1	(19-38)	≤0.01
Ofloxacin (5μg)	22.6 ±1.5	(19-26)	27.7 ±2.6	(19-41)	≤0.01

It was also observed that all isolates which were nalidixic acid resistant by both disc diffusion and MIC methods had their MIC values for ofloxacin ≥0.125μg/ml as compared to the nalidixic acid sensitive strains having MIC values of ≤ 0.06μg/ml (Figure 1). When ofloxacin MIC of ≥0.125μg/ml was adopted as a breakpoint, nalidixic acid screening led to the detection of all *S. typhi* isolates with decreased fluoroquinolone susceptibility (p-value 0.01).

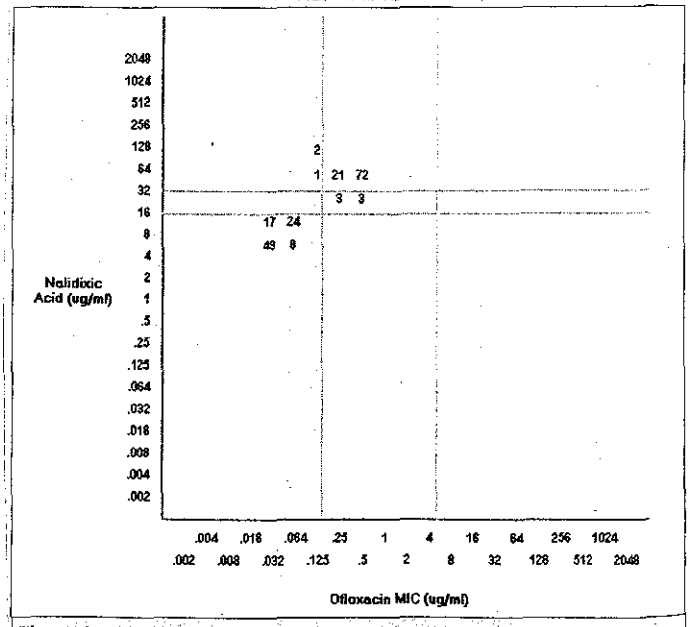

Figure 1: MIC values ofloxacin versus nalidixic acid.

Correlation between ofloxacin MICs and zone diameters around 5-mg disk of ofloxacin are shown in Figure 2. Ninety-eight out of 100 *S. typhi* strains for which ofloxacin MICs were between 0.125-0.5 μg/ml had inhibition zone diameter of ≤ 25 mm. Remaining two isolates had MIC of 0.125 μg/ml with the

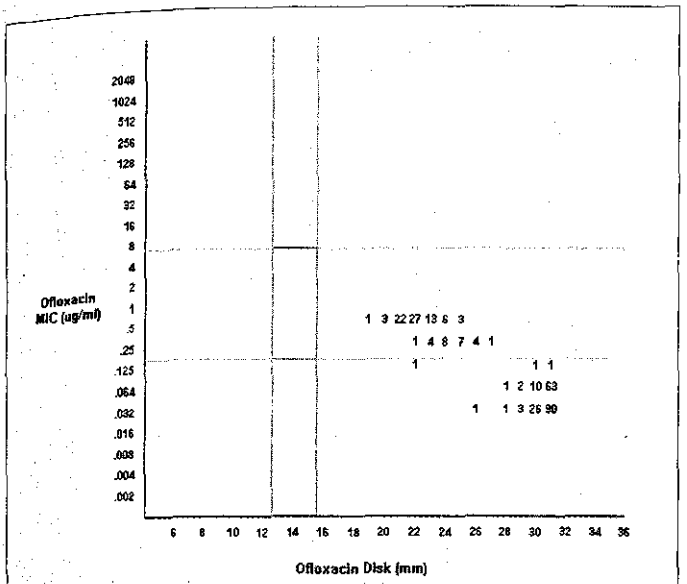

Figure 2: Zone diameter versus MIC values for ofloxacin.

inhibition zone diameter of 30-32mm by disk diffusion method. Correlating the MIC of ofloxacin with that of nalidixic acid for the above mentioned isolates, all *S. typhi* isolates with ofloxacin MIC of ≥0.125 mg/ml were nalidixic acid resistant (MIC ≥32 μg/ml), whereas, all *S. typhi* isolates with MIC of ofloxacin ≤ 0.06ug/ml had inhibition zone diameter of ≥ 25mm around ofloxacin disk and had MIC values of ≤8 μg/ml for nalidixic acid and, therefore, considered sensitive. This correlation between nalidixic acid disk and ofloxacin MICs for all *S. typhi* isolates tested as determined by Pearson's correlation study was also statistically significant (Pearson Coefficient value of ≤0.01). Correlation studies also showed an inverse relationship between antimicrobial susceptibilities by both the above described methods (Table II). Reduced zone diameters by disc diffusion method correlate well with the increasing MIC values for the given antibiotic even if the isolate is shown to be fluoroquinolone susceptible by disk diffusion method as per NCCLS recommendations.

Table II: Correlation between nalidixic acid disk and ofloxacin MIC values. (*Significance, **N. Number of isolates,***Correlation is significant at the level of 0.01 level (2-tailed).

		Nalidixic acid disc(mm)	Ofloxacin MIC (μg/ml)
Nalidixic acid disc(mm)	Pearson correlation	1	-0.924***
	Sig*. (2-tailed)		0.00
	N**	200	200
Ofloxacin MIC (μg/ml)	Pearson correlation	-0.924***	1
	Sig*. (2-tailed)	0.00	
	N**	200	200

Table III: Correlation between ofloxacin disk and ofloxacin MIC values. (*Significance, **N. Number of isolates, ***Correlation is significant at the level of 0.01 level (2-tailed).

		Ofloxacin disc(mm)	Ofloxacin MIC (μg/ml)
Ofloxacin disc(mm)	Pearson correlation	1	-0.899***
	Sig*. (2-tailed)		0.00
	N**	200	200
Ofloxacin MIC (μg/ml)	Pearson correlation	-0.899***	1
	Sig*. (2-tailed)	0.00	
	N**	200	200

All the isolates for which ofloxacin MICs were ≥0.125 μg/ml showed no zone of inhibition around nalidixic acid by disc diffusion test. MIC of nalidixic acid for these isolates were >32μg/ml. In contrast 99 out of 100 isolates which had MIC value of ofloxacin <0.125 μg/ml (0.3-0.6 μg/ml) had inhibition zone diameter around nalidixic acid disk ≥20mm. MIC value of nalidixic acid for these isolates were ≤ 8 μg/ml. Based on the MIC value and disk diffusion results, screening for nalidixic acid resistance has led to the detection of all *S. typhi* strains with reduced susceptibility to ofloxacin (MIC values ≥0.125μg/ml). These results fairly correlate the higher MIC values for isolates resistant to nalidixic acid by both the screening methods. Sensitivity of nalidixic acid screening method was calculated to be 100% in detecting *S. typhi* isolates with decreased fluoroquinolone susceptibility (MIC ≥ 0.125μg/ml) with the specificity approaching 97%.

Discussion

Fluoroquinolones and third generation cephalosporins (ceftriaxone, cefixime) are found to have maximum activity against multidrug resistant *S. typhi* isolates with the reported resistant rate of less than 2%.[13,14,22]

With the emergence of these MDR strains, fluoroquinolones became the agent of choice in treatment of uncomplicated typhoid fever. Several studies have compared the efficacy of short course oral treatment with fluoroquinolone (ofloxacin, ciprofloxacin) and third generation cephalosporin (cefixime, ceftriaxone).[5,8-10] In almost all of the studies short course treatment with oral ofloxacin (5 days) was reported to be significantly better than ceftriaxone particularly in areas where MDR *Salmonella typhi* is prevalent thus favoring the widespread use of fluoroquinolone in the community for treating enteric fever.

Investigators from Vietnam first reported the isolation of nalidixic acid resistant *S. typhi* in 1993.[17] The MICs of ciprofloxacin for these nalidixic acid resistant isolates were higher as compared to nalidixic acid sensitive isolates, although, the MICs were still within the current NCCLS range for susceptibility (i.e 0.125-0.5μg/ml). Disk diffusion method failed to detect these isolates as all were interpreted as fluoroquinolone susceptible. A growing body of clinical and microbiological evidence indicates that such nalidixic acid resistant *Salmonella* infections exhibit a decreased clinical response to fluoroquinolones.[18,23,24] Cure rates achieved by fluoroquinolone treatment in patients infected with nalidixic acid resistant strains were only 50% as compared to 97% for patients infected by nalidixic acid sensitive strains.[17] Hence, authors recommended that laboratories must test extraintestinal *Salmonella* isolates for nalidixic acid resistance and NCCLS fluoroquinolone breakpoints for *Salmonella* should be re-evaluated.[24]

Screening test using nalidixic acid disk diffusion test as evaluated further by various authors[18-20] is demonstrated to be highly sensitive and specific for detecting *Salmonella* isolates with decreased susceptibility to fluoroquinolones (MIC ≥ 0.125ug/ml).

The primary objective of this study was to explore and validate nalidixic acid susceptibility as a screening method to detect *S. typhi* strains with high MIC to ofloxacin. Our findings were consistent with the previously published results.[19-21] Among 200 *S. typhi* isolates evaluated, two distinct sub-populations were identified when isolates susceptible to ofloxacin by NCCLS criteria were tested for nalidixic acid susceptibility by disc diffusion method, nalidixic acid sensitive and nalidixic acid resistant *S. typhi*. The MIC of ofloxacin for nalidixic acid resistant isolates were higher (≥ 0.125µg/ml) than those for nalidixic acid sensitive isolates (≤ 0.06µg/ml). All nalidixic acid resistant isolates had no zone of inhibition around 30µg nalidixic acid disk.

Based on these observations nalidixic acid screening test was found to be highly sensitive in the detection of strains with decreased susceptibility to fluoroquinolones. Studies with similar results have recommended that in addition to the categories of resistant and fully susceptible, a category of decreased fluoroquinolone susceptibility could be useful in the susceptibility determinations of *Salmonella*.[18] Recent recommendations by working party BSAC (British Society of Antimicrobials and Chemotherapy) for standard disc susceptibility testing method also states that in testing *Salmonella* species for ciprofloxacin susceptibility there is clinical evidence to indicate poor response in systemic infections caused by *Salmonella* species with reduced susceptibility to fluoroquinolones (ciprofloxacin MIC 0.125-1 mg/L). This reduced susceptibility is most reliably detected with nalidixic acid 30µg discs as strains with reduced susceptibility show no zone of inhibition.[25]

Our second objective was to correlate zone diameters of ofloxacin for both nalidixic acid sensitive and resistant strains to define a cut-off value that can fairly be associated with decreased fluoroquinolone susceptibility with MIC value of ≥0.125µg/ml. All *S. typhi* isolates which were nalidixic acid sensitive had inhibition zone diameter of ≥25mm around 5µg ofloxacin disk with MIC ofloxacin 0.03-0.06µg/ml, hence ofloxacin susceptible. All these isolates were also susceptible to nalidixic acid (mean zone diameter of 24.9 ± 2.1). In contrast, all 100 isolates with inhibition zone diameter of ≤25mm by disk diffusion method had an MIC of ≥ 0.125µg/ml for ofloxacin (Figure 2). These isolates were resistant to nalidixic acid with no zone of inhibition around nalidixic acid disk. The mean zone of inhibition around 5µg ofloxacin disk for 100 nalidixic acid sensitive isolates, as observed in this study was 27.7 ± 2.6 mm, a size significantly greater than nalidixic acid resistant strains with inhibition zone diameter 22.6 ± 1.5mm. These results establish the association between nalidixic acid resistance, higher MIC values and a significant difference in zone diameter for ofloxacin. It is important for microbiological laboratories to recognize these less susceptible isolates by performing nalidixic acid susceptibility as a screening method for all *S. typhi* strains isolated from blood culture samples in order to help clinicians to initiate appropriate alternative antimicrobial therapy.

Conclusion

Nalidixic acid susceptibility as a screening methodology was a highly sensitive and specific in identifying *S. typhi* isolates with MIC value of ≥0.125µg/ml in our series. A zone diameter of ≤ 25 mm around 5µg ofloxacin disk is appropriate as a selection criterion to detect almost all *Salmonella* isolates with decreased susceptibility to fluoroquinolone.

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