Original Research

DETECTION OF RESISTANCE DETERMINANTS BY MOLECULAR ANALYSIS AMONG THE QUINOLONE RESISTANT *SALMONELLA ENTERICA* SEROVAR TYPHI ISOLATES FROM KANCHIPURAM

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ABSTRACT

Aim: To study the antimicrobial susceptibility pattern and detection of plasmid mediated quinolone resistance in *Salmonella enteric* serovar Typhi.

Method: We collected 62 isolates from a tertiary care hospital in Kanchipuram from 2009–2011. All the isolates were subjected to antimicrobial susceptibility testing by Kirby-Bauer disk diffusion method and broth microdilution method was used to determine minimum inhibitory concentration of ciprofloxacin and nalidixic acxid. Then the strains were amplified for the presence of plasmid mediated quinolone resistance (*PMQR*) gene.

Result: All the 62 isolates were found to be nalidixic acid resistant strains (NARST) and susceptible to ciprofloxacin. Few isolates showed resistance to ampicillin, co-trimoxazole, tetracycline and chloramphenicol. None of the isolates amplified for *PMQR* gene.

Discussion: The present study indicates the higher prevalence of NARST and there may be a low-level rate of dissemination of Qnr determinants among human *Salmonella* isolates.

Keywords: Antimicrobail resistance Salmonella spp, PMQR

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INTRODUCTION

Enteric fever poses a serious threat to developing countries where they remain as the most threatening endemic infection [1]. The global incidence of Salmonella has been estimated as 21 million with 7,00,000 each year mainly in countries such as South East Asia, Africa and Latin America [2]. *Salmonella enterica* serovar Typhi [*Salmonella typhi*] remains the most common etiological agent of enteric fever followed by *S. enterica* serovar Paratyphi A. Failure of primary drugs [ACCoT] and concurrent reports on Nalidixic acid- resistant *Salmonella* [NARST] from various countries around the globe, leading to multidrug-resistant *Salmonella*, brought fluoroquinolones and third-generation cephalosporins into the treatment scenario [3,4,5,6,7]. Multidrug resistant (MDR) *Salmonella* are restraining the therapeutic options of enteric fever. They are known as multidrug resistance when they are resistant to ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracycline [8,9].

The targets of fluoroquinolones are the two enzymes DNA gyrase and topoisomerase IV, which are encoded by *gyrA*, *gyrB* and the *parC*, *parE* genes, respectively. Mutation in a quinolone resistance-determining region (QRDR), efflux pump associated resistance, bacterial permeability, qnr plasmid and up/down regulation of operon genes have been documented as the various reasons for resistance [10,11,12]. The first report on plasmid mediated quinolone resistance [PMQR] was reported in UK in 2007 by Hopkins *et al* [13]. The PMQR is mediated by *qnr* gene harbored by the bacteria in their plasmid. The qnr families consist of *qnr A*, *qnr S*, *qnr* B, *qnr* C and *qnr D* that confer resistance to members of fluoroquinolone.

The present study was undertaken to analyze the current status of antibiotic susceptibility pattern and presence of plasmid mediated quinolone resistance gene (*qnr*) in *Salmonella enterica* serovar Typhi isolates from people presenting with enteric fever in a tertiary care centre in Kanchipuram.

MATERIALS AND METHODS

Nearly 615 blood samples from patients presenting with fever in a tertiary care hospital in Kanchipuram from 2009 to 2011 were screened for serovar Typhi. We isolated a total of 62 clinical isolates of

Salmonella spp. The isolates were identified using the routine laboratory methods. Preliminary identification included Gram's staining, test for motility and oxidase, followed by biotyping and serotyping. Biotyping included a battery of standard biochemical test, ability to ferment sugars and aminoacid decarboxylation. Serotyping was done with the antisera, from *King Institute of Preventive Medicine, Guindy, Chennai* to confirm the serotype of the *Salmonella* isolates.

Antimicrobial susceptibility testing

All the confirmed strains of *Salmonella enterica* serovar Typhi were subsequently tested to detect their antibiogram pattern by disc diffusion method as per CLSI guidelines [14]. In brief, the isolates were subcultured 24 hours before performing the susceptibility testing and the concentration was adjusted to 0.5 McFarland standards. Lawn was made on Muller-Hinton agar (MHA), and the discs were placed on it and incubated overnight at 37°C. The antibiotics used were: ciprofloxacin (5µg), norfloxacin (10µg), nalidixic acid (30 µg), cefotaxime (30µg), ceftriaxone (30µg), ampicillin (10µg), chloramphenicol (30µg), co-trimoxazole (25µg), tetracycline (30µg), gentamicin (10µg). The zone of inhibiton was measured and analyzed as per the CLSI guidelines. Broth microdilution was done to determine the minimal inhibitory concentrations of nalidixic acid and ciprofloxacin.

Dectection of *qnr* gene among *Salmonella enterica* serovar Typhi isolates

Plasmid isolation from the isolates was done by alkaline lysis method. In short, about 1.5 ml of 24 hours fresh culture was taken in an eppendorf tube and centrifuged for 5 minutes at 1000 rpm. The supernatant was then discarded and the pellets were dissolved in ice cold solution I, containing EDTA, tris Hcl and sucrose. Then about 200µl of solution II, containing sodium hydroxide and SDS, was added and inverted for few times and stored at 4° C for 4–5 minutes. Later 150µl of solution III, containing sodium acetate, acetic acid and distilled water, was added and inverted for few minutes and stored at 4° C for 4–5 minutes. Then the tubes were centrifuged at 10,000 rpm for 10 minutes.

The supernatant was transferred to a sterile eppendorf tube and 100% ethanol was added twice the volume and vortexed and allowed to stand for 5 minutes. Then the tubes were centrifuged for 10 minutes. The supernatant was discarded and the pellets were resuspended in 70% ethanol and the tube was inverted for several times and centrifuged for 5 minutes. The supernatant was discarded and the pellets were dried until the ethanol evaporated. 50µl of sterile tris EDTA buffer was added to the pellet and stored in 4° C.

The plasmids were subjected to PCR for the detection of quinolone resistant gene (*qnr*) using the specific primers, Fwd 5'-GGG TAT GGA TAT TAT TGA TAA AG-3' Rvs M13 5'-CTA ATC CGG CAG CAC TAT TA-3' (Sigma Oligos, Bangalore). The amplicons were resolved at 100V for 20 minutes in 1% agarose gel with ethidium bromide. The gel was documented in the Bio-Rad gel documentation system.

RESULTS

Of the 615 blood samples that were screened, we obtained 62 isolates of *Salmonella enteric* serovar Typhi.

Antimicrobial susceptibility testing

The drug resistance pattern of Salmonella was found to quiet variable. Out of the 62 isolates 100% sensitivity was observed with ciprofloxacin, ceftriaxone, cefotaxime, gentamicin and 100% resistant to nalidixic acid by Kirby-Bauer disk diffusion method. Chloramphenicol, co-trimoxazole norfloxacin, ampicillin and tetracycline showed 93.5%, 90.3%, 91.9%, 85.5% and 95.2% sensitivity, respectively. Among the resistance pattern to other antibiotics 4 isolates (6.5%) were resistant to ampicillin, chloramphenicol, 3 isolates (4.8%) were resistant to tetracycline, and 6 isolates (9.7%) were resistant to co-trimoxazole (Table I). Better sensitivity was observed for ACCOT group of drugs. Since nalidixic acid showed 100% resistance, both nalidixic acid and ciprofloxacin were subjected for MIC test by broth micro dilution method. Few isolates that showed intermediate resistance to ceftriaxone and cefotaxime were also tested for MIC by broth microdilution.

Table-1: Antibiotic Susceptibility pattern of *Salmonella enterica* serovar Typhi-Disc diffusion

Antibiotics	Sensitivity (%)	Intermediate (%)	Resistant (%)
Ciprofloxacin	100% (62/62)	-	-
Norfloxacin	91.9% (57/62)	8.1% (5/62)	-
Nalidixic acid	-	-	100 % (62/62)
Ceftriaxone	100% (62/62)	-	-
Cefotaxime	100% (62/62)	-	-
Ampicillin	85.5% (53/62)	8.1% (5/62)	6.5% (4/62)
Chloramphenicol	93.5% (58/62)	-	6.5% (4/62)
Co-trimoxazole	90.3% (56/62)	-	9.7% (6/62)
Tetracycline	95.2% (59/62)	-	4.8% (3/62)
Gentamicin	100 % (62/62)	-	-

Table 2: MIC values of *Salmonella enterica serovar Typhi* to Nalidixic acid and Ciprofloxacin

Sr. No	Drug dilution range for Nalidixic acid (µg/mL)	Isolates in *Salmonella Typhi*	Drug dilution range for Ciprofloxacin (µg/mL)	Isolates in *Salmonella Typhi*
1	256	62	64	-
2	128	-	32	-
3	64	-	16	-
4	32	-	8	-
5	16	-	4	-
6	8	-	2	-
7	4	-	1	32
8	2	-	0.5	24
9	1	-	0.25	6
10	0.5	-	0.125	-
11	-	-	0.0625	-

All the 62 isolates tested by broth micridilution showed 100% resistance to nalidixic acid and 100% senstivity to ciprofloxacin (Table 2). All the strains had a MIC value greater than 256 for nalidixic acid. The MIC value for ciprofloxacin was in the range 0.25–1µg/mL. MIC determinations of few intermediately resistant Ceftriaxone and cefotaxime strains were found to be sensitive by MIC studies (data not shown).

Detection of *qnr* gene by PCR

Out of the 62 isolates that were analysed for the presence for plasmid mediated *qnr* gene none of the isolate amplified for the *qnr* gene. All were negative for ***PMQR*** (Fig. 1).

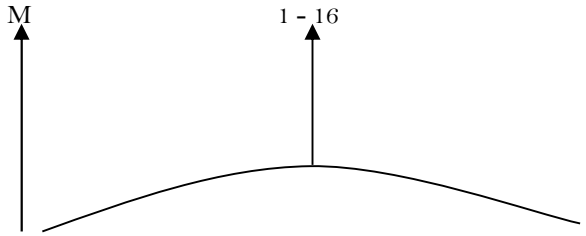

Fig 1: Representative photograph showing the absence of *qnr* gene among the quinolone resistant isolates

DISCUSSION

The recent trend in increasing antimicrobial resistance among the members of *Salmonella enterica* has alarmed us the need to relentlessly assess the rationally used antibiotics for management of enteric fever. Among our isolates 3 our isolates were resistant to ampicillin, chloramphenicol, tetracycline and nalidixic acid and hence were considered as multidrug resistant Salmonella. Two isolates were resistant to all the above drugs and co-trimoxazole. In the present study 8.1% of our isolates were found to be MDR strains. Previous studies by Sivakumar *et al.* [9] and Sandvang et al. [15] reports higher prevalence of MDR and the results our study has differed by lesser prevalence rate.

The infections caused by the *Salmonella* spp are increasing. Fluoroquinolones are widely used in treating these infections. Mutation in topoisomerase

is the important source of resistance against fluoroquinolones among *Salmonella* spp. Plasmid mediated resistance had been widely reported among these organisms.

The first report on plasmid mediated quinolone resistance in *Salmonella* spp was reported by Hopkins in 2007 [13]. Later in a prevalence study conducted in France out of 516 nalidixic acid resistant isolates of *Salmonella typhi* only 1 showed positive for *qnr* A i.e. 0.2%. Similar prevalence studies conducted in South Korea among 216 isolates, all the isolates were negative for *qnr* A, B, C, D or S among the nalidixic acid resistant community acquired *Salmonella* species [16].

In our present study we tried to monitor the prevalence of plasmid mediated quinolone resistance among nalidixic acid resistant *Salmonella enterica* serovar Typhi in Kanchipuram. But like the previous study we also did not obtain any positive result for plasmid mediated *qnr* gene.

Cattoir *et al*., [16] showed in his study that though the isolates showed resistance to nalidixic acid they were still sensitive to ciprofloxacin. In our study also all our isolates showed 100% resistance to nalidixic acid and 100% sensitive to ciprofloxacin.

When correlating the present study with the previous study it indicates there may be a low-level rate of dissemination of Qnr determinants among human *Salmonella* isolates. This study being only a preliminary study could be further continued by looking the PMQR among large number of fluoroquinolone resistant *Salmonella* isolates and also at the other mechanisms leading to resistance in *Salmonella* that could help in proper disease management.

REFERENCE:

1. Raveendran R, Wattal C, Sharma A, Oberoi JK, Prasad KJ, and Datta S. High level ciprofloxacin resistance in *Salmonella enterica* isolated from blood. *Indian Journal of Medical Microbiology* 2008;26(1):50–53.
2. Prajapati B, Rai GK, Rai SK, Upreti HC, Thapa M, Singh G, Shrestha RM. Prevalence of *Salmonella typhi* and *paratyphi* infection in children: a hospital based study. *Nepal Medical College Journal* 2008; 10(4):238–241.
3. Gaind R, Paglietti B, Murgia M, Dawar1 R, Uzzau S, Cappuccinelli P, Deb M, Aggarwal P and Rubino S. Molecular characterization of ciprofloxacin-resistant *Salmonella enterica* serovar Typhi and Paratyphi A causing enteric fever in India. *Journal of Antimicrobial Chemotherapy* 2006;58:1139–1144.
4. Surg Capt Misra RN, Surg Capt Bawa KS (Retd), Lt Col Magu SK, Surg Cdr Bhandari S, Col Nagendra A, and Wg Cdr Menon PK (Retd). Outbreak of Multi-drug Resistant *Salmonella Typhi* Enteric Fever in Mumbai Garrison. *MJAFI* 2005;61(1).
5. Wain J, Hoa NT, Chinh NT, Vinh H, Everett MJ, Diep TS, Day NP, Solomon T, White NJ, Piddock LJ, and Parry CM. Quinolone-resistant *Salmonella typhi* in Vietnam: molecular basis of resistance and clinical response to treatment. *Clincal Infectious Diseases* 1997;25:1404–1410.
6. Threlfall EJ, Ward LR. Decreased susceptibility to ciprofloxacin in Salmonella enterica serotype Typhi, United Kingdom. *Emerging Infectious Diseases* 2001;7:448–50.
7. Ackers ML, PuhrND, TauxeRV, Mintz ED. Laboratory based surveillance of *Salmonella* serotype Typhi infections in United States—antimicrobial resistance on the rise. *JAMA* 2000; 283:2668–2673.
8. Ashraf MA, Hiroyoki N, Tadashi S. Molecular characterization of ontegrons in non typhoid Salmonella serovars isolated in Japan. *Journal of Antimicrobial Chemotheraphy* 2005; 55:371–374.
9. Sivakumar T, Saravanavel NA, Prabhu D, Shankar T, Vijayabaskar P. Characterization of Multidrug Resistant Patterns of Salmonella sp. *World Journal of Medical Sciences* 2012; 7(2): 64–67.
10. Crump JA, Barrett TJ, Nelson JJ, Angulo FJ. Re-evaluating fluoroquinolone breakpoints for *Salmonella enterica* serotype Typhi and for Non-Typhi Salmonellae. *Clincal Infectious Diseases* 2003;37:75–81.
11. Nair S, Unnikrishnan M, Turner K, Parija SC, Churcher C, Wain J, Harish BN. Molecular analysis of fluoroquinolone-resistant Salmonella Paratyphi A isolate, India. *Emerging Infectious Diseases* 2006;12:489–491.
12. Hirose K, Hashimoto A, Tamura K, Kawamura Y, Ezaki T, Sagara H, Watanabe H. DNA sequence analysis of DNA gyrase and DNA topoisomerase in the quinolone resistance determining regions of Salmonella enterica serovar Typhi and serovar Paratyphi A. Antimicrobial Agents and Chemotheraphy 2002;46:3249–3252.
13. Hopkins KL, Wootton L, Day MR and Threlfall EJ. Plasmid-mediated quinolone resistance determinant qnrS1 found in Salmonella enterica strains isolated in the UK. *Journal of Antimicrobial Chemotherapy* 2007;59:1071–1075.
14. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; 17th informational supplement. MS100-S17, 2007. CLSI, Wayne, PA.
15. Sandvang D, Aarestrup EM, Jensen LB. Characterization of integrons and antibiotic resistance genes in Danish multiresistant *Salmonella typhimurium* DT 104. FEMS Microbiology Letter 2008;160:37–41.
16. Cattoir V, Weill FX, Poirel L, Fabre L, Soussy CJ, Nordmann P. Prevalence of *qnr* genes in Salmonella in France. *Journal of Antimicrobial Chemotherapy* 2007;59:751–754.