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Original Article

Detection Of Mutated *gyrA* Gene From Nalidixic Acid Resistant *Salmonella Typhi And Paratyphi A* Isolated From Enteric Fever Patients In A Tertiary Care Hospital Of Bangladesh

Maiz-ul Ahad Suman[1], Md. Abdullah Siddique[2], SM Shamsuzzaman[3], Abu Rayhan Khandakar[4] , Faiz Ahmed Khondaker[5] Sharmin Akter Sumi[6], Roushan Jahan[7]

[1]Department of Microbiology, Gonoshasthaya Samaj Vittik Medical College, Dhaka. [2]Department of Microbiology, Barind Medical College, Rajshahi. [3]Department of Microbiology, Dhaka Medical College. [4]Department of Biochemistry, Rajshahi Medical College. [5]Department of Hepatology, Shaheed Suhrawardy Medical College. [6]Department of Burn and plastic surgery, Dhaka Medical College. [7]Department of Pediatrics, BSMMU

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ABSTRACT

A cross sectional study for the detection of empirically used antibiotic resistant *Salmonella* was carried out in the department of microbiology of Rajshahi Medical College (RMC) and PCR and DNA sequencing were done in the department of microbiology of Dhaka Medical College during the period from August 2014 to July 2015.Total 323 blood samples were collected from suspected enteric fever patients from medicine and paediatric units of RMC hospital and cultured on brain heart infusion broth for isolation of *Salmonella*. Identification of *Salmonella* was done by biochemical tests and final identification was done by specific antisera. Antimicrobial sensitivity test was done by disc diffusion technique. PCR was used to detect gyrA gene. Sequencing of the gyrA gene was done to see mutation. Culture yielded growth of *Salmonella typhi* in 11 (36.67%) and *Salmonella paratyphi A* in 19(63.33%) samples. Among 11 *Salmonella typhi*, 8(72.73%) were resistant to nalidixic acid, 2(18.18%) to ciprofloxacin and one (9.09%) to ofloxacin. Among 19 *Salmonella paratyphi A*, all (100%) were resistant to nalidixic acid, 4(21.05%) to ofloxacin, and 3(15.79%) to ciprofloxacin. All the *Salmonella typhi* and *Salmonella paratyphi A* were gyrA gene positive. All the nalidixic acid resistant strains of *Salmonella typhi* and *Salmonella paratyphi A* were mutated at amino acid position 83 by replacing serine with phenylalanine. None of the nalidixic acid sensitive *Salmonella* strain had mutated gyrA gene.

Key word: Bangladesh, *gyrA* gene mutation, nalidixic acid resistance, *Salmonella typhi, Salmonella paratyphi A.*

INTRODUCTION

Enteric fever (typhoid and paratyphoid fever) is an endemic disease in developing countries[1]. The etiological agent of typhoid fever is *Salmonella typhi* and paratyphoid fevers are *Salmonella paratyphi A, B* and *C*[2]. Among these *Salmonella, Salmonella typhi and Salmonella paratyphi A* are mainly responsible for enteric fever. Antibiotic is the main therapeutic option for the treatment of enteric fever and mortality rate may reach up to 30% in the absence of effective antibiotic therapy[3]. Since *Salmonella* are resistant to conventional antibiotics (ampicillin, chloramphenicol and co-trimoxazole), ciprofloxacin and ofloxacin had been the drugs of choice in Bangladesh4. But rampant use of ciprofloxacin made *Salmonella typhi* and *Salmonella paratyphi A* resistant to nalidixic acid by mutation in *gyrA* gene which replace nalidixic acid from the target site in gyrase A[5,3]. These nalidixic acid resistant *Salmonella* are also less responsive to ciprofloxacin due to less hydrogen bonding formed between ciprofloxacin and altered gyrase A. Resistance of *Salmonella* to nalidixic acid is mediated by alteration of amino acid in DNA gyrase which is result of mutation in *gyrA* and *gyrB* chromosomal genes and by alteration of amino acid in tropoisomerase which is result of mutation in *parE* and *perC* genes or by decreased permeability of drug or over-expression of efflux pump. However, mutation in *gyrA* gene is more common than *gyrB, parE* and *perC* genes[6]. Resistance mutations in *gyrA* gene is clustered in a region of gene product 67-106 amino acids called quinolone resistance-determining region and most common mutations associated with quinolone resistance are located in amino acid, serine-83 or aspartic acid-87[7]. Single mutation in *gyrA* gene (either amino acid serine-83 or aspartic acid-87) is associated with resistance to nalidixic acid and decreased susceptibility to

✉ ***Correspondence:***
Dr. Maiz-ul Ahad Suman
Senior Lecturer
Department of Microbiology
Gonoshasthaya Samaj Vittik Medical College, Dhaka
Phone number: 01798638986
E-mail: sumanmaiz@yahoo.com

fluoroquinolone[8].

A good number of quinolone resistant *Salmonella* have been detected in Rajshahi Medical College Hospital but the reason behind this resistance has not been clear. So this study has been carried out to find out the mutations in *gyrA* gene from nalidixic acid resistant *Salmonella*.

MATERIALS AND METHODS

Microbiological study was carried out in the department of microbiology of Rajshahi Medical College (RMC) and PCR and sequencing was performed in the department of microbiology of Dhaka Medical College from August 2014 to July 2015. Blood samples were collected from 323 suspected enteric fever patients of both outpatient and inpatient departments of medicine and pediatric units of RMC hospital. Patients presented with fever ≥102° F for more than 3 days and associated constipations, diarrhea, diffuse abdominal pain or coated tongue were clinically diagnosed as enteric fever and were included in this study.

About 5ml blood from adult and 1 to 5 ml blood (according to body weight) from young children were collected following preparation of the skin site with 70% alcohol, then providone iodine and transferred to blood culture bottles containing 50 and 25ml brain heart infusion broth respectively. The bottles containing blood were sent to laboratory promptly and incubated at 37°C.

The culture bottles were examined daily for visible turbidity and subcultured every alternate day on blood, MacConkey and Salmonella-Shigella agar up to 14 days. Bacterial growth was indicated by the presence of turbidity in the media. If visible growth was detected a little amount of the broth was dispensed by sterile disposable syringe and inoculated on blood, MacConkey and Salmonella-Shigella agar and incubated at 37°C for 24 hours. If growth observed on blood, MacConkey and Salmonella-Shigella agar media, bacteria were identified as *Salmonella typhi* and *Salmonella paratyphi A* by nonlactose fermenting colony on MacConkey agar media, gram negative bacilli in Gram stained smear, motile, indole, urease and citrate negative, alkaline slant and acid butt with or without H_2S production in triple sugar iron agar media, negative oxidase test and finally agglutination with *Salmonella* polyvalent O and specific H antisera. Antimicrobial susceptibility was determined by modified Kirby-Bauer disc diffusion method and following Clinical and Laboratory Standards Institute (CLSI) guide line using Mueller-Hinton agar plate[9]. Both *Salmonella typhi* and *paratyphi A* were tested for susceptibility with ciprofloxacin (5µg), ofloxacin (5µg) and nalidixic acid (30µg). A representative disc of each batch was tested with ATCC 25922 strain of *Escherechia coli* to assess the quality of the test. Disc diffusion test was interpreted according to CLSI (2013) and European Committee on Antimicrobial Susceptibility Testing, 2015.

Preservation of bacteria: The bacteria was streaked on nutrient agar slope and incubated at 37°C. When visible growth was observed, sterile liquid paraffin was poured on slope to cover the agar. The tube containing growth was then preserved at 4°C[10].

Detection of *gyrA* gene by polymerase chain reaction:

Formation of pellet of *Salmonalla*[11]: The nalidixic acid resistant and sensitive *Salmonella typhi* and *Salmonella paratyphi A* were tested for *gyrA* gene by PCR using previously used primer, forward 5′-TGTCCGAGATGGCCTGAAGC-3′ reverse 5′-CGTTGATGACTTCCGTCAG-3′. The bacteria were cultured in brain heart infusion broth within micro centrifuge tube and incubated at 37°C for 24 hours. The microcentrifuge tube was centrifuged at 4000 x g for 10 minutes and supernatant was discarded. The pellets were then preserved at -20°C for DNA extraction.

DNA extraction: The *Salmonella* pellets were suspended with 300µl of sterile distilled water and vortexed well. The microcentrifuge tube was placed on heat block at 100°C for 10 minutes and then immediately placed on ice block for 5 minutes. The microcentrifuge tube was then centrifuged at 13000 x g at 4°C for 6 minutes and the supernatant was transferred to another fresh microcentrifuge tube. This supernatant contains extracted DNA which was used as template DNA for PCR.

Mixing of master mix, primer and extracted DNA: A total of 25µl of reaction mixture was prepared in a PCR tube by using 12.5µl of master mix (a mixture of dNTP, taq polymerase, $MgCl_2$, PCR buffer), 1 µl of forward primer, 1 µl of reverse primer, 3 µl of extracted DNA and 7.5µl nuclease free water.

Amplification in thermal cycler: After initial denaturation at 94°C for 3 minutes, total 30 cycles of each cycle consists of denaturation at 94°C for one minute, annealing at 55°C for one minute and extension at 72°C for one minute with one cycle of final extension at 72°C for 10 minutes.

Gel electrophoresis: Electrophoresis of the amplicons was done at 100 volts for 35 minutes in 1X Tris-borate-EDTA buffer in 1.5% agar gel, stained with ethidium bromide and visualized by UV transilluminator. Band size was assessed by comparing with bands of 100 bp DNA ladder.

DNA sequencing: Amplified DNA was purified by PCR Purification Kit (Favorgen, Biotech Corp,Taiwan) according to manufacturer's instruction. Purified DNA was then sequenced in ABI C500 gene sequencer. The sequence of the amplified DNA was compared with gyrA DNA sequence available in gene bank and mutation site of DNA and subsequently mutated protein was identified.

RESULT

Of the 323 blood samples, 11(3.41%) *Salmonella typhi* and 19(5.88%) *Salmonella paratyphi A* were isolated. On antimicrobial susceptibility tests, 8(72.73%) *Salmonella typhi* and 19(100%) *Salmonella paratyphi A* were resistant to nalidixic acid (Table-1). All the *Salmonella typhi* and *Salmonella paratyphi A* were positive for gyrA gene (fig.1) DNA sequencing of quinolone resistance-determining region of *gyrA* gene of nalidixic acid sensitive (fig.2) and nalidixic acid resistant *Salmonella typhi* and *Salmonella paratyphi A* (fig.3) showed mutation at amino acid position 83(TCC, coded for serine was replaced by TTC, coded for phenylalanine). None of the nalidixic acid sensitive strains showed mutation of amino acid at position 83 (Table-2).

Table-1 Antimicrobial resistance pattern of *Salmonella typhi and S. Paratyphi A.*

Antimicrobial agent	*S. typhi* n(%)	*S. paratyphi A* n(%)
Ciprofloxacin	2(18.18)	3(15.79)
Nalidixicacid	8(72.73)	19(100)
Ofloxacin	1(9.09)	4(21.05)

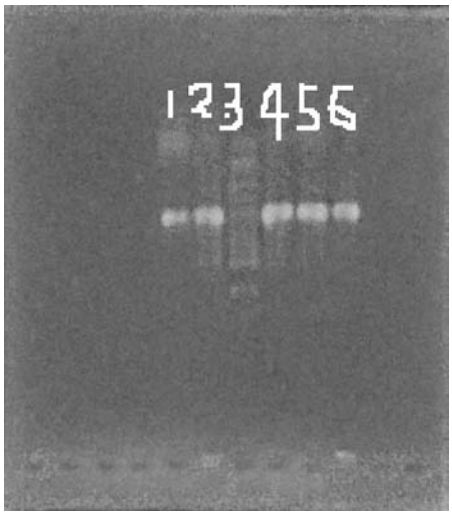

Figure 1. Photograph of gyrA gene amplified from *Salmonella typhi* (lane 1& 2), *Salmonella paratyphi A (lane 4,5&6)* and 100 bp DNA ladder (lane 3)

136 NNNGGGAATCCGACTTACGCATGACGTATTGGGCATGAC
175 TGGAACAAAGCCTATAAAAAATCTGCCCGTGTCGTTGGTG
216 ACGTAATCGGTAAATACCATCCCCACGGCGAT**TCC**GCAGT
256 GTATGACACCATCGTTCGTATGGCGCAGCCATTCTCGCTG
296 CGTTACATGCTGGTGGATGGTCAGGGTAACTTCGGTTCTA
336 TTGACGGCGACTCCGCGGCGGCAATGCGTTATACGGAGAT
376 CCGTCTGGCGAAAATCGCCCACGGACTGATGGCCGATCT
415 CGAAAAAGAGACGGTGGATTTCGTGGATAACTATGACGG
454 TACGGAAAAAATTCCGGACGTCATGCCGACCAAAATTCC
493 GAATCTGCTGGTGAACGGTTCTTCCGGTATCGCAGTAGGT
533 ATGGCGACGAATATCCCGCCGCACAACCTGACGGAAGTC
572 ATCAACGANNN

Fig-2. DNA sequence of quinolone resistance-determining region of *gyrA* gene of nalidixic acid sensitive *Salmonella typhi* with highlighted base sequence coded for amino acid (serine) at position 83 (mutation of which causes nalidixic acid resistance).

136 NNNGGGAATCCGACTTACGCATGACGTATTGGGCATGAC
175 TGGAACAAAGCCTATAAAAAATCTGCCCGTGTCGTTGGTG
216 AATCGGTAAATACCATCCCCACGGCGAT**TTC**GCAGT
256 TGACACCATCGTTCGTATGGCGCAGCCATTCTCGCTG

Fig-3. Partial DNA sequence of quinolone resistance-determining region of *gyrA* gene of nalidixic acid resistant *Salmonella typhi and Salmonella paratyphi A* with highlighted area of mutation at amino acid position 83. Due to point mutation, cytosine (C) is substituted with thymine (T), as a result, TCC, which is coded for serine was replaced by TTC, which is coded for phenylalanine.

Table-2 Detection of mutated *gyrA* gene from nalidixic acid resistant *S. typhi* and *S. paratyphi A* by sequencing of amplified DNA.

DNA	category	Position of mutation	Changes in base sequence	Changes in amino acid
S.typhi	***sample***	Serine-83	TCC to TTC	Serine to phenylalanine
S.paratyphi A	***sample***	Serine-83	TCC to TTC	Serine to phenylalanine
S.typhi/S.paratyphiA	***Control***	Serine-83	TCC-on charge	Serine-no charge

Note: T=Thymine, C=Cytosine, *S. typhi* and *S. paratyphi A* sample = nalidixic acid resistant strains, *S. typhi/S. paratyphi A* control = nalidixic acid sensitive strains.

DISCUSSION

Despite the use of antibiotics and development of newer antimicrobial agents, enteric fever remains major public health problem in developing countries due to resistance to conventional antibiotics (ampicillin, chloramphenicol and co-trimoxazole) and treatment failure with fluoroquinolones.

Increased isolation of nalidixic acid resistant *Salmonella* indicates that fluoroquinolones are no more treatment option for enteric fever.

In this study culture yielded growth of *Salmonella typhi* in 3.41% and *S.paratyphi A* in 5.88% of the clinically suspected enteric fever cases which is similar to other studies in Nepal[12] and India[13].

In consistent with other studies of India [14,15], 72.73% *Salmomella typhi* were resistant to nalidixic acid in the present study. In addition, 18.18% *Salmonella typhi* were resistant to ciprofloxacin which is similar to published data from India[3] and Nepal[16]. In contrast, 34.5% ciprofloxacin resistant strains were reported from New Delhi[17]. About 9.09% *Salmonella typhi* were resistant to ofloxacin in this study which agrees with other reports from India[14,18] but does not correlate with data of Pakistan where 88.2% *Salmonella* were resistant to ofloxacin[19]. The dissimilarity of resistance pattern of *Salmonella typhi* to ciprofloxacin and ofloxacin between our study and other studies might be due to difference in use of antibiotics to treat enteric fever in different countries and different areas of the same country. Moreover, these are orally administered drugs which are prescribed by the physicians and used by the people due to its easy availability for treatment of other infections, which may play role in development of drug resistance against *Salmonella*.

All (100%) *Salmonella paratyphi A* were resistant to nalidixic acid in this study which is similar to other studies in India and Nepal [17,20]. Dissimilarity was noted by Shetty et al (2012) in India who reported 62.5% *Salmonella paratyphi A* were resistant to nalidixic acid. About 15.79% strains were resistant to ciprofloxacin which is similar to studies in India[3,21]. In contrast, 1.56% strains resistant to ciprofloxacin was reported from India[22]. Ofloxacin was ineffective in 21.05% *Salmonella paratyphi* strains in this study which is similar with a study in Pakistan[23] but does not correlate with data of another study in Pakistan[19] where 83.9% S*almonella paratyphi* were resistant to ofloxacin.

From these findings it seems that proportion of ciprofloxacin resistance is less *in vitro*, but as most of the *Salmonella typhi* and 100% *Salmonella paratyphi A* are resistant to nalidixic acid in this study, so ciprofloxacin and other quinolones will not be effective *in vivo*.

In both *Salmonella typhi* and *Salmonella paratyphi A*, *gyrA* gene was mutated on amino acid position 83 and serine was replaced by phenylalanine. This data is similar to data reported from Vietnam[24], some parts of Asia[25] where same mutation at position 83 of *gyrA* gene was observed in 78.9 to 100% *Salmonella typhi* strains but dissimilar with a report from China[26] where 30 % *Salmonella* were found mutated at amino acid position 87 of *gyrA* gene. For *Salmonella paratyphi A*, similar data were reported from different regions where 95 - 100% *Salmonella paratyphi A* were mutated at amino acid position 83[27,28]. In contrast to the present findings it was reported from China that 16% *Salmonella paratyphiA* were mutated at amino acid position 87 of gyrA gene[28]. The amino acids serine at position 83 and aspartic acid at position 87 of gyrase are necessary for sensitivity with nalidixic acid[29]. We, however, did not find any mutation at position 87.

CONCLUSION

Salmonella paratyphi A was predominant over *Salmonella typhi* as a causative agent of enteric fever. Most of the *Salmonella typhi* and 100% *Salmonella paratyphi A* were resistant to nalidixic acid and the resistance was due to mutation at amino acid position 83 of *gyrase protein* by replacing serine with phenylalanine.

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