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Original Article

MOLECULAR ANALYSIS OF *gyrA*MUTATIONS IN *SALMONELLA PARATYPHI A*BY PCR-RFLP AND SEQUENCING METHOD

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ABSTRACT

Objective: Enteric fever is an endemic disease, causing major public health problems with high morbidity and mortality in India and other developing countries. The present study to investigate the prevalence of antimicrobial resistance and detection of mutation

Methods: Detection fluroquinolone resistance and detection of *gyrA*genemutation by PCR-RFLP and confirmation of mutation by the gene sequencing method.

Results: 41 clinical isolates of *Salmonella paratyphi A* were collected from tertiary care hospitals andwere included in this study. Antimicrobial susceptibility testing was performed by disc diffusion method and MIC estimated by agar dilution method. Detection of ser83 mutation in*the gyrA gene* by PCR-RFLP method and mutations were confirmed by sequencing. Out of 41 clinical isolates of *S.paratyphiA, 92.5*% were resistance to nalidixic acid, 12.5% to ciprofloxacin, 10 % to cefotaxime and 4.5% to ceftriaxone. ACCo-T group of drugs were showed better sensitivity to S. *paratyphi A.* 100 % of isolates showed *gyrA* mutation by PCR-RFLP, which was further confirmed by sequencing.

Conclusion: Different geographical enteric isolates were own specific mutation on gyrase gene.New fluoroquinolones types are needed with enhanced ability to counter resistance conferred by gyrase gene mutation

Keywords: *Salmonella paratyphi* A, PCR-RFLP, *gyrA,* Antimicrobial susceptibility.

INTRODUCTION

Enteric fever continues to remain a major public health problem in many developing countries and S.paratyphiA [1], being the main agent for increasing the infection. Fluoroquinolones achieve high concern in the bile,bowel, urinarytract and macrophages, it is coupled with ease of oral administration has made the quinolone the drug of choice for enteric fever treatment.

Patient with enteric fever have responded to ciprofloxacin and ofloxacin with more than 90% cure rate,Kadhiravanet al2005 reported treatment failure with strains resistant to nalidixic acid and having a reduced susceptibility to ciprofloxacin (0.125-1mg/ml) [2]. High-level ciprofloxacin resistance in *S. paratyphi A* reported by Adachi et al2005 [3].

Reduced susceptibility to fluoroquinolones results in a poor treatment response among Salmonellosis patient and prolonged bacterial shedding [4]. The rise in resistance to fluoroquinolones is observed commonly in the regions where fluoroquinolones are used empirically to treat enteric fever. Quinolone and fluoroquinoloneresistance commonly arise via mutations in the genes encoding DNA gyrase (*gyr A* and *gyr B*) and DNA topoisomerase IV (*par C* and *par E*) [5].

A number of resistance mechanisms to quinolones have beenidentified, including point mutations that result in amino acidsubstitutions in the topoisomerases, reduced outer membrane permeability, increased efflux of antibiotics and other harmfulcompounds, and the plasmid-encoded quinolone-resistance (*qnr*) genes [6,7,8]. Point mutations in the topoisomerase genes are generallyrestricted to certain cordons within the 'quinolone resistancedetermining region' (QRDR) [9]. In Salmonella, some of the morecommon point mutations found to be associated with resistance toquinolones occur in the *gyrA* gene resulting in substitutions at theSer-83 position, often to Tyr, Phe or Ala, and Asp-87 substitutionsto Asn, Gly or Tyr. The most common amino acid substitutionreported in ParC is Thr-57Ser, with Thr-66, Ile or Ser-80 Argbeing observed as occasional second substitutions [10].*SalmonellaTyphi* and *Salmonella Paratyphi A* strains showing resistance or reduced susceptibility to fluoroquinolones have been reported, andthese almost invariably have point mutations in *gyrA* [3,11,12]. The present study revealed the fluoroquinolones and quinolone resistance at aroundChennai, among salmonella species due to point mutation in gyrasegene.

MATERIALS AND METHODS

A total of 41 clinical isolates of *Salmonella paratyphiA* were collected during October 2007 to December 2009from tertiary care Hospital and other clinical laboratories in Chennai. All the strains were isolated and identified by biochemically using standard procedures and confirmed by slide agglutination test using specific antisera procured from King Institute of Preventive Medicine Chennai, India.All the isolates were stored in brain heart infusion broth with 15% glycerol at -80ºC until further use.

Antimicrobial susceptibility testing of the Salmonella isolates to various routinely used antibiotics was determined by disc diffusion technique on Muller Hinton Agar using commercially available discs following Clinical Laboratory Standard Institute (CLSI, 2011) guidelines [22].

The panels of antimicrobial agents included were ciprofloxacin (5µg), norfloxacin (10µg), nalidixic acid (30µg), ampicillin (10µg), chloramphenicol (30µg), co-trimoxazole (1.25/23.75µg), tetracycline (30µg), cefotaxime (30µg), ceftriaxone (30µg) and gentamicin (10µg). MIC against ampicillin, nalidixic acid, ciprofloxacin, cefotaxime and ceftriaxone was determined by agar dilution method following CLSI guidelines 2011.

Preparation of Template DNA

A single bacterial colony from the overnight grown culture were suspended in 100µl of 50mM NaOH and kept it in a water bath at

97ºC for 3mins and then kept into refrigerator for 5mins.After refrigeration added 16µl of 1MTris-HCl and centrifuged at 8000rpm for 2mins.The supernatant containing bacterial DNA was separated and stored at-20˚C [13].

Amplification of *gyrA*and*parC*genes

Template DNA isolated from bacterial strains as described above was amplified by PCR using gene specific primers for*gyrA* Forward 5'-ATGAGCGACCTTGCGAGAGAAATTACACCG-3'and Reverse 5'-TTCCATCAGCCCTTCAATGCTGATGATGTCTTC-3' procured from Sigma-Oligos, India. The PCR was performed using GeneAmpgold9700 (ABI USA) in a total reaction volume of 50µl containing 5µl of 10x PCR bufferwith 25mM MgCl2,5µl of 2.5mM dNTP mix,2µl of 2µm primer stocks each, 0.25µlof 5U/µl *Taq* DNA polymerase (NEB, USA) and 2.5 µl of template DNA.

The thermal cycling protocol for amplification is programmed in GeneAmpgold9700 (ABI, USA) as follows; initial denaturation at 95ºC for 2min followed by 35 cycles of *i)* Denaturation at 94ºC for 45sec; *ii)* primer annealing at 60ºC for 45sec&*iii)* primer extension at 72ºC for 30sec followed by a final extension at 72ºC for 7min. The PCR product was electrophoresed in 1% (w/v) agarose gel (Sigma - USA) prepared in 0.5xTris-Acetate EDTA (TAE) buffer at 100V for 15min using Mupid-Ex (Takara, Japan) and visualized by ethidium bromide staining using Gel documentation system (Bio Rad -USA).

Restriction digestion using *Hin*fI

The PCR amplified 630bp fragment of *gyr A* gene has three *Hin*fI restriction sites one of which lies at Ser83. Restriction digestion was performed using the 20µlof PCR product and 10U of *Hin*fI (NEB, USA) at 37 ºC for 16hrs.

After complete digestion the tubes were short spun. Two percent agarosegel (w/v) with ethidium bromide (50µg/ml) was made in 0.5xTAE buffer, 20µl of the *Hinf*Idigested PCR product was mixed with 2µl of 6x gel loading dye and resolved at 100V for 25mins using 0.5xTAE as tank buffer in Mupid-Ex (Takara, Japan) 5µl of the 100bp DNA ladder (Fermentas, USA) was resolved along with the digest to refer the size of the digested fragments. The gel was documented using gel documentation system (Bio Rad-USA) and based on the restriction pattern the mutation status of each bacterial strain has been determined.

DNA sequencing for *gyr A*

The *gyr A* gene was amplified by PCR and sequencing was performed by outsourcing the samples to MacrogenInc, Korea for selective*S. paratyphiA*strains (5 out of 41) representing the Ser83 mutation. These were selected on the basis of ciprofloxacin and nalidixic acid MIC so that the entire MIC level of ciprofloxacin0. 0625µg/µl to 0.5 µg/µl and nalidixic acid 16 µg/µlto 256 µg/µl.

RESULTS

41 *Salmonella paratyphiAwas collected* in the present study. One isolate from stool and others from blood isolates respectively. The antimicrobial susceptibility pattern of *Salmonella paratyphi A* against various antimicrobials such as chloramphenicol 95%, co-trimoxazole 95%, ciprofloxacin 87.5%, ceftriaxone 95.5% and tetracycline 92.5%.

The ACCo-T groups of drugs were showed better sensitivity to *S. paratyphi A* probably due to increased use of quinolone and cephalosporins. 92.5% of nalidixic acid resistance to *S. paratyphi A*wasobserved.

MIC studies of *S. paratyphiA*, ampicillin show 95% (39/41) of sensitivity by MIC studies. Only 5% of the isolates were (2/41) found to be resistant by MIC method. Thirty eight out of forty one (38/41) isolates of *S. paratyphi A* were resistant to nalidixic acid by MIC with a range of 256µg/µl for 35 isolates and 128 µg/µl for 3 isolates, 3 strains showed sensitivity according to CLSI guidelines with a MIC of 16µg/µl.

The MIC range of ciprofloxacin for *S. paratyphiA*is as follows; 25 isolates showed 0.5 µg/µl, 14 isolates showed 0.25 µg/µl and one isolate showed 0.125 µg/µl and all are sensitive as per CLSI guidelines. Only one isolate was (0.0625µg/µl) sensitive by both MIC and disc diffusion method.

Thirty nine out of forty one isolates of *S. paratyphi A* were sensitive to ceftriaxone by disc diffusion and MIC with a (range of 0.5 µg/µl to 0.0625 µg/µl). The one intermediately resistant and another fully resistant isolate by disc diffusion were turned out to be sensitive by MIC methods, all the isolates of *S. paratyphi A* were found to be sensitive to cefotaxime with a MIC range of 0.5-0.0625 µg/µl; though few isolates showed resistance by disc diffusion.

In general the present study showed that *S. paratyphi A* isolate exhibited better sensitivity to the routinely used antibiotics viz, ampicillin, chloramphenicol, co-trimoxazole and tetracycline (ACCo-T) by disc diffusion except nalidixicacid, which showed only 7.5% of sensitivity by disc diffusion 4.5% of *S. paratyphi A* isolates were MDR in our study.

PCR –RFLP and nucleotide sequence analysis

A 630bp fragment containing the QRDR of the *gyrA* gene of the 41 clinical isolates were amplified by PCR and sequenced.The 630bp PCR based fragment of *gyr A* gene has three *Hin*fIrestriction sites, one of which lies at Ser83. The codon for Serine (TTC) is mutated to phenylalanine (TCC) there by leading to the loss of restriction site for *Hin*fI. Restriction analysis of the PCR product revealed that 100% (41 isolates) of *S.paratyphi A* were showing Ser 83 mutation in *the gyrA gene* (Figure 1).

The enzyme*Hin*fI cuts the 630bp of *gyrA* gene product at three sites, therefore the*gyr A* gene product from NAR strains showed *a HinfI restriction pattern* consisting of three fragments of sizes 343bp, 144bp and 138bp (Figure 2).

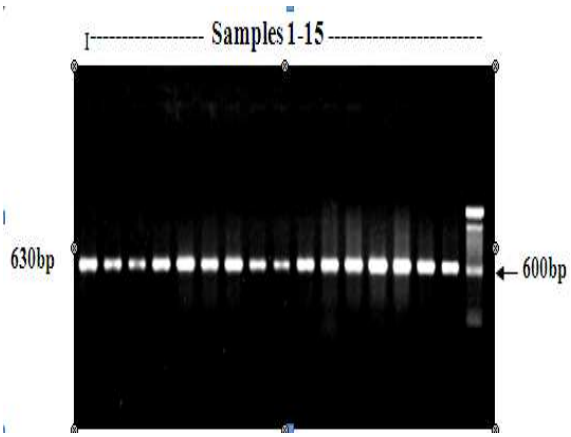

Fig. 1: Ethidium bromide stained UV document of Agarose gel representing the *gyrA* PCR product (630bp) in *S.paratyphiA*strains

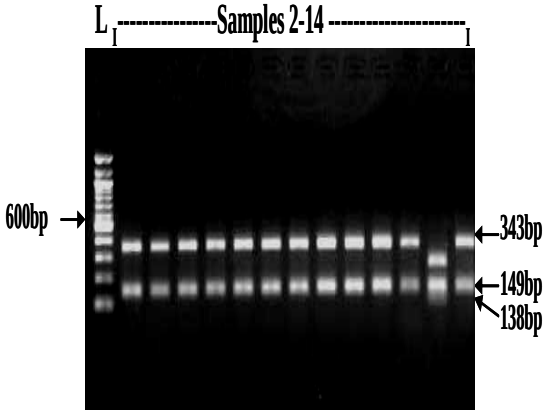

Fig. 2: A representative RFLP electrophorogram showing the *gyr A* PCR product digested with *HinfIc*

DISCUSSION

Enteric fever is a major public health problem in our country. Proper sanitation, public health education and vaccination are continuing preventive measures that would improve this situation. The emergence of antibiotic resistant strains of bacteria is closely linked to the irrational use of antibiotics in treating human infections, especially ciprofloxacin [14].

Strains that are already resistant to nalidixicacid may require fewer exposures to fluoroquinolones to develop high-level resistance to ciprofloxacin, than the strains that are fully ciprofloxacinsusceptible [15].

Single mutations in the *gyrA*gene have been found to be sufficient for high-level nalidixic acid resistance in *Salmonella* [16]. Increased efflux of the antimicrobial has also been reported to be a common mechanism and generally represents the first step in the acquisition of fluoroquinoloneresistance [17]. AcrAB/TolC is the main efflux pump involved in determining the intrinsic level of resistance in Enterobacteriaceae, according to basal levels of expression, and confers quinolone resistance when over expressed [18].

The most important mechanisms producing nalidixic acid resistance are point mutations in the *gyrA*gene and increased efflux contribution. In this study, all of the isolates resistant to nalidixic acid had a single mutation in *gyrA*. However, efflux pump activity could be less important in decreasing ciprofloxacin susceptibility levels, atleast the low level of resistance observed in these isolates. It seems likely that over expression of the AcrABTolC efflux pump is responsible for this effect, due to the high prevalence of the efflux pump among clinical isolates and in vitro mutants of *Salmonella* [12, 19, and 20]. Although a concomitant over expression of another efflux pump cannot be ruled out.

According to the results of present study necessities the nalidixicacid susceptibility testing combined with PCR-*Hinf*Idigestion, provide useful and simple methods to identify Salmonella strains that can result in an infection not responding to ciprofloxacin therapy.Present study results showed that the majority of patients with severe disease and documented ciprofloxacin failure were infected with NAR strains, has also been observed by other workers [21].

Investigation of isolates by the ser83 mutation in *gyr A* by PCR-RFLP and sequencing can promptlyindicate that,different geographical enteric isolates were own specific mutation on gyrase gene. Detailed study will be needed for gene variations screening on fluoroquinolones resistant strains.Empiric administration of fluoroquinolones also seems to play a role in the expansion of resistance,appropriate therapy, mortality and morbidity of the disease can be reduced.This aspect of the disease wants further study as it has far reaching implications,it would be possible to design newer antibiotics to target these mechanisms as well. The development of such drugs would play a major role to alleviate the suffering caused by this disease. It would also be possible to design fluoroquinolones with an enhanced ability to counter resistance conferred by gyrase gene mutation.

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