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Detection of *gyrA* ser83 mutation and *parC* gene amplification in *E.coli* and *Klebsiella pneumoniae* from tertiary care hospital, Puducherry

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Abstract

Introduction: Fluoroquinolones are the broad spectrum antibiotics and recently the clinical isolates of *Enterobacteriaceae* species had slowly extend the high resistance towards them. The point mutation in *gyrA* gene had showed decreased susceptibility to ciprofloxacin and resistant to nalidixic acid. The aim of the present study was to identify *gyrA* and *parC* genes and *gyrA ser83* mutation among clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* by RFLP.

Methods: A total of 100 clinical strains of both *Escherichia coli* and *Klebsiella pneumoniae* were collected from tertiary care hospital from Pondicherry. All the strains were subjected to antimicrobial susceptibility pattern testing by disc diffusion method as per the Clinical Laboratory Standard Institute (CLSI2012). PCR based screening of *gyrA* and *parC* was carried out and additional analysis of *gyrA* ser83-phe mutation in *E.coli* and *K.pneumoniae* by agarose gel electrophoresis.

Results: Variation in both ciprofloxacin and nalidixic acid resistance were showed in the (100) clinical isolates of *E.coli* and *K. pneumoniae* were reported earlier. *GyrA* and p*arC* genes were detected in both susceptible and resistant isolates of *E.coli* and *K.pneumoniae*. Among them, *E.coli showed* 100% (50/50) and *K.pneumoniae* showed 84% (42/50) positivity for *gyrA* while 78% (39/50) and 64% (32/50) positivity for *parC.* PCR-RFLP results indicated gyrA ser83 mutation in 84% (42/50) of *E.coli* and 66% (33/50). The results also shows that *E.coli* is less susceptible to fluoroquinolones compared to *K.pneumoniae.*

Conclusion: The overall resistance were increased for the ciprofloxacin and nalidixic acid among clinical isolates of *E.coli* and *K. pneumoniae due* to *gyrA* ser83-phe mutation. The antimicrobial resistance to fluoroquinolone drugs indicate the rapid genetic modifications undertaken by *Enterobacteriaceae* species and need for routine screening for resistance to develop next generation drugs.

Keywords: E.coli, Klebsiella species, Ciprofloxacin, gyrA, parC and PCR-RFLP.

Introduction

Among the *Enterobacteriaceae species*, the prevalence of resistance to fluoroquinolone is becoming a major problem in India. Fluoroquinolones and third and fourth generation cephalosporins are the most frequently prescribed antimicrobial agents for both gram negative and positive bacterial infections in all over the world.(1) However, antimicrobial resistance to drugs at present is increasing at an alarming rate, and is a global health concern, posing distinctive challenges to clinical microbiologists, clinicians, infection control professionals.

Fluoroquinolones resistance has increased in number in almost all gram negative bacteria, particularly in *E.coli*, *Klebsiella species*, *Salmonella species* etc.(2,3) In recent years ESBL producing *E.coli* and *Klebsiella pneumoniae* have been increasing owing to infection and providing treatment to become a serious issue and also due to multi drug resistance (MDR). Eventhough fluoroquinolones are the effective drug for extended spectrum betalactamases producing *E.coli* and *Klebsiella pneumoniae* and also to treat the nosocomial, urinary tract infection, enteric fever and other bacterial infections.

Moreover, many studies reported that the ESBL-EK strains became susceptible to the fluoroquinolones drugs, also aspect of treatment these agents are very effective to them.(4) Although a current studies have established that 40–45% of such isolates are resistant to fluoroquinolones.(5,6)

An exposure and risk factors for fluoroquinolone resistant among ESBL-EK infection is most important and looks at the reason for the emergence of resistance. Fluoroquinolones are the most important drug which can have main role in treating ESBL-EK infection. In addition, consequently assist to reduce the dependence on other drugs like carbapenem and it can be limit the emergency of resistance in carbapenem drugs.

ESBL-producing strains have been shown to be significantly more frequent among ciprofloxacin-resistant *E. coli* than among ciprofloxacin-susceptible *E. coli* strains.(7) Moreover, prior use of fluoroquinolones, an indwelling urinary catheter, and an invasive procedure within 72 hr prior to bacteraemia have been identified as independent risk factors for ciprofloxacin resistance in bloodstream infections due to ESBL *E. coli* and *Klebsiella* spp.(8-10)

Fluroquinolones exert an antibacterial effect through inhibiting DNA synthesis by interacting with

DNA gyrase and topoisomerase IV. DNA gyrase which encoded by the genes such as *gyrA*, *gyrB* and topoisomerase IV encoded by *parC* and *parE*. One of the most important mechanisms of quinolone and fluoroquinolone resistance is to the substitutions of amino acid in the DNA gyrase and topoisomerase IV[11,12] or to active efflux of the agent via antibiotic efflux pumps.[13]

Production of β-lactamase is the most common mechanism responsible for resistance to β-lactam antibiotics among the members of the *Enterobacteriaceae* family, which in turn is related to a single base mutation in gyrA, subunit of DNA gyrase that leads to aminoacid and alteration of codons in the quinolone resistance determining regions (QRDR). Fluroquinolones contain C-8 methoxy group and exhibit stronger antibacterial activity against bacteria that are resistant to quinolone due to gyrA mutation.[14]

The main objective of the present study is to find out the presence of *gyrA ser83* mutation and *parC* gene amplification in clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae*.

Materials and Methods

Collection of clinical isolates of *E.coli* and *Klebsiella Pneumoniae*:

A total of 100 clinical isolates of *E.coli* and *Klebsiella Pneumoniae* were collected during 2012-2013 from SMV Medical College and Hospital, Pondicherry. Isolation and identification was carried out by standard microbiological techniques from 186 faecal specimens. Antibiotic susceptibility testing was carried out by disc diffusion method followed by Clinical Laboratory Standard Institute (CLSI 2012). The strains were stored in 0.5% of semisolid brain heart infusion medium along with 15% of glycerol at -20°C.

Extraction of genomic DNA: A single bacterial colony from overnight culture was suspended in 100µL of 50mM NaoH and incubated at 97°C for 3 minutes and immediately transferred to 4°C. After 5-10 minutes, 16µl of 1M of Tris-HCL was added and centrifuged at 8000rpm for 2 minutes. The supernatant containing bacterial genomic DNA was transferred to sterile tubes and stored at-20°C.[15]

Amplification of *gyrA* and *parC* genes by PCR: PCR Amplification of *gyrA* and *parC* genes: Template DNA prepared from bacterial strains as described above was amplified by PCR. The following primers were modified from,[16] GyrA F: 5' ATG AGC GAC CTT GCG AGA GAA ATT ACA CCG 3' and GyrA R: 5' TTC CAT CAG CCC TTC AAT GCT GAT GAT GTC TTC 3' amplified gyrA gene fragment of length 630bp. The *parC* gene sequence was retrieved from NCBI database and sequence specific primers, ParC F: 5'ATG AGC GAT ATG GCA GAG CGC CTT GCG CTA 3' and ParC R: 5' ACG CGC CGG TAA CAT TTT CGG TTC CTG CAT 3' were designed using Primer 3 online tool to amplify a 480bp gene fragment of *parC*. The primers were procured from Sigma Oligos, India. PCR was performed using Gene Amp 9700 thermal cycler (ABI USA) in final reaction volume of 50 µL containing 5 µL of 10x PCR buffer with MgCl2 {NEB, USA}, 5 µL of 10mM dNTP Mix (Takara, Japan), 2 µL of 2µM forward and reverse primers, 0.25 µL of Taq DNA polymerase (5U/µL) {NEB, USA}, 2.5 µL of template DNA and 35.25 µL of sterile water. The thermal conditions for the amplification of both *gyrA* and *parC* gene comprised one cycle of 2 min at 95°C, followed by 35 cycles of 45 sec at 94°C, 45 sec at 60°C and 30 sec at 72°C, with a final extension of 7 min at 72°C. The 10 µL of PCR product was resolved in 1% (w/v) agarose gel (Sigma, India) prepared in 0.5X Tris Acetic acid EDTA (TAE) buffer and detected by ethidium bromide staining after electrophoresis (BioRad, USA). The 1000bp DNA ladder (Gibco BRL, USA) was used to refer the size of the PCR product in the gel. The gel was documented using gel documentation system (BioRad, USA).

Restriction digestion gyrA by *HinfI* enzyme: The amplified 630bp fragment of the gyrA gene has three *HinfI* restriction sites one of which lies at codon Ser83. The PCR product was digested for the detection of gyrA Ser83 mutation by *HinfI* enzyme. A volume of 20 µL of precipitated PCR product was digested with 10 units of *HinfI* (New England Biolab-USA) at 37ºC for 16hrs. The tubes were short spun after the complete digestion. Two percent agarose gel with ethidium bromide (50µg/ml) was prepared using 0.5xTAE buffer and 10 µL of the *HinfI* digested PCR product was mixed with 2 µL of 6x gel loading dye and resolved at 100V for 25 minutes using 0.5xTAE as a tank buffer; 5 µL of 1000bp DNA ladder (Gibco Brl-USA) was used to refer the size of the digested fragments and documented using a gel documentation system (Bio Rad USA).

Result and Discussion

Analysis of ser83 mutation in *gyrA* and *parC* gene amplification: The *gyrA*(630bp)PCR product was detected in all *E.coli* isolates showing 100% (50/50) positivity and 84% (42/50) positivity in *K.pneumoniae* strains(Fig.1). While *parC* (480bp) were identified only in 78% (39/50) of E.coli and 64% (32/50) in *K.pneumoniae* strains (Fig-2). The gyrA (630bp) gene contains three *HinfI* restriction sites, one of which present at the codon 83 serine (TCC), when mutated to phenylalanine (TTC) leads to loss of the restriction site. The gyrA (630bp) PCR product with codon83 mutation due to loss of one restriction site, *HinfI* digestion results in three fragment of length 343bp, 149bp and 138bp involved in nalidixic acid resistant strains (Fig-3). Analysis of gyrA product digested with *HinfI* enzyme

which showed that 84% (42/50) of *E.coli* and 66% (33/50) of *K.pneumoniae*.

Resistance rate of ESBL producing as well as fluoroquinolone resistant isolates of *E.coli* and *K.pneumoniae* are increasing in hospital and community settings. Moreover, resistance to imipenem and meropenem have been reported, which would further complicate the treatment regimen, as these are the latest generation of broad spectrum antibiotics used against drug-resistant pathogenic bacteria, as well as in the empiric therapy of critically ill patients. Resistance percentages of 32 to 89% have confirmed by the MIC values against five fluoroquinolone antibiotics.

Enterobacteriaceae family of organisms were more resistance to quinolone and fluoroquinolone drugs and patients with intra abdominal infections, acquiring resistance with nosocomial infections in order to cover the entire spectrum of potential pathogens. In Asian countries, an alarming resistance of >60% to ampicillin-sulbactam or a quinolone and ESBL-producing strain is observed.[17-19] ESBL producing E.coli and K.pneumonia were highly reported with different rate of prevalence ranging from New Zeland (4.4%), 77.4% in India and vastly varied in Asian Pacific regions. Whereas the ESBL production was detected in USA in different frequency from 4.7% and 17.5% of *E. coli* and *K. pneumoniae* isolates, respectively. The other studies they have reported that 17% and 27% of the strains of E.coli and Klebsiella pneumoniae were producing ESBL respectively, were susceptible to ciprofloxacin.[17] We have earlier reported, 48% nalidixic acid and 32% ciprofloxacin resistance in *E.coli* and *Klebsiella pneumoniae* that is concordant with 32 to 89% of resistance documented by other findings.[20]

Moreover, the decreased susceptibility to ciprofloxacin and resistance to nalidixic acid clinical isolates of *E.coli* and *Klebsiella pneumoniae* were fall on to gyrA serine 83 mutation and which is most frequently altered amino acid and phenyl alanine is the most common substitution.[12]

It is hereby confirm that the single aminoacid substitution at position 83 confers increased resistance than at position 87, however the further mutations in *gyrA* or other genes are essential for the development of high-level fluoroquinolone resistance.[21]

Conclusion

Among the Enterobacteriaceae, both *E.coli* and *Klebsiella species* had enormous infection dynamics and resistance to fluoroquinolones. Most predominantly both *E.coli* and *Klebsiella sps* strains were resistant to a greater number of antibiotics, and the percentage of resistance values for each antibiotic were higher with the isolated strains. The present study confirms the emerging trend of fluroquinolone resistance due to single point mutation in *gyrA* gene. We also observed *E.coli* is comparatively less susceptible to fluroquinolone. Further studies are required to alter the mutated gene to its wild type and to study the interrelationships between compensatory mutations, fluroquinolone resistance and DNA supercoiling.

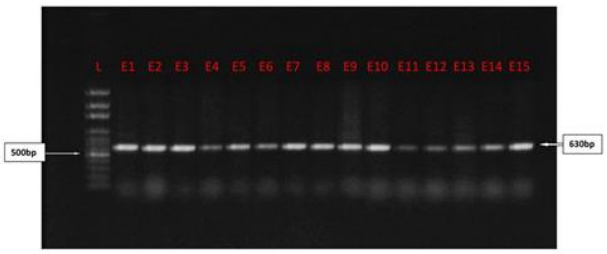

Fig. 1: Agarose showing the gyrA PCR product (630bp)

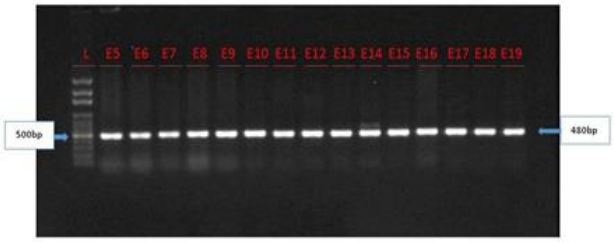

Fig. 2: Agarose gel showing the *parC* PCR product (480bp)

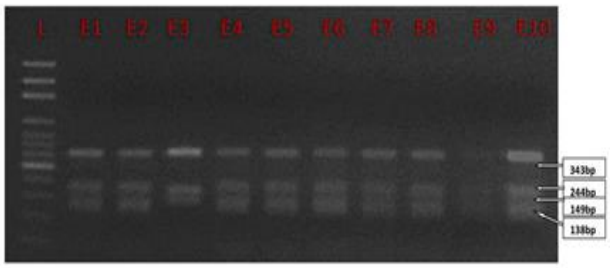

Fig. 3: RFLP electrophoretic gel showing *gyrA* PCR product digested with HinfI

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