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A study on prevalence of plasmid mediated quinolone resistant genes in Klebsiella pneumoniae

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Introduction: Fluoroquinolone resistance is increasingly been reported in *Enterobacteriaceae*. Resistance to quinolones among *Enterobacteriaceae* is mediated by point mutations in chromosomal genes that encode the subunits of DNA-gyrase and topoisomerase IV. **Objectives:** The present study was done to identify quinolone resistant *Klebsiella pneumoniae* isolates from clinical samples and to detect the prevalence of plasmid mediated quinolone resistance genes. **Materials & Methods**: A total of 63 isolates from various clinical samples were identified by standard biochemical methods. Antimicrobial resistance pattern of these isolates were performed by disk diffusion method as per guidelines of CLSI. **Results:** By disk diffusion, 90.99% isolates were found to be resistant to Ciprofloxacin. The sensitivity of isolates to Imipenem was found to be 99.10%. About 50.74% isolates were resistant to Aminoglycosides and 61.19% were resistant to third generation Cephalosporins. By E test, 80.95% isolates were resistant for Ciprofloxacin with MIC of >1µg/ml. By PCR, out of 63 isolates, 6 (9.52%) showed aac (6') – Ib - cr gene, 7 (11.11%) were positive for qnr A gene, 29 (46.03%) for both qnr B and aac (6') - Ib-cr gene and 8 (12.70%) for qnr B gene. **Conclusion:** The transferrable nature of Plasmid mediated fluoroquinolone resistant genes favours the dissemination of the resistant genes between different bacterial genera and limit therapeutic options available. The prevalence of plasmid mediated quinolone resistance mediated by qnr A, qnrB and aac (6') - Ib-cr gene results in multidrug resistance among *Klebsiella pneumoniae*.

Keywords: Fluoroquinolone resistant genes, Klebsiella pneumoniae, E test, High level quinolone resistance

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Introduction

Quinolones are synthetic antibiotics with broad spectrum activity against clinically important pathogens responsible for urinary tract infection, gastrointestinal infection, respiratory tract infection, sexually transmitted diseases and skin diseases [1,2].

Fluoroquinolones show excellent activity against *Enterobacteriaceae,* fastidious Gram negative bacteria and *Pseudomonas aeruginosa,* moderate activity against *Staphylococci*, *Mycobacteria*, *Chlamydia*, *Mycoplasma* and *Ureaplasma*, less activity against *Streptococci* and anaerobic bacteria [3, 4, 5]. Ciprofloxacin resistance has been observed in *Enterobacteriaceae* and is most often

Seen in strains of *Escherichia coli*, *Enterobacter* and *Klebsiella spp.* Plasmid mediated quinolone resistance was first reported among clinical isolates of *Klebsiella pneumoniae* in 1998. In *E. coli* and *Klebsiella pneumoniae*, qnr like genes are identified in conjugative plasmids that varied in size from 54 to > 180 kb [6].

These genes are located in sul1 type integrons that may possess genes which may acts as recombinase for mobilization of antibiotic resistance genes located nearby (bla CTX-M, amp C). Genes encoding for plasmid mediated cephalosporinase (FOX-5), Clavulanic acid inhibited Extended spectrum class A β lactamases, SHV-7 and CTX M-9 and narrow spectrum Pencillinase PSE I are associated with qnr positive plasmids [7, 8, 9].

Resistance to quinolones among *Enterobacteriaceae* is mediated by point mutations in the quinolone resistance determining regions (QRDR) of the DNA gyrase and topoisomerase IV genes leading to a target modification [8].

Other mechanisms such as efflux pump, target protection encoded by qnr genes and enzymatic modifications encoded by aac (6') Ib-cr have been found to contribute to quinolone resistance.

Aim and Objectives

01. To isolate and identify Klebsiella pneumoniae from clinical samples.
02. To determine the antibiotic susceptibility pattern of Klebsiella pneumoniae.
03. To detect Quinolone resistance in Klebsiella pneumoniae.
01. To determine molecular characterization of quinolone Resistant Klebsiella pneumoniae.

Materials and Methods

Study population: Patients attending Vinayaka Mission Kirupananda Variyar Medical College and Hospitals, Salem

Study design: Cross-sectional study

Inclusion criteria: Patients in all age group

Exclusion criteria

01. Patients on antibiotic therapy
02. Immunocompromised patients

Methodology: The present study was done from October 2018 to January 2019 in Department of Microbiology,Vinayaka Mission's Kirupananda Variyar Medical College and Hospitals, Salem.

During this period, a total of 63 isolates of *Klebsiella* were collected for the study. The samples were cultured on Blood agar and Mac Conkey agar.

Identification was made based on colony morphology, microscopy, motility and biochemical reactions [10].

Antibiotic susceptibility test was done on Muller Hinton agar by Kirby Bauer's disc diffusion method. The antibiotic panel used were: Amikacin (30 µg/ml), Amoxycillin / clavulanic acid (10/20 µg/ml), Co-trimoxazole (25 µg/ml), Ampicillin (10 µg/ml), Cefotaxime (30 µg/ml), Ceftazidime (30 µg/ml), Cefepime (30 µg/ml), Gentamicin (30 µg/ml), Nitrofurantoin (100 µg/ml), Ciprofloxacin (5µg/ml) and Imipenem (10 µg/ml). *Escherichia coli* ATCC 25922 was used as control organism [11].

Disc diffusion method: Morphologically similar colonies were inoculated into peptone water and incubated at 37^{0}C for 2 hours. 0.5 McFarland standard was used to check the density of the suspension and Muller Hinton agar was inoculated by lawn culture method.

After placing antibiotic disks, the plates were incubated at 37^{0}C for 16-18 hours. The diameter of the zone size was measured with a calibrated scale and results were interpreted in accordance with CLSI guidelines [11].

Epsilometer test (E-test): The Minimum Inhibitory Concentration for Ciprofloxacin was determined by E strip containing concentration gradient of 0.002-32 µg/ml.

Colonies of *Klebsiella pneumoniae* were inoculated into peptone water and the density of the suspension was compared to 0.5 McFarland standards.

Using an applicator stick the E strips were placed on lawn culture on Muller Hinton agar and incubated at 37^{0}C for 18-24 hour.

The point where the eclipse zone of inhibition intersects with the strip is taken as MIC value. MIC value ≥ 1 µg/ml was considered as resistant to Ciprofloxacin.

Polymerase Chain Reaction: Approximately, 100ng/µl of total DNA was taken for conventional PCR. 25µL reaction mixture was taken for PCR amplification.

It consist of forward and reverse primers (≈25ng, 1µl each), PCR reaction buffer (10X. 2.5µl), dNTPs (10Mm 0.5µl), Taq DNA polymerase (1U/µl. 1.0µl), Template DNA (≈100ng/µl) and nuclease free water (18.0µl).

The PCR profile was as follows: Initial denaturation at 95^{0}C for 5 minutes followed by 35 cycles of denaturation at 94^{0}C for 1 minute, annealing at 68^{0}C for 55 seconds, extension at 72^{0}C for 1 minute and final extension at 72^{0}C for 5 minutes.

The amplified products were mixed with 6X loading dye along with buffer and electrophoresed through 1.5% of agarose gel (containing 0.5µl/ml ethidium bromide) in TBE buffer at 100V for 1 hour. The gel was visualized under UV transillumination.

Results

Out of 63 isolates studied, 18 were from sputum, 15 from pus sample, 24 from urine and 6 were from blood (**Figure 1).**

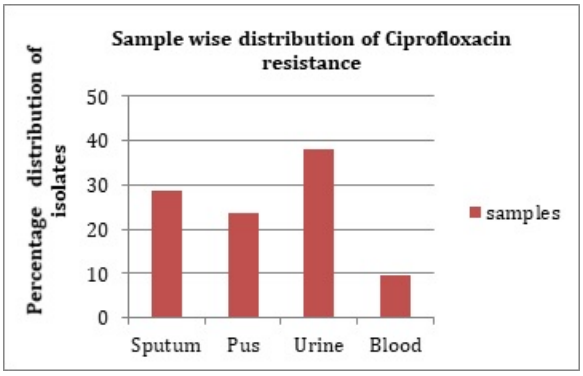

Figure-1: Distribution of Ciprofloxacin resistant isolates from clinical sample

Male patients (68%) were predominant in the present study when compared to female patients (32%) **(Figure 2).**

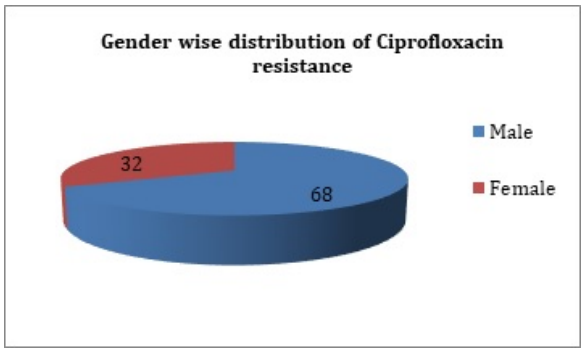

Figure-2: Gender wise distribution of Ciprofloxacin resistance

Out of 63 samples, 43 (60%) were from Outpatients and 20 (40%) were from inpatients (Figure 3).

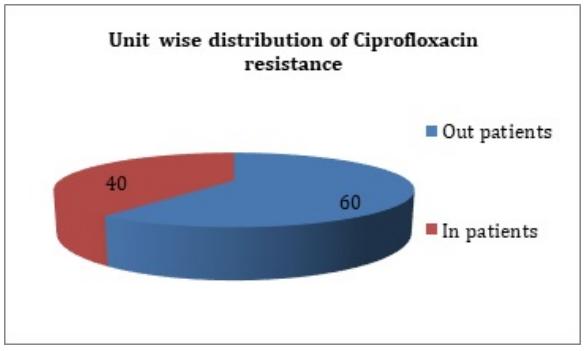

Figure-3: Unit wise distribution of Ciprofloxacin resistance

By disk diffusion, 90.99% isolates were resistant to Ciprofloxacin. 99.10% isolates showed sensitivity to Imipenem. About 50.74% isolates were resistant to Aminoglycosides and 61.19% were resistant to third generation Cephalosporins **(Table: 1).**

Table-1: Antibiotic susceptibility pattern of isolates.

Antibiotics	Sensitive	Resistant
Amoxyclav	27.12%	72.88%
Gentamicin	41.73%	58.27%
Ciprofloxacin	09.01%	90.99%
Co-trimoxazole	17.92%	82.08%
Amikacin	56.19%	43.81%
Cefotaxime	40.35%	59.65%
Ceftazidime	37.28.%	62.72%
Cefipime	15.63%	84.37%
Amoxicillin	14.81%	85.19%
Imipenem	99.10%	00.90%

Nitrofurantoin	12.18%	87.82%

By E test, 12 isolates (19.05%) were sensitive with MIC of 1µg/ml, 51 isolates (80.95%) were resistant for Ciprofloxacin with MIC of >1µg/ml **(Table 2).**

Table-2: Minimum Inhibitory Concentration for Ciprofloxacin resistance.

Ciprofloxacin MIC	Number of isolates
1µg/ml	12 (19.05%)
4 µg/ml	11 (17.46%)
8 µg/ml	15 (23.81%)
16 µg/ml	16 (25.39%)
>32 µg /ml	09 (14.29%)

By PCR, 6/63 (9.52%) isolates showed aac (6') - Ib-cr gene, 7/63 (11.11%) of the isolates were positive for qnr A gene, 29/63 (46.03 %) for both qnr B and aac (6') - Ib-cr gene and 8/63 (12.70%) for qnr B gene **(Fig: 4).**

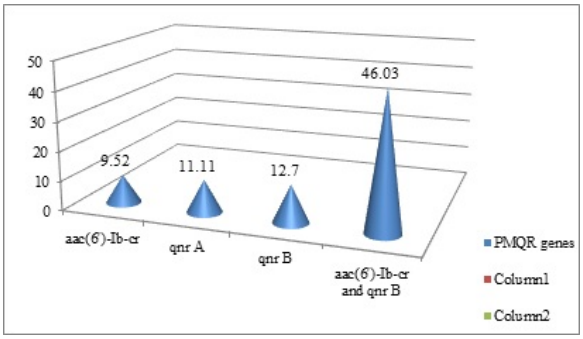

Figure-4: Distribution of quinolone resistant genes among *Klebsiella pneumoniae*

Out of 63 patients, 47% resistance was seen between 40-50 years of age, 33% between 50-60 years of age, 3% resistance in age group 60-70 years of age, 7% resistance between 30-40 years of age and 10% resistance in age group 20-30 years.

Discussion

Resistance to qnr determinant is increasingly reported worldwide in isolates of *Enterobacteriaceae* [12,13,14, 15,16,17]. Resistance arises as a result of chromosomal mutation responsible for the modification of target enzymes or decreased intracellular drug accumulation by the upregulation of efflux pumps and modification of outer membrane proteins. Patients who are receiving quinolone prophylaxis are at a risk of developing infection by resistant strains [18]. Emergence of multidrug resistance due to co-transmission of qnr genes with aac(6)-Ib-cr, ESBL and plasmid mediated AmpC have been reported among clinical isolates of *Klebsiella pneumoniae*.

The transferable plasmid mediated quinolone resistance genes are associated with low level resistance to fluoroquinolones, but their presence could probably facilitate higher levels of resistance by mutational alterations of type II topoisomerase [19].

In the present study, Ciprofloxacin resistant isolates were obtained from urine samples (38.10%) followed by sputum (28.57%), pus (23.81%) and blood (9.52%). In an Indian study, Shilpa K et al have reported 31.03 % Ciprofloxacin resistant *Klebsiella pneumoniae* isolates from sputum sample with highest isolation rate among patients above the age of 60 years [20]. In the present study, 47% resistant isolates were from patients in the age group of 40-50 years.

By disk diffusion, 90.99% isolates were resistant to Ciprofloxacin in the present study. In an Indian study by NW Nandhihal among UTI, 79% *Klebsiella* isolates were resistant to Ciprofloxacin [21]. In the present study, 51 isolates (80.95%) were resistant for Ciprofloxacin with MIC of >1µg/ml. The presence of aac (6') - Ib-cr and qnr S gene associated with high MIC value of ≥ 64µg/ml was reported by Yugendran T et al [22]. A study by Soundarajan et al, have reported 32% Ciprofloxacin resistant *Klebsiella* isolates with aac (6') - Ib-cr as predominant quinolone resistant genes [23].

The present study showed predominance of qnr B and aac (6') - Ib-cr gene among *Klebsiella pneumoniae*. By PCR, 9.52% isolates showed aac (6')-Ib-cr gene, 11.11% of the isolates were positive for qnr A gene, 46.03% for both qnr B and aac (6')-Ib-cr gene and 12.70% for qnr B gene. Presence of qnr B genes in isolates of *K. pneumoniae* was reported by Hu FP et al [24]. These isolates were found to be susceptible to Ciprofloxacin by disk diffusion method.

In another study, Andrea Endimiani et al have reported quinolone resistance due to qnr B and aac (6')-Ib gene in *Klebsiella pneumoniae* [25]. In a South Indian study, prevalence of PMQR mediated by qnrA (48%) and qnr A and qnr B (24%) in multidrug-resistant *K. pneumoniae* isolates have been reported [26].

Among the quinolone resistant genes, qnr genes are frequently isolated and found to confer low level resistance.

Apart from the qnr genes, a variant of an aminoglycoside 6'-N-acetyltransferase, aac(6')-Ib which confers resistance to Tobramycin, Amikacin and Kanamycin can confer an incremental resistance to fluoroquinolones [27]. In a similar study from North East India, aac (6')-Ib-cr gene was detected in 23.08% *Klebsiella pneumoniae* isolates [28].

High level resistance in *Enterobacteriaceae* due to aac (6')-Ib-cr gene have been reported [29].

The aac(6')-Ib-cr gene among *Enterobacteriaceae*, may create an environment facilitating the selection of more highly resistant determinants, especially those harbouring topoisomerase mutations.

Limitations of the study: In the present study, Ciprofloxacin resistant isolates of *Klebsiella pneumoniae* were screened for quinolone resistant genes. The degree of resistance to other quinolone drugs and their association with resistance genes can be further analysed.

Author contributions

- **Indra Priyadharsini. R:** Designed and guided the study.
- **Venkata Raghavendra Rao. A*:** Assisted in performing the tests and its organization. Prepared the manuscript and analysed the data.
- **Dr. Kavyashri. J:** Performed the required test and organized the data.

Conclusion

There is an increasing trend in the frequency of plasmid mediated quinolone resistance among *Klebsiella pneumoniae*. The mechanism of resistance in fluro-quinolones is mediated through plasmids favouring the dissemination of the resistant genes between different bacterial genera.

Thus, it is the prime role of hospital infection control team to establish an antibiotic policy which paves way to prevent drug resistance in future.

What does this study add to existing knowledge?

This study showed the presence of multidrug resistant *Klebsiella pneumoniae* isolates from clinical samples. Low level resistance to fluoroquinolones conferred by plasmid mediated quinolone resistance genes reduce bactericidal activity to Ciprofloxacin in vivo and can result in therapeutic failures.

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