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Identification of Plasmid-Mediated Quinolone Resistance Genes among Clinical Isolates of *Klebsiella species* in a Tertiary Care Center at South Karnataka

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ABSTRACT

Introduction: fluoroquinolone resistance is widespread in many Gram-negative bacteria which produce extended-spectrum beta-lactamase (ESBLs), including *Klebsiella pneumoniae.* This study aims to perform antimicrobial susceptibility testing and to investigate the presence of the plasmid-mediated quinolone resistance (PMQR) genes in clinical isolates of extended-spectrum β-lactamases (ESBLs) producing Klebsiella isolates.
Materials and Methods: A total of 201 Klebsiella species isolated from various non-repeated clinical samples were collected from Yenepoya Medical College Hospital and identified by biochemical method. An antibiotic susceptibility test was performed for fluoroquinolone (FQ) drugs and ESBL detection was done by combined disc diffusion method according to the CLSI guidelines. The isolates were screened for the PMQR genes i.e. *qnrA, qepA, aac(6')Ib-cr*, and *oqxA&B* by polymerase chain reaction (PCR).
Results: Out of 201 ESBL positive *Klebsiella* isolates, 153 were ESBL positive with fluoroquinolone resistance confirmed by PCR. The antibiotic susceptibility pattern showed that most strains were multi drug resistant. Out of 153 fluoroquinolones resistant *Klebsiella* isolates, MIC of 142 isolates were resistant to fluoroquinolone drug, whereas 11 isolates had MIC for levofloxacin below 12 µg/ml. Among 142 FQ-resistant strains: PMQR genes i.e., *qnrA* was present in 60 (42.3%), followed by *aac(6')Ib-cr* 137 (96.5%), *qepA* 05 (3.5%), *oqxA* 120 (84.5%) and *oqx* B 130 (91.6%) isolates.
Conclusion: The resistant profile was found to be quite high to ESBL production with FQ resistance in *Klebsiella* isolates infection, which is emerging because of an improper antibiogram schedule. This study highlights the increasing incidence of *aac(6')Ib-cr* and *oqxA & B* among ESBL-producing *Klebsiella* isolates.
Keywords: PMQR, ESBL, Klebsiella pneumoniae, PCR

INTRODUCTION

Quinolones represent a significant category of antimicrobials that prove effectiveness against both gram-negative and gram-positive bacteria. Multi-drug resistant *Klebsiella pneumoniae* is one of the important nosocomial and community acquired opportunistic bacteria causing urinary tract infection, pneumonia, septicemia etc.[1, 2] *Klebsiella* species is one

of the major gram-negative organisms causing urinary tract infection, pneumoniae, septicemia etc. *Klebsiella* species is emerging as a multidrug-resistant organism by acquiring resistance genes against β-lactam antibiotics. Plasmid mediated quinolone resistance (PMQR) in *Klebsiella* spp. results mainly from mutations in DNA gyrase, topoisomerase IV and/or changes in the expression of outer membrane and efflux pumps.[3] Aim of the study to perform antimicrobial susceptibility testing and to investigate the presence of the PMQR genes in clinical isolates of ESBL producing *Klebsiella* isolates.

MATERIALS & METHODS

A total of 201 non-repeating *Klebsiella* species strains were collected from Yenepoya Medical College Hospital, Yenepoya University. Samples were processed for gram-stain and culture. Inoculation was done on Blood agar and MacConkey's agar media and incubated overnight at 37 ºC. Identification of *K. pneumoniae* isolates was done by standard biochemical methods.[4]

Susceptibility assay for beta lactam and quinolone Antibiotics:

Antibiotic susceptibility of these *Klebsiella* isolates was determined according to Kirby–Bauer disc diffusion method and results were interpreted according to guidelines of Clinical and Laboratory Standard Institute. The following beta-lactam and quinolone antibiotics were used (disc: 6 mm: lg): ceftazidime (30), ceftazidime and clavulanic acid (30/10), nalidixic acid (30), ciprofloxacin (5), and levofloxacin (5), Sparfloxacin (SPX), and Moxifloxacin (MO) (5) (HiMedia Lab Ltd., India). *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were used as positive and negative controls respectively in each phenotypic ESBL test.[5]

Detection of PMQR genes:

DNA was extracted from the *Klebsiella* isolates (n=142) according to the Qiagen kit literature provided with the QIAamp DNA mini kits. Extracted DNA was collected and stored at -20°C till further processing.

DNA from each sample was amplified by PCR with the primer sets [Table 1]. The oligonucleotide primers were procured from Eurofins Genomics Private India Limited, Bangalore. PCR was performed using the in-house standardized PCR protocols in a thermal cycler (Applied Biosystems, USA). Conventional PCR was used for the detection of *bla-CTX-M, aac (6') Ib-cr, qepA*, and *qnrA* and multiplex PCR was used for the detection of *oqxA, oqxB* genes. PCR was performed in the thermal cycler (BIORAD CFX096) by the following steps as shown in Table 2.

Table 1. Primers used for the detection of blaCTX-M, PMQR (*qnrA, aac(6')Ib-cr, qepA, oqxA, oqxB*) genes [6-10]

SI. No.	Genes	Primer sequence	Size of amplified product (bp)
1	*blaCTX-M*	F: 5'- TTTGCGATGTGCAGTACCAGTAA-3' R: 5'- CGATATCGTTGGTGGTGCCATA-3'	544 bp
2	*qnrA*	F: 5'- TTTGCGATGTGCAGTACCAGTAA-3' R: 5'- CGATATCGTTGGTGGTGCCATA-3'	619 bp
3	*aac (6') Ib-cr*	F: 5'-TTGCGATGCTCTATGAGTGGCTA 3' R: 5'-CTCGAATGCCTGGCGTGTTT-3'	482 bp
4	*qepA*	F: 5'-CTGCAGGTACTGCGTCATG-3' R: 5'-CGTGTTGCTGGAGTTCTTC-3'	403 bp
5	*oqxA*	F: GACAGCGTCGCACAGAATG R: GGAGACGAGGTTGGTATGGA	339 bp
6	*oqxB*	F: 5'-CGAAGAAAGACCTCCCTACCC-3' R: 5'-CGCCGCCAATGAGATACA-3'	240 bp

Abbreviations used: F: Forward, R: Reverse, bp- Base pair, *blaCTX-M*: β-lactamase gene; *qnrA*: Quinolone resistant gene; *aac (6') Ib-cr*: Aminoglycoside acetyltransferase gene; *qepA, oqxA* and *oqxB*: Quinolone efflux pump gene.

Table 2. Conditions for PCR of *blaCTX-M, qnrA, aac (6') Ib-cr, qepA, oqxA and oqxB* genes

SI. No.	Primer name	Hot start	Denaturation	Annealing	Extension	Final extension	Cycles
1	*blaCTX-M*	95°C, 5 min	95°C, 30 s	51°C, 30 s	72°C, 30 s	72°C, 5 min	35
2	*qnrA*	95°C, 5 min	94°C, 30 s	57°C, 30 s	72°C, 30 s	72°C, 5 min	35
3	*Aac (6') Ib-cr*	95°C, 5 min	94°C, 45 s	55°C, 45 s	72°C, 45 s	72°C, 5 min	35
4	*qepA*	95°C, 5 min	94°C, 45 s	60°C, 45 s	72°C, 1 min	72°C, 5 min	35
5	*oqxA/ oqxB*	95°C, 15 min	94°C, 30 s	62°C, 1 min	72°C, 1 min	72°C,10 min	30

Abbreviations used: *blaCTX-M*: β lactamase gene, *qnrA*: quinolone resistant gene, *aac(6')Ib-cr*: Amino-glycoside acetyltransferase gene, *qepA*: Quinolone efflux pump gene, *oqxA and oqxB*: Efflux pump gene, min: Minutes, s: Seconds.

Positive PCR products (1 µL) were purified by QIAquick PCR purification kit (Qiagen) and sent for sequencing to Applied Biosciences, Bangalore. Sequence analysis was performed with blast program of NCBI GenBank database http://blast.ncbi.nlm.nih.gov/. Both purification and sequencing were performed at Applied Biosciences, Bangalore. [11-13]

Statistical Analysis

PMQR genes detection among ESBLs producing *Klebsiella* isolates was shown as percentages. The data arrived from the results of the study was analysed by descriptive statistics and presented in the form of percentages.

RESULT

The antimicrobial susceptibility pattern has been studied for all 201 ESBL producing *Klebsiella* isolates as per standard protocol. All isolates were found 100% resistant to ceftazidime, followed by 192 (95.5%) cefepime, 184 (91.5%) amoxiclav, 184 (91.5%) piperacillin, 150 (74.6%) piperacillin/tazobactam, 165 (82%) co-trimoxazole, and 153 (76.1%) were resistant to all fluoroquinolones. Out of 153 fluoroquinolones resistant *Klebsiella* isolates- MIC of 142 isolates were resistant to fluoroquinolone drug, whereas 11 isolates had MIC for levofloxacin below 12 µg/ml. [Table 3].

Table 3. Antimicrobial susceptibility pattern of ESBL producing *Klebsiella* isolates

Antibiotics (µg)	AST for ESBL *Klebsiella* species (n=201)		
	Sensitive	Intermediate	Resistant
Amikacin (AK) (10)	82 (40.8%)	3 (1.5%)	116 (57.7%)
Ceftazidime (CAZ) (30)	-	-	201 (100%)
Cefepime (CPM) (30)	9 (4.5%)	-	192 (95.5%)
Amoxiclav (AMC) (30)	15 (7.5%)	02 (1%)	184 (91.5%)
Piperacillin/tazobactam (PIT) (100/10)	47 (23.4%)	04 (2%)	150 (74.6%)
Imipenem (IPM) (10)	105 (52.2%)	03 (1.5%)	93 (46.3%)
Meropenem (MRP) (10)	103 (51.3%)	02 (1%)	96 (47.7%)
Ciprofloxacin (CIP) (5)	45 (22.4%)	03 (1.5%)	153 (76.1%)
Levofloxacin (LEV) (5)	46 (22.9%)	02 (1%)	153 (76.1%)
Sparfloxacin (SPX) (5)	46 (22.9%)	02 (1%)	153 (76.1%)
Moxifloxacin (MO) (5)	46 (22.9%)	02 (1%)	153 (76.1%)
Co-trimoxazole (COT) (25)	36 (18%)	-	165 (82%)
Tetracycline (TE) (10)	56 (27.9%)	02 (1%)	143 (71.1%)
Nitrofurantoin (NIT) (300) (Only for urine Sample)	9 (22.5%)	01 (2.5%)	30 (75%)

The data is expressed as frequency with percentage in parenthesis. Abbreviations used: AST- Antibiotic susceptibility testing, ESBL- Extended spectrum beta lactamase.

Detection of PMQR genes by PCR:

Out of 201 ESBL producing *Klebsiella* isolates, 142 fluoroquinolone resistant isolates were screened for the presence of

ESBL gene, i.e., *bla CTX-M* and plasmid mediated quinolone resistance (PMQR) genes, i.e., *qnrA, aac (6') Ib-cr, qepA, oqxA* and *oqxB* as shown in table 4 and figure 1.

Table 4. Detection of drug resistance genes in ESBL producing *Klebsiella* isolates (n=142) by polymerase chain reaction

SI. No.	FQR samples (n= 142)	*Bla CTX-M* n (%)	*qnrA* n (%)	*aac(6') Ib-cr* n (%)	*oqxA* n (%)	*oqxB* n (%)	*qepA* n (%)
1	Pus (54)	44 (31)	23 (16.2)	51 (36)	42 (29.6)	47 (33.1)	-
2	Urine (33)	27 (19)	11 (7.8)	32 (22.5)	29 (20.4)	32 (22.5)	01 (0.7)
3	Spt (19)	17 (12)	09 (6.3)	19 (13.4)	17 (12)	18 (12.7)	03 (2.1)
4	BC (16)	15 (10.6)	08 (5.6)	16 (11.3)	13 (9.2)	14 (9.9)	-
5	VA (8)	08 (5.6)	03 (2.1)	07 (4.9)	07 (4.9)	07 (4.9)	01 (0.7)
6	ETA (4)	03 (2.1)	02 (1.4)	04 (2.8)	04 (2.8)	04 (2.8)	-
7	HVS (2)	02 (1.4)	-	02 (1.4)	02 (1.4)	02 (1.4)	-
8	BF (4)	04 (2.8)	03 (2.1)	04 (2.8)	04 (2.8)	04 (2.8)	-
9	BAL (2)	02 (1.4)	01 (0.7)	02 (1.4)	02 (1.4)	02 (1.4)	-
	Total	122 (85.9)	60 (42.2)	137 (96.5)	120 (84.5)	130 (91.5)	05 (3.5)

The data is expressed as frequency with percentage in parenthesis. Abbreviations used: Drug resistance genes- *blaCTX-M, qnrA, aac (6') Ib-cr, qepA, oqxA & oqxB*, Spt- sputum, BC- blood culture, VA- ventilator aspirate, ETA- endotracheal aspirate, HVS- high vaginal swab, BF-body fluid, BAL- bronchoalveolar lavage, FQR- fluoroquinolone resistant.

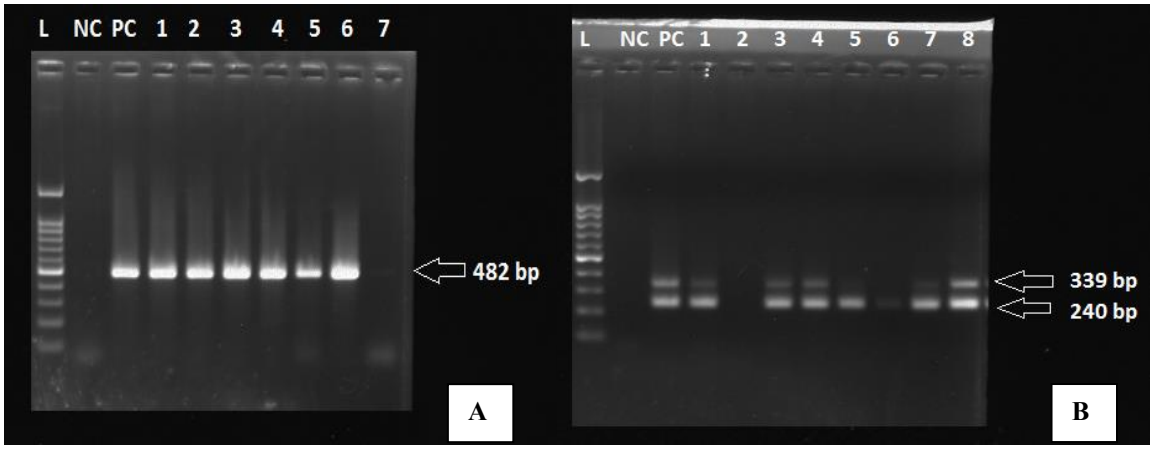

Figure 1. Agarose gel of the amplified products of PMQR genes from fluoroquinolone resistant ESBL producing *Klebsiella* isolates. *aac (6') Ib-cr* (482bp) gene done by conventional PCR (A); *oqxA* (339 bp) & *oqxB* (240 bp) genes done by multiplex PCR (B)

Abbreviations used: *aac (6') Ib-cr*: Aminoglycoside acetyltransferase, *oqxA* and *oqxB*: Efflux pump gene, L- 100 base pair DNA ladder; NC: Negative control; PC- Positive control;

Figure 1 (A) Lane 1- 6: Positive Samples; 7: Negative sample for *aac (6') Ib-cr* gene

Figure 1 (B) Lane 1, 3-5,7,8: Positive samples; 6: Weak positive; 2: Negative samples for *oqxB* gene (240bp). Lane 1,3,4,8: Positive samples; 7: Weak positive; 2,5,6: Negative samples for *oqxA* gene (339bp).

DISCUSSION

Plasmid-mediated resistance mechanisms play a significant role in fluoroquinolone resistance. The resistance mechanisms against antimicrobial agents can take place in four major ways: by altering drug targets, protecting drug targets, enzymatic drug modification, and reducing drug accumulation.[14] *Klebsiella* resists quinolone antibiotics by all these ways. While chromosomal mutations in genes coding for DNA gyrase, topoisomerase IV and genes coding for outer membrane, and efflux proteins are also responsible for the resistance.[15] Plasmid-mediated quinolone resistance PMQR involves, the *qnr* proteins

protecting DNA gyrase and topoisomerase IV from quinolones and the *aac(6')Ib-cr* enzyme, which is able to modify some fluoroquinolone antibiotics.[16] In the present study, the aim was to detect drug resistance PMQR genes, i.e., *qnrA, aac(6')Ib-cr, qepA, oqxA* and *oqxB* among ESBL producing *Klebsiella* species. Among 201 isolates, 153 were resistant to CIP, LEV, SPR, and MOX by antimicrobial susceptibility testing. A total of 142 isolates were identified for the PMQR genes, of which maximum PMQR genes were *aac(6')Ib-cr* gene, i.e., 96.5% (137/142), followed by 91.5% (130/142) *oqxB*, 84.5% (120/142) *oqxA* gene, 42.2% (60/142) *qnrA,* and only 3.5% (5/142) were *qepA* gene positive. A study done by Tripathi *et al.,* (2012), from Kolkata,[17] has reported that PMQR genes, i.e., *qnrA* were detected in 37% (27/73), and *qnrB* were 56% (41/73) of the *Klebsiella* isolates, which is concordance to the present study. Another study from Chennai, (2011), has reported among 23 ESBL producing *K. pneumoniae* isolates, 70% (16/23) were *qnrA* and 48% (11/23) *qnrB* genes positive and 34.7% (8/23) isolates had both *qnrA* and *qnrB* genes, and *aac(6')Ib-cr* was observed in 56.5% (13/23) isolates,[2] which is higher than the present study in case of *qnrA* gene, i.e., 42% were positive and less in case of *aac(6')Ib-cr* gene, i.e., 96.5%. The most commonly detected PMQR gene through PCR analysis was *aac (6') Ib-cr,* present in 68.8% of samples, followed by oqxA at 56.7%, oqxB at 54.6%, qnr at 6.4%, and qepA at 2%. Additionally, a study from Puducherry reported aac (6') Ib-cr at a prevalence of 64.5%. [11, 18] which is concordance to the present study reports that maximum isolates were positive for *aac(6')Ib-cr* gene (96.5%) in ESBL producing *K. pneumoniae* isolates, followed by *oqxB* (91.5%), *oqxA* (84.5%), *qnrA* (42.2%), *and qepA* (3.5%). A study conducted in Assam, (2016), reported that 73.08% isolates of *Klebsiella* species were positive for *aac(6')Ib-cr* gene, The prevalence of *aac (6') Ib-cr* was highest among other PMQR determinants,[19] which is similar to the present study.

DNA sequencing was done in 10 randomly selected samples in order to find out if any novel mutation has occurred. The amplified products were sequenced to confirm the alleles. After comparing with the known alleles in NCBI's GenBank, it was observed that in the isolates studied in the present study that no novel mutations have been identified among all alleles, as all the nucleotide sequences showed 100% similarity with the other sequences. Similarly, a study done in Puducherry has also not shown any novel mutation among the PMQR study.[19]

CONCLUSION

To conclude molecular analysis of this study showed that, ESBL producing *K. pneumoniae* isolates acquired from a tertiary care hospital in Karnataka, India, had high ESBL occurrence with predominant *blaCTX-M* type, and high frequency of PMQR genes. The *aac (6') Ib-cr, oqxA,* and *oqxB* genes were widely distributed among the *K. pneumoniae* isolates than any other PMQR genes. The screening of ESBL producing *K. pneumoniae* for PMQR carriage could be helpful in both treatment and prevention of the spread of resistant strains

Declaration by Authors

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