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Original Article

Identification of plasmid-mediated quinolone resistance genes *qnrA1*, *qnrB1* and *aac(6')-1b-cr* in a multiple drug-resistant isolate of *Klebsiella pneumoniae* from Chennai

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Abstract

Purpose: Resistance to fluoroquinolones, a commonly prescribed antimicrobial for Gram-negative and Gram-positive microorganisms, is of importance in therapy. The purpose of this study was to screen for the presence of Plasmid-Mediated Quinolone Resistance (PMQR) determinants in clinical isolates of *Klebsiella pneumoniae*. **Materials and Methods:** Extended-Spectrum Beta-Lactamase (ESBL) isolates of *K. pneumoniae* collected during October 2009 were screened by the antimicrobial susceptibility test. The plasmids from these isolates were analysed by specific Polymerase chain Reaction (PCR) for *qnrA*, *qnrB* and *aac(6')-1b*. The amplified products were sequenced to confirm the allele. **Results:** Our analysis showed that 61% out of the 23 ESBL *K. pneumoniae* isolates were resistant to ciprofloxacin and 56% to levofloxacin. The PMQR was demonstrated by transforming the plasmids from two isolates P12 and P13 into *E. coli* JM109. The PMQR gene *qnrA* was found in 16 isolates and *qnrB* in 11 isolates. The plasmid pKNMGR13 which conferred an minimum inhibitory concentration (MIC) of more than 240 µg/ml in sensitive *E. coli* was found to harbour the *qnrA1* and *qnrB1* allele. Furthermore, the gene *aac(6')-1b*-cr encoding a variant aminoglycoside 6'-N Acetyl transferase which confers resistance to fluoroquinolones was found in the same plasmid. **Conclusions:** Our report shows the prevalence of PMQR mediated by *qnrA* and *qnrB* in multidrug-resistant *K. pneumoniae* isolates from Chennai. A multidrug-resistant plasmid conferring high resistance to ciprofloxacin was found to harbour another PMQR gene, *aac(6')–1b-cr* mutant gene. This is the first report screening for PMQR in *K. pneumoniae* isolates from India.

Key words: *Aac(6')-1b-cr, Klebsiella pneumoniae, plasmid-mediated quinolone resistance, qnrA, qnrB*

Introduction

Quinolones constitute an important group of antimicrobials active against Gram-negative and Gram-positive bacteria.[1] Fluoroquinolones are the third largest selling drug class with sales of US$ 7.1 billion, accounting for 17% of the world antibiotic market in 2009.[2] Since the introduction of fluoroquinolones for therapy in 1962, resistance of the *Enterobacteriaceae* to these agents has become common, widespread and generally non-clonal.[3] Quinolone resistance can be due to mutations in the chromosomal genes for the DNA gyrase and topoisomerase IV, the targets of quinolone action; and by changes in expression of efflux pumps and porins that control the accumulation of these agents inside the bacterial cell.[4] Plasmid-mediated quinolone resistance (PMQR) was first reported in 1988 in a fluoroquinolone-resistant strain of *Klebsiella pneumoniae* in Alabama, USA.[5] This gene encoding quinolone resistance, named *qnrA1,* belongs to a pentapeptide repeat family. Two other transferable quinolone resistance determinants have also been described, *aac*(*6'*)*-Ib-cr* which encodes a variant aminoglycoside acetyltransferase with two amino acid alterations allowing it to inactivate ciprofloxacin[6] and *qepA* genes which encode efflux pumps that extrude quinolones.[7]

The *qnr* genes show a high level of diversity. Five main types of the *qnr genes*, *qnrA,*[5] *qnrB,*[8] *qnrC,*[9] *qnrS*[10] and *qnrD,*[11] have been identified. The *qnrB gene* was identified first in an isolate of *K. pneumoniae* from South India, and subsequently has been found in isolates in the USA, Korea, Kuwait, France and Taiwan.[3,8] The *qnrA* genes were also reported in *K. pneumoniae* from South Indian isolates in the same paper.[8]

Since the report of Jacoby *et al.*, there has not been any screening for the prevalence of the *qnr* genes in *K. pneumoniae* isolates in India. In a study from our laboratory,

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we analysed 23 isolates of *K. pneumoniae* collected in October 2009 and found them to be Extended-Spectrum Beta-Lactamase (ESBL) isolates with plasmids containing different combinations of bla_{SHV} , bla_{OXA-1} and bla_{CTX-M} genes. Since ESBL isolates are frequently resistant to quinolones, and the plasmids containing ESBL genes also have the genes conferring quinolone resistance, we undertook this study to screen for *qnr* genes in these 23 ESBL isolates of *K. pneumoniae* collected in October 2009. This is the first report from India demonstrating plasmid-mediated quinolone resistance (PMQR) mediated by *qnr* genes and the *aac(6')-1b-cr* allele in *K. pneumoniae*.

Materials and Methods

Bacterial isolates

Twenty-three clinical isolates of *K. pneumoniae* collected from tertiary care hospitals during Oct 2009 were subjected to routine culture and antibiotic susceptibility testing.

Antimicrobial susceptibility testing

Antibiotic susceptibility testing was performed according to standard methods on Mueller Hilton agar (Himedia Laboratories Pvt. Ltd., Mumbai, India). The antibiotics Norfloxacin (10 µg $disk^{-1}$, Himedia Laboratories Pvt. Ltd., Mumbai, India), Levofloxacin (5 µg $disk^{-1}$, Himedia Laboratories Pvt. Ltd., Mumbai, India) and Ciprofloxacin (5 µg $disk^{-1}$, Himedia Laboratories Pvt. Ltd., Mumbai, India) were used for antibiotic susceptibility screening. The results were interpreted as per the CLSI guidelines.[12] Minimum inhibitory concentration of Ciprofloxacin was determined by the HiComb™ E-Strip from Himedia Laboratories Pvt. Ltd., Mumbai, India, following the manufacturer's instructions.

PCR amplification and sequence analysis

Amplification of the *qnrA*, *qnrB* and *qnrS* genes was performed for all the *K. pneumoniae* isolates using the primer sets described in a previous report.[13] PCR experiments were carried out according to standard conditions (annealing temperature at 55°C [for *qnrA*], 60°C [for *qnrB* and *qnrS*] and extension 1 min at 72°C, 35 cycles) using primers synthesised by Eurofins Genomics India Pvt. Ltd, Bangalore, India. Taq DNA polymerase (Biotools, B and M Labs, Madrid, Spain) and dNTP (Cinna Gen Inc. Tehran, Iran) were used as per standard protocols. For detecting the *aac(6')-1b,* primers were chosen to amplify all known *aac(6')-Ib* variants.[14] The primers used were 5'-TTGCGATGCTCTATGAGTGGCTA-3' and 5'-CTCGAATGCCTGGCGTGTTT-3' which produce a 482-bp product. PCR conditions were 94°C for 45 s, 55°C for 45 s and 72°C for 45 s for 34 cycles. The *aac(6')-Ib* variants allele was identified by direct sequencing of the PCR product with primer 5'CGTCACTCCATACATTGCAA 3'.

DNA was prepared for the PCR reaction by suspending a single colony of the clinical isolate in 500 µl of sterile Millipore water in a 1.5 eppendorf tube, followed by boiling at 100ºC for 5 minutes and centrifuged at 5,000 rpm for 10 minutes.[15] Two µl of supernatant was used as DNA source for the PCR reaction. PCR experiments were carried out according to conditions given. The PCR products were analysed on a 1.5% agarose gel (Himedia Laboratories Pvt. Ltd., Mumbai, India) and the DNA bands were visualised by staining with Ethidium Bromide (Himedia Laboratories Pvt. Ltd., Mumbai, India). The 100 bp DNA Marker from Medox Biotech India Pvt. Ltd., Chennai, India, was used for sizing the PCR bands. The PCR-amplified products were sequenced by SciGenom Labs Pvt. Ltd., Cochin, Kerala, India. The sequence from the chromatogram were analysed by BLAST and compared with known alleles to identify the correct allele.

Plasmid isolation and transformation

Plasmids from the clinical isolates of *K. pneumoniae* were isolated using HiPurA™ Plasmid DNA Mini and Midi prep purification Spin Kit (HiMedia Laboratories Pvt. Ltd., Mumbai, India). For transformation experiments, plasmid DNA was isolated from two fluoroquinolone isolates of *K. pneumoniae* (Isolate P12 and P13) and transformed into a recipient strain (*E. coli* JM109). *E. coli* JM109 is resistant to Nalidixic acid (Sigma-Aldrich Co., St. Louis, MO, USA) but sensitive to ciprofloxacin. The plasmid DNA was transformed into *E. coli* by electroporation using PEP™ (Personal Electroporation Pak Electroporator - BTX® Genetronics Inc) giving two electric pulses of 180 V at an interval of two seconds of each. The transformants were selected in LB agar (Himedia Laboratories Pvt. Ltd., Mumbai, India) containing Ciprofloxacin (Sigma-Aldrich Co., St. Louis, MO, USA) at a concentration of 1 µg/ml. These transformants were analysed further for antibiotic resistance pattern and PCR amplification.

Results

Fluoroquinolone resistance of Klebsiella pneumoniae clinical isolates from Chennai

Clinical isolates of *K. pneumoniae* collected from Chennai in October 2009 were analysed for their resistance to the most commonly used fluoroquinolone antibiotics—ciprofloxacin and levofloxacin. It was found that 14 out of the 23 isolates (61%) of the ESBL isolates were resistant to ciprofloxacin, five out of 23 (22%) intermediately resistant and four (17%) were sensitive. The antibiotic levofloxacin was marginally better with 52% isolates resistant. The high resistance to fluoroquinolones is worrying and can compromise antimicrobial treatment.

Association between MIC to Ciprofloxacin and the presence of qnr genes

PMQR can be mediated by the *qnr* genes. In order to find out if these isolates harboured the *qnrA* and *qnrB* genes, a specific PCR was carried out from plasmids isolated from these isolates. In 16 out of 23 (70%) ESBL isolates, *qnrA*-specific PCR product [Figure 1a] was observed. In 11 isolates (48%), *qnrB* gene was observed [Figure 1b]. We were unable to amplify any product for the *qnrS* genes. Eight out of the 23 isolates (24%) had both *qnrA* and *qnrB* genes. In four isolates (12%), none of the *qnr* genes were present. The *qnrA* and *qnrB* amplified product from the isolate P13 was sequenced to confirm that it was the *qnrA1* [Figure 2]: GenBank Acc. No. HQ675013.1 and *qnrB1* [Figure 3]: GenBank Acc. No. HQ675012.1 allele, respectively.

In order to correlate the fluoroquinolone resistance in the isolates with the *qnr* gene determinants, the minimal inhibitory concentration of ciprofloxacin for 19 resistant isolates was determined [Table 1]. We found that two isolates showed MIC >240 µg/ml, while nine showed MIC >120 µg/ml. All the isolates which had an MIC >120 µg/ml were associated with either *qnr*A or *qnr*B or both genes. However, there were two isolates with an MIC of >60 µg/ml which were not associated with *qnr* genes. In addition,

Table 1: The MIC of ciprofloxacin for *Klebsiella pneumoniae* isolates and the detection of *qnrA* and *qnrB* genes is shown

Culture No.	Ciprofloxacin MIC (µg)	*qnrA*	*qnrB*	*aac(6')-1b-cr*
UT 01	>240	+	+	+
UT 02	>240	+	-	+
UT 06	>120	-	+	+
UT 08	>120	+	+	+
UT 09	>120	+	-	+
UT 10	>120	+	-	ND
PUS12	>120	+	+	+
PUS13	>120	+	+	+
PUS14	>120	+	+	+
PUS16	>120	+	-	+
PUS18	>120	+	+	+
UT 05	>60	-	-	+
UT 07	>60	+	+	ND
PUS21	>60	+	-	-
PUS25	>60	-	-	ND
PUS23	>10	+	-	-
PUS15	>5	+	-	+
PUS19	>5	-	-	-
PUS22	>5	+	+	ND
UT 03	S	-	+	ND
UT 04	S	-	+	ND
PUS20	S	+	-	+
PUS24	S	-	-	ND

The MIC level was determined by HiComb MIC test for each isolate and the *qnr A* and *qnr B* were detected by specific PCR reactions. ND - Not determined. S – Sensitive. + indicates PCR amplification and – indicates absence of PCR amplification

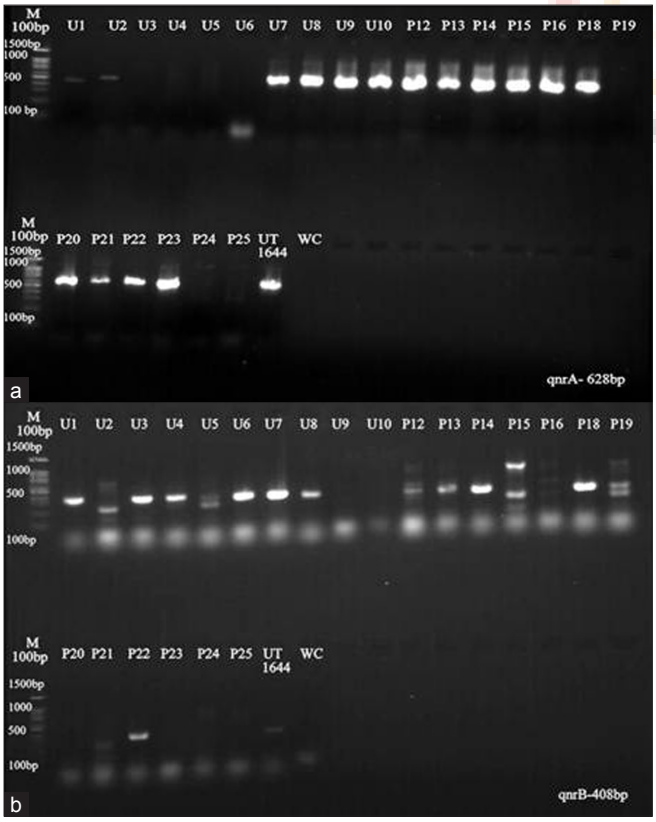

Figure 1: Amplification of *qnrA* and *qnrB*: Single colony PCR amplification of 628 bp *qnrA* gene (a) and 408 bp of *qnrB* gene (b) from the clinical isolates of *Klebsiella pneumoniae* in 1.5% agarose gel

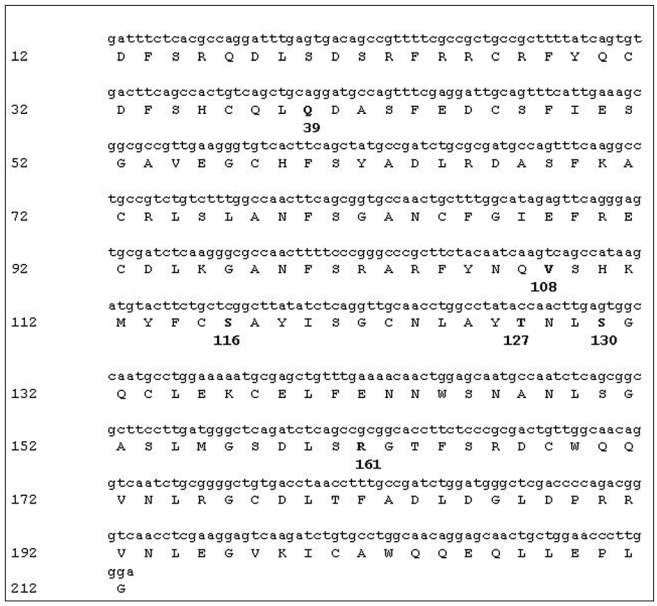

Figure 2: Partial sequence of *qnrA1* from pKNMGR13 (GenBank Accession Number HQ675013.1). The amino acid sequences from 12 to 212 are given. The amino acids which are variant and used to identify the *qnrA* alleles are given in bold

two fluoroquinolone-sensitive isolates harboured the *qnr* genes. Therefore, we wanted to find out if fluoroquinolone resistance was plasmid mediated and if there were other genes which could confer cumulative resistance.

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          ctgagcggcactgaatttatcggctgtcagttctatgatcgtgaaagccagaaagggtgc
35         L  S  G  T  E  F  I  G  C  Q  F  Y  D  R  E  S  Q  K  G  C

          aattttagtcgtgcgatgctgaaagatgccatttttaaaagctgtgatttatccatggcg
55         N  F  S  R  A  M  L  K  D  A  I  F  K  S  C  D  L  S  M  A
              55                60
          gattttcgcaattccagtgcgctgggcattgaaattcgccactgccgcgcacaaggcgca
75         D  F  R  N  S  S  A  L  G  I  E  I  R  H  C  R  A  Q  G  A
                       79                                          94
          gatttccgcggcgcaagctttatgaatatgatcaccacgcgcacctggttttgtagcgca
95         D  F  R  G  A  S  F  M  N  M  I  T  T  R  T  W  F  C  S  A

          tatatcacgaataccaatctaagctacgccaatttttcgaaagtcgtgttggaaaagtgt
105        Y  I  T  N  T  N  L  S  Y  A  N  F  S  K  V  V  L  E  K  C

          gagctgtgggaaaaccgttggataggtgcccaggtactgggcgcgacgttcagtggttca
115        E  L  W  E  N  R  W  I  G  A  Q  V  L  G  A  T  F  S  G  S

          gatctctccggcggcgagttttcgactttcgactggcgagcagc
125        D  L  S  G  G  E  F  S  T  F  D  W  R  A
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Figure 3: Partial sequence of *qnrB1* from pKNMGR13 (GenBank Accession Number HQ675012.1). The amino acid sequences from 35 to 138 are given. The amino acids which are variant and used to identify the *qnrB* alleles are given in bold

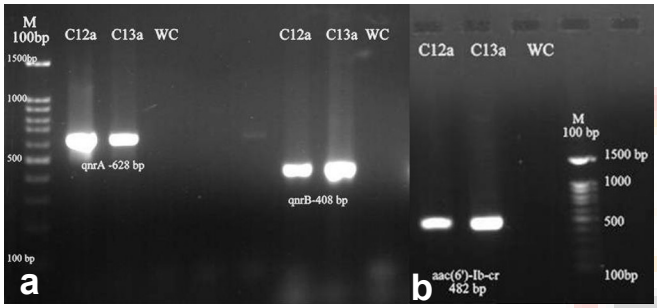

Figure 4: PMQR-specific amplification in *E. coli* transformants. (a) shows *qnrA* and *qnrB* gene-specific PCR on the *E. coli* JM109 transformants with the plasmids isolated from *Klebsiella pneumoniae* isolate P12 and P13. The *E. coli* JM109 transformants with the plasmid pKNMGRP12 is C12a and that with plasmid pKNMGRP13 is C13a. (b) shows the *aac(6')-Ib* gene-specific PCR on plasmids pKNMGRP12 and pKNMGRP13

Transfer of quinolone resistance

Since fluoroquinolone resistance can be chromosomal, plasmid mediated or a combination of both, we wanted to find out if the resistance in these isolates was plasmid borne. We chose two isolates P12 and P13 which carried both *qnr*A and *qnr*B genes and an MIC >120 µg/ml for further studies. Plasmid DNA from these two isolates was isolated and transformed into *E. coli* JM109 by electroporation. The transformants were selected on ciprofloxacin (1 µg/ml). Plasmid isolated from the transformants was analysed by *qnr*-specific PCR. [Figure 4] shows the specific amplification of both *qnrA* and *qnrB* in the transformants.

Furthermore, the minimum inhibitory concentration of ciprofloxacin in the transformants was determined. It was found that the *E. coli* transformants with the plasmid either from the P12 or P13 *K. pneumoniae* isolate showed an MIC of >240 µg/ml [Figure 5]. The *E. coli* JM109 strain without the plasmids was sensitive to ciprofloxacin. This clearly shows that ciprofloxacin resistance is mediated by plasmids from these isolates. The transformants were also resistant to cefotaxime, a third-generation cephalosporin, suggesting that the ESBL resistance determinants may be carried in the transferred plasmid.

Since the level of PMQR was high, we investigated whether any other quinolone resistance genes were present. Reports have shown that 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.[14] A mutant allele with the *Trp102Arg* and *Asp179Tyr* changes can acetylate ciprofloxacin resulting in resistance. To find out if the high level of fluoroquinolone resistance is due to the presence of this gene, we analysed the presence *of aac(6')-Ib by PCR.*

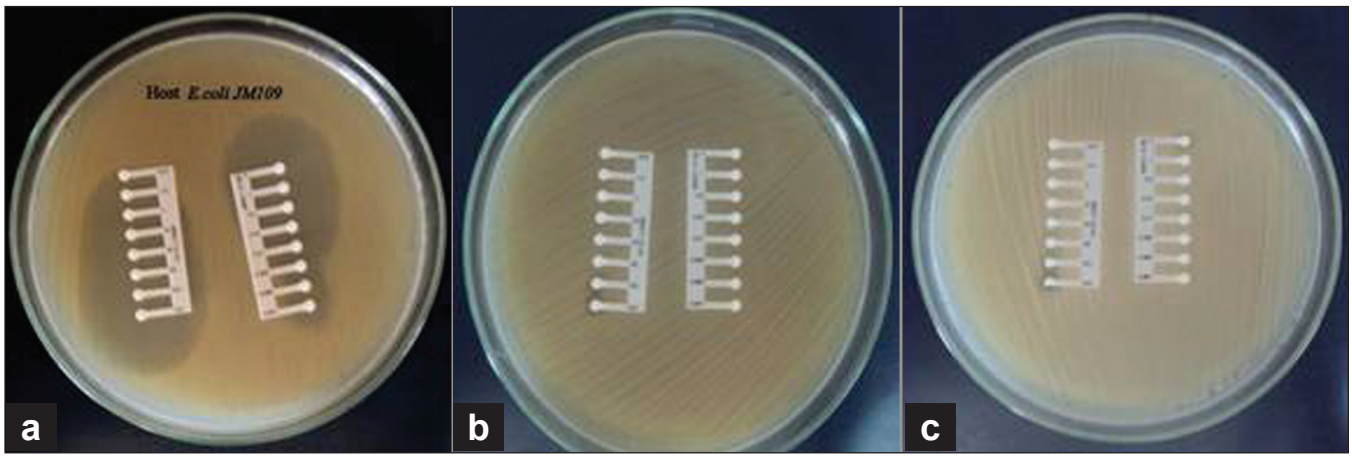

Figure 5: MIC of Ciprofloxacin for *E. coli* JM109 and the transformants. Transformed colonies were tested for Ciprofloxacin MIC value by E-Strip Test. (a) Host *E. coli* JM109, (b) - The plasmid pKNMGR12 transformed in *E. coli* JM109 (c)- The plasmid pKNMGR13 transformed in *E. coli* JM109

Screening for the aac(6')-1b gene in Klebsiella pneumoniae isolates and identification of the cr mutant

Plasmids isolated from 16 isolates including one ciprofloxacin-sensitive isolate was analysed by *aac (6') – 1b*-specific PCR. Thirteen isolates showed the *aac (6') – 1b* gene and three isolates did not amplify the gene [Figure 6]. The *E. coli* JM109 transformants containing the plasmid pKNMGR12 or pKNMGR13 also showed the specific amplification. The amplified product of the *aac (6') – 1b* from the plasmid pKNMGR13 was sequenced using an internal primer [Figure 7]. This sequence contained the Trp102Arg and Asp179Tyr mutation confirming that this pNMGR13 contained the *aac(6') 1b-cr* mutant which can confer ciprofloxacin resistance. The presence of this gene along with the *qnrA* and *qnrB* in the plasmid pKNMGR13 could be responsible for conferring higher level of ciprofloxacin resistance. It remains to be seen whether the *aac(6')-1*b gene in other isolates carry the cr mutant of *aac(6') 1b*. This is the first study reporting the presence of the aminoglycoside acetyl transferase gene *aac(6')-1b* and the presence of the bifunctional ciprofloxacin-resistant variant in *K. pneumoniae* isolates from Chennai.

Discussion

This study screening for fluoroquinolone resistance in ESBL isolates of *K. pneumoniae* from Chennai collected in October 2009 showed that 61% isolates were resistant to ciprofloxacin and 52% to levofloxacin. In an earlier study from our laboratory analysing 188 isolates of *K. pneumoniae*, collected during November 2007 to October 2008, we had observed 52% isolates resistant to ciprofloxacin. A study on 48 ESBL *K. pneumoniae* isolates collected in January 1999 to December 2003 in a University hospital located in Rome, Italy, reported 48% isolates resistant to Ciprofloxacin.[16] Together, these data clearly show that fluoroquinolone resistance is increasing among *K. pneumoniae* isolates.

The frequency of *qnr* genes in this study was found to be 69.5% for *qnr*A and 47.8% for *qnr*B. An analysis of the *K. pneumoniae* isolates obtained in the United States from 1999 to 2004 detected 14% *qnrA* and 6% *qnrB* in ceftazidime-resistant.[17] More recently, a study in clinical isolates of Enterobacteriaceae collected between 2001 and 2006 at Korea, 56.9% carried the *qnr* genes.[18] This is the first report after the discovery of the *qnrA* and *qnrB* gene from South India where a screening for both *qnrA* and *qnrB* has been reported from India.

Although the resistant isolates did show the presence of the *qnr* alleles, among the four sensitive isolates, two harboured the *qnrB* allele and one harboured the *qnr A* allele. This could be due to the following reasons. The resistance to quinolones due to the presence of *Qnr A* is the result of *Qnr* binding to gyrase or topoisomerase IV at a site overlapping the DNA binding site for ciprofloxacin. The level of resistance conferred by the genes is believed to be a result of the copy number and the transcriptional level of the *qnr* genes.[19] Furthermore, the resistance can be cumulative due to the presence of other quinolone resistance genes.

The plasmid borne nature of the quinolone resistance was shown for the isolates P12 and P13. Plasmids from the isolates P12 and P13 gave a very high MIC of 240 µg/ml to ciprofloxacin when transformed into the *E. coli* strain JM109. Since *qnr* genes are reported to give only a low level of quinolone resistance,[20] it is highly likely that these plasmids contain additional genes conferring quinolone resistance. AAC(6′)-Ib-cr, a variant aminoglycoside acetyltransferase capable of modifying ciprofloxacin and reducing its activity, has been reported to be widely prevalent and circulated together with *qnr* genes.[21] The complete nucleotide sequence of plasmid pKP96, which carries the PMQR determinants *qnrA1*, *aac(6')-Ib-cr* and the $bla_{CTX-M-24}$ ESBL coding gene from *K. pneumoniae* isolated in China[22] has been reported. This plasmid was found to contain a complex class 1 integron consisting of *aac(6')-Ib-cr*, *qacEdelta1*, *sul1*, IS*CR1*, *qnrA1*, *ampR*, *orf5* and *orf6.*

Our analysis of the isolates revealed that 13 isolates out of 19 tested harboured the *aac-(6')-1b* gene encoding

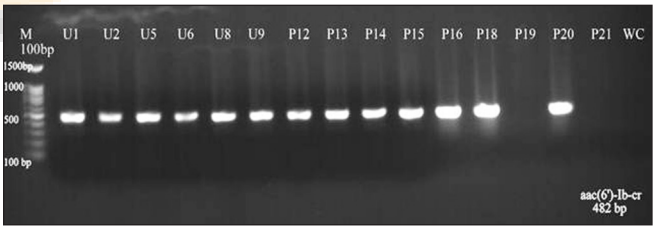

Figure 6: Amplification of *aac(6')-Ib*: PCR amplification of 482 bp *aac(6')-Ib* gene from plasmids isolated from the clinical isolates of *Klebsiella pneumoniae* is shown. WC - water control. The plasmid isolated from isolate P23 also did not amplify this gene (data not shown)

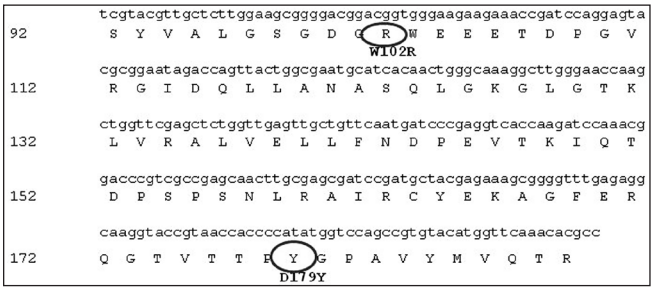

Figure 7: Partial sequence of *aac(6') -1b-cr* from pKNMGR13. The amino acid sequences from 92 to 189 are given. The amino acids which are variants in the bifunctional ciprofloxacin-resistant mutant are circled

aminoglycoside 6'-*N*-acetyltransferase, which confers resistance to amikacin, kanamycin and tobramycin and found to be broadly distributed geographically and present in many clinically important Gram-negative rods.[3] A variant, *aac(6')-Ib-cr*, with the additional property of acetylating and inactivating fluoroquinolones with an accessible amino nitrogen on the piperazine ring, was described in 2006, encoded by a plasmid isolated in 2000-2001 from Shanghai.[6] The enzyme encoded by *aac(6')-Ib-cr* differs from *aac(6')-Ib* by only two amino acid substitutions, both of which are required for the enhanced substrate recognition. The "cr" variant was subsequently found to be widely distributed around the world.[3] Our results show the presence of the *aac(6')-1b* gene in the *Klebsiella* isolates from Chennai and the presence of the cr variant in at least one of the isolates.

This report is significant since this is the first report showing that PMQR is prevalent in *K. pneumoniae* in Chennai. Our earlier analysis on these isolates had shown the presence of the CTX-M gene and the IncF1C replicon in the plasmid pKNMGRP13.[23] Further complete sequencing of the resistance plasmid will be very important to identify the presence of genes such as *qepA*, an efflux protein conferring resistance to ciprofloxacin; the genetic context of the resistance genes which can give clues to the transfer mechanisms across species and the plasmid backbone carrying the resistance determinants.

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