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High incidence of plasmid-mediated quinolone resistance genes among ciprofloxacin-resistant clinical isolates of Enterobacteriaceae at a tertiary care hospital in Puducherry, India.

Thiyagarajan Yugendran, Belgode Narasimha Harish

Background: Plasmid-mediated quinolone resistance (PMQR) has received considerable attention recently. Data analysis in JIPMER revealed 75% of the Enterobacteriaceae isolates to be ciprofloxacin-resistant in 2012. Few reports regarding the prevalence of PMQR are available from India. Hence, the present study was carried out to ascertain the prevalence of PMQR genes among clinical isolates of ciprofloxacin-resistant Enterobacteriaceae in JIPMER.

Methods: The study included 642 ciprofloxacin-resistant clinical Enterobacteriaceae isolates. JIPMER hospital's annual consumption data for fluoroquinolones were retrieved from the Department of Pharmacy. The test isolates were screened for the presence of *qnr A*, *B*, *D*, *S* and *aac(6')-Ib-cr* genes. PMQR-positive isolates alone were tested for the presence of class I (*intI1*) and class II (*intI2*) integrons. Randomly selected PCR amplicons were sequenced and analysed using MEGA software. A total of 30 PMQR strains chosen at random were assessed for the transferability of the PMQR genes.

Results: Majority of the strains exhibited high MIC values with 106 strains exhibiting MIC value ˃256µg/mL. The *aac(6')-Ib-cr* gene had the highest prevalence at 64% (414) while, *qnrB* and *qnrS* genes were present in 15% (97) and 10% (64) of the isolates respectively. None of the strains were positive for *qnrA* and *qnrD*. All PMQR-positive isolates were screened for class I (*intI1*) and class II (*intI2*) integrons. Class I integron was found to be predominant among the test isolates with a few of them carrying both the classes of integrons. Transferability of PMQR genes to transconjugants was identified.

Discussion: PMQR genes were found to exhibit an increasing trend of prevalence among the clinical isolates in this study. Thus, the need for rational usage of fluoroquinolones and reconsideration of their clinical breakpoints has arisen.

High incidence of plasmid-mediated quinolone resistance genes among ciprofloxacin-resistant clinical isolates of Enterobacteriaceae at a tertiary care hospital in Puducherry, India.

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Abstract

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Methods: The study included 642 ciprofloxacin-resistant clinical Enterobacteriaceae isolates. JIPMER hospital's annual consumption data for fluoroquinolones were retrieved from the Department of Pharmacy. The test isolates were screened for the presence of *qnr A*, *B*, *D*, *S* and *aac(6')-Ib-cr* genes. PMQR-positive isolates alone were tested for the presence of class I (*intI1*) and class II (*intI2*) integrons. Randomly selected PCR amplicons were sequenced and analysed using MEGA software. A total of 30 PMQR strains chosen at random were assessed for the transferability of the PMQR genes.

Results: A majority of the strains exhibited high MIC values with 106 strains exhibiting MIC value >256µg/mL. The *aac(6')-Ib-cr* gene had the highest prevalence at 64% (414) while, *qnrB* and *qnrS* genes were present in 15% (97) and 10% (64) of the isolates respectively. None of the strains were positive for *qnrA* and *qnrD*. All PMQR-positive isolates were screened for class I (*intI1*) and class II (*intI2*) integrons. Class I integron was found to be predominant among the test isolates with a few of them carrying both the classes of integrons. Transferability of PMQR genes to transconjugants was identified.

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Keywords: PMQR, *qnr*, *aac(6')-Ib-cr*, fluoroquinolone resistance, Enterobacteriaceae

Introduction

Fluoroquinolone resistance among the Enterobacteriaceae is a serious public health problem as it is responsible for the rise in respiratory tract infections worldwide whereas, in Asia UTI and intra-abdominal infections are on the rise (Dalhoff, 2012). Accumulation of mutations within target DNA gyrase enzyme was thought be the only mechanism of fluoroquinolone resistance until in the year 1998 when *qnr*, a plasmid-borne fluoroquinolone resistance gene, was reported (Strahilevitz *et al.,* 2009). Similarly, there are a few more reports on fluoroquinolone resistance mechanism/s that involve enzymatic degradation of fluoroquinolones and efflux pump activities (Robicsek *et al.,* 2006a; Vetting *et al.,* 2008; Robicsek *et al.,* 2006b; Strahilevitz *et al.,* 2009). In recent years, Enterobacteriaceae isolates have exhibited a higher level of fluoroquinolone resistance (Redgrave *et al.,* 2014). Due to the increase in fluoroquinolone resistance, plasmid-mediated quinolone resistance (PMQR) has received considerable attention in recent years. The *qnr* gene alleles *A*, *B*, *C*, *D* and *S*, encode for a pentapeptide repeat His6 protein capable of protecting DNA gyrase from fluoroquinolones (Robicsek *et al.,* 2006b; Strahilevitz *et al.,* 2009). Integrons are mobile genetic elements that have been identified in plasmids harbouring PMQR genes allowing them to spread horizontally for which they are widely feared (Pazhani *et al.,* 2011).

PMQR genes have been stressed upon in many studies (Strahilevitz *et al.,* 2009; Pazhani *et al.,* 2011; Mendez *et al.,* 2009). Prevalence reports from India regarding these genes are very few in contrast to reports available from other countries. Moreover, the frequency of quinolone resistance in clinical isolates of gram-negative bacilli is very high in India (Hariharan *et al.,* 2015). Data analysis in Department of Microbiology, JIPMER revealed 75% of the Enterobacteriaceae isolates to be resistant to ciprofloxacin in the year 2012 (T. Yugendran, unpublished data). Therefore, in this study ciprofloxacin-resistant isolates belonging to the family Enterobacteriaceae from the samples of patients attending JIPMER hospital were collected and screened for PMQR determinants and integrons with an aim to ascertain the PMQR prevalence in the hospital.

Materials & Methods

1. Bacterial strains

A total of 642 clinical isolates belonging to the family Enterobacteriaceae resistant to ciprofloxacin by Kirby-Bauer disk diffusion method subsequently confirmed by agar dilution MIC were part of the study. Standard methods were followed for isolation and identification of the bacteria from clinical specimens like blood, pus, CSF, etc. (Forbes, Sahm & Weissfeld, 2007). *Esch. coli* (J53), *Shigella boydii* (IDH738), *Esch. coli* (BCH1108), *Morganella morganii* (500914) and *Esch. coli* (TC145) harbouring *qnrA*, *qnrB*, *qnrS*, *qnrD* & *aac(6')-Ib* and *qnrA* & *aac(6')-Ib-cr* were used as positive controls in the PCR assay. The ATCC strain *Esch. coli* 25922 served as the quality control in the antimicrobial susceptibility test.

2. Antibiotic Susceptibility Test

The antibiotics included in the panel were amikacin (30µg), ceftriaxone (30µg), ceftazidime (30µg), ciprofloxacin (5µg) and gentamicin (10µg), meropenem (10µg) and commercially available cefoperazone-sulbactam disk. In particular cases, bacteria were also tested for imipenem, piperacillin-tazobactam using the commercially available disk. The antibiotic susceptibility of the test isolates were interpreted as per CLSI (2015) guidelines. MIC values were determined for ciprofloxacin alone by agar dilution method and *Esch. coli* ATCC 25922 was included as the quality control. The lowest concentration of antibiotic at which the growth of bacteria had been completely inhibited was recorded as MIC value.

3. Fluoroquinolone consumption data

JIPMER hospital's annual consumption data for fluoroquinolones were retrieved from Department of Pharmacy, JIPMER.

4. PCR Assay

DNA templates were prepared from the overnight inoculum of test strains grown on Nutrient HiVeg™ Agar (Himedia Laboratories, India) resuspended in MiliQ water after three rounds of washing. Crude template DNA was prepared by boiling lysis method. The reactions were performed in Flexilid Mastercycler PCR system (Eppendorf, Germany). The target genes, primer sequences, PCR conditions and amplified product sizes are given in Table I. PMQR-positive isolates alone were screened for the class I (*intI1*) and class II (*intI2*) integrons. Electrophoresis and staining analysed the PCR products with ethidium bromide.

5. Nucleotide Sequencing

Sequencing of PCR products was carried out at Xcelris Genomics, Ahmedabad. Nucleotide sequences were analysed over BLAST server (ww.ncbi.nlm.nih.gov/blast) against the GenBank database of the National Center for Biotechnology Information. The nucleotide and deduced protein sequences were examined with MEGA software for gathering phylogenetic details.

6. Conjugation assay

PMQR-positive strains numbering 30 were randomly selected for assessing the transferability of the PMQR genes following a previously described method (Jacoby *et al.,* 1996) with *Esch. coli* (J53) AziR (sodium azide-resistant) as the recipient strain. Transconjugants were selected on MacConkey agar containing sodium azide (100 µg/ml) and ciprofloxacin (0.5 µg/ml) and confirmed based on the results of biochemical and antimicrobial susceptibility tests carried out for transconjugants, recipient and donor bacterial cells. Screening of the transconjugants by PCR assay determined the transferability of PMQR genes.

RESULTS

1. Antibiotic Susceptibility Test

Out of 642 Enterobacteriaceae isolates, 43 isolates were MDR showing resistance against the entire antibiotic panel. Resistance to ceftriaxone, ceftazidime and cefoperazone-sulbactam were seen in 398, 381 and 351 isolates respectively. All the isolates were resistant to ciprofloxacin. Resistance to ciprofloxacin was confirmed by the agar dilution method. The MIC values of all the strains against ciprofloxacin ranged from 2 µg/mL to >256 µg/mL as summarized in Fig. I. It is notable that 106 (~16%) strains had MIC values >256µg/mL. A total of 112 isolates were resistant to meropenem, and it was most effective among all the other antibiotics.

2. Fluoroquinolone Consumption Data

Data from the Department of Pharmacy revealed that ciprofloxacin is the most extensively used fluoroquinolone in JIPMER hospital followed by levofloxacin and ofloxacin (supplementary data, S1).

3. PMQR Prevalence

Remarkably, majority of the isolated strains 414 (64.5%) harboured *aac(6')-Ib-cr*. While *qnrB* and *qnrS* genes were present in 97 (15%) and 64 (10%) isolates respectively (Fig. II). The proportion of *aac(6')-Ib-cr*, *qnrB* and *qnrS* among the clinical isolates was found to be 64.49, 15.1 and 9.96 with a confidence interval of 60.72 – 68.12, 12.5 -18.04 and 7.82 – 12.47 respectively. *Esch. coli* had the maximum frequency of *aac(6')-Ib-cr* and *qnrB* genes. On the other hand, the frequency of the *qnrS* gene was highest among *K. pneumoniae* isolates with *Klebsiella spp.* altogether accounting for more than half of the total *qnrS* gene identified. *Esch. coli* constituted almost half of the total *aac(6')-Ib-cr* positive isolates. None of the strains were positive for *qnrA* & *qnrD*, indicating the absence of these *qnr* alleles among the clinical isolates included in the study. Interestingly, of the 106 test isolates with MIC >256µg/mL only, three isolates were negative for PMQR genes.

The majority of the strains were found to carry one of the PMQR genes. But a few clinical isolates were found positive for multiple PMQR genes constituting about 7% of the total isolates. All these isolates either carried *qnrB* or *qnrS* along with *aac(6')-Ib-cr* gene. None of the isolates harboured *qnrB* and *qnrS* simultaneously. *Esch. coli*, *Klebsiella spp.*, *Enterobacter spp.* and *Proteus mirabilis* were the organisms carrying multiple PMQR genes but, the association of *qnrS* with *aac(6')-Ib-cr* was seen only in Esch. coli and K. pneumoniae. The association of *aac(6')-Ib-cr* and *qnrS* with MIC values was statistically significant with a p-value <0.0000001 and 0.006261 respectively.

Of the total PMQR positive isolates 212 were found to carry class I integron whereas, 95 isolates were found to carry class 2 integron whereas, 47 isolates were positive for both the classes of integrons. However, we must admit that the study neither included the integron sequence analysis nor screened the integron-positive isolates for the presence of contiguous resistance gene cassettes.

4. Transfer of PMQR

Conjugation experiments were done on 30 randomly selected PMQR strains. However, only 18 transconjugants were successfully achieved. Among the transconjugants 11 were positive for *aac(6')-Ib-cr*, four were positive for *qnrB*, and two were positive for *qnrS* genes. It is interesting to note that one particular transconjugant was found positive for *aac(6')-Ib-cr* as well as *qnrB*.

5. Nucleotide sequencing

The nucleotide sequences of the PMQR genes reported in our study are available in GenBank. The accession numbers assigned are: KR080534 to KR080543 for *aac(6')-Ib-cr*, KR080544 & KR080545 for *qnrB* and KR080546 for *qnrS*. All the identified PMQR genes were found to be closely related based on the pair-wise distance matrix value. The overall distance matrix for *aac(6')-Ib-cr* was found to be 2.642 whereas the pair-wise distance matrix for the *qnrB* gene sequences was found out be 1.255. The study did not attempt to identify variants of *qnr* genes. The pair-wise distance matrix of the *aac(6')-Ib-cr* gene has been summarised in supplementary data S2.

DISCUSSION

The preceding decade has witnessed a very high usage of fluoroquinolones (Geetha *et al.,* 2014). This extensive usage of fluoroquinolones has led to the emergence of Enterobacteriaceae isolates with reduced susceptibility to them. Interestingly, neither the fluoroquinolone consumption in the hospital nor the frequency of PMQR isolates varied much in the four years of the study (Fig. III). In Enterobacteriaceae, the three major groups of *qnr* determinants are *qnrA*, *qnrB* and *qnrS* (Geetha *et al.,* 2014) with *qnrD* having a prevalence of negligible extent. *qnrC* gene was not included in this study as it has got the least prevalence (Kim *et al.,* 2009).

In India, very few reports regarding the prevalence of PMQR are available compared to the number of PMQR prevalence findings reported from other countries even though, there are reports concerning the prevalence of PMQR among Enterobacteriaceae in India, a detailed prevalence report with a large sample size of clinical isolates is not available. This study included ciprofloxacin-resistant Enterobacteriaceae clinical isolates with high MIC values for the detection of PMQR determinants and has reported the prevalence of PMQR determinants from a large sample size for the first time in India.

A consistent rising trend of resistance among Enterobacteriaceae against fluoroquinolones particularly ciprofloxacin has been demonstrated in this study similar to previous reports. But there are also a few striking differences. Firstly, a variety of Enterobacteriaceae species were included in this study compared to the previous reports that are mostly limited to *Esch. coli* and *Klebsiella pneumoniae* with rare inclusions of *Proteus spp.* and *Enterobacter spp.* (Veldman *et*

al., 2011; Wang *et al.,* 2003; Pasom *et al.,* 2013; Yang *et al.,* 2014) [Fig. – IV]. This study reports the presence of PMQR in *Providencia rettgeri* for the first time. Secondly, we detected *aac(6')-Ib-cr* genes in a vast number of clinical isolates. Finally, the frequency of the *qnrB* & *qnrS* genes among the bacterial population studied is higher compared to previous reports. The first PMQR gene to be identified and reported was *qnrA* (Strahilevitz *et al.,* 2009) astonishingly, this allele was found to be absent among our clinical strains. It is remarkable to note that all the *Esch. coli* resistant to ciprofloxacin were found to carry one of the PMQR genes. This is worrisome because PMQR genes are capable of horizontal transfer thereby, accelerating the spread of this resistance mechanism among various clinical pathogens.

It is known that QRDR mutations induce high-level MICs while, PMQR genes induce low-level MICs (Strahilevitz *et al.,* 2009; Robicsek *et al.,* 2006b). But, of the 528 PMQR positive test isolates found in this study, 329 (62.2%) had MICs ≥64 µg/mL, while only 19 (16.5%) of the isolates lacking a PMQR gene had MICs ≥64 µg/mL (p value <0.0001). Therefore, there was a significant association between increased MIC and the presence of PMQR genes, opening up the possibility that PMQR genes could have contributed to high MICs among the test isolates. But, we must agree that the efflux pump activities of these test isolates were not elucidated and their QRDR mutation profile was also not identified. Thus, this particular finding of the study is inconclusive as to how instrumental PMQR genes are in increasing the MIC of a strain against ciprofloxacin. With future investigation of these clinical isolates for QRDR mutations and efflux mechanisms, the prominence of PMQR in fluoroquinolone resistance can be elucidated.

CONCLUSION

In the present study, we have elucidated the prevalence rate of the plasmid-mediated quinolone resistance genes among clinical Enterobacteriaceae isolates recovered from a tertiary care hospital in Puducherry, India. Resistance to fluoroquinolones has predominantly increased with a majority of the isolates exhibiting high MIC values. However, the significant finding of our study is that the prevalence of PMQR genes is on the rise.

Moreover, the majority of the literatures on *qnr* gene are on prevalence rates from around the world and reports on mechanistic aspects at the molecular level are very few. Future research should focus more the molecular mechanism of the PMQR genes and its encoded proteins.

Ethics Approval

The study was approved by JIPMER Institute Ethics Committee, Jawaharlal Institute of Post Graduate Medical Education and Research, Puducherry, India (ECR/324/Inst/PY/2013).

Grant Disclosures

This study was supported by JIPMER Institute Research Council intramural grant.

Competing Interest

The authors declare that they have no conflict of interest.

Authors Contributions

Thiyagarajan Yugendran and Belgode Narasimha Harish conceived and designed the study. Thiyagarajan Yugendran performed the experiments, analyzed the data and prepared the manuscript. Belgode Narasimha Harish reviewed the manuscript.

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Table 1(on next page)

Table - 1

Table 1 - List of PCR primer pairs used in this study.

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Gene	Primer Sequence (5'-3')		Amplicon Size (bp)	Reference	PCR Condition	
					T†	T‡
qnrA	Forward	CAGCAAGAGGATTTCTCACG	630	8	58	30
	Reverse	AATCCGGCAGCACTATTACTC				
qnrB	Forward	GGCTGTCAGTTCTATGATCG	488	8	59.1	30
	Reverse	SAKCAACGATGCCTGGTAG				
qnrD	Forward	CGAGATCAATTTACGGGGAATA	581	9	57	30
	Reverse	AACAAGCTGAAGCGCCTG				
qnrS	Forward	GCAAGTTCATTGAACAGGGT	428	10	55.6	30
	Reverse	TCTAAACCGTCGAGTTCGGCG				
aac(6')-Ib-cr	Forward	TTGGAAGCGGGGACGGAM	260	11	58	30
	Reverse	ACACGGCTGGACCATA				
intI1	Forward	GTTCGGTCAAGGTTCTG	920	4	55	45
	Reverse	GCCAACTTTCAGCACATG				
intI2	Forward	ATGTCTAACAGTCCATTTTT	420	4	55	30
	Reverse	AAATCTTTAACCCGCAAAC				

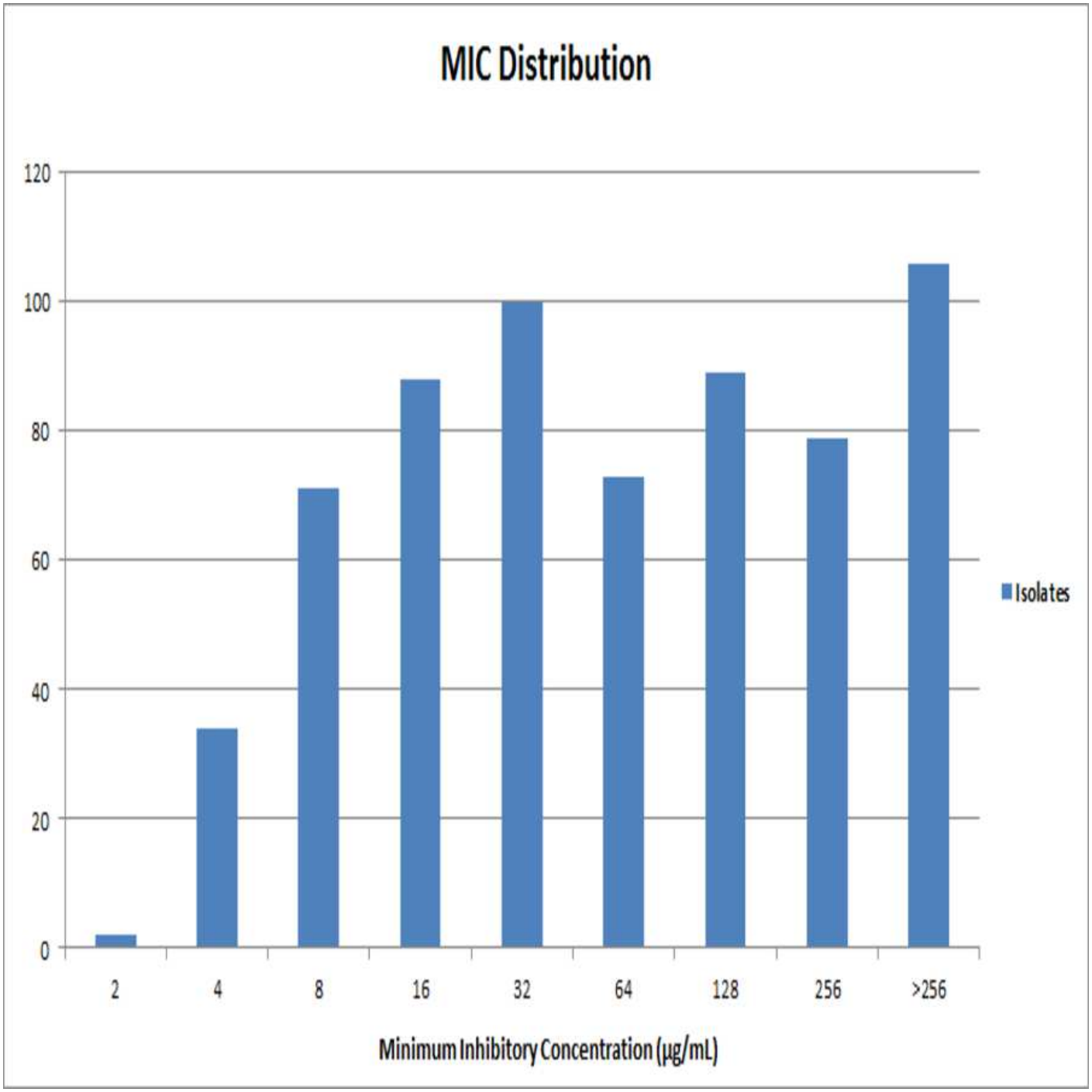
1

2 †annealing temperature in ˚C; ‡extension time in sec

1

Figure- 1

Fig 1 - Minimum Inhibitory Concentration (MIC) distribution of the test isolates

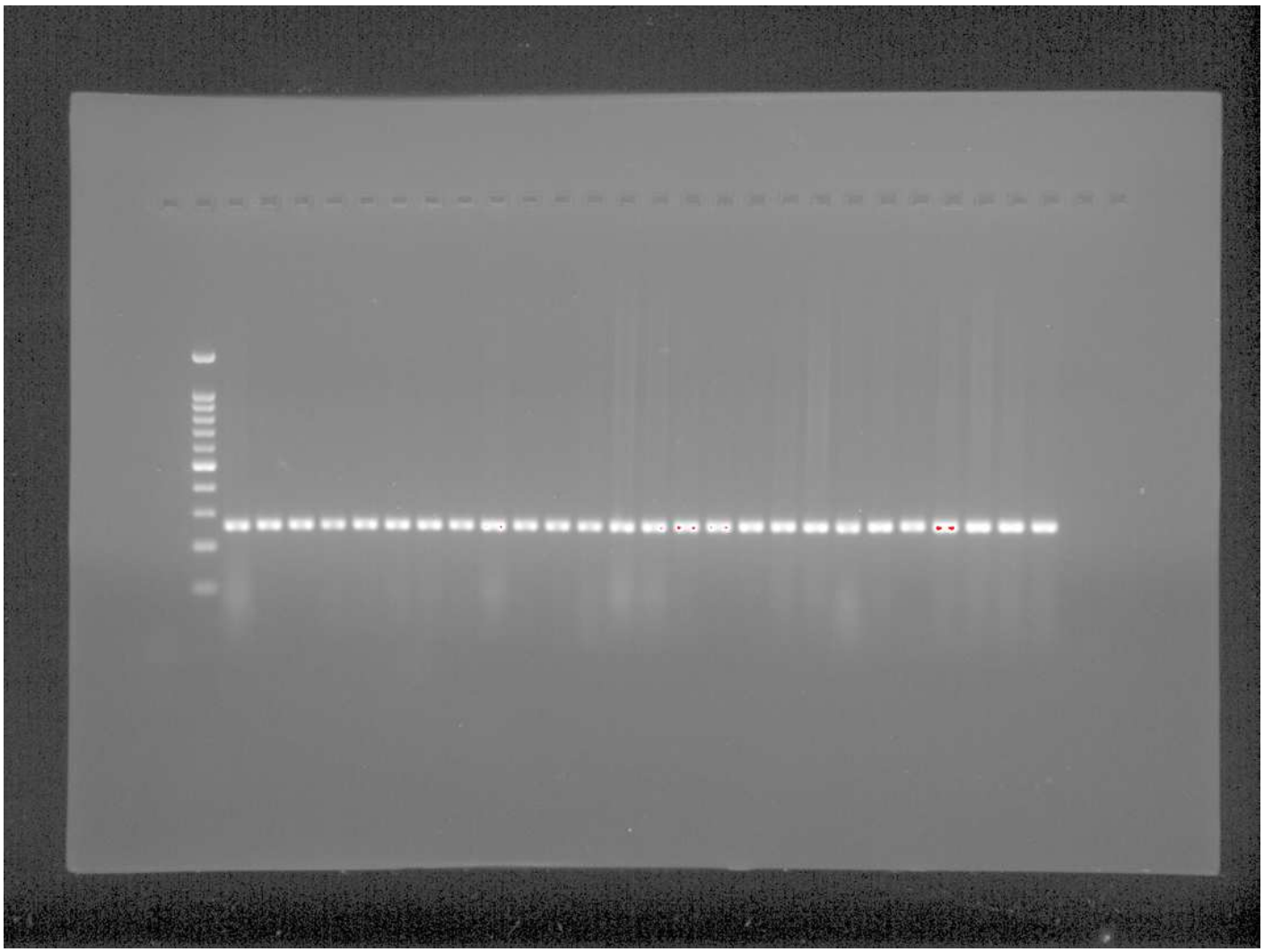


2

Figure - 2A

Fig 2A - Gel documentation of PCR assay for aac (6')-Ib-cr gene. Lane 2: 100 bp DNA ladder, Lane 3: Positive control (amplicon size – 260 bp), Lane 4-28: aac(6')-Ib-cr positive isolates, Lane 29: Negative control

**Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.*

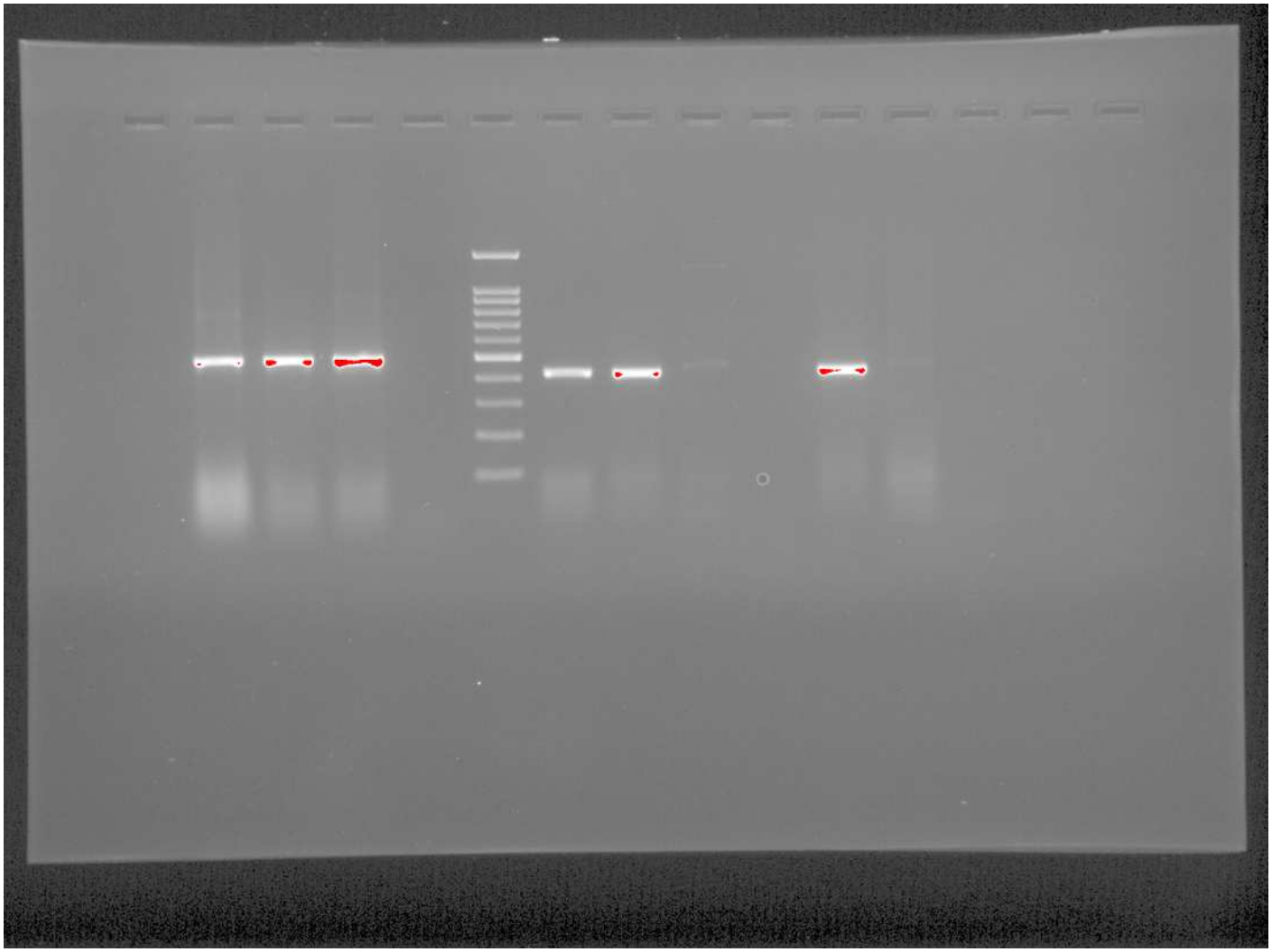


3

Figure - 2B

Fig 2B -Gel documentation of PCR assay for *qnr* alleles. Lane 2: *qnrB* Positive control (amplicon size – 488 bp), Lane 3-4: *qnrB* positive isolates, Lane 5: Negative control, Lane 6:100 bp DNA ladder, Lane 7: *qnrS* Positive control (amplicon size – 428 bp) , Lane 8,11: *qnrS* positive isolates, Lane 9-10: *qnrS* negative isolates, Lane 12: Negative control

**Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.*



4

Figure - 3

Fig 3 - Graphical representation of year-wise consumption of ciprofoxacin tablets in JIPMER hospital and the annual frequency of PMQR isolates for four years.

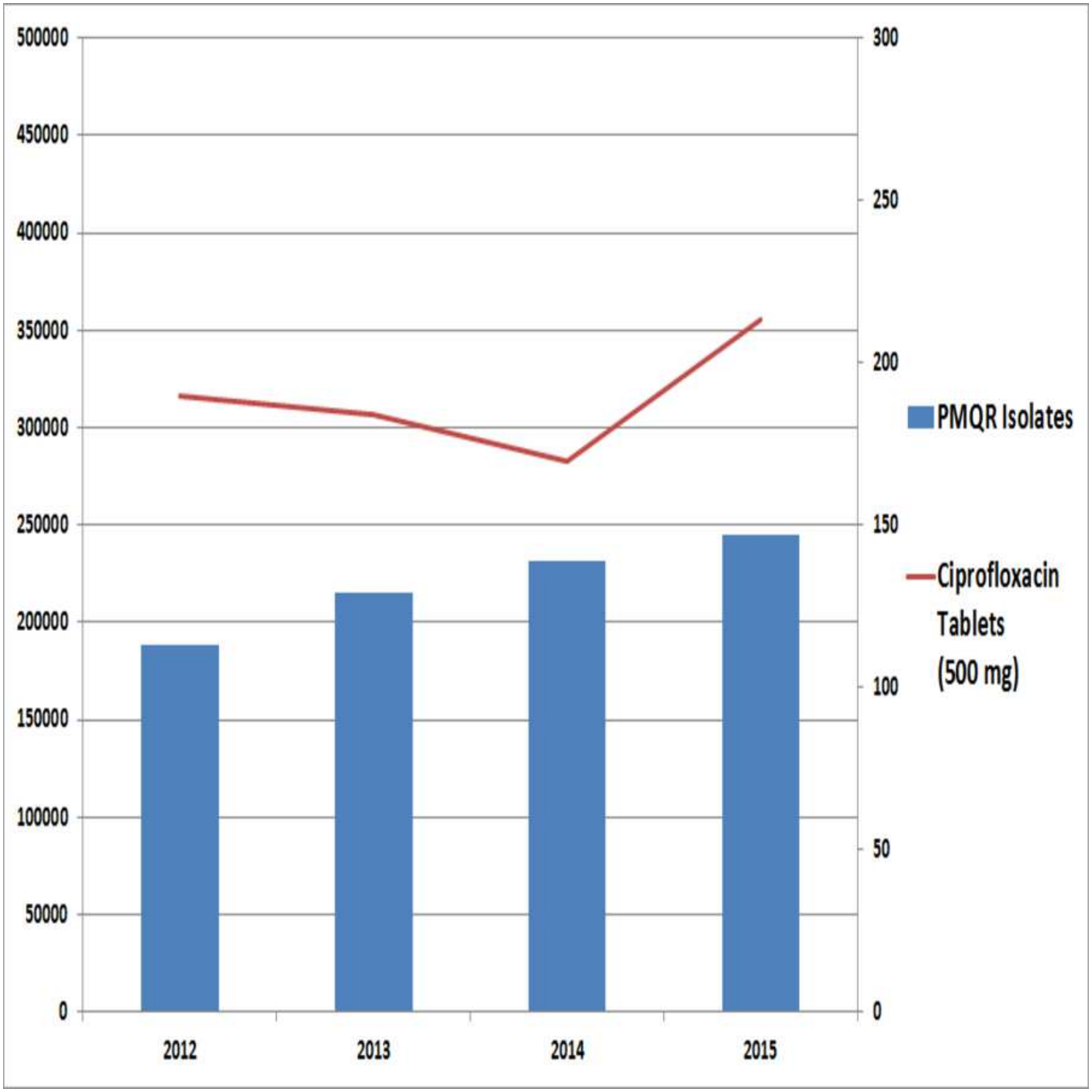


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Figure - 4

Fig 4 - Prevalence distribution of the identified PMQR genes among the test isolates.