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A STUDY ON PLASMID MEDIATED CIPROFLOXACIN RESISTANCE IN PATHOGENIC ENTEROBACTERIACEAE ISOLATED IN A TERTIARY CARE HOSPITAL

Microbiology

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ABSTRACT

Introduction: Widespread use of fluoroquinolones has resulted in the emergence of resistance to fluoroquinolones. There are many loci in bacterial genomes which are associated with quinolone resistance. This study aims at detecting two quinolone resistant plasmids in pathogenic Enterobacteriaceae isolates from various clinical samples received in the microbiology lab.

Materials and methods: This study was carried out in a tertiary care hospital for a period of 6 months. All the Enterobacteriaceae isolated from the clinical samples in the lab were identified and subjected to antibiotic sensitivity testing by Kirby Bauer's disc diffusion technique according to CLSI guidelines. MIC of these ciprofloxacin resistant isolates were determined and those with MIC >0.5mg/L were subjected to polymerase chain reaction for the detection of qepA and aac-(6^{I})-Ib-cr plasmids.

Results and discussion: Escherichia coli was the most common isolate harbouring the plasmids of interest. 98% of the ciprofloxacin resistant isolates harboured the aac-(6^{I})-Ib-cr plasmid while only 31 % of the ciprofloxacin resistant isolates harboured the qepA plasmid.

Conclusion: Since these quinolone resistance plasmids were discovered after 1998, not many studies conducted in India to detect these plasmids. It is necessary to study the phenotypic and other changes associated with fluoroquinolone resistance to initiate suitable control measures.

KEYWORDS

Ciprofloxacin resistance, Enterobacteriaceae, qepA and aac-(6^{I})-Ib-cr plasmids.

INTRODUCTION:

Fluoroquinolones have a broad spectrum of activity and are not very expensive- this has enabled the widespread use of this drug in human as well as in veterinary practice. Ciprofloxacin was introduced in 1987 [1]. Since then it is on World Health Organization's List of Essential Medicines, which includes the most important medications needed in a basic health system[2]. The indiscriminate use of this antibiotic has resulted in the rapid emergence of resistance worldwide. Quinolones were accidentally discovered as byproducts during the synthesis of chloroquine. The primary target of quinolones is Topoisomerase IV in Gram positive bacteria and DNAgyrase in Gram negative bacteria thereby resulting in topological stress and DNA damage during cell division resulting in activation of apoptotic pathway. There are four main mechanisms involved in quinolone resistance –

1. **Mutations in QRDR (Quinolone Resistance Determining Region)**-the region occupied by
- gyrA, gyrB and oriA genes coding for DNA gyrase -Gram negative bacteria
- parC and parE genes coding for Topoisomerase IV -Gram positive bacteria

2. **Over expression of endogenous MDR efflux pumps** which result in the decrease of drug concentration within the bacteria

3. **Decrease in the number of porins** can confer resistance

4. **PMQR (Plasmid mediated Quinolone Resistance)-**
- **quinolone resistance plasmids –qnr A, qnrB, qnrC, qnrD, qnrS**- protects the complex of DNA and DNA gyrase or topoisomerase IV enzymes from the inhibitory effect of quinolones
- **oqxAB and qepA** encode efflux pumps that extrude quinolones
- **aac(6′)-Ib-cr** encodes a variant aminoglycoside acetyltransferase with two amino acid alterations allowing it to inactivate ciprofloxacin through the acetylation

The first plasmid for quinolone resistance qnr A1 was discovered in 1998 [4]. Since then many plasmids mediating quinolone resistance have been discovered. This study aims at detecting two quinolone resistant plasmids- aac-(6^{I})-Ib-cr plasmid and the qep A plasmid- in pathogenic Enterobacteriaceae isolates (except Salmonella species and Shigella species) from various clinical samples (except blood and stool) received in the microbiology lab.

MATERIALS AND METHODS:

This study was carried out in a tertiary care hospital for a period of 6 months after obtaining clearance from the institutional ethical committee. All the Enterobacteriaceae isolated from the clinical samples (except blood and stool) in the lab were identified using the appropriate biochemical tests and subjected to antibiotic sensitivity testing by Kirby Bauer's disc diffusion technique according to CLSI guidelines. The samples included in this study were urine, respiratory samples like sputum, endotracheal aspirates, throat swab, bronchial washings, broncho-alveolar lavage, pleural fluid and exudates like pus, wound swab, csf, vaginal swab, ear swab, ascitic fluid, etc. Minimum inhibitory concentration (MIC) of ciprofloxacin for those isolates which were ciprofloxacin resistant by disc diffusion was determined by E-test using ciprofloxacin Ezy MIC strip (Paper strip which is coated with ciprofloxacin in a concentration gradient manner).Those showing high level resistance MIC > 256mcg/ml were subjected to polymerase chain reaction for the detection of qep A and aac-(6^{I})-Ib-cr plasmids.

The DNA was extracted using DNA purification kit (Pure Fast® Bacterial Genomic DNA purification kit). The aac-(6^{I})-lb-cr gene was detected using primers **F**: 5'-CCCGCTTTCTCGTAGCA-3'**R:** 5'-TTAGGCATCACTGCGTCTTC-3' and qep A was detected using **F**: 5'-CGTGTTGCTGGAGTTCTTC-3' **R:** 5'- CTGCA GGTA CTGCG TCATG-3'

obtained from Helini Biomolecules, Chennai, India. The reaction mixture contained Master mix **(** 25µl of Master Mix contains: 10X Taq buffer, 2mM Mgcl2, 0.4mM dNTPs mix, and 2U Proofreading Taq DNA polymerase)- 10µl, Primer – forward (10pmoles/µl)- 5µl, Primer- reverse (10pmoles/µl)-5µl, Genomic DNA - 5µl. The reaction was performed in a thermal cycler programmed for initial denaturation at 94ºC for 3 min, followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 58ºC for 1 minute and extension at 72ºC for 1min. Final extension was performed at 72º C for 5 min. The amplified products were then analysed by agarose gel electrophoresis using gel loading dye along with 10µl Helini 100bp DNA Ladder. The gel was then viewed in UV Transilluminator and the band pattern was observed.

RESULTS:

During the period of study, 4161 urine samples, 1203 exudate samples and 621 respiratory samples were received. 4161 urine samples were collected, among them 1124 were culture positive and 831 of these belonged to Enterobacteriaceae and 411 of them were Ciprofloxacin

resistant. Similarly among the respiratory samples, of the 621 samples received, 273 were culture positive, 123 were Enterobacteriaceae and 56 ciprofloxacin resistant. Finally among the 1203 exudates samples received in the lab, 686 samples were culture positive, of which 226 were members of the Enterobacteriaceae family and 115 were ciprofloxacin resistant. This is illustrated in **Table** 1. The identification and distribution of these organisms among the different samples is shown in the **Figure 1**.

Table 1: Sample wise distribution of organisms

Organism	Urine Total samples received:4161 Culture positive:1124		Exudates Total samples received:1203 Culture positive :686		Respiratory samples Total samples received: 621 Culture positive:273		Total	Ciprofoxacin Resistant isolates
	Total	Ciprofoxacin Resistant isolates	Total	Ciprofoxacin Resistant isolates	Total	Ciprofoxacin Resistant isolates		
Escherichia coli	515	350	97	56	34	24	646	430
Klebsiella species	183	59	68	29	69	21	320	109
Citrobacter species	75	27	9	8	15	8	99	43
Enterobacter species	25	0	13	7	5	3	43	10
Proteus mirabilis	17	0	25	15	0	0	42	15
Proteus vulgaris	16	5	14	0	0	0	30	5
Total	831	411	226	115	123	56	1180	612

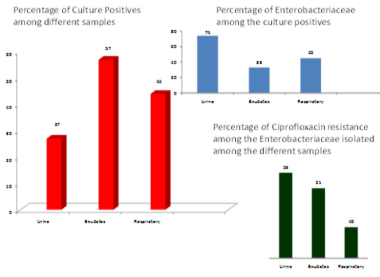

Figure 1: Percentage of culture positives and ciprofloxacin resistant Enterobacteriaceae among each group of samples

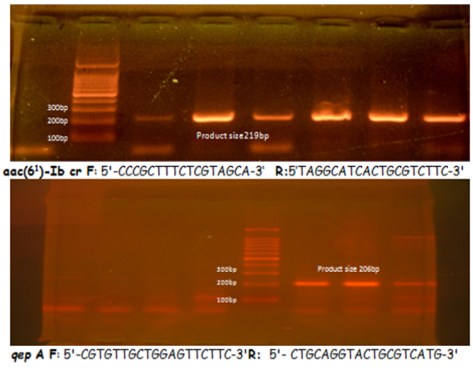

Figure 2: PCR analysis of aac(61)-Ib cr and qep A gene

Table 1 shows that the commonest organism to be isolated is Escherichia coli followed by Klebsiella species, Citrobacter species, Enterobacter species, Proteus mirabilis and Proteus vulgaris. Ciprofloxacin resistance is common in Escherichia coli – 66.5% of isolates were found to be resistant. The next commonest organism found to be ciprofloxacin resistant was Citrobacter species(43.4%), followed by Proteus mirabilis (35.7%), Klebsiella species (34.1%), Enterobacter species (23.3%) and Proteus vulgaris (16.7%). MIC of > 256mcg/ml for Ciprofloxacin was seen in 108 of the total 612 ciprofloxacin resistant isolates. The others showed MICs ranging between 16mcg/ml - 128mcg/ml. Of the 108 isolates, 106 isolates (98%) harboured the aac-(6^{1})-Ib-cr plasmid and 33 isolates (31%) harboured the qep A plasmid. **Figure 2** shows the gel documentation of the two plasmids of interest. The distribution of the plasmids of interest is as shown in **Table 2.**

Table 2: Distribution of aac - (61)- Ib- cr and qep A plasmids among the various organisms isolated

Organism	aac - (61)- Ib- cr	qep A
Escherichia coli	63	23
Klebsiella species	18	7
Citrobacter species	8	2
Enterobacter species	8	1
Proteus mirabilis	4	-
Proteus vulgaris	2	-

DISCUSSION

Members of the Enterobacteriaceae are the commonest Gram negative organisms to be isolated in clinical samples in our lab. Escherichia coli shows the highest incidence among them. A high prevalence of resistance to fluoroquinolones has been noted in Enterobacteriaceae owing to the extensive use of these drugs in clinical practice right from the time these drugs were introduced. This study has recorded an overall resistance of about 52 % amongst all the Enterobacteriaceae isolated from various clinical samples over a period six months, of which Escherichia coli recorded the highest incidence of ciprofloxacin resistance of about 67%. A study from Shanghai has reported that the percentage of ciprofloxacin resistance in E.coli has exceeded 50%. This resistance is highly disheartening. Most of the isolates showed high level resistance to ciprofloxacin.

When quinolones were introduced there were no pre-existing natural resistance genes because they were purely synthetic drugs. Literature and studies [5-8] in the past suggest that high level quinolone resistance is possible only due to two or more simultaneous chromosomal mutations in the QRDR. This kind of resistance can be passed on only vertically to progeny bacteria. The plasmids which were discovered later and responsible for low level ciprofloxacin resistance resulted in horizontal transfer of resistance at a much faster rate and also to unrelated bacteria resulting in rapid spread of multidrug resistance genes. So it is possible that chromosomal mutations as well as plasmid mediated mechanisms can act together resulting in the spread of high level ciprofloxacin resistance at a rapid rate. All these changes can cause selection of mutants with high level quinolone resistance [9].However this study has not elucidated the QRDR mutation profile. The qnr genes were also not analysed because there were many studies which had studied the prevalence of this gene. This study mainly analysed the prevalence of aac(6^{1})-Ib cr and qep A gene which were not exploited in many studies carried out in South India. It revealed that 98% of those isolates which showed high level ciprofloxacin resistance harboured the aac(6^{1})-Ib cr gene while 31% harboured the qep A gene. The MIC of ciprofloxacin was severalfold higher for those isolates which harboured the qepA gene. It was also noted that most of the isolates which harboured this plasmid were simultaneously resistant to other groups of antibiotics like beta lactams and aminoglycosides. Reports[10] suggest that dissemination of qep A plasmid along with other well known plasmids known to be responsible for ESBL production, aminoglycoside resistance offer the bacteria a survival advantage in livestock breeding environment as well as in clinical settings. However this study has not analysed the presence of co-existence of other plasmids responsible for antibiotic resistance of other groups of antibiotics, which might be transmitted simultaneously.

CONCLUSION:

Quinolone resistance is very common in our hospital as in other hospitals. Lot of plasmids and chromosomal mutations are involved in quinolone resistance. This has led to the great speed with which resistance has disseminated. Moreover plasmids can be transferred horizontally which can lead to spread of resistance among diverse group of organisms. It is therefore necessary to study phenotypic and other changes associated with quinolone resistance in order to curtail the spread of resistance and initiate control measures.

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