Status of qnr and aac(6')-lb-cr Genes in Quinolone-Resistant Non-Fermenters

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Abstract— *Non-fermenters are aerobic non-spore forming Gram-negative bacilli that either incapable of catabolized carbohydrates as a source of energy or degrade them via oxidative rather than fermentative metabolic pathways. In the group of non-fermenters Pseudomonas aeruginosa is eminent pathogen followed by Acinetobacter baumanni. Both of these organisms are reported for their intrinsic resistance against antibiotics and their ability to acquire gens that encode resistance. Resistant against quinolones like ciprofloxacin is common against Gram's negative non-fermenters. For most of the mechanism of resistant in both of these organisms against quinolone, is the production of aminoglycoside modifying enzymes, mutations in topoisomerases, up-regulation of efflux pumps and presence of plasmid mediated qnr genes. In this study, during the study period total 94 non-fermenters were isolated from 378 numbers of different clinical specimens. Out of 94; 21 isolates were found resistant against quinolone antibiotics like ciprofloxacin-5 µg, levofloxacin-5 µg , norfloxacin-10 µg, which were subjected for PCR studies to detect resistant genes, only five (23.81%) isolates were found to be associated with aac(6')-lb-cr and two (9.52%) were found positive for carrying qnrD gense as additional with aac(6')-lb-cr genes. Organisms that processed qnrD and aac(6')-lb-cr gens were, Pseudomona aeruginosa, Acinetobacter baumanni , Alcaligenes faecalis , Acinetobacter calcoaceticus, and Pseudomonas sp.*

Key word: Non-fermenters, Quinolone resistant, PCR studies, QNR, aac(6')-Ib-cr.

I. INTRODUCTION

Because of wide spread use of antibiotics now a day's resistance is a global problem. Resistant bacterial infection is always typical because of the high antibiotic selection pressure and concentration of susceptible patients. Problem has started to reach into the community setting as exemplified by the recent emergence of community-acquired quinolones resistant non-fermenters in many parts of the world. [1]

Families of broad-spectrum synthetic antibacterial drug that drive from quinoline compounds are called quinolones. It is a class of antibiotics that acts by interrupting the replication of DNA molecules in bacteria. The action involves inhibition of the bacteria's gyrase so that positively super coiled DNA cannot be relaxed for DNA transcription and replication [*Hawkey PM. (2003). Mechanisms of quinolone action and microbial response. J antimicrobe chemother, 51 (Suppl 1):29-35]*. These quinolones are like Norfloxacin, Ciprofloxacin, Levofloxacin etc.

By definition group of aerobic non-spore forming Gram's negative bacilli that either incapable of catabolized carbohydrates as source of energy or degrade them via oxidative rather than fermentative metabolic pathways are called non-fermenters.[3] In human infection non-fermenters play critical role due

to their multidrug resistant property against all common used antibiotics.[4] In the group of non-fermenters *Pseudomonas aeruginosa* is eminent pathogen followed by *Acinetobacter baumanni*.[5,6]

In *P. aeruginosa* quinolones resistance is mostly related to changes in topoisomerases, whereas permeability altered and efflux pumps production is also responsible, in *Acinetobacter baumanni* resistance to quinolones due to the changes in the protein targets and also to the over expression of efflux pumps. [6] *qnr* genes carried by plasmid DNA are found to mediate quinolone resistance.[7] Rate of *qnr* mediated resistance is very low; it can present a higher level if it interacts with genomic determinants.[8]

qnr genes are very common in almost all genera of the family *Enterobacteriaceae*.[9] Five classes of these genes are currently identified and they are *qnrA, qnrB, qnrC, qnrD* and *qnrS.* [10,11] Proteins driven from this gens are known *Qnr*A, *Qnr*B, *Qnr*S, *Qnr*C and *Qnr*D proteins which protect DNA gyrase and topoisomerase IV from inhibition by quinolones. Gene *aac (6')–Ib–cr* the aminoglycoside acetylteransferase variant capable of acetylating and subsequently reducing the activity of ciprofloxacin and norfloxacin. [12]

Therefore, the current study, aimed to detect the quinolones resistant organisms through Antibiotic sensitivity test and to identify the responsible genes for quinolones resistant through PCR studies.

II. METHODOLOGY

A laboratory based observational study was conducted on 378 numbers of different clinical specimens were collected from different Govt. hospitals, Private hospitals, and various pathological laboratories of Beed and Akola districts of Maharashtra, India.

2.1 Identification of Non-fermenters

Identification was carried out according to Koneman's color atlas and text book of Diagnostic Microbiology.[3] (Sixth edition).

2.2 Antibiotic Susceptibility

Susceptibility test were performed by using disk diffusion method where antibiotic disc of Ciprofloxacin -5µg, Levofloxacin-5µg, Norfloxacin-5µg were used.

2.3 Isolation of DNA sample

For DNA preparation bacterial cell were collected in 1.5 ml eppendrof tube containing 500µl of distilled water. The tubes were given heat shock treatment in hot water bath at 80°C for 20 minutes. After heat shock treatment the tubes were centrifuged at 1320 rpm for 10 min. The supernatant was collected and used as crude DNA or total DNA samples.

2.4 Characterization of quinilone resistant gene

PCR assay was performed to detect qunolone resistant gene according to Cano ME. *et al.* 2009[14] and Cavaco ML. *et al.* 2009.[15]

2.5 Oligonucleotides used as primers in the study

Primer pairs of Qnr-1 and Qnr-2 that targeting different regions were designated, Qnr-1 was designated to detected *qnrA, qnrB, qnrS,* and *qepA* and Qnr-2 was designated to detected *aac(6_)-lb-cr, qnr-D, and gyra-A.*

Primer Pairs	Type	Target	Sequence (5'-3')	Amplified product size (BP)	Gene Bank accession number	Reference
qnrA-1A:- qnrA-1B:-	Qnr-1	qnrA	TTCAGCAAGAGGATTTCTCA GGCAGCACTATTACTCCCAA	628	AY070235.1	Trans, J.H. *et al* (2002)[16]
qnrB- FQ1:- qnrB- FQ2:-	Qnr-1	qnrB	ATGACGGCCATTACTGTATAA GTTTGCTGCTCGCCAGTCGA	546	DQ351241.1	Jcoby, G.A. *et al.* (2006)[17]
qnrS-1A:- qnrS-1B:-	Qnr-1	qnrS	CAATCATACATATCGGCACC TCAGGATAAACAACAATACCC	675	AB187515.1	Hata, M. & Suzuki, M *et al.* (2005)[18]
qepA-F:- qepA-R:-	Qnr-1	qepA	GCAGGTCCAGCAGCGGGTAG CACGATACTCGGGCAGGAAG	260	NC_010558.1	Perichon, B. *et al.* 2008[18]
aac(6_)-lb-cr-F aac(6_)-lb-cr-R	Qnr-2	aac(6_)-lb-cr	ATG ACT GAG CAT GAC CTT GC TTA GGC ATC ACT GCG TGT TC	519	KJ488983.1	Cano. M. E. *et al.* 2009[14]
qnrD-F qnrD-R	Qnr-2	qnrD	CGAGATCAATTTACGGGGAATA AACAAGCTGAAGCGCCTG	582	EU692908	Cavaco, M.L. *et al.* 2009[15]
qnrC-F qnrC-R	Qnr-2	qnrC	GGGTTGTACATTTATTGAATC TCCACTTTACGAGGTTCT	447	EU917444	Wang. M. *et al.* 2009[19]

2.6 Reaction mixtures

Amplification reaction was performed in 25µl volume containing 1µl each primer, 12.5µl Taq mixture, 1.5 µl of template DNA and rest amount is filled with nucleous free water (3 µl).

2.7 Reaction conditions

Reaction was performed in a DNA thermal cycler (Bio-Rad). Initial denaturation was performed at 95°C for 2 minutes followed by 32 cycle of denaturation at 95°C for 20 seconds, annealing at 57°C for 30 seconds and extension at 72°C for 1 minute and then final extension at 72°C for 5 minutes.

2.8 Gel electrophoresis and analysis

After PCR amplification 5µl each of amplicon was separated by electrophoresis in 1% agarose gel, 10µl of Ethidium bromide was added to the agarose. Both assay products were electrophoresed for one and half hour at 50 V in 1XTBE buffer. The gel was imaged in gel doc EZ imager (Bio-Rad). PCR amplicon size was calculated by comparison to molecular weight size marker. (100BP and 1000 BP hyper ladder).

2.9 Transformation study

To observe the horizontal gene transfer capability of quinolone resistant genes, positive isolates were further subjected for plasmid DNA isolation and purification by using qiagen plasmid DNA isolation and purification kit. It was then further transferred to *E.coli* JM107 competent cell where plate containing 0.5µg/ml concentration of ciprofloxacin

III. RESULTS

In present study out of 378 numbers of different clinical specimens, 94 (24.86%) non-fermenters were isolated. Majority of these non-fermenters isolates founds were *Pseudomona aeruginosa* followed by *Pseudomonas fluorescens, Acinetobacter baumanni, Acinetobacter calcoaceticus, Alcaligenes faecalis* and *Pseudomonas sp.* (Table 1)

Table 1
Frequency distribution of isolated non-fermenters from different clinical specimens

S. No.	Isolates	Number of specimens	% of specimens
1	*Pseudomona aeruginosa*	**78**	**80.41**
2	*Pseudomonas fluorescens*	**08**	**8.25**
3	*Acinetobacter baumanni*	**05**	**5.15**
4	*Acinetobacter calcoaceticus*	**01**	**1.03**
5	*Alcaligenes faecalis*	**01**	**1.03**
6	*Pseudomonas sp.*	**01**	**1.03**
	Total	**94**	**100**

Out of these 94 non-fermenters isolated, 21 (21.65%) isolates were found resistant against quinolone antibiotics like ciprofloxacin-5 µg, levofloxacin-5 µg , norfloxacin-10 µg. (Table 2)

Table 2
Frequency of the quinolone resistant organisms

S. No.	Isolates	Number of specimens	% of specimens
1	*Pseudomona aeruginosa*	**13**	**61.90**
2	*Acinetobacter baumanni*	**05**	**23.81**
3	*Acinetobacter calcoaceticus*	**01**	**4.76**
4	*Alcaligenes faecalis*	**01**	**4.76**
5	*Pseudomonas sp.*	**01**	**4.76**
	Total	**21**	**100**

Out of these 21 non-fermenters resistant against quinolone antibiotics which were subjected for PCR studies to detect resistant genes, only five (23.81%) isolates were found to be associated with aac(6')-lb-cr and two (9.52%) were found positive for carrying qnrD gense as additional with aac(6')-lb-cr genes. (Table 3)

Table 3
Frequency of the qnr Genes

S. No.	Isolates	Genes
1	*Pseudomona aeruginosa*	**aac(6')-Ib-cr, qnr D**
2	*Acinetobacter baumanni*	**aac(6')-Ib-cr**
3	*Acinetobacter calcoaceticus*	**aac(6')-Ib-cr**
4	*Alcaligenes faecalis*	**aac(6')-Ib-cr**
5	*Pseudomonas sp.*	**aac(6')-Ib-cr, qnr D**

3.1 Finding of polymerase chain reactions

After PCR reaction five (5) out of twenty one (21) quinolone resistant organisms were found to be associated with *aac(6')-lb-cr* and two (2) were associated with *qnrD* gens. Organisms that processed *aac(6')-lb-cr* and *qnrD* gens were, *Pseudomona aeruginosa, Acinetobacter baumanni , Acinetobacter calcoaceticus, Alcaligenes faecalis* , and *Pseudomonas sp.*

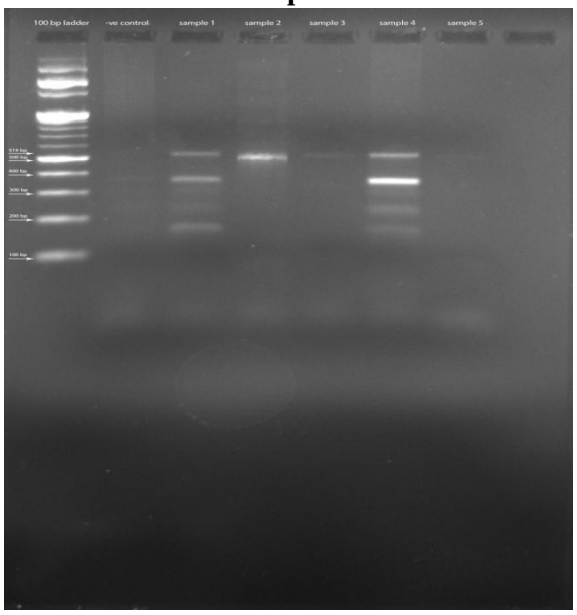
From this study it was confirmed that there were no detection of Qnr-A, Qnr-B, Qnr-S and QepA gene and aac(6')-lb-cr Genes. Multiplex PCR result of QNR-2 class gene confirm the presence of *aac(6')-lb-cr* gene in all five isolates and *QnrD* gene in sample 1 and 5. Presence of *aac(6')-lb-cr* gene was further justified by using simplex PCR studies by using *aac(6')-lb-cr* as primer.

3.2 Findings of transformations study

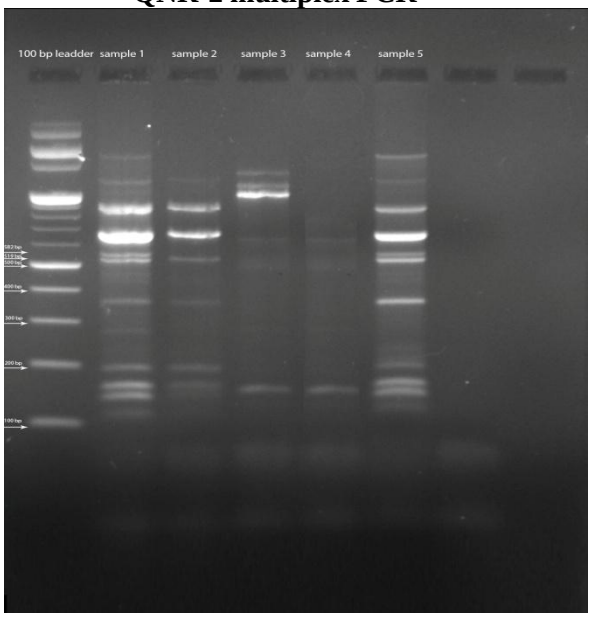
It was found that *pseudomonas aeruginos, Alcaligenes faecalis* and *Pseudomonas sp.* were found positive for horizontal gene transfer. Which may indicate the presence of *qnrD* and aac(6')-lb-cr genes either encoded by plasmid DNA or may coexistent with chromosomal DNA.

Figure 1
PCR Amplification Products of aac (6')-lb-cr simplex and *aac* (6') Genes (Photo) Table 3)

aac simplex



QNR-2 multiplex PCR



IV. DISCUSSION

In present study The most frequently encountered non-fermentative Grams's negative bacilli were found to be *Pseudomonas aeruginosa,* and it was the predominant bacterial strain in all specimens. It is similar to other study of Rit *et al*. (2013).

In this study 21 quinolone resistant organisms were examined for the presence of *qnr* and *aac(6')-lb-cr* genes by PCR. It was found that *aac(6')-lb-cr* gene was more prevalent than *qnr* classes of gene in isolated non-fermenters. Cayci *et. al.* 2014 reported the presence of *aac(6')-lb-cr* gene in *Pseudomonas aeruginosa* from Turkey and it is similar to the present study. Multiplex PCR reaction of qnr2 sets gene indicate the presence of *qnrD* gene and very clear band observed at 582 bp in *Pseudomonas aeruginosa* and *Pseudomonas sp.*

It has been described previously that chromosomal mutation in bacterial topoisomerase gene and genes regulating expression of efflux pump or both responsible for quinolone resistance. At present Plasmid-mediated quinolone resistance has been reported in several parts of the world (Oktem *et al.* 2008). Wide spread use of quinolones lead to the reduced susceptibility to quinolones. In India very few reports are available regarding plasmid encoding qnr mediated quinolones resistance. However, numerous

additional qnr genes have recently been identified, suggesting that they have existed in nature for many years. *aac(6')-lb-cr* are the main enzymes responsible for aminoglycoside acetylation in *Pseudomonas aeruginosa.*

V. CONCLUSION

Clinical isolates of non-fermenters, along with others are constantly exposed to hospital environments where they have gained resistance to most antibiotics by various mechanisms. In this study it was found that isolated non-fermenters process *aac(6')-lb-cr* and *qnrD* type gens that mostly encoded by plasmid DNA. These non-fermenters could pose a serious health hazardous if given a chance to infect the community.

CONFLICT

None declared till date.

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