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RESEARCH ARTICLE

Molecular detection of *aac(6')-Ib-cr* among clinical Enterobacterial isolates conferring quinolone resistance:- A study from North east India

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ABSTRACT

Objective: Fluoroquinolones are the commonly prescribed antimicrobial for Gram-negative and Gram-positive microorganisms. The resistance against these antibiotics is of importance in therapy. The current study aimed at determination of *aac(6')-Ib-cr* gene within the Enterobacterial isolates and their transmission dynamics.

Materials and Methods: Ninety-seven isolates of *Enterobacteriaceae* collected during May 2013 to October 2013 were screened by the antimicrobial susceptibility test. The plasmids from these isolates were analysed by specific Polymerase chain Reaction (PCR) for *qnr*A, *qnr*B, *qnrD*, *qnrC*, and *aac(6')-1b-cr* and the horizontal dissemination of these plasmid mediated quinolone determinants and *aac(6')-1b-cr* genes were determined by transformation.

Results: Our analysis showed that 94 out of 97 phenotypically screened positive isolates, 85.57% (n = 83), 84.53% (n=82) were resistant to norfloxacin, ciprofloxacin respectively. Coexisting *qnr*D genes was identified in 11isolates carrying *aac(6')-Ib-cr* gene. The PMQR and *aac(6')-Ib-cr* was demonstrated by transforming the plasmid carrying *qnr*D and *aac(6')-Ib-cr* gene into *E. coli* strain DH5α. PCR assay confirmed that *aac(6')-Ib-cr* was present in all the transformants.

Conclusion: Our report shows the presence of heterogenous resistance mechanism can lead to worrisome condition at the nosocomial level and community acquired infection. So *aac(6')-Ib-cr* containing strains should be promptly detected and referred to clinicians so that the treatment with non-hydrophilic FQs, such as levofloxacin and ofloxacin, or other classes of antibiotics to prevent high level resistance onset and spread. *J Microbiol Infect Dis 2016;6(3): 97-102*

Keywords: *Aac(6')-1b-cr; Enterobacteriaceae;* plasmid-mediated quinolone resistance*; qnrD; qnrC*

INTRODUCTION

Resistance to quinolone among the *Enterobacteriaceae* is related primarily to the mutation of chromosomal genes encoding DNA gyrase, topoisomerase IV, regulatory efflux pumps, and/or porins [1], reports indicate that quinolone resistance may also be related to plasmid-mediated genes. Since plasmid-mediated quinolone resistance (PMQR) was first described in 1998 [2], four types of PMQR determinants have been identified: *qnr, aac(6')-Ib-cr, qepA*, and *oqxAB* [3]. The last PMQR gene, *aac(6')-Ib-cr*, encodes a bifunctional aminoglycoside 6'*N*-acetyltransferase capable of acetylating both aminoglycosides and fluoroquinolones [4,5]. This gene is a variant of the classic *aac(6')-Ib-cr* gene, which confers resistance to certain aminoglycosides (amikacin, isepamicin, and tobramycin). This *aac(6')-Ib-cr* enzyme includes two mutations (Trp102Arg and Asp179Tyr) that reduce aminoglycoside resistance but confer resistance to ciprofloxacin and norfloxacin. *aac(6')-Ib-cr* is often found as part of a complex class 1 integron including other antibiotic resistance genes. The fact that strains of *Enterobacteriaceae* harboring *aac(6')-Ib-cr* have been described worldwide [6]. Ciprofloxacin MICs increases 2-to 4-fold due to the acquisition of *aac(6')-Ib-cr* genes; however, ciprofloxacin MICs remain below the susceptibility breakpoint according to the Clinical and Laboratory Standards Institute (1 µg/ml) [7]. The current study aimed at determination of *aac(6')-Ib-cr* gene within the Enterobacterial and their transmission dynamics.

METHODS

Bacterial isolates

Consecutive non-duplicate isolates of *Enterobacteriaceae* were collected from community health cen-

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tres in and around Silchar town and from the patient who have attended the clinics or were admitted to Silchar Medical College and Hospital (SMCH) during May 2013 to October 2013 were included in this study. Isolates were identified based on conventional biochemical testing and cultural characteristics [8].

Screening of quinolone resistance

Susceptibility to antibiotics was determined by the disc diffusion method using the CLSI recommendations [7] using the following antibiotics nalidixicacid (30µg), norfloxacin (10µg), ciprofloxacin (5 µg), ofloxacin (5 µg), lomefloxacin (5 µg), gatifloxacin(5 µg), gemifloxacin (5 µg), sparfloxacin (5 µg), levofloxacin (5 µg). *E. coli* ATCC 25922 was used as the quality control for antimicrobial susceptibility tests. Antibiotic susceptibility tests were done on Muller Hinton agar plate.

Characterization of quinolone resistance by multiplex PCR assay

DNA extraction was performed using an improved boiling centrifugation method [9]. Presence of *qnr*D, *qnr*C and *aac* (6')-*Ib-cr* genes were detected by PCR based technique using the primers shown in Table 1. Each single reaction mixture (25 µl) contained 1µl (100 ng) of DNA suspension, 15 pmol of each primer, 12.5 µl of 2x Go green master mix (Promega, Madison, USA) and nuclease free water is added to make the volume 25 µl. Previously screened *aac* (6')-*Ib-cr* was taken as positive control and *E.coli* ATCC 25922 was taken as negative control. Reactions were run under the following conditions; initial denaturation at 95°C for 2min; 35 cycles of 95°C for 50 sec, 53°C for 40 sec and 72°C for 1.20 min; and a final extension at 72°C for 5 min. The amplified PCR products were resolved by electrophoresis in 1 % agarose gel and visualized after staining with ethidium bromide.

Table 1. List of primers.

Primer pair	Target	Sequence (5'→3')	Product size (bp)	Reference
*qnr*A-1A *qnr*A-1B	*qnr*A	TTCAGCAAGAGGATTTCTCA GGCAGCACTATTACTCCCAA	628	24
*qnr*B-CS-1A *qnr*B-CS-1B	*qnr*B	CCTGAGCGGCACTGAATTTAT GTTTCCTGCTCGCCAGTCGA	546	24
*qnr*S-1A *qnr*S-1B	*qnr*S	CAATCATACATATCGGCACC TCAGGATAAACAACAATACCC	675	24
*qnr*C-F *qnr*C-R	*qnr*C	GGGTTGTACATTTATTGAATC TCCACTTTACGAGGTTCT	447	24
*qnr*D-F *qnr*D-R	*qnr*D	CGAGATCAATTTACGGGGAATA AACAAGCTGAAGCGCCTG	582	25
aac(6')-Ib-cr-F *aac(6')-Ib-cr-R*	*aac*(*6*')-*Ib cr*	ATG ACT GAG CAT GAC CTT GC TTA GGC ATC ACT GCG TGT TC	519	26

Plasmid analysis, transformation and conjugation assay

Plasmid DNA was extracted and purified by Qiagen mini prep kit. The plasmid was transferred into *E. coli* DH5α by the heat shock method [10] and transformants were selected by incubation on Luria-Bertani (LB) (Himedia, Mumbai, India) agar plates containing 0.25 µg/ml and 0.5 µg/ml of norfloxacin, ciprofloxacin, ofloxacin and levofloxacin each. Transformants were screened for their plasmid content and resistance phenotype. To investigate the transferability of plasmid encoding quinolone resistance, conjugation experiments using the streptomycin-resistant *E. coli* recipient strain B (Genei, Bangalore, India) were performed as described previously [11].

Plasmid characterization

Plasmid incompatibility was determined for wild type, transformant and transconjugants by PCR based replicon typing method [6]. The PCR condition was described earlier [6].

Antibiotic susceptibility

Susceptibilty were done by disc diffusion method for the following antibiotics**:** ampicillin (10 mcg), cefotaxime (30 mcg), ceftriaxone (30 mcg), ceftazidime (30 mcg), imipenem (10 mcg), cotrimoxazole (25 mcg), tigecycline (15 mcg), gentamicin (120 mcg), amikacin (30 mcg), polymixinB (300 U) (Hi-media, Mumbai, India).

In addition, the MICs of ciprofloxacin (Cipla Ltd, Sikkim), levofloxacin (Ajanta Pharma Limited, Mumbai), norfloxacin (Cipla Ltd, Mumbai), ofloxacin (Micro Labs Ltd, Bangalore), were determined by the agar dilution method using the CLSI recommendations (CLSI 2012). The quality control strains used for this part of the study were *E. coli* ATCC 25922.

Typing of isolates

All *qnr* and *aac(6')-Ib-cr* positive isolates were analyzed for clonality by enterobacterial repetitive intergenic consensus (ERIC)-PCR. Each single reaction mixture (25 µl) contained 1µl (10 ng) of DNA suspension, 15 pmol of each primer, 12.5 µl of 2x Gogreen master mix (Promega, Madison, USA) and nuclease free water is added to make the volume 25 µl. Primers used and reaction condition was as described earlier [12].

The work was approved by Assam University Institutional Ethical committee vide no IEC/AUS/C/2014-002dt-14/08/14.

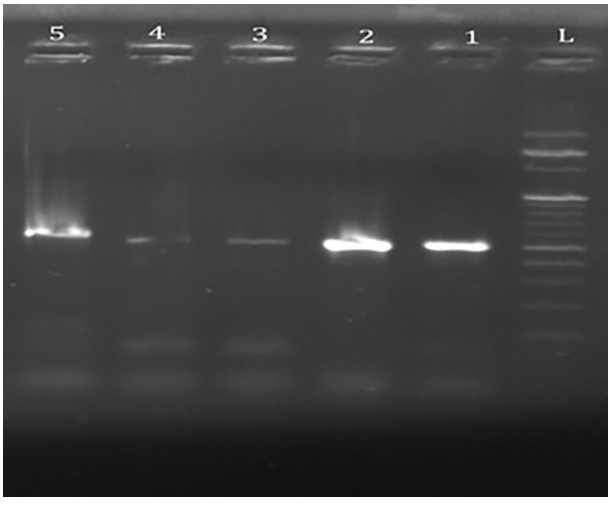

Figure 1. PCR results with the *aac(6')-Ib-cr* primer and *qnr*D primer Lane 1 and 2 showing positive for *aac(6')-Ib-cr* genes (519bp). Lane 3, 4 and 5 showing positive for *qnr*D genes (582bp). Abbreviations: L= Ladder(100bp)

RESULTS

A total of 97 isolates of *Enterobacteriaceae* were collected during May 2013- August 2013, which were *E. coli* (n =74), *K. pneumoniae* (n =14), *K.oxytoca* (n =2) and *P. vulgaris* (*n*=7). Ninety four isolates were screened phenotypically as quinolone resistant, showed non-susceptibility to at least one of the tested quinolone antibiotics. Table 2 summarises the quinolone resistant patterns of the isolates included in the study. Out of total number of 97 isolates of Enterobacteriaceae, 49 (51.04%) were associated with urinary tract infection (UTI), 26 (27.08%) with gastrointestinal tract infection, 22(22.68%) wound infection and abscess.

The prevalence of *aac(6')-Ib-cr* was highest among other plasmid mediated quinolone resistant determinants. Twenty three of ninety four isolates were positive for *aac(6')-Ib-cr*, of which 11 isolates carried the cr-variant. Coexisting *qnr*D genes was identified in eleven isolates carrying *aac(6')-Ib-cr* gene. The *qnr*A, *qnr*B and *qnr*C were not detected in the isolates examined (Table 3).

aac(6')-Ib-cr gene was successfully transferred into *E. coli* strain DH5α by transformation. The transformants were selected against norfloxacin, ciprofloxacin. Quinolone resistance could be transferred by conjugation from nine of eleven *qnr* positive donors and 19 of the twenty three *aac(6')-Ib-cr* gene. PCR assay confirmed that the transconjugant harboured the same plasmid mediated quinolone determinants and *aac(6')-Ib-cr* gene as their donors. Plasmid encoding *aac(6')-Ib-cr* were subjected to replicon typing. The analysed plasmid belong to IncP,HI2,FI Is,K/B,F repB replicon type. DNA fingerprinting with ERIC-PCR showed ten different clones exist in *E.coli*, eight different clones in *K. pneumoniae* and two different clones in *P.vulgaris* in isolates harbouring *aac(6')-Ib-cr* gene. The *aac(6')-Ib-cr* positive strain showed good susceptibility to Polymixin B followed by Imipenem, Ceftazidime, Ceftriaxone, Amikacin, Cefotaxime, Gentamicin, Tigecycline, Ampicilin, Cotrimoxazole (Table 4). The MIC results showed that all isolates have high MIC values for norfloxacin (MIC50 and MIC90- ≥256 µg/ml), ciprofloxacin (MIC50- 128 µg/ml and MIC90- ≥256 µg/ml), ofloxacin (MIC50-64 µg/ml and MIC90-256 µg/ml),and Levofloxacin (MIC50-64 µg/ml and MIC90-≥256 µg/ml) were above the breakpoint.

Table 2. Quinolone resistance pattern of the isolates.

Antibiotics	*E. coli* (n=74) n (%)	*K. pneumoneae* (n=14) n (%)	*K. oxytoca,* (n=2) n (%)	*P. vulgaris* (n=7) n (%)
Nalidixic acid	70 (94.6)	10 (71.4)	1(50.0)	5 (71.4)
Norfloxacin	69 (93.2)	9 (64.3)	1(50)	4 (57.1)
Ciprofloxacin	67 (90.5)	10 (71.4)	1(50)	4 (57.1)
Ofloxacin	67 (90.5)	8 (57.1)	0 (0.0)	3 (42.9)
Levofloxacin	59 (79.7)	11 (78.6)	0 (0.0)	1 (14.3)
Lomefloxacin	63 (85.1)	11 (78.6)	1 (50.0)	3 (42.9)
Sparfloxacin	60 (81.1)	10 (71.4)	1(50)	2 (28.6)
Gemifloxacin	60 (81.1)	10 (71.4)	0 (0.0)	3 (42.9)

n=Total no of isolates, n=no. of resistant isolates, %=percentage.

Table 3. Prevalence of *qnr* and *aac(6')-Ib-cr* genes in the selected Enterobacteriaceae isolates.

Organism	No. of isolates with *qnr*/total no. of isolates (%)	
	*qnr*D	*aac(6')-Ib-cr*
E. coli	9/72 (12.5)	16/72 (22.22)
K. pneumoniae	2/13 (15.38)	3/13 (23.08)
K. oxytoca	0/2 (0.0)	1/2 (50)
P. vulgaris	0/7 (0.0)	1/7 (14.28)

Table 4. Antibiotic susceptibility of quinolone resistant isolates.

Antibiotics	*E. coli,* n=72 n (%)	*K. pneumonia,* n=13 n (%)	*K. oxytoca,* n=2 n (%)	*P. vulgaris,* n=7 n (%)
Ampicillin	28 (38.9)	3 (23.1)	1 (50)	0 (0.0)
Cotrimoxazole	25 (34.7)	2 (15.4)	1 (50)	1 (14.3)
Gentamicin	32 (44.4)	4 (30.8)	0 (0.0)	1 (14.3)
Amikacin	47 (65.3)	9 (69.2)	2 (100)	1 (14.3)
Polymixin B	61 (84.7)	10 (76.9)	2 (100)	4 (57.1)
Tigecycline	28 (38.9)	4 (30.8)	1 (50)	2 (28.6)
Imipenem	61 (84.7)	9 (69.2)	2 (100)	4 (57.1)
Cefotaxime,	43 (59.7)	7 (53.9)	2 (100)	3 (42.9)
Ceftazidime	54 (75)	10 (76.9	2 (100)	3 (42.9)
Ceftriaxone	58 (80.6)	10 (76.9)	2 (100)	3 (42.9)

n=Total no of isolates, n=no. of sensitive isolates, %=percentage.

DISCUSSION

Plasmid-mediated quinolone resistance may facilitate the spread and the increase of the prevalence of quinolone-resistant strains [13]. In recent years, increasing occurrence of aminoglycoside resistant strains has imposed a major threat not only because of their ability to cause serious infections but also because of their increasing resistance to antimicrobial agents [14]. This study examined the plasmid-mediated mechanisms of fluoroquinolone resistance in 97 isolates of *Enterobacteriaceae*, *E. coli* (n =74), *K. pneumoniae* (n=14), *K.oxytoca* (n=2) and *P. vulgaris* (n=7) isolates. Our results found that the occurance of *aac(6')-Ib-cr* genes was 24.5% which is higher than that reported in fluoroquinolone resistant *S.flexneri* in China [15]. Whereas the incidence of prevalence of *aac(6')-Ib-cr* gene is quite higher in Middle Atlantic region of United states [16]. Although the *aac(6')-Ib-cr* gene appears to be more prevalent than any other qnr genes [17], we also detected these genes at the

same frequency in this study. These findings is in consistent with the results of with previous studies, which indicated that *aac(6')-Ib-cr* genes was the most widespread PMQR at Chennai in India [18]. The findings of this study conflict with the results of other studies conducted in India, which indicated that *qnr*B was the most widespread PMQR [19] or *aac(6')-Ib-cr* gene coexist with *qnr*B [20]. The antibiotic susceptibility test revealed that all of those 28 isolates were susceptible to imipenem but resistant to most β-lactams, aminoglycosides and including cefotaxime, ceftazidime, amikacin and so on. The MIC of the *aac(6')-Ib-cr* positive isolates were higher for all the antibiotics tested except 8 phenotypically screened positive isolates had non-active gene *aac(6')-Ib-cr* whose MIC values were located in a criterion of the susceptibility. This confirms that the prevalence of PMQR may be underestimated when resistance breakpoints are used as marker for detection of these determinants [21]. The presence of many subgroup demonstrated by ERIC indicates that these isolates are not derived from clonal expansion. Finally, the successful transformation of *aac(6')-Ib-cr* positive indicates efficient mechanism of horizontal transferability of resistant strain. The conjugation experiments were also successful which implies that the strains are much threatening for horizontal dissemination of these genes.

In conclusion, this finding may have clinical implications, since eleven isolates possessing both *qnr*D *aac(6')-Ib-cr* genes may promote further selection to high-level resistance when quinolones are used [22]. The *aac(6')-Ib-cr* gene has spread rapidly among *Enterobacteriaceae*, and although only conferring a low-level resistance, it may create an environment facilitating the selection of more highly resistant determinants, especially those harbouring topoisomerase mutations [23]. So the presence of heterogeneous resistance mechanism can lead to worrisome condition at the nosocomial level and community acquired infection. So *aac(6')-Ib-cr* containing strains should be promptly detected and referred to clinicians so that the treatment with non-hydrophilic FQs, such as levofloxacin and ofloxacin, or other classes of antibiotics to prevent high level resistance onset and spread. However, since the level of resistance conferred is low, it is often difficult to detect the gene's presence based on the observed phenotype on a routine basis in the laboratory. Molecular detection of *aac(6')-Ib-cr* among clinical bacterial isolates would be of clinical interest.

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