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Phenotypic Detection of AAC(6 □)-Ib-Cr Expression in Enterobacteriacae Isolates

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Abstract

Background:
Quinolone resistance in Enterobacteriaceae usually results from mutations in genes carried by chromosomally encoded type II topoisomerases, efflux pumps, or porin-related proteins. Since 1998, plasmid-mediated quinolone resistances (PMQR) have been described. Recently, a new mechanism of transferable quinolone resistance was reported: enzymatic inactivation of certain quinolones. AAC(6')-Ib-cr is a variant of AAC(6')-Ib which encodes an aminoglycoside acetyltransferase that confers reduced susceptibility to ciprofloxacin or norfloxacin.. PMQR determinants are often combined with extended-spectrum beta-lactamases (ESBLs) leading to organisms possessing multi drug resistance.

Aims & Objectives: *To study the incidence of AAC(6')-Ib-cr expression in Enterobacteriacae isolates by phenotypic method and to find any correlation between the genetic expression of AAC(6')-Ib-cr along with ESBL production.*

Materials and methods: *The 430 clinical Enterobacteriaceae isolates exhibiting ciprofloxacin and norfloxacin resistance were included in this study. They were grown in Luria-Bertani Broth (LB broth) containing norfloxacin (8 µg/ml), with intermittent shaking for 18 h at 35°C. Ten microliters of each culture medium was applied on the blank disk set on a Mueller-Hinton agar plate inoculated with E. coli ATCC 25922 and incubated for 18 h at 35°C. A significant decrease of a growth-inhibitory zone by ≥ 10 mm was considered as a positive test for the production of aac(6')-Ib-cr, while aac(6')-Ib-cr production was considered not to be produced in strains which did not show any reduction in the zone of inhibition.*

Results: *The overall expression of the AAC(6')-Ib-cr did not show much variation with almost half of the isolates from all the different samples showing AAC(6')-Ib-cr production with blood isolates showing a production as high as 66.77%.). A significant association was noted with ESBL production and the simultaneous expression of AAC(6')-Ib-cr in both E.coli (P value <0.0001) and K.pneumoniae isolates (P value = 0.0137)which were the two most predominant isolates in the study. A significant association (P value 0.0442) was observed between aminoglycoside resistance and AAC(6')-Ib-cr expression in E.coli isolates only, while no significant association was observed (P value= 0.7763) in K. pneumonia isolates.*

Conclusion: *AAC(6')-Ib-cr, though being a recently described gene has high degree of expression in Enterobacteriaceae isolates, which not only confers resistance to fluroquinolones and aminoglycosides but also is coexpressed with ESBL genes, thus producing a scenario of multi drug resistant organisms, further increasing the burden on our limited armamentarium against these bacteria.*

Keywords: *AAC(6')-Ib-cr, ESBL, Enterobacteriacae*

I. Introduction

Quinolone resistance in Enterobacteriaceae *usually results from mutations in genes carried by chromosomally encoded type II topoisomerases, efflux pumps, or porin-related proteins. Since 1998, plasmid-mediated quinolone resistances (PMQR) have been described. They have been reported worldwide in unrelated Enterobacterial species and are usually associated with mobile elements.*[1] *Quinolones and beta-lactams are among the three most commonly used antimicrobials in human therapeutics. PMQR determinants confer low-level resistance, but their presence could potentially facilitate evolution of the bacterial host toward higher levels of resistance by mutational alterations in type II topoisomerases. PMQR determinants are often combined with extended-spectrum beta-lactamases (ESBLs). This suggests that there is the potential for selection of PMQR by the use of beta-lactams and of beta-lactam resistance by the use of quinolones. The Qnr proteins protect DNA from quinolone binding to gyrase and topoisomerase IV. QepA1 and QepA2* are quinolone efflux pump proteins .[2]

Recently, a new mechanism of transferable quinolone resistance was reported: enzymatic inactivation of certain quinolones. AAC(6′)-Ib-cr is a variant of AAC(6′)-Ib which encodes an aminoglycoside acetyltransferase that confers reduced susceptibility to ciprofloxacin or norfloxacin by N acetylation of a

piperazinyl amine. AAC(6′)-Ib-cr differs from AAC(6′)-Ib by the following two amino acid substitutions: Trp102Arg and Asp179Tyr which together are necessary and sufficient for the enzyme's ability to acetylate ciprofloxacin.[3,4]

When both *qnrA* and AAC(6′)-Ib-cr are present in the same cell, the level of resistance is increased fourfold more than that conferred by *qnrA* alone, with an MIC of ciprofloxacin of 1.0 µg/ml, a value near the clinical breakpoint for susceptibility. In addition, the presence of AAC(6′)-Ib-cr alone increased substantially the frequency of selection of chromosomal mutants upon exposure to ciprofloxacin.[3]

Since the fluoroquinolone-modifying enzyme gene, AAC(6′)-Ib-cr, was first reported in 2006, it has rapidly spread among *Enterobacteriaceae* clinical isolates worldwide. The presence of AAC(6′)-Ib-cr is associated with decreased susceptibility to aminoglycosides (kanamycin, amikacin, and tobramycin) and to norfloxacin and ciprofloxacin. This allelic variant of *aac(6′)-Ib* was found to be linked to the extended-spectrum β-lactamase (ESBL) gene $bla_{CTX-M-15}$ in isolates from many countries, while association of *aac(6′)-Ib* with the $bla_{CTX-M-2}$ ESBL gene has been widely reported in Uruguay and Argentina.[5]

AAC(6′)-Ib-cr may be more widespread than Qnr-determinants. Both, Qnr- and AAC(6′)-Ib-cr production are associated with the ESBL production, thus, representing a second mechanism of co-selection of drug-resistance due to exposure to chemically unrelated agents.[5].

Detection of AAC(6′)-Ib-cr has so far depended mainly on genotyping, PCR, and sequencing. Recently, simultaneous high-resolution melting analysis and pyrosequencing were developed for detection. However, these methods are costly and need specialized equipment. Therefore, the availability is limited to highly advanced institutions, such as research laboratories and university hospitals. A phenotypic test validated by Wachino *et al*. for the detection of AAC has been recently described.. In the present study this costeffective and practical disk-based method was used to look for the presence of AAC(6′)-Ib-cr variant and to detect its association with ESBL production and in clinical isolates of enterobacteria showing resistance or intermediate resistance to ciprofloxacin and norfloxacin.[6]

II. Material & Methods

Inclusion criteria: Enterobacteriaceae isolates exhibiting ciprofloxacin and norfloxacin resistance or intermediate resistance as per Clinical Laboratory Standards Institute (CLSI) guidelines by the Kirby Bauer disc diffusion method were included in the study.[7]

The 430 clinical isolates included in this study were grown in Luria-Bertani Broth (LB broth) containing norfloxacin (8 µg/ml), with intermittent shaking for 18 h at 35°C. The MIC's of norfloxacin for these strains was ≥16 µg/ml, and they showed visible growth in liquid broth containing 8 µg/ml of norfloxacin. The broth containing the same concentration of norfloxacin as the other tubes, but lacking any bacteria, was used as the control in this study. Ten microliters of each culture medium was applied on the blank disk set on a Mueller-Hinton agar plate inoculated with *E. coli* ATCC 25922 and incubated for 18 h at 35°C. The result is shown in Figure 1. When the control medium was applied, an 18-millimeter growth-inhibitory zone (corresponding to 80 ng norfloxacin per disk) was observed. A significant decrease of a growth-inhibitory zone by ≥ 10 mm was considered as a positive test for the production of *aac(6′)-Ib-cr*, while *aac(6′)-Ib-cr* production was considered not to be produced in strains which did not show any reduction in the zone of inhibition.

The decrease in zone diameter is an indicator of *aac(6′)-Ib-cr* production and would be attributed to the inactivation of norfloxacin in the culture medium by the AAC(6_)-Ib-cr enzyme produced during growth.

ESBL detection; ESBL detection was performed as per the CLSI guidelines using ceftazidime, ceftazidime + clavulanic acid and cefotaxime , cefotaxime+ clavulanic acid and by the disc approximation technique.[7]

III. Results

Of the 430 isolates included in the study, most of the isolates in the study were from pus samples 231(53.72%) followed by urine 14(33.72%) , respiratory samples 34(7.9%), body fluids 14(3.26%) and blood cultures 6(1.40%)(Table1). The overall expression of the AAC(6′)-Ib-cr did not show much variation with almost half of the isolates from all the different samples showing AAC(6′)-Ib-cr production with blood isolates showing a production as high as 66.77%. (Table1).

Among the different isolates *E. coli* was the predominant isolate(256/444) followed by *K.pneumoniae* (106/444), *P. mirabilis, Citrobacter spp. K.oxytoca and Enterobacter spp.*. All the isolates showed a high incidence of AAC(6′)-Ib-cr production, with almost half of all the isolates producing the fluroquinone modifying enzyme, with *P.mirabilis* being the only exception which showed a low incidence of 14.29% only. The overall expression of the AAC(6′)-Ib-cr was 218/ 430(50.70%). (Table 2).

ESBL production was noted in 142/256 (55.47%) of E. coli isolates and 62/106(58.49%) of K. pneumonia isolates. A significant association was noted with ESBL production and the simultaneous expression of AAC(6′)-Ib-cr in both E.coli (P value <0.0001) and K.pneumoniae isolates (P value = 0.0137)which were the two most predominant isolates in the study (Table 3).

Aminoglycoside resistance was noted in 79/256 (30.86%) of the E.coli isolates and 63/106 (59.43%) of K. pneumonia isolates but a significant association (P value 0.0442) was observed between aminoglycoside resistance and AAC(6')-Ib-cr expression in E.coli isolates only, while no significant association was observed (P value= 0.7763) in K. pneumonia isolates (Table 4).

The overall incidence of AAC(6')-Ib-cr expression of 50.70% and 53.91% in E. coli was noted in this study, which corresponds to the findings of Robicsek AJ et al. In China. The study found an incidence of 53.91% in E. coli and 52.83% in K. pneumonia, which is much higher than the findings of 32% of E. coli, 16% of K. pneumoniae, 7.5% of Enterobacter, reported by Park et al. The study finds a very high association with ESBL production similar to the results of Warburg et al. who observed that 68.8% of aac(6')-Ib-cr +ve isolates were ESBL producer. Table 5 denotes the different studies that have been carried out worldwide regarding incidence of AAC(6')-Ib-cr expression

IV. Discussion

The emergence of new mechanisms of quinolone resistance, namely, those caused by the horizontal transfer of resistance genes on mobile genetic elements, are of great concern since they bring new possibilities for the spread of resistance. [8]

According to data from the European surveillance of antimicrobial consumption, as the defined daily dose (DDD)/1,000 inhabitants per day of ciprofloxacin increased slightly from 0.5 DDD/1,000 inhabitants per day in 1997 to 0.6 DDD/1,000 inhabitants per day in 2003, there was a constant increase in resistance to fluoroquinolones. In the isolates included in this study, the percentage of ciprofloxacin -resistant ESBL positive isolates was high and a decrease of the ciprofloxacin intermediate resistance phenotype was observed along with the emergence of the *aac(6_)-Ib-cr* gene as detected by phenotypic methods. This suggests, a role for this low-level PMQR gene in enhancing the selection of chromosomal mutations and resulting in the occurrence and dissemination of clinically relevant resistance levels.[9]Additionally, prior quinolone use is a risk factor for subsequent infection with quinolone-resistant, ESBL-producing organisms.[10]

The overall incidence of AAC(6')-Ib-cr expression of 50.70% and 53.91% in E. coli was noted in this study, which corresponds to the findings of Robicsek AJ et al. [11]In China. The study found an incidence of 53.91% in E. coli and 52.83% in K. pneumonia, which is much higher than the findings of 32% of E. coli, 16% of K. pneumoniae, 7.5% of Enterobacter, reported by Park et al.[3] The study finds a very high association with ESBL production similar to the results of Warburg et al. [12]who observed that 68.8% of AAC(6′)-Ib-cr isolates were ESBL producer

In this study a significant association of aminoglycoside resistance with AAC(6′)-Ib-cr was noted in case of E. coli but similar association was not noted in case of Klebsiella probably because of the fact that the AAC(6′)-Ib-cr confers resistance to kanamycin but not to gentamicin which was not tested in this particular study.[10] A high incidence of 42% of 31 gentamicin-resistant isolates harboring AAC(6′)-Ib-cr, was also noted by Park et al. [3]

The most striking finding of our study was the wide penetration of the AAC(6′)-Ib-cr allele in nearly half of all the Enterobacteriacaea isolates, E. coli (53.91%), Klebsiella pneumonia (52.83%), Klebsiella oxytoca (57. 14%), Enterobacter sp. (60%). Although this variant gene was not reported until 2006, it was already present in more than half of multidrug-resistant E. coli isolates in Shanghai, China, in 2000 to 2001, and it is now present in the majority of census regions of the United States. The various antibiograms and the range of species of the isolates studied suggest that the dissemination of AAC(6′)-Ib-cr does not occur through clonal spread or the spread of a single plasmid. The diversity of plasmids on which this gene circulates is not yet known, but its presence as part of an integron cassette suggests that it could be widely mobile among plasmids. Selection pressures from the use of aminoglycosides that are enzyme substrates (kanamycin, tobramycin, and amikacin) and the use of quinolones with a piperazinyl amine that is subject to *N*-acetylation by the *cr* variant enzyme would be predicted to promote gene prevalence but have not yet been studied in clinical settings.[3]

Warburg et al. had also demonstrated an epidemiologic link between AAC(6′)-Ib-cr and CTX-M ESBL. Our findings support earlier suggestions of a linkage between AAC(6′)-Ib-cr and ESBLs and raise the possibility that the use of ciprofloxacin—a widely prescribed fluoroquinolone in the world—is a driver of both fluoroquinolone resistance and the emergence of CTX-M ESBLs. The present increase in multi drug resistance could be hypothesised to the fact that increase in β lactams consumption leads to increase in ESBL production, which in turn is increases the *aac(6')-Ib-cr* gene expression and vice a versa and the *aac(6')-Ib-cr* gene expression is in turn linked with aminoglycoside resistance, thus by administering any class of quinolones or β

lactams is in turn leading to development of multidrug resistance organisms in the community as well as the hospital setup (Figure 2). [11]

There have been studies which indicate horizontal transfer of a*ac(6_)-Ib-cr* among the *Enterobacteriaceae*. The *aac(6')-Ib-cr* gene showed a high association with β lactamase genes, including OXA-1, CTX-M-3 or -15, and TEM-1, in isolates from Korea. This is an alarming finding indicating that the spread of this gene would only increase in the years to come.[[4]

Larger studies could be undertaken to see the geographical distribution of the fluroquinolone modifying enzyme.

There have been reports of *qnrB* and *aac(6')-Ib-cr* coexpression in clinical *Enterobacteriaceae* isolates from a Bulgarian hospital. The study also concluded that *aac(6')-Ib-cr* might already be widespread and substantially more prevalent than *qnr* genes. If the incidence of coexpression of both these genes were to increase then that could lead to very high incidence of quinolone resistance. [13]

There have been various methods described for identification of *aac(6_)-Ib-cr* including genotyping, PCR, and sequencing. Recently, simultaneous high-resolution melting analysis and pyrosequencing, which have restraints of the cost associated with the tests and the fact that these tests are usually available with reference laboratories. We conclude that this phenotypic method is a simple method for identification of *aac(6')-Ib-cr* producers and that it would be of great assistance in screening for *aac(6')-Ib-cr* producers and their epidemiology in clinical microbiology laboratories, with resource limited settings.

V. FIGURES & TABLES

Significant decrease of a growth-inhibitory zone (>10mm) signified aac(6')-Ib-cr-positive isolates.

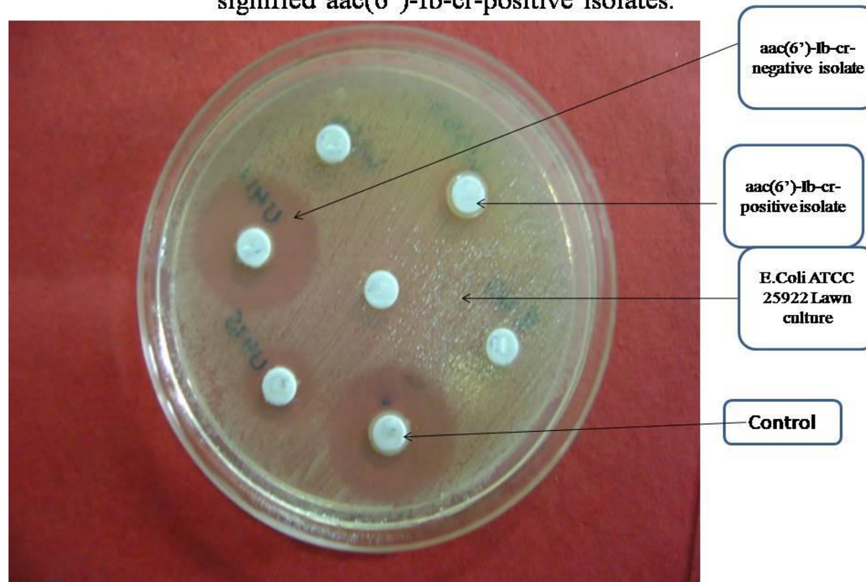

Figure 1: Significant decrease of a growth-inhibitory zone (>10mm) signified aac(6')-Ib-cr-positive isolates.

Table 1: Sample wise distribution of Isolates producing Fluoroquinolone – Modifying Enzyme AAC(6')-Ib-cr

Samples	Total N=430	Isolates producing Fluoroquinolone – Modifying Enzyme AAC(6')-Ib-cr	Isolates not producing Fluoroquinolone – Modifying Enzyme AAC(6')-Ib-cr
Urine	145(33.72%)	80(55.17%)	65
Wound / Pus	231(53.72%)	102(44.16%)	129
Throat Swab / Sputum / ET tube suction	34(7.9%)	18(52.94%)	16
Blood	6(1.40%)	4(66.67%)	2
Body cavity fluids (e.g. pleural fluid)	14(3.26%)	6(42.86%)	8

Among the 430 isolates included in this study, most of the isolates showing fluroquinolone resistance or intermediate resistance were obtained from pus samples (53.72%), followed by urine (33.72%)., but the overall expression of the AAC(6')-Ib-cr did not show much variation with almost half of the isolates from

different samples showing AAC(6')-Ib-cr production, with blood isolates showing a production as high as 66.77%.

Table 2: Organism wise distribution of **AAC(6')-Ib-cr expression.**

Quinolone resistant Organisms	**Total number of isolates (N=430)**	**Isolates producing Fluoroquinolone – Modifying Enzyme AAC(6')-Ib-cr**	**Isolates without producing Fluoroquinolone – Modifying Enzyme AAC(6')-Ib-cr**
Escherichia coli	256	138/256 (53.91%)	118
Klebsiella pneumoniae	106	56/106 (52.83%)	50
Klebsiella oxytoca	14	8/14 (57.14%)	6
Enterobacter sp.	10	6/10 (60%)	4
Proteus mirabilis	28	4/28 (14.29%)	24
Citrobacter sp.	16	6/16 (37.5%)	10

Among the different isolates *E. coli* was the predominant isolate(59.53%) followed by *K.pneumoniae* (24.65%). All the isolates showed a high incidence of **AAC**(6')-Ib-cr production, with almost half of all the isolates producing the fluroquinone modifying enzyme, with P.mirabilis being the only exception which showed a low incidence of 14.29% only. The overall expression of AAC(6')-Ib-cr among all the isolates was 218/ 430(50.70%).

Table 3: Distribution of ESBL production observed in different isolates

Escherichia coli (Total 256)	Isolates producing Fluoroquinolone – Modifying Enzyme AAC(6')-Ib-cr	Isolates without producing Fluoroquinolone – Modifying Enzyme AAC(6')-Ib-cr	P value <0.0001
ESBL +	106 (41.4%)	36 (14.06%)	
ESBL -	32 (12.5%)	82(32.03%)	
Chi-square 55.21, df - 1	OR-7.545	95% C.I.= 4.324 to 13.17	
Klebsiella pneumoniae (Total 106)	Isolates producing Fluoroquinolone – Modifying Enzyme AAC(6')-Ib-cr	Isolates without producing Fluoroquinolone – Modifying Enzyme AAC(6')-Ib-cr	P value 0.0137
ESBL +	39(36.79%)	23(29.69%)	
ESBL -	17(16.04%)	27(25.47%)	
Chi-square 6.082, df – 1	OR – 2.693	95% CI = 1.215 to 5.971	

ESBL production was noted in 142/256 (55.47%) of *E. coli* isolates and 62/106(58.49%) of *K. pneumonia* isolates. A significant association was noted with ESBL production and the simultaneous expression of **AAC(6')-Ib-cr** in both *E.coli* (**P value <0.0001)** and *K.pneumoniae* isolates (**P value = 0.0137**)which were the two most predominant isolates in the study.

Table 4: Distribution of aminoglycoside resistance and **AAC(6')-Ib-cr** production observed in different isolates

Escherichia coli (Total 256)	Isolates producing Fluoroquinolone – Modifying Enzyme AAC(6')-Ib-cr	Isolates without producing Fluoroquinolone – Modifying Enzyme AAC(6')-Ib-cr	P value 0.0442
Aminoglycoside resistance +	50(19.53%)	29 (11.33%)	
Aminoglycoside resistance -	88(34.38%)	89(34.76%)	
Chi-square 4.050, df - 1	OR = 1.744	95%CI = 1.012 to 3.005	
Klebsiella pneumoniae (Total 106)	Isolates producing Fluoroquinolone – Modifying Enzyme AAC(6')-Ib-cr	Isolates without producing Fluoroquinolone – Modifying Enzyme AAC(6')-Ib-cr	P value 0.7763
Aminoglycoside resistance +	34(32.08%)	29(27.36%)	
Aminoglycoside resistance -	22(20.75%)	21(19.81%)	
Chi-square 0.08072, df - 1	OR = 1.119	95%CI = 0.5147 to 2.433	

Aminoglycoside resistance was noted in 79/256 (30.86%) of the E.coli isolates and 63/106 (59.43%) of K. pneumonia isolates but a significant association P value 0.0442 was observed between aminoglycoside resistance and AAC(6')-Ib-cr expression in E.coli isolates only, while no significant association was observed P value 0.7763 in K. pneumonia isolates.

Table 5: Comparison of various studies conducted for detection of AAC(6')-Ib-cr incidence

Author	AAC(6')-Ib-cr	Year	Place	Others
Stefana Sabtcheva *et al.*	52.5% isolates of 163 ESBL producer	2005	Bulgaria	

Chi Hye Park *et al.*	32% of E. coli, 16% of K. pneumoniae, 7.5% of Enterobacter	1999 - 2004	USA	42% of gentamicin-resistant isolates harbored aac(6')-Ib-cr
Robicsek AJ *et al.*	51% of E. coli	2000 - 2001	China	
Gabriela Warburg *et al.*	1991 -1997, none,1998 - 2005 onwards, 7.1% of the total isolates	1991 - 2005	Israel	68.8% of aac(6')-Ib-cr +ve isolates were ESBL producer.
This study	2012-2013 53.91% of *E. coli* and 52.83 % *of K. pneumonia*, overall 218/ 430(50.70%)	2012-2013	India	73.98 % of aac(6')-Ib-cr +ve isolates were ESBL producer

The overall incidence of **AAC(6')-Ib-cr expression of** 50.70% and 53.91% in E. coli was noted in this study, which corresponds to the findings of Robicsek AJ *et al.* In China. The study found an incidence of 53.91% in E. coli and 52.83% in *K. pneumonia*, which is much higher than the findings of 32% of E. coli, 16% of K. pneumoniae, 7.5% of Enterobacter, reported by Park *et al.* The study finds a very high association with ESBL production similar to the results of Warburg *et al.* who observed that 68.8% of aac(6')-Ib-cr +ve isolates were ESBL producer

VI. Conclusion

AAC(6′)-Ib-cr, though being a recently described gene has high degree of expression in Enterobacteriaceae isolates, which not only confers resistance to fluroquinolones and aminoglycosides but also is coexpressed with ESBL genes, thus producing a scenario of multi drug resistant organisms, further increasing the burden on our limited armamentarium against these bacteria.

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