SHORT REPORT

Prevalence of aac(6′)-lb-cr plasmid-mediated and chromosome-encoded fluoroquinolone resistance in Enterobacteriaceae in Italy

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Abstract
The spread of aac(6′)-lb-cr plasmid-mediated quinolone resistance determinants was evaluated in 197 enterobacterial isolates recovered in an Italian teaching hospital. The aac(6′)-lb-cr gene was found exclusively in Escherichia coli strains. The gene was located on a plasmid which presented additional ESBL genes. Most of the clinical strains were clonally related and displayed three point mutations at the topoisomerase level which conferred high resistance to fluoroquinolones.

Keywords: aac(6′)-lb-cr, fluoroquinolones, plasmid-mediated resistance, Gram-negative

Findings
The aminoglycoside acetyltransferase Aac(6′)-Ib-cr variant, an enzyme usually encoded by a plasmid-borne gene, extends its drug targets to include fluoroquinolones (FQs) in addition to aminoglycosides. It is characterized by amino acid changes at codon 102 (Trp→Arg) and codon 179 (Asp→Tyr). The Aac(6′)-Ib-cr protein is able to specifically acetylate hydrophilic FQs presenting a free piperazinyl amine (i.e. ciprofloxacin and norfloxacin) [1].

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. This fact is particularly worrisome at the nosocomial level, where aac(6′)-Ib-cr containing strains should be promptly detected and treated with non-hydrophilic FQs, such as levofloxacin or ofloxacin, or other classes of antibiotics to prevent high-level resistance onset and spread.

In this work we determined the prevalence of the aac(6′)-Ib-cr gene variant among clinical isolates of Enterobacteriaceae collected at the teaching Hospital of Padua, Italy. In the time period of March-May 2008, 197 non-duplicate clinical isolates were collected. These displayed the whole range of MIC of ciprofloxacin; in particular, 104 samples were susceptible (MIC ≤ 1), 35 intermediate (1 < MIC < 4), and 58 resistant (MIC ≥ 4) to ciprofloxacin. Exact MIC values were measured by means of E-test strips (AB Biodisk, Solna, Sweden). Bacterial isolates were: 145 E. coli, 38 K. pneumoniae, 5 Proteus mirabilis, 5 Enterobacter aerogenes, 2 Enterobacter cloacae and 2 Citrobacter freundii. Sample identity and results are reported in Table 1.

The presence of the aac(6′)-lb or aac(6′)-lb-cr genes was assessed by PCR amplification and subsequent sequencing with primers: aacF 5’-ATGACTGAGCATGCCTTG-3’; aacR 5’-AACCATGTACACGGCTGG-3’; aacSEQ 5’-CGTCACCTCCATATTGAATCACGAA-3’ [2]. Twenty-five samples out of 197 (13%) were positive for the aac(6′)-lb gene and of these 16 (8%) displayed the aac (6′)-lb-cr variant. In particular, aac(6′)-lb-cr was found exclusively in E. coli, while aac(6′)-lb was present mostly in K. pneumoniae (8 K. pneumoniae, 1 E. coli). Out of the 16 aac(6′)-lb-cr-positive samples, 15 were collected from urine and 1 from skin; 13 were from inpatients (81%) and 3 from outpatients.

All aac(6′)-lb were tested for other plasmid-mediated quinolone-resistance genes, i.e. qnr and qepA, by PCR amplification and sequencing using published procedures [3]. None of these was found in the aac(6′)-lb-cr-positive samples, while 5 out of 9 aac(6′)-lb-positive strains presented the qnrB19 gene, indicating that just one of the...
| Isolate ID | Bacterial species | qnr gene | aac(6')-Ib gene | MIC (mg/L) | GyrA | ParC | ESBL<sup>1</sup> | ESBL genotypic | ERIC |
|------------|-------------------|----------|-----------------|------------|------|------|----------------|----------------|------|
| 6          | *Escherichia coli* | cr       |                | ≥ 4 ≥ 4 ≥ 16 ≥ 16 ≥ 16 | S83L, D87N | E84V | -              | -              | 1    |
| 6<sup>T</sup> | *E. coli TOP10* | cr       | 0.008 0.008 0.016 0.004 0.006 | - | - | nd | -              | -              |      |
| 13         | *E. coli*         | cr       | ≥ 4 ≥ 4 ≥ 16 ≥ 16 ≥ 16 | S83L, D87N | E84V | - | CTX-M-1       | -              | 1    |
| 13<sup>C</sup> | *JS3AzKanNaR* | cr       | 0.064 0.064 0.047 0.047 0.023 | - | - | nd | CTX-M-1       | -              |      |
| 19         | *E. coli*         | non-cr   | nd             | nd            | nd | nd | nd            | nd            |      |
| 23         | *E. coli*         | cr       | ≥ 4 ≥ 4 ≥ 16 ≥ 16 ≥ 16 | S83L, D87N | E84V | X | CTX-M-1+TEM-1 | 2              |      |
| 23<sup>C</sup> | *JS3AzKanNaR* | cr       | 0.047 0.047 0.047 0.047 0.032 | - | - | nd | CTX-M-1+TEM-1 | -              |      |
| 37         | *E. coli*         | cr       | 0.006 0.006 0.016 0.004 0.006 | - | - | nd | CTX-M-1+TEM-1 | 1              |      |
| 37<sup>T</sup> | *E. coli TOP10* | cr       | 0.006 0.006 0.016 0.004 0.006 | - | - | nd | CTX-M-1+TEM-1 | 3              |      |
| 39         | *E. coli*         | cr       | ≥ 4 ≥ 4 ≥ 16 ≥ 16 ≥ 16 | S83L, D87N | E84V | X | CTX-M-1       | -              | 1    |
| 39<sup>T</sup> | *E. coli TOP10* | cr       | 0.006 0.006 0.016 0.004 0.006 | - | - | nd | CTX-M-1       | -              |      |
| 40         | *E. coli*         | cr       | ≥ 4 ≥ 4 ≥ 16 ≥ 16 ≥ 16 | S83L, D87N | E84V | X | CTX-M-1+TEM-1 | 1              |      |
| 40<sup>T</sup> | *E. coli TOP10* | cr       | 0.006 0.006 0.016 0.004 0.006 | - | - | nd | CTX-M-1+TEM-1 | 1              |      |
| 44         | *E. coli*         | cr       | 0.047 0.047 0.032 0.047 0.023 | - | - | X | CTX-M-1       | 3              |      |
| 44<sup>C</sup> | *JS3AzKanNaR* | cr       | 0.047 0.047 0.047 0.047 0.023 | - | - | nd | CTX-M-1       | -              |      |
| 51         | *E. coli*         | cr       | ≥ 4 ≥ 4 ≥ 16 ≥ 16 ≥ 16 | S83L, D87N | E84V | X | CTX-M-1       | 1              |      |
| 51<sup>T</sup> | *E. coli TOP10* | cr       | 0.006 0.006 0.016 0.004 0.006 | - | - | nd | CTX-M-1       | 1              |      |
| 52         | *E. coli*         | cr       | ≥ 4 ≥ 4 ≥ 16 ≥ 16 ≥ 16 | S83L, D87N | E84V | X | CTX-M-1       | -              | 1    |
| 52<sup>T</sup> | *E. coli TOP10* | cr       | 0.004 0.004 0.016 0.004 0.006 | - | - | nd | CTX-M-1       | -              |      |
| 53         | *E. coli*         | cr       | ≥ 4 ≥ 4 ≥ 16 ≥ 16 ≥ 16 | S83L, D87N | E84V | X | CTX-M-1       | 1              |      |
| 53<sup>T</sup> | *E. coli TOP10* | cr       | 0.004 0.004 0.016 0.004 0.006 | - | - | nd | CTX-M-1       | -              |      |
| 111        | *Klebsiella*      | non-cr   | gmr819         | 1.5 1.5 4 2 nd | nd | nd | X | TEM-1+SHV-12   |      |
| 128        | *K. pneumoniae*   | non-cr   |                | 1.5 1.5 3 4 2 | 2 | 2 | nd | nd | nd | 1    |
| 137        | *K. pneumoniae*   | non-cr   | qnrB819        | 1.5 1.5 4 1.5 1.5 | 1.5 | nd | X | TEM-1         |      |
| 143        | *K. pneumoniae*   | non-cr   | qnrB819        | 1.5 1.5 4 1.5 1.5 | nd | nd | - | TEM-150+SHV-12| 1    |
| 144        | *K. pneumoniae*   | non-cr   | qnrB819        | 1.5 1.5 6 1.5 1.5 | nd | nd | X | TEM-150       |      |
| 164        | *E. coli*         | cr       | ≥ 4 ≥ 4 ≥ 16 ≥ 16 ≥ 16 | S83L, D87N | E84V | X | CTX-M-1+TEM-1 | 1              |      |
| 164<sup>T</sup> | *E. coli TOP10* | cr       | 0.004 0.004 0.016 0.004 0.006 | - | - | nd | CTX-M-1       | -              |      |
| 175        | *E. coli*         | cr       | ≥ 4 ≥ 4 nd ≥ 8 ≥ 8 | S83L, D87N | E84V | X | CTX-M-1       | 1              |      |
| 175<sup>T</sup> | *E. coli TOP10* | cr       | 0.006 0.006 0.016 0.004 0.006 | - | - | nd | CTX-M-1       | -              |      |
Resistance ranges: nalidixic acid S = negative

nd = not determined

phen = ESBL phenotypic determination.

amikacin, imipenem, piperacillin/tazobactam; an equal
tant to

samples. Accordingly, most of the samples were resis-
tantly measured according to the CLSI M100-S18 and

or AmpC were detected. ESBL production, phenotypi-
coupled with TEM-1 in 7 strains. No other

samples presented the CTX-M-1 gene, which was

acquired/maintained in the clinical isolates.

reported plasmid-encoded mechanisms of FQ resistance
was acquired/maintained in the clinical isolates.

The presence of mechanisms of chromosomal resist-
tance to FQ was assessed on aac(6’)-Ib-cr-positive
strains. The genotypic analysis, performed with universal
primers [4], revealed that all samples, but #44, coded for
two mutations in GyrA (S83L, D87N) and one mutation
in ParC (E84V). Phenotypic analysis of resistance to

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primers [4], revealed that all samples, but #44, coded for

strains. The genotypic analysis, performed with universal
primers for the detection of ESBL, i.e. bla

lat, bla

ACT-M-1, 2, 8, 9, and AmpC genes, i.e. bla

MOX-

1, 2, bla

CMY-1-1, 11, bla

LAT-1-4, 1, bla

BL, 1, bla

DHA-1, 2, bla

ACC,

bla

MIR-1T, 1, 2, bla

FOX-1, 5b [5-8]. Fifteen out of 16
samples presented two main electrophoretic band corre-
responding to > 100 Kbp and 25 Kbp (Figure 1A). In each
case the aac(6’)-Ib-cr gene was located in the > 100 Kbp
band, as demonstrated by PCR gene amplification, using
DNA extracted from each band as template. Plasmid
localization of the aac(6’)-Ib-cr gene was further con-
firmed by successful transformation into E. coli Top10
strain of all 16 sample-plasmid DNA, extracted accord-
ing the Kieser protocol [10]. Transferability of the resis-
tance gene of three clinical isolates was tested by

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tance gene of three clinical isolates was tested by

Plasmid DNA was extracted from aac(6’)-Ib-cr-positive
strains and run on agarose gel, to confirm the presence
of the aac(6’)-Ib-cr gene on a mobile element: all sam-
pies presented two main electrophoretic band corre-
ponding to > 100 Kbp and 25 Kbp (Figure 1A). In each
case the aac(6’)-Ib-cr gene was located in the > 100 Kbp
band, as demonstrated by PCR gene amplification, using
DNA extracted from each band as template. Plasmid
localization of the aac(6’)-Ib-cr gene was further con-
firmed by successful transformation into E. coli Top10
strain of all 16 sample-plasmid DNA, extracted accord-
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tance gene of three clinical isolates was tested by

176 177 178 178T 180 182 182T 183 184 184T 185 185T 186 187
K. pneumoniae non-cr qnrB19 1 1 2 15 ≥ 8 nd nd - TEM-150
E. coli cr - ≥ 4 ≥ 4 nd ≥ 8 ≥ 8 S83L, D87N E84V X CTX-M-1+TEM- 1
E. coli TOP10 cr - 0.006 0.006 0.016 0.004 0.006 - - nd CTX-M-1+TEM- 1
K. pneumoniae non-cr - 0.25 0.25 0.75 0.25 0.38 nd nd X nd
E. coli cr - ≥ 4 ≥ 4 ≥ 8 ≥ 8 ≥ 8 S83L, D87N E84V X CTX-M-1 1
E. coli TOP10 cr - 0.006 0.006 0.016 0.004 0.006 - - nd CTX-M-1
Enterobacter aerogenes non-cr - 0.75 0.75 2 1 0.75 nd nd nd nd
E. coli cr - ≥ 4 ≥ 4 nd ≥ 8 ≥ 8 S83L, D87N E84V X CTX-M-1+TEM- 1
E. coli TOP10 cr - 0.006 0.006 0.016 0.004 0.006 nd CTX-M-1+TEM- 1
E. coli cr - ≥ 4 ≥ 4 nd ≥ 8 ≥ 8 S83L, D87N E84V X CTX-M-1+TEM- 1
E. coli TOP10 cr - 0.006 0.006 0.016 0.004 0.006 - - nd CTX-M-1+TEM- 1
E. coli TOP10 - - 0.016 0.016 0.047 0.047 0.023 - - nd -
J334AzKanR - - 0.002 0.002 0.016 0.004 0.006 - - nd -

* = transformant strain; ** = transconjugant strain. ⁶NA = nalidixic acid, ⁷CPF = ciprofloxacin, ⁸OFX = ofloxacin, ⁹LVF = levofloxacin, ¹⁰MXF = moxifloxacin. ESBL phen = ESBL phenotypic determination.

nd = not determined

- = negative

Resistance ranges: nalidixic acid S = 6 mg/L, R = 32 mg/L; ciprofloxacin S = 1 mg/L, I = 2 mg/L, R = 4 mg/L; ofloxacin, levofloxacin, moxifloxacin S = 2 mg/L, I = 4 mg/L, R ≥ 8 mg/L.

Table 1 Properties of aac(6’)-Ib-positive clinical isolates and transconjugant strains (Continued)
MIC analysis of FQs in transformants and transconjugants compared to the wild type isolates showed a drastic decrease in the MIC values of all tested antibiotics. However, MIC of ciprofloxacin increased of 2-4 times in transformants and transconjugants, compared to the wild type recipient strains, *E. coli* Top10 and J53. These results are in line with the notion that the aac(6\')-Ib-cr alone does not confer high level resistance to the drugs, but it stimulates chromosomal mutations on the FQ targets, i.e. gyrase and topoisomerase IV, which in turn dramatically increase resistance to these drugs.

To assess the clonal relationship between aac(6\')-Ib-cr-positive isolates, the “enterobacterial repetitive intergenic consensus” (ERIC)-PCR genomic DNA profiles was analysed with specific primers [5]. Three different main subgroups were identified (Figure 1B). Most aac(6\')-Ib-cr-positive samples were clonally related (82%), while 1 (6%) (#23) and 2 (12%) (#39 and #44) samples belonged to two different subgroups (Table 1).

We have shown for the first time the presence of the aac(6\')-Ib-cr gene limited to *E. coli* species in North-East Italy. Like other plasmid-mediated resistance genes, i.e. *qnr*, the aac(6\')-Ib-cr gene does not significantly increment MIC values, but it seriously increases the mutant prevention concentration (MPC) with final production of remarkably resistant strains upon treatment with standard FQ dosage [1]. Indeed, we found that all but one clinical isolate presented three mutations at the topoisomerase level (2 in GyrA and 1 in ParC) with consequent generation of very resistant strains (MIC of ciprofloxacin $\geq$ 32). These mainly derived by clonal expansion, as demonstrated by ERIC subgrouping. Interestingly, sample #44 did not show any mutation in the topoisomerase genes, and so retained full susceptibility to FQs. However, #44 resulted clonally related to sample #39 which instead was fully resistant, indicating that transition from susceptibility to resistance probably occurred in a very limited time interval.

Finally, aac(6\')-Ib-cr-positive strains, which were strongly associated with ESBL, were collected mainly by inpatients and the proven plasmid localization and conjugation underline a very efficient mechanism of

Figure 1 aac(6\')-Ib-cr-positive strain analysis. A) Analysis of plasmids extracted from aac(6\')-Ib-cr-positive strains by the Kieser method. Plasmids were run on 0.7% TAE agarose gel at 50 V for 5 h. The numbers above each lane indicate the clinical strains ID. M stands for marker. B) Clonal relationship based on the repetitive element PCR fingerprinting method, using “enterobacterial repetitive intergenic consensus” (ERIC) primers. Amplified PCR products representative of the three identified subgroups were loaded on 1% TAE agarose gels and run at 100 V for 2 h. Both gels were stained with ethidium bromide.
horizontal transferability of these multiresistant strains. Therefore, the presence of aac(6')-Ib-cr-positive strains must be promptly detected and referred to clinicians in order to avoid use of FQs which would augment drug resistance and impair therapy.

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Authors’ contributions
IF carried out the molecular genetic studies and plasmid analysis. AC participated in the design of the study and in the selection of the clinical strains. CB performed the phenotypic analysis. SNR conceived of the study and participated in its design and coordination and drafted the manuscript. GP conceived of the study and participated in the coordination of the study. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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