First Detection of the Plasmid-Mediated Quinolone Resistance Determinants qnrB, qnrS and aac(6’)-Ib-cr in Extended Spectrum Beta-Lactamases-producing Klebsiella pneumonia in Bouaké, Côte d’Ivoire

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Authors’ contributions

This work was carried out in collaboration among all authors. Authors CAK and SPAN designed the study. Authors MNT, AEA, ZOW, BC, PM, NFW, EMS and SLB performed the experiments, analyzed the data and drafted the manuscript. Authors MNT, AEA and CAK were involved in critically reviewing the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

**Aims:** The aims of the present study were to investigate the presence of Plasmid-Mediated Quinolone Resistance (PMQR) determinants and the association of these determinants with Extended Spectrum Beta-Lactamases (ESBLs) genes in ESBL-producing *Klebsiella pneumoniae* isolates from Teaching Hospital of Bouaké, Côte d’Ivoire.

**Study Design:** It is a retrospective study.

**Place of Study:** Bacteriology-Virology Laboratory of Teaching Hospital, Bouaké, Côte d’Ivoire.

**Methodology:** From January 2015 to December 2016, 96 ESBL-producing *Klebsiella pneumoniae* isolates were collected from several specimens. Antimicrobial susceptibility of isolates was tested using the standard disk-diffusion method on Mueller-Hinton and interpretation according to recommendations of the 2017 EUCAST. These isolates analyzed for the detection of ESBL (bla<sub>CTX-M</sub>, bla<sub>TEM</sub> and bla<sub>SHV</sub>) and PMQR genes (aac(6')-Ib-cr, qnrB and qnrS) using simplex PCR.

**Results:** Of the 96 ESBL-producing strains, 85 (88.55%) harbored at least one of the ESBL genes tested. Out of the 85 strains encoding ESBL genes, 96.47% carried bla<sub>CTX-M</sub> and 92.94% bla<sub>SHV</sub> and bla<sub>TEM</sub> genes. Eighty nine (89.6%) of the 96 ESBL producing-isolates were resistant to ciprofloxacin and 84.4% to norfloxacin. Among the 96 strains, 80 (83.33%) were found harboring at least one PMQR gene consisting of 78 (81.3%) aac(6')-Ib-cr, 61 (63.5%) qnrB and 15 (15.6%) qnrS. Among the PMQR-positive strains, 68.4% coharbored qnrB+acc(6')-Ib-cr genes, 10.5% qnrB+qnrS+acc(6')-Ib-cr and 6.6% qnrS+acc(6')-Ib-cr. The qnrB gene was always linked to aac(6')-Ib-cr gene. Aac(6')-Ib-cr gene showed the highest association with three ESBL genes (87.6%), followed by qnrB gene (70.6%), then qnrS (17.7%).

**Conclusion:** The PMQR genes were highly prevalent in ESBL-producing *Klebsiella pneumoniae*, primarily the aac(6')-Ib-cr gene. The high associated was observed between ESBL and PMQR genes, notably with the aac(6')-Ib-cr gene.

*Keywords:* *Klebsiella pneumoniae*; resistance; genes; plasmid-mediated quinolone resistance; beta-lactamases.

### ABBREVIATIONS

| Abbreviation | Definition |
|--------------|------------|
| DNA          | Deoxyribo Nucleic Acid |
| PMQR         | Plasmid-Mediated Quinolone Resistance |
| Qnr          | Quinolone resistant |
| ESBL         | Extended Spectrum Beta-Lactamases |
| ATCC         | American Type Culture Collection |
| MALDI-TOF    | Matrix-Assisted Laser Desorption/Ionization Time of Flight |
| USA          | United States American |
| UV           | Ultra Violet |
| SPSS         | Statistical Package for the Social Sciences |

### 1. INTRODUCTION

After several decades of overuse of antibiotics, human pathogenic bacteria have reached alarming levels of resistance to many antibiotics. Fluoroquinolones, antibiotics that should have been used as a last resort in human medicine, have not been spared by this phenomenon. Two chromosomal mechanisms are responsible for clinical resistance to fluoroquinolones: the accumulation of mutations in the genes that code for DNA gyrase and IV topoisomerase, and the decrease in their intracellular concentration through increased efflux pump activity and/or decreased membrane permeability [1].

The discovery in 1998 of subclinical plasmid resistance to fluoroquinolones in a multi-resistant beta-lactamase-producing strain of *Klebsiella pneumoniae* lifted the veil on this phenomenon [2]. This resistance plasmid was then shown to host the qnrA gene coding for a 218-amino acid protein, qnrA, belonging to the family of proteins with repeated pentapeptide motifs that protects the DNA-gyrase complex from inhibition by quinolones [3]. Four other plasmid determinants also involved in quinolone resistance have been reported (qnrB, qnrS, qnrC and qnrD). Another mechanism of plasmid resistance has been related in strains isolated in China, namely inactivation of Fluoroquinolones by acetylation.
The determinant of this resistance is a variant of an aminoside N-acetyltransferase [4]. Another novel mechanism of plasmid resistance has been discovered, by active excretion of fluoroquinolones via the qepA efflux pump [5,6].

These PMQR mechanisms, in addition to conferring sub-clinical levels of resistance, also increase the likelihood of emergence of clinically resistant strains [7]. The co-expression of other multi-resistance genes by PMQR carrier strains and their dissemination in hospitals and in the community is a risk factor for therapeutic deadlock. Several studies, based on molecular approach, have reported a strong association between positive PMQR and ESBL positive Enterobacteriaceae isolates [8-11]. ESBLs are enzymes that generate resistance to the majority of beta-lactam antibiotics, the most widely used antibiotics in human medicine. These enzymes are commonly produced by *Klebsiella pneumoniae*, an enterobacterium responsible for high morbidity and mortality worldwide. It can cause nosocomial pneumonia, sepsis, urinary tract infections, wound infections, bacteremia and liver infections [12].

*Klebsiella pneumoniae* is one of the multi-resistant bacteria identified as an urgent threat to human health by the World Health Organization. The purpose of this study were to investigate the presence of PMQR determinants (aac(6')-Ib-cr, qnrB and qnrS) and to examine the association of these determinants with ESBLs genes in ESBL-producing *Klebsiella pneumoniae* isolated from Teaching Hospital of Bouaké.

2. METHODOLOGY

2.1 Strains Collection

From January 2015 to December 2016, 96 ESBL-producing *Klebsiella pneumoniae* were collected at the Bacteriology-Virology Laboratory of Teaching Hospital of Bouaké, Côte d'Ivoire. These strains were isolated from various biological products including urine, blood, pus, pleural fluid, bronchial specimen, urinary catheter, joint fluid and cephalo-rachidian fluid for diagnostic Purpose. Their identification was first carried out by the Minor's mini at Teaching Hospital of Bouaké and then confirmed at the Institute of Medical Microbiology, University Hospital Münster (Germany) by using Matrix-Assisted Laser Desorption/Ionization Time Of Flight mass spectrometry (MALDI-TOF) (Microflex, Bruker Daltonics, Bremen, Germany) [13].

2.2 Antimicrobial Susceptibility Testing

Antibiotic susceptibility of *Klebsiella pneumoniae* was tested using the standard disk-diffusion method on Mueller-Hinton agar and interpreted according to the 2017 recommendations of European Committee on Antimicrobial susceptibility Testing [14]. To do this, eighteen antibiotics were used: ampicillin (AMP, 10 μg), amoxicillin/clavulanic acid (AMC, 20/10 μg), piperacillin/tazobactam (PTZ, 30/6 μg), cefalexin (CFX, 30 μg), cefuroxim (CMX, 30 μg), cefotixin (FOX, 30 μg), cefotaxim (CTX, 5 μg), ceftazidim (CAZ, 10 μg), cefixim (CFM, 5 μg) cefepim (FEP, 30 μg), meropenem (MEM, 10 μg), imipenem (IPM, 10 μg) aztreonam (ATM, 30 μg), gentamicin (GM, 10 μg), ciprofloxacin (CIP, 5 μg), norfloxacin (NOR, 10 μg), trimethoprim/sulfamethoxazole (SXT, 1.25/23.75 μg), chloramphenicol (C, 30 μg). Antibiotic disk were purchased from Oxoid Ltd (Basingstoke, Hampshire, UK). *Escherichia coli* ATCC 25922 (American Type Culture Collection) strain was used as a control for antibiotic susceptibility testing.

2.3 Extraction of Bacterial DNA

*Klebsiella pneumoniae* isolate DNA was extracted by the simple boiling method. Briefly, *Klebsiella pneumoniae* isolates were cultured on Mueller Hinton agar (Merck, Germany) and incubated at 37°C. After 24 hours, one to five colonies were suspended in distilled water, and suspension was boiled for 30 min at 95°C in water bath [15]. The suspension was centrifuged at 14,000 rpm for five min and the supernatant transferred to filter columns. After a final centrifugation at 1200 rpm for five min, the supernatant was transferred to a new microtube and stored at -20°C.

2.4 Molecular Detection of ESBLs and PMQR Genes

Presence of *bla*TEM, *bla*SHV, *bla*CTX, *qnr*B, *qnr*S and aac (6')-Ib-cr genes were detected by simplex PCR using primers specific for each gene (Table 1) [16-19]. PCR amplification was performed in a reaction mixture of 25 μL, which contained 5 μL template DNA, 12.5 μL of 2x GoTaq® G2 Hot Start Colorless Master Mix.
2.5 Statistical Analysis

The data were analyzed using the statistical package for windows v.19.0 (SPSS). The relationships between ESBL production and PMQR determinants were evaluated by the Pearson chi 2 test. p value <0.05 was considered statistically significant.

### Table 1. Primers used for the detection of ESBL and PMQR genes

| Genes       | Primers              | Sequences (5′→3′)                  | Size (bp) | References |
|-------------|----------------------|------------------------------------|-----------|------------|
| bla<sub>TEM</sub> | TEM-F                | ATGAGATTCAACATTTCGCTG             | 840       | [16]       |
|             | TEM-R                | TTACCATGCTTATACGTCG               | 1051      | [17]       |
|             | SHV-F                | TTATGGCGCTTACCTTGACC              | 544       | [18]       |
|             | SHV-R                | ATTTGCTGCTTCTTTAATCCTCCG          |           |            |
| bla<sub>CTX-M</sub> | CTX-F              | TTTGCATGATGCACTAGCCTACGG          |           |            |
|             | CTX-R                | CGATATCGGTTGATGCGGCCCAT          |           |            |
| qnrB        | qnrB-F               | TGGCGAAAATTTTAGAAGAAG             | 594       | [19]       |
| qnrS        | qnrS-F               | GACGTGCTAATCTTGGGTGAT             | 388       | [19]       |
|             | qnrS-R               | AAACCTCGACTTAAGTCTGTA             |           |            |
| aac(6')-lb-cr | aac(6')-lb-cr-F     | TTTGCGATGCTCTATGGTAGGCCTA        | 482       | [20]       |
|             | aac(6')-lb-cr-R      | CTCGAATGCCTGCGGTGTTTC            |           |            |

(Promega, Madison, USA), 2.5 µL of each primer (10 µM) and 2.5 µL nuclease free water. The amplification of all these resistance genes was performed in a thermal cycler (Applied Biosystems, Inc., CA).

For bla<sub>TEM</sub>, bla<sub>SHV</sub> and bla<sub>CTX</sub> genes, thermal cycling conditions of consisted of an initial denaturation cycle of amplification at 94°C for 15 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 50 s, extension at 72°C for 90 s, and a final cycle of amplification at 72°C for 7 min. PCR conditions for qnr genes (qnrB and qnrS) were as follows: initial denaturation at 94°C for 5 min followed by 30 cycles consisting of 1 min at 94°C for denaturation, 1 min at 57°C for annealing and 1 min at 72°C for extension, then a final extension step at 72°C for 10 min. Primary denaturation at 95°C for 5 min; 35 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min; and, a final extension at 72°C for 10 min were used as thermal-cycling conditions for aac(6')-lb-cr genes.

PCR products were analyzed on 1.5% agarose gel at 115 V for 80 min in TBE 1X containing GelRed® Nucleic Acid Stain 10,000X using 100-bp DNA ladder (Promega, USA) as a size marker. The visualization of the bands was done under UV (ultra violet) in a Gel Documentation System.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Antibiotic susceptibility profile

All strains were resistant to cefalexin, cefuroxim, ceftazidim, cefepime and aztreonam. Resistance was 99% for cefotaxim and cefixim, 25% for cefoxitin, 4.2% for imipenem and meropenem, 91.7% for amoxicillin + clavulanic acid and 69.8% for piperacillin-tazobactam. The resistance rates to fluoroquinolones were 89.6% and 84.4% for ciprofloxacin and norfloxacin respectively. Regarding aminoglycosides, 84.3% of the strains were resistant gentamicin. Resistance to chloramphenicol and trimethoprim/sulfamethoxazole was 25% and 97.9% respectively (Fig. 1).

3.1.2 Prevalence of ESBL and PMQR genes

Of the 96 ESBL-producing strains, 85 (88.55%) harbored at least one of the ESBL genes tested in the present study. Out of the 85 strains encoding ESBL genes, 96.47% carried bla<sub>CTX-M</sub> and 92.94% bla<sub>SHV</sub> and bla<sub>TEM</sub> genes (Fig. 2). Moreover, the 3 genes were present simultaneously in 85.06% of the strains encoding ESBL genes.

Regarding the presence of PMQR genes, electrophoresis analysis revealed 83.33% (80) of 96 ESBL-producing strains harbored at least one PMQR gene which consisted of 63.5% for qnrB, 15.6% for qnrS and 81.3% for aac(6')-lb-cr genes. However, any PMQR genes were not detected in 16 strains (Fig. 3). There were statistically significant differences regarding the association between ESBL production and the presence of PMQR genes (p<0.001). The concomitant presence of 3 PMQR genes was detected in 8.33% of ESBL-producing strains.
resistance genes in clinical strains of Klebsiella pneumoniae isolated from patients at Teaching Hospital Bouaké. To do this, we again performed an antibiotic susceptibility test for all strains, following by the detection of resistance genes by PCR.

3.1.3 Distribution of PMQR determinants among encoding ESBL genes strains

Among strains encoding ESBL genes, 89.41% carried PMQR determinants. About the distribution of the different 3 PMQR genes among strains encoding ESBL genes, aac(6')-Ib-cr gene showed the highest association with three ESBL genes (87.6%), followed by qnrB gene (70.6%), then qnrS (17.7%).

3.2 Discussion

Bêta-lactams and fluoroquinolones have been considerably used since their acceptance in therapy. Inappopriate use is responsible for the rapid emergence of resistance to these two classes of antibiotics [21,22]. This study was trying to describe the co-existence of fluoroquinolone (PMQR) and bêta-lactam (ESBL) resistance genes in clinical strains of Klebsiella pneumoniae.

From this investigation, it appeared that bla_{CTX-M} was the most prevalent ESBL gene followed by bla_{TEM} and bla_{SHV} with the same prevalence. Our results are in accordance with studies by Shahid et al. [23], Ahmed et al. [24], Goudarzi et al. [25] Abdallah et al. [26], Ouedraogo et al. [27], Kpoda et al. [28] and Amer et al. [29] but disagreed with those by Pishtiwan a et al. [30], Bajpai et al. [31], Ghorbani-Dalini et al. [32], Yazdi et al. [33] and Eftekhar et al. [34] where bla_{TEM} and bla_{SHV} were most prevalent. This discrepancy could be explained by regional variation. The co-production of ESBLs genes were observed in most of the isolates meanwhile others isolates contained not none gene. The coexistence of ESBL genes were mentioned in many studies as those of Salah et al. [35], Amer et al. [36] and Amer et al. [29]. Absence of ESBL genes in some isolates would explain the presence another type of ESBL gene such as the VEB, OXA, TLA types.
Fig. 2. Agarose gel electrophoresis of PCR ESBLs genes of clinical isolates of *Klebsiella pneumoniae*

Lane M: DNA Marker (100-1500 bp), T+: positive control, T-: negative control, A) PCR amplification of \( \text{bla}_{\text{SHV}} \) gene showing single band at 1051 bp (lanes No. 89, 90, 92, 94, 95, 96 and 99 positives isolates for \( \text{bla}_{\text{SHV}} \) isolates; lanes No. 91, 93, 97 and 98 negatives isolates for \( \text{bla}_{\text{SHV}} \)). B) PCR amplification of \( \text{bla}_{\text{CTX-M}} \) gene, showing single band at 544 bp (lanes No. 1, 9, 12, 51, 54, 57, 58, 60, 61, 67: positives isolates for \( \text{bla}_{\text{CTX-M}} \) gene; lane 63: negatives isolates for \( \text{bla}_{\text{CTX-M}} \) gene). C) PCR amplification of \( \text{bla}_{\text{TEM}} \) gene, showing single band at 840 bp (lanes No. 14, 15, 16, 17, 18, 19, 20, 21, 23, 25, 26: positives isolates for \( \text{bla}_{\text{TEM}} \) gene; lanes 22 and 27: negatives isolates for \( \text{bla}_{\text{TEM}} \) gene).
Fig. 3. Agarose gel electrophoresis of PCR PMQR genes of isolates of *Klebsiella pneumoniae*

[Lane M: DNA Marker (100-1500bp), T+: positive control, T-: negative control, A) PCR amplification of *qnrB* gene showing single band at 594 bp (lanes No. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 13 positives isolates for *qnrB*, lanes No. 13 negatives isolates for *qnrB*, B) PCR amplification of *aac(6')-Ib-cr* gene, showing single band at 594 bp (lanes No. 1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13 positives isolates for *aac(6')-Ib-cr* gene; lane 2: negative isolate for *aac(6')-Ib-cr* gene), C) PCR amplification of *qnrS* gene, showing single band at 388 bp (lanes No. 6 and 11: positives isolates for *qnrS* gene; lanes 1, 2, 3, 4, 5, 7, 8, 9, 10, 12 and 13 negatives isolates for *qnrS* gene)]
The presence of resistance to fluoroquinolones in ESBL-producing isolates has been previously related [37]. In this study, high level of resistance to fluoroquinolones was observed. This prevalence was higher than those in Abidjan, Côte d’Ivoire (73.3%), Iran (68%) and Spain (19.4%) [38-40].

This high level of resistance to fluoroquinolones thus confirms high prevalence of PMQR determinants obtained in this study. These results were comparable with those noted in Tunisia (80%) [41] but higher than those reported in 2017 by Tahou et al. in Abidjan (Côte d’Ivoire), in 2016 by Domokos et al. in Hungary, in 2015 by Eftekhar and Seyedpour in Tehran (Iran), in 2014 by Guillard et al. in eastern France and in 2004 by Wang et al. which were 50.54%, 75%, 59.5%, 27.3% and 11% respectively [42-45]. A lower prevalence of PMQR determinants was also noted in Argentina (66.6%), Mexico (61.1%) and China (13.5%) [46-48]. However, a high prevalence (88.5%) that ours was observed in a study conducted in Iran [49].

In our investigation, the most prevalent gene among all PMQR genes was aac(6’)-Ib-cr, followed by qnrB and qnrS. Several studies have reported high levels of aac(6’)-Ib-cr genes compared to qnr genes [42-44,49-52]. Yet, the results of studies conducted in India, which indicated that qnrB was the most widespread PMQR [53]. This difference could be due to the variation in the type of samples processed. The high prevalence of aac(6’)-Ib-cr among Klebsiella pneumoniae isolates in our study may be related to clonal spread of a single clone.

Frequencies of qnr (B, S) genes found in this study are lower than those reported by other in Côte d’Ivoire in 2017 which were 71.73% for qnrB and 26.08% for qnrS [38] but higher than those obtained in Morocco where qnrB was found at 23% and qnrS at 3% [19]. Different prevalence of qnr genes were reported (64.3% for qnrS and 26.2% for qnrB) in Niger as well as in Togo (47.74% for qnrB and qnrS 47.10%) [54,55]. The low prevalence of qnrS was observed in our series was similar in a study conducted in Hungary [42]. We observed in this study that qnrS and aac(6’)-Ib-cr genes existed alone contrary to qnrB gene which was always linked to aac(6’)-Ib-cr reason for the high prevalence of the association qnrB+aac(6’)-Ib-cr. These two genes would exist on the same plasmid.

High frequency of PMQR determinants among our isolates carrying ESBL genes supports suggestions in which PMQR genes are co-associated with genes encoding ESBLs. Previous reports showed that PMQR genes were associated with diverse ESBL types. Korean researchers noted that SHV-12 and DHA-1 were co-produced with qnrA1 and qnrB4 [56]. In a Taiwanese Hospital, all qnr-positive ESBL-producing Enterobacter cloacae isolates possessed the bla<sub>SHV</sub>-12 and qnrB2-like genes [57]. In the current study, we noted a strong association between three ESBLS genes and aac(6’)-Ib-cr gene. Our findings corroborate those of Guillard et al. [44]. The presence of three ESBL genes and aac(6’)-Ib-cr genes in the same mobile genetic elements could explain the co-resistance of these genes. It could be the same strain of bacteria circulating. Molecular typing could confirm that, hence the limitation of our study.

Other potential limitations of this study were the absence of data on minimal inhibiting concentration (MIC) for norfloxacin and ciprofloxacin to determine the level of bacterial resistance to these antibiotics, exclusion of non ESBL-producing strains and sequence analysis of the different genes.

4. CONCLUSION

ESBLs have been extensively reported for the past years in both community and nosocomial settings and a strong linkage between their production and fluoroquinolones resistance has been described in Enterobacteriaceae. Our findings indicated high prevalence of PMQR determinants in ESBL-producing Klebsiella pneumoniae from Bouaké, Côte d’Ivoire. The high associated was observed between ESBL and PMQR genes, notably with the aac(6’)-Ib-cr gene.

ETHICAL APPROVAL

The study was carried out with the approval of the Medical and Scientific Department of Bouaké Teaching Hospital, who acts as the institutional ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.
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