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Antimicrobial Resistance of *Klebsiella pneumoniae* - ESBL Producing Strains Isolated from Clinical Specimens in Abidjan (Cote de Ivoire)

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Authors' contributions

This work was carried out in collaboration between all authors. Authors JET, NKG and PDS participated in the design of the study, in the collection and analysis of the data. Authors VG, FK and JK analyzed and interpreted the data. Authors KKG and BMO coordinated the study. The manuscript was designed by author JET. All authors contributed to the revision and approved the final version.

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ABSTRACT

Aims: The objective of this study is to detect the prevalence of qnr genes in broad-spectrum beta-lactamase producing *Klebsiella pneumoniae*, involved in hospital and community-acquired infections.
Study Design: It is a retrospective study.
Place and Duration of Study: Bacteriology and virology laboratorie of Institut Pasteur, Abidjan, Côte d'Ivoire.
Methodology: From January 2011 to June 2016, 350 *Klebsiella pneumoniae* were isolated from various clinical specimens and identified by conventional bacteriological tests. Antibiotic resistance (beta-lactams, quinolones and aminoglycosides) and detection of broad-spectrum beta-lactamases

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were assessed by the diffusion method and the synergy test, respectively. Molecular characterization of quinolone resistance genes (qnr A, qnr B and qnr S) was performed by the conventional polymerase chain reaction (PCR).
Results: Of the 350 *Klebsiella pneumoniae* isolates, 91(26%) were detected as ESBL producer, 36.2% (n=33) recovered from urine, 24.2% (n=22) from pus and 20.8% (n=19) from blood culture respectively. 46(33%) strains were idenifed to carry qnr genes, qnrB predominate 33(71%), followed by qnr 12(26.1%) and qnrA 1(2.2%). The strains exhibited high resistance to most of the agents tested, expect imipemen, low resistance to amikacin 4.1%, moderate to cefoxitin 31.8% and 54.9% with amoxicillin-clauvanic acid.
Conclusion: Although these observed prevalences are small proportions, this can be seen as a warning signal for the future. The emergence and dissemination of resistance genes in Côte d'Ivoire could pose a public health problem. Thus, the establishment of a relevant resistance surveillance policy to better control the circulation of multidrug-resistant strains is necessary.

Keywords: Klebsiella pneumonia; genes; resistance; quinolone; beta-lactamase; emergence.

1. INTRODUCTION

Quinolones and fluoroquinolones are molecules of choice, used to treat a wide variety of bacterial infections in humans [1]. The abusive use of these antibiotics has led to the progressive emergence of resistant bacteria, causing therapeutic failures that represent a real public health problem [2]. Support for quinolone resistance other than the chromosome was described for the first time in *Klebsiella pneumoniae* [3]. In addition, three mechanisms of plasmid resistance have recently been described. These include the quinolone-specific efflux pump [4], the protection of quinolone targets by qnr proteins [3] and the hydrolysis of quinolones By a protein derived from an enzyme responsible for aminoglycoside resistance (AAC (6) -1b-cr) [5]. The genetic determinant of this resistance is borne by mobile genetic elements such as plasmids and transposons. These elements allow the dissemination of resistance genes through horizontal transfer, resembling the genetic engineering methods developed in the laboratory [6]. Three types of qnr genes are known, qnr A genes with six (06) subtypes, qnr B with 19 subtypes and qnr S with three (03) subtypes [7]. In the world and particularly in Côte d'Ivoire, the resistance of bacteria to quinolones and fluoroquinolones has become a real concern for healthcare workers as well as for patients, as Guessennd and al. [8] and Ouattara and al. [9]. However, in Côte d'Ivoire, very little data are available on the qnr genes, hence the interest of this study whose main objective is to determine the prevalence of qnr A, B and S genes in *Klebsielle pneumoniae* producer of beta-lactamases isolated in patients hospitalized in Abidjan (Côte d'Ivoire).

2. MATERIALS AND METHODS

2.1 Strain Collection

Klebsiella pneumoniae strains isolated from various clinical specimens were collected from January 2011 to June 2016. The strains were isolated by direct inoculation of Drigalski medium containing 2 mg / L ceftazidime (homemade medium) [10]. The identification of the stains was carried out by the gallery of the reduced rack of LEMINOR in the laboratory of bacteriology and virology of Institut Pasteur of Ivory Coast (IPCI).

2.2 Bacterial Sensitivity

2.2.1 Antibiogramme

Sensitivity to antibiotics was determined by the diffusion method of discs in agar medium (Müeller-Hinton) according to the recommendations of the Antibiogram Committee of the French Society of Microbiology [11]. The antibiotic discs tested were amikacin (30 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), norfloxacin (10 µg), cefotaxime (30 µg), ceftazidime), Ceftriaxone (30 µg), aztreonam (30 µg), cefalotin (30 µg) cefoxitin (30 µg) tobramicin (10 µg) and amoxicillin-clavulanic acid (20/10 µg).

2.2.2 Detection of broad spectrum beta-lactamase production (ESBL)

The double synergy method was used for the detection of *Klesbiella pneumoniae* ESBL according to [12]. This consisted in placing the cephalosporin discs of 3rd generation (cefotaxime, ceftriaxone and ceftazidime) and

aztreonam at 30 mm around a central disc of amoxicillin clavulanic acid according to the recommendations of the French Committee of Antibiogramme of the French Society of Microbiology [11]. The presence of ESBL is represented by a distortion of the inhibition zone, with respect to the disc containing the clavulanic acid, thus describing a "champagne stopper" image. Only the isolates of *Klebsiella pneumoniae* ESBL which showed resistance to aminoglycosides and fluoroquinolones were taken into account in this study.

The reference strain *E. coli* ATCC 25922 was included in the course of the antibiograms in order to carry out the positive control.

2.3 Genotyping of Strains of *Klebsiella pneumoniae* ESBL

Extraction of the plasmid DNA from the *Klebsiella pneumoniae* strains and the reference strains (Table 1) was carried out by the alkaline lysis method with phenolyzation (homologous protocol). Conventional PCR detected the quinolone resistance genes (qnr A, B and S). Pairs of specific primers were used to amplify the fragments of the genes encoding each resistance (Table 2). The PCR amplification was carried out in a volume of 50 µl with the thermocycler (Perkin® Elmer Gen Amp Lapplied Biosystems 9700). The PCR amplification conditions used to detect qnr, included a pre-denaturation step at 94℃ for 5 min followed by 30 cycles at 94℃ for 30 s, 55℃ for 30 s and 72℃ For 60 s, with polishing at 72℃ for 10 minutes. The reaction medium is composed of 5 µl of plasmid DNA, 0.3 U of Taq polymerase (Promega), 10 µM dNTP mixture, 10 µM MgCl2, 10 µM each target primer, and 5X PCR buffer (final concentration). Another DNA-free reaction mixture was used for the negative control. The amplified products were analyzed by electrophoresis in a 1.5% agarose gel (Invitrogen) stained with ethidium bromide. The reading was carried out on the ultraviolet plate (Gel doc).

3. RESULTS AND DISCUSSION

3.1 Bacterial Strains

Out of a total of 350 strains of *Klebsiella pneumoniae* collected from January 2011 to June 2016, 91 were ESBL producers. The prevalence observed for these ESBL isolates with antibiotics was 26%. The distribution of ESBL-producing strains according to clinical specimens is shown in Fig. 1. *Klebsiella pneumoniae* is elevated in urine (36.2%), followed by pus (24.2%) and blood (20.8%).

3.2 Sensitivity of Strains of *Klebsiella pneumoniae* ESBL

3.2.1 For beta-lactams

The strains tested were all susceptible to imipenem (99%) with the exception of one strain. Resistance was 31.8% for cefoxitin, 92.3% for ceftazidin and cefotaxime, 93.4% for ceftriaxone, and 100% for cefalotin. As for aztreonam, the level of resistance was 90.1% and finally 54.9% for amoxicillin and clavulanic acid.

Table 1. Characteristics of reference strains taken as controls

Bacteria	Numbers	Characteristics	Positive control
E. coli	U2A 2118	*qnrA1*	Controls
E. coli	U2A 2119	*qnrB1*	Positives genes *qnr*
E. coli	U2A 2120	*qnrS1*	*A, B, S*

qnr = Quinolone résistance

Table 2. List of primers to be used for detection

Genes		sequences 5'-3'	References	Size of the amplicon (bp)
qnrA	Amorce F	TTCTCACGCCAGGATTTGAG	Seyed *et al*. (2014) Jundishapur	571
	Amorce R	TGCCAGGCACAGATCTTGAC	J Microbiol. 2014 july;7 (7) e11136	
qnrB	Amorce F	TGGCGAAAAAATTGAACAGAA	Seyed *et al*. (2014) Jundishapur	594
	Amorce R	GAGC AAC GATCGCCTGGTAG	J Microbiol. 2014 july;7 (7) e11136	
qnrS	Amorce F	GACGTGCT AAC TTGCGTGAT	Seyed *et al*. (2014) Jundishapur	388
	Amorce R	AAC ACCTCGACTTAAGTCTGA	J Microbiol. 2014 july;7 (7) e11136	

3.2.2 For floroquinolones

The rate of resistance to quinolones was 67% for ciprofloxacin, 50.5% for nalidixic acid and 73.6% for norfloxacin.

3.2.3 For aminoglycosides

The resistance of the strains to aminoglycosides revealed that only amikacin was the most active molecule with only 4.4% resistance. As for tobramicin and gentamicin, all strains were resistant to 84.6% and 74.7%, respectively.

3.3 Search for Qnr Genes

The PCR technique allowed the detection of 46 strains of the 91 analyzed with the qnr gene, ie a prevalence of 50.54% with a predominance of type B. Thus, on the 46 strains 33, the qnr B gene (594 bp) 71.73%, 12 strains possess qnr S (388 bp) or 26.08% and a single strain possesses qnr A (571 bp), that is to say 2.17%. Fig. 2 shows the genes qnr B and S with respective positive bands 594 and 388 base pairs.

Klebsiella sp are a group of microorganisms frequently isolated from biological products following a bacterial infection. It has been reported according to the publications of Bao et al. [13] and Rangaiahagari et al. [14] that *E. coli* and *Klebsiella sp* are the most commonly encountered species.In this report, 36.2% of *Klebsiella pneumoniae* was isolated from urine. This observed isolation predominance was reported by Hashemi et al. [15] in Iran with 69.3% and Raji et al. [16] with 64.7% in Nigeria. However, high rates of positive blood cultures with *K. pneumoniae* ESBL have been described by some authors [17,18].

The sensitivity of strains of *Klebsialla pneumoniae* is characterized by a high level of resistance to antibiotics, namely beta-lactams, aminoglycosides and quinolones. Thus, a prevalence of 26% of ESBL-producing strains and up to 90% resistance to third-generation cephalosporins has been observed. This situation is related to the hospital origin of the strains studied. According to several studies, antibiotic-resistant *Klebsiella pneumoniae* is very often involved in nosocomial infections [19,20].

With respect to the qnr gene data, this study detected 46 strains of the 91 analyzed with the qnr gene, ie a prevalence of 50.54% with a predominance of type B. This frequency is higher than that reported by Guessennd et al. [8] in Côte d'Ivoire, who reported a prevalence of 14.6% for the qnr B gene that was most frequent, 9.9% for qnr A and 2.7% for the qnr S gene. Ouattara et al. [9] also detected the qnr S gene in Côte d'Ivoire in isolates from different ecosystems with a very high prevalence of 95% in animals. However, the qnr A and qnr B genes have not been detected. Studies in China, the USA and Slovenia have found low prevalences between 0 and 12% [21,22,23]. The prevalence rates of the qnr genes observed by these different authors are lower than the

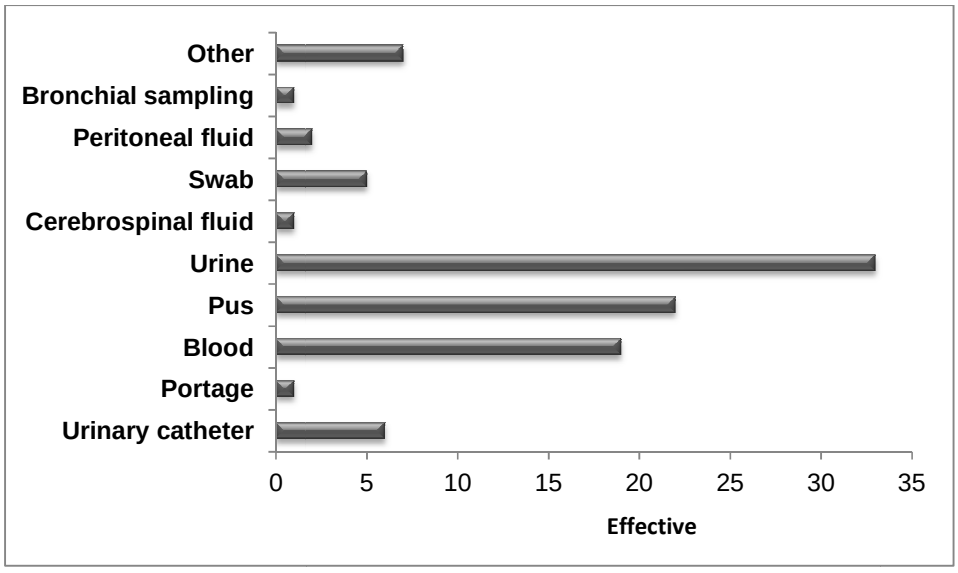

Fig. 1. Distribution of strains of *Klebsiella pneumoniae* in the various pathological products

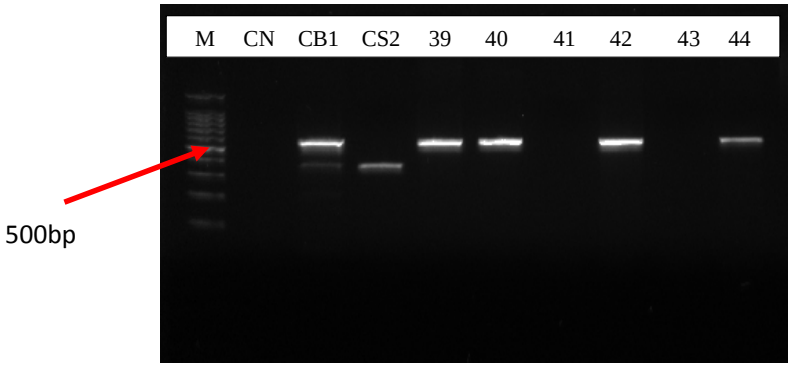

Fig. 2. 1.5% agarose gel electrophoresis showing multiplex PCR for the simultaneous detection of the qnr, B and S genes

Lane M: molecular weight marker (Invitrogen, 100 bp DNA Ladder); Lane CN: Negative control; Lane CB1: Positive control qnr B (594 bp); Lane CS2: Positive control qnr S (388 bp); Lane 39, 40, 42 and 44: positive samples for qnr B

prevalence obtained in this study. This situation is not only a major concern in the therapeutic management of patients, because the presence of this gene (qnr) is a potential risk of horizontal transfer by mobile genetic elements (plasmid and transposon) from one bacterium to another. It therefore appears necessary to put in place measures to monitor bacterial resistance to antibiotics in different ecosystems of countries. In addition, qnr-positive isolates show resistance to aminoglycosides and beta-lactams. This may be explained by the fact that plasmid support of quinolone resistance is associated with integrons bearing resistance determinants to several other antibiotics such as beta-lactams and aminoglycosides [24]. The coexistence of genes of resistance to various families of antibiotics in the same bacterium can pose a diagnosis and therapy problem according to Roh et al. [25]. Previous studies have also shown that most ESBL genes are located on transposons and on self-transferable plasmids, which generally co-exist with the plasmid-mediated quinolone resistance determinants [26,27]. This phenotype could reflect the excessive use of beta-lactam and Quinolones and the absence of strict antimicrobial policies in health care facilities.

Moreover, the conventional PCR technique used in this study has shown limits, although it has made it possible to identify the genes qnr A, B and S. In fact, the primers used do not make it possible to screen all the variants qnr described so far. Other quinolone plasmid resistance genes have recently been described: qnr C and qnr D [28,29].

The presence of genes of resistance to quinolones and fluoroquinolones at the level of strains of human origin raises the question of the origin of these strains carrying the qnr genes. Would they be strains carried by medical personnel or patients from another service? Whatever the response to these problems, the presence of the qnr gene in hospitals or in the community, is a threat of diffusion of multiresistant bacteria for which a solution must be considered.

4. CONCLUSION

The emergence of mechanisms of quinolone plasmid resistance is a real threat to the efficacy of fluoroquinolones at several levels. First, their presence on conjugative plasmids promotes their mobility and their dissemination in the various pathogenic bacteria. Moreover, their association with other determinants of resistance, contributes to a cosmelection of resistance to antibiotics. Plasmid mechanisms of quinolone resistance independently confer a low level of resistance to fluoroquinolones, but their presence could also promote evolution towards a higher level of resistance by mutation selection in the targets of these molecules. The emergence of this combination of resistance genes in Côte d'Ivoire could pose a public health problem. Hence the need for a relevant resistance surveillance policy to better control the circulation of multidrug-resistant strains. Moreover, for future studies of other amplification primers (qnr C and qnr D) could finally be used to have an overview of the qnr genes. Therefore, the sequencing of the qnr detected in this study would make it possible to

know the different variants existing in Côte d 'Ivoire.

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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