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First report of qnr genes in multidrugs resistant (ESBL) enterobacteria isolated from different ecosystems in Abidjan, Ivory Coast

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Abstract
Aim: To show the prevalence of qnr genes in Enterobacteria producing a beta-lactamase resistant to nalidixic acid extended spectrum and ciprofloxacin isolated from various ecosystems such as human, animals and the environment. Materials and methods: The multiresistant Enterobacteria strains (n = 155) were isolated from a selective homemade medium (Drigalski + 2mg /l of ceftazidime) of diverse ecosystems (humans, animals and environments). Resistance to antibiotics (beta-lactams and quinolones) has been evaluated by the diffusion method and detection of beta-lactamases extended spectrum (ESBL) was carried out as recommended by NCCLS. Characterization of quinolone resistance genes (qnr A, qnr B and S qnr) was performed by multiplex PCR. Results: high levels of percent resistance to quinolones and fluoroquinolones (35% to 95%) and 35% to 93.2% respectively to nalidixic acid and ciprofloxacin were observed. The qnr gene S was detected in isolates from different ecosystems with very high prevalence strains (95%) in animals. However qnrA and qnr B genes were not detected. Conclusion: The emergence of new resistance mechanisms adopted by some strains of Enterobacteria especially in human and in the environment represents a threat to public health.

1. Introduction

Quinolones and fluoroquinolones are the antibiotics used worldwide for the treatment of infectious diseases [1]. They are employed in both human and veterinary medicine [2]. The Misuses of any others families of antibiotics has contributed to the selection and the emergence of resistant bacterias to fluoroquinolones [1]. The carrier quinolone resistance other than chromosome was first described in *Klebsiella pneumoniae* [3]. Several mechanisms are involved in fluoroquinolones resistance particulary the action of the qnr protein, the inactivation of the antibiotic activity (Acetylase AAC (6 ') - cr-Ib) and decrease in intracellar concentration of the antibiotic (efflux pump Qep A) [4-5]. The

prevalence of qnr genes in resistance to quinolones and fluoroquinolones remains increasing high with prevalences ranging from 0.2 to 94% [6]. The genetic determinant of this resistance is the qnr gene whose characteristic is to be beared by different types of integrons. The importance of these integrons remains in they portability and ability to accelerate the spread of quinolone resistance [3]. Quinolone resistance is frequently associated with genes of beta-lactam resistance such as SHV, TEM, PER, VEB, CTX-M among Enterobacteria producing extended spectrum beta-lactamases (ESBL). Three types of qnr genes are known: qnrA genes with 06 subtypes, qnr B with 19 subtypes and qnr S (3 subtypes) [7]. Qnr genes have been described for the first time in Ivory Coast in isolated clinical strains of inpatient and outpatient [8].

Given the human-animal-environment interactions and the potential effect of quinolone resistance on these ecosystems, the main objective of this work is to show the prevalence of genes qnrA, B and S hold by enterobacteria producing beta lactamases isolated in diverse ecosystems.

2. Materials and Methods

2.1. Collection of Human Strains

The Enterobacteria strains producing extended spectrum beta-lactamases (ESBL) were obtained from the clinical bacteriology unit (CBU) of the *Institut Pasteur of Côte d'Ivoire*. These strains were isolated from biological products (urine, blood and pus) and consisted in 09 *Escherichia coli*, 08 *Klebsiella pneumoniae* and 05 *Enterobacter cloacae* strains.

2.2. Collection of Animal Strains

The enterobacteria ESBL's strains in animals (cattle, sheep and pig) were isolated from stools freshly emitted by animals or from rectal swabs made directly on these animals. These saddles were collected in sterile jars containing saline. The strains isolated consisted primarily of *Escherichia coli* strains. They were distributed as follows 21 strains from cattle, 20 from sheep, and 23 from pigs.

2.3. Collection of Environmental Strains

The environmental enterobacteria ESBL's producers were isolated from hospital effluents of three health centers (CHU Cocody, CHU Treichville, general Abobo hospital) and municipal effluents of 04 municipalities of Abidjan city.

Strains from hospital effluents were distributed as follows: *Escherichia coli* (28); *Klebsiella pneumoniae* (08); *Enterobacter aerogenes* (10) and strains of municipal wastewater were distributed as follows: *Escherichia coli* (11); *Klebsiella pneumoniae* (04); *Klebsiella oxytoca* (04) and *Enterobacter aerogenes* (04).

2.4. Isolation and Identification of Enterobacteria Strains

The sampling, the identification, the conservation and the genotypic characterization of strains were made in different specialized units from the Institut Pasteur of Côte d'Ivoire The strains were isolated by direct inoculation of Drigalski medium containing 2 mg / L ceftazidime (homemade medium)[9]. The identification of strains was done on API20E galleries.2.5. Antibiotic Susceptibility of Strains of Enterobacteria ESBL

According to standardized flood susceptibility, the aspiration technique was performed on all strains as recommended by the Antibiogram's Committee of the French Society for Microbiology (CASFM 2012). The extended spectrum beta-lactamase production was confirmed by a standard double-disk synergy test illustrating by a specific image of synergy between antibiotic disks as described by the National Committee for Clinical Laboratory Standards [10].

This technique consists of adding the disk associated with amoxicillin clavulanic acid, the aztreonam disk and those of the third-generation cephalosporin, at a distance of 2 cm from center to center. Negative tests were repeated with disks of 1.5 cm center to center or on Mueller Hinton medium containing 250 mg / ml of cloxacillin. Only were included in the study, ESBL enterobacteria showing resistance to quinolones and fluoroquinolones.

The tested antibiotics were given in Table 1.

Table 1. *List of antibiotics used in the study and their break point (according to CA-SFM 2012).*

Antibiotics (load en µg)	Acronym	S≥	I	R<
Beta-lactam				
Amoxicillin+clavulanic acid (20/10)	AMC	21		16
Piperacillin (75)	PIP	20		16
Ceftazidime (30)	CAZ	26		21
Ceftriaxone (30)	CRO	26		23
Cefepime (30)	FEP	24		24
Aztreonam (30)	ATM	27		21
Cefotaxime (30)	CTX	26		23
Cefoxitine Cefoxitin(30)	FOX	22		15
Imipenem (10)	IPM	24		17
Meropenem (10)	MEM	22		15
Quinolones and fluoroquinolones				
Nalidixic acid (30)	NA	20		15
Ciprofloxacin (5)	CIP	25		22

The *Escherichia coli* strain 25922 was used as positive control when performing susceptibility test.

2.6. Genotyping of ESBL Strains of Enterobacteria

Detection of genes qnr A, B and S was done using a multiplex PCR method. Plasmids DNA were extracted from strains by alkaline lysis with phenolyzation. The PCR amplification was performed in a final reaction volume of 50 µl. Primers used in this study were given in Table 2. The reaction mixture contained a PCR Reaction Buffer, 10x concentrated with 20 mM $MgCl_2$, PCR Grade Nucleotide Mix (2.5 mM each), specific primers for each target (20 pmol)

and a FastStart Taq DNA Polymerase, 5 U/µl (Roche).

Reference strains provided by the collection of the Institut Pasteur de Paris (CIP) were used as positive controls for the PCR (Table 4) and a reaction mixture without DNA served for PCR negative control.

The PCR conditions (Table 3) were carried out in a thermalcycler UNOII (BIOMETRA®). Amplification products were analyzed by electrophoresis in a 2% agarose gel (Invitrogen) stained with ethidium bromide. Playback is performed on Ultra Violet plate.

Table 2. *Primers used in the study*

Target Genus	Primers	Sequences (5'- 3')	Position on genome	size of PCR product (pb)	accession number
qnrA	*qnrA*-1A	TTCTCACGCCAGGATTTGAG	339-358	571	AY070235
	qnrA-1B	TGCCAGGCACAGATCTTGAC	910-891		
qnrB	*qnrB*-cs-1A	TGGCGAAAAAATT(GA)ACAGAA	54-73	594	DQ351241
	qnrB-cs-1B	GAGCAACGA(TC)GCCTGGTAG	648-630		
qnrS	*qnrS*-1A	GACGTGCTAACTTGCGTGAT	101-120	388	DQ485529
	qnrS-1B	AACACCTCGACTTAAGTCTGA	489-469		

Table 3. *qnr's genes amplification conditions*

Amplification steps	Condition/Time
Initial denaturation	94°C/5min
Cyclic denaturation	94°C/1min
Annealing	60°C/45sec
Cyclic elongation	72°C/1min
Final Elongation	72°C/1min
Cycles number	30

Table 4. *Bacterial reference strains used for the PCR reaction control*

Bacteria specimen	CIP code	Resistance gene harboring	positif control using for
E. coli	UAA2118	*qnrA1*	*qnrA*
	UAA2119	*qnrB1*	*qnrB*
	UAA2120	*qnrS1*	*qnrS*

3. Results

3.1. Antibiotic Susceptibility of strains of Enterobacteria ESBL

At the level of beta-lactams, only carbapenems (imipenem and meropenem) had a total efficiency (100% sensitivity) on ESBLs enterobacteria whatever the origin. A lower rate of resistance was observed for cephamycins especially for animal strains.

All strains of ESBL producing Enterobacteria showed a high level of resistance to conventional quinolones (nalidixic acid) from 35% to 95 cattle strains, 2% for human strains. As far as concerns the fluoroquinolones (ciprofloxacin) resistance rate was as high and also went from 35% for bovine strains to 93.2% for human stem

The rate of antibiotic resistance of the main species isolated from different ecosystems is reported in Table 5.

Table 5. *Enterobacteria Resistance rates according to their isolation origin*

	Resistance rates (I+R %)					
Antibiotics (load en µg)	**human strains**	**Animal strains**			**environmental strains**	
Beta-lactam		**bovine strains**	**sheep strains**	**porcine strains**	**hospital effluents**	**Municipal effluents**
Amoxicillin+ clavulanic acid (20/10)	87.1	40	51.7	21.3	51.2	60.5
Piperacillin (75)	100	100	100	100	100	100
Ceftazidime (30)	100	NT	NT	NT	100	100
Ceftriaxone (30)	100	100	100	100	100	100
Cefepime (30)	100	NT	NT	NT	97.6	90.7
Aztreonam (30)	100	100	100	100	100	100
Cefotaxime (30)	100	100	100	100	100	100
Cefoxitin(30)	36.1	5	13.8	2.1	58.5	27.9
Imipenem (10)	0	0	0	0	0	0
Meropenem (10)	0	0	0	0	0	0
Quinolone						
Nalidixic acid (30)	95.2	35	41.4	55.3	80.5	67.4
Ciprofloxacin (5)	93.2	35	37.9	44.7	73.2	60.2

NT = Not Tested

3.2. Search Qnr Genes

PCR results showed a high prevalence of qnrS gene (388 bp) in the animal strains with frequencies of 90.5%; 80% and 91.3% respectively in cattle, sheep and pigs. qnrA and qnr B genes were not detected during this study. All of the genes identified are summarized in Table 6.

Table 6. *Distribution of qnr's genes according to the origin of ESBL strains*

Strain origin	Number of strains tested	*qnrA* (%)	*qnrB* (%)	*qnrS* (%)
Human	22	0(0)	0(0)	2 (9.1)
Bovine	21	0(0)	0(0)	19 (90.5)
Sheep	20	0(0)	0(0)	16 (80)
Porcine	23	0(0)	0(0)	21 (91.3)
Hospital effluents	46	0(0)	0(0)	5 (10.9)
Municipal effluents	23	0(0)	0(0)	5 (21.7)

Figure 1 and Figure 2 show qnr genes A, B and S with respective positive bands 571, 594 and 388 base pairs.

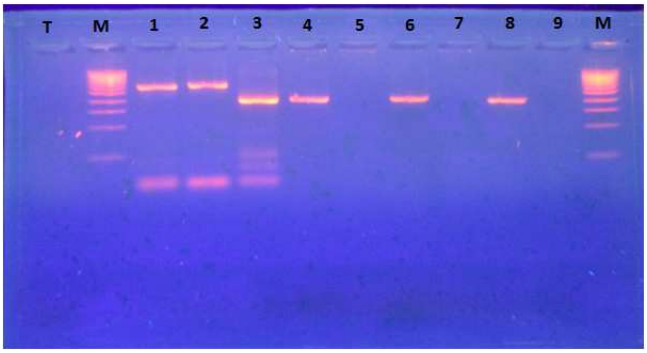

Figure 1. *2% agarose gel electrophoresis showing multiplex PCR amplification products for simultaneous detection of genes qnr A, B and S.Lane T: Negative control*

Lane M: molecular weight marker (Invitrogen, 100 bp DNA Ladder).
Lane 1: Positive control qnr A (571 bp)
Lane 2: Positive control qnr B (594 bp)
Lane 3: Positive control qnr S (388 bp)
Lane 4, 6 and 8: positive samples for qnr S

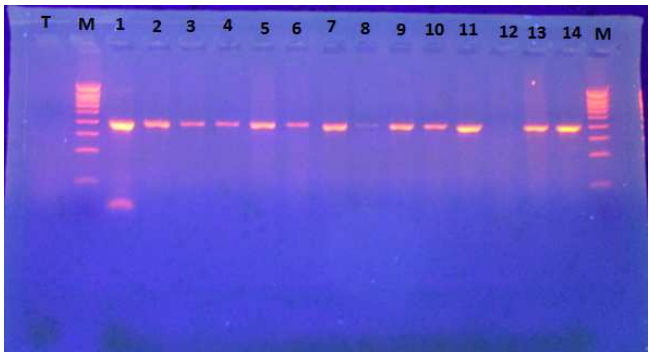

Figure 2. *2% agarose gel electrophoresis showing simplex PCR amplification products for detection of the gene qnr S.*

Lane T: Negative control
Lane M: molecular weight marker (Invitrogen, 100 bp DNA Ladder).
Lane 1: Positive control qnr S (388 bp)
Lane 2, 11, 13 and 14: positive samples for qnr S

4. Discussion

In this study, *Escherichia coli* (*E. coli*) were the most isolated strain. The predominance of this specy and *Klebsiella pneumoniae* had already been noted out in an earlier study on the prevalence and resistance profiles of Enterobacteria producing ESBL causing human infections in Abidjan [11]. Some studies affirmed that *Klebsiella* strains were usually carriers of resistance genes. The extended spectrum beta-lactamase was generally found on large plasmids carrying resistance genes and was mostly isolated from *Klebsiella* strains than from *Escherichia coli* [12]. *Klebsiella* strain's survival time is longer than other enteric bacteria on the hands and in the hospital environment. This is facilitating its dissemination around hospitals [13].The emergence of multidrug-resistant Escherichia coli in animal domain (beef and pig) was reported in other studies. The analysis of some of these E. coli showed they were producers of extended spectrum beta-lactamase (ESBL) carrying the CTX-M gene well knowed in human medicine [14]. In several studies in Africa (Benin, Morocco) and Europe (Spain, France), some authors also demonstrated a bacterial flora of hospital effluents dominated by Gram-negative bacteria belonging to the Enterobacteria (*E. coli*, *K. pneumoniae*) [15-16-17-18].

In the family of betalactam class antibiotics, ESBL's enterobacteria are usually resistant to cephalosporins 3rd and 4th generation and to aztreonam, but susceptible to carbapenems. In our study all strains were susceptible to carbapenems (meropenem and imipenem). These antibiotics are considered as drugs of choice for treatment infectious diseases related to resistant enterobacteria [19-20]. The ESBL's enterobacteria by their genetic determinism are often resistant to other classes of antibiotics due to the mobile genetic elements on their genome including plasmids, transposons and integrons [21]. In addition, the combination of these resistant genes carried by these mobile elements with the genes of chromosome resistance is the result of multiresistance to major classes of antibiotics used in therapy [22-23]. Based on this observation, we noted levels of resistance to quinolones (nalidixic acid) from 35% for animals (cattle) to 95.2% for human and rates of fluoroquinolone resistance of 35% for cattle to 93.2% for human. These high rates of fluoroquinolone resistance in human were also been reported by Guessennd and al., [8] with rates of 76.8% for nalidixic acid and 70.2% for ciprofloxacin in human strains. In animals especially in the poultry industry, Blanco and al.; [24] obtained rates ranging from 37% to nalidixic and a relatively low rate for ciprofloxacin (7%) for all quinolones. In Iceland, a rate of 42.5% for ciprofloxacin and nalidixic acid was obtained by Thorunn and al., [25]. The two studies have been realized on *E. coli*'s strains. Quinolone resistance in Enterobacteria is

usually the result of a chromosomal mutation, leading to alteration of the target enzyme or the accumulation of the antimicrobial. However, it was also reported plasmid-mediated quinolone resistance (PMQR), reflecting an acquisition of qnr genes, qepA, and aac (6 ') - Ib cr [26-27]. The PMQR is frequently associated with the presence of ESBLs resistance genes and aminoglycosides on the same plasmid. In Ivory Coast, the first study of qnr genes was realized by Guessennd and al., [8] on 151 human strains producing ESBL isolated from clinical specimens. This study showed a qnr prevalence of 27.2% with a predominance of qnr B gene (14.6%) followed by qnr A gene (9.9%). In our study only the qnr S gene was identified with a prevalence of 9.2% among human strains. The qnr genes A and B were not been identified. This lower rate of qnr S gene detection in human strain could be explained by the fact that these strains use another sort of qnr resistance genes. To date new qnr genes have been identified: these are the qnr genes C and D [28-29-30-31]. showing new resistance mechanisms like the genes coding for proteins qepA, and aac (6 ') - Ib-cr. Regarding hospital and municipal effluents, rates of 10.9% and 21.7% for qnr S gene were respectively detected. These rates are similar to that's founds by Vishal and al., [32] in India where 90% of isolated E. coli producing extended spectrum beta-lactamase and fluoroquinolone resistance had the aac (6 ') - Ib-cr gene. The last 10% were holders of the qnr A or B genes. No qnr S gene were detected in their study.

The High levels of qnr S genes involved in the resistance to fluoroquinolones were observed in farm animals.

5. Conclusion

This study showed high resistance prevalence in Enterobacteria producing broaded spectrum beta-lactamase to quinolones and fluoroquinolones especially in human strains. The search for genes of fluoroquinolone resistance revealed a high rate of qnr S genes in animals. The emergence of new resistance mechanisms are fears in Ivory Coast especially in human and environmental strains because despite the high quinolones and fluoroquinolones prevalence of resistance genes (qnr A, qnr B resistance rates and qnr S) which have been the subject of previous studies are far from being achieved. It would be interesting to focus research on new resistance genes (qnr C and qnr D) and to the mechanisms of resistance (QEP A and AAC (6 ') - Ib-cr).

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