Current Research in Microbiology and Biotechnology
Vol. 5, No. 1 (2017): 953-957
Research Article
Open Access

ISSN: 2320-2246

Quinolone Resistance (qnr) genes in Fecal Carriage of Extended Spectrum beta-Lactamases producing Enterobacteria isolated from Children in Niger

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Received: 23 November 2016 Accepted: 21 December 2016 Online: 02 January 2017

ABSTRACT

Qnr genes are known to confer a low level resistance to fluoroquinolone in *Enterobacteriaceae*. Their presence with other antibiotic resistance mechanism as extended spectrum β-lactamase (ESBL) on the same plasmid are common and can spread widely. This study aimed to detect the presence of *qnr* genes in ESBL-producing commensal *Enterobacteriaceae* in 443 children aged 6 to 59 months included in a clinical trial conducted by Doctors without Borders/Epicentre at Maradi, Niger. A total of 78 strains among 163 ESBL-producing commensal *Enterobacteriaceae* with phenotypic resistance to quinolone were selected and identified with the API 20E identification system. A multiplex PCR with specific primers was then performed to detect the presence of *qnrA, qnrB* and *qnrS* genes. These strains included 63 *E. coli* and 15 *Klebsiella spp.* We found 42 (53.9%) of them harboring at least one *qnr* gene: *E. coli* (n=28) and *Klebsiella spp* (n=14) : 4 (9.5%) *qnrA*, 11 (26.2%) *qnrB* and 27 (64. 3%) *qnrS*. This is the first identification of *qnr* genes in ESBL producing commensal *E. coli* and *Klebsiella spp* in children without antibiotic therapy in the seven precedent days in Niger. These genes could be involved in the resistance to quinolones in Niger.

Keywords: *Enterobacteriaceae*, ESBL, quinolone resistance, Niger.

1. INTRODUCTION

Quinolones and β-lactams are the most used antibiotics in the world to treat bacterial infectious diseases [1]. Quinolones are synthetic antibiotics used for infections involving gram negative bacteria such as *Enterobacteriaceae*. Fluoroquinolones, quinolone derivatives, have a large spectrum antibacterial activity and their intrinsic activity is superior to quinolone's one [2].

Three main mechanisms of quinolone resistance have been described: i) an accumulation of mutations in the gene coding for DNA gyrase and toposisomerase IV, targets of quinolones; ii) a decreasing of intracellular concentration of fluoroquinolones by the reduction of porins production or by the modification of the efflux pumps activity, iii) an acquisition of plasmid resistance gene [2]. The plasmid mechanism of quinolone resistance leads to a low level of fluoroquinolone

resistance and facilitate the emergence of high level of resistance when the antibiotic is used in therapeutic conditions [3]. The acquisition of plasmid-mediated quinolone resistance (PMQR) genes can lead to the protection of quinolone's target by *qnr* (quinolone resistance) proteins, the hydrolysis of quinolone by *aac(6')-Ib-cr* protein, involving in the aminoside resistance and the quinolone efflux pumps [2,3]. The *qnr* proteins belong to the repeated pentapeptidique protein (RPR) family characterized by a serial repetition of five amino acids. They protect gyrase and topoisomerase IV against fluoroquinolone's action. About 100 different *qnr* variants have been described mainly from *Enterobacteriaceae*, and grouped in 5 distinct families *qnrA, qnrB, qnrC, qnrD and qnrS* [3,4]. Previous studies in Europe, United States, Asia and Africa described *Enterobacteriaceae* with both extended spectrum β-lactamase (ESBL) and *qnr* genes [3,5].

However no data are available in Niger about the prevalence of *qnr* genes among ESBL producing *Enterobacteriaceae* (ESBL-E). The aim of our study was to characterize *qnr* genes among fecal commensal ESBL producing *E. coli* and *Klebsiella spp* in children aged 6 to 59 months. These children were included in a randomized clinical trial conducted from 2012 to 2013 by Doctors without Borders/Epicentre at Maradi, Niger, where the main objective was to assess the effect of routine amoxicillin use on nutritional recovery in children with uncomplicated severe acute malnutrition [6].

2. MATERIALS AND METHODS

2.1 Selection of bacterial strains

Bacterial strains were selected from a collection of strains isolated from stools of 443 children included in a sub-study from March to July 2013 of the parent clinical trial [6]. The aim of the sub-study was to describe the potential effect of amoxicillin on emergence of fecal ESBL-E. Fresh stool samples or swab samples were collected from children in 4 health centers of Madarounfa district in Maradi region (Madarounfa, Gabi, Dan Issa and Tofa). The samples were stored at 4°C in Cary-Blair tube and transferred to the Epicentre bacteriological laboratory at Maradi for bacteriological analysis. Briefly, stool samples were plated on chromogenic selective media (CHROMagar ESBL, CHROMagar Company, Paris, France) to identify ESBL-E: pink, dark blue and white colonies suspecting respectively *E. coli, Klebsiella spp* and *Salmonella spp*. Antibiotic susceptibility tests were performed by the disc diffusion method on Muller Hinton agar according to the recommendations of the Antibiogram Committee of the French Society of Microbiology [7]. The following antibiotic discs (Biorad, Marne-La-Coquette, France) were used: amoxicillin (25µg), amoxicillin+clavulanic acid (20µg+10µg), tircarcillin (75µg), cefalotin (30µg), cefoxitin (30µg), cefotaxim (30µg), ceftazidim (30µg), cefepim (30µg), Imipenem (10µg), ertapenem (10µg), nalidixic acid (30µg), ofloxacin (5µg), amikacin, (30µg) gentamicin (15µg), tetracyclin (30UI), trimethroprim/sulfamethoxazole (1,25+23,75µg). ESBL phenotype was confirmed by the resistance to third generation cephalosporins and the double disc test synergy method. *E. coli CIP 7624* was used as a control strain in susceptibility test. All ESBL-E were then conserved at +20°C in preservation medium (Biorad, Marne-la-coquette, France). ESBL producing suspected *E. coli* and *Klebsiella spp* resistant to quinolone (nalidixic acid and ofloxacin) were randomly selected., packed in a triple packaging system and transferred to the bacteriology laboratory of Saint Camille Hospital of Ouagadougou, Burkina Faso for further strains identification and molecular biology. The strains identification was done with API 20E (BioMérieux, Marcy l'Etoile, France).

2.2 Qnr genes detection

2.2.1 DNA extraction

Bacterial strains were plated on Trypticase soy agar overnight and a colony was inoculated into 2 mL of Luria-Bertani broth (Biorad, Marne la Coquette, France) and incubated at 37°C for 24 hours. Cells from this culture were harvested by centrifugation at 10000 RPM for 10 min. The pellet obtained was suspended in 500 µL of phosphate buffer (100 mH, pH 7) and incubated at 100°C during 15 min. The mix was centrifugate at 1000 RPM for 10 min and the DNA in the supernatant was precipitated in 250 µL of ethanol. The pellet obtained after centrifugation at 1000 RPM for 10 min was washed twice with 1000 µL of ethanol 75°C. The pellet was dried, resuspended in 100 µL of sterile water and stored at -20°C for PCR analysis.

2.2.2 *Qnr* genes amplification and detection

The presence of *qnr* genes, *qnrA, qnrB and qnrS* were amplified by multiplex PCR as previously described [8] with the following specific primers from ABI (Applied Biosystem, California, USA):

qnrA, 516 pb: for 5′-ATTTCTCACGCCAGGATTTG/ Rev 5′-GATCGGCAAAGGTTAGGTCA
qnrB, 469 pb*:* For 5′-GATCGTGAAAGCCAGAAAGG/ Rev 5′-ACGATGCCTGGTAGTTGTCC
qnrS 417 pb*:* For 5′-ACGACATTCGTCAACTGCAA/ Rev 5′-TAAATTGGCACCCTGTAGGC

PCR reactions performed in the GeneAmp PCR System 9700 (Applied Biosystems, California USA) were: 32 cycles of denaturation at 94°C for 45s, annealing at 53°C for 45s and extension at 72°C for 1mn.

The amplification products were visualized under UV illumination after electrophoresis at 100 V for 1 hour on a 2% agarose gel containing ethidium bromide. A 100 bp DNA ladder (Promega, USA) was used as a marker size.

2.3 Statistical analysis

Data were analyzed with the SPSS Statistics V21.0 software package. The Pearson chi 2 test was used to compare the distribution of *qnr* genes among strains

susceptible and resistant to nalidixic acid. A p value < 0.05 was statistically significant.

2.4 Ethical consideration

This study was approved by the ethical committee of Niger (Comité Consultatif National d'Ethique du Niger) and received the authorization for strains transfer from Niger to Burkina Faso. The protocol was reviewed and accepted by the local ethical committee of CERBA/LABIOGENE University of Ouagadougou I, Prof Joseph Ky Zerbo, Burkina Faso.

3. RESULTS AND DISCUSSION

A total of 100 strains were randomly selected among 163 ESBL-producing commensal *Enterobacteriaceae* with phenotypic resistance to quinolone. Finally, due to contamination of some isolated, a total of 78 were assessed for the quinolone resistance study. The mean age of the children was 15.8 months (SD 8.2). The distribution of strains was 63 (80.8%) *E. coli* and 15 (19.2%) *Klebsiella spp* (13 *Klebsiella pneumoniae* and 02 *Klebsiella oxytoca*). All strains were resistant to ofloxacin and 21 (26.9%) susceptible to nalidixic acid.

3.1 Distribution of *qnr* genes

The analysis of PCR products revealed 42 strains harboring at least one *qnr* gene: 4 (9.5%) *qnrA*, 11 (26.2%) *qnrB* and 27 (64.3%) *qnrS*. However, any *qnr* genes was detected in 36 strains. The concomitant presence of two *qnr* genes was not detected. The prevalence of *qnr* genes was 44.44% (28/63) among *E. coli* and 93.33% (14/15) among *Klebsiella spp*. Table 1 shows the distribution of *qnr* genes in bacterial species.

Table 1: Distribution of *qnr* genes.

	Number	Absence of *qnr* genes	*qnr genes*		
			qnrA	*qnrB*	*qnrS*
E. coli	63	35	3	2	23
K. pneumoniae	13	1	1	7	4
K. oxytoca	2	0	0	2	0
Total	**78**	**36**	**4**	**11**	**27**

Table 2: Distribution of *qnr* genes according to the susceptibility to nalidixic acid.

	Number	Absence of *qnr* genes	*qnr genes*		
			qnrA	*qnrB*	*qnrS*
NA-S	21	1	1	3	16
NA-R	57	35	3	8	11
Total	**78**	**36**	**4**	**11**	**27**

NA-S: nalidixic acid susceptible, NA-R: nalidixic acid resistant

3.2 *Qnr* genes distribution according to the susceptibility to nalidixic acid

Among the strains susceptible to nalidixic acid, 95.24% (20/21) had one *qnr* genes. Among those resistant to nalidixic acid, 38.60% (22/57) had *qnr* genes ($p<0.001$). Table 2 shows the distribution of *qnr* genes according to the susceptibility to nalidixic acid.

Our study has shown that in Niger, *qnr* genes associated with resistance to fluoroquinolone were mostly present among ESBL producing *E. coli* (44.4%) and *Klebsiella spp.* (93.3%) isolated from the fecal commensal flora of children aged 6 to 59 months with uncomplicated severe acute malnutrition. The high proportion of these plasmid resistance genes in ESBL-E could be explained by two main factors. On the one hand, the selection of mutant variants due to fluoroquinolone drug pressure [9] because these are part of the most widely used antibiotics in Africa such as ciprofloxacin which is commonly used in pediatric care in Niger [10]. The use of other classes of antibiotics, β-lactams, cephalosporins and macrolides also induces the emergence of bacterial strains resistant to fluoroquinolones [11]. In addition, these plasmid resistance genes are transmitted not only vertically when new generation inherit antibiotic resistance gene but also horizontally when bacteria share or exchange sections of genetic material with other bacteria by the conjugation mechanism [12]. On the other hand, some studies have reported the presence of the PMQR genes in the environment, especially in certain domestic animals such as chickens, traditionally breed in rural areas in West Africa [13], suggesting another possible way of acquisition of these genes. The consequence is that the constant and permanent presence of these genes, will be observed in the commensal flora, increasing the mutant prevention concentration (MPC) and selecting the mutant strains having high levels of resistance to fluoroquinolone [14].

The presence of bacteria resistant to antibiotics in the commensal flora is involved in the spread of the resistance mechanisms. In fact, commensal strains resistant to antibiotics can either become pathogens by translocation or transfer their mechanism of resistance to pathogenic strains or be involved in the inter-individual dissemination of resistance [15].

The presence of *qnr* genes was determined by PCR in the commensal flora of children aged 6 to 72 months in

South America, Peru and Bolivia respectively with a prevalence of 65% and 23% for *qnrB*, 41% and 5% for *qnrS* [16]. Also in south America, in a more recent study, Armas-Freire et al. found a prevalence of 35.3% of commensal strains of *E. coli* resistant to ciprofloxacin isolated from a population of adults and children in rural areas and among them 11.3% carried the gene *qnrB* [17]. This high prevalence of *qnr* genes contrast with those found in Asia, mainly in Vietnam in a mixed population of adults and children living in urban areas. In this Vietnamese study, the prevalence of *qnr* genes differed among the *Enterobacteriaceae* species isolated from the commensal flora, resistant to gentamicin, ceftazidime, nalidixic acid and 63.6% of them had an ESBL. Among *E. coli*, the prevalence of *qnrA* and *qnrS* were respectively 0.6%, 9.2% and within *Klebsiella pneumoniae*, only *qnrS* was present with a rate of 33.3% [18]. The presence of *qnr* genes in ESBL-E was reported on clinical strains in several countries in the world with varying prevalence [5]. It was 4.9% in Europe, Spain [19], 27% in West Africa, Ivory Coast [20], and 36% in adults in North Africa, Morocco [21]. In Central America, Mexico, the prevalence of *qnr* genes was 32.1% in children aged 0-4 years [22].

The presence of *qnr* genes was significantly more frequent in strains susceptible to nalidixic acid and resistant to ofloxacin than in those resistant to both nalidixic acid and ofloxacin. The same trend was observed in the study of Betitra et al in Algeria where only 10% of 30 nalidixic acid resistant *E. coli* were *qnr*-positive [23].

One limitation of this study was the absence of data on minimal inhibiting concentration (MIC) for quinolone (nalidixic acid) and fluoroquinolones (ciprofloxacin, ofloxacin) which could determine the level of resistance to these antibiotics and also the lack of molecular characterization of ESBL genes. Therefore, it has been difficult to interpret in greater detail these results. However, in addition to the *qnr* genes, the likely presence of mutations in the gyrase and topoisomerase IV genes could explain the strain sensitivity to nalidixic acid and resistance to ofloxacin. For these last strains the presence of *qnr* genes has not sufficiently increased the nalidixic acid MIC conferring a resistance to this specific antibiotic [5]. Despite these limitations, the results of this study alerts us to i) the emergence and spread of antibiotic resistance from young children, ii) the presence of *qnr* genes encountered in Niger. Moreover, the absence of *qnr* genes from certain strains suggested the presence of other quinolone resistance mechanisms.

In conclusion, this study characterized some *qnr* genes circulating in Niger for the first time that are more prevalent among others due to their easy transfer between bacteria. These results should contribute to the implementation of antibiotic resistance surveillance system in Niger.

Acknowledgments: The authors thank the nongovernmental organization EPICENTRE in Niger for their support to this study. We thank the bacteriology laboratory team of EPICENTRE at Maradi Niger for their involvement in the isolation of bacterial strains: Bachir Assao, Aissa Moumouni, Ibrahim Iddo, Souley Iro, Seini Inoussa, Chou-ayb Yahaya and Ali Amadou. We also thank M. Aristide Bado for statistical guidance. We are also grateful to the West African Economic and Monetary Union (WAEMU) Commission for their financial support in the biomolecular analysis at CERBA/LABIOGENE.

Funding: This work has been supported by the West African Economic and Monetary Union (WAEMU) Commission, Ouagadougou, Burkina Faso

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