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

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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 topoisomerase 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 aminosaldehyde resistance and the quinolone efflux pumps [2,3]. The qnr proteins belong to the repeated pentapeptidique protein (RPP) 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 Antiibiogram 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), ticarcillin (75μg), cefatolin (30μg), cefotixin (30μg), cefotaxim (30μg), cefazidim (30μg), cefepim (30μg), Imipenem (10μg), ertapenem (10μg), nalidixic acid (30μg), ofloxacine (5μg), amikacine, (30μg) gentamicin, (15μg), tetracycline (30μg), trimethoprim/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 ofloxacine) 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):

- 5′-ATTTTCTCACGCCAGATTGTC/ Rev 5′-GATCCGAAAAGGTTAGGCTCA
- qnrB, 469 pb: For 5′-GATCGTGAAAGCCAGAAAGG/ Rev 5′-ACGATGCCCTGTAGTGTGC
- qnrS 417 pb: For 5′-ACGACATTTGCAACTGCAA/ Rev 5′-TAAATGCGAACCCTGTAGGC

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 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 pneumoniana and 02 Klebsiella oxytoca). All strains were resistant to oloxac 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>
<thead>
<tr>
<th></th>
<th>Number</th>
<th>Absence of qnr genes</th>
<th>qnr genes</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>qnrA</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>63</td>
<td>35</td>
<td>3</td>
</tr>
<tr>
<td><strong>K. pneumoniae</strong></td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>K. oxytoca</strong></td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>78</td>
<td>36</td>
<td>4</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th></th>
<th>Number</th>
<th>Absence of qnr genes</th>
<th>qnr genes</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>qnrA</td>
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<tr>
<td><strong>NA-S</strong></td>
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<td>1</td>
</tr>
<tr>
<td><strong>NA-R</strong></td>
<td>57</td>
<td>35</td>
<td>3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>78</td>
<td>36</td>
<td>4</td>
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NA-S: nalidixic acid susceptible, NA-R: nalidixic acid resistant

3.3 qnr genes prevalence
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 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.

http://crmb.aizeonpublishers.net/content/2017/1/crmb953-957.pdf
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, cefazidime, 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 gysrse 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.

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**4. REFERENCES**


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