Phenotypic and molecular characterization of quinolone resistance of enteropathogens isolated from diarrhea in young children in Koula-Moutou/Gabon

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ABSTRACT

In the face of the growing phenomenon of multidrug-resistant bacteria, studies on the sensitivity profiles of enteric pathogens are required to better manage the remaining antibiotic capital. The objective of this study was to investigate the sensitivity and to determine the genetic basis of quinolone-resistant enteric diarrheal pathogens isolated from children in the city of Koula-Moutou, Gabon. Thirty strains of the Salmonella genus, five strains of Shigella and two strains of Yersinia were analyzed. The Kirby–Bauer method was used to determine the sensitivity of these isolates to five molecules in the quinolone family. The presence of chromosomal (gyrA) and plasmid (PMQR) quinolone resistance genes, including the QNR complex (qnrA, qnrB, and qnrS) were investigated by PCR. These enteric pathogens exhibited high levels of resistance to quinolones, particularly ofloxacin (100%) and ciprofloxacin (97.3%). Most isolates of Salmonella were found to be resistant to nalidixic acid (86.7%), however all isolates of the Shigella and Yersinia genera were susceptible to this antibiotic. Among the 37 strains, 21.6% had the gyrA gene, 13.5% had the qnrB and qnrS genes but the qnrA gene was not detected. Enteric pathogens isolated from the diarrheal feces of the children in Koula-Moutou exhibited high levels of phenotypic resistance to quinolones. This resistance is especially due to mutations occurring on the chromosomal determinant gyrA which also played a role in the reduced sensitivity of these strains to fluoroquinolones. The resistance is also linked to a lesser extent to the acquisition of the PMQR genes, notably qnrB and qnrS. In conclusion, this study highlights the high prevalence rate of enteric pathogens isolated from diarrheal feces of children in Gabon and shows the presence of the QNR complex genes (qnrB and qnrS) in the strains.

Introduction

The current challenge in the treatment of bacterial infections is to overcome the phenomenon of resistance, or even multidrug resistance, to different antibiotic molecules, particularly those of ‘last resort’ antibiotics with a broad spectrum [1]. Quinolones and fluoroquinolones (FQ) are the first line of treatment for enterobacteriaceae urinary tract infections. Their use has been expanded to include...
treatments for systemic infections caused by most aerobic Gram-negative bacteria and some Gram-positive bacteria such as staphylococci [2,3]. In addition, this family of antibiotics is also recommended for the treatment of diarrheal diseases, particularly against enteric pathogenic bacteria (Escherichia coli, Shigella, Salmonella, Aeromonas, and Vibrio) [4]. Compared to other pathogens, enterobacteriaceae have very low minimum inhibitory concentrations (less than 0.25 µg/ml) and low acquired FQ resistance [5]. The pharmacological properties, broad spectrum of action and oral bioavailability of quinolones and FQ have certainly contributed to a rather abusive use of these molecules. This has unfortunately increased the resistance of enterobacteriaceae to this family of antibiotics [6,7].

Resistance to quinolones in enterobacteriaceae is mainly due to point chromosomal mutations in antibiotic targets: gyrase coded by the gyrA and gyrB genes and topoisomerase IV coded by the parC and parE genes. This resistance is often associated with impermeability or active flow leading to different resistance phenotypes [8,9]. The second classic FQ resistance mechanism is an efflux mechanism with positive regulation of native efflux pumps. In the particular case of acquired bacterial resistance to FQ, it is done in stages, with the progressive accumulation of the different resistance mechanisms ‘in steps’, favored especially by the repeated prescriptions of FQ [10]. In general, after several mutations have combined, a high level of resistance is expressed leading to a clinical effect in enterobacteria [3,8]. Regarding these mechanisms, recent studies report resistance of the plasmid type. Indeed, new mechanisms of quinolone resistance are described such as the presence of the Quinolones Resistant complex (QNR). The genes qnrA, qnrB, qnrS, qnrC and qnrD act by protecting the target while the qepA, and qpxAB genes code for proteins forming active efflux pumps. Moreover, the aac(6)-Ib-cr gene is involved in target modification [6].

Currently, resistance to quinolones and FQ is increasingly reported and the global epidemiological situation is constantly evolving [6]. In the case of enterobacteriaceae, the prevalence of resistant strains is increasing. Indeed, 25% of clinical isolates of E. coli were resistant in Spain and 45% in Hong Kong, China [11]. Numerous studies report several cases of Shigella strains resistant to FQ, particularly in Asia [5,12]. Strains of FQ-resistant Salmonella have been isolated in Africa and their geographical distribution described in several countries such as Kenya, Tanzania, Malawi, South Africa, Zambia, the Democratic Republic of the Congo and Nigeria [13].

There is little evidence available in Africa on the prevalence of these chromosomal and plasmid resistance genes in enterobacteriaceae, including enteric pathogens responsible for infantile diarrhea. This prospective and descriptive study isolated enteric pathogens from diarrheal feces of children in the city of Koula-Moutou in Gabon. It aims to characterize the genetic profile of resistance of enterobacteriaceae to quinolones.

**Material and methods**

**Study design and origin of isolates**

The various enteric pathogen isolates from this study come from the Laboratory of Molecular and Cellular Biology (LABMC) at the University of Science and Technology in Masuku, Gabon. These strains were isolated from the feces of 102 children aged 0 to 5 years old suffering from diarrhea in the city of Koula-Moutou. These strains included 30 strains of the genus Salmonella, 5 strains of the genus Shigella and 2 strains of the genus Yersinia.

**Phenotypic evaluation of susceptibility profiles of enteropathogens to quinolones**

The evaluation of the sensitivity of enteric pathogens to quinolones was carried out by the Mueller Hinton (MH) gel medium antibiotic disc
diffusion technique according to the Kirby-Bauer technique. Five [5] molecules in this family were tested: nalidixic acid (NAL, 30 μg), (1st generation quinolones), ciprofloxacin (CIP, 5 μg), norfloxacin (NXN, 10 μg), ofloxacin (OFX, 5 μg) (2nd generation FQ) and levofloxacin (LVX, 5 μg) (3rd generation FQ). In this study, 0.5 Mcfarland standardized inoculi were prepared from 18 to 24 hours pure colonies in sterile physiological water (0.9% NaCl), then seeded to the surface of the Mueller Hinton agar medium and incubated at 37°C for 18–24 hours. Finally, the interpretation of the results was made according to the standards of the Antibiogram Committee of the French Microbiology Society (Comité de l’Antibiogramme de la Société Française de Microbiologie, CASFM): recommendations 2019 v.2.0.

Molecular research of quinolone resistance genes

Total DNA extraction was performed from bacterial colonies, resulting in 400 μl of a solution containing 1X DNA/RNA reagent (Biolabs New England), 2X lysis buffer (1% SDS, 20 mM NaCl, 20 mM Tris pH 8, 20 mM EDTA) and K protein concentrated at 100 μg/ml. The preparations were incubated at 56°C for 1 hour, centrifuged at 12,000 rpm, and then added to 400 μl with a mixture of cold isopropanol and 3 M sodium acetate. The preparations were then centrifuged at 14,000 rpm for 15 minutes, and the different caps were resuspended in 70 μL of ultra-pure water. DNA concentrations were determined by measuring sample absorbance at a wavelength of 260 nm via a spectrophotometer (Thermofisher).

Specific primers (Table 1) were used for the detection of gyrA genes encoding for gyrase and those of the QNR complex (qnrA, qnrB and qnrS) encoding for plasmid resistance to quinolones [9,14].

The different PCR reactions were performed in a 20 μl final reaction volume containing the primer mix concentrated at 0.3 μM, the multiplex PCR master mix at 1X (Qiagen® Multiplex PCR Kit) and 2 μl of DNA matrix diluted at the thousandth for the QNR complex. In contrast, the classic PCR mix consisted of 10 μl master mix at 2X (Amplitaq Golden) of Dgyra primers concentrated at 0.5 μM and the same volume of diluted DNA. The amplification was performed using a thermocycler (Bio-RAD, T100TM, USA) under the following conditions: the initial denaturation step was performed at 95°C for 15 min, followed by 30 cycles consisting of a denaturation step at 94°C for 1.5 min, a hybridization step at 54°C for 1 min, an elongation step at 72°C for 1 min; and finally, a final elongation step at 72°C for 7 min. The various amplicons were separated by electrophoretic migration on agarose gel at 1.5% (m/v) and added to the concentrated ethidium bromide at 10 μg/ml at a voltage of 300 V. The different sizes

| Genes | Primer | Primer Sequence (5’ – 3’) | Heigth (bp) | References |
|-------|--------|--------------------------|-------------|------------|
| gyrA  | DgyrA-F | AACGGTGATGACTTCCGTC      | 511         | [9]        |
|       | DgyrA-R | TATGGATATGCAGTGATTG      |             |            |
| qnrA  | QNR-F  | GATCGGCGAGGGTTAGGCA      | 516         | [9]        |
|       | QNR-R  | ATTTCTCACGCCAGGATTG      |             |            |
| qnrB  | QNR-F  | GATCGGCAAGGACAGAAAG     | 383         | [9]        |
|       | QNR-R  | ACGATGCCCTGGAATGGATCC   |             |            |
| qnrS  | QNR-F  | ACGACATTCTGAACTGCAA     | 417         | [9]        |
|       | QNR-R  | TAAATTGGGACCCCTGTAGGC   |             |            |
were evaluated with a 100 bp molecular weight marker (Qiagen®, Gelpilot) and the strips viewed and photographed under UV light.

**Ethical considerations**

The study was approved by the Gabonese National Ethics Committee for Research and the Ministry of Health (PROT N° 0020/2015/SG/CNE). The study protocol was approved and authorized by the Center-East General Direction of Health, the Direction of the Ogooué-Lolo Provincial Academy and the Provincial Direction of Family and Social Welfare. Stool samples were collected from children after obtaining their parents’ or guardians’ written informed consent.

**Statistical analyses**

The data collected during this study were captured and processed using the software Microsoft Office Excel 2016. The one-factor variance (1-factor Anova) was analyzed and used to observe the differences in resistance which may exist between the different antibiotics (quinolones) to the strains studied. In addition, the Tukey parity test made it possible to make pairwise comparisons of the different antibiotics. All these statistical analyses were carried out at risk $\alpha = 5\%$.

**Results**

The assessment of the susceptibility profiles of enteric pathogens isolated from diarrheal feces to the quinolone family showed an overall resistance prevalence of 82.7%. The details of the profiles obtained in relation to the different molecules of this family are recorded in Table 2.

Based on their analysis, the results in Table 2 show a high prevalence of phenotypic resistance of enteric pathogens to the five quinolone molecules tested. Overall, the highest levels of resistance recorded were observed against second generation FQ with rates of 100%, 97.3% and 78.4%, for ofloxacin, ciprofloxacin and norfloxacin, respectively. These were followed by first generation quinolones, nalidixic acid with 70.3% and 67.6% for levofloxacin, and third generation FQ. Furthermore, these results indicate a variation of this resistance depending on the pathogen. The resistance to nalidixic acid is 86.7%, 0% and 0% for the genus *Salmonella*, *Shigella* and *Yersinia*, respectively. Levels of 100% resistance to ciprofloxacin and ofloxacin are recorded for *Salmonella* and *Shigella*. The levels of resistance for *Salmonella* and *Shigella* are 83.3% and 60.0% for norfloxacin and 76.7% and 40.0% for levofloxacin, respectively. The different resistance rates recorded for *Yersinia* strains are 100% for ofloxacin, 50% for ciprofloxacin and norfloxacin, respectively. No isolates of this species have phenotypic resistance to nalidixic acid and levofloxacin.

| Names of strains       | Number | 1st generation NAL | 1st generation CIP | 1st generation NXN | 1st generation OFX | 2nd generation LFV | 3rd generation nAL | 3rd generation CIP | 3rd generation NXN | 3rd generation OFX | 3rd generation LVX | p-value |
|-----------------------|--------|---------------------|--------------------|--------------------|--------------------|---------------------|---------------------|---------------------|--------------------|--------------------|---------------------|---------|
| *Salmonella enterica* | 6      | 4 (66,7)            | 6 (100,0)          | 6 (100,0)          | 6 (100,0)          | 6 (100,0)           | 6 (100,0)           | 0,00540             |                    |                    |                     |         |
| *Salmonella Paratyphi A* | 3   | 2 (66,7)            | 3 (100,0)          | 3 (100,0)          | 3 (100,0)          | 0 (0,0)             | 61,200              |                     |                    |                    |                     |         |
| *Salmonella Typhi*     | 4      | 4 (100,0)           | 4 (100,0)          | 4 (100,0)          | 4 (100,0)          | 4 (100,0)           | 0,00540             |                     |                    |                    |                     |         |
| *Salmonella spp*       | 17     | 16 (94,1)           | 17 (100,0)         | 12 (70,6)          | 17 (100,0)         | 13 (76,5)           | 0,00038             |                     |                    |                    |                     |         |
| **Total**              | 30     | **26 (86,7)**       | **30 (100,0)**     | **25 (83,3)**      | **30 (100,0)**     | **23 (76,7)**       |                     |                     |                    |                    |                     |         |
| *Shigella sonnei*      | 2      | 0 (0,0)             | 2 (100,0)          | 2 (100,0)          | 2 (100,0)          | 2 (100,0)           | 2 (100,0)           | 0,00300             |                    |                    |                     |         |
| *Shigella spp*         | 3      | 0 (0,0)             | 3 (100,0)          | 1 (33,3)           | 3 (100,0)          | 0 (0,0)             | 0,04200             |                     |                    |                    |                     |         |
| **Total**              | 5      | **0 (0,0)**         | **5 (100,0)**      | **3 (60,0)**       | **5 (100,0)**      | **2 (40,0)**        |                     |                     |                    |                    |                     |         |
| *Yersinia pestis*      | 2      | 0 (0,0)             | 1 (50,0)           | 1 (50,0)           | 2 (100,0)          | 0 (0,0)             | 0,53000             |                     |                    |                    |                     |         |
| **Global Prevalence**  | 37     | **26 (70,3)**       | **36 (97,3)**      | **29 (78,4)**      | **37 (100,0)**     | **25 (67,6)**       |                     |                     |                    |                    |                     |         |
Moreover, the variability of intraspecies resistance shows that strains of *Salmonella enterica* have 100% resistance to ciprofloxacin, norfloxacin, ofloxacin and levofloxacin and a rate of 66.7% for nalidixic acid with a significant difference (p = 0.0054). In addition, the Tukey parity test shows that this variability in behavior is more pronounced in the presence of norfloxacin and ciprofloxacin (p = 0.05) and in the presence of ofloxacin and ciprofloxacin (p = 0.0071). Similarly, heterogeneity of behavior of *Salmonella spp* isolates in the presence of the five molecules were recorded (p = 0.00038), particularly between nalidixic acid and ciprofloxacin (p=0.001), norfloxacin (p = 0.00264) and levofloxacin (p = 0.0136). However, *Salmonella typhi* strains are all resistant to the five molecules tested without any significant difference (p = 0.766). Strains of *Salmonella paratyphi* A are all sensitive to levofloxacin, 66.7% of strains are resistant to nalidixic acid and all are resistant to second generation FQ. However, no significant differences in behavior were observed (p = 0.612). For the various species of *Shigella*, a difference in behavior in the presence of the three generations of quinolones is recorded with statistical significance (Table 2). Indeed, *Shigella sonnei* strains are all sensitive to first generation quinolones and by contrast, they are all resistant to second and third generation quinolones. Furthermore, the Tukey parity test reveals a distinction between ciprofloxacin and three second generation FQ molecules: ofloxacin (p = 0.00241), norfloxacin (p = 0.01511) and levofloxacin (p = 0.01064). *Shigella spp* strains are all susceptible to first and third generation quinolones. In contrast, they are all resistant to two of the three second generation quinolone molecules, namely ciprofloxacin (100.0%) and ofloxacin (100.0%). *Yersinia pestis* isolates showed no significant differences in behavior for the three generations of quinolones tested (p = 0.5300).

The genotypic origin of phenotypic resistance patterns to different generations of observed quinolones was evaluated by looking for possible determinants. Among the 37 strains analyzed, 78.4% (n = 29) were carriers of the chromosomal determinant *gyrA* and 13.5% were carriers of genes of the QNR complex with 5.4% carrying the *qnrB* gene and 8.1% the *qnrS* gene. None of the 37 strains carried the *qnrA* gene and 21.6% did not carry the *gyrA* gene. Details of their distribution within the different species obtained are recorded in Table 3.

The results in Table 3 show that 35.1% of bacterial strains eventually possess the quinolone resistance determinants. Indeed, *Salmonella* spp strains are the main carriers of these determinants (46.2%) followed by *Salmonella enterica*, *Salmonella Typhi* and *Shigella sonnei* with a prevalence of 15.4%

|               | *Salmonella enterica* n (%) | *Salmonella Paratyphi A* n (%) | *Salmonella Typhi* n (%) | *Salmonella spp* n (%) | *Shigella sonnei* n (%) | *Shigella spp* n (%) | *Yersinia pestis* n (%) |
|---------------|-----------------------------|-------------------------------|--------------------------|------------------------|----------------------------|------------------------|------------------------|
| **Mono-detection** |                             |                               |                          |                        |                            |                        |                        |
| *gyrA*        | 24 (64.9)                   |                               |                          |                        |                            |                        |                        |
| *qnr B*       | 4 (16.7)                    | 3 (12.5)                      | 2 (8.3)                  | 11 (45.8)              | 0 (0.0)                    | 2 (8.3)                 | 2 (8.3)                |
| *qnr S*       | 0 (0,0)                     | 0 (0,0)                       | 0 (0,0)                  | 0 (0,0)                | 0 (0,0)                    | 0 (0,0)                 | 0 (0,0)                |
| **Co-detection** | 5 (13.5)                    |                               |                          |                        |                            |                        |                        |
| *gyrA-qnr B*  | 0 (0,0)                     | 0 (0,0)                       | 1 (20,0)                 | 1 (20,0)               | 0 (0,0)                    | 0 (0,0)                 | 0 (0,0)                |
| *gyrA-qnr S*  | 0 (0,0)                     | 0 (0,0)                       | 0 (0,0)                  | 2 (40,0)               | 0 (0,0)                    | 1 (20,0)                | 0 (0,0)                |
| **Global Prevalence** | 29 (78.4)                   | 4 (13.8)                      | 3(10.3)                  | 3 (10.3)               | 14 (48.3)                  | 0 (0,0)                 | 3 (10.3)               | 2 (6.9) |
each. The less represented is the *Shigella spp* strain with a detection rate of 7.6%. In addition, the *qnrB* gene is detected in *Salmonella Typhi* and *Salmonella spp* with prevalence rates of 25.0% and 5.9%, respectively. Finally, the *qnrS* gene is detected in *Salmonella spp* (11.8%) and *Shigella spp* (33.3%).

**Discussion**

In this study, the different levels of resistance to quinolones and FQ and the genetic supports of this resistance were analyzed within heterogeneous strains of enteric pathogens isolated from the diarrhea of children in the city of Koula-Moutou in Gabon.

**Phenotypic resistance profile to 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> generation quinolones**

In the present study, a strong resistance to quinolones is recorded which corroborates the spread of enterobacteriaceae resistant to this family of antibiotics as well as in enteric pathogens responsible for diarrhea, such as *Salmonella spp* and *Shigella spp* [3,9,14]. The prevalence rates recorded are similar to those obtained in several other studies, even though the phenotypic profile is heterogeneous. In the present study, the prevalence of resistance of *Salmonella Typhi* and *Paratyphi* A is similar to those obtained in Nepal [15]. Similarly, a 100% FQ resistance rate is recorded for *Salmonella Typhi*, which is similar to that reported in a study in Pakistan, which ranged from 84.7% to 91.7% [16]. Resistance prevalence rates of *Salmonella Paratyphi* A to nalidixic acid (66.7%) and to ofloxacin (100.0%) are similar to those obtained by Dutta *et al.*, in India [17]. In contrast, those of non-typical *Salmonella* in this study are significantly higher than those in southern Tanzania [18], Iran [19] and Ethiopia [20]. *Shigella* isolates were all sensitive to nalidixic acid as reported in southern Ethiopia [21] and in Bangui in the Central African Republic [22]. However, these results stand in constrast to a profile obtained in Iran in which 50.4% of strains were resistant [23]. The increased resistance of *Shigella spp* strains to ciprofloxacin in Latin America [24] is consistent with the results of this study, in which all isolates of *Shigella spp* are resistant to ciprofloxacin. The same results are also obtained for the other FQ. The levels of resistance for ciprofloxacin and ofloxacin obtained in this study corroborate those in India [25]. However, the prevalence of resistance to norfloxacin (78.4%), ciprofloxacin (97.3%) and levofloxacin (67.6%) was significantly higher than in other studies [8,12,20,23,26]. A high resistance to FQ is extensively due to excessive, inappropriate and uncontrolled use of first and second line antibiotics which are used to treat a range of common infections in the tropics [15,17,27]. These phenotypes could possibly be explained by the presence of extended-spectrum β-lactamases, which to date remain the main resistance mechanism in enterobacteriaceae [28]. These enzymes, particularly CTX-M, would confer cross-resistance to antibiotics in the aminoside and quinolone families through genes located primarily on plasmids or other mobile genetic elements [29]. In addition, Jamali *et al.* demonstrated the co-existence of EβLS and PMQR genes on quinolone-resistant enterobacteriaceae strains in their study [30]. These facts would clearly explain the high prevalence of phenotypic resistance recorded in our study.

**Identification of genes for chromosomal and plasmid resistance to quinolones**

The occurrence of even single amino acid changes in target genes in bacteria is one of the common pathways for antibiotic resistance [31]. In this study, an absence of amplification of chromosomal resistance marker genes (*gyrA*) to quinolones is observed in most of the bacterial strains phenotypically resistant to quinolones. These results clearly support that the resistance of diarrheal enteropathogens to quinolones in children in the city of Koula-Moutou in Gabon is
High levels of phenotypic resistance to recorded quinolones may be explained by the presence of other chromosomal and plasmid determinants, and as such, the lack of characterization in this study is its main limitation. Indeed, although the gyrA gene appears to be the main chromosomal determinant of quinolone resistance, the parC and parE genes could also be involved and should be examined in our region. Further studies are required to characterize other plasmid determinants in order to understand their expansion and the genetic determinism of resistance to quinolones.

**Conclusion**

In conclusion, this study highlights the high prevalence of resistance to quinolones by enteric pathogens isolated from diarrheal stools of children in Gabon. In addition, it appears that the observed phenotypic resistance could be genotypically mediated by the presence of QDRR and PMQR type genes. These PMQR determinants are a potential factor in increasing horizontal transmission of clinical resistance to quinolones as their expansion on the intestinal microbiota constitute potential reservoirs of resistance. Therefore, the sensible use of FQ by health workers as well as the implementation of a monitoring network for multidrug-resistant strains is indispensable, to preserve the antibiotic capital and the sustainability of their clinical efficacy against pathogens.

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**Disclosure statement**

No potential conflict of interest was reported by the author(s).
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