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First Detection of Fluoroquinolones and Aminoglycosides Resistance Genes in Multiresistant *Enterobacteriaceae* from Urinary Tract Infections

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Abstract Aminoglycosides and fluoroquinolones are among the most used antibiotics in synergy with beta-lactams. The objective of this work was to detect genes associated with resistance to fluoroquinolones and aminoglycosides in multiresistant strains of enterobacteria responsible for urinary tract infections. The diffusion method was used for antimicrobial sensitivity test of strains. *qnr* and *aac* genes were detected by Polymerase Chain Reaction. A total of 128 enterobacterial strains were studied. Resistance to the antibiotic families tested was observed with a frequency greater than 50%. Aminoglycosides were the least active family among the antibiotics used, with a resistance rate of 69.53%. *aac*(6')-Ib-cr and *qnr*S genes were detected at rates of 34.09% and 22.72%, respectively. 13.63% of the strains showed coexpression of the *qnr* and *aac* genes. The detection of resistance genes to fluoroquinolones and aminoglycosides in enterobacterial strains isolated at the National Public Health Laboratory in Brazzaville could constitute a major concern in the therapeutic management of patients because it could lead to high costs for prolonged hospitalization.

Keywords: *Enterobacteriaceae, multidrug resistance, fluoroquinolones and aminoglycosides resistance genes, urinary tract infections*

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1. Introduction

In combination with beta-lactams, aminoglycosides and fluoroquinolones are currently among the most widely used antibiotics in world. This preference is justified by their safety, efficacy, and low cost [1]. However, the use of aminoglycosides in monotherapy is rare and essentially corresponds to the management of certain urinary tract infections (UTI). This satisfaction, long maintained by the discovery and disclosure of new molecules, came to a halt towards the end of 1990. The lack of discovery of new classes of antibiotics and the abusive and inappropriate use of existing ones in human medicine, veterinary medicine, or animal feed has resulted in an overall increase in the level of resistance of bacteria. According to the 2014 WHO report on global surveillance of antimicrobial resistance, antimicrobial resistance is a reality all over the world and is now a serious threat to public health [2]. This fact is all the more worrying these days as emerging mechanisms are appearing everywhere, sometimes confronting medicine with therapeutic impasses.

The mechanisms of resistance of *Enterobacteriaceae* to aminoglycosides generally boil down to enzymatic inactivation by their modifying enzymes. These mechanisms confer high-level resistance to all clinically relevant aminoglycosides [3]. As for the fluoroquinolones, their mechanisms of resistance to enterobacteria are either of the chromosomal type, essentially due to the modification of type II topoisomerases, or else of the plasmid type by protection of the Qnr target or by modification of the *aac*(6')-Ib-cr target., followed by QepA (quinolone efflux pump) active efflux [4].

Furthermore, although the production of beta-lactamases represents the most widespread antimicrobial resistance mechanism in *Enterobacteriaceae*, the spectrum of antimicrobial resistance has widened over the years and now extends to aminoglycosides and the latest generation of fluoroquinolones. Thus, in the face of antimicrobial resistance, infections due to *Enterobacteriaceae* resistant

to fluoroquinolones and aminoglycosides constitute a real public health problem. Indeed, these pathogens are implicated in the expansion of nosocomial and community infections, thus leading to prolonged hospitalizations, an increase in morbidity and mortality, and consequently to high costs of health care [5].

The resistance of *Enterobacteriaceae* to the multiple antibiotics prescribed in the hospital, more specifically to fluoroquinolones and aminoglycosides, continues to grow, in particular by the acquisition of enzymes for resistance to aminoglycosides and fluoroquinolones. Faced with this global challenge, the WHO, in collaboration with the Food and Agriculture Organization (FAO) and the World Organization for Animal Health (WOAH), developed in 2015 a "global", encouraging each Member State to build a national plan to combat antibiotic resistance with a "One Health" perspective [6]. The emergence of antimicrobial resistance was only belatedly recognised by the scientific community, despite the frequent appearance of antibiotic-resistant mutant pathogens [7]. Many bacterial infections caused by these pathogens lead to increased costs of care, prolonged hospitalizations, ineffective therapeutic treatments, and a high rate of morbidity and mortality [8].

The emergence and dissemination of resistant bacteria are due to the power of adaptation, which manifests itself in their ability to appropriate new properties or by modifying their genome (mutations) [9]. Stopping or at least controlling the problem of resistance requires several approaches: better use of antibiotics; better compliance with hygiene measures in order to limit the spread of these bacteria; and finally, a better understanding of the mechanisms of acquisition of resistance to antibiotics in order to identify new therapeutic targets. Recently, a study of Bartley *et al.*, [10] indicated the presence of genes associated with resistance to fluoroquinolones and aminoglycosides in *Enterobacteriaceae* isolated from samples from an urban lake in Brazil. The presence of *qnr* and *aac* genes was also reported in Togo during the same year [11], as well as in Egypt [12]. This work aims to understand the genetic mechanisms involved in the transfer of resistance genes, in order to put in place the necessary measures for better therapeutic management of infections caused by *Enterobacteriaceae* in Congo and to allow effective epidemiological surveillance to best counter the disease. the progression of this resistance.

2. Methods

2.1. Isolation and Identification of Enterobacteria

In this study, a total of 438 biological samples consisting of urine were aseptically collected at the Bacteriology Department at the National Public Health Laboratory in Brazzaville during the period from august to november, 2021. Thus, thanks to the use of conventional microbiological techniques, enterobacteria were isolated from these samples using Eosin Methylene Blue (EMB) agar medium after incubation aerobically for 24 h at 37°C. A count was made beforehand to confirm the positivity of the samples. After purification, the Gram type was determined by staining. The identity of the strains was finally confirmed by the use of the biochemical tests present in the Api 20 E bacterial identification system. After isolated and identified, these strains were stored in Soy Tryptocase agar at room temperature for later use.

2.2. Antimicrobial Susceptibility Testing

The sensitivity of the strains to antibiotics was tested by referring to the recommendations of the Clinical and Laboratory Standards Institute « CLSI » [13], using the standard method of Kirby and Bauer, based on the diffusion of antibiotic discs on Mueller-Hinton Agar (MHA). The bacterial inoculum was adjusted to a turbidity of McFarland Standards. 11 antibiotic discs were used, including cefepime (30 µg), ceftazidime (10 µg), cefotaxime (5 µg), aztreonam (30 µg), amoxicillin + clavulanic acid (20 µg), imipenem (10 µg), norfloxacin (10 µg), levofloxacin (5 µg), ciprofloxacin (5 µg), gentamicin (10 µg) and amikacin (30 µg). The diameter of the inhibition zone for each antibiotic disk was measured after 18 h of incubation at 35°C ± 2°C, and the results were confirmed as susceptible or resistant by the use of a control strain of *E. coli* ATCC25922.

2.3. Detection of *aac* and *qnr* genes by PCR

2.3.1. Extraction of Plasmid DNA

Plasmid DNA was obtained using the Nucleospin Plasmid kit (Macherey-Nagel, Germany) and by referring to the manufacturer's instructions.

Table 1. Primers used for gene detection by PCR

Genes	Primers	Sequences	Size (bp)	References
***qnr*S**	*qnr*S -F	GACGTGCT AAC TTGCGTGAT	388	[14]
	*qnr*S -R	AACACCTCGACTTAAGTCTGA		
***qnr*A**	*qnr*A-F	ATTTCTCACGCCAGGATTTG	516	[15]
	*qnr*A-R	GATCGGCAAAGGTTAGGTCA		
***qnr*B**	*qnr*B-F	GATCGTGAAAGCCAGAAAGG	469	[15]
	*qnr*B-R	ACGATGCCTGGTAGTTGTCC		
aac(6')-Ib-cr	*6'*-Ib-F	TATGAGTGGCTAAATCGAT	395	[16]
	6'-Ib-R	CCCGCTTTCTCGTAGCA		
aac(3')-Ib-cr	3'-Ib-F	TATGAGTGGCTAAATCGAT	372	[16]
	3'-Ib-R	CCCGCTTTCTCGTAGCA		
***Aad*A**	aadA-F	CCCAATTTGTGTAGGGCTTA	812	[17]
	aadA-R	TTGTACGGCTCCGCAGTG		

2.3.2. Gene Amplification by PCR

The 88 strains showing resistance to fluoroquinolones and aminoglycosides were used to perform the PCR reactions in a final volume of 50 µL containing 25 µL of the master mix, 16 µL of molecular biology ultrapure water, 2 µL of each primer (forward and reverse), and 5 µL of DNA. For each isolate, six PCR reactions were done using the specific primers (Table 1). The microtubes are then placed in a thermocycler (Biorad, Singapore). The program used for the amplifications includes an initial denaturation at 95°C for 5 minutes, followed by 30 cycles each comprising denaturation at 95°C for 1 minute, hybridization at 55°C for 45 seconds, elongation at 72°C for 1 minutes, and a final elongation at 72°C for 7 minutes.

2.4. Statistical Analysis

Data has been processed using Excel 2013 software (Microsoft Corporation, USA). This tool was used for the statistical analysis. The percentages were calculated for the measured inhibition diameters. GraphPad Prism (Version 7.0.0.159, USA) was also used to process resistance data by antibiotic family. The comparison of variables was statistically significant using the Chi-square test.

3. Results

3.1. *Enterobacteriaceae* Isolation

Using urine samples, the enterobacterial strains were isolated and identified in this study. From these results, it appears that the 128 strains mainly include the species *Escherischia coli*, with 34 strains, followed by 24 strains of *Enterobacter aerogenes*. Finally, *Citrobacter freundii* and *Enterobacter clocae* are the least representative species, with 2 strains each (Figure 1).

3.2. Antimicrobial Susceptibility Results

The results of the antimicrobial susceptibility are presented in Table 2 below, taking into account the resistance. Depending on the antibiotic molecules tested, the strains showed varying levels of resistance. Among molecules used, gentamicin from the aminoglycoside family was the least inactive on the 128 strains of *Enterobacteriaceae* studied, with a resistance rate of 89.06%. This rate is followed by that of cefepime, which is a 4th generation cephalosporin, with a resistance rate of 85.94%. As for the fluoroquinolones, norfloxacin was the molecule for which the strains were most insensitive, with a resistance rate of 75%, followed by ciprofloxacin (73.43%). On the other hand, beta-lactams showed resistance rates of 59.38% for imipenem, which is a carbapenem, and 57.81% for ceftazidime, which is also a 3rd generation cephalosporin.

3.3. Resistance According to Antibiotic Families

According to the families of antibiotics tested, the enterobacterial strains were more resistant to the family of aminoglycosides in the present study, with a rate of 69.53%, followed by beta-lactams with 68.33%. Fluoroquinolones come last with a rate of 64.58% (Figure 2). It is important to note that the majority of the strains showed simultaneous resistance to all three families of antibiotics tested.

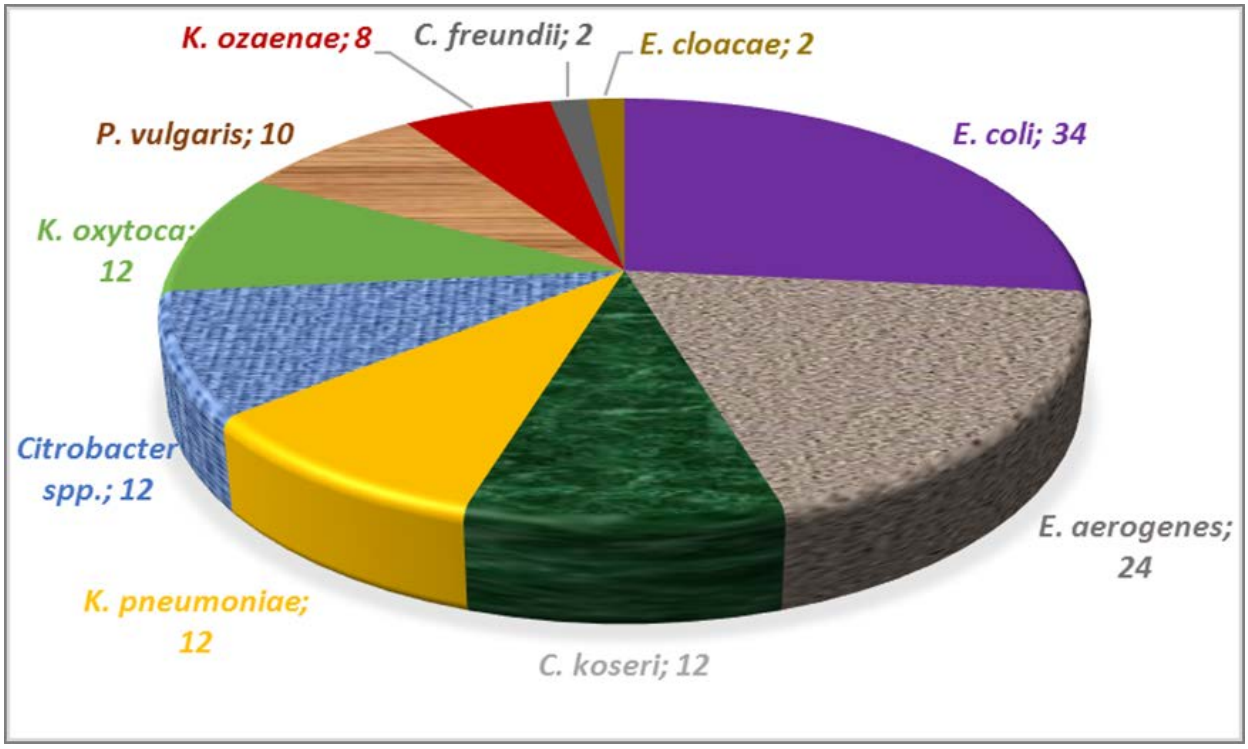

Figure 1. Enterobacterial isolated strains

Table 2. Antibiotic resistance percentage of *Enterobacteriaceae* strains

Families	Antibiotic	Strains										Average resistance by molecule (%)
		E. coli n=34	*E. aerogenes* n=24	*C. koseri* n=12	*K. pneumoniae* n=12	*Citrobacter spp.* n=12	*K. oxytoca* n=12	*P. vulgaris* n=10	*K. ozaenae* n=8	*C. freundii* n=2	*E. cloacae* n=2	
		R (%)	**R (%)**	**R (%)**	**R (%)**	**R (%)**	**R (%)**	**R (%)**	**R (%)**	**R (%)**	**R (%)**	
Beta-lactams	AUG	28 (82,35)	22 (91,66)	6 (50)	8 (66,67)	10 (83,33)	8 (66,67)	8 (80)	6 (75)	2 (100)	2 (100)	79,5
	CTX	28 (82,35)	22 (91,66)	8 (66,67)	4 (33,33	6 (50)	4 (33,33)	6 (60)	2 (25)	2 (100)	0 (0)	54,2
	AT	28 (82,35)	22 (91,67)	8 (66,67)	6 (50)	6 (50)	4 (33,33)	6 (60)	4 (50)	2 (100)	2(100)	68,4
	CAZ	22 (64,71)	22 (91,66)	8 (66,67)	4 (33,33)	6 (50)	4 (33,33)	6 (60)	2 (25)	2 (100)	0 (0)	52,4
	FEP	32 (94,12)	24 (100)	12 (100)	10 (83,33)	10 (83,33)	10 (83,33)	6 (60)	4 (50)	2 (100)	2 (100)	85,4
	IMI	44 (64,71)	32 (66,67)	4 (16,67)	16 (66,67)	16 (66,67)	16 (66,67)	4 (40)	6 (75)	0 (0)	2 (100)	56,3
Fluoroquino-lones	NOR	30 (88,24)	22 (91,67)	8 (66,67)	6 (50)	8 (66,67)	6 (50)	8 (80)	6 (75)	2 (100)	0(0)	66,8
	CIP	30 (88,24)	22 (91,67)	8 (66,67)	4 (33,33)	8 (66,67)	6 (50)	8 (80)	6 (75)	2 (100)	0 (0)	65,1
	LEV	24 (70,59)	2 (8,33)	2 (16,67)	6 (50)	8 (66,67)	6 (50)	8 (80)	2 (25)	2 (100)	0 (0)	46,7
Aminoglycosides	CN	30 (88,24)	22 (91,67)	8 (66,67)	8 (66,67)	12 (100)	12 (100)	10 (100)	8 (100)	2(100)	0 (0)	81,3
	AK	16 (47,06)	16 (66,67)	4 (33,33)	6 (50)	4 (33,33)	6 (50)	6 (60)	6 (75)	0 (0)	0 (0)	41,5

Legend: AUG: amoxicillin + clavulanic acid, AT: aztreonam, CAZ: ceftazidime, CTX: cefotaxime, FEP: cefepime, IMI: imipenem, CIP: ciprofloxacin, LEV: levofloxacin, NOR: norfloxacin, CN: gentamicin, AK: amikacin, n: number of strains per species, %R: percentage of strains per species, %R: percentage of resistance.

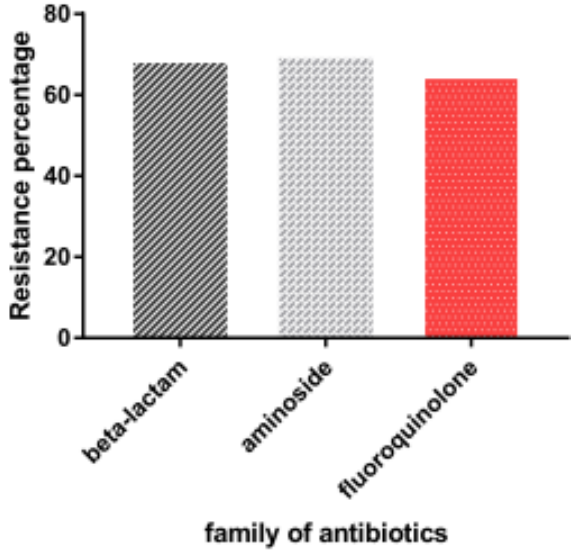

Figure 2. Antimicrobial resistance percentages of strains by antibiotic family

3.4. Detection of Resistance Genes

Among the 128 strains of *Enterobacteriaceae*, 88 showed multidrug-resistance. These strains were selected with the aim to perform the PCR in order to search for the genes which confer resistance to aminoglycosides and quinolones.

3.4.1. Aminoglycoside Resistance Genes

PCR analysis by 1.5% agarose gel electrophoresis showed a single type of specific band around 395 bp, confirming amplification of the *aac*(6')-Ib-cr gene (Figure 3). In total, 30 strains were positive by PCR, i.e. a rate of 34.09%.

12 strains of *E. coli*, or 13.64%, amplified this gene. However, the *aac*(3')-Ib-cr and *aad*A genes were found to be negative in these strains.

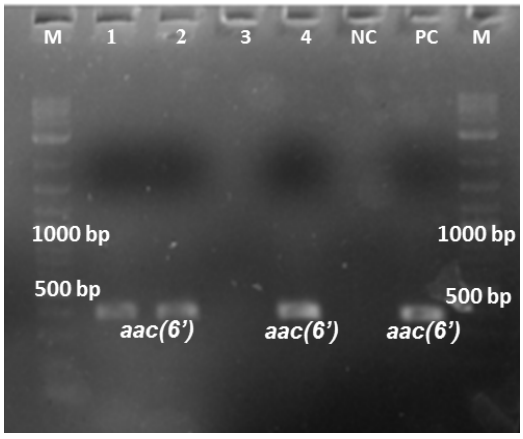

Figure 3. Amplification of the *aac*(6') gene (Legend: M: Molecular weight marker; wells 1, 2, and 4: strains positive for the *aac*(6') gene; well 3: strain negative for the *aac*(6') gene; NC: negative control; PC: positive control)

3.4.2. Fluoroquinolone Resistance Gene

The *qnr* genes were sought in this work as fluoroquinolone resistance genes. Among the 44 strains tested, 10 amplified the *qnr*S gene, i.e., had a frequency of 22.73%. Analysis of this PCR by 1.5% agarose gel electrophoresis showed a single type of specific band at 388 bp for this gene. The other genes (*qnr*A and *qnr*B) were found to be negative (Figure 4).

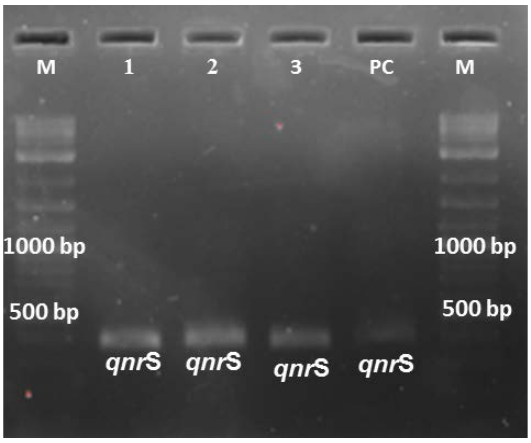

Figure 4. Amplification of the *qnr*S gene (Legend: M: molecular weight marker; wells 1, 2, and 3: strains positive for the *qnr*S gene; PC: positive control)

3.5. Coexpression of Aminoglycoside and Fluoroquinolone Resistance Genes

Among the ten (10) strains having amplified the *qnr*S gene, six (13.63%) also amplified the *aac*(6')-Ib-cr gene. In total, there are 4 strains of *E. coli* and 2 of *Klebsiella pneumoniae* that showed co-expression of these two types of genes, i.e., 4.5% of the strains (Figure 5).

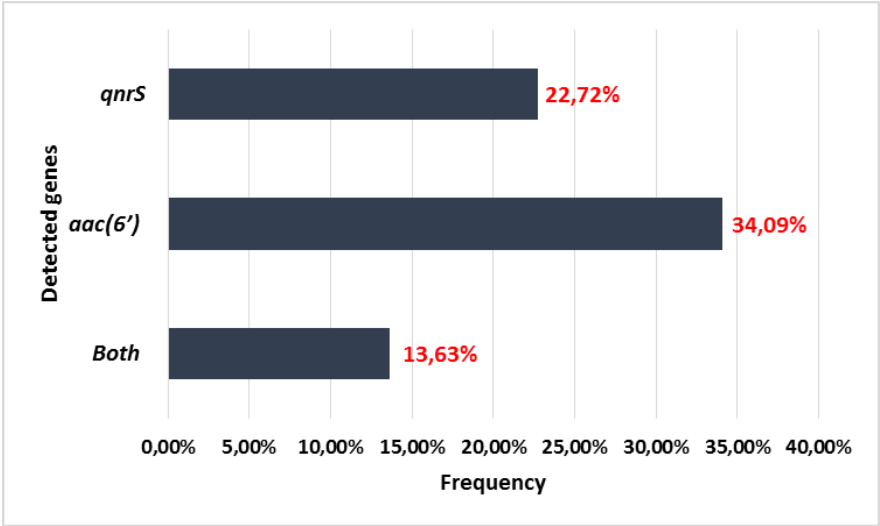

Figure 5. Expression levels of the *qnr* and *aac* genes within the strains

4. Discussion

This work aims to study the antibioresistance to aminoglycosides and fluoroquinolones in strains of *Enterobacteriaceae* isolated at the National Public Health Laboratory of Brazzaville, in Republic of Congo. A total of 128 strains of *Enterobacteriaceae* were isolated from the urine from patients of different ages and sexes. Using this biological material, strains of *Enterobacteriaceae* were frequent in the urine (84.4%). This predominance was also shown by Anago in Togo [18]. This finding is also similar to these results previously obtained by Bahmani in Iran, where urine was the majority sample, allowing the isolation of *Enterobacteriaceae* [19].

The 128 isolates of *Enterobacteriaceae* can be grouped into five genera: *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, and *Proteus*. These genera include ten species, of which *Escherichia coli* is the predominant represented by a frequency of 26.56%, followed by *Enterobacter aerogenes* with 18.75%. Finally, *Citrobacter freundii* and *Enterobacter clocae* come last, with a frequency of 1.56% for each of them. The predominance of *Escherichia coli* has been reported in previous work [20,21,22]. The high predominance of *E. coli* in patients with urinary tract infections (UTI) is expected and it is well known by physicians and researchers all over the world as this bacterium represents a normal component of the intestinal microbiota of humans and animals and has strains with the potential of causing UTI and other extraintestinal infections. This bacterium has adhesins capable of binding the bacterium to the urinary epithelium and preventing its elimination through bladder emptying [23,24].

The antibiotics tested belong to beta-lactams, fluoroquinolones, and aminoglycosides. The strains studied showed resistance greater than 50%. Similar resistance patterns were published in Morocco by Romeli *et al*., who obtained a resistance rate of 72% to aminoglycosides and fluoroquinolones in *Enterobacteriaceae* producing beta-lactamases isolated from urine [25].

Among the families of antibiotics tested, the resistance was higher among aminoglycosides, with an average rate of 69.53%. Gentamicin was the least active aminoglycoside on *Enterobacteriaceae* because 89.06% of strains showed resistance to it. Our results are similar to those of Romeli, who reported an 86% enterobacterial resistance rate to gentamicin in Morocco [25]. A rate of 50% for resistance to amikacin was observed in this study. Indeed, the resistance to amikacin continues to grow because it has been reported that amikacin constitutes the most active aminoglycoside on *Enterobacteriaceae*. Okalla reported a

resistance rate of *Enterobacteriaceae* to amikacin of 13.4% in Cameroon [26]. This is because amikacin is frequently used for infections caused by *Enterobacteriaceae* due to the ineffectiveness of other aminoglycosides. Resistance to aminoglycosides could be mainly due to the production of enzymes of the acetylase, adenylase, and phosphorylase transferase types. It can also be associated with active efflux mechanisms [27].

Beta-lactams showed high resistance rates of around 68.33%. In this family, cefepime was the least active antibiotic with a resistance rate of 85.94%, and ceftazidime was the most active molecule with a resistance rate of 57.81%. Similar to that obtained by [26] in Cameroon, who reported a resistance rate of 75% to 3rd generation cephalosporins in strains of *Enterobacteriaceae*. This could be explained by the use of these molecules in probabilistic treatments. This resistance could be attributed to the production of a cephalosporinase [28].

Fluoroquinolones come last with an average resistance rate of 64.58%. Norfloxacin was the least active fluoroquinolone, with a resistance rate of 75%, followed by ciprofloxacin, which is 65.1%. Levofloxacin was the most active fluoroquinolone with a rate of 45.31%. Similar results were also reported in Iran by Amirkamali, with respective rates of 68% for ciprofloxacin [29]. The resistance to fluoroquinolones observed in this study could be attributed to the coexistence of several mechanisms, including the efflux mechanism, the acquisition of resistance genes, and the modification of the target [30].

Globaly, the strains studied showed simultaneous resistance to all families of antibiotics tested. Indeed, *E. coli*, like some other species, showed resistance to both fluoroquinolones, beta-lactams and aminoglycosides, which is worrying because this species is more prevalent in the context of our work. The predominant and multi-resistant character constitutes a health threat. This finding could be the consequence of selection pressure due to the misuse of broad-spectrum antibiotics in hospital and community settings, as well as the cross-transmission of acquired resistance to plasmid determinism [31].

The genetic determinants encoding modifying enzymes (AMEs) are important sources of resistance to aminoglycosides in bacteria. The corresponding genes are highly mobile and can be transported by integrons, transposons, plasmids, and other transposable gene elements, often together with other resistance genes [32]. Studies conducted in different regions in word have reported that the *aac*(6')-Ib-cr gene is together with the *ant*(2´)-I gene, with both AME genes having a high prevalence [33,34,35]. In our study, we detected the *aac*(6')-Ib-cr gene at a rate of 34.09%. The detection of this gene in the present study is a first in the Republic of Congo. The study conducted in Egypt by Abo-State reported similar rates of around 30% prevalence of the *aac*(6')-Ib-cr gene [20]. The detection of this gene in this study could explain the observed resistance to amikacin and gentamicin. It has also been reported that the *aac*(6')-Ib-cr gene can also induce low-level resistance to ciprofloxacin and promote the selection of resistant mutants by chromosomal mutation mechanisms. Thus, the *aac*(6')-Ib-cr gene confers resistance to all aminoglycosides, except streptomycin [36].

With regard to resistance to fluoroquinolones, the plasmid that induces this resistance involves the *qnr* genes, which encode the proteins that protect DNA gyrase and topoisomerase IV [37]. Among non-hospital sources, *qnr* genes have been reported in *Enterobacteriaceae* isolated from pigs, cattle, and poultry [38]. Here, we detect the *qnrS* gene at a rate of 22.72%. This is the first demonstration of this gene in *Enterobacteriaceae* in the Republic of Congo. This result is similar to that obtained by Guessennd in Côte d'Ivoire, who detected the *qnrS* gene in isolates from different ecosystems but with a very high prevalence in animals [39]. However, Makaya reports the detection of *qnrA*, *qnrB*, and *qnrS* genes at respective rates of 13%, 21.8%, and 8.7% in *Pseudomonas aeruginosa* in Côte d'Ivoire [40]. In the latter case, the difference could be explained by the fact that she studied a different Gram-negative bacterium than *Enterobacteriaceae*. In our study, the most representative species that allowed this gene to be amplified in our work was *Escherichia coli*. Similarly, Tahou detected the *qnr*B, *qnr*A, and *qnr*S genes at rates of 71.73%, 26.08%, and 2.17%, respectively, in Côte d'Ivoire [41]. The difference in rates could be explained by the conditions of the study environments and the nature and size of the sampling.

Among the strains studied, 4.5% showed co-expression of the *qnr*S and *aac*(6') genes. The coexistence of plasmids carrying these genes could increase the chances of survival for these isolates in hospital and community settings. The facility of transfer of drug resistance in these isolates is also a cause for concern [42]. Over time, these isolates could serve as efficient genetic vehicles for the transfer of antimicrobial resistance genes. The diffusion of these genes could be linked to the existence of genetic mobile elements that can be transmitted between bacteria of the same species or different species.

5. Conclusion

Antibiotic resistance has become a global public health problem that does not exclude the Republic of Congo. Indeed, over the past ten years, a significant increase in antibiotic resistance has been observed nationwide, particularly among Gram-negative bacilli (GNB). The resistance mechanisms that these germs develop and the worldwide spread of resistance genes are also a major concern. This work has shown an increase in the resistance of *Enterobacteriaceae* strains to the most commonly used antibiotics. This resistance is due to the misuse of antibiotics, which develops resistance mechanisms in *Enterobacteriaceae*. Also, the reported presence of *qnr* and *aac* genes is a concern in the therapeutic management of patients because it could lead to high costs for prolonged hospitalization and increased morbidity and mortality.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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