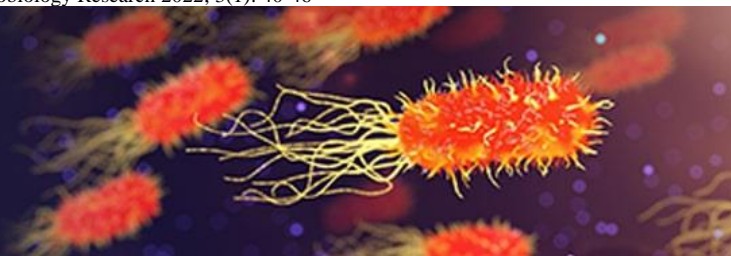


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Nicole Prisca Makaya Danguï Nieko

a) Service de Bactériologie, Laboratoire National de Santé Publique, BP 120, Brazzaville, Congo

b) Laboratoire de Microbiologie, Infectiologie et Immunologie, Ecole Normale Supérieure, Université Marien Nguouabi, BP 69, Brazzaville, Congo

Cyr Jonas Morabandza

a) Service de Bactériologie, Laboratoire National de Santé Publique, BP 120, Brazzaville, Congo

b) Laboratoire de Microbiologie, Infectiologie et Immunologie, Ecole Normale Supérieure, Université Marien Nguouabi, BP 69, Brazzaville, Congo
c) Faculté des Sciences et Techniques, Université Marien Nguouabi, BP 69, Brazzaville, Congo

Moïse Doria Kaya-Ongoto

a) Service de Bactériologie, Laboratoire National de Santé Publique, BP 120, Brazzaville, Congo

b) Faculté des Sciences et Techniques, Université Marien Nguouabi, BP 69, Brazzaville, Congo

Duchel Jeanedvi Kinouani Kinavouidi

a) Service de Bactériologie, Laboratoire National de Santé Publique, BP 120, Brazzaville, Congo

b) Faculté des Sciences et Techniques, Université Marien Nguouabi, BP 69, Brazzaville, Congo

Haman Judel Mikia

Laboratoire de Microbiologie, Infectiologie et Immunologie, Ecole Normale Supérieure, Université Marien Nguouabi, BP 69, Brazzaville, Congo

Fabien Kangoula-Dia-Kikouidi-Kia-Louzala

Service de Bactériologie, Laboratoire National de Santé Publique, BP 120, Brazzaville, Congo

Isaac Samuel Onyankouang

a) Service de Bactériologie, Laboratoire National de Santé Publique, BP 120, Brazzaville, Congo

b) Faculté des Sciences et Techniques, Université Marien Nguouabi, BP 69, Brazzaville, Congo

Fabien Roch Niama

a) Service de Bactériologie, Laboratoire National de Santé Publique, BP 120, Brazzaville, Congo

b) Faculté des Sciences et Techniques, Université Marien Nguouabi, BP 69, Brazzaville, Congo

Corresponding Author:

Nicole Prisca Makaya Danguï Nieko

a) Service de Bactériologie, Laboratoire National de Santé Publique, BP 120, Brazzaville, Congo

b) Laboratoire de Microbiologie, Infectiologie et Immunologie, Ecole Normale Supérieure, Université Marien Nguouabi, BP 69, Brazzaville, Congo

Phenotypic and genotypic characterization of metallo-Beta-lactamase and extended-spectrum Beta-lactamase among enterobacteria isolated at national public health laboratory of Brazzaville

Nicole Prisca Makaya Danguï Nieko, Cyr Jonas Morabandza, Moïse Doria Kaya-Ongoto, Duchel Jeanedvi Kinouani Kinavouidi, Haman Judel Mikia, Fabien Kangoula-Dia-Kikouidi-Kia-Louzala, Isaac Samuel Onyankouang and Fabien Roch Niama

Abstract

The improper use of antimicrobials against infectious diseases has allowed microorganisms to develop defense mechanisms that give them insensitivity to these agents. All bacteria are concerned by this phenomenon. This work aimed to assess prevalence of beta-lactamase produced by enterobacterial isolates. Then, disc diffusion, double disc synergy test (DDST) and combined disc test (CDT) were respectively used for antimicrobial resistance, detection of Extended-Spectrum Beta-Lactamases (ESBL) and Metallo-Beta-Lactamases (MBL). *bla* genes were detected by PCR. A total of 132 enterobacterial strains were studied. Resistance to antibiotic families was observed with a greater frequency than 50%. Gentamicin was the least active beta-lactam antibiotic, with a resistance rate of 88%. 40.9% of strains show an ESBL phenotype and 16.6% were MBL. An overall prevalence of 74% (40/54) and respectively rates of 29.6%, 27.7% and 16.7% for *bla*SHV, *bla*CTX and *bla*TEM genes were observed. SHV, CTX, CTX/SHV/TEM, CTX/TEM, SHV/TEM and CTX/SHV were different ESBL genotypes observed. ESBL-producing enterobacteria isolation worried about the future of antimicrobial therapy in the Republic of Congo. This is a public health problem that requires careful monitoring and implementation of a policy of rational antibiotics use.

Keywords: Enterobacteria, antibiotic resistance, ESBL, MBL, *bla* genes

Introduction

The antimicrobials introduction into the therapeutic arsenal of infectious diseases and their improper use have allowed microorganisms to develop defenses that give them insensitivity to these agents. All bacterial species are concerned by the phenomenon of antibiotic resistance, a real therapeutic problems (Bryskier, 1999) [8]. Thus, the isolation in hospital and community environment of multidrug-resistant Gram-negative bacilli (GNB) in general and beta-lactamases producers in particular has become a global health problem (Meletis, 2015) [23]. This reality was recently recognized by the World Health Organization (WHO) in its report on antimicrobial resistance (WHO, 2014) [26]. The last ten years have been marked by the emergence and dissemination of new resistance genes and the major challenges of this resistance have been encountered mainly in different species of enterobacteria (Patricia, 2001) [27]. Among multidrug-resistant bacteria (MRBs), enterobacteria producing β -lactamases have taken an important place due to their high frequency and high pathogenic potential. β -lactamases are the most inactivation enzymes frequently encountered and are the main mechanism of acquired resistance to β -lactam antibiotics. Based on the nature of their amino acids, there are four classes of beta-lactamases, A-D (Ambler, 1980) [2]. Classes A, C and D are β -lactamases comprising a serine with a hydrolytic mechanism, where we distinguish Extended Spectrum Beta-Lactamases (ESBLs). The first reported ESBLs concerned *Klebsiella pneumoniae*, but gradually other species such as *Escherichia coli*, *Enterobacter* sp. have been the cause of epidemics described in hospitals (Kansaye, 2020). Currently, several ESBL groups are described including TEMs, SHVs, CTX-M and many others. All of these enzymes are not resistant to carbapenems. Carbapenems resistance appeared two decades later (Bebrone, 2007) [7] and involves class B beta-lactamases, those

that contain a zinc ion active site, hence their name, Metallo-Beta-Lactamases (MBL) containing subclasses B1, B2 and B3 (Walsh *et al.*, 2005) [38]. They are characterized by a wide spectrum of activity towards β -lactams antibiotics, with the exception of monobactams, they are able to hydrolyze all classes of β -lactams, including carbapenems (Poirel *et al.*, 2007) [29], antibiotics used in intensive therapy in clinical environment as a means of combating severe infections caused by MRBs, also thanks to their broad spectrum of action (Nicolau, 2008) [25]. The emergence of MBLs is a serious problem in the community and clinical environment. MBLs are able to hydrolyze all classes of β -lactams, including carbapenems (Poirel *et al.*, 2007) [29], antibiotics used in intensive therapy in clinical environment as a means of combating severe infections caused by MRBs, also thanks to their broad spectrum of action (Nicolau, 2008) [25]. No effective inhibitor of these enzymes has yet been found. They are inactivated by metal chelators such as Ethylene Diamine Tetra-Acetic (EDTA), dipicolonic acid, phenantroline. It is also interesting to note that the majority of genes encoding these enzymes are of plasmid origin and that they are easily transferable between bacterial strains by conjugation or transposition. MBLs can spread easily and allow the spread of resistance to carbapenems (Bebrone, 2007) [7]. Bacteria producing carbapenemases have been reported in several countries, including Turkey (Karabay *et al.*, 2016) [22], China (Wang *et al.*, 2018) [39], Mexico (Torres-González *et al.*, 2015) [37] and Brazil (Rossi Gonçalves *et al.*, 2016) [31].

In Africa, several studies report that the continent is not on the margins of this reality (Elouennas *et al.*, 2008) [11]. Recently, a study reported the detection of MBL in *Pseudomonas aeruginosa* isolated from hospitalized patients in Abidjan, Côte d'Ivoire (Gba *et al.*, 2018) [15].

In the Republic of Congo, the study of MBL and ESBL enterobacterial is not sufficiently documented. To contribute to this problem, we proposed to conduct this study in order to explore the enterobacteria producing these enzymes giving them the resistance.

Methods

Enterobacterial isolation

From august to november 2021, a total of 438 urine samples were aseptically collected at the Bacteriology department of National Public Health Laboratory of Brazzaville. Thus, by using conventional microbiology techniques, enterobacteria were isolated in Eosin Methylene Blue (EMB) agar medium after aerobic incubation at 37 °C for 24 h. Initially, the colonies were counted to confirm the sample positivity. After purification, Gram type of bacteria was determined by staining. Identification of strains was confirmed by using biochemical tests with "Integral System Enterobacteria" (Liofilchem).

Antimicrobial susceptibility testing

The antimicrobial resistance profile of all isolates was determined by using the standard Kirby and Bauer method, based in antibiotics disc diffusion on Mueller-Hinton Agar (MHA). The antibiotics used were: Cefepime (30 μ g), Ceftazidime (10 μ g), Cefotaxime (5 μ g), Aztreonam (30 μ g), Amoxicillin + clavulanic acid (20 μ g), Imipenem (10 μ g), Norfloxacin (10 μ g), Levofloxacin (5 μ g),

Ciprofloxacin (5 μ g), Gentamicin (10 μ g) and Amikacin (30 μ g). The diameter of the inhibition zone for each antibiotics disc was measured and results were defined in accordance Clinical and Laboratory Standards Institute guidelines (CLSI, 2021) [9].

Phenotypic screening of MBL

All strains previously resistant to imipenem were selected to perform imipenem/imipenem + EDTA combined disc test (CDT) in order to detect metallo-beta-lactamase (MBL) producing strains. Briefly, a bacterial inoculum with an optical density of 0.5 McFarland was prepared with 0.9% NaCl medium suspension. Two imipenem discs were placed 30 mm apart on MHA previously seeded with the bacterial inoculum. On one of disc, 4 μ L of EDTA (0.5 M, pH = 8) were added. The diameter of inhibition zone around these discs are measured and compared after incubation of 18 h at 37 °C.

The result is considered as positive if inhibition diameter around imipenem + EDTA is greater than that obtained with the imipenem disc alone by at least 7 mm (Yong *et al.*, 2002) [41].

Determination of ESBL producing isolates

ESBL production was detected by Double Disc Synergy Test (DDST), which consists of placing discs of ceftazidime (10 μ g), cefotaxime (5 μ g), cefepime (30 μ g), imipenem (10 μ g) and aztreonam (30 μ g) at a distance of 30 mm (center to center) from a disc of amoxicillin + clavulanic acid (30/10 μ g). The increase of the inhibition zone between amoxicillin + clavulanic acid disc and aztreonam, cefotaxime indicates the production of ESBL (Fracas *et al.*, 2008) [14]. The reference strain *E. coli* ATCC 25922 was used.

Detection of *bla* genes by PCR

After DNA plasmid extraction using Nucleospin Plasmid Kit (Macherey-Nagel, Germany), different types of PCR were performed depending on the targeted gene using the primers shown in Table 1. A simplex PCR was performed for *bla*CTX gene detection. The PCR reaction was performed in a final volume of 50 μ L containing 19 μ L of sterile distilled water, 2 μ L of DNA, 2 μ L of each primer (forward and reverse), 25 μ L of Promega PCR Master Mix. The amplification program used comprises: an initial denaturation at 95 °C for 5 min, followed by 30 cycles each comprising a denaturation at 95 °C for 1 min, hybridization at 55 °C for 45 s, an extension at 72 °C for 1 min and a final extension at 72 °C for 7 min.

Multiplex PCR using four primers was also performed to simultaneously detect *bla*SHV and *bla*TEM genes. PCR reaction was performed in a final volume of 50 μ L containing 15 μ L of sterile distilled water, 2 μ L of DNA, 2 μ L of each primer (forward and reverse), 25 μ L of Promega PCR Master Mix. The used program for the amplifications comprises: an initial denaturation at 95 °C for 5 min, followed by 30 cycles each comprising a denaturation at 95 °C for 1 min, hybridization at 60 °C for 1 min, an extension at 72 °C for 1 min and a final extension at 72 °C for 7 min. The 0.2 mL microtubes are then placed in the thermocycler (Biorad, Singapore). Amplification products was analysed by agarose gel electrophoresis at 1.5%.

Table 1: Specific primers used for *bla* genes detection

Genes	Primer name	Sequences (5'-3')	Product size (pb)	References
CTX	CTX-F	TCTTCCAGAATAAGGAATCCC	909	Sturenburg <i>et al.</i> , 2004
	CTX-R	CCGTTTCCGCTATTACAAAC		
SHV	os-5 (+)	TTTATGGCGTTACCTTTGACC	795	Yagi <i>et al.</i> , 2000
	os-6 (-)	ATTTGTGCGCTTCTTTACTCGC		
TEM	a216 (+)	ATGAGTATTCAACATTTCCGTG	1079	Essack <i>et al.</i> , 2004
	a217 (-)	TTACCAATGTCTTAATCAGTGAG		

Results

Enterobacterial isolates: During the study, a total of 132 samples tested were positive, representing an isolation rate of 30%.

Isolates identification: The use of conventional

microbiology methods made possible the identification of the 132 enterobacterial isolates including, 33 strains of *Escherichia coli* which is the most representative species, followed by 23 strains of *Enterobacter aerogenes*. Finally, *Enterobacter cloacae* is the least representative species with 4 strains (Figure 1).

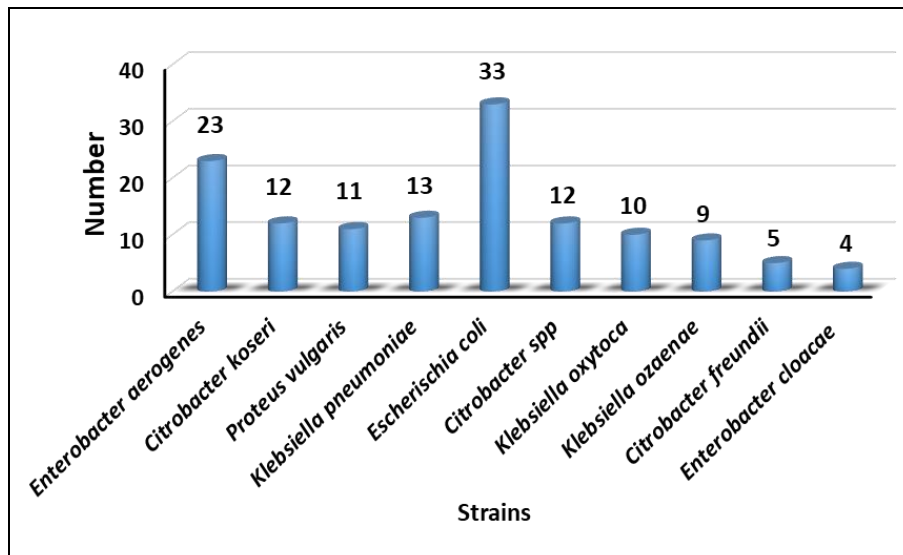


Fig 1: Distribution of identified enterobacterial strains

Antimicrobial susceptibility results

Different levels of resistance were observed to antibiotics tested in enterobacterial strains. For 132 strains studied, the results revealed good levofloxacin activity with a sensitivity rate of 55%, followed by amikacin with a sensitivity rate of

45%. Gentamicin was the least active aminoglycoside antibiotic in the strains studied with a high resistance rate of 87%. However, cefepime was the least active antibiotic in the β -lactam family with a high resistance rate of 82% (figure 2).

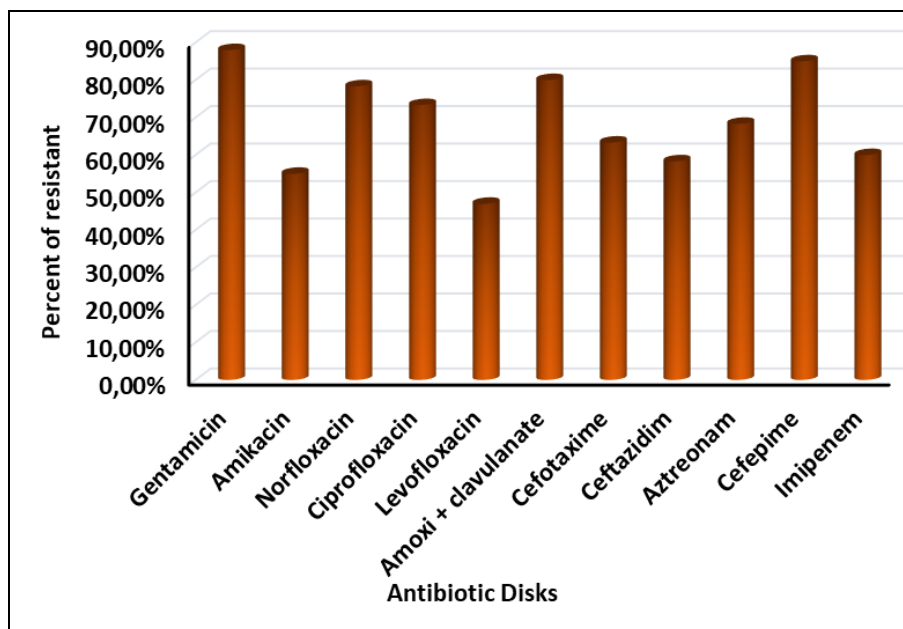


Fig 2: Antibiotics resistance profile

MBL-producing strains

Among the 132 studied strains, 78 were resistant to imipenem. These strains were selected for MBL screening, 22 were positive, a percentage of 16.6% taking into account the initial sample (figure 3). A predominance of MBLs was obtained with *E. coli* species, with 8 positive strains, 6% (Table 2).

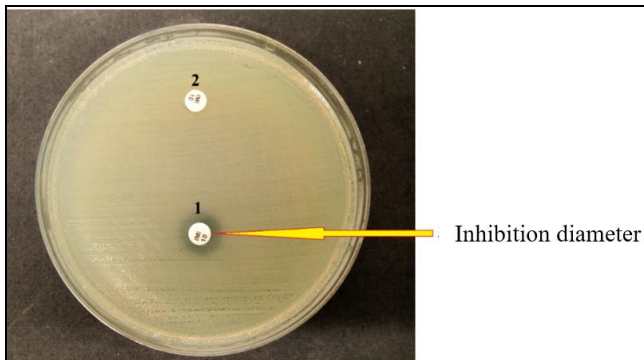


Fig 3: Double Disc Synergy Test 1: imipenem + EDTA, 2: imipenem

ESBL-producing strains

The result of the synergy test for phenotypic detection of ESBLs is illustrated in figure 4. Among 132 strains tested, 54 were positive, a rate of 40.9%. 12.8% of ESBL-producing strains are *E. coli* (Table 2). *K. pneumoniae* represents the least representative ESBL-producing species in this work, with a rate of 2.3%.

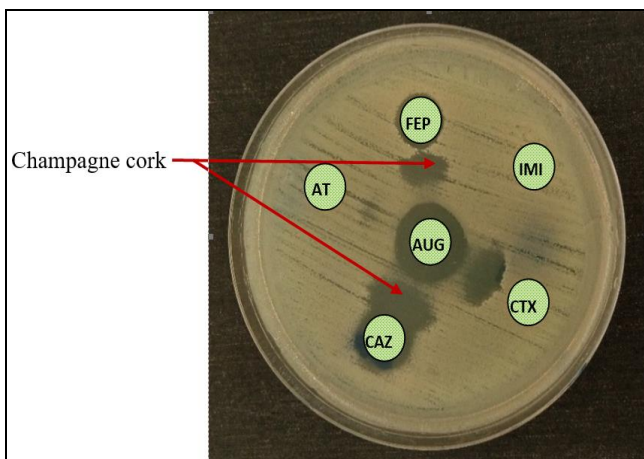


Fig 4: ESBL phenotypic test

Table 2: Prevalence of beta-lactamase-producing strains (n=132)

Strains	ESBL No (%)	MBL No (%)	Total No (%)
<i>E. coli</i>	17 (12,9)	8 (6)	25 (18,9)
<i>K. pneumoniae</i>	3 (2,3)	2 (1,5)	5 (3,8)
<i>E. aerogenes</i>	14 (10,6)	4 (3)	18 (13,6)
<i>K. ozaenae</i>	0 (0)	3 (2,3)	3 (2,3)
<i>P. vulgaris</i>	5 (3,8)	2 (1,5)	7 (5,35)
<i>C. koseri</i>	6 (4,5)	1 (0,8)	7 (5,35)
<i>K. oxytoca</i>	4 (3)	2 (1,5)	6 (4,5)
<i>Citrobacter spp</i>	5 (3,8)	0 (0)	5 (3,8)
Total No (%)	54 (40,9)	22 (16,6)	76 (57,6)

bla gene detection

Detection of CTX gene by simplex PCR

Fifty-four (54) ESBL-producing strains were selected for molecular analysis. After PCR, results showed the presence of CTX gene, around 909 bp (figure 5). This gene was detected in 15 strains, a rate of 27.7% (Table 3).

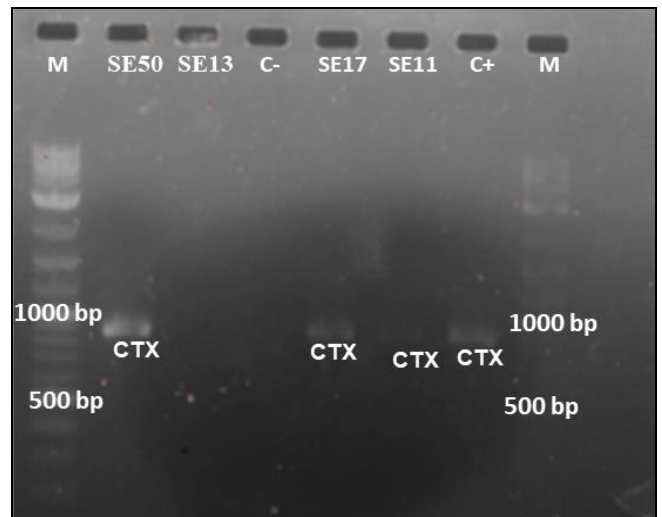


Fig 5: Amplification of the CTX gene.

MP: Molecular size marker; C-: Negative control; C+: Positive control. SE50, SE13, SE17 and SE11: Enterobacterial isolates

Detection of SHV and TEM genes by multiplex PCR

Multiplex PCR was performed with the same samples (54 strains). The results of multiplex PCR showed after agarose electrophoretic analysis, the presence of SHV and TEM genes with respective bands around 800 and 1079 bp (figure 6). SHV gene is observed in 16 (29.6%) strains tested. The TEM gene is detected in 9 strains (16.7%). These results are detailed in Table 3.

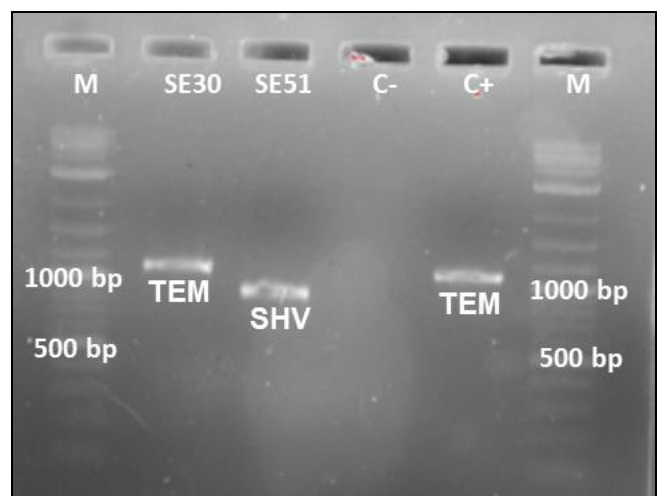


Fig 6: Multiplex PCR result for SHV and TEM genes detection.

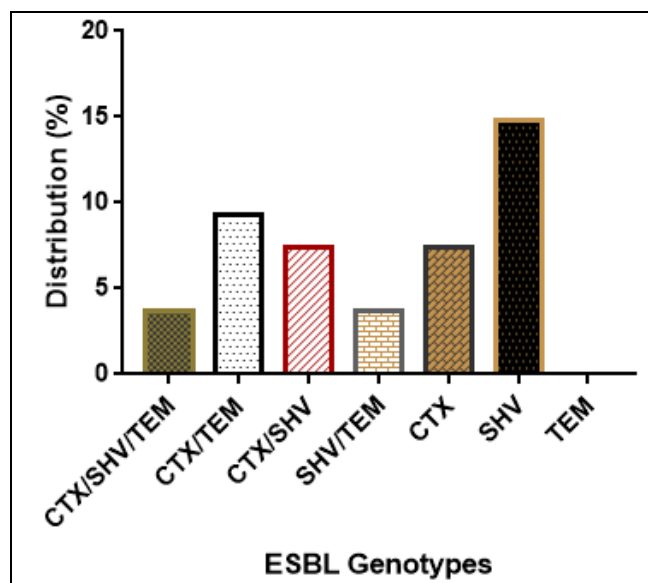
M: molecular size marker; C-: Negative control; C+: Positive control. SE30, SE51: Enterobacterial isolates

Table 3: Distribution of *bla* genes in enterobacterial isolates (n=54)

<i>bla</i> gene	Strains					Total No (%)
	<i>E. coli</i> No (%)	<i>E. aerogenes</i> No (%)	<i>K. ozaenae</i> No (%)	<i>K. pneumoniae</i> No (%)	<i>Citrobacter spp</i> No (%)	
CTX	8 (14,8)	4 (7,4)	3 (5,5)	0 (0)	0 (0)	15 (27,7)
SHV	6 (11,1)	0 (0)	5 (9,3)	3 (5,5)	2 (3,7)	16 (29,6)
TEM	5 (9,3)	0 (0)	4 (7,4)	0 (0)	0 (0)	9 (16,7)
Total	19 (35,2)	4 (7,4)	12 (22,2)	3 (5,5)	2 (3,7)	40 (74)

Genotypic profiles

Analysis of genotypic profiles shows that one or more types of ESBL genes are detected in enterobacterial strains (figure 7). These genes are detected alone or in combination. No strains carrying only TEM gene were observed in this study. SHV and CTX genes detected alone were obtained at the respectively rates of 14.8% and 7.4%. CTX/SHV/TEM genotype was found at a rate of 3.7%. CTX/TEM, CTX/SHV and SHV/TEM genotypes were respectively observed at rates of 9.25, 7.4 and 3.7%. There is a predominance of SHV genotype in this study.

**Fig 7:** Genotypic profiles of ESBL associated genes

Discussion

This study aimed to assess prevalence of beta-lactamase produced by enterobacteria isolated at the National Public Health Laboratory of in Brazzaville. Phenotypic and genotypic characterization of ESBL was done, as well as the phenotypic characterization of MBL. All isolates were from urine samples. 132 enterobacterial isolates have been identified and divided into 5 genera: *Escherischia*, *Enterobacter*, *Klebsiella*, *Citrobacter* and *Proteus*. These genera have been divided into 10 species.

In this study, all strains were obtained from urine as biological material. These results are similar with those obtained by Bahmani (2019) [4] in Iran, who isolated majority 67.6% of enterobacteria from urine samples.

Escherischia coli is the predominant species among studied strains, with a frequency of 25%. These results are similar to those obtained by Abo-State (2018) [1] in Egypt and Jamali *et al.*, (2014) [20] in Morocco, who reported a predominance of *E. coli*. This predominance is explained by the fact that *E. coli* has adhesins capable of bacterium binding to the urinary epithelium and preventing its elimination by bladder emptying (Bakiri and Amamra, 2009) [5].

About strains susceptibility to antibiotics, there is significant resistance for each family of antibiotics tested. This resistance is greater than 50% for each disc, except for levofloxacin of the fluoroquinolone family.

For aminoglycoside, gentamicin with a high resistance rate of 88% was the least active antibiotic. The aminoglycoside resistance could be mainly due to the enzymes production, such as acetylases, adenylases and phosphorylases transferase. It can also be associated with active efflux mechanisms (Decré, 2012) [10].

In β -lactam antibiotics, cefepime was the least active, bacteria showed resistance of 84.4% with this molecule. This could be attributed to cephalosporinase production (Bradford, 2001) [6].

In fluoroquinolones, norfloxacin was the antibiotic at which bacteria showed the most resistance with a rate of 78%. The high resistance to this molecule could be explained by the massive use in the treatment of urinary tract infections (Thabet *et al.*, 2010) [36].

Globally, studied strains showed simultaneous resistance to all tested antibiotic families. In addition to being predominant in this work, *E. coli* has shown resistance to both fluoroquinolones, beta-lactams and aminoglycosides, which is worrying. The predominant and multidrug-resistant nature constitutes a public health threat to the success of the antibiotics arsenal currently available. It could be the consequence of selection pressure due to the improper use of broad-spectrum antibiotics in hospital and community settings, as well as the cross-transmission of resistance acquired to plasmid determinism (Ferjani *et al.*, 2011) [13].

In this study, 27.2% of the strains studied were resistant to imipenem. This rate is higher than that reported in Ivory Coast by Guessennnd (2013) [17] which obtained 10.4% of strains resistant to imipenem. However, the work of Asadolah-Malayeri *et al.*, (2016) [3] reported a imipenem resistance rate of 97%. Resistance to imipenem can be explained by the selection of resistant bacteria favored by the frequent use of this antibiotic during the probabilistic treatment of severe nosocomial infections (Grall *et al.*, 2011) [16]. It could also be due to the loss of porin D (*oprD*) and/or the carbapenemase production (Mesaros *et al.*, 2005) [24].

Detection of MBL production was achieved by using combined disc test. 22 strains tested positive for combined discs, with a rate of 16.6%. This rate is higher than that obtained by Bahmani (2019) [4] in Iran, who obtained a rate of 13% of MBLs.

A total of 54 strains among 132 tested produced the extended-spectrum β -lactamases, a rate of 40.9%. This rate is higher than those obtained by Jamali *et al.*, (2014) [20] and Sbiti *et al.*, (2017) [32] with respectively ESBL production rates of 3% and 12.2%.

In this study, the majority of the extended-spectrum β -lactamases-producing strains consist of *Escherichia coli* with a rate of 12.9% and *Enterobacter aerogenes* (10.6%).

These results are similar to those reported in the literature (Henniche *et al.*, 2021) ^[19], where *E. coli* was the predominant ESBL strain.

CTX, SHV and TEM genes were respectively detected at the rates of 27.7%, 29.6% and 16.7% in ESBL strains. These results are similar with those of Shahid *et al.*, (2011) ^[33], who worked in northern India hospitals and amplified CTX, SHV and TEM. However, they obtained rates of 28.8% for CTX, 13.7% for SHV and 10.9% for TEM. In addition, Paveza *et al.*, (2019) ^[28] obtained higher results in order of 84% for CTX, 81% for SHV and 73% for TEM in southern Chile.

The genotypic profile of the studied strains showed different genotypes. CTX/TEM and CTX/SHV are respectively observed at rates of 9.25% and 7.4%. These observed rates are higher than those reported by Raji *et al.*, (2015) ^[30] whose work in Nigeria and showed CTX/TEM and CTX/SHV genotypes at rates of 3.7% each in *E. coli*. On the other hand, CTX/SHV/TEM genotype was observed with a rate of 3.7%. The presence and expression of these genes in the same strain could increase the spectrum of enterobacterial antibiotics resistance of and confer the phenomenon of multidrug resistance (Mesaros *et al.*, 2005) ^[24]. This could lead to therapeutic failures (Hagel *et al.*, 2015). This co-resistance is the result of the dissemination of various resistance genes via conjugative plasmids or transposons between bacteria of the same species or different species (Skurnik and Andremont, 2006) ^[34].

Conclusion

This study shows a high level of resistance for most antibiotics usually prescribed in clinical environment. That calls into question conventional therapeutic strategies in community infections. The unregulated sale and non-rational and widespread consumption of antibiotics raise fears of a rapid spread of multi-resistant bacteria within all ecosystems. Isolation of beta-lactamase-producing enterobacteria worries for the future of antimicrobial therapy in the Republic of Congo. This is a public health problem that requires careful monitoring and the implementation of a policy of rational use of antibiotics both in community and hospital. Bacterial infections cannot be completely avoided, but strict compliance with hygiene rules reduces the risk. Similarly, the development of rapid tests for the detection of beta-lactamase-producing strains could help to prevent their spread.

Data Availability

The Excel sheets including the data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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