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Phenotypic Characterization of Carbapenemase-Producing Enterobacteriaceae Strains in a Referral Teaching Hospital in Yaoundé, Cameroon

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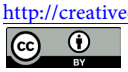
Abstract

Background: Carbapenemase-producing Enterobacteriaceae (CPE) are an important and increasing threat to global health. They are nowadays more encountered routinely in hospitals and cause high morbidity and mortality due to limited therapeutic alternatives. This study sought to determine the prevalence of CPE in Yaoundé teaching hospital, Cameroon, and the associated risk factors. **Materials and Method:** To achieve this goal, a descriptive cross-sectional study coupled to an analytical component with consecutive collection of Enterobacteria strains was carried out during a three-month period (from 27th July to 24th October 2018) in the University Teaching Hospital of Yaoundé, Cameroon. The oxidase and biochemical identification tests using a miniaturized Api 20 E system were performed on colonies grown on Eosin Methylene Blue (EMB) medium and subcultured on nutrient agar. Drug susceptibility testing was carried out according to the Antibiogram Committee of the French Society of Microbiology (CA-SFM 2018.V.2.0). The detection of carbapenemase production was performed by the CA-SFM 2018



algorithm for the screening of carbapenemase-producing enterobacteriaceae and its classification by inhibitory synergy tests. **Results:** Out of the 104 isolates, *Escherichia coli* (50%) was the most prevalent species, followed by *Klebsiella pneumoniae* (37.5%) and *Citrobacter frendii* (12.5%). Drugs susceptibility patterns showed a high resistance to penicillins group (97.4% to amoxicillin), cephalosporins (68.4% to cefotaxim, 58.1% to cefixim, 60.7% to ceftazidim, 57.1% of cefoxitin) and aztreonam (55.7%). However, 11.9% carbapenems related resistance was noticed: 14.4% to imipenem, 13.8% to ertapenem and 7.5% to meropenem. Numerous co-resistance to quinolones (65.8%), fluoroquinolones (49.6%), aminoglycosides (49.6%) and cotrimoxazole (71.8%) were also observed. From 104 isolates, AmpC production represented 23.08% (25/104) and 36.54% (38/104) were ESBL-isolates. The overall prevalence of CPE was 25% (26/104) with *K.pneumoniae* predominant (61.53%). Besides, Class A and class B carbapenemase were mainly produced with respectively 20% (21/104) and 5% (5/104). Univariate analysis revealed a significant association of carbapenemase production to *Klebsiella pneumoniae* ($p = 0.01$), ESBL and AmpC production (($P = 0.01$ and $P = 0.001$ respectively) while that association was only significant to *Klebsiella spp* ($p = 0.04$) and AmpC production ($p = 0.02$) in multivariate analysis. **Conclusion:** The multi-resistance of Enterobacteriaceae to antibiotics in Cameroon has considerably increased. More attention should be paid to those bacteria to stall antimicrobial resistance spread.

Keywords

Enterobacteriaceae, Antibiotics, Carbapenemase, Resistance

1. Introduction

According to WHO, Drug resistance is one of the most serious health threats facing humanity [1]. It could cause 10 million deaths per year and an overall cost of $100 trillion to the global economy by 2050 [1]. Among them, the increasing bacteria resistance to antibiotics which has become nowadays a major public health concern, raises fears of epidemic and endemic situations and therapeutic impasses [1] [2]. The urgency of the situation is such that international health authorities are actively mobilizing to safeguard antibiotics so that the progress they have made in the treatment and prevention of bacterial infections over the past 70 years should not be wiped out so easily in only a few years. This is particularly the case for beta lactamines, a mainstay of antibiotic therapy for enterobacteriaceae infections. Some potentially highly pathogenic enterobacteriaceae such as *Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae* or *aerogenes,* have become resistant to available molecules, including carbapenems [3] [4]. This resistance remains much more common in hospitals than in the community due most often to the presence of plasmids that promotes rapid development of resistant genes after frequent exposure to suboptimal antibio-

tics concentration [5]. Three characteristics define CPE as Emerging Antibiotic-Resistant Bacteria: emergence, multiresistance to antibiotics with plasmid transfer of resistance genes between species of commensal Enterobacteriaceae of the digestive tract and the risk of epidemic spread in the hospital environment, as well as community [6]. Thecarbapenemases that confer carbapenem resistance are made up of 3 different classes among the 4 classes of β-lactamases defined by the Ambler classification system: class A, class B (metallo-beta-lactamases) and class D (oxacillinases) [7]. Several outbreaks of carbapenemase-producing enterobacteriaceae strains have been reported worldwide, in Europe especially in the southern part of the continent (Italy, Spain, Greece) [8], USA [9], China [10] and Africa [11].

According to previous studies, the resistance to carbapenems is variable, always more pronounced in *Enterobacter cloacae* and *Klebsiella pneumonaie* than in *E. coli* or *Proteus mirabilis* [12] [13]. The etiological and antibiotic sensitivity profiles of those germs are likely to vary in space and time. In Cameroon, the epidemiological data concerning carbapenemase producing enterobacteria are scarce. However, a better understanding of the epidemiology of carbapenem resistant bacteria could help to always provide therapeutic alternatives and most interestingly to detect and foresee newly emergent strains or outbreak. The intended pilot study was to determine the phenotypic characterization of carbapenemase-producing *Enterobacteriaceae* strains in a referral teaching hospital in ya-oundé, Cameroon.

2. Material and Method

2.1. Study Design, Duration and Location

A cross-sectional descriptive and analytical study was carried out during a three-month period (from 27th July to 24th October 2018) in the University Teaching Hospital of Yaoundé, Cameroon.

2.2. Sampling Method and Study Population

During the study period, we performed a convenience sampling of all suspected Enterobacteria strains or Enterobacteria identified from routine bacteria culture of usual pathological products (stool, urine, blood, ascite, pus, …) by the bacteriology laboratory personnel using conventional identification system. These strains were systematically collected, labelled, conserved and included in the study for upcoming re-identification and analyses. Non-confirmed cases of *Enterobacteriaceae* and strains with lack of useful clinical informations were excluded from the study.

2.3. Conservation of Enterobacteria

This was performed according to the guide to bacteria preservation (https://opsdiagnostics.com/notes/ranpri/aguidetobacteriapreservation.htm). In all, each *Enterobacteria* isolated during the study period (from 27th July to 24th

October 2018) at the University Teaching Hospital of Yaoundé, Cameroon were systematically conserved in cryotubes containing 1.5 mL of brain-heart infusion broth supplemented with 10% glycerol, which acts as a cryoprotectant. The strains were then stored at −20˚C until their re-identification.

2.4. Samples Processing and Re-Identification

Subculture and isolation: collected and preserved strains were initially seeded on nutrient agar using the quadrant streaking method to obtain isolated colonies. Only pure cultures (those having only one type of colony) were considered for re-identification as previously reported [14].

Culture and Macroscopic identification: Giving that the colonies were large enough, we then have to seed one single colony from pure culture in an EMB agar. After growth, their morphology, diameter and colour were recorded following the description guide provided by the EMB manufacturer (Bio-RAD, France).

Biochemical identification: The oxidase test was carried out on target colonies thereafter and a bacterial suspension (0.5McFarland turbidity) of each target colony was directly seeded in API20E system (Biomerieux, France) for their re-identification based on the miniaturized biochemical tests [15].

Drug susceptibility testing: The Antibiotic susceptibility testing of each isolate was perfomed on Mueller Hinton agar by disk diffusion method according to the standard of the Antibiogram Committee of the French Society of Microbiology (CA-SFM 2018-v.2.0). In all, 18 discs of antibiotics from different antibiotics families including B-lactams (amoxicillin 20 µg, amoxicillin+clavlanic acid 10 µg, ticarcillin 75 µg, ticarcillin+clavulanic acid 20 µg, temocillin 30 µg, cefoxitin 30 µg, cefotaxim 5 µg, cefixim 30 µg, ceftazidim 10 µg, cefepim 30 µg, aztreonam 30 µg, ertapenem 10 µg, imipenem 10 µg, meropenem 10 µg), Aminoglycosides (gentamicin 10 µg), Fluoroquinolones (ciprofloxacin 5 µg, nalidixic acid 30 µg) and Cotrimoxazole were used. The detection of extended spectrum beta-lactamases (ESBL) was indicated by the formation of a champagne cork characterizing a synergy between a B-lactamase inhibitor and a third or fourth generation cephalosporin (30 mm apart). The probable plasmid AmpC hyperproduction was indicated by a reduced sensitivity to cefotaxim and/or cefixim and/or ceftazidim and/or aztreonam in the absence of synergy between these molecules and clavulanic acid.

Detection and classification of carbapenemases: Giving its high sensitivity to CPE, Ertapenem, a carbapenem was used as standard and any strain having a reduced sensitivity to ertapenem [an inhibition diameter (10 µg/ml disk) < 25 mm] during agar diffusion test was subjected to the screening algorithm of carbapenemase-producing enterobacteriaceae (**Figure 1**). According to the algorithm, the confirmation of carbapenemase production and the classification of suspected enterobacteria strains were performed by inhibitory synergy tests, in cluding Boronic acid and Chelating agent (EDTA) tests for class A and class B (Metallo-B-Lactamases) respectively.

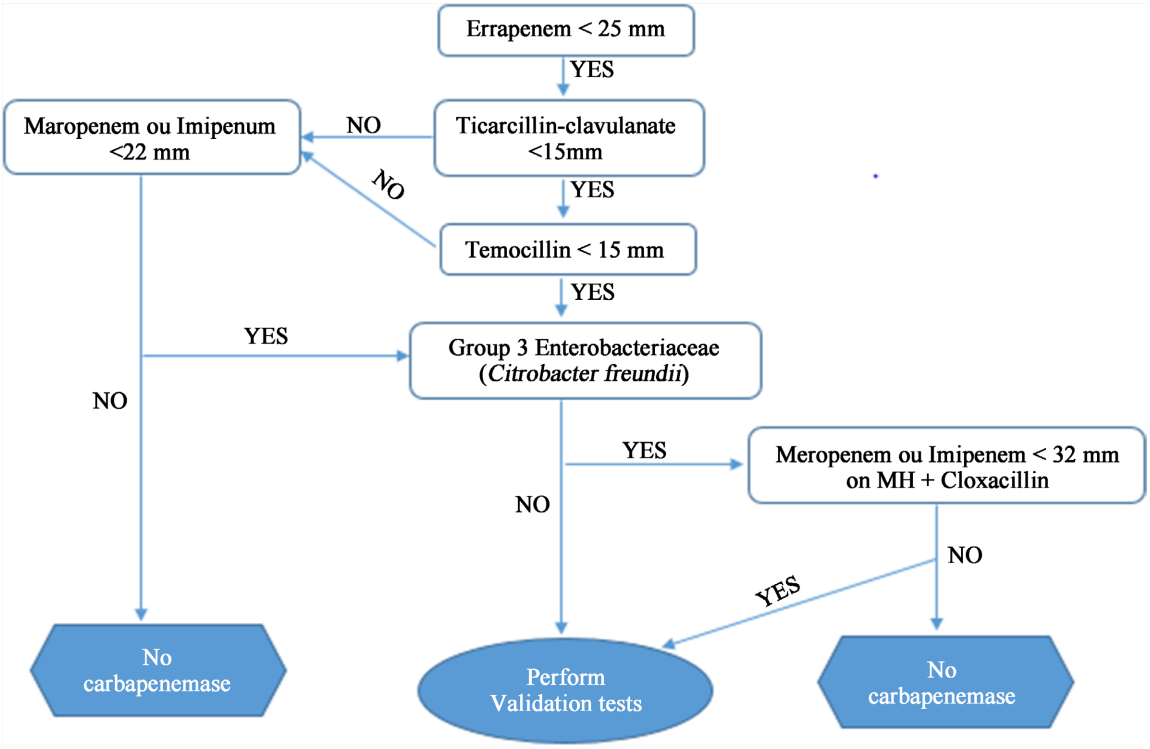


Figure 1. Algorithm for the Screening of carbapenemase-producing enterobacteriaceae (CPE) [16].

Determination of multiple antibiotic resistance (MAR) index: Multiple antibiotic resistance (MAR) index was determined for each isolate by using the formula MAR = a/b, where a represents the number of antibiotics to which the test isolate depicted resistance and b (18) represents the total number of antibiotics to which the test isolate has been evaluated for susceptibility [17].

2.5. Long Term Conservation of CPE Strains

Once the analysis was completed, each pure colony of CPE isolates were placed in cryotubes containing 1.5 mL of brain-heart infusion broth supplemented with 20% glycerol and then stored at −80˚C.

2.6. Data Evaluation and Analysis

The different variables and results obtained after verification of their compliance were recorded in Excel 2013 software, then analyzed with STATVIEW statistical software. The main analysis included the calculation of frequencies and their 95% confidence intervals (for the qualitative variables), and the mean or median (for quantitative variables). Univariate and multivariate analyzes by logistic regression made it possible to determine the factors influencing the occurrence of a carbapenemase production in enterobacteriaceae (P-value < 0.05).

2.7. Ethical Consideration

For this study, an ethical clearance was issued by the institutional research ethics committee of the Université des Montagnes (N˚2018/206/UdM/PR/CIE) and a

research authorization by the director of the University Teaching Hospital of Yaoundé (N˚207/AR/CHUY/DG/DGA/CAPRC) was also obtained.

3. Results

3.1. General Characteristics of the Origin of the *Enterobacteriaceae* Strains

Out of 104 *Enterobacteriaceae* strains, 67 (64.4%) were from female patients and 37 (35.6%) from male. The sex ratio M/F was about 1/2. The mean age of the study population was 39.23 ± 27.14 years (min: 6 days; max: 92 years). The most represented age group was [18 - 55] years old (47.1%) and those of more than 55 years (32.7%) (Table 1).

Besides, 35.6% (37/104) were community-based patients and 64.4% (67/104) were hospitalized. *Enterobacteriaceae* were from urine: 37.5% (39/104), blood: 24.0% (25/104), stools: 16.4% (17/104), vaginal swab: 13.5% (14/104) and pus: 8.7% (9/104) (Table 1).

Table 1. General characteristics of participants.

Characteristics	Total number (n)/104	Percentage (%)
Sex		
Female	67	64.4
Male	37	35.6
Sex ratio	0.55	
Age group		
≥55 years	34	32.7
[18 - 55 years]	49	47.1
[5 - 18 years]	3	2.9
[3 months - 5 years]	6	5.8
[0 - 3 months]	12	11.5
Mean age:	39.23 ± 27.14 years	
Hospitalization		
Yes	67	64.4
No	37	35.6
Specimen		
Urine	39	37.5
Blood	25	24.0
Stools	17	16.4
Vaginal swab	14	13.5
Pus	9	8.7

3.2. Frequency of Different *Enterobacteria* Species

Figure 2 shows that three different microorganisms were identified, of which *Escherichia coli* (50%) was the most prevalent followed by *Klebsiella pneumoniae* (37.5%) and *Citrobacter freundii* (12.5%).

3.3. Assessment of Susceptibility of *Enterobacteriaceae* Isolates to Beta-Lactam Antibiotics and Others Families of Antibiotics

The susceptibility profile of *Enterobacteriaceae* isolates to different families of antibiotics is shown on Figure 3 and Figure 4.

Among the 14 antibiotics from 4 different families tested in this study, 97.4% of isolates were resistance to amoxicillin, 48.6% to amoxicillin clavulanate, 68.4% to cefotaxim, 58.1% to cefixim, 60.7% to ceftazidim, 57.1% of cefoxitin and 5.7% to aztreonam. However, 11.9% related resistance was found to carbapenems: 14.4% to imipenem, 13.8% to ertapenem and 7.5% to meropenem (Figure 3).

Figure 4 shows the resistance to aminoglycosides (gentamicin: 49.6%),

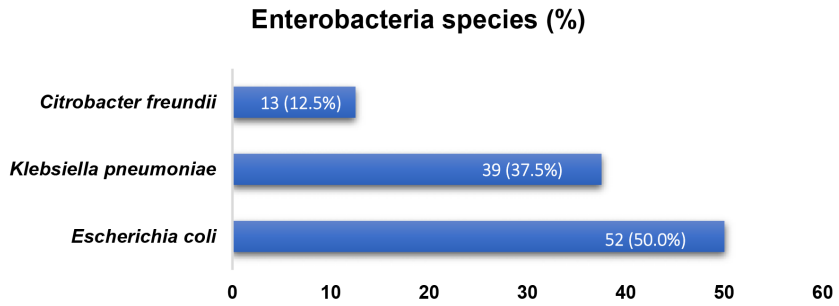


Figure 2. Frequency of different Enterobacteria species.

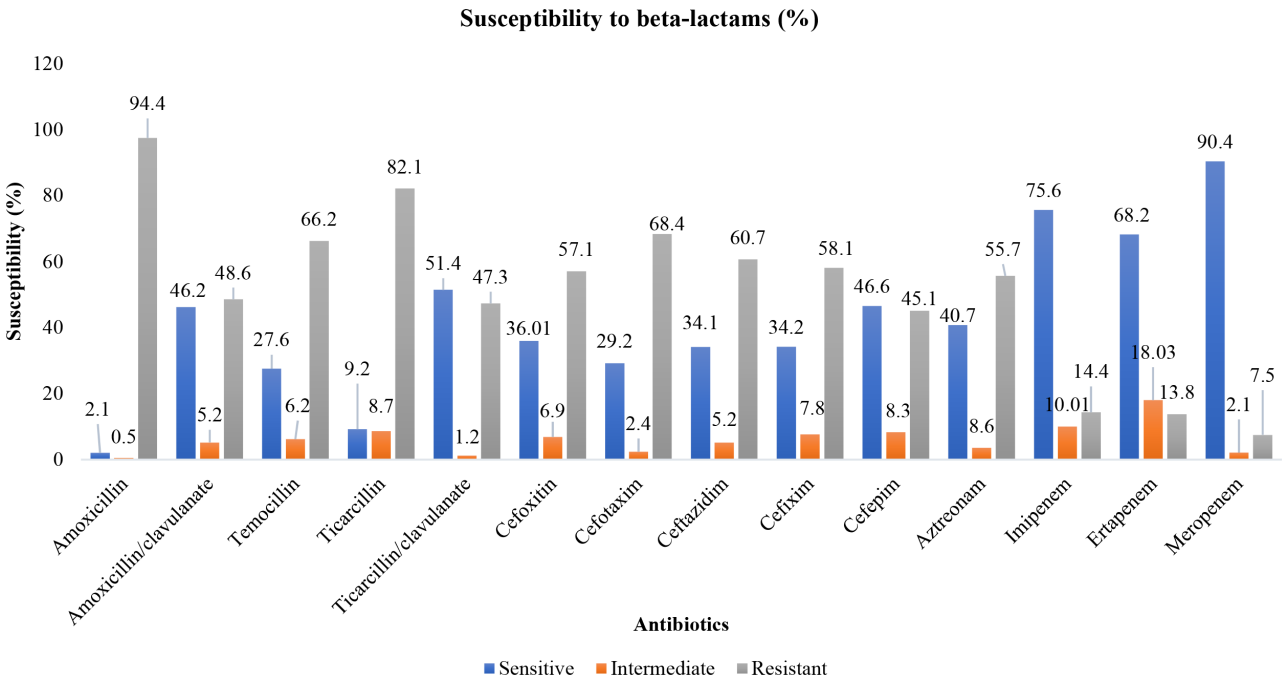


Figure 3. Beta-lactam antibiotics susceptibility profile.

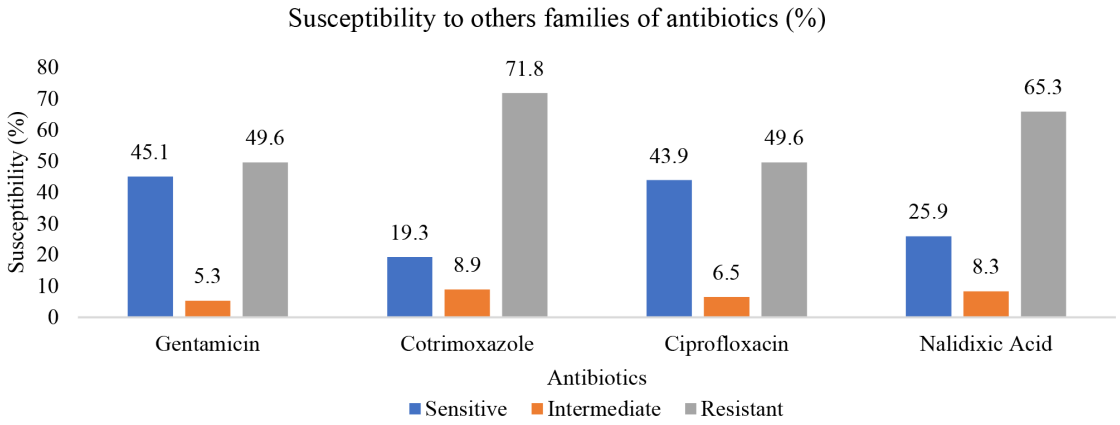


Figure 4. Susceptibility profile to others antibiotics families.

Table 2. MAR indices of *Enterobacteria* strains.

MAR index	Number of isolates/104	Percentage
00	13	12.5%
0.1	35	37.7%
0.2	20	19.2%
0.3	14	13.5%
0.5	12	11.5%
0.8	10	9.6%

first-generation quinolone (nalidixic acid: 65.8%), second-generation fluoroquinolone (ciprofloxacin: 49.6%) and co-trimoxazole (71.8%).

3.4. MAR Indices of the *Enterobacteria* Strains

In Table 2, MAR indices for different isolates of enterobacteriaceae revealed 68 (69.4%) with a MAR index less than or equal to 0.2 and 36 (34.6%) greater than 0.2.

3.5. Assessment of Extended-Spectrum Beta-Lactamases (ESBL) and AmpC Production and Resistance Profile of *Enterobacteriaceae* Producing ESBL (E-ESBL) to Aminoglycoside, Quinolone, Fluroquinolone and Cotrimoxazole

Of the 104 strains, 36.5% (38/104) were probably producing ESBL and 23.1% (24/104) were producing AmpC.

From Figure 5, the resistance rate of E-ESBL was: 56.6% resistance to gentamicin (aminoglycoside), 57.2% to ciprofloxacin (quinolones) and 62.7% to nalidixic acid (quinolone) and 88.8% to cotrimoxazole.

3.6. Prevalence of Carbapenemase-Producing *Enterobacteriaceae* (CPE) and Classification of Carbapenemases

As all isolates with a reduced sensitivity to ertapenem were submitted to the al-

gorithm for the screening of carbapenemase-producing enterobacteriaceae, 25% (26/104) were cabapenemase productive of class A: 80.8% (21/26), followed by class B: 19.2% (5/26) (Figure 6).

3.7. CPE According to Species

Table 3 shows that, of the 26 CPE obtained, the most represented species was Klebsiella pneumoniae: 65.4% (17/26), followed by Escherichia coli: 7/26 (26.9%) and Citrobacter freundii: 2/26 (7.7%).

3.8. Risk Factors Associated to CPE

Logistic regression showed that the following factors: *Klebsiella spp* [p-value:

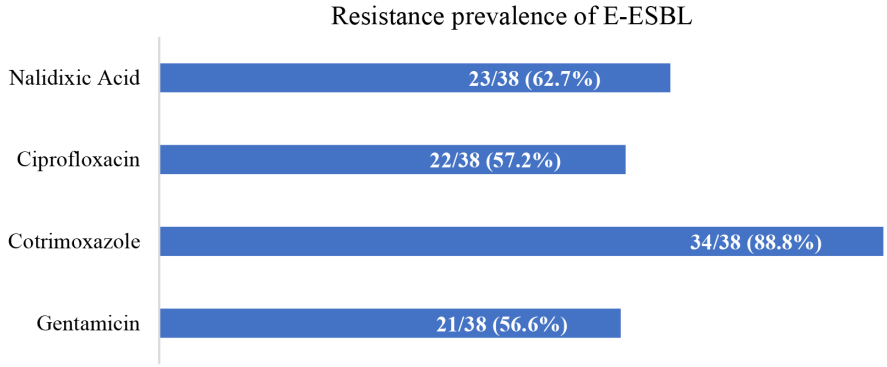


Figure 5. Resistance profile of E-ESBL to aminoglycosides, quinolones, fluroquinolones and cotrimoxazoles.

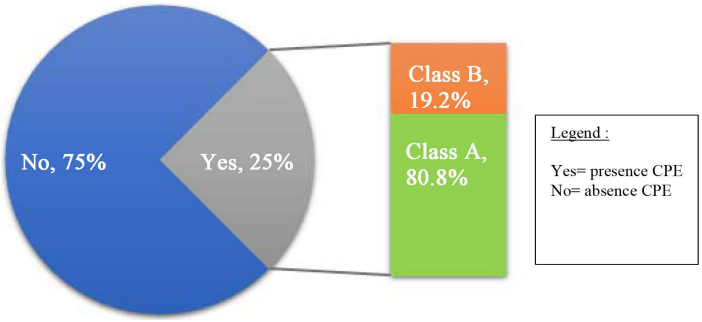


Figure 6. Prevalence and breakdown of carbapenemase classes.

Table 3. CPE according to species.

	Carbapenemases production (n = 26)		
	Class A	Class B (MBL)	n (%)
Escherichia coli (n = 7)	3 (42.8%)	4 (57.1%)	7 (26.9%)
Klebsiella pneumoniae (n = 17)	16 (94.1%)	1 (5.9%)	17 (65.4%)
Citrobacter freundii (n = 2)	2 (100%)	0 (0%)	2 (7.7%)
n (%)	21 (80.8%)	5 (19.2%)	26 (100%)

MBL = metallo-betalactamase.

0.04, OR: 4.87 (1.07 - 22.1)] and AmpC-production [p-value: 0.02, OR: 8.86 (1.7 - 17.9)] were still significantly associated to the production of carbapenemases (Table 4, Table 5).

Table 4. Univariate analysis of risk factors associated to CPE.

Variables	Carbapenemase production		OR brute	IC 95% OR	p-value
	No	Yes			
Sex					
F	52 (77.61%)	15 (22.39%)	1	Ref	
M	26 (70.27%)	11(29.73%)	1.46	[0.36; 9.59]	0.47
Age (year)					
Middle age ± SD	40.83 ans ± 5.66	33.91 ans ± 10.80	0.99	[0.97; 1.01]	0.33
Hospitalization					
No	28 (75.68%)	9 (24.32%)	1	Ref	
Yes	50 (74.63%)	17 (25.37%)	1.09	[0.57; 3.65]	0.44
Microorganisms					
C. freundii	11 (84.62%)	2 (15.38%)	1	Ref	
K. pneumoniae	22 (56.4%)	17 (43.6%)	3.82	[3.15; 7.80]	0.01
E. coli	45 (86.54%)	7 (13.46%)	0.86	[0.12; 3.65]	0.63
AmpC-production					
No	66 (82.50%)	14 (17.50%)	1	Ref	
Yes	12 (50.00%)	12 (50.00%)	4.71	[3.92; 5.05]	0.001
ESBL-production					
No	53 (80.30%)	13 (19.70%)	1	Ref	
Yes	25 (65.79%)	13 (34.21%)	2.12	[1.82; 3.65]	0.01

P-value is significant if < 0.05.

Table 5. Multivariate analysis of risk factors associated to CPE.

Independent variables	Adjusted OR	CI 95% OR	P-value
AmpC-Production			
No	1	Ref	
Yes	8.86	[1.67; 17.92]	0.02
Microorganism			
Citrobacter freundii	1	Ref	
Escherichia coli	0.2	[0.03; 1.31]	0.09
Klebsiella spp	4.87	[1.07; 22.11]	0.04
ESBL-Production			
No	1	Ref	
Yes	0.96	[0.19; 2.55]	0.58

P-value is significant if < 0.05.

4. Discussion

To contribute to the fight against multi-drug resistant bacteria, a cross-sectional and analytical study was carried out in the Yaoundé Teaching Hospital, Cameroon. The aim of this study was to determine the prevalence of CPE and the associated risk factors.

Female were the most represented (64.4%) and the strains were most frequently isolated from the urine (37.5%). Similar results have been found in Iran in 2020 (more of the isolates were cultured from urine: 80.65%) [18]. This predominance of women was probably due to urinary tract infection as shown earlier in a cross-sectional study in Douala [19] and likely related to their anatomy: shortness of the urethra, proximity of orifices such as anus and vagina; inadequate hygiene practices or pregnancy.

E. coli was the most prevalent bacteria specie (50%) followed by *Klebsiella pneumoniae* (37.50%). Similar results has been also reported in 2015 in Cameroon where the most represented species was *E. coli* (48.5%) followed by *Klebsiella pneumoniae* (32.8%) [20]. This result could be explained by the predominance of urine specimen and *E. coli* is known to be the most incriminated specie in urinary tract infections [21].

A high resistance to β-lactams especially penicillins and cephalosporins was shown in this study. However, about 11.9% related resistance was found to carbapenems. A study conducted in Alger some years ago in 2011 found a lower resistance to β-lactam [22]. Resistance of Enterobacteriaceae to β-lactams is probably increasing worldwide and in particularly Africa. In fact, penicillins are nowadays sold abusively and third-generation cephalosporins are massively used as treatment in humans and animals. Moreover, due to poverty in Africa, people choose auto-medication rather than undergoing normal consultation in hospital and also, get their medications at lower cost along the street rather than in the pharmacy.

The co-resistances of these strains were also studied and a high co-resistances to aminoglycosides (49.6%), quinolones (65.8%), second-generation fluoroquinolones (49.6%) and sulfamides (71.8%) was found. Those results are similar to those reported earlier in the country [23]. Equally in 2015 in Algeria, Lagha *et al* experienced a 54% resistance to co-trimoxazole [24], though lower compared to that (71.8%) in our study. This difference could reflect that *Enterobacteria* resistance mecanisms are dynamics. Most of enterobacteria are not only resistant to the majority of β-lactams but also to many antibiotics of other families such as fluoroquinolones, aminoglycosides, cotrimoxazole and quinolones [20]. This multi-resistance could result either from over-consumption of antibiotics or from the natural resistance to other antibiotics, often associated with genes encoding for carbapenemases [25]. Equally, the lack of hygiene and the prosmicuity of beds are factors that exacerbate the transmission of antibiotic-resistant strains in hospitals.

Of the 104 Enterobacteriaceae strains, 25% were CPE (with 80.8% carbapenem

resistant class A, 19.2% class B). *K. pneumoniae w*as the most predominant CPE, followed by *E. coli* and *Citrobacter freundii*. In fact, in 2015, the National Agency for Drug and Health Product Safety revealed that *Klebsiella pneumoniae* were the most concerned species of Enteriobactericaeae-producing Carbapenemases (EPCs), while *E. coli* strains rarely hosts carbapenemase genes [25]. These results differ from those obtained in a study conducted in Cameroon in 2011 [26], where out of 58 strains of carbapenemase-producing enterobacteria, 33% were class A, 27% class B and 31% class C and D. This difference is probably due to the larger samples size from this study.

The research and classification of carbapenemases during our study were performed by the synergy tests with inhibitors of carbapenemases (EDTA, Boronic acid). We obtained 80.8% (21/26) carbapenem resistant of class A and 19.2% (5/26) of class B. The most common carbapenemases found in enterobacteriaceae are KPC (class A), NDM (class B) and OXA-48 (class D) and the most common species involved is *K. pneumoniae*, which is the major reservoir of these enzymes and the leading cause of both community and healthcare associated infections. The capsule, as a critical virulence factor in this organism, plays an important role in its pathogenesis and avoids phagocytosis [27]. The main KPC reservoirs are *K. pneumoniae* in the United States, Israel and Greece, NDM's and *E. coli* in India, and those of OXA-48 are *K. pneumoniae* and *E. coli* in North Africa and Turkey [28].

The production of ESBL/carbapenemases predominantly by nosocomial infections related strains constitute the main enzymatic mechanisms evolved to escape antibiotic treatment [24]. In these favorable hospital environments, numerous conditions such as the abusive consumption of broad-spectrum antibiotics, the weakness of patients' immune system or the manual handling by the nursing staff, are factors enhancing the emergence of multidrug resistant strains [29].

In a multivariate logistic regression analysis, *Klebsiella pneumoniae* and AmpC-beta lactamase production were selected as risk factors for the production of carbapenemases in Enterobacteriaceae. In Tunisia, Sallem *et al* showed wide variation in the distribution of ESBLs, AmpC, carbapenemase and other plasmid-mediated resistance determinants. Isolates of *Klebsiella pneumoniae* producing carbapenemase activity carried variants of the blaNDM-1 (n = 11), blaOXA-48 (n = 11), blaNDM-1 + blaOXA-48 (n = 1), blaNDM-1 + blaVIM-1 (n = 1), blaOXA-204 (n = 1), as well as variants of the blaCTX-M, blaOXA, blaTEM, blaCMY, blaDHA and blaSHV genes on conjugative plasmid [30]. This could be explained by the fact that, *Klebsiella* easily host carbapenemases genes. Increasing detection of carbapenemase producing *Klebsiella pneumoniae,* including class A (KPC), class B (IMP, VIM AND NDM) and class D (OXA-48-like enzymes) has led to international concern, as they are carried on transposable elements in association with other resistance determinants, such as Extended spectrum β-lactamases (ESBLs), AmpC cephalosporinases and 16S Rrna Methyltransferases [31].

5. Limitation of the Study

A limitation of this study was the absence of the molecular characterisation of CPE due to lack of funding. This aspect makes it possible later on to confirm the phenotypes obtained in the current study by molecular analyses and deepen the investigations for a complete assessment of resistance related genes.

6. Conclusion

This study revealed a high production of carbapenemases in *Klebsiella pneumoniae* among enterobacteria. Those strains were resistant to almost all β – lactams but and groups of antibiotics such as aminoglycosides, quinolones, and cotrimoxazole. The multi-resistance of Enterobacteriaceae to antibiotics in Cameroon has considerably increased. More attention should be paid to those bacteria to stall antimicrobial resistance spread across the country.

Consent for Publication

All authors consented for publication.

Availability of Data and Material

All data generated or analysed in the course of this study are included in this manuscript.

Authors' Contributions

CID conceived the project and designed the study. CID and PDDD searched relevant literature, scrutinized all relevant information and draft the manuscript. CID, RKW and BDTP conducted and coordinated the field study. PDDD, CNT, CPK, MCT, VMN, BKM, HKT and CDSN collected and processed the samples and data. PDDD and RKW analyzed the data and wrote the article. CID, NB, HGK and BDTP revised the manuscript. All authors read and approved the final manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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