Chapter

Phenotypic Characterisation of Carbapenemases Produced by Enterobacteria Isolated from Patients of the Medico-Social Centre of the National Social Insurance Fund of Maroua: Cameroon

Daoudou Bakari, Jojo Mbala Ekamba, Mamoudou Hamadou, Yaya Garga, Martin-Paul Baane and Félicité Obono

Abstract

The aim of this study is to determine the types of carbapenemases moving around the city of Maroua with a view to contribute to the development of a control strategy against the enterobacteria that produce them. The investigation carried out on the biological samples showed that 5.97% of the sample contained carbapenem-resistant microorganisms. This includes 2.20% of urine samples, 0.94% of osteitis samples, 0.63% of wound pus samples, 1.26% of stool samples and 0.94% of blood samples. The microorganisms responsible for this resistance to carbapenems are 5.26% for each of species *Arizona*, *Citrobacter braakii*, *Enterobacter gergoviae*, *P. vulgaris*, and Serratia ficaria, 26.32% for the species *E. gergoviae* and *P. mirabilis* and 21.05% for the species *S. odorifera* 1. All these enterobacteria produce at least one carbapenemase, which 36.84% are of the KPC type, 10.53% of the OXA-48 or OXA-181 type and 52.63% of types that could not be determined by the algorithm proposed by Nordmann et al. used for this purpose. The types of carbapenemases determined in this revealed 11 substrates and inhibition profiles associated with their production. This highlighted the difficulty of applying an inhibition law in situ in the context of probabilistic antibiotic therapy.

Keywords: carbapenemases, enterobacteriaceae, substrate profile

1. Introduction

The increasing complexity of the phenomenon of resistance of enterobacteria against beta-lactamines in Cameroon will be a major public health problem if

nothing is done. The study of the enzymatic systems involved in inducing these resistances in the cities of Yaoundé, Ngaoundéré and Douala has shown evolutions in space and time [1–3]. In the city of Maroua, the alarm was raised with the identification of multi-resistant microorganisms in dairy products and raw meat sold in the city [4, 5]. These resistances have been attributed to the production of high-level cephalosporinases and b-lactamases including Carbapenemases [5, 6]. The concern in relation to these observations is that, microorganisms with similar profiles have started to be isolated from biological samples at the National Social Insurance Fund (MSC-NSIF) of Maroua. Moreover, cases of death were noted due to infections by this category of Enterobacteriaceae before the end of the analysis of the samples, although routine antibiotics were administered (MSC-NSIF patient files). The emergence of carbapenemase-producing Enterobacteriaceae (EPC) in the city of Maroua could in the future, become the main factor of therapeutic failures. In a socio-economic context where probably antibiotic therapy remains the most widely used strategy, knowledge of the enzyme systems involved in these enterobacteria could make patient management more effective. The objective of this study is to determine the types of carbapenemases moving around in the city of Maroua with a view to developing a strategy control to fight against the enterobacteria that produce them.

2. Materials and methods

2.1 Study site

The study took place at the MCS-NSIF (Medico-Social Centre of National Social Insurance Fund of Maroua) in Maroua, which is located between 10°35'North latitude and 14°19′East longitude [7]. The MCS-NSIF is one of the hospitals in the distric of Maroua I first. It is adjacent to the road that connects the *'Djarma'* crossroads to the third and northernmost entrance of the SODECOTON company. This hospital receives among its patients, those who clinical examinations require the realisation of the antibiogram.

The requirement of an antibiogram by the clinician was the criteria retained for the choice of the samples to be analysed. In compliance with this requirement, a sample of 318 biological samples was taken from patients received at the MCS-NSIF laboratory in Maroua. This sample consisted of 123 urine samples, 71 vaginal samples, 70 stool samples, 27 blood samples, 13 osteitis pus samples, 9 urethral samples and 5 wound pus samples. The material collection were done between the month of january and febuary in 2018.

2.2 Isolation, purification and selection of resistant carbapenem strains

2.2.1 Isolation of enterobacterial strains

The plating technique carried out near a flame maintained by a Bunsen burner was used to isolate the strains of interest [8]. Biological material that remained attached to the sterile loop handle was streaked onto MacConkey agar in a Petri dish. For the microorganisms to be isolated from the blood, a pre-culture in bovine heart-brain infusion incubated at 37°C for 18 hours in an oven preceded the implementation of the technique.

2.2.2 Purification of enterobacterial strains

The quadrant method was used to purify the strains of interest [8]. One of the colonies from among those having the same appearance during the isolation phase was used for this purpose. The first quadrant was formed by inoculation in tight streaks using a sterile loop. The loop used at this stage of the operation is flamed to red and cooled by touching an unused area of the agar. The Petri dish is rotated at an angle of 90° and the loop is passed once through the first quadrant to form the second quadrant. The same procedure is used to form the other two quadrants.

2.2.3 Selection of strains of interest

The standardised method for determining the susceptibility of bacteria to antibiotics using ertapenem 10 μg, imipenem 10 μg and meropenem 10 μg was used to select the strains of interest [9]. First, a suspension containing 10^6 CFU/mL of bacteria for each of the purified strains was prepared. Swabbing for each of the prepared suspensions was performed on Müller-Hinton (MH) contained in a petri dish. The carbapenem discs were placed at a distance of 3 cm from each other in each of the seeded Petri dishes. All prepared Petri dishes were incubated at 37°C for 18 hours in an oven. It should be noted that the disc quality test was validated on E. coli ATCC 29522 reference strains classified as susceptible. The determination of resistant, intermediate or susceptible traits was based on the comparison between the inhibition diameters obtained and those of the EUCAST reference [9].

2.3 Determination of the enzymatic character of carbapenem resistance

The Carba NP test which is a biochemical colorimetric test was used to demonstrate the enzymatic activity of carbapenem resistance in the strains of interest [10]. A 100 L volume of Tris–HCl B-PER II (Bacterial Protein Extraction Reagent) lysis buffer, 20 mM, pH 7.5 and one colony of bacteria were introduced into each of two 1.5 mL Eppendorf tubes prepared for each strain to be tested. The resulting mixture was homogenised using a 1000 L micropipette. Subsequently, 100 L of solution (A) containing 0.54% (W/V) phenol red and 0.2 mM zinc sulphate was introduced into control tube 1. The same volume of solution (A), this time containing concentrated carbapenem 6 mg/mL, was introduced into test tube 2. Both tubes were incubated at 37°C in the incubator for 2 hours. The appearance of a yellow coloration was interpreted as positive and therefore the presence of carbapenemase, whereas the red coloration was interpreted as negative and therefore the absence of carbapenemase.

2.4 Determination of carbapenemase classes produced by the strains of interest

The classes of carbapenemases produced by the Enterobacteriaceae were determined using phenotypic inhibition and synergy tests [9]. After swabbing on MH, the antibiotics were arranged with a distance of 3 cm between them. These were imipenem (IMP), ertapenem (ETP), meropenem (MRP), amoxicillin (AMX), cefotaxime (CTX), ceftazidime (CAZ), EDTA, clavulanic acid (CMA), cloxacillin (CXC), cefepime (CFP), piperacillin-tazobactam (PIT) and aztreonam (AZT). The elements used in the algorithm to identify the types of carbapenemases produced by the strains of interest were arranged as follows (**Figure 1**):

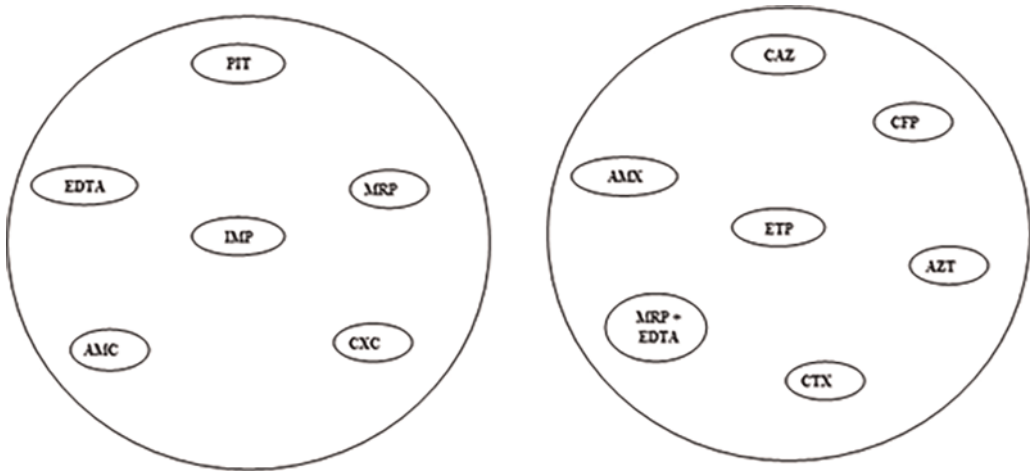

Figure 1.
Layout of the elements of the carbapenemase class identification algorithm.

CLASS	TYPE	AMX	AMC	PIT	CTX	CAZ	IMP	ETP	MRP	AZT
A	KPC	R	S/I	R	R	R	S/I/R	I/R	S/I/R	R
	KPC + BLSE	R	I/R	R	R	R	I/R	I/R	I/R	R
B	IMP/VIM/NDM	R	R	I/R	R	I/R	S/I/R	I/R	S/I/R	S
	IMP/VIM/NDM + BLSE	R	R	I/R	R	R	I/R	R	S/I/R	R
D	OXA-48/OXA-181	R	R	S/I/R	S/I	S	S/I	S/I	S/I	S
	OXA-48/OXA-181 + BLSE	R	R	I/R	R	R	I/R	I/R	I/R	R

Legend: R = Resistant I = Intermediate S = Sensitive [11].

Table 1.
Resistance phenotypes resulting from the expression of carbapenemases reported in Enterobacteriaceae without or with extended-spectrum lactamases.

The classes of carbapenemases produced by the isolated Enterobacteriaceae were determined from the algorithm (**Table 1**).

2.5 Determination of minimum inhibitory concentrations of carbapenems

The E-test, an agar diffusion technique, was used to determine the minimum inhibitory concentrations (MICs) [12]. The commercially available strip was adapted with easily accessible blotting paper. This blotting paper, cut to the size of 10 cm x 1 cm, was divided into 10 zones of equal size (widthwise) by lines obtained with a pencil. This paper was sterilised in an autoclave at 125°C for 15 minutes. To determine the MICs of the ETP, 640 g of antibiotic was introduced into a sterile 10 mL volumetric flask. This mass was dissolved in 5 mL of sterile distilled water measured with a pipette. After complete dissolution, the volume was made up to the mark to obtain a concentrated solution C1 64 mg/L. Solution C1/2 was obtained by removing 1 mL of solution C1 and adding it to a tube containing the same volume of sterile distilled water. Concentrated solution C1/2n was obtained by pipetting 1 mL of the prepared concentrated solution C1/n into 1 mL of distilled water. Once the 10 dilutions had

been obtained, 25 L was taken from each tube using a 50 L micropipette and arranged along with the blotting paper in the corresponding zones respecting the gradient (C1, C1/2, C1/4, C1/8, C1/16, C1/32, C1/64, C1/128, C1/256, C1/512). The same procedure was adopted for the determination of MICs for MRP and IMP.

2.6 Identification of carbapenemase-producing strains

The identification of carbapenemase-producing strains of Enterobacteriaceae has followed a three-stage procedure [13].

2.6.1 Orientation of the diagnosis by observing particularly discriminating features of the Enterobacteriaceae

Characteristics such as pigmentation and mucoid of colonies, invasion of solid media by colonies, appearance of small colonies were observed directly on the culture medium after incubation at 37°C for 24 h. For mobility, 20 L of a suspension from a colony dissolved in 1 mL of peptone water and incubated at 37°C for 30 minutes was placed on a slide using a 50 L micropipette and covered with a coverslip. This mount was viewed under the 40X objective of the microscope to observe the movement of the bacteria. Suspicion of enterobacteria was made when the bacteria were either immobile or showed peritrich-like mobility.

For Gram staining, a colony from the culture medium was placed in a thin layer on the slide using a platinum loop. The layer formed was fixed by the flame maintained by the Bunsen burner. This layer was covered with gentian violet for 45 seconds, rinsed with water and then covered again with Lugol's. This Lugol's was cleaned after 45 seconds with 95° alcohol in a wash bottle and then rinsed with water. The washed slide was then covered with Fuchsin for 45 seconds, rinsed again with water, dried and read under a 100X microscope objective. The presence of an enterobacterium was confirmed if a Gram-negative bacillus was observed with bipolar staining.

2.6.2 Revelation of the biochemical characteristics that characterise their metabolism

The biochemical characteristics of the metabolism of the enterobacteria retained after diagnostic orientation were obtained using the API 20E gallery. Firstly, the tubes were moistened by introducing 10 mL of sterile distilled water. A bacterial suspension for each species was prepared by diluting the colonies from MH in 5 mL of sterile distilled water. Each tube in the gallery was inoculated with the corresponding suspension using a sterile Pasteur pipette. They were filled by pressing the Pasteur pipette inwards and to the side to avoid bubbles. The wells for citrate (CIT), Voges Prauskauer (VP), gelatinase (GEL) traits were filled completely (tube and cup) for aerobic conditions. For the Arginine dehydrogenase (ADH), Lysine decarboxylase (LDC), Ornitine decarboxylase (ODC), Hydrogen sulphide (H2S) and Urease (URE) wells, the filling was done only at the level of the tube and the well was filled with paraffin oil to create anaerobic conditions. The whole set was incubated at 37°C in the incubator for 22 hours and then a drop of developer was introduced in some wells. These were FeCl3 in the Tryptophan deaminase (TDA) well, Kovacs reagent in the Indole (IND) well, —naphthol and NaOH in the VP well and Nit1, Nit 2 in the BNit well. The staining obtained in each well provided guidance on the positivity or negativity of the reaction.

2.6.3 Identification

The result of the reactions obtained in each well is fed into the Enterobacteriaceae identification software which displays the species of Enterobacteriaceae responsible for the biochemical properties obtained in the API 20 E gallery wells.

2.7 Data analysis

The data obtained were analysed using SPSS 20 and API 20 E Enterobacteriaceae identification software. The SPSS 20 software was used to convert the experimental results into percentages. This software was also used to calculate Pearson's correlation values between inhibition diameters and carbapenem MICs. The second software was used to determine the species of enterobacteria from the results obtained from the API 20 E gallery.

3. Results and discussion

3.1 Results

3.1.1 Identification of microorganisms and mechanism of resistance to carbapenems

The proportion of biological samples containing carbapenem-resistant microorganisms was 5.97%. This proportion is distributed between urine samples, which represent 2.20%, osteitis pus 0.94%, wound pus 0.63%, stool 1.26% and blood 0.94%. The presence of carbapenem-resistant microorganisms was not observed in urethral and vaginal swabs. The percentage of samples that did not contain carbapenem-resistant microorganisms was 94.03%. This frequency was distributed among urine samples 36.48%, osteitis pus 0.63%, wound pus 3.46%, urethral 2.83%, vaginal 22.33%, stool 20.75% and blood 7.55% (**Table 2**).

The species of enterobacteria responsible for carbapenem resistance in biological samples are variously distributed. Urine samples contain 36.84% of carbapenem-resistant microorganisms. This percentage is distributed between the species

Biological samples	**Frequency (%)**		**Totals**
	Containing resistant carbapenem enterobacteria	**Not containing resistant carbapenem enterobacteria**	
Urine	2.20	36.48	38.68
Pus from osteitis	0.94	0.63	1.57
Pus from wounds	0.63	3.46	4.09
Urethra	0.00	2.83	2.83
Vaginal	0.00	22.33	22.33
Stool	1.26	20.75	22.01
Blood	0.94	7.55	8.49
Totals	**5.97**	**94.03**	**100.00**

Table 2.
Frequency of biological samples with and without carbapenem-resistant Enterobacteriaceae.

Enterobacter gergoviae 10.53%, Enterobacter asburiae 5.26%, Proteus mirabilis 5.26%, Proteus vulgaris 5.26%, Serratia ficaria 5.26% and Serratia odorifera 1 5.26%. The proportion of 21.05% of carbapenem-resistant microorganisms was obtained in osteitis pus. This proportion is represented by the microorganisms *Arizona* 5.26%, *P. mirabilis* 10.53% and *S. odorifera* 1 21.05%. Carbapenem-resistant microorganisms identified in wound pus samples account for 10.53%. These were *E. gergoviae* 5.26% and *P. mirabilis* 5.26%. In blood samples, the proportion of microorganisms of interest is 10.53%. It is represented by *E. gergoviae* 5.26% and *S. odorifera* 1 5.26%. Carbapenem-resistant microorganisms in stool samples represent a proportion of 21.05%. These are Citrobacter braakii 5.26%, *E. gergoviae* 5.26%, *P. mirabilis* 5.26% and *S. odorifera* 1.5.26% (**Table 3**).

3.1.2 Phenotypes of identified carbapenemases

3.1.2.1 Dissemination of identified carbapenemases among enterobacteria

The carbapenemases circulating in the city of Maroua are of several types and in different proportions. The KPC type which represents 36.84% of identified carbapenemases is produced at 5.26% by each of the species *Arizona*, *E. asburiae, P. mirabilis, P. vulgaris, S. ficaria* and at 10.53% by the species *E. gergoviae*. Type OXA-48 or OXA 181 represents 10.53% of all these carbapenemases. The species *P. mirabilis* and *S. odorifera 1* each contributes 5.26% of the production. Eight types of carbapenemases produced by enterobacteria do not fit into the reference algorithm. These are non-determined types (NDPs) 1, 2, 3, 4, 5, 6, 7 and 8. NDE 1 represents 10.53% of all carbapenemases and is produced by *E. gergoviae* and *S. odorifera 1*, which each contributes 5.26%. TND 2, which accounts for 10.53%, is produced by the microorganism *P. mirabilis*. TND 3 produced by a single microorganism, *S. odorifera* 1, is present at 5.26%. TND 4, produced by the microorganism *P. mirabilis* only, occupies

Biological samples	**Enterobacteriaceae species**								
	Arizona	***Citrobacter braakii***	***Enterobacter gergoviae***	***Enterobacter asburiae***	***P. vulgaris***	***P. mirabilis***	***Serratia ficaria***	***S. odorifera 1***	**Totals**
Urine	0.00	0.00	10.53	5.26	5.26	5.26	5.26	5.26	**36.84**
Pus from osteitis	5.26	0.00	0.00	0.00	0.00	10.53	0.00	5.26	**21.05**
Pus from wounds	0.00	0.00	5.26	0.00	0.00	5.26	0.00	0.00	**10.53**
Urethra	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**0.00**
Vaginal	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**0.00**
Blood	0.00	0.00	5.26	0.00	0.00	0.00	0.00	5.26	**10.53**
Stool	0.00	5.26	5.26	0.00	0.00	5.26	0.00	5.26	**21.05**
Totals	**5.26**	**5.26**	**26.32**	**5.26**	**5.26**	**26.32**	**5.26**	**21.05**	**100.00**

Table 3.
Frequency of Enterobacteriaceae species identified in the biological samples taken.

Enterobacteria species	Carbapenemase types										Totals
	KPC	OXA-48 ou OXA 181	Undetermined 1	Undetermined 2	Undetermined 3	Undetermined 4	Undetermined 5	Undetermined 6	Undetermined 7	Undetermined 8	
Arizona	5.26	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**5.26**
Citrobacter braakii	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.26	0.00	0.00	**5.26**
Enterobacter gergoviae	10.53	0.00	5.26	0.00	0.00	0.00	0.00	0.00	5.26	5.26	**26.32**
Enterobacter asburiae	5.26	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**5.26**
P. mirabilis	5.26	5.26	0.00	10.53	0.00	5.26	0.00	0.00	0.00	0.00	**26.32**
P. vulgaris	5.26	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**5.26**
Serratia ficaria	5.26	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**5.26**
S. odorifera 1	0.00	5.26	5.26	0.00	5.26	0.00	5.26	0.00	0.00	0.00	**21.05**
Totals	**36.84**	**10.53**	**10.53**	**10.53**	**5.26**	**5.26**	**5.26**	**5.26**	**5.26**	**5.26**	**100.00**

Table 4.
Dissemination of circulating carbapenemase types between the identified enterobacteria species.

5.26%. The TND 5, 6, 7, 8 each represents 5.26% produced respectively by the micro-organisms *S. odorifera* 1, *C. braakii*, and *E. gergoviae* for the last two types (**Table 4**).

3.1.2.2 Distribution of carbapenemase types in biological samples

The carbapenemases circulating in the city of Maroua are differently distributed in biological samples. The KPC type was found in 21.05% of urine samples, 5.26% of osteitis pus samples and 10.53% of wound pus samples. For type OXA-48 or OXA-181, 5.26% is present in osteitis pus and 5.26% in blood samples. TND 1 is only found in urine samples at a proportion of 10.53%. TND 2 was present in 5.26% of urine samples and in the same proportion of osteitis pus samples. TND 3 was present in 5.26% of the osteitis pus samples only. TND 4, 5, 6, 7 are only found in stool samples and represent 5.26% each. TND 8 is only found in blood samples and represents 5.26% (**Table 5**).

3.1.3 Substrate and inhibitor profiles

The results of the Carba NP test showed that all the Enterobacteriaceae identified in the biological samples use an enzymatic mechanism as a means of resistance to carbapenems. On the other hand, the study of the substrate and inhibitor profiles highlighted three cases, namely enzymatic activity implying resistance (R), decreased enzymatic activity leading to intermediate resistance (I) and a complete absence of enzyme activity implying sensitivity (S).

3.1.3.1 Carbapenemase substrate and inhibitor profiles

The carbapenemase KPC has described two different profiles defined as (P1 and P2). The P1 profile is observed with the microorganisms *Arizona* isolated from osteitis pus

Biological samples	Carbapenemase types										Totals
	KPC	OXA-48 or OXA 181	Undetermined 1	Undetermined 2	Undetermined 3	Undetermined 4	Undetermined 5	Undetermined 6	Undetermined 7	Undetermined 8	
Urine	21.05	0.00	10.53	5.26	0.00	0.00	0.00	0.00	0.00	0.00	36.84
Pus from osteitis	5.26	5.26	0.00	5.26	5.26	0.00	0.00	0.00	0.00	0.00	21.05
Pus from wounds	10.53	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	10.53
Blood	0.00	5.26	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.26	10.53
Stool	0.00	0.00	0.00	0.00	0.00	5.26	5.26	5.26	5.26	0.00	21.05
Totals	36.84	10.53	10.53	10.53	5.26	5.26	5.26	5.26	5.26	5.26	100.00

Table 5.
Distribution of carbapenemases in biological samples.

samples, *P. mirabilis* isolated from wound pus samples, *S. ficaria, E. gergoviae, E. asburiae, P. vulgaris* isolated from urine samples. The P1 profile, which is characterised by enzymatic activity on all substrates (ETP, IMP, MRP, AMX, CAZ, CTX, CFP) is not inhibited by the carbapenemase inhibitors used (AMC, CXC, AZT, EDTA, PIT). The MICs in the P1 profile are greater than 64 mg/L for ETP, 16 mg/L for IMP and 64 mg/L for MRP.

The second P2 profile was observed with *E. gergoviae* isolated from wound pus samples. Enzymatic activity towards all substrates was maintained in the presence of the inhibitors used, except for AMC, for which it was rather reduced. The MIC values here are above 64 mg/L for ETP and 16 mg/L for IMP and MRP respectively.

The identified OXA carbapenemases describe a single substrate and inhibition profile. This P3 profile is observed with *P. mirabilis* and *S. odorifera* 1 isolated from osteitis pus and blood samples respectively. The activity of the enzyme in this profile is observed on certain substrates (AMX, CFP) and in the presence of inhibitors (CXC, AMC, PIT). It is decreased on the substrates (ETP, IMP, MRP) as well as on one of the inhibitors, EDTA. Another characteristic of this profile is the absence of enzymatic activity towards the substrates CAZ, CTX and one of the inhibitors, AZT. The MICs for these microorganisms are greater than 64 mg/L for ETP, 4 mg/L for IMP, 4 mg/L and 8 mg/L for MRP in *P. mirabilis* and *S. odorifera* 1, respectively.

The P4 profile characterising the TND 1 carbapenemase was identified in *E. gergoviae* and *S. odorifera* 1, all isolated from urine samples. The activity of the enzyme is observed towards the substrates ETP, MRP, AMX, CAZ, CFP and in the presence of the inhibitors CXC, AMC, AZT, PIT. This enzymatic activity is diminished in the presence of IMP, one of the inhibitors, EDTA and is absent in the presence of the substrate CTX. The MICs are greater than 64 mg/L for ETP in both microorganisms, equal to 16 mg/L in *E. gergoviae* for IMP and MRP respectively, and 4 mg/L for IMP and 32 mg/L for MRP in *S. odorifera* 1.

Only *P. mirabilis* isolated from urine samples and osteitis pus expressed the P5 profile. This P5 profile associated with the production of the TND 2 carbapenemase shows an absence of enzymatic activity on the CAZ substrate. However, this activity is observed with regard to the substrates (ETP, IMP, MRP, AMX, CTX, CFP) and in the

Enzymes	Espèces d'entérobactéries	Biological samples	Differentiation	ETP	IMP	MRP	AMX	CAZ	CTX	CXC	AMC	AZT	MRP + EDTA	CFP	PIT	Profiles
KPC + BLSE	*Arizona*	Pus from osteitis	Effect on substrate	R	R	R	R	R	R	R	R	R	R	R	R	P1
			DIC (mm)	10	0	13	0	0	18	0	10	12	13	7	12	
			MIC (mg/L)	>64	32	64	/	/	/	/	/	/	/	/	/	
	Enterobacter asburiae	Urine	Effect on substrate	R	R	R	R	R	R	R	R	R	R	R	R	
			DIC (mm)	10	11	13	0	15	0	0	12	0	13	0	11	
			MIC (mg/L)	>64	64	32	/	/	/	/	/	/	/	/	/	
	Enterobacter gergoviae	Urine	Effect on substrate	R	R	R	R	R	R	R	R	R	R	R	R	
			DIC (mm)	10	16	13	8	0	16	0	13	14	13	10	11	
			MIC (mg/L)	>64	16	32	/	/	/	/	/	/	/	/	/	
	Proteus mirabilis	Wound pus	Effect on substrate	R	R	R	R	R	R	R	R	R	R	R	R	
			DIC (mm)	11	13	15	7	16	20	0	15	18	16	14	12	
			MIC (mg/L)	>64	16	64	/	/	/	/	/	/	/	/	/	
	Proteus vulgaris	Urine	Effect on substrate	R	R	R	R	R	R	R	R	R	R	R	R	
			DIC (mm)	8	11	12	0	0	14	0	14	0	13	0	10	
			MIC (mg/L)	>64	>64	64	/	/	/	/	/	/	/	/	/	
	Serratia ficaria	Urine	Effect on substrate	R	R	R	R	R	R	R	R	R	R	R	R	
			DIC (mm)	8	13	12	0	0	13	0	10	12	12	10	16	
			MIC (mg/L)	>64	64	32	/	/	/	/	/	/	/	/	/	
KPC or KPC + BLSE	*E. gergoviae*	Wound pus	Effect on substrate	R	R	R	R	R	R	R	I	R	R	R	R	P2
			DIC (mm)	6	13	15	10	15	15	0	16	0	15	10	12	
			MIC (mg/L)	>64	16	16	/	/	/	/	/	/	/	/	/	

Enzymes	Espèces d'entérobactéries	Biological samples	Differentiation	ETP	IMP	MRP	AMX	CAZ	CTX	CXC	AMC	AZT	MRP + EDTA	CFP	PIT	Profiles
OXA-48 or OXA-181	*P. mirabilis*	Pus from osteitis	Effect on substrate	I	I	I	R	S	S	R	R	S	I	R	R	P3
			DIC (mm)	12	20	18	15	23	27	0	18	27	20	20	13	
			MIC (mg/L)	>64	4	4	/	/	/	/						
	Serratia odorifera 1	Blood	Effect on substrate	I	I	I	R	S	S	R	R	S	I	R	R	
			DIC (mm)	12	20	20	0	23	23	0	12	29	20	22	9	
			MIC (mg/L)	>64	4	8	/	/	/	/	/	/	/	/	/	
Undetermined 1	*E. gergoviae*	Urine	Effect on substrate	R	I	R	R	R	S	R	R	R	I	R	R	P4
			DIC (mm)	16	14	13	0	15	20	0	10	15	16	12	15	
			MIC (mg/L)	>64	16	16	/	/	/	/	/	/	/	/	/	
	S. odorifera 1	Urine	Effect on substrate	R	I	R	R	R	S	R	R	R	I	R	R	
			DIC (mm)	12	21	16	7	15	20	0	18	20	17	19	11	
			MIC (mg/L)	>64	4	32	/	/	/	/	/	/	/	/	/	
Undetermined 2	*P. mirabilis*	Urine	Effect on substrate	R	R	R	R	S	R	R	R	R	R	R	R	P5
			DIC (mm)	6	12	12	0	20	7	0	12	0	12	0	12	
			MIC (mg/L)	>64	32	>64	/	/	/	/	/	/	/	/	/	
		Pus from osteitis	Effect on substrate	R	R	R	R	S	R	R	R	R	R	R	R	
			DIC (mm)	6	11	14	0	20	16	0	12	7	14	11	12	
			MIC (mg/L)	>64	32	32	/	/	/	/	/	/	/	/	/	
Undetermined 3	*S. odorifera 1*	Pus from osteitis	Effect on substrate	R	I	I	R	R	S	R	R	I	I	R	R	P6
			DIC (mm)	12	20	18	0	12	22	0	16	21	19	18	10	
			MIC (mg/L)	>64	4	4	/	/	/	/	/	/	/	/	/	

Enzymes	Espèces d'entérobactéries	Biological samples	Differentiation	ETP	IMP	MRP	AMX	CAZ	CTX	CXC	AMC	AZT	MRP + EDTA	CFP	PIT	Profiles
Undetermined 4	*P. mirabilis*	Stools	Effect on substrate	R	I	I	R	S	S	R	S	R	I	I	R	P7
			DIC (mm)	11	20	20	10	25	21	0	19	20	20	25	12	
			MIC (mg/L)	>64	4	4	/	/	/	/	/	/	/	/	/	
Undetermined 5	*S. odorifera 1*	Stools	Effect on substrate	R	S	I	R	R	S	R	S	I	I	I	R	P8
			DIC (mm)	12	25	20	11	16	26	0	19	22	21	21	11	
			MIC (mg/L)	>64	0,125	2	/	/	/	/	/	/	/	/	/	
Undetermined 6	*Citrobacter braakii*	Stools	Effect on substrate	R	I	S	R	S	S	R	S	S	I	I	R	P9
			DIC (mm)	13	25	22	10	21	28	0	19	27	22	22	12	
			MIC (mg/L)	>64	0,5	4	/	/	/	/	/	/	/	/	/	
Undetermined 7	*E. gergoviae*	Stools	Effect on substrate	R	S	I	R	I	S	R	S	S	I	I	R	P10
			DIC (mm)	12	23	21	12	17	26	0	20	28	21	21	8	
			MIC (mg/L)	>64	0,5	4	/	/	/	/	/	/	/	/	/	
Undetermined 8	*E. gergoviae*	Blood	Effect on substrate	R	R	S	R	S	S	R	R	R	S	I	I	P11
			DIC (mm)	13	16	22	0	22	27	0	16	17	25	22	19	
			MIC (mg/L)	>64	16	0,5	/	/	/	/	/	/	/	/	/	

Table 6.
Substrate and inhibition profiles associated with the production of carbapenemases circulating in Maroua.

presence of all inhibitors (AMC, CXC, AZT, EDTA, PIT). MICs are greater than 64 mg/L for ETP, equal to 32 mg/L for IMP and greater than 64 mg/L for MRP when isolated from urine and equal to 32 mg/L for MRP when isolated from osteitis pus.

The P6 profile is expressed by the *S. odorifera* 1 microorganism isolated from osteitis pus which produces TND 3 carbapenemase. The enzymatic activity of this profile is observed on the substrates ETP, AMX, CAZ and in the presence of the inhibitors CXC, AMC, PIT. This decreased enzymatic activity on the substrates IMP, MRP and in the presence of the inhibitors AZT and EDTA are absent with respect to CTX. The MIC values here are above 64 mg/L for ETP and 4 mg/L for IMP and MRP.

The *P. mirabilis* microorganism isolated from stool samples and producing the TND 4 carbapenemase expresses the P7 profile. This profile is characterised by an enzymatic activity towards each of the two substrates ETP, AMX and towards three inhibitors CXC, AZT, PIT. This enzymatic activity is decreased on the substrates IMP, MRP, CFP and in the presence of the inhibitor EDTA. Finally, no enzymatic activity was observed on the substrates CAZ, CTX and in the presence of the inhibitor AMC. The MIC values for this microorganism are greater than 64 mg/L for ETP and equal to 4 mg/L for IMP and MRP respectively.

The P8 profile described by TND 5 carbapenemase is observed with *S. odorifera* 1 isolated from stool samples. It is characterised by an enzymatic activity towards the substrates ETP, AMX, CAZ and in the presence of the inhibitors CXC, PIT. This enzymatic activity, which is diminished in the presence of the substrates MRP, CFP and the inhibitors AZT, EDTA, is absent on two substrates IMP, CTX and on an inhibitor AMC. The MICs here are greater than 64 mg/L for ETP, equal to 0.125 mg/L for IMP and 2 mg/L for MRP.

The *C. braakii* species producing the TND 6 carbapenemase isolated from stool samples express the P9 profile. The enzymatic activity here is observed on ETP and AMX substrates and in the presence of the inhibitors CXC and PIT. This enzymatic activity is decreased on both substrates MRP, CFP and in the presence of the inhibitor EDTA. It is absent on the substrates IMP, CAZ, CTX and in the presence of the inhibitors AMC, AZT. The MICs here are greater than 64 mg/L for ETP, equal to 0.5 mg/L for IMP and 4 mg/L for MRP.

The P10 profile is observed with the TND 7 carbapenemase produced by *E. gergoviae* isolated from stool samples and is characterised by enzymatic activity on the substrates ETP, AMX and in the presence of the inhibitors CXC, PIT. This enzymatic activity is decreased in the presence of two substrates MRP, CAZ and the inhibitor EDTA. There is no enzymatic activity on two substrates IMP, CTX and on two inhibitors AMC, AZT. The MICs are above 64 mg/L for ETP, 0.5 mg/L for ETP, and 4 mg/L for MRP.

Finally, the P11 profile is always found in *E. gergoviae* isolated from blood samples but which produces the TND 8 carbapenemase. It is characterised by enzymatic activity on three substrates ETP, IMP, AMX and on three inhibitors CXC, AMC, AZT. This enzymatic activity is decreased in the presence of the substrates MRP, CFP and in the presence of the inhibitor PIT. On the other hand, it is absent on the substrates CAZ, CTX and in the presence of the inhibitor EDTA. The MICs are greater than 64 mg/L for ETP, 16 mg/L for IMP and 0.5 mg/L for MRP (**Table 6**).

3.2 Discussion

Biological samples containing carbapenem-resistant Enterobacteriaceae represented 5.97%. This percentage is distributed between urine samples (2.20%),

osteitis pus (0.94%), wound pus (0.63%), blood (0.94%) and stool (1.26%). Carbapenem-resistant Enterobacteriaceae were not identified in urethral and vaginal swabs. The high proportion of carbapenem-resistant microorganisms in urine could be explained by the fact that this medium is potentially an extra-digestive reservoir for ESBL-producing Enterobacteriaceae [14]. The emergence of carbapenem resistance in some of the biological samples taken reflects the increasing complexity of the phenomenon in enterobacteria [15]. This complexification of the resistance phenomenon in the city of Maroua had already been observed in bacteria contaminating the food sold there [4, 5]. Several explanations can be found for the emergence of carbapenem resistance in the city of Maroua. The emergence of carbapenem resistance could be the consequence of exponential and uncontrolled use of antibiotics [6, 16–18]. The flow of populations between risk areas (Europe, Asia) and the city of Maroua could also contribute to the importation of strains expressing these types of resistance [19]. The opening of the University of Maroua, which contributes enormously to the migration of populations from various origins to the city, is also a major risk factor for the transport of multidrug-resistant strains of bacteria. The emergence of this type of resistance may finally be due to an exchange of the genes responsible for their expression between bacterial species from the digestive tract or the environment [20]. This exchange can take place via the phenomena of transduction [21], conjugation [22], or transformation [23].

Using API 20 E galleries, *Arizona*, *C. braakii, E. gergoviae, E. asburiae, P. mirabilis, P. vulgaris, S. ficaria* and *S. odorifera* 1 were identified as the carbapenem-resistant Enterobacteriaceae in the specimens. These Enterobacteriaceae are variously distributed in the samples. The species *E. gergoviae, E. asburiae, P. mirabilis, P. vulgaris, S. ficaria, S. odorifera* 1 were identified in urine specimens. Those found in osteitis pus were *Arizona, P. mirabilis, S. odorifera* 1. Two microorganisms, *E. gergoviae and P. mirabilis* were isolated from wound pus samples. The microorganisms isolated from blood were *E. gergoviae, S. odorifera 1. Finally, C. braakii, E. gergoviae, P. mirabilis* and *S. odorifera* 1 were identified in stool samples. The proportions of carbapenem-resistant Enterobacteriaceae in biological samples were 36.84% in urine samples, 21.05% in osteitis pus and stools respectively, and 10.53% in blood and wound pus samples. This distribution in biological samples shows that Enterobacteriaceae are likely to cause deleterious effects in the organism from a variety of environments [24]. The diversity of environments where these enterobacteria have been identified can be explained by the great power of adaptation that characterises them [25] and the multi-resistance to antibiotics that does not facilitate their elimination [16, 17].

The enzymatic mechanism of resistance to carbapenems was demonstrated in 100% of the Enterobacteriaceae that were identified. This observation is in agreement with the fact that enzymatic inactivation of carbapenems is the main mechanism used by enterobacteria to resist their bactericidal effects [26]. The yellow colour change of phenol red used as a colour indicator to show the presence of enzymatic activity on carbapenems has been interpreted as the result of acidification of the reaction medium [27, 28]. This acidification of the reaction medium is a consequence of hydrolysis of the -lactam ring at the amide bond which produces a carboxyl function [29]. The level of expression of this reaction confers certain characteristics to enterobacteria. These characteristics were assessed indirectly on culture media using the inhibition diameters-MIC relationship [9]. The inhibition diameters-MIC correlation for selected carbapenems ($r = 0.578$, $p < 0.01$ for IMP and $r = 0.858$, $p < 0.01$ for MRP) allowed three characteristics to be defined. The first characteristic is resistance to carbapenem, which indicates the presence of enzymatic activity (R). The second characteristic is

intermediate resistance which is the result of decreased enzyme activity (I). The third characteristic, marked by an absence of enzyme activity (S), defines the susceptibility of the enterobacteria to carbapenem [9].

The interpretation of the characteristics expressed by the enterobacteria in the presence of the substrates and inhibitors defined by the algorithm used made it possible to highlight three types of carbapenemases in these enterobacteria isolated from biological samples. These are carbapenemases of the KPC, OXA-48 or OXA-181 type and TNDs. The dominant proportion of KPC carbapenemases (36.84%) can be explained by the fact that they are the most abundant and widespread among enterobacteria [30]. They are also characterised by the existence of several variants that differ only by the substitution of one or two amino acids [31]. In contrast, the low percentage of OXA-41 or OXA-181 carbapenemases (10.53%) in the samples can be justified by the fact that this is an enzyme produced from a single auto transferable plasmid that does not carry additional resistance genes [32]. The low proportion of each of the TNDs can be explained by the fact that they are new phenotypes of point synthesis due to the presence of integrons. Integrons sometimes contain transposons from which some transposase-containing Enterobacteriaceae can be naturally genetically engineered to form highly expressed resistance operons [33].

The types of carbapenemases identified are differently distributed in biological samples and between enterobacteria. This random distribution within species of Enterobacteriaceae could be justified by the ease with which resistance-conferring genes diffuse between microorganisms [11]. It is this random distribution that may explain the difficulty in effectively applying probabilistic and/or therapeutic antibiotic therapy in cases of infection with resistant carbapenem enterobacteria [34]. The enzymatic activity of carbapenemases, which is manifested by hydrolysis at the amide bond of the said ring, has made it possible to describe 11 different substrates and inhibition profiles.

The first substrate and inhibition profile, P1, is characterised by enzymatic activity on all carbapenems including monobactam (AZT) used. The fact that this enzymatic activity is not influenced by the presence of EDTA proves that the enzyme does not need a heavy metal to hydrolyse the substrates. These characteristics are unique to KPC-type class A carbapenemases produced from plasmids [35]. It was also observed that the activity of this enzyme is maintained in the presence of its inhibitors PIT and AMC. This observation highlights a synergy of action between the carbapenemase KPC and an ESBL. Indeed, in the presence of a "suicide" inhibitor that serves as a decoy, such as clavunate or tazobactam, the bacteria compensate for the enzymatic deficit by amplifying the synthesis of ESBLs [6, 36]. This hyperproduction can be mediated by mutations in the promoter of the gene and/or by an increase in the number of plasmids carrying the bla gene. These ESBLs would therefore play the known role of multiplying the targets of antibiotics to limit their effectiveness [37]. From the above, it appears that bacteria of the P1 profile have the capacity to produce both KPC-type carbapenemases and ESBLs, all of which are class A.

Measurement of MICs for this profile using the E-test showed that variations are only observable between *P. vulgaris*, *E. asburiae*, *E. gergoviae* and *P. mirabilis*. From 16 mg/L for *P. vulgaris* and *E. asburiae*, it increases to 64 mg/L for *E. gergoviae* and *P. mirabilis*. The fluctuations obtained with the MIC values for carbapenems in these microorganisms could be explained by the existence of two KPC variants between these identified enterobacterial species [31].

The second substrate and inhibition profile (P2) is associated with the carbapenemase identified in *E. gergoviae* isolated from wound pus. The enzymatic

activity here shows several similarities with the P1 profile. The only difference is the decrease in enzyme activity in the presence of AMC. The MIC values do not differ from those obtained with *E. gergoviae* isolated from urine samples. This slight variation in MIC suggests that the same KPC is produced in the P1 profile by this microorganism in both urine and wound pus. The decrease in enzyme activity in the presence of clavunate may be due to insufficient ESBL production to contain all the suicide inhibitor molecules. The consequence is a decrease in the number of enzyme molecules available for substrate hydrolysis which would then lead to a decrease in enzyme activity.

The third substrate and inhibition profile (P3) is expressed by OXA-type carbapenemases (48 or 181) produced by *P. mirabilis* and *S. odorifera* 1 isolated from osteitis pus and blood samples respectively. This profile is characterised by enzymatic activity on CXC, decreased on the three carbapenems and not observed at all on AZT. EDTA has no discernible influence on this activity. All these characteristics are consistent with the description of a class D carbapenemase [35, 38]. Another observation on this profile is that the activity of the enzyme resumes on AZT in the presence of CTX. The resumption of enzyme activity on AZT in the presence of CTX illustrates the theory that the combination of two -lactams can be antagonistic if one of them is an -lactamase inducer. CTX would therefore induce the production of ESBLs that could hydrolyse AZT. This illustrates the fact that *P. mirabilis* and *S. odorifera* 1 are likely to produce inducible ESBLs in addition to OXAs. Analysis of the MICs obtained in these two species shows that there are no differences in the activity of this enzyme either at the level of the microorganisms or the samples. This suggests that the OXA produced by these microorganisms originates from the same plasmid that has migrated from one species to another [39].

The P4 profile is only found in *E. gergoviae* and *S. odorifera* 1 isolated from urine samples. It is characterised by an enzymatic activity on ETP and MRP but diminished with respect to IMP. The inhibitors clavunate and tazobactam have no effect on this enzymatic activity. This observation can be explained by the fact that these bacteria produce class B carbapenemases or, a combination of ESBL and chromosomal type A and/or B carbapenemases [16, 17]. The decrease in enzymatic activity in the presence of EDTA validates the hypothesis of the presence of a class B carbapenemase [38]. The combination of these observations leads us to believe that the genes coding for the synthesis of both class A and B carbapenemases, both chromosomal, are present in these bacteria. It is, therefore, the inhibition of class B carbapenemase by EDTA that would be at the origin of the decrease in enzymatic activity. In this context, the decrease in enzymatic activity would then be due to the reduction in the quantity of carbapenemases potentially active on carbapenems. In view of the above, it is possible that the bacteria *E. gergoviae* and *S. odorifera* 1 possess in their chromosomes both genes coding for the synthesis of class A and B carbapenemases. The MIC measurements for these microorganisms did not show any differences apart from that obtained with IMP (16 mg/L and 4 mg/L in *E. gergoviae* and *S. odorifera* 1 respectively). This difference in MICs can be explained by mutations that may occur in the amino acid sequence homology or by the level of production of one or the other of these carbapenemases.

The P5 substrate and inhibition profile is found in *P. mirabilis* isolated from urine samples and osteitis pus. This profile is characterised by enzymatic activity on all substrates except CAZ. This activity is maintained in the presence of all inhibitors. This suggests a most likely plasmid hyper production of KPC associated with cephalosporinase. The different profiles for this microorganism (P1 when derived from wound pus and P5 when derived from either urine samples or osteitis pus),

although suspected of producing all the KPCs, would be the result of the difference in the enzyme that accompanies the production of these KPCs.

The P6 profile is found in *S. odorifera* 1 isolated from osteitis pus. It is characterised by an enzymatic activity towards ETP. This activity decreases on IMP and MRP. The presence of inhibitors has no visible effect on the enzymatic activity. The analysis of this P6 profile shows several similarities with the P3 profile. The same is true for the MIC values, which are close to those of the P3 profile. The great similarity observed between the P6 and P3 profiles suggest that *S. odorifera* 1 and *P. mirabilis*, both isolated from osteitis pus samples, produce carbapenemases of types OXA-48 or OXA-181. However, the increase in enzymatic activity observed with the P6 profile of *S. odorifera* 1 is thought to be the result of possible mutations in the OXA carbapenemases and the production of a cephalosporinase that activates the hydrolysis of CAZ [9].

The P7 profile expressed by *P. mirabilis* isolated from stools is characterised by an enzymatic activity on ETP and decreased on IMP and MRP substrates. The presence of clavunate shows inhibition of the enzymatic activity. This enzymatic activity, the extent of which varies from one carbapenemase to another, can be explained by the fact that it is the product of genes carried by the chromosomes [35, 40, 41]. The inhibition of the latter by clavunate validates the hypothesis of a class A carbapenemase.

The P8 profile identified in *S. odorifera* 1 isolated from stools shows enzymatic activity on ETP. This activity decreases on MRP and disappears on IMP. The inhibitor clavunate causes a loss of enzyme activity while EDTA has no effect on this activity. The MIC values show that the enzyme activity is distinct from one substrate to another. The fact that the enzyme activity is distinct on carbapenems and cephalosporin (CTX) sensitivity shows that this bacterium produces a chromosomal carbapenemase [35]. The inhibition of enzyme activity in the presence of clavunate supports the hypothesis of a class A carbapenemase [38]. Suspected carbapenemases may be SME, IMI-1 [40, 41]. The multiple similarities observed between the P7 and P8 profiles suggest that the carbapenemase produced in profile P8 may be a mutated form of that produced by *P. mirabilis* isolated from stool samples.

The P9 profile observed with the *C. braakii* microorganism isolated from stools is characterised by enzymatic activity on the ETP. This activity decreases with respect to MRP and disappears with respect to IMP and cephalosporins (CAZ, CTX). The presence of the inhibitor EDTA has no effect on the enzymatic activity contrary to clavunate and AZT which inhibit this activity. The strong similarity between the P9 and P8 profiles suggests that the same carbapenemase is mutated between *C. braakii* and *P. mirabilis* isolated from stool samples.

The P10 profile found in *E. gergoviae* isolated from stools always shows an enzymatic activity that varies from one carbapenem to another. With a few exceptions, this P10 profile is similar to the P9 profile. The observations show that the two profiles are similar and the few differences observed could reflect the presence of mutations in the genes producing these enzymes.

The last profile P11 is the fourth substrate and inhibition profile obtained with *E. gergoviae* isolated from blood. It is characterised by an enzymatic activity on ETP and IMP. This activity is diminished in the presence of MRP. The disappearance of this activity in the presence of EDTA indicates that the activity of this enzyme requires the presence of heavy metal [42]. No inhibition of the enzyme activity is observed with classical class A carbapenemase inhibitors. All these observations point to a class B carbapenemase [43]. The difficulty in typing this carbapenemase from this substrate and inhibition profile is the demarcation observed with other classical class B

carbapenemases. This demarcation comes from the fact that the enzymatic activity here decreases towards MRP whereas class B carbapenemases exhibit enzymatic activity on all carbapenems [43]. The decreased enzymatic activity of this P11 profile on MRP can be explained by the presence of mutations in the primary amino acid sequence homology at the active site [44]. The presence of these mutations may be a consequence of being produced from integrons carrying 'cassette' genes from which several genes can be assembled [10]. The fact that this P11 profile shows activity on AZT and towards CAZ and CTX suggests the presence of an ESBL.

4. Conclusion

The objective of this work was to determine the types of carbapenemases moving around the city of Maroua in order to contribute to the development of a control strategy against the enterobacteria multidrug resistance. It was found that *Arizona, C. braakii, E. asburiae, E. gergoviae, P. mirabilis, P. vulgaris, S. ficaria* and *S. odorifera* 1 are the species of enterobacteria that produce carbapenemases in biological samples (urine, wound pus, osteitis pus, blood, stools). The carbapenemases identified are of the KPC, OXA and undetermined types. 11 different substrates and inhibition profiles are expressed by these microorganisms, some of which are able to produce two different classes of carbapenemases, others of producing a carbapenemase-BLSE or cephalosporinase combination. These 11 profiles have shown the difficulties of applying law of inhibition of these carbapenemases in situ in the context of probabilistic antibiotic therapy. This observation is valid whether the enterobacterium is identified or the biological medium of isolation is known.

Author details

Daoudou Bakari[1]*, Jojo Mbala Ekamba[1], Mamoudou Hamadou[1,2], Yaya Garga[1], Martin-Paul Baane[1,3] and Félicité Obono[2]

1 Faculty Science, Department of Biological Sciences, University of Maroua, Maroua, Cameroon

2 National Advanced School of Engineering of Maroua, University of Maroua, Maroua, Cameroon

3 HSWC-NSIF Biological Analysis Laboratory, Maroua, Cameroon

*Address all correspondence to: bkimou3@gmail.com

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