British Microbiology Research Journal
9(1): 1-9, 2015, Article no.BMRJ.18250
ISSN: 2231-0886

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Phenotypic Detection of Extended Spectrum Beta-Lactamase and Carbapenemases Produced by *Klebsiella* spp Isolated from Three Referrals Hospitals in Yaounde, Cameroon

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Authors' contributions

This work was carried out in collaboration with all authors. Authors ACB, HGK and SK-S designed the study. Authors ACB, HGK and EEL wrote the protocol and first draft of the manuscript and managed literature searches. Authors ACB, MT, CDM, EEL and SB managed the analysis of the study and literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BMRJ/2015/18250
Editor(s):
(1) Eggehard Holler, Cedars-Sinai Medical Center, Department of Neurosurgery, Los Angeles, USA and University of Regensburg, Germany.
Reviewers:
(1) Anonymous, Ghana.
(2) Patrick Akpaka, Paraclinical Sciences, The University of the West Indies, Jamaica.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=1216&id=8&aid=9675

Original Research Article

Received 11th April 2015
Accepted 30th April 2015
Published 9th June 2015

ABSTRACT

Aims: The main objective of this study was to determine the resistance phenotype of β-lactamines by *Klebsiella* in three hospitals in Yaoundé.
Study Design: A cross-sectional descriptive study.

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Place and Duration of Study: Bacteriology laboratories of three referrals hospitals in Yaounde (University Teaching Hospital, General Hospital and Gynaeco-Obstetric and Pediatric Hospital) between May and November 2013.
Methodology: A cross-sectional descriptive study was carried out over a 6 month-period from May to November 2013 in the bacteriology laboratories of three referrals hospitals in Yaounde. 99 strains of *Klebsiella* spp. were collected for the study. Antimicrobial susceptibility testing was done using the disc diffusion method. The antibiotics tested were the β-lactams, other inhibitors like clavulanic acid, tazobactam, Ethylene-Diamine-Tetra-Acetic Acid (EDTA), cloxacillin and 3-aminophenyl boronic acid hydrochloride. The minimum inhibitory concentration (MIC) was also determined to enable the classification of the different resistance phenotypes.
Results: Ninety-nine *Klebsiella* spp. were identified from urine (52.5%), blood (21.2%), pus (15.2%) and others sites (11.1%). The distribution of the *Klebsiella* spp. was: *Klebsiella pneumoniae pneumoniae* (78.7%), *Klebsiella oxytoca* (12.12%), *Klebsiella pneumoniae ozaenae* (5.05%), and *Klebsiella pneumoniae rhinoscleromatis* (4.04%). The isolates were most resistant to piperacillin (76%) and cephalothin, (85%). The most active antibiotics were imipenem (99%), and ertapenem (77%). The phenotypic tests revealed the following resistance phenotypes: extended-spectrum beta-lactamase (30.30%), wild (27.27%), penicillinase resistant to inhibitors (16.16%), carbapenemase (11.11%). Out of these 99 *Klebsiella* spp., 5 were carbapenemases producers of class C and 6 of class D. The MIC were variable with different antibiotics tested but the MIC of imipenem were always lower than 1 µg/ml.
Conclusion: The interpretation of the antimicrobial susceptibility testing has enabled the establishment of a high prevalence of expanded spectrum β-lactamase and consequently leading to an increase in the presence of carbapenemase producing *Klebsiella* spp. This could lead to therapeutic failure in case of treatment with beta-lactamines antibiotics. Therefore this trend needs to be monitored.

Keywords: Klebsiella spp; resistance phenotypes; ESBL; carbapenemases.

1. INTRODUCTION

Bacterial infections acquired in the hospital as well as in the community settings are a serious public health concern. These infections are mostly caused by Gram-negative bacilli which are nowadays increasingly implicated in the development of antibiotic resistance. The resistance of *Klebsiella* to antibiotics has in recent years experienced an alarming global increase [1].

Since 1980s, the third generation cephalosporins (3GC) belong to the group of antibiotic used for the treatment of severe enterobacterial infection. Quickly the extended-spectrum beta-lactamases (ESBLs), Plasmid transmitted enzymes responsible for resistance to penicillins, cephalosporins and to monobactams began to develop. Due to the broad spectrum activity of the carbapenemes, they have recently become the alternatives to the 3GC [2].

The ESBLs are broad spectrum enzymes conferring resistance to bacteria against most of the β-lactam antibiotics except cephamycins and carbapenemes [2]. The resistance of gram-negative bacilli to carbapenems, particularly by the production of transmissible carbapenemases is a major public health problem [3]. Most of these Carbapenemases have been identified in *Klebsiella pneumoniae* [4]. The emergence and spread of carbapenemase-producing Enterobacteriaceae in different parts of the world is a major threat especially in nosocomial infections [2]. The isolation of carbapenemase-producing *Klebsiella* spp. is increasing worldwide [4]. In Cameroon, some phenotypic studies presumptively reveal the existence of carbapenemase-producing *Klebsiella* spp. (C Mbakop, University of Yaounde 1, Cameroon, Unpublished results).

Klebsiella spp. are involved in many nosocomial infections and many strains are resistant to antibiotics. Studies carried out at the Yaounde Central Hospital found a resistance rate of 18.8% of ESBL -producing *Klebsiella* [5]. However, this observation is unknown in many other hospitals. This study aimed at determining the resistance and the phenotypes of *Klebsiella* spp., isolated from three clinical laboratories in three referrals hospitals in Yaounde.

2. MATERIALS AND METHODS

Ninety-nine *Klebsiella* spp. were isolated from all clinical specimens at three bacteriology laboratories from three referrals hospitals in Yaounde from May to November 2013. The identification of the *Klebsiella* isolates was done using standard bacteriological procedures and biochemically using the API 20E gallery (BioMérieux SA, Lyon, France).

Antimicrobial susceptibility testing (AST) was performed on Mueller-Hinton agar according to "Comité d'Antibiogramme de la Société Française de Microbiologie (CASFM)" [6] using antibiotics of the β-lactam family and inhibitors such as clavulanic acid, tazobactam, Ethylene-Diamine-Tetra-Acetic Acid (EDTA), cloxacillin and 3-aminophenyl boronic acid hydrochloride. The antibiotics tested for all isolates were: amoxicillin (25 µg), amoxicillin + clavulanic acid 25/10 ug), ticarcillin (75 µg), ticarcillin + clavulanic acid (75/10 ug), piperacillin (100 ug), piperacillin-tazobactam (100/10 ug) cephalothin (30 µg), cefoxitin (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefepime (30 µg), aztreonam (30 µg), imipenem (10 ug) and ertapenem (10 ug).

The minimum inhibitory concentration (MIC) was determined using the E-test. The phenotypes were determined from the interpretation of the antimicrobial susceptibility testing as recommended by the CA-SFM. Detection of ESBLs was done using the synergy test by the combination of amoxicillin and clavulanic acid disks, ceftazidime, aztreonam and cefepime.

The detection and classification of carbapenemase were performed by the modified Hodge test and the use of different inhibitors (cloxacillin, boronic acid and EDTA)

3. RESULTS AND DISCUSSION

This study was conducted in the bacteriology laboratories of three public referrals hospitals in Yaounde: University Teaching Hospital, Yaoundé (UTHY), Yaounde General Hospital (YGH) and Gynaeco-Obstetric and Pediatric Hospital, Yaounde (HGOPY). Among the 99 strains of *Klebsiella* spp. isolated from 99 patients, most of them were from hospitalized patients (n = 85 or 85.85%), mainly from the pediatrics unit, n = 34 (35.35%) and intensive care unit n = 27 (27, 27%). These results are similar to those obtained in a study carried out in Cameroon which revealed a predominance of *Klebsiella* in the pediatric unit (48.4%) and medical intensive care unit (21.3%) (C Mbakop, University of Yaounde 1, Cameroon, Unpublished results). This could be partly due to misuse of antibiotics or poor application of hygiene in these units. On the other hand it may be due to a high concentration of patients in these units of intensive care which could enhance the transmission of germs between patients [3].

Table 1 shows that all strains were resistant to amoxicillin (aminopenicillin) and ticarcillin (carboxypenicillin).

The wild type was found in all strains. The isolated bacterial strains expressed high resistance to ureidopenicillins (piperacillin, 76%) and first-generation cephalosporins (1GC: cephalothin, 85%).

The action of inhibitors (clavulanic acid, tazobactam) enabled the restoration of penicillins activities such as amoxicillin, piperacillin and ticarcillin (39%, 38%, 22% respectively). A 55.5% (n=55) resistance was observed for cefoxitin. For the 3GCs the resistance was variable: cefotaxime 52% and ceftazidime 54%; for monobactams (aztreonam) 51% and 56% of the strains were resistant 4GCs (cefepime). The most active antibiotics were imipenem (99%) and ertapenem (77%).

Regarding the sensitivity of strains to antibiotics, they expressed a high level of resistance with respect to most of the antibiotics tested. The wild phenotype was confirmed for all of our strains as recommended by CASFM, [6]. Overall, there has been a slight increase in the level of antibiotic resistance as comparison to a 2011 study (C Mbakop, University of Yaounde 1, Cameroon, Unpublished results) with a p-value = 0.001. The resistance of the 1GC (cephalothin), 2GC (cefoxitin), 3GC (ceftazidime), 4GCs (cefepime), monobactam (aztreonam) and imipenem were 85%, 55%, 54%, 56%, 51% and 1% respectively. They also found 84%, 52%, 45%, 50.3%, 38% and 12% respectively. This increased level of resistance to β-lactam antibiotics could probably be as a result of inappropriate prescribing and indiscriminate use of this antibiotic family [7]. The high prevalence of "resistant" phenotypes acquired primarily within hospital strains (strains 66/85 or 77.64%) shows the possibility of an increased antibiotics selection pressure [8,9,10]. In addition, *Escherichia coli* and *Klebsiella* spp. have usually been cited as being the Enterobacteriaceae species presenting the most

resistant phenotypes [11,12]. The frequency of the "wild type" (27.27%) obtained in our study was low and well below the frequency of the known wild type of *Klebsiella* spp. (70%) [13,14].

The interpretation of the AST enabled us to highlight the different resistance phenotypes expressed by the isolates (Fig. 1, Table 2, Table 3, Fig. 2).

The phenotypic classification was based on the susceptibility profile of the different antibiotics tested which enabled us to obtain the following phenotypes: expanded spectrum β-lactamase (ESBL) n = 30 (30.3%), low spectrum penicillinase / wild n = 27 (27.27%), penicillinase resistant to inhibitors n = 16 (16.16%), carbapenemase n=11(11.11%), high-level penicillinase n = 8 (8.08%), HyperOXY n = 4 (4.04%), high-level cephalosporinase + extended spectrum β -lactamase to n = 2 (2.02%) and high-level cephalosporinase n = 1 (1.01%). The most observed phenotype was the ESBL (30.3%), followed by the wild-type (27.3%). The phenotype of high-level cephalosporinase was the least (1.01).

Nearly a third of the *Klebsiella* spp. were ESBL producers (30.3%), showing a remarkable increase in the frequency of these strains in Cameroon, as previous studies in the Yaounde Central Hospital and UTHY had revealed frequencies of 18.8% in 2005 [15] and 29% in 2012 respectively (N Kouya, School of Health Sciences / Catholic University of Central Africa, Cameroon, Unpublished results). However, the Alert, Investigation and Surveillance of Nosocomial Infections Network in France indicated in 2008 that 17% of *K. pneumoniae* responsible for bacteremia were ESBL-producing. The high frequency of ESBL-producing *Klebsiella* in our context would be the consequence of regular use of β-lactam antibiotics or prolonged hospitalization of patients [16]. Resistance related to the production of carbapenemase by *E. coli* and *Klebsiella* spp. have been described [17,5]. Search and classification of carbapenemase in our study were performed by the modified Hodge test and the synergy test. All Carbapenemases producing strains showed a negative modified Hodge test. The results of this test are variable in detecting carbapenemases in Class B, C and D [18]. Among the 11 carbapenemases-producing *Klebsiella* isolates, 45.45% were Class C and 54.54% class B. These results are different from those of Mbakop et al. in Cameroon with 58 carbapenemase-producing Enterobacteriaceae, 33% were class A, 27% of class B and 31% class C and D. This difference is due to the fact that the previous study (C Mbakop, University of Yaounde 1, Cameroon, Unpublished results) included all Enterobacteriaceae spp. and in addition all inhibitors were not used for the classification of the carbapenemases.

Table 1. Susceptibility of bacterial isolates to antibiotics

Antibiotics	Susceptibility (%)		
	Sensitive	Intermediate	Resistant
Amoxicillin	0	0	100
Amoxicillin + clavulanic acid	39	32	29
Ticarcillin	0	0	100
Ticarcillin + clavuanic acid	22	18	60
Piperacillin	24	13	63
Piperacillin+ tazobactam	62	29	9
Cephalothin	15	25	60
Cefoxitin	45	17	38
Cefotaxime	49	1	51
Ceftazidime	46	12	42
Cefepime	44	30	26
Aztreonam	49	8	43
Imipenem	99	1	0
Ertapenem	77	19	4

NB : Resistant bacterial strain by definition is a non- susceptible strain , that is categorized "resistant " or "intermediate"

The distribution of these carbapenemases with respect to the status permitted us to see that all carbapenemase - producing strains were isolated from hospitalized patients (shown on Table 2). There was an association between ESBL-producing strains and carbapenemase, hospitalized status and hospital units. There was a high prevalence of ESBL-producing strains and/or carbapenemase in patients hospitalized in the pediatric wards and intensive care unit.

The high prevalence of ESBL and carbapenemase-producing strains in hospitalized patient in the pediatric (36.36%) and intensive care units (36.36%) may be due to the fact that ESBL and carbapenemase enzymes are produced mostly by nosocomial strains responsible for some hospital outbreaks [19] and in the units with prolonged hospitalization [20]. All these suggest that frail individuals are the most exposed.

Indeed, it has been observed that the acquisition of ESBL and carbapenemase concerns patients with prolonged hospitalization and exposure to invasive devices [21]. Other risk factors may include malnutrition, hemodialysis, total parenteral nutrition, ICU admission or prior hospitalization. Some studies have reported that the restriction of the use of antibiotics has

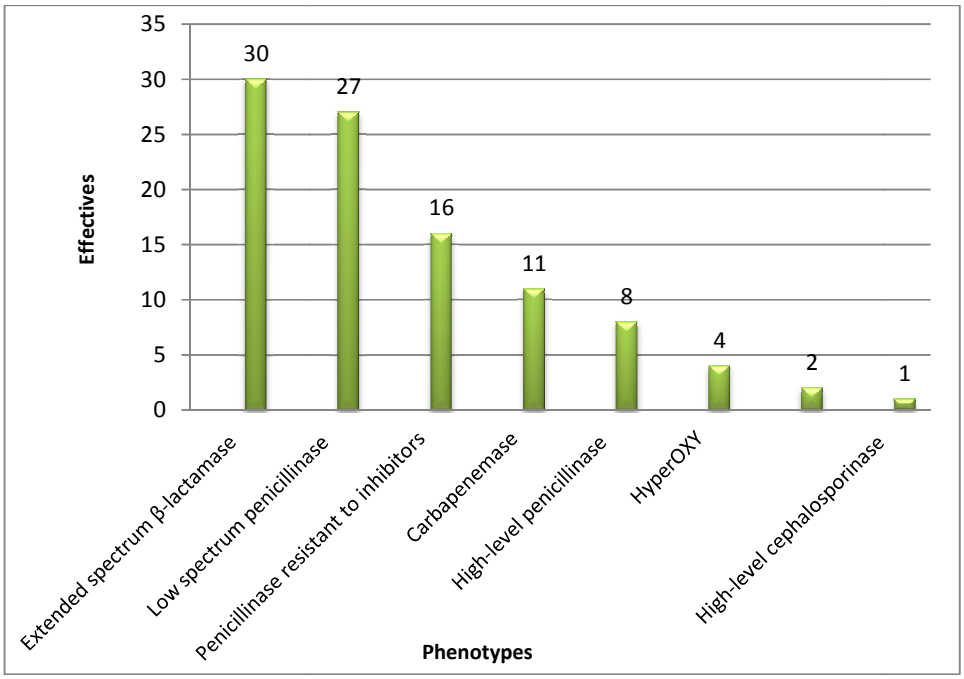

Fig. 1. Phenotypic classification of strains

Table 2. Distribution of ESBL and carbapenemase-producing and non-producing bacteria with respect to the hospital units

Hospital units	ESBL		Total	Carbapenemases		Total
	Producing strains	Non-producing strains		Producing strains	Non-producing strains	
Pediatric	9	26	35	4	31	35
Intensive care	8	19	27	4	23	27
Medical	3	4	7	1	6	7
Gynaeco-obstetric	2	4	6	0	6	6
Surgical	2	3	5	2	3	5
Emergency	3	2	5	0	5	5
Total	27	45	85	11	74	85

contributed to the decrease in the number of ESBL producing strains [22].

Table 3 shows that ESBL phenotypes n = 27 (90%) and carbapenemase n = 9 (81.8%) were the most observed by *Klebsiella pneumoniae pneumoniae.*

The class D carbapenemase (OXA-48) was first described in *K. pneumoniae* and was often associated with other β-lactamases, especially ESBL [23]. In Turkey, several outbreaks of nosocomial infections have been associated with strains producing this type of carbapenemase [20,23]. To our knowledge, no data are available on the clinical impact of cephalosporinases whose spectrum is partially expanded to carbapenemes (Class C).

As shown on Fig. 2, the sensitivity of strains to antibiotics depends on the method used. Out of the 29 strains resistant to cefotaxime by agar diffusion method, only 23 were confirmed by determining the minimum inhibitory concentration (MIC); the other 6 were categorized as "sensitive" or intermediate by the latter.

Table 4 describes the characteristics of some *Klebsiella* strains with respect to the specimens, the hospital unit from which it was isolated, the diameter of the zone of inhibition and MIC values of some beta lactams (ceftazidime, cefotaxime, cefepime and impenem).

Table 3. Distribution of *Klebsiella* spp. based on ESBL and carbapenemase production

Bacteria species	ESBL		Total	Carbapenemases		Total
	Producing strains	Non-producing strains		Producing strains	Non-producing strains	
Klebsiella pneumoniae pneumonia	27	51	78	9	69	78
Klebsiella oxytoca	0	12	12	2	10	12
Klebsiella pneumoniae ozaenae	3	2	5	0	5	5
Klebsiella pneumoniae rhinoscleromatis	0	4	4	0	4	4
Total	30	69	99	11	88	99

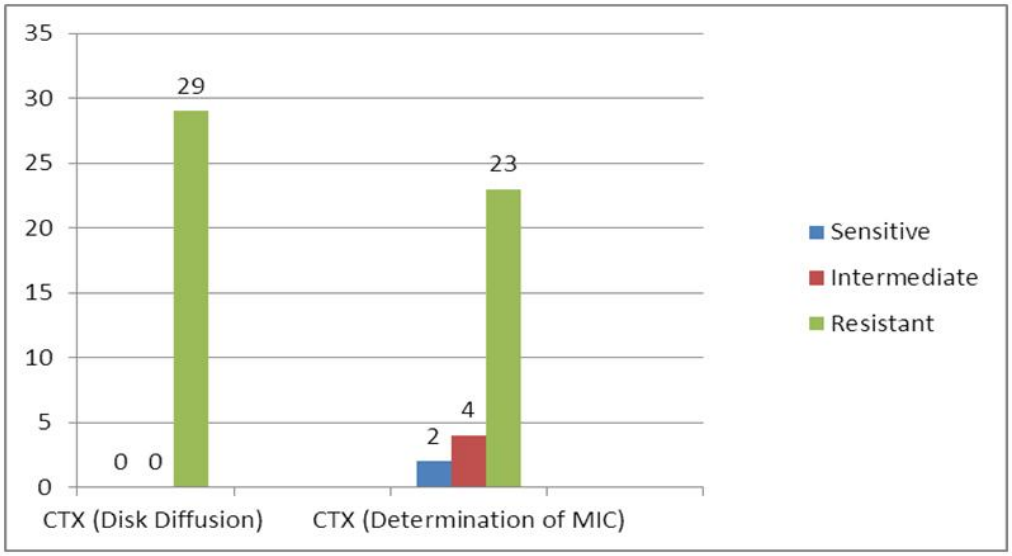

Fig. 2. Comparison of Minimum Inhibitory Concentration (MIC) and the diameter of the inhibition zone of cefotaxime (CTX)

Table 4. Characteristics of the *Klebsiella* spp resistant to 3GC (CAZ: ceftazidime, CTX

Code	Unit	Specimen	Diameter of the zone of inhibition (mm)				MIC (µg/ml)			
			CTX	CAZ	FEP	IMP	CTX	CAZ	FEP	IMP
K. 19	Eme	Pus	15(R)	27(R)	21(I)	29(S)	>256(R)	16(R)	8(I)	0.25(S)
K. 36	Ext	Pus	12(R)	14(R)	20(I)	29(S)	32(R	24(R)	6(I)	0.25(S)
K. 51	Med	Urine	14(R)	16(R)	20(I)	31(S)	48(R)	8(I)	7(I)	0.25(S)
K. 52	ICU	Urine	15(R)	18(R)	23(I)	28(S)	64(R)	4(I)	3(I)	0.19(S)
K. 53	Gynaeco	Pus	13(R)	14(R)	22(I)	30(S)	>256(R)	16(R)	6(I)	0.25(S)
K. 54	Med	Blood	00(R)	11(R)	15(R)	31(S)	256(R)	48(R)	32(R)	0.25(S)
K. 55	Eme	Urine	15(R)	16(R)	18(I)	31(S)	32(R)	6(I)	6(I)	0.25(S)
K. 56	ICU	Urine	24(I)	21(I)	29(S)	31(S)	2(I)	4(I)	1(S)	0.12(S)
K. 57	Sur	Pus	00(R)	13(R)	16(R)	31(S)	256(R)	32(R)	12(R)	0.25(S)
K. 58	Ped	Urine	14(R)	21(I)	23(I)	30(S)	16(R)	3(I)	3(I)	0.25(S)
K. 85	Ped	Urine	11(R)	11(R)	15(R)	28(S)	>256(R)	32(R)	16(R)	0.38(S)
K. 86	Eme	Urine	00(R)	00(R)	15(R)	31(S)	128(R)	32(R)	16(R)	0.12(S)
K. 87	Ped	Urine	13(R)	17(R)	20(I)	30(S)	32(R)	12(R)	4(I)	0.25(S)
K. 88	Med	Pus	13(R)	17(I)	22(I)	27(S)	>256(R)	8(I)	8(I)	0.75(S)
K. 89	Med	Pus	11(R)	12(R)	22(I)	26(S)	32(R)	16(R)	6(I)	0.75(S)
K. 90	ICU	Vaginal secretion	08(R)	10(R)	14(R)	29(S)	>256(R)	96(R)	48(R)	0.75(S)
K. 91	ICU	Urine	11(R)	13(R)	18(I)	30(S)	32(R)	12(R)	6(I)	0.19(S)
K. 92	ICU	Urine	14(R)	14(R)	19(I)	26(S)	0.06(S)	6(I)	6(I)	0.19(S)
K. 93	Ped	Urine	22(R)	20(I)	34(S)	22(I)	2(I)	0.25 I	0.064(S)	0.75(S)
K. 94	Ped	Urine	12(R)	00(R)	16(R)	25(S)	12(R)	1(S)	2(I)	0.12(S)
K. 96	Sur	Urine	11(R)	13(R)	16(R)	26(S)	>256(R)	48(R)	16(R)	0.38(S)
K. 97	Ped	Blood	21(R)	21(I)	23(I)	31(S)	1.6(I)	2(I)	2(I)	0.25(S)
K. 98	Ped	Urine	00(R)	09(R)	15(R)	25(S)	128(R)	48(R)	24(R)	0.38(S)
K. 99	ICU	Pus	10(R)	12(R)	16(R)	25(S)	96(R)	24(R)	12(R)	0.38(S)
K.6	Ped	Blood	08(R)	10(R)	16(R)	32(S)	0.06(S)	0.25 S	0.06(S)	0.38(S)
K.76	Ped	Blood	12(R)	15(R)	18(I)	31(S)	64(R)	12(R)	8(I)	0.19(S)
K.78	ICU	Urine	13(R)	00(R)	14(R)	27(S)	256(R)	256(R	32(R)	0.38(S)

cefotaxime), 4GC (FEP:cefepime) and carbapenemes (IMP: imipenem) Eme=Emergency ; Ext=Out-Patients ; Med=Medical ; ICU=Intensive Care Unit ; Gynaeco= Gynaecological ; Ped=Paediatric

4. CONCLUSION

The interpretation of the AST has enabled the establishment of a high prevalence of expanded spectrum β-lactamase and consequently leading to an increase in the presence of carbapenemase producing *Klebsiella* spp. This could lead to therapeutic failure in case of treatment with beta-lactamines antibiotics. Therefore this trend needs to be monitored.

CONSENT

All the patients recruited for the study signed the consent form.

ETHICAL APPROVAL

An ethical clearance for this study was obtained from the National Ethic Committee for Human Research in Cameroon.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
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