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RESEARCH ARTICLE

Phenotypic Detection of Extended Spectrum β-Lactamase and AmpC producing *Enterobacteriaceae* Isolated in A General Hospital

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ABSTRACT

Objective: The antibiotic resistance of *Enterobacteriaceae* is a worldwide preoccupation, and misuse antibiotics of beta-lactam group allowed the development of bacteria producing extended spectrum beta-lactamase and cephalosporinase AmpC enzymes type resistance. The aim of this study was to determine the frequency of these enzymes among strains isolated at the General Hospital in Douala, Cameroon.

Methods: We conducted a cross-sectional study. For phenotypic detection of resistance enzymes, MASTDISCS™ test impregnated third and fourth generation cephalosporin's was used by diffusion on Mueller Hinton agar. Measuring the inhibition areas and comparing the inhibition diameters determined the nature of the resistance mechanism.

Results: This study included 195 strains of *Enterobacteriaceae*. The most frequent species were *Escherichia coli* and *Klebsiella pneumoniae*, with a frequency of 49.2% and 31.3% respectively. After determination of resistance phenotypes, 101 (51.8%) isolates were found to be producing resistance enzymes. The frequency of ESBL-producing *Enterobacteriaceae* was 19.5%; AmpC producing was 14.3% and both enzymes (AmpC + ESBL) 17.9%. *E. coli* and *K. pneumoniae* resistance rates were 90% and 83.7% for Cotrimoxazole, 82.5% and 78.3% for ciprofloxacin, 20% and 13.5% for Amikacin, respectively. Imipenem, Amikacin and Fosfomycin were the most active molecules with 4.9%, 19.8% and 33.6%, out of 101 resistant strains, respectively.

Conclusion: This study showed a high frequency of resistance enzyme producing strains. This situation leads to resistance to antibiotics most commonly used. This finding justifies a change in prescription habits for protection of molecules that are still active. *J Microbiol Infect Dis 2018; 8(3):113-119*

Keywords: AmpC, Cameroon, *Enterobacteriaceae,* ESBL, Resistance

INTRODUCTION

Bacterial resistance to antibiotics is one of the most important problems in the anti-infective therapeutics worldwide; among the offending germs, *Enterobacteriaceae* have an important place in infectious diseases [1]. These infectious diseases are responsible for 45% of deaths in low-income countries and 70% of all cases of mortality due to microorganisms worldwide [2]. According to the World Health Organisation (WHO) and the National Institute of Health (NIH), they would be the second cause of death and the major cause of loss of productive years of life [3,4]. For the treatment of these infections, beta-lactams are from the most prescribed antibiotic molecules particularly in Africa [5]. Bacteria have developed different mechanisms to counteract the action of these molecules; one of the most common is the enzymatic resistance through β-lactamase production [6,7]. The main groups of β-lactam antibiotics have a β-lactam cycle whose amine linkage may be hydrolysed by these resistance enzymes, resulting in a microbiologically inactive compound [8,9]. Several studies have shown that the emergence of these resistances is the consequence of the

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overuse of antibiotics resulting in a selection pressure; due to persistent exposure of the bacterial strains to antibiotics, creating excessive productions and mutations of β-lactamases. These new enzymes called extended spectrum β-lactamases have the property to confer resistance to Penicillin's and 1st, 2nd, 3rd, and 4th generation cephalosporins to bacteria [10]. Another class of β-lactamase, the AmpC is also clinically significant by the fact that it confers resistance to cephalosporin, and is not affected by β-lactamase inhibitors [11].

Studies concerning the production of ESBL and AmpC around the world have shown that, the resistance phenomenon is observed both in developed countries and in Africa, particularly in Cameroon where according to a study done at the Yaoundé Central Hospital, the prevalence of ESBL-producing strains was 12%, observed in all species of *Enterobacteriaceae* studied (*Klebsiella spp.* 18.8%, *Citrobacter* spp. 17.6%, *E. coli* 14.3%, Proteus spp. 1.8%) [12]. Another study performed in Ngaoundere shows the emergence of community strains of ESBL-producing *Enterobacteriaceae*, favoured by the empirical use of antibiotics, including amoxicillin, ciprofloxacin and Trimethoprim/ sulfamethoxazole [13]. In 2015 in General Hospital Douala (GHD), we observed high rates of resistance of *Enterobacteriaceae* to antibiotics, including the beta lactams, these rates were in continuous progression over the years [14].

This study intended to determine the frequency of ESBL and AmpC in *Enterobacteriaceae* isolated in Laboratory of Clinical Biology at the GHD (Cameroon), using simple phenotypic method.

METHODS

Location and type of study

This was a cross sectional study, conducted within the Bacteriology Unit of Clinical Biology Laboratory at the General Hospital, Douala, Cameroon, over a five months period from 1st August to 31st December 2015.

Studied strains

All isolates of *Enterobacteriaceae* from patients hospitalized in different services of the GHD, and outpatient diagnostic levies, were included in the study. The stains obtained during the study period, were consecutively and exhaustively sampled. The isolates considered contaminants and those from stools have been excluded.

Identification of strains and study of susceptibility to antibiotics

The samples were cultured and incubated for 24 hours at 37 ^{0}C, on Cysteine Lactose Electrolyte Deficient (CLED) agar for urines samples, MacConkey and Eosin Methylene Blue (EMB) for other specimen (pus, puncture fluids, sputum, catheters, urinary probes, and blood cultures).

The identification of the isolated bacterial species, based on the study of biochemical and enzymatic characters, was made by seeding ID32E® galleries (bioMérieux), followed after 24 hours of incubation, by automatic colorimetric reading on Mini API™ (bioMérieux, Marcy l'Etoile, France).

The study of susceptibility to antibiotics was done by the dilution technique on ATB G - EU™ and ATBUR EU™ galleries (bioMérieux) incubated at 37 ^{0}C for 24 hours, then automatic turbidinephelometric reading and expertisation of results on Mini API™, according to the breakpoints of Committee of susceptibility to antibiotics of the French Society of Microbiology (CASFM).

The following antibiotics molecules have been studied in the susceptibility test galleries: Amoxicillin (AMO), Amoxicillin + Clavulanic acid (AMC), Ticarcillin (TIC), Piperacillin (PIC), Piperacillin + Tazobactam (TZP), Cefalotin (CFT), Cefuroxime (CXM), Cefixime (CFM), Cefotaxime (CTX), Cefoxitine (CXT), Ceftazidime (CAZ), Cefepime (CMP), Imipenem (IMI), Meropenem (MERO), Gentamicin (GEN), Tobramycin (TOB), Amikacin (AKN), Tetracyclin (TET), Nalidixic Acid (NAL), Ofloxacin (OFL), Levofloxacin (LEVO), Ciprofloxacin (CIP), Sulfamethoxazole + Trimethoprim (TSU), Fosfomycin (FOS).

ESBL and AmpC detection

Phenotypic detection of enzymes-hydrolysing-Beta lactams production was made by the diffusion method on Mueller-Hinton agar, using the Mastdiscs ID™ kits.

The confirmation kit for production of extended spectrum β-lactamases without chromosomal, inducible or dereprimed AmpC (D52C) consisted of six discs (Z_1 to Z_6) made up as follows: Z_1=CAZ 30 µg, Z_2=CAZ 30 µg + Clavulanic Acid (CLAV) 10 µg, Z_3=CTX 30 µg, Z_4=CTX 30 µg + CLAV 10 µg, Z_5=CPD 30 µg, Z_6=CPD 30 µg + CLAV 10 µg.

The confirmation kit for extended-spectrum-β-lactamases production with chromosomal AmpC (D63C) contained two discs: Z'_1=CMP 30 µg and Z'_2=CMP 30 µg + CLAV 10 µg.

The discs in each kit were applied on 4 mm thickness Mueller-Hinton agar on Petri dishes of 90 mm diameter, seeded by a bacterial suspension of 0.5 McFarland.

A 30 mm distance between two discs, and 15 mm between the discs and the edge of the Petri dishes was respected in order to avoid overlapping zones of inhibition. The dishes were then incubated at 37 ^{0}C for 18 hours.

Reading has consisted in the measure with caliper and the comparison of different discs inhibition diameters.

Interpretation

According to the manufacturer, ESBL production was positive when the difference between the diameters of inhibition of discs [Z_2-Z_1], [Z_3-Z_4] or [Z_5-Z_6] was equal or greater than 5 mm, then AmpC production was positive when [Z'_2-Z'_1] was equal or greater than 5 mm.

Ethics approval

This study was conducted in accordance with ethics directives related to research in Cameroon. We obtained the research authorizations of the Regional Delegation of the Health of Littoral Region, the Director of the GHD, and ethical clearance from the Institutional Ethics Committee of Research for Human Health from University of Douala.

Statistical Analyses

All statistical analyses were done using Sphinx software version 5 (Sphinx Development, Chavanod, France). Word processing was done on the Microsoft Word version 2013. The charts and graphs were made with data base software Microsoft Excel version 2013. The analysis software Featured was achieved according to proportions calculations (frequency and percentage) for categorical variables and calculations of means and standard deviations for quantitative variables.

RESULTS

A total of 195 strains of *Enterobacteriaceae* were isolated from all samples collected in the Bacteriology Unit of the Clinical Laboratory of the GHD, during the study period.

The samples came from outpatients (51.3%) and patients hospitalized in the various departments of the Hospital, in particular Pediatrics (15.4%), Medicine (13.3%), Surgery (10.8%) and Intensive Care Unit (6.2%). Among these patients, we noted a female predominance (55.9%), and ages ranging from 0 to 93 years with an average of 37.5 years. Regarding the type of sampling, 71.8% of the strains were isolated from the urine, and 21.5% from suppurations.

The isolated strains were constitutes mostly of *E. coli* (n=96), followed by *K. pneumonia* (n=61), *Enterobacter spp.* (n=13), *Serratia spp.* (n=7), *Proteus mirabilis* (n=6), *Citrobacter spp.* (n=5), *Providencia stuartii* (n=4), *Morganella morganii* (n=2) and *Yersinia intermediae* (n=1) (Table 1).

Resistance to betalactamines

There is a high overall resistance of strains to Penicillins and Cephalosporins; resistance levels ranging from 38.5% for Piperacillin + Tazobactam, to 85.5% for Ticarcillin. The most active molecules were Imipenem (6 resistant strains or 3.1%), and Meropenem (24 resistant strains or 12.3%) (Figure 1).

ESBL and AmpC Enzymes production

Enterobacteriaceae that developed an ESBL-type or AMPC-type enzymatic resistance accounted for 51.8% of all isolated strains, i.e. 101 strains. Among the 101 strains, 38 have been showed ESBL production (19.5%), 28 AmpC (14.3%), and 35 the two types of enzymes (17.9%) (Table 1). Overall, the *E. coli* was the most represented, but the *Klebsiella* showed a higher rate of enzyme production (41.6% and 59% respectively). Of all the isolated species, only *Morganella* (2 strains) did not show any enzyme production. For some species, all isolated strains produced at least one resistance enzyme, namely *Citrobacter*

freundii, *Providencia stuartii*, and *Yersinia intermediae.*

Table 1. Characteristics of 195 *Enterobactericeae* strains.

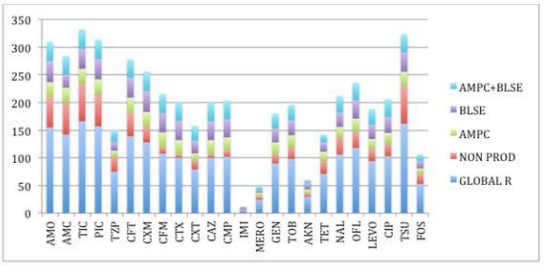
Characteristics		Total n (%)	Non Prod n=94	REP* n=101	AmpC n=28	ESBL n=38	AmpC + ESBL n=35	P
Age (y)	≤20	54 (27.7)	36	18	4	7	7	0.03
	20-40	54 (27.7)	22	32	10	9	13	
	40-60	47 (24.1)	15	32	7	15	10	
	>60	40 (20.5)	21	19	7	7	5	
Gender	Male	86 (44.1)	33	53	16	21	16	0.01
	Female	109 (55.9)	61	48	12	17	19	
Bacteria	*E. coli*	96 (49.2)	56	40	11	21	8	0.04
	K. pneumoniae	61 (31.3)	24	36	8	12	17	
	Enterobacter spp.	13 (6.7)	3	10	6	1	3	
	S. odorifera	7 (3.6)	3	4	1	2	1	
	P. mirabilis	6 (3.1)	4	2	1	0	1	
	Citrobacter spp.	5 (2.6)	2	3	0	0	3	
	P. stuartii	4 (2.1)	0	4	1	1	2	
	Y. intermediae	1 (0.5)	0	1	0	1	0	
	M. morganii	2 (1.0)	2	0	0	0	0	
Wards	Medicine	26 (13.3)	12	14	2	2	10	0.05
	Surgery	21 (10.8)	3	18	7	7	4	
	Intensive Care	12 (6.2)	1	11	3	5	3	
	Burn Unit	2 (1.0)	0	2	1	0	1	
	Pediatrics	30 (15.4)	22	8	3	2	3	
	Gynecology	4 (2.1)	2	2	2	0	0	
	Outpatients	100 (51.3)	54	46	10	22	14	
Sample	Urine	140 (71.8)	81	59	15	23	21	0.02
	Pus	42 (21.5)	10	32	10	11	11	
	Urinary probe	7 (3.6)	2	5	2	3	0	
	Puncture liquid	2 (1.0)	0	2	1	0	1	
	Catheter	2 (1.0)	0	2	0	1	1	
	Blood	2 (1.0)	1	1	0	0	1	

Regarding services, strains isolated in pediatrics showed the lowest production rate of ESBL and AmpC (26.6%), these rates ranged from 46% for outpatients to 91.6% for patients hospitalized in the Intensive Care Unit. Strains isolated produced 76.1% resistance enzymes in the suppurations and 42.1% in the urine.

DISCUSSION

This study concerning the detection of ESBL and AmpC production in Enterobacteriaceae isolated at the GHD shows the predominance of E. coli and K. pneumoniae among the isolated strains. This predominance had already been observed in our laboratory in similar proportions, with a high overall resistance to betalactamines, other authors have observed the same distribution [12,14-16]. Since their discovery, the production of ESBL and AmpC enzymes in

Enterobacteriaceae has continued to grow; we found high levels of production of these resistance enzymes alone, and even of the two associated. Gangoué in Yaoundé, Cameroon between 1995 and 1998, Raji in Nigeria in 2011, and Singhal in India found ESBL production rates of 12%, 37.3% and 64% respectively [12,15,16]. These rates may vary depending on the methods used for detection, and geographic regions [17].



AMO = Amoxicillin; AMC = Amoxicillin + Clavulanic acid; TIC = Ticarcillin; PIC = Piperacillin; TZP = Piperacillin + Tazobactam; CFT = Cefalotin; CXM = Cefuroxime; CFM = Cefixime; CTX = Cefotaxime; CXT = Cefoxitine; CAZ = Ceftazidime, CMP = Cefepime; IMI = Imipenem; MERO = Meropenem; GEN = Gentamicin; TOB = Tobramycin; AKN = Amikacin; TET = Tetracyclin; NAL = Nalidixic Acid; OFL = Ofloxacin; LEVO = Levofloxacin; CIP = Ciprofloxacin; TSU = Sulfamethoxazole + Trimethoprim; FOS = Fosfomycin.

Figure 1. Antibiotics resistance levels.

The discs used in this study make it possible to carry out a quick and simple phenotypic test, and especially to conclude that there is no production of ESBL by *Enterobacteriaceae* [18]. Black et al. also demonstrated the reliability of this simple and inexpensive technique for the detection of producing AmpC *Enterobacteriaceae* on genotypically identified strains [19]. Detection of AmpC enzymes is a problem for clinical laboratories, they should be able to detect important antibiotics resistance mechanisms in time, with sensitive and specific methods like found by Nassim et al; to contribute to clinical and infection control decision making [20]. Optimal disc spacing is necessary with the double disk test to avoid falsely negative or inconclusive results [21].

Isolates that coproduce both an ESBL and a high level of AmpC are becoming more comon; in 2002, Moland et al found that 2% of the *K. pneumoniae* strains isolated in US hospitals had this two enzymes; Manoharan et al. showed in 2012 that 92% of the AmpC-producing Enterobacteriacea strains isolated in Indian hospitals also produced ESBL [22,23].

K. pneumoniae and *E. coli* were the major species producing ESBL and AmpC, a finding also observed in other studies [12,16]. Regarding Enterobacter, the isolated strains were more productive of AmpC than ESBL because of their constitutive chromosomal production of AmpC [24]. These strains are most often found in the pus and urine and medical devices of patients hospitalized in the Intensive Care Unit and in Surgery [12,24]. At General Hospital of Douala, the lowest rates were observed for strains from Pediatric samples.

We observed higher levels of antibiotic resistance of ESBL and AmpC producing strains than non-producing strains; this is true for betalactamines as well as for other families of antibiotics. ESBL-producing strains are usually multidrug-resistant; plasmids inducing the production of these enzymes are mobile genetic elements that also frequently carry the genes coding for resistance to Aminoglycosides, Fluoroquinolones and Cotrimoxazole [24,25]. AmpC betalactamase production is also frequently accompanied by multi-resistance to antibiotics [26].

The Piperacillin-Tazobactam combination, which is usually active on ESBL-producing strains, has been shown to be ineffective in many cases (between 39% and 60% resistance), despite the low use of this combination compared to Amoxicillin-Clavulanic acid.

High levels of resistance to third-generation Cephalosporins should exclude these molecules from antibiotic regimens. According to Paterson et al., restricting their use would reduce the incidence of ESBL production by *K. pneumoniae* [24].

This multidrug resistance of strains isolated at the General Hospital, also affects Quinolones, with high levels. This high rate is the consequence of the acquisition of antibiotic resistance factors, following the therapeutic habits, the protocols used in the services and the pressure of selection; these molecules should be excluded as an alternative to the management of third-generation Cephalosporins-resistant bacteria, including urinary tract infections for which these antibiotics are widely used [24].

Isolated strains showed good sensitivity to Imipenem and Amikacin. These antibiotics are active on *Enterobacteriaceae* producing ESBL

and AmpC isolated in several countries [23,27,28]. AmpC hydrolyzes all Betalactamines with the exception of Cefepime and Carbapenems [29]. However, the AmpC-producing strains in our study, although all susceptible to Imipenem, showed resistance to Meropenem and Cefepime. These strains may have developed other mechanisms involving Carbapenem resistance.

Lack of confirmation of the detection of resistance enzymes identified by molecular methods represents a limitation in this study, as well as the small sample size.

Conclusion

This study showed a high frequency of ESBL and AmpC producing strains at the GHD. This situation leads to multidrug resistance to antibiotics most commonly used in the treatment protocols of infections in patients. These resistance rates are due to poor prescription habits and excessive consumption of antibiotics. This finding justifies a change in prescription habits for the protection of molecules that are still active and for the reduction of the production rate of these resistance enzymes.

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Authors' contribution: COE and DA coordinated the study and drafted the manuscript, COE, JPNM, RNM and ERM collected data and participated in its design, COE and ET performed the statistical analysis. All the authors read and approved the final manuscript.

Declaration of Conflicting Interests: The authors declare that they have no conflict of interest.

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