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DIVERSITY AND DISTRIBUTION OF BIOTYPES AND ANTIBIOTIC RESISTANCE PHENOTYPES OF *ENTEROBACTER* SSP. ISOLATED FROM PATIENTS IN YAOUNDE, CAMEROON

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Abstract
Since the prevalence of *Enterobacter* spp. as a nosocomial pathogen is on the increase and the prevalence of β-lactams resistant strains is on the rise, a study was designed to analyze the diversity and distribution of *Enterobacter* spp. at the Yaounde General Hospital with regard to biochemical and antibiotic resistance profiles. From December 2006 to August 2007, a prospective study on *Enterobacter* strains isolated from clinical samples was conducted in the bacteriology laboratory of the Yaounde General Hospital. In addition to phenotypes derived from the commercial biochemical kit API 20E (BioMèrieux), 3 biochemical tests were performed. Antibiotics resistances were determined on Mueller Hinton agar by standard disks diffusion procedures to 12 antibiotics. There was a great variability in biotypes within the species *cloacae* with 9 distinct biotypes identified. Phenotypes characterized by a high level of resistance (more than 6 antibiotics tested) were present both in community and hospitalized patients. 7 strains of *Enterobacter* were identified as strains producing extended spectrum betalactamases. For all cephalosporins, the percentages of resistance were greater among hospitalized patients, children and new-borns being the most affected. The resistance were higher in blood cultures than in other samples. The distribution of resistance within the *Enterobacter* genera was different depending on whether the patient was hospitalized or not, the category of age to which he belongs and the specimen from which the strains were isolated. There was no association between biotypes isolated and the origin or the type of the specimen and the patient's age.
Keywords: *Enterobacter spp., biotypes, antibiotic resistance*

RÉSUMÉ

Enterobacter spp. a été reconnu comme un pathogène nosocomial de plus en plus important au cours de ces dernières années. Sa résistance intrinsèque à l'ampicilline et aux céphalosporines de 1ère et 2e génération ainsi que la sélection fréquente de mutants résistants aux céphalosporines à large spectre ou aux aminoglycosides a contribué à l'augmentation de la prévalence de cette espèce, en particulier chez les patients immunodéprimés. La connaissance de l'épidémiologie des infections bactériennes est essentielle dans la mise en œuvre des politiques de contrôle appropriées.

A notre connaissance, il n'existe pas de données publiées sur l'épidémiologie des infections à *Enterobacter* à l'Hôpital Général de Yaoundé. C'est fort de ce constat que nous nous sommes proposés d'analyser la diversité et la distribution au sein de ce genre bactérien au regard de deux marqueurs phénotypiques (profil biochimique et profil de résistance aux antibiotiques).

Pour atteindre cet objectif, une étude prospective sur les souches d'*Enterobacter* isolées des produits pathologiques a été réalisée au laboratoire de bactériologie de l'Hôpital

Général de Yaoundé de Décembre 2007 à Août 2008. En plus des phénotypes dérivés des tests biochimiques de la galerie API 20E (Bio-Mèrieux), 3 tests biochimiques ont été réalisé. Les profils de résistance aux antibiotiques ont été déterminés sur gélose Mueller Hinton par la méthode standard de diffusion des disques vis-à-vis de 12 antibiotiques. Les disques étaient déposés de manière à mettre en évidence la production de bétalactamases à spectre élargi (BLSE). Les résultats ont été interprétés selon les recommandations du CLSI.

Durant la période d'étude, au total 23 souches d'*Enterobacter* spp. ont été isolées des différents spécimens cliniques. De ces isolats, 65% provenaient des malades hospitalisés. *Enterobacter cloacae* et *Enterobacter aerogenes* étaient les espèces les plus fréquentes. Les urines et les hémocultures étaient les principaux sites d'isolement, regroupant respectivement 52,2% et 21,74%.

On a observé une grande variabilité des biotypes au sein de l'espèce *cloacae* avec 9 biotypes distincts identifiés. Cependant, on n'a pas noté d'association entre les biotypes identifiés et la catégorie d'âge du patient ou la nature du produit pathologique. Dans l'ensemble, les fréquences de résistance aux β-lactamines étaient de 25% pour la ticarcilline, 56,5% pour l'amoxycilline/acide clavulanique et variaient de 39,5 - 43,5% pour les céphalosporines de 3^{e} génération. Pour les quinolones, les pourcentages de résistance étaient de 17,4 et 30% pour la ciprofloxacine et l'acide nalidixique respectivement. Aucune souche isolée n'a présenté une résistance à l'imipenem. La production de BLSE a été détectée chez 7 (30,43%) souches. Les phénotypes caractérisés par un haut niveau de résistance (plus de 3 familles d'antibiotiques) sont présents aussi bien chez les patients hospitalisés que chez les patients externes. La distribution des résistances selon le statut du patient a montré que pour toutes les céphalosporines, les pourcentages de résistance étaient plus importants chez les patients hospitalisés ; les enfants et les nouveaux-nés étant les plus affectés. Les résistances aux céphalosporines, aux quinolones, à la gentamicine, au trimethoprime sulfamethoxazole, à l'amoxycilline et à l'amoxycilline/acide clavulanique étaient plus élevées dans les hémocultures que dans les autres produits pathologiques. Aucune souche isolée des nouveaux-nés n'a présenté une résistance à l'acide nalidixique.

L'ensemble des résultats de cette étude souligne l'importance d'*Enterobacter* comme pathogène associé aux infections hospitalières et la dissémination des souches résistantes aux β-lactamines. Il ressort que, la distribution de la résistance au sein des espèces du genre *Enterobacter* à l'Hôpital Général de Yaoundé est différente selon que le patient est hospitalisé ou non, la catégorie d'âge à laquelle il appartient et la nature du produit pathologique. Il n'existe aucune association entre les biotypes isolés et l'origine ou la nature du prélèvement et l'âge du patient.

INTRODUCTION

Enterobacter spp. are among the most Gram negative pathogens associated with nosocomial infections. It has been found to account for 4-12% of sepsis caused by Gram negative organisms, accompanied by a mortality rate ranging from 15 – 87% (1). As an opportunistic pathogen, *Enterobacter* primarily attacks immunocompromised individuals who are hospitalized and suffer from severe underlying disease (2). Its intrinsic resistance to ampicillin and narrow spectrum cephalosporins as well as the frequent selection of mutants resistant to extended cephalosporins or to aminoglycosides has contributed to the increase in prevalence of this species(3). More recently, it appears that *Enterobacter* spp., including multiple resistant strains, have spilled over into community. This spread presents a serious threat to favorable outcome in the treatment of *Enterobacter* infections in community and hospital settings. Knowledge about epidemiology of *Enterobacter* infections is essential to guide appropriate control policies (4).

Although DNA based techniques are the gold standard for epidemiological studies, antibiotics profiles and biochemical results, are routinely employed in clinical laboratories because they are easy to perform and to interpret and relatively inexpensive (4).

The aim of this study was to compare *Enterobacter* strains isolated from hospitalized and community patients by looking at their antibiotic resistance patterns and biochemical profiles with regard to the type of specimen and the category of age to which the patient belongs.

MATERIAL AND METHODS

Bacterial strains. *Enterobacter* ssp. strains were recovered from hospitalized and community patients received in the Yaounde General Hospital over a 9-months period (from December 2006 to August 2007). Presumptive *Enterobacter* ssp. isolates were identified on the basis of conventional microbiological procedures (5).

Full biochemical identification was determined by the API 20E system (6). Isolates were maintained at -70°C in skimmed milk. *Escherichia coli* 25922 was used as control strain.

Biotyping. In addition to phenotypes derived from commercial biochemical test kits according to manufacturer's instructions, the following tests were performed using conventional manual methods. Production of DNase was determined in DNA medium incubated for 24 hrs at 37°C. The plates were observed against a white background. DNA producing- strains appeared with a distinct clear zone surrounding spot inoculums.

Esculine hydrolysis was assessed by inoculating a bile esculine plate with the organism. Presence of a dark brown to black colour indicated esculine splitting.

Hemolysis test was performed by observation of zone of hemolysis after growth for 24 hrs on blood agar medium.

For strains presenting the same identification number, any difference in the results from positive to negative and from negative to positive with at least one of the biochemical test characterized a different biotype.

Antibiotyping. Antibiotic resistance profiles were determined on Mueller Hinton by standard disk diffusion procedures (7) to the following antibiotics: amoxycilline (25µg), amoxycillin-clavulanic acid (20/10µg), ceftazidime (30µg), ceftriaxone (30 µg), cefotaxime (30µg), ciprofloxacine (5µg), trimethoprim-sulfamethoxazole (1,25/23,75µg), gentamicin (10µg), nalidixic acid (30 µg), imipenem (10µg) and ticarcillin (75 µg) agar (oxoïd; Basingstoke, UK and Becton Dickinson Microbiology system, Cockesville).

The control strain *Escherichia coli* ATCC 25922 was run simultaneously with the test organisms. Results were interpreted according to the CLSI guidelines.

Isolates were reported as resistant if the diameters of the inhibition zone were within the intermediate range.

Screening test for ESBL producing strains. Amoxicillin-clavulanic acid (20/10 ug) disc was placed toward the center of the plate, a ceftazidime (30 ug) was placed 15 mm out from the edge of amoxicillin-clavulanic acid disc at 90° angle, so that its inner edge was 15 mm from it. The same was performed with ceftriaxone (30 ug) or cefotaxime (30 ug) discs so that they were spaced 90° apart and 15 mm from amoxicillin-clavulanic acid disc. A cefoxitin (30 ug) disc known as inducer of ESBL was also placed. Plates were incubated at 37°C aerobically for 18-24 hrs. Extension of the zone of inhibition toward the Amoxicillin-clavulanic acid disc indicated the presence of an ESBL (8).

Data analysis. Antibiotic resistance profiles and frequencies were determined with WHONET software version 5.0 provided by WHO.

For statistical analysis, the Chi square test was employed. Differences were considered statistically significant with p-value < 0,05.

RESULTS

General epidemiology. A total of 23 *Enterobacter* strains (16 *Enterobacter cloacae*, 3 *Enterobacter aerogenes*, 2 *Enterobacter sakazakii* and 2 *Enterobacter asburiae*) were isolated during the study period. Among these, 15 (65%) were recovered from hospitalized patients (in-patients) and 8 (35%) from community patients (out-patients). Specimens from which they were isolated included urine (52,2%), blood (21,74%) and others (26,09%). In hospitalized patients, strains from the pediatric unit represented nearly 66,7% of the isolates.

Biotyping. The biochemical profiles obtained for each strain allowed the differentiation in biotypes. The defining tests were hydrolysis of esculine, production of DNase and production of hemolysis.

All the *Enterobacter* isolates were DNase negative and non hemolytic. Hydrolysis of esculine was observed in all the *Enterobacter asburiae* and *Enterobacter aerogenes* isolates, where as 3 of the 16 *Enterobacter cloacae* as well as 1 of the 2 *Enterobacter sakazakii* did not show ability to split esculine.

The 16 *Enterobacter cloacae* isolates were divided into 9 biogroups according to their biochemical profiles. *Enterobacter aerogenes* and *Enterobacter asburiae* were classified each into 2 biotypes, while *Enterobacter sakazakii* isolates belonged to the same biotype.

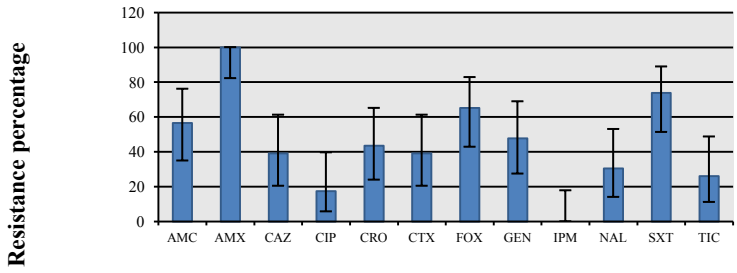
However, there was no association between biotypes identified and the type of the specimen

TABLE 1: Distribution of resistance phenotypes

Variable	Resistance phenotypes		p-value
	Resistant to 6 or more antibiotics	Resistant to less than 6 antibiotics	
Patient's status			
in-patients (%)	7 (46,7 %)	8 (53,3 %)	0,4125
out-patients (%)	4 (50 %)	4 (50 %)	
Type of specimen			
urine (%)	4 (33,4 %)	8 (66,6 %)	
blood (%)	4 (80 %)	1 (20 %)	0,5572
others (%)	4 (66,6 %)	1 (33,4 %)	
Age group of the patient			
adult (%)	6 (46,2 %)	7 (53,8 %)	
infant (%)	3 (100 %)	0 (0 %)	0,527
new-born (%)	2 (28,6 %)	5 (71,4 %)	

or the category of age to which the patient belonged.

Antibiotic resistance. Disk diffusion susceptibility tests showed all the strains to be resistant to amoxicillin, while there was 100% susceptibility to imipenem. Results of the susceptibility testing are summarized in figure 1. The frequency of resistance to β-lactam antibiotics were 25% for ticarcilline, 56.5% for amoxycillin/ clavulanic acid and ranged from 39.5-43.5% for the 3rd generation cephalosporins. For quinolones, resistance percentages were 17.4% and 30% for ciprofloxacin and nalidixic acid respectively.



Abbreviations: AMX: amoxycilline (25µg), AMC: amoxycillin-clavulanic acid (20/10µg), CAZ: ceftazidime (30µg), CRO: ceftriaxone (30 µg), CTX: cefotaxime (30µg), CIP: ciprofloxacine (5µg), SXT: trimethoprim-sulfamethoxazole (1,25/23,75µg), GEN: gentamicin (10µg), NAL: nalidixic acid (30 µg), IPM: imipenem (10µg), TIC: ticarcillin (75 µg).

Using double disk synergy test, ESBL production was detected in 7 (30,43%) of the 23 *Enterobacter* isolates.

Table 1 shows the comparison of the distribution of resistance phenotypes with regard to patient's status, types of specimen and age group of the patient. Phenotypes characterized by a high level of resistance (more than 5 antibiotics tested) are present in both community and hospitalized patients; 4 (50%) of the 8 community strains were resistant to more than 5 antibiotics while these phenotypes represented 7 isolates of the 15 hospital strains. However the difference observed was not significant (p>0,05). Differences in the distribution of these high level of resistance phenotypes were not statistically significant (p>0,05) depending on whether the specimen was urine, blood or others. Like for the specimen type, this difference was not statistically significant (p>0,05) between the age group to which the patient belonged.

Table 2 shows the comparison of antibiotic resistance among hospitalized and community patients. For all cephalosporins, the percentages of resistance were greater among hospitalized patients.

TABLE 2: Comparison of antibiotic resistance according to patient's status, type of specimen and age group of the patient

Antibiotics	Variables							
	Patient's status		**Type of specimen**			**Age group of the patient**		
	in-patients	out-patients	urine	blood	others	adult	infant	new-borns
Amx	100 (74.7-100)	100 (59.8-100)	100 (69.9-100)	100 (46.3-100)	100 (51.7-100)	100 (71.7-100)	100 (31.0-100)	100 (56.1-100)
Tic	20 (5.3-48.6)	37,5 (10.2-74.1)	33,3 (11.3-64.5)	20 (1.1-70.1)	16,7(0.9-63.5)	30,8 (10.4-61.1)	0 (0.0-69.0)	28,6 (5.1-69.8)
Amc	53,3 (27.4-77.7)	62,5 (25.9-89.8)	25 (6.7-57.2)	100 (46.3-100)	83,3 (36.5-99.1)	53,8 (26.1-79.6)	0 (12.5-98.2)	57,1 (20.2-88.2)
Caz	40 (17.5-67.1)	37,5 (10.2-74.1)	25 (6.7-57.2)	80 (29.9-98.9)	33,3 (6.0-75.9)	30,8 (10.4-61.1)	66,7 (12.5-98.2)	42,9 (11.8-79.8)
Cro	46,7 (22.3-72.6)	37,5 (10.2-74.1)	25 (6.7-57.2)	80 (29.9-98.9)	50 (13.9-86.1)	38,5 (15.2-67.8)	66,7 (12.5-98.2)	42,9 (11.8-79.8)
Ctx	40 (17.5-67.1)	37,5 (10.2-74.1)	25 (6.7-57.2)	80 (29.9-98.9)	33,3 (6.0-75.9)	30,8 (10.4-61.1)	66,7 (12.5-98.2)	42,9 (11.8-79.8)
Fox	66,7 (38.7-87.0)	62,5 (25.9-89.8)	33,3 11.3-64.5)	100 (46.3-100)	100(51.7-100)	53,8 (26.1-79.6)	66,7 (12.5-98.2)	85,7 (42.0-99.2)
Imp	0 (0.0-25.3)	0 (0.0-40.2)	0 (0.0-30.1)	0 (0.0-53.7)	0 (0.0-48.3)	0 (0.0-28.3)	0 (0.0-69.0)	0 (0.0-43.9)
Gen	46,7 (22.3-72.6)	50 (17.4-82.6)	33,3 (11.3-64.5)	80 (29.9-98.9)	50 13.9-86.1)	46,2 (20.4-73.9)	66,7 (12.5-98.2)	42,9 (11.8-79.8)
Nal	20 (5.3-48.6)	50 (17.4-82.6)	33,3 (11.3-64.5)	40 (7.3-83.0)	16,7 (0.9-63.5)	30,8 (10.4-61.1)	100 (31.0-100)	0 (0.0-43.9)
Cip	6,7 (0.4-34.0)	37,5 (10.2-74.1)	25 (6.7-57.2)	0 (0.0-53.7)	16,7 (0.9-63.5)	23,1 (6.2-54.0)	33,3 (1.8-87.5)	0 (0.0-43.9)
Sxt	66,7 (38.7-87.0)	87,5 (46.7-99.3)	75 (42.8-93.3)	80 (29.9-98.9)	66,7 24.1-94.0)	76,9 (46.0-93.8)	100 (31.0-100)	57,1 (20.2-88.2)

Abbreviations: AMX: amoxycilline (25µg), AMC: amoxycillin-clavulanic acid (20/10µg), CAZ: ceftazidime (30µg), CRO: ceftriaxone (30 µg), CTX: cefotaxime (30µg), CIP: ciprofloxacine (5µg), SXT: trimethoprim-sulfamethoxazole (1,25/23,75µg), GEN: gentamicin (10µg), NAL: nalidixic acid (30 µg), IPM: imipenem (10µg), TIC: ticarcillin (75 µg).

The comparison of antibiotic resistance with regard to specimen type showed that resistance to cephalosporins, quinolones, gentamicin, trimethoprim-sulfamethoxazole, amoxicillin and amoxicillin/clavulanic acid was higher in blood cultures than other specimens.Resistance to 3rd generation cephalosporins was slightly higher in children and new-borns. No strains isolated from new-borns were resistant to nalidixic acid.

DISCUSSION

The present study was motivated by the fact that to our knowledge, there are no published data on this topic at the Yaounde General Hospital. In absence of data on prior hospitalization of patients, we tried to distinguish between hospital and community infections based on the hospitalization or not of the patients at the moment of samples collection. Samples from internal services and intensive care unit reflect hospital infections whereas those from external consultations reflect community cases.

The biochemical profiles obtained from the 23 isolates allowed the differentiation of biotypes. One reason for the great variability in biotypes observed in *Enterobacter cloacae* strains may be the great number of their isolates compared to the others species.

The variability observed in the defined biochemical tests agrees with that reported in the literature. Due to this metabolic diversity in *Enterobacter*, biotyping has been shown to be a useful approach for strains identification (9).

Resistance of *Enterobacter* strains to each of the major groups of antimicrobial agents varies widely among published reports. All the *Enterobacter* isolates were found to be sensitive to imipenem which again advocates the usage of carbapenem antibiotics as a therapeutic alternative to β-lactams resistant strains. This observation is similar to that of Paterson (10) who showed that the vast majority of *Enterobacteriaceae* including strains producing ESBL remained susceptible to carbapenems

The evolution of *Enterobacteriaceae* resistance to the 3rd generation cephalosporins is related to the enzymatic production of ESBLs. The frequency of EBL producing strains observed in this study (30, 43%) is considered high compared to that of others parts in Africa like Nigeria where the rate of BLSE is around 20% (11). This geographic variation in the prevalence of resistance may be explained by the variability of epidemiological factors, hospital hygiene and antibiotics use among various institutions (12).

Although the phenotypes characterized by a high level of resistance were isolated from hospital patients, they are also present in community patients, highlighting the fact that the problem of resistance is not restricted to hospitalized patients. European and American studies reported an increase in incidence of cephalosporins resistance in *Enterobacteriaceae* strains isolated in community setting (13). In fact, a significant consumption of antibiotics is done outside the hospital, since it has been reported a consumption of 275 tonnes of antibacterial agents in ambulatory patients in Spain, a country with rates of very high resistance in community strains (14). The majority of 3rd generation cephaloporins are pharmaceutically available in injections which justifies their greater prescription in hospitalised patients and the even greater prevalence of resistance in these patients. The relative low resistance percentages observed for the other antibiotics may be due to the fact that these are available in oral forms and therefore are more flexibly administered in out-patients.

The multi-resistance pattern of strains isolated from blood cultures could be justified by the fact that all blood samples were recovered from hospitalized patients, usually carriers of intravenous devices which are a source of contamination by multi-resistant strains of the hospital environment.

In summary, this study emphasizes the importance of *Enterobacter* spp. as pathogen associated with hospital infections and the extensive spread of β-lactams resistant strains in particular to extended spectrum cephalosporins. In spite of the low number of isolates, it came out of this study that the distribution of resistance within the *Enterobater* genera at the Yaounde General Hospital is different depending on whether the patient is hospitalized or not, the age group of the patient and the specimen from which the strains were isolated.

Routine detection of extended spectrum β-lactamases should be done by laboratories to control the spread of infections associated with *Enterobacter*. Sweet control measures should also include judicious use of antibiotics based on antibiogram results and implementation of a monitoring program for resistance to institute proper therapeutic strategies in the light of emergence of resistance.

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