

# $\beta$ -lactamase producing enterobacteria isolated from surveillance swabs of patients in a Cardiac Intensive Care Unit in Rio de Janeiro, Brazil

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## ABSTRACT

There is a high incidence of infections caused by betalactamase-producing Gram-negative microorganisms in Brazil. These organisms are of clinical and epidemiological importance, since their mobile genetic elements facilitate cross-infection. The present study was conducted in sentinel rectal swabs from patients admitted to a cardiac surgery hospital in Rio de Janeiro, from January through December 2007, in a consecutive manner. The aim of the study was to characterize the genotype and phenotype of these isolates from colonized patients. Biochemical tests, antimicrobial susceptibility tests, a confirmatory test for the expression of extended spectrum betalactamase (ESBL) production and polymerase chain reaction for the blaTEM, blaSHV, CTX-M1, Toho-1 and AmpC genes were performed at the University Hospital of *Universidade do Estado do Rio de Janeiro* (UERJ). The most frequently isolated bacteria were *Escherichia coli* 9/41 (21.95%) and *Klebsiella pneumoniae* 14/41 (34.1%). In 24/41 (58%), the ESBL genotype was confirmed. The most prevalent genes in samples that expressed ESBL were blaTEM 13/24 (54%), AmpC 12/24 (50%), blaSHV 6/24 (25%), CTX-M1 7/24 (29%), and Toho-1 6/24 (25%). Of these, 14/24 (58%) presented more than one genotype for the tested primers. In nine (37%) samples other than *E. coli*, *K. pneumoniae* or *Proteus* spp., the phenotype for ESBL was found and confirmed by PCR. The most sensitive substrate in the approximation test in ESBL positive samples was ceftriaxone (83%). Fifty percent of the samples expressed AmpC were associated with other genes. Intermediate susceptibility to ertapenem was found in 2/41 (5%).

**Keywords:** polymerase chain reaction; infection control; enterobacteriaceae infections.

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## INTRODUCTION

The *Enterobacteriaceae* family constitutes the largest and most heterogeneous group of Gram-negative rods of clinical importance, and includes several genera, species and subspecies. In humans, they can cause a variety of diseases, and can be isolated from several different sites in clinical samples.<sup>1</sup> Many of these microorganisms produce enzymes that hydrolyze penicillins, cephalosporins and monobactams. The most frequently isolated betalactamases are extended spectrum (ESBL), AmpC and carbapenemases. ESBL are enzymes that result in resistance to large spectrum cephalosporins (oxymino cephalosporins), penicillins and monobactams, while sensitivity to cephamycins (cefoxitine) and carbapenems is preserved. Betalactamase inhibitors (BLI)

are compounds that bind reversibly or irreversibly to betalactamases, avoiding antibiotic degradation by them. The three most important compounds are sulbactam, clavulanic acid and tazobactam. Some enzymes are resistant to the three BLI.<sup>2</sup> AmpC betalactamases are coded by plasmids, which may contain genes that encode resistance to aminoglycosides, quinolones and others. Clinical relevant bacteria that produce AmpC encoded  $\beta$ -lactamases are the CESP group (*Citrobacter* spp., *Enterobacter* spp., *Serratia* spp., *Providencia* spp.), *M. morgani* and *P. aeruginosa*. It may be difficult for the routine clinical laboratory to identify resistance patterns since this expression may be inducible, that is, it shows during and/or after antimicrobial treatment with  $\beta$ -lactams.<sup>3</sup> These microorganisms are sensitive to carbapenems, but these drugs are strong induc-

ers of AmpC enzyme production. There is no standardization by the *Clinical and Laboratory Standards Institute* (CLSI) for the detection of these isolates.<sup>4</sup> Phenotypic tests use the resistance to cephamycins as the resistance marker related to cephalosporins. Cefoxitin induces the enzyme, so a narrowing of the growth inhibition zone will be noticed.<sup>4</sup> Carbapenemases are  $\beta$ -lactamases that hydrolyze carbapenems, promoting resistance to them. Infections caused by these bacteria are a challenge to treat, since carbapenems are considered the antibiotics of choice to treat severe infections due to ESBL producing strains.<sup>5,6</sup>

The presence of carbapenemases is also found in *Enterobacteriaceae*.<sup>7,8</sup> Patient transfer between hospitals has been reported as a cause of its dissemination. As yet, there is no consensus on which infection control measures need to be implemented to avoid dissemination of these bacteria. Some authors suggest screening tests in hospitalized patients for the detection of rectal carriage.<sup>9</sup> For *K. pneumoniae*, the most important resistance mechanism is the production *bla*<sub>kpc</sub> carbapenemases.<sup>10</sup> The gene that codifies for the *bla*<sub>kpc</sub> enzyme is carried by mobile genetic elements, the transposons, which facilitate further resistance dissemination. The difficulty in detecting the *bla*<sub>kpc</sub> gene resides in the fact that some bacterial strains show susceptibility to carbapenems within the cut-off point established for carbapenems, but with high minimal inhibitory concentrations (MIC). Therefore, they are reported as sensitive to carbapenems, and no infection control measures are applied.<sup>9</sup> Some investigators have suggested ertapenem as the best susceptibility marker, as its action is not inoculum dependent.<sup>11</sup> *Clinical and Laboratory Standards Institute* (CLSI) published in January 2009 recommendations for samples of enterobacteria that were susceptible to carbapenems with a high MIC or with reduced zone diameter in the disk diffusion method to be tested for carbapenemase resistance by the Modified Hodge Test (MHT).<sup>12</sup>

The goal of the present study was to determine the genotype and phenotype of consecutive surveillance rectal swabs isolates cultured from patients admitted to a cardiac surgery hospital in Rio de Janeiro, so as to review and adopt the necessary infection control measures.

## METHODS

This study was submitted to the Ethics Committee at the *Instituto Nacional de Cardiologia* (INC) and approved under number 0138/01.03.07. It was a cohort study, including all consecutive rectal swabs from patients admitted to the ICU in INC in the year 2007. Surveillance of patients admitted to the hospital is part of infection control measures routinely established by the Infec-

tion Control Committee. This surveillance model aims to detect patients who are colonized by multiresistant organisms, both Gram-positive and Gram-negative. Patients with risk factors for such colonization, such as those transferred from other hospitals or long term residence facilities or home care settings, or with a history of hospital admission in the previous six months, are submitted to collection of rectal swabs, to identify ESBL producing microorganisms. The patient is maintained under contact precaution until culture results. If the culture is positive, the patient, already under contact precaution, has not, theoretically, served as a source for hospital transmission. The established protocol in our institution is testing exclusively for ESBL production. Rectal swabs are cultured in selective media (MacConkey agar). If there is growth on MacConkey, a disk diffusion test is performed on Mueller Hinton agar. Antimicrobials tested routinely at INC microbiology lab are cefepime, ceftazidime, cefotaxime, ceftriaxone and aztreonam. Results are given as "positive for ESBL" or "negative for ESBL", and identification of the microorganism is not performed.

Samples included in this study were obtained from frozen, stocked samples kept by the microbiology lab in INC, dating from January to December 2007.

INC is a public tertiary referral hospital for cardiac surgery in the city of Rio de Janeiro. It has 186 beds, and is responsible for 90% of pediatric cardiac surgery in the city of Rio, and 80% of cardiac surgery in adults. The present study characterized genotypically and phenotypically 41 available stocked screening stool samples from patients referred or not from other hospitals in their admission. As part of the study, bacteria from stools were identified in the research lab in UERJ. ESBL expression was confirmed by disk diffusion test followed by PCR for the detection of *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, CTX-M-1, Toho-1 and AmpC genes. For confirmation of AmpC  $\beta$ -lactamase production, the samples were directly submitted to PCR. The primers used for PCR are shown in Table 1.<sup>13-15</sup> The presence of carbapenemases was also sought for by the MHT in those samples showing reduced sensitivity to ertapenem. Samples from rectal swabs were not routinely identified, but only tested for ESBL production. With the change in the world scenario, with growing prevalence of multiresistant Gram-negatives, and as part of the present aim of this study, the microorganisms isolated were identified and carbapenemase production sought for. This study also aimed to test the most prevalent resistance genes in these colonization samples.

## RESULTS

In this study, 41 stool samples were included to characterize antibiotic and genotypic profile. In 30/41 (73.2%) ESBL phenotypic expression was found, as well as in 24/41

**Table 1.**

Gene	Primers	Product	References
bla <sub>TEM</sub>	F:5'-TTG GGT GCA CGA GTG GGT TA -3' R:5'-TAA TTG TTG CCG GGA AGC TA -3'	503bp	[13]
bla <sub>SHV</sub>	F:5'-TCG GGC CGC GTA GGC ATG AT -3' R:5'-AGC AGG GCG ACA ATC CCG CG -3'	625bp	[13]
Toho-1	F:5'-GCGACCTGGTAACTACAATCC-3' R:5'-CGGTAGTATTGCCCTTAAGCC- 3'	351bp	[14]
CTXM-1	F:5'-GACGATGTCACTGGCTGAGC- 3' R:5'-AGCCGCCGACGCTAATACA-3'	499bp	[14]
AmpC	F:5'-CGT TTG TCA GGC ACA GTC AAA TCC A-3' R:5'- TTC CAC TGC GGC TGC CAG T3'	567bp	[15]

(58.5%) genotypic expression with the test primers. All samples tested susceptible to imipenem and 2/41 (5%) presented intermediate susceptibility to ertapenem. Susceptibility to quinolones was present in 13/41(31%). Table 2 shows the antibiotic susceptibility pattern of the isolates. Samples that were shown to be ESBL positive were submitted to polymerase chain reaction assays (PCR). The most frequently encountered genes were: bla<sub>TEM</sub> 15/30 (13/24), 50% (54%), AmpC 15/30 (12/24), 50% (50%), bla<sub>SHV</sub> 10/30 (6/24), 33.3% (25%), CTX-M1 10/30 (7/24), 33,3% (29%), and Toho-1 7/30 (6/24), 23.3% (25%).

ESBL production was shown in strains in which testing is not routinely recommended. These enterobacteria presented a phenotype (ghost zone) by disk diffusion testing, and were studied by PCR for ESBL presence. Even when a strong AmpC enzyme inducer was used (clavulanic acid), the samples, mostly with *Enterobacter* spp. (CESP group), expressed ESBL. Eight samples of *Enterobacter* sp. were identified and presented the ESBL phenotype. In all these, AmpC expression was shown by PCR.<sup>16</sup> Of these eight samples, six (75%) were categorized as within cut-off point with zone diameters between 17 and 20 mm for ertapenem (CLSI, 2008), and 2/8 (25%) had intermediate sensitivity, with zone diameter of 16 mm. The modified Hodge test was negative in all tested samples.

## DISCUSSION

Some members of the *Enterobacteriaceae* family, especially *E. coli*, *K. pneumoniae*, *Enterobacter* spp. and *Serratia marcescens*, are strongly associated with health care related infections, and this is particularly true in Brazil.<sup>17</sup> The importance of identifying enterobacteria and implementing infection control measures when resistant strains are identified is paramount. The present study reinforces the importance of

identifying all bacterial species that produce β-lactamases since colonization precedes infections in most cases involving multiresistant *Enterobacteriaceae*, and has been identified as an important risk factor in hospitalized patients.<sup>3</sup> As expected, *K. pneumoniae* and *E. coli* predominated in the ESBL producing species identified, as described in the literature.<sup>12,18,19</sup> CLSI should prompt searching for ESBL in strains of *Klebsiella* spp., *E. coli* and *Proteus mirabilis*, but we found that 9/24(37%) of the identified bacteria in this study belonged to strains which are not routinely investigated for ESBL even by the present recommendations.<sup>20</sup> Data from the literature focus on ESBL production in isolates of *K. pneumoniae* and *E. coli*; we, however, also showed that these samples of ESBL producers were from the CESP group of enterobacteria. CLSI has tried to develop tests to validate ESBL production in species other than *Klebsiella*, *E. coli* and *Proteus*. However, since the 2010 recommendations of reducing the cut-off point for cephalosporins so as to minimize interpretation errors when reporting resistance in confirmatory tests.<sup>21</sup> In our institution, it was not an established protocol to identify ESBL producers in routine sentinel rectal swabs, since the purpose of these swabs was to recommend contact precautions or not. The identity of these isolates was not known until the present study, where *Enterobacter* spp. was found. This served as an alert to us, and with the CLSI guidelines, led us to adopt a new protocol where all isolates are identified, even in colonization samples, in order to define our local epidemiology and adopt appropriate infection control measures.<sup>12,22</sup>

Therefore, had this been a routine practice, these patients would have been placed on contact precaution measures, avoiding dissemination of carbapenemase producers in INC. The recognition of the first case of carbapenemase in a patient colonized by a carbapenemase producing *enterobacterium* occurred in September 2009, and dissemination,

Table 2.

Sample number	Bacterial species	Antimicrobial resistance
25173	<i>Escherichia coli</i>	Am;cf;ctr; st;
46150	<i>Proteus</i> spp.	Am; cf; ctr; cip;st
59782 A	<i>Enterobacter cloacae</i>	Am; amc; tzp; cf;fox; ctr; cep;cip;st
39757	<i>Enterobacter cloacae</i>	Am;amc;cf;fox;ctr;taz;cip;st
91876	<i>Klebsiella pneumoniae</i>	Am; amc; cf; ctr
56467	<i>Enterobacter</i> spp.	Am; amc; cf; fox; ctr; cip; st
3659	<i>Escherichia coli</i>	Am; tzp; cf; ctr; cep; st
46153 A	<i>Klebsiella pneumoniae</i>	Am; cf; ctr; cep;st
28998	<i>Enterobacter cloacae</i>	Am; amc; cf; fox; ctr; st
52548 A	<i>Klebsiella pneumoniae</i>	Am; cf; ctr;cep;cip;st
52548 B	<i>Klebsiella pneumoniae</i>	Am; tzp; cf; ctr;taz;cep; cip;st
47163	<i>Enterobacter</i> spp.	Am; amc; tzp; cf; fox; ctr; cip; st
96412 A	<i>Escherichia coli</i>	Am; cf; ctr;
96412 B	<i>Klebsiella pneumoniae</i>	Am; cf; ctr;cep
94631	<i>Serratia marcescens</i>	Am; amc; cf; fox;cip;st; ctr
21951	<i>Escherichia coli</i>	Am; tzp; cf; ctr; cep;st
93802	<i>Enterobacter</i> spp.	Am; amc; cf; fox; st; ctr; taz
96368	<i>Klebsiella pneumoniae</i>	Am; cf; ctr; taz;st; an
46177	<i>Klebsiella pneumoniae</i>	Am; cf; taz;st
38383	<i>Klebsiella pneumoniae</i>	Am; cf; ctr; st
28398	<i>Proteus</i> spp.	Am; tzp; cf; cip; st; ctr
52467	<i>Enterobacter</i> spp.	Am; amc; cf; fox; ctr; cip
39299	<i>Klebsiella pneumoniae</i>	Am; cf; ctr; taz
41045	<i>Proteus</i> spp.	Am; tzp; cf; fox; ctr; cep; cip;st
58251	<i>Enterobacter cloacae</i>	Am; amc; tzp;cf; fox; tr;taz;cep;cip;st
58592	<i>Enterobacter cloacae</i>	Am; amc; cf; fox; st; ctr
90111	<i>Escherichia coli</i>	Am;ctx;st
69739	<i>Escherichia coli</i>	Am; cf; ctr; cep; cip; st
69748	<i>Escherichia coli</i>	Am; tzp; cf; ctr;cep;cip;st
40827 B	<i>Klebsiella pneumoniae</i>	Am; cf; ;ctr
54614	<i>Escherichia coli</i>	Cf;ctr
14939	<i>Enterobacter</i> spp.	Am; cf; ctr; st
71393 A	<i>Klebsiella pneumoniae</i>	Am; cf;ctr; st
71393 B	<i>Klebsiella pneumoniae</i>	Am; cf; ctr;cep;st; an
47750	<i>Enterobacter cloacae</i>	Am; amc; cf; fox;ctr
20173	<i>Morganella morganii</i>	Am; amc; cf; ctr; fox;ctr;cip;st;an
54447	<i>Proteus mirabilis</i>	Am; cf; ctr;cip;st
26046	<i>Klebsiella oxytoca</i>	Am; cf; taz;st
71286	<i>Klebsiella pneumoniae</i>	Am;cf;tzp;ctr
71429	<i>Enterobacter</i> spp.	Am; amc; cf; fox;
72947	<i>Escherichia coli</i>	Am; cf;ctr;cip; st

Antimicrobials resistance: am, ampicillin; amc, amoxicillin/clavulanic acid; tzp, piperacillin/tazobactam; cf, cephalotin; fox, ce-foxitine; ctr, ceftriaxone; taz, ceftazidime; cep, cefepime; cip, ciprofloxacin; st, trimethoprim-sulphamethoxazole; na, amikacin.



culminating with an outbreak in the adult ICU, occurred in January 2010. This has resulted in a revised protocol, implemented in January 2010, whereas all patients are screened for ESBL and carbapenemase producing strains in rectal swabs they should belong to risk groups, as mentioned in the methods section. The Modified Hodge Test is a phenotypic test that may be used to detect reduced susceptibility to carbapenems. It has been considered sensitive and specific for the detection of carbapenemases by MMWR.<sup>9,23,24</sup> It may be part of routine practice in microbiology labs, since it is neither difficult to perform nor expensive. However, its interpretation depends on the observer, as there is no standardization of its reading.<sup>9,11</sup> For the clinical microbiologist and infection control team, it is important to have quick answers as to whether or not patients are colonized by ESBL and/or by Hodge positive bacteria. However, we believe it is also important to further study these isolates, determining their species and evaluate, by molecular studies, its resistance genes. For ESBL and carbapenemase production expression, the gold standard is PCR.<sup>4,14</sup>

In a study from 2006, carbapenemase producing strains from rectal swabs were described.<sup>8</sup> An outbreak in Puerto Rico in 2008 was reported, with 39 cases of infection with carbapenemase producing strains, where two colonized, asymptomatic patients who should have been under contact precautions were not. These patients acted as reservoirs in the outbreak.<sup>9</sup>

Inadequate antimicrobial therapy in the treatment of severe infections in hospitalized patients is associated with increased mortality, and some authors emphasize the major impact of non-anticipated resistance.<sup>25</sup> In the present study, 100% of the samples showed sensitivity to imipenem and 2/41 (5%) showed intermediate susceptibility to ertapenem. Ertapenem has been used for ESBL producing strains as its administration is once daily and it is not active against non-fermenters, having theoretically no impact on resistance induction in this group.<sup>26</sup> However, as shown in this study, there is growing resistance of *Enterobacteriaceae* to ertapenem. Resistance to ertapenem was not detected by disc diffusion, however two samples showed intermediate sensitivity in this method.

Disk diffusion tests used for confirmation for ESBL production detected 83.3% for ceftriaxone, 6.6% for ceftazidime, and 10% for both. PCR used to broaden the epidemiological study showed several samples (58%) expressing more than one gene for the test primers, which is in accordance with the literature that reports that enterobacteria may carry several genes that encode for ESBL.<sup>5</sup> Our study also identified the presence of ESBL genes in strains for which the phenotypic test is not recommended by CLSI. This group found 30% (9/30) of the total samples that expressed ESBL, eight of the samples being *Enterobacter* spp. and one *Serratia marcescens*. The controversies about microorganisms other than *E. coli*,

*Klebsiella* spp. or *P. mirabilis* relate to the use of BLI clavulanic acid. Clavulanic acid, especially for members of the CESP group, may act as AmpC inducer, making the recognition of ESBL production difficult and yielding false negative ESBL results. However, in the present study CESP group member expressing ESBL by the disk diffusion method, when submitted to PCR for the detection of the AmpC gene, also presented this gene. Thus, there was no interference in the phenotypic expression of ESBL by the presence of the AmpC gene.

## CONCLUSION

The identification of *Enterobacteriaceae* involved in colonization of hospitalized patients during the study period resulted in greater awareness as to the presence of other resistance mechanisms, not so far routinely sought for in our institution. Owing to the study results and analysis, new rules for microbiological screening and infection control measures were put into practice. In other words, testing for carbapenemase production and isolation precautions for patients in whom these microorganisms were identified was implemented. The microbiology lab, at the present moment, has reduced the cut-off points for cephalosporins, as recommended by the CLSI, 2010, but kept the phenotypic tests for ESBL and carbapenemases expression.

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