CHARACTERIZATION OF CTX-M-TYPE EXTENDED-SPECTRUM BETA-LACTAMASES (ESBLS) AMONG *ENTEROBACTERIACEAE* FROM A PORTUGUESE HOSPITAL

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RESUMO

Na última década tem-se assistido à disseminação de enzimas do tipo CTX-M em diversos países, determinando alterações significativas na epidemiologia das beta-lactamases de espectro alargado (BLEA). A monitorização da disseminação de *Enterobacteriaceae* produtoras de CTX-M em Portugal foi efectuada em isolados clínicos recentes de um hospital Português. bla_{CTX-M} e ISs associadas ao seu ambiente genético foram pesquisados por PCR. A elevada prevalência de variantes de CTX-M do grupo I confirma a mudança na distribuição dos tipos de BLEA em Portugal.

PALAVRAS-CHAVE

Beta-lactamases de espectro alargado, ESBLs, CTX-M, *Enterobacteriaceae*, Portugal

ABSTRACT

During the last decade CTX-M enzymes have become widespread in several countries, causing a shift in the ESBL epidemiology of specific areas. In order to monitorize the dissemination of CTX-M-producing *Enterobacteriaceae* in Portugal, we characterized ESBL producers recovered from a Portuguese hospital. bla_{CTX-M} and specific ISs and genes associated with bla_{CTX-M} genetic environment were searched by PCR. Detection of a high occurrence of CTX-M cluster I variants confirms a shift in ESBL distribution towards CTX-M enzymes in Portugal.

KEYWORDS

Extended-Spectrum Beta-Lactamases, ESBLs, CTX-M, *Enterobacteriaceae*, Portugal

1. INTRODUCTION

Beta-lactam antibiotics are first-line therapeutic option to treat infections caused by *Enterobacteriaceae*, but resistance to these compounds has been increasingly reported in the last decades (Rossolini and Mantengoli, 2008). Different mechanisms of resistance to this antibiotic family have been described and include porin alterations, Penicillin Binding Proteins (PBPs) modifications, efflux systems and/or production of enzymes capable of beta-lactams hydrolysis (beta-lactamases) (Bradford, 2001). In *Enterobacteriaceae*, production of Extended-Spectrum Beta-Lactamases (ESBLs) constitutes the most common resistance mechanism to beta-lactam antibiotics (Rossolini and Mantengoli, 2008). ESBL enzymes have hydrolytic capacities over several beta-lactams, including penicillins, monobactams and extended-spectrum cephalosporins (except cephamycins), and are inactivated by classic beta-lactamase inhibitors such as clavulanic acid (Gniadkowski, 2001). Only carbapenems (e.g. imipenem) are stable to these enzymes, constituting the unique therapeutical option in the group of the beta-lactams (Gniadkowski, 2001). ESBL-producing bacteria are also frequently resistant to non-beta-lactam antibiotics, as genes encoding to these patterns are also frequently located in the same mobile genetic platform (plasmid, transposon and/or integron) carrying bla_{ESBL} genes (Cantón *et al.*, 2008; Paterson and Bonomo, 2005). This situation makes more difficult the therapy of some infections caused by ESBL-producing organisms.

Since the first report of an ESBL-producing *Klebsiella pneumoniae* in 1983 in Germany, several nosocomial outbreaks of ESBL-producing organisms have been described worldwide (Cantón *et al.*, 2008). Prevalence of ESBLs is high in nosocomial environments mainly because of long-term exposure to antibiotics and catheterization, among other factors (Bradford, 2001). However, spread of ESBLs has also been demonstrated in other ecological niches such as healthy humans, animals, and aquatic environments (Bradford, 2001; Carattoli, 2008; Machado *et al.*, 2004; Machado *et al.*, 2008; Machado *et al.*, 2009).

Several ESBL types have been created, according to their amino acid sequences, and their hydrolytic ability over several beta-lactam antibiotics (Bradford, 2001; http://www.lahey.org/studies/webt.htm). TEM and SHV families have evolved from TEM-1, TEM-2, or SHV-1 enzymes, respectively, by stepwise mutations that affect the active site of the enzyme extending its spectrum of activity (Bradford, 2001), and have been traditionally considered the most widespread ESBLs (Bradford, 2001; Cantón *et al.*, 2008; Gniadkowski, 2001). They are mainly found among *Enterobacteriaceae*, although recent reports describe their presence among non-*Enterobacteriaceae* species (e.g. *Pseudomonas aeruginosa*) (Bradford, 2001). Non-TEM/-SHV-type ESBLs, include CTX-M, OXA, GES, IBC, VEB, PER and other variants, and until recently these enzymes were rarely reported (Bradford, 2001).

During the last decade CTX-M enzymes have become widespread in several countries, causing a shift in the ESBL epidemiology of specific areas (Cantón and Coque, 2006; Hawkey, 2008; Livermore *et al.*, 2007; Walther-Rasmussen and Hoiby, 2004). In Europe, CTX-M enzymes are becoming the predominant ESBLs, as stated recently by Euro Surveillance (Coque *et al.*, 2008). These enzymes were firstly designated as CefoTaXiMases (CTX-M) due to their higher hydrolytic activity over cefotaxime than ceftazidime (Walther-Rasmussen and Hoiby, 2004). However, CTX-M enzymes able to efficiently hydrolyze ceftazidime have also been described (Bonnet, 2004). These enzymes are grouped in five clusters (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25) on the basis of their amino acid sequences (Figure 1) (Bonnet, 2004). Chromosomal beta-lactamase genes from some *Kluyvera* species (widely dis-

tributed in the environment) have been suggested as the ancestors of each CTX-M-cluster and mobilization of bla_{CTX-M} genes to other bacterial genera seems to have occurred through recombinatorial events mediated by IS*CR1*, IS*Ecp1* or phage related elements (Bonnet, 2004; Lartigue *et al.*, 2004; Oliver *et al.*, 2001; Tolemman *et al.*, 2006). CTX-M enzymes remain mainly detected in *Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella enterica* serovar Typhimurium (Bonnet, 2004; Bradford, 2001), but they were also recently identified in *Vibrio cholerae*, *Acinetobacter baumannii* or *Aeromonas hydrophila* (Walther-Rasmussen and Hoiby, 2004).

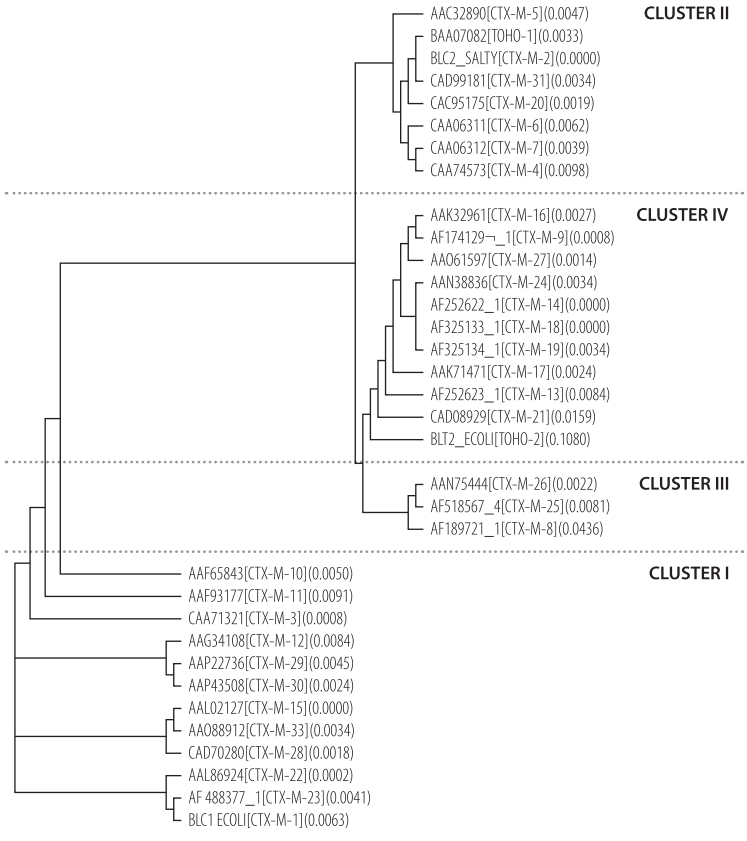

Figure 1. Phylogenetic tree for CTX-M enzymes (adapted from Lahey Clinic) (http://www.lahey.org/studies/webt.htm)

The rapid and wide spread of CTX-M enzymes worldwide is mainly due to the frequent association of bla_{CTX-M} genes with genetic mobile elements, such as epidemic plasmids, transposons, and/or insertion sequences located upstream bla_{CTX-M} also responsible for the expression of gene (e.g. $bla_{CTX-M-15}$) (Cantón and Coque, 2006; Coque *et al.*, 2008; Lartigue *et al.*, 2004). However, CTX-M epidemiology is much more complex, involving not only horizontal gene transfer, but also the clonal dissemination of CTX-M-producing organisms (e.g. CTX-M-15-producing epidemic clones) (Cantón and Coque, 2006; Cantón *et al.*, 2008). All of these factors contribute to the increasing dissemination of CTX-M ESBLs in nosocomial and community environments (Cantón and Coque, 2006), placing CTX-M-producing organisms as a serious clinical issue.

Overall, CTX-M-15 is achieving great dominance in Europe (Cantón and Coque, 2006; Coque *et al.*, 2008) and in a previous survey (2003-2005) performed in tertiary care Portuguese hospitals dissemination of epidemic strains and plasmids coding for CTX-M-15 enzymes were also reported (Machado *et al.*, 2007). Shifts in ESBL-epidemiology might impose new therapeutic and infection control approaches, justifying their periodic surveillance, and hence a continuous surveillance in a bigger scale, comprising hospitals from diverse geographic areas is imperative (Coque *et al*., 2008). In this way, the objectives of the present study were to characterize and analyse the diversity the CTX-M ESBLs among *Enterobacteriaceae* from a Portuguese hospital not previously evaluated, over a period of nine months, in order to gather useful epidemiological data that might contribute to the design of interventions to control the dissemination of CTX-M-producing organisms and/or CTX-M enzymes in our country.

2. **MATERIALS AND METHODS**

2.1. **BACTERIAL ISOLATES**

A total of sixteen *Enterobacteriaceae* isolates recovered at Hospital A from the Centre region of Portugal and previously identified as presumptive ESBL producers were included in the present work. The isolates were obtained from clinical samples recovered from patients at several hospital wards between May 2007 and January 2008. Species identification, preliminary susceptibility testing and presumptive detection of ESBL production were performed at the hospital using the Vitek Automatic System (bioMérieux, Marcy-l'Étoile, France).

2.2. **ANTIMICROBIAL SUSCEPTIBILITY TESTING**

Further antibiotic susceptibility testing was performed by the disc diffusion method following the CLSI guidelines (CLSI, 2007). Antimicrobial agents tested were the following: amoxicillin/clavulanic acid (30 µg), cefoxitin (30 µg), cefoxatime (30 µg), ceftazidime (30 µg), cefepime (30 µg), aztreonam (30 µg), imipenem (10 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), gentamicin (10 µg), amikacin (30 µg), tobramycin (10 µg), streptomycin (10 µg), spectinomycin (25 µg), netilmicin (30 µg), kanamycin (30 µg), trimethoprim (5 µg), sulfonamides (300 µg), tetracyclines (30 µg), and chloramphenicol (30 µg). Discs were purchased from OXOID (Basingstoke, England). All intermediate-susceptible isolates were considered as non-susceptible.

2.3. **DETECTION OF ESBL PRODUCERS**

The presence of an ESBL phenotype was confirmed by the standard double-disc synergy test (DDST), using cefoxatime, ceftazidime, cefepime, and aztreonam discs placed 25 mm apart from an amoxicillin/clavulanic acid disc (Jarlier *et al.*, 1988; Machado *et al.*, 2007).

2.4. MOLECULAR CHARACTERIZATION OF CTX-M ESBLS AND THEIR GENETIC ENVIRONMENT

2.4.1. POLYMERASE CHAIN REACTION (PCR) AMPLIFICATIONS

Molecular characterization of CTX-M ESBLs was performed by PCR using genomic DNA from wild type strains. Bacterial DNA was extracted by boiling a bacterial suspension in 300 μL of water during 10 minutes, followed by centrifugation. Amplification of beta-lactamase (*bla*) genes coding for CTX-M was achieved using primers and amplification conditions for detection of a conserved sequence of bla_{CTX-M} (Edelstein *et al.*, 2003). Further amplification of other regions of the bla_{CTX-M} gene which allow the determination of the CTX-M enzyme cluster and/or type was carried out using another set of primers specific for genetic regions that characterize each CTX-M cluster (Pitout *et al.*, 2004). For selected cases, pairs of primers were designed for sequencing reactions of specific bla_{CTX-M} genes (Machado *et al.*, 2006). Presence of specific ISs and genes associated with bla_{CTX-M} genetic environment were also searched by PCR (IS*Ecp1*, bla_{OXA}, bla_{TEM}) (Bert *et al.*, 2002; Machado *et al.*, 2004; Machado *et al.*, 2007). Primers and conditions used for all amplification reactions are presented in Table 1.

Table 1. Primers sequences and PCR conditions used in this study.

Primer	Sequence (5'-3')	Gene	Size (bp)	Amplification Conditions	[$MgCl_2$]	Reference
CTX-M-F'	TTT GCG ATG TGC AGT ACC AGT AA	CTX-M	590	1 cycle of 10 min at 94ºC; 35 cycles of 1 min at 94 ºC, 1 min at 51ºC, 1 min at 72ºC; 1 cycle of 10 min at 72 ºC	1.7	Edelstein *et al.*, 2003
CTX-M-R'	CGA TAT CGT TGG TGG TGC CAT A					
CTXM1-F3	GAC GAT GTC ACT GGC TGA GC	CTX-M Cluster I	499	1 cycle of 10 min at 94ºC; 35 cycles of 1 min at 94 ºC, 1 min at 55ºC, 1 min at 72ºC; 1 cycle of 10 min at 72 ºC	1.7	Pitout *et al.*, 2004
CTXM1 R2	AGC CGC CGA CGC TAA TAC A					
TOHO1-2F	GCG ACC TGG TTA ACT ACA ATC C	CTX-M Cluster II	351	1 cycle of 10 min at 94ºC; 35 cycles of 1 min at 94 ºC, 1 min at 55ºC, 1 min at 72ºC; 1 cycle of 10 min at 72 ºC	1.7	Pitout *et al.*, 2004
TOHO1-1R	CGG TAG TAT TGC CCT TAA GCC					
CTXM825-F	CGC TTT GCC ATG TGC AGC ACC	CTX-M Cluster III	307	1 cycle of 10 min at 94ºC; 35 cycles of 1 min at 94 ºC, 1 min at 55ºC, 1 min at 72ºC; 1 cycle of 10 min at 72 ºC	1.7	Pitout *et al.*, 2004
CTXM825-R	GCT CAG TAC GAT CGA GCC					
CTXM914-F	GCT GGA GAA AAG CAG CGG AG	CTX-M Cluster IV	474	1 cycle of 10 min at 94 ºC; 35 cycles of 1 min at 94 ºC, 1 min at 62 ºC, 1 min at 72 ºC; 1 cycle of 10 min at 72 ºC	1.7	Pitout *et al.*, 2004
CTXM914-R	GTA AGC TGA CGC AAC GTC TG					
CTX-M 10F deg	ATG GTT AAA AAA TCA CTG CGT C	CTX-M (*sequencing*)	876	1 cycle of 10 min at 94 ºC; 35 cycles of 1 min at 94ºC, 1 min at 55,5 ºC, 1 min at 72 ºC; 1 cycle of 10 min at 72ºC	1.7	Machado *et al.*, 2006
CTX-M 10R deg	TTA CAA ACC GTY GGT GAC G					
TEM-F	ATG AGT ATT CAA CAT TTC CG	TEM	847	1 cycle of 10 min at 94 ºC; 35 cycles of 1 min at 94 ºC, 1 min at 58 ºC, 1 min at 72 ºC; 1 cycle of 10 min at 72 ºC	1.7	Machado *et al.*, 2007
TEM-R	CTG ACA GTT ACC AAT GCT TA					
OXA-Group III-F	TTT TCT GTT GTT TGG GTT TT	OXA	427	1 cycle of 10 min at 94 ºC; 35 cycles of 1 min at 94ºC, 1 min at 54 ºC, 1 min at 72 ºC; 1 cycle of 10 min at 72ºC	1.2	Bert *et al.*, 2002
OXA-Group III-R	TTT CTT GGC TTT TAT GCT TG					
IS*Ecp1*-F	ATC TAA CAT CAA ATG CAG G	IS*Ecp1*	1391	1 cycle of 10 min at 94 ºC; 35 cycles of 1 min at 94ºC, 1 min at 59 ºC, 5 min at 72 ºC; 1 cycle of 10 min at 72ºC	1.5	Machado *et al.*, 2004
IS*Ecp1*-R	AGA CTG CTT CTC ACA CAT					

2.4.2. VISUALIZATION OF PCR RESULTS

After amplification reactions, PCR products were analysed in 2% agarose gels, using the following electrophoresis conditions: 100 V, 35 minutes, TAE buffer 1X. SYBR Safe™ DNA Gel Stain (Invitrogen, Paisly, United Kingdom) at 0.1 μL/mL was used as a nucleic acid intercalator for detection of the amplified products under an UV light.

2.4.3. **PURIFICATION OF PCR PRODUCTS**

Selected PCR products were purified using the Wizard® SV Gel and PCR Clean-UP System (PROMEGA Corporation, USA) following the manufacturer instructions.

2.4.4. **SEQUENCING**

Sequencing reactions of purified PCR products were performed using the ABI 3700 automated sequencer (Applied Biosystems, Perkin-Elmer, Foster City, CA). Nucleotide sequences were compared to sequences in the GenBank and EMBL databases by using the BLASTN local alignment search tools. Protein sequences derived from the genes were aligned using CLUSTAWL and analysed in order to identify the CTX-M-type according to sequences published in http://www.lahey.org/studies/webt.htm.

3. **RESULTS AND DISCUSSION**

Presence of CTX-M ESBLs was observed in twelve isolates (75%, 12/16) identified as *E. coli* (n=8), *K. pneumoniae* (n=1), *P. mirabilis* (n=2), and *E. cloacae* (n=1) (Table 2). The CTX-M-producing isolates were obtained from clinical samples of patients located at the following hospital wards: Medicine I (n=5), General Urgency (n=2), Medicine II (n=2), Men Medicine (n=1), Orthopedy (n=1), and Gastroenterology (n=1). These isolates were recovered from urine (n=10), pus (post-surgery) (n=1), and exsudates (n=1). Most of the CTX-M-producing isolates exhibited non-susceptibility profiles to several non-beta-lactam antibiotics (Table 2). *E. coli* was the species most frequently found as ESBL producer (56%, 9/16), which is in accordance with reports from other authors (Cantón *et al.*, 2008). CTX-M-producing *E. coli* showed a clear spread among different wards of the studied hospital, being more prevalent in Medicine I ward (Table 2).

Table 2. Characterization of CTX-M-producing *Enterobacteriaceae* isolates included in this study.

Isolate	Species	Date of isolation (mo/day/yr)	Origin	Specimen	Ward	Beta-Lactamases (detected and/or sequenced *bla* genes)	IS*Ecp1*	Non-β-lactam resistance phenotype [a]
H559	*E. coli*	5/13/07	Hospital A	Urine	General Urgency	OXA, CTX-M cluster I	+	Tb, Cp, Na, Te, Km
H560	*E. coli*	6/30/07	Hospital A	Urine	Medicine I	TEM, OXA, CTX-M cluster I	+	Tb, Cp, Na, Km
H567	*E. coli*	5/30/07	Hospital A	Urine	Medicine I	TEM, CTX-M cluster I	+	Gm, Tb, Cp, Na, Te, Nt, Km
H580	*E. coli*	1/4/08	Hospital A	Urine	Men Medicine	TEM-1*, OXA, **CTX-M-15-variant***	+	Gm, Tb, Ak, Sm, Sp, Cp, Na, Te, Nt, Km
H588	*E. coli*	11/29/07	Hospital A	Urine	General Urgency	TEM, OXA, CTX-M cluster I	+	Gm, Tb, Ak, Sm, Cp, Na, Te, Nt, Km
H596	*E. coli*	12/29/07	Hospital A	Urine	Medicine I	OXA, CTX-M cluster I	+	Gm, Tb, Sm, Sp, Cp, Na, Su, Te, Cm, Nt, Km
H602	*E. coli*	1/8/08	Hospital A	Urine	Orthopedy	TEM, CTX-M cluster III, **CTX-M-1***	+	Gm, Tb, Sm, Sp, Na, Su, Tp, Te, Nt, Km
H607	*E. coli*	12/19/07	Hospital A	Urine	Gastroenterology	TEM, OXA, CTX-M cluster I	+	Gm, Tb, Sm, Cp, Na, Te, Nt, Km
H565	*K. pneumoniae*	5/24/07	Hospital A	Urine	Medicine I	CTX-M cluster I	–	Sm, Sp, Cp, Na, Su, Tp, Te, Cm
H585	*P. mirabilis*	12/2/07	Hospital A	Pus (surgery)	Medicine II	OXA, **CTX-M-15-variant***	–	Gm, Tb, Ak, Sm, Sp, Cp, Na, Tp, Te, Nt, Km
H627	*P. mirabilis*	12/29/07	Hospital A	Exsudate	Medicine II	TEM, OXA, CTX-M cluster I	+	Gm, Tb, Ak, Sm, Sp, Cp, Na, Te, Nt, Km
H605	*E. cloacae*	11/29/07	Hospital A	Urine	Medicine I	TEM, OXA, CTX-M cluster I	+	Gm, Tb, Ak, Sm, Cp, Na, Te, Nt, Km

[a] **Tb**, tobramycin; **Ak**, amikacin; **Sm**, streptomycin; **Nt**, netilmicin; **Km**, kanamycin; **Su**, sulphonamides; **Tp**, trimethoprim, **Te**, tetracycline; **Cp**, ciprofloxacin; **Na**, nalidixic acid; **Cm**, chloramphenicol; **Sp**, spectinomycin; **Gm**, gentamicin

Among the CTX-M enzymes, CTX-M belonging to cluster I were the most frequently detected, being identified among all CTX-M-producing isolates (Table 2). CTX-M enzymes of clusters III were only found in one *E. coli* isolate also harbouring a TEM and a CTX-M enzyme of cluster I. Isolates belonging to CTX-M clusters II or IV were absent. For specific amplified bla_{CTX-M} genes, a detailed characterization of the nucleotide sequence was carried out. Sequencing results revealed two distinct CTX-M-types: CTX-M-1 and CTX-M-15-variants. The presence of CTX-M-15-variants was expected, since the most common CTX-M enzyme in Europe and Portugal has been CTX-M-15. bla_{TEM} and bla_{OXA} genes were commonly found in isolates also harbouring bla_{CTX-M} genes of cluster I (8/12 and 9/12, respectively) (Table 2). These genes have been detected in the same epidemic plasmids carrying the $bla_{CTX-M-15}$, also associated to the ST131 Complex Type Clone (Coque *et al.*, 2008). The presence of the insertion sequence IS*Ecp1* was also confirmed in all but one CTX-M producers of cluster I. The CTX-M-15 enzyme was previously found in Portugal (Machado *et al.*, 2006; Mendonça *et al.*, 2007), and the presence of bla_{TEM} and bla_{OXA} in these bacteria could indicate an involvement of the same epidemic plasmid associated to CTX-M-15 enzymes. Concerning CTX-M-1, this enzyme was only found in our country among *Enterobacteriaceae* recovered from poultry meat or seagulls faeces (Machado *et al.*, 2008; Poeta *et al.*, 2008). Further studies will be necessary to analyse the potential of horizontal transfer of CTX-M-1 and CTX-M-15 variants, and characterize the plasmid involved, in order to evaluate the presence and/or maintenance of epidemic plasmids carrying these CTX-M ESBLs in our country.

4. **CONCLUSIONS**

We described a high occurrence of CTX-M enzymes among ESBL-producing *Enterobacteriaceae* from a Portuguese hospital. The detection of CTX-M-15-variants- and CTX-M-1-producing organisms confirms a shift in ESBL distribution towards CTX-M enzymes, a finding previously observed in other regions of our country and in Europe (Coque *et al.*, 2008; Machado *et al.*, 2007). The twelve CTX-M-producing *Enterobacteriaceae* in a total of eighty *Enterobacteriaceae* that were recovered during the analysis period, can be considered a devastating threat to public health, as plasmidic and/or clonal dissemination might be present and would amplify the level of danger to the community. Further analysis will better highlight the current epidemiology of CTX-M-producing *Enterobacteriaceae* in this hospital. The present epidemiological information will be useful in the design of interventions to control the dissemination of CTX-M-producing organisms and/or CTX-Ms in this hospital and, ultimately, in our country.

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