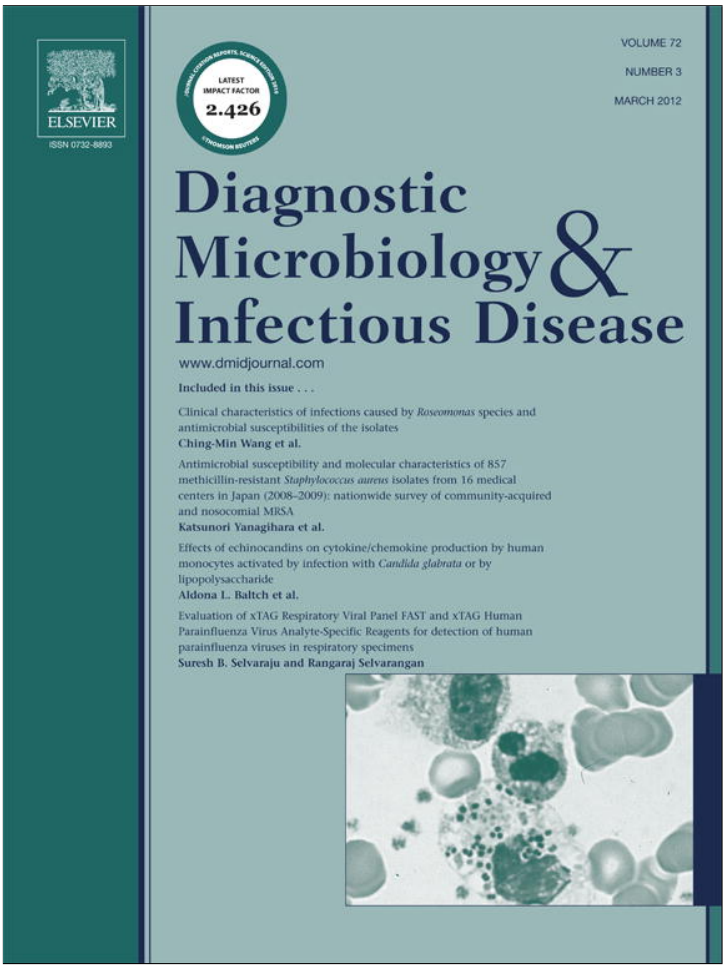

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Diagnostic Microbiology and Infectious Disease 72 (2012) 272–277

DIAGNOSTIC MICROBIOLOGY AND INFECTIOUS DISEASE

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Notes

Emergence and risk factors of β-lactamase–mediated resistance to oxyimino-β-lactams in *Enterobacteriaceae* isolates☆,☆☆

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Received 4 July 2011; accepted 12 November 2011

Abstract

We studied 193 *Enterobacteriaceae* isolates presenting diminished susceptibility to oxyimino-cephalosporins recovered in a Portuguese hospital (2004–2008). CTX-M-3 producers, firstly detected in Portugal, were associated with a *Klebsiella pneumoniae* microepidemic clone. Production of CTX-M–type enzymes (CTX-M-1/-3/-9/-14/-15/-32), age ≥65 years, and nosocomial infection were risk factors for higher nonsusceptibility to oxyimino-β-lactams. CMY-2 and DHA-1 β-lactamases were only identified in 1% of isolates.

Keywords: Oxyimino-β-lactams; *Enterobacteriaceae*; Portugal; ESBL; CTX-M-3; CTX-M-15

Some groups of β-lactamases, such as the extended-spectrum β-lactamases (ESBLs) and plasmid-mediated AmpC β-lactamases (PMAβ), are of particular significance in the failure of treatment for infections (Jacoby and Munoz-Price, 2005).

The geographic spread of CTX-M enzymes has increased rapidly, and these enzymes are currently the main cause of resistance to oxyimino-β-lactams, namely in Portugal (Hawkey and Jones, 2009; Mendonça et al., 2007, 2009). However, there is no information available on risk factors associated with resistance mechanisms to oxyimino-β-lactams and on the dissemination of PMAβ in the country, as well as its association with ESBL production; this is presented here for 1 hospital providing diverse health care facilities. The newly detected CTX-M-3 variant in Portugal is also reported.

Between the first semesters of 2004 to 2008, 193 *Enterobacteriaceae* (109 *Escherichia coli*, 81 *Klebsiella pneumoniae* and 3 *Proteus mirabilis*) isolates were collected consecutively (1 per patient), at Hospital Garcia de Orta, in Portugal and identified as ESBL producers. It is a 600-bed tertiary-level hospital (including a 28-bed intensive care unit), with a catchment population of about 390,000 citizens. Nosocomial (107/193, 55.4%) or community-acquired infections (75/193, 38.9%) were identified according to Centers of Disease Control and Prevention criteria (Garner et al., 1988); 11 (5.7%) of 193 were of unknown origin. The isolates were reached from different biological samples: urine (127, 65.8%), blood (27, 14.0%), wounds (17, 8.8%), others (18, 9.3%), and unknown origin (4, 2.1%).

Among all isolates, 106 (54.9%) were from women and 84 (43.5%) from men: 120 (62.2%) from patients ≥65 years old, 41 (21.2%) from patients 41–64 years old, 21 (10.9%) from patients 19–40 years old, and 8 (4.1%) from ≤18 years

☆ This work was presented in part at the 10th β-Lactamase Meeting, Eretria, Greece, 2008.

☆☆ V. Manageiro was supported by grant SFRH/BD/32578/2006 from Fundação para a Ciência e a Tecnologia, Lisbon, Portugal. D. Jones-Dias was supported by grant BRJ/02/DG/2009 from NIH Dr. Ricardo Jorge, Lisbon, Portugal.

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0732-8893/$ – see front matter
doi:10.1016/j.diagmicrobio.2011.11.009

Table 1
Antimicrobial susceptibility of the 193 *Enterobacteriaceae* ESBL-, non–ESBL-, and PMAβ-producing isolates

Antibiotic (disk content, μg)	Non-ESBL (n = 24)		PMAβ (n = 2)		CTX-M* (n = 154)		Non–CTX-M-ESBL† (n = 13)		Total ESBL (n = 167)		Total (N = 193)	
	R (%)	IR (%)	R (%)	IR (%)	R (%)	IR (%)	R (%)	IR (%)	R (%)	IR (%)	R (%)	IR (%)
Amoxicillin (25)	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Amoxicillin plus clavulanic acid (20 + 10)	58.3	87.5	100.0	100.0	40.9	84.4	23.1	53.8	39.5	82.0	40.4	82.9
Ticarcillin (75)	91.7	91.7	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.0	99.0
Piperacillin (75)	87.5	91.7	50.0	100.0	98.7	100.0	92.3	100.0	98.2	100.0	96.4	99.0
Piperacillin plus tazobactam (75 + 10)	62.5	66.7	0.0	0.0	16.2	53.9	23.1	38.5	16.8	52.7	22.3	53.9
Cephalothin (30)	66.7	95.8	100.0	100.0	100.0	100.0	92.3	100.0	99.4	100.0	95.3	99.5
Cefuroxime (30)	20.8	20.8	100.0	100.0	100.0	100.0	61.5	61.5	97.0	97.0	87.6	87.6
Cefepime (30)	12.5	25.0	0.0	0.0	69.5	98.1	7.7	61.5	64.7	95.2	57.5	85.5
Cefoxitin (30)	8.3	12.5	100.0	100.0	4.5	13.0	0.0	0.0	4.2	12.0	5.7	13.0
Ceftazidime (30)	25.0	91.7	50.0	100.0	81.8	95.5	92.3	100.0	82.6	95.8	75.1	95.3
Cefotaxime (30)	16.7	25.0	50.0	50.0	100.0	100.0	61.5	76.9	97.0	98.2	86.5	88.6
Ceftriaxone (30)	25.0	29.2	0.0	0.0	100.0	100.0	84.6	100.0	98.8	100.0	88.6	90.2
Aztreonam (30)	12.5	20.8	0.0	100.0	88.3	98.1	69.2	92.3	86.8	97.6	76.7	88.1
Imipenem (10)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Meropenem (10)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ciprofloxacin (5)	16.7	20.8	100.0	100.0	80.5	81.8	69.2	69.2	79.6	80.8	72.0	73.6
Norfloxacin (5)	12.5	16.7	100.0	100.0	81.8	81.8	69.2	69.2	80.8	80.8	72.5	73.0
Gentamicin (15)	12.5	20.8	0.0	0.0	56.5	57.8	76.9	76.9	58.1	59.3	51.8	53.9
Amikacin (30)	0.0	0.0	0.0	0.0	5.8	21.4	7.7	23.1	6.0	21.6	5.2	18.7
Trimethoprim–sulfamethoxazole (1.25 + 23.75)	29.2	50.0	50.0	100.0	63.0	63.6	38.5	46.2	61.1	62.3	57.0	61.1

R = Resistant; IR = nonsusceptible.

* Two CTX-M-producing isolates co-expressed PMAβ (CMY-2 enzymes).

† Non–CTX-M-ESBL, including enzymes of the SHV, TEM, and GES families.

Table 2
MICs of antibiotics for representative CTX-M–producing *E. coli* isolates, transformants* and the recipient†

E. coli strain	MIC (μg/mL) of antibiotic‡														
	AMX	AMC	TIC	CF	CAZ	CCAZ	CTX	CCTX	CFE	FOX	IMP	MER	CIP	GEN	TMP
E. coli K12 C600	≤4	≤4	≤4	4	≤1	≤1	≤1	≤1	≤1	2	≤0.5	≤0.5	≤0.5	0.25	0.25
INSRA7733 (CTX-M-1 + TEM-1)	>4096	64	4096	>1024	4	≤1	>1024	≤1	1024	8	≤0.5	≤0.5	≤0.5	2	>128
EcK12 C600 (CTX-M-1 + TEM-1)	>4096	32	4096	>1024	8	≤1	>1024	≤1	512	8	≤0.5	≤0.5	≤0.5	2	>128
INSRA6490 (CTX-M-3 + TEM-1 + SHV-1)	>4096	128	4096	>1024	16	≤1	>1024	≤1	>1024	8	≤0.5	≤0.5	≤0.5	2	>128
EcK12 C600 (CTX-M-3)	>4096	128	4096	>1024	8	≤1	>1024	≤1	1024	4	≤0.5	≤0.5	≤0.5	0.5	>128
INSRA7604 (CTM-M-9)	4096	16	4096	>1024	≤1	≤1	512	≤1	16	8	≤0.5	≤0.5	32	1	>128
EcK12 C600 (CTX-M-9)	2048	8	4096	1024	≤1	≤1	512	≤1	8	≤1	≤0.5	≤0.5	≤0.5	0.5	>128
INSRA5776 (CTX-M-14 + TEM-1)	>4096	32	4096	>1024	8	≤1	1024	≤1	32	64	≤0.5	≤0.5	>512	64	>128
EcK12 C600 (CTX-M-14)	4096	32	4096	>1024	2	≤1	512	≤1	8	4	≤0.5	≤0.5	≤0.5	1	0.25
INSRA7199 (CTX-M-15)	4096	32	4096	>1024	16	≤1	1024	≤1	256	4	≤0.5	≤0.5	≤0.5	2	0.25
EcK12 C600 (CTX-M-15)	>4096	8	4096	>1024	32	≤1	>1024	≤1	512	4	≤0.5	≤0.5	≤0.5	1	≤0.125
INSRA7751 (CTX-M-32 + TEM-1)	>4096	16	4096	>1024	64	≤1	>1024	≤1	1024	4	≤0.5	≤0.5	≤0.5	4	>128
EcK12 C600 (CTX-M-32 + TEM-1)	>4096	32	4096	>1024	64	≤1	>1024	≤1	512	2	≤0.5	≤0.5	≤0.5	0.5	>128

* EcK12 C600 harboring CTX-M-1 plus TEM-1, CTX-M-3, CTX-M-9, CTX-M-14, CTX-M-15, CTX-M-32 plus TEM-1 are the transformants of the clinical *E. coli* isolates INSRA7733 (CTX-M-1 + TEM-1), INSRA6490 (CTX-M-3 + TEM-1 + SHV-1), INSRA7604 (CTM-M-9), INSRA5776 (CTX-M-14 + TEM-1), INSRA7199 (CTX-M-15), and INSRA7751 (CTX-M-32 + TEM-1), respectively.

† *E. coli* K12 C600 was the recipient.

‡ AMX = Amoxicillin; AMC = amoxicillin plus clavulanic acid; TIC = ticarcillin; CF = cephalothin; CAZ = ceftazidime; CCAZ = ceftazidime plus clavulanic acid; CTX = cefotaxime; CCTX = cefotaxime plus clavulanic acid; CFE = cefepime; FOX = cefoxitin; IMP = imipenem; MER = meropenem; CIP = ciprofloxacin; GEN = gentamicin; TMP = trimethoprim–sulfamethoxazole.

Table 3
Odds ratios (OR) and 95% confidence intervals (CI) from the analysis of risk factors for β-lactamase–mediated nonsusceptibility to oxyimino-β-lactams in *Enterobacteriaceae* isolates, 2004–2008*

Antimicrobial agent (no. of nonsusceptible isolates)	Risk factor	OR†	95% CI	*P* value
Cefotaxime (171)	Age group (≤18)	0.11 (P)	0.02–0.47	.013
	Age group (≥65)	6.98	2.45–19.89	<.001
	Genotypic group (non-ESBL)	0.01 (P)	0–0.03	<.001
	Ward (internal medicine)	13.2	1.74–100.5	.001
	Year of isolation (2008)	0.36 (P)	0.14–0.88	.041
Ceftriaxone (174)	Age group (≥65)	3.18	1.19–8.48	.034
	Genotypic group (non-ESBL)	0.005 (P)	0.001–0.028	<.001
	Ward (internal medicine)	5.07	1.13–22.65	.028
Aztreonam (170)	Age group (19–40)	0.27 (P)	0.09–0.8	.048
	Age group (≥65)	3.62	1.45–9.04	.009
	Genotypic group (ESBL CTX-M)	52.98	14.38–195.2	<.001
	Genotypic group (non-ESBL)	0.01 (P)	0.002–0.03	<.001
	Ward (internal medicine)	13.96	1.84–106	.001
Gentamicin and/or Amikacin (129)	Age group (19–40)	0.33 (P)	0.13–0.82	.030
	Age group (≥65)	2.37	1.28–4.39	.009
	Genotypic group (ESBL CTX-M)	3.96	1.91–8.23	<.001
	Genotypic group (non-ESBL)	0.1 (P)	0.03–0.27	<.001
	Community acquired	0.48 (P)	0.26–0.89	.029
	Nosocomial	2.1	1.14–3.86	.024
Ciprofloxacin and/or Norfloxacin (147)	Age group (≤18)	0.11 (P)	0.02–0.55	.010
	Age group (≥65)	7.78	3.78–16.02	<.001
	Genotypic group (ESBL CTX-M)	8.79	4.04–19.15	<.001
	Genotypic group (non-ESBL)	0.13 (P)	0.05–0.33	<.001
	Nosocomial	2.83	1.46–5.48	.003
	Source (urine)	2.65	1.37–5.13	.006
Trimethoprim–sulfamethoxazole (118)	Age group (19–40)	0.35 (P)	0.14–0.88	.042
	Community acquired	0.47 (P)	0.26–0.85	.019
	Nosocomial	2.22	1.23–4.01	.012
	Ward (surgery)	4.91	1.08–22.27	.037
MDR (134)	Age group (19–40)	0.26 (P)	0.1–0.66	.008
	Age group (≥65)	2.89	1.55–5.38	.001
	Genotypic group (ESBL CTX-M)	5.27	2.49–11.13	<.001
	Genotypic group (non-ESBL)	0.07 (P)	0.02–0.22	<.001
	Community acquired	0.48 (P)	0.26–0.89	.029
	Nosocomial	2.31	1.26–4.26	.011

* Differences in antimicrobial resistance were simultaneously tested for association with age categories, β-lactamase genotypic group, gender, site of infection, source, ward, and year of specimen. Only significant associations are presented: *P* values ≤ 0.05 and confidence limits excluding null values (0, 1, or [*n*]).

† (P) indicates an OR value for a protective or negative association; otherwise, values are for a positive association.

old. Information regarding the age and sex of the patient was lacking for 3 isolates. As concerns the wards, 67 (34.7%) of 193 isolates were collected in the internal medicine service, 31 (16.1%) of 193 in the emergency room or ambulatory (outpatients), 16 (8.3%) of 193 in surgery, 14 (7.2%) of 193 in nephrology/urology, 13 (6.7%) of 193 in intensive care unit, 10 (5.2%) of 193 in observation room, 6 (3.1%) of 193 in hematology/oncology, 5 (2.6%) of 193 in gastroenterology, 4 (2.1%) of 193 in neurology, 3 (1.6%) of 193 in obstetrics/gynecology, and 24 (12.4%) of 193 in other hospital wards.

Disk diffusion method and MICs were used to test the antimicrobial susceptibility of clinical isolates, and transformants plus their clinical isolates, respectively (SFM; http://www.sfm-microbiologie.org/) (Tables 1 and 2). All *Enterobacteriaceae* presented diminished susceptibility to at least 1 oxyimino-cephalosporin and 88.1% to aztreonam. In this set of strains, 69.4% (134/193) were multidrug-resistant (MDR), as presenting a reduced susceptibility to 3 or more structurally unrelated antibiotics, among which were fluoroquinolones (97%), aminoglycosides (91.8%), and trimethoprim–sulfamethoxazole (72.4%).

Fig. 1. Distribution of 193 isolates expressing β-lactamase–mediated resistance to oxyimino-cephalosporins (first semester from 2004 to 2008), and genetic relatedness of 21 *K. pneumoniae* and 28 *E. coli* isolates by pulsed-field gel electrophoresis. *K. pneumoniae* isolates with profile types Kp0001 and Kp0006, and *E. coli* isolates with profiles Ec0006, Ec0010, and Ec0013 were defined as forming clusters KpI, KpIII, EcI, EcII, and EcIII, respectively.

Clinical isolates were tested for the presence of bla_{ESBL} and $bla_{PMA\beta}$ genes, by using polymerase chain reaction and sequencing, as described previously (Mendonça et al., 2009; Pérez-Pérez and Hanson, 2002). Overall, the presence of ESBLs was confirmed in 167 of 193 isolates and the bla_{CTX-M} genes in 154 of 167 isolates (CTX-M-ESBL group), such as $bla_{CTX-M-1}$ (6, 3.9%), $bla_{CTX-M-3}$ (13, 8.4%), $bla_{CTX-M-9}$ (2, 1.3%), $bla_{CTX-M-14}$ (4, 2.6%), $bla_{CTX-M-15}$ (123, 79.9%), and $bla_{CTX-M-32}$ (6, 3.9%). Among the CTX-M group, 76.0% of isolates were MDR, 62.4% of them with a nosocomial origin, which is a cause of concern (Pitout et al., 2005). Two bla_{CMY-2} genes were identified in *E. coli* isolates in association with $bla_{CTX-M-15}$ gene (Table 1). Indeed, as reported in Taiwan (Chen et al., 2007), the coexistence of ESBL and AmpC β-lactamases not only limits treatment options, but also complicates routine phenotypic detection of ESBLs, causing a growing problem for clinical microbiology laboratories (Hanson, 2003; Pai et al., 2004).

In the non–CTX-M-ESBL group (n = 13), we detected β-lactamase genes of the SHV (bla_{SHV-2a}, n = 3; bla_{SHV-12}, n = 7; $bla_{SHV-115}$, n = 1), TEM (bla_{TEM-10}, n = 1; bla_{TEM-52}, n = 1; $bla_{TEM-107}$, n = 1), and GES (bla_{GES-1}, n = 1) families, alone or associated with each other, with non-ESBL genes (bla_{SHV-11}, bla_{TEM-1} and bla_{OXA-30}) or with inhibitor-resistant SHV genes (bla_{SHV-26}). Two non–ESBL PMAβ-producing isolates were identified: 1 *E. coli* expressing the bla_{CMY-2} gene and 1 *K. pneumoniae* expressing the $bla_{DHA-1-type}$ gene. PMAβ-producing isolates were nonsusceptible to almost all β-lactams tested, except the combination of piperacillin plus tazobactam, cefepime, ceftriaxone, and carbapenems (Table 1). No ESBL enzymes of the CTX-M family were detected in 1999 in this hospital, in contrast with the identification of a CMY-2-type–producing isolate (data not shown). However, here, there was a substantial emergence in the number of ESBLs found. Although ESBLs from SHV, TEM, and GES families are important, because they confer β-lactamase–mediated resistance to oxyimino-β-lactams on *Enterobacteriaceae*, the ability of CTX-M enzymes to spread between different pathogens has led to it becoming the most significant problem (Cantón and Coque, 2006; Hawkey and Jones, 2009; Livermore et al., 2007).

Conjugation, performed by liquid and solid mating assays (Mendonça et al., 2006), showed that all CTX-M–type determinants tested were found to be carried on conjugative plasmids, facilitating their spread (Cantón and Coque, 2006). In general, the transformants had antibiotic resistance profiles similar to those of their parental clinical isolates (Table 2).

We tested for risk factors associated with nonsusceptibility to oxyimino-β-lactams, mediated by the production of β-lactamases, in *Enterobacteriaceae* isolates (Table 3; only factors identified as significant are shown: $P \leq 0.05$). OpenEpi software, version 2.3.1 (Sullivan et al., 2009), was used for statistical analysis. Fisher exact test was used to assess differences in antibiotic resistance between different groups. Two-sided P values of ≤0.05 were considered to be statistically significant. Associations were determined by calculation of odds ratios with 95% confidence intervals. The null hypothesis was rejected for P values of ≤0.05. Higher nonsusceptibility rates were consistently associated (for at least 4 of the 6 classes of antibiotics tested and MDR) with an age of ≥65 years, nosocomial infection, and production of CTX-M–type enzymes (Table 3). Long-term hospitalizations leading to nosocomial infections and exposure to antibiotics have also been described by others as risk factors for the acquisition of ESBL-producing isolates (Ben-Ami et al., 2009; Lavigne et al., 2007). A protective association was found for other age groups than ≥65 years, for the acquisition of infections due to non–ESBL-producing isolates and for the community-acquired infections. The internal medicine ward was a risk factor for nonsusceptibility to oxyimino-β-lactams and surgery for nonsusceptibility to trimethoprim–sulfamethoxazole. *Enterobacteriaceae* isolates collected from urine were significantly associated with nonsusceptibility to fluoroquinolones, but not to the other drugs tested. We also found that nonsusceptibility to oxyimino-β-lactams, aminoglycosides, and quinolones was associated with CTX-M-ESBL isolates, and this may be related to the dissemination of a single plasmid or to other mobile genetic elements (Cantón and Coque, 2006; Nicolas-Chanoine et al., 2008; Woodford et al., 2009).

The clonal relationship between 49 *E. coli* and *K. pneumoniae* isolates representative of all CTX-M β-lactamase groups, years, community/nosocomial origin, and wards was studied by pulsed-field gel electrophoresis as previously described (Mendonça et al., 2007) (Fig. 1). The persistent *E. coli* CTX-M-15–producer clone (cluster EcII) was widespread across diverse hospital wards and over several years. *K. pneumoniae*–producing CTX-M-15 (cluster KpI) and CTX-M-3 (cluster KpII) were mostly isolated in internal medicine and included both nosocomial and community-acquired isolates (Fig. 1). Clonality suggests that a micro-epidemic clone emerged in this hospital in 2006, in both pathogens, and disappeared in 2008, revealing the first description of CTX-M-3 in Portugal, an enzyme initially reported in 1995 in *Citrobacter freundii* and in *E. coli* isolates from Poland (Gniadkowski et al., 1998). Other outbreaks associated with Gram-negative isolates producing CTX-M-3 have been described all over the world (Dutour et al., 2002; Empel et al., 2008; Literacka et al., 2009; Moriguchi et al., 2007; Ramdani-Bouguessa et al., 2006; Yan et al., 2000).

Given the spread of both CTX-M-3 and CTX-M-15 β-lactamases in this hospital and the recent demonstration that CTX-M-3 variants (such as CTX-M-15) confer greater resistance to extended-spectrum cephalosporins (Novais et al., 2010), monitoring isolates for the presence and evolution of bla_{ESBL}, and particularly bla_{CTX-M}, is now imperative.

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