Evaluation of Occurrence and Characterization of Carbapenemases among Enterobacteriaceae Isolated by the SENTRY Antimicrobial Surveillance Program Worldwide (2000-2004)

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SENTRY ANTIMICROBIAL SURVEILLANCE

AMENDED ABSTRACT

Background: Enterobacteriaceae (ENT) strains with decreased susceptibility (S) to carbapenems (CRB) were evaluated for the production of carbapenemases (CPase).
Methods: ENT strains collected through the SENTRY Program were tested for S by CLSI broth microdilution methods against imipenem (IMI), meropenem (MER) and >25 antimicrobials. Isolates with elevated MIC values for CRB (≥ 2 µg/ml for IMI and MER), except indole+ *Proteae* and *P. mirabilis*, were screened for production of metallo-ß-lactamase (M8L) and group 2f ß-lactamases (2f BL) by disk approximation (DA) tests using EDTA and 2-MPA as inhibitors of M8L and clavulanate as an inhibitor of 2f BL. Isolates with positive DA tests were evaluated by PCR using generic primers for IMP, VIM and SPM when DA was positive for M8L; and for KPC, SME, IMI and NmcA when DA was positive for 2f BL. Sequencing was performed in selected strains to characterize the enzyme. CPase-producing strains were epidemiologically typed by automated ribotyping and PFGE.
Results: Among 37,577 strains evaluated, 117 (0.31%) had increased CRB MIC values and a CPase was identified in 49 (42%) strains. The results are summarized in the table.

Organism	No. of isolates: Tested	Increased CRB MIC	PCR +	CPase (n)	Country (State)
Citrobacter spp.	1,061	3	2	KPC-3 (2)	USA (NY)
Enterobacter spp.	5,206	57	19 (*E. cloacae*)	IMP-1 (11)	Turkey
				VIM-1 (2)	Italy
				KPC-2/3 (5)	USA (AK, NY, VA)
				IMI-1 (1)	USA (NY)
E. coli	20,138	4	2	KPC-3 (2)	USA (NY)
Klebsiella spp. (KSP)	8,977	44	23 (19 *K. pneumoniae* and 4 *K. oxytoca*)	VIM-1 (9)	Greece
				KPC-2/3 (14)	USA (NY, AK)
Serratia spp.	2,175	9	3	KPC-3 (1)	USA (NY)
				SME-2 (2)	USA (TX)

In general, strains with M8L were observed in Europe (EU) while strains with 2f BL were observed in the USA. KPC-like enzymes were identified in 7 species and in 5 USA medical centers (MC), but most were identified in KSP isolated from 2 MC in NY. Dissemination was mainly clonal and gene sequencing demonstrated bla_{KPC-2} and bla_{KPC-3}.
Conclusions: The occurrence of CPase-producing ENT is still rare but appears to be increasing in some regions, mainly M8Ls in the Mediterranean EU and KPC in the NY area.

INTRODUCTION

Enterobacteriaceae comprises a major group of pathogens causing hospital-acquired infections. "Third-generation" cephalosporins are considered excellent choices for treating infections caused by these enteric bacilli. However, the emergence and dissemination of extended spectrum ß-lactamases (ESBL's) has compromised the use of these agents in certain geographic regions. As a consequence, the use of carbapenems has increased significantly in some hospitals and carbapenem-resistant Gram-negative bacilli have started to emerge.

Gram-negative bacteria can become resistant to carbapenems by over-production of AmpC ß-lactamases usually associated with loss of outer membrane porins and/or efflux pumps. The production of metallo-ß-lactamases (M8Ls) or carbapenemases of Bush group 2f may also confer high-level resistance to carbapenems. The fact that these ß-lactamase genes are generally located on plasmids or transposons facilitates the dissemination of these potent resistance mechanisms.

Carbapenemases with a metal ion (typically Zn^{++}) at the active site, commonly called M8Ls, were initially detected in non-fermentative Gram-negative species, such as *Stenotrophomonas, Pseudomonas* and *Acinetobacter,* mainly in Asia, Europe and South America. In the last few years these carbapenemases have been described in some enteric bacilli, such as *Klebsiella pneumoniae*, *Enterobacter* spp. and *Serratia* spp.

The serine carbapenemases (Bush group 2f) have been sporadically reported from Europe and the United States, until recent reports of outbreaks of KPC-producing *K. pneumoniae* in the New York City area. These enzymes have been confined to very few species of Enterobacteriaceae, but are rapidly emerging in many medical centers in other parts of the United States (USA).

The degree of resistance to carbapenems may vary significantly among carbapenemase-producing Enterobacteriaceae. Among M8L-producing *P. aeruginosa,* carbapenem MIC values are usually high, but carbapenemase-producing Enterobacteriaceae may show imipenem and meropenem MIC values within the susceptible category when using the Clinical and Laboratory Standards Institute (CLSI, formerly National Committee for Clinical Laboratory Standards [NCCLS]) breakpoints. In the present study, we screened Enterobacteriaceae with reduced susceptibility to carbapenems for carbapenemase genes among SENTRY Antimicrobial Surveillance Program isolates collected in the 2000-2004 period.

MATERIALS AND METHODS

Bacterial isolates. In the 2000-2004 period the SENTRY Program collected 44,219 Enterobacteriaceae isolates from medical centers located in North America, Latin America and Europe. The isolates were consecutively collected from bloodstream infections, skin and soft tissue infections, urinary tract infections and pneumonia in hospitalized patients according to a common protocol. Only isolates considered clinically significant were included in the study. Species identification was confirmed by standard biochemical tests and Vitek cards, where necessary.

Susceptibility testing. The Enterobacteriaceae isolates were susceptibility tested against more than 25 antimicrobials by the broth microdilution procedure as described by the CLSI using validated dry-form panels manufactured by Trek Diagnostics (Cleveland, OH, USA). Interpretations of susceptibility to all antimicrobials tested were by CLSI (2005) criteria, where available. *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212 and *Pseudomonas aeruginosa* ATCC 27853 were routinely included in the testing for quality assurance.

Screening for carbapenemases. Enterobacteriaceae isolates with reduced susceptibility to imipenem and meropenem (MIC ≥ 2 µg/ml) were tested for production of carbapenemases. Indole-positive proteae and *Proteus mirabilis* were excluded since these species are inherently less susceptible to carbapenems.

- Disk approximation. Potential carbapenemase producers were screened using disk approximation techniques. M8L screens were performed using imipenem, meropenem and ceftazidime as substrates and EDTA as well as 2-mercaptopropionic acid (2-MPA) as ß-lactamase inhibitors. Screening for serine carbapenemases was achieved by a method described by Pottumarthy et al. (2002) in which imipenem and meropenem were used as substrates and clavulanic acid as the ß-lactamase inhibitor.
- PCR. Isolates with positive disk approximation test for M8L were screened for bla_{IMP}, bla_{VIM} and bla_{SPM} using PCR primers described elsewhere. Because some strains producing serine carbapenemases may have a negative disk screening test result, isolates with elevated carbapenem MIC values and negative PCR results for M8L genes were screened for IMI, KPC, NmcA and SME genes.

Gene sequencing. PCR amplicons for the carbapenemase genes were sequenced using a Sanger-based dideoxy sequencing strategy involving the incorporation of fluorescent-dye-labeled terminators into the sequencing reaction products. Sequences obtained were compared to the available sequences via NCBI BLAST search.

Epidemiological studies. Multiple isolates from the same medical center harboring carbapenemases belonging to the same family were typed using the Riboprinter™ Microbial Characterization system. Isolates with identical ribotypes were further characterized by pulsed-field gel electrophoresis (PFGE).

RESULTS

- Overall susceptibility and resistance patterns of Enterobacteriaceae isolates to key ß-lactams, aminoglycosides and fluoroquinolone compounds are presented in Table 1. Imipenem and meropenem remained the most active agents against enteric bacilli with virtually no resistance in *E. coli* (only three isolates with imipenem MIC of ≥ 8 µg/ml, and only one resistant), 0.1% in *Citrobacter* spp., 0.2% in *Klebsiella* spp. and *Enterobacter* spp., and 0.3-0.4% in *Serratia* spp.
- *E. coli* ranked highest in the frequency of isolation compared to other enteric bacilli and generally exhibited low resistance rates to most classes of antimicrobials, except for fluoroquinolones (13.4% resistance to ciprofloxacin; Table 1).
- *K. pneumoniae* was the second most frequently isolated Enterobacteriaceae, and 18.7 - 19.4% of isolates showed an ESBL phenotype. In addition, 13.3 and 8.1% of strains were resistant to gentamicin and ciprofloxacin, respectively (Table 1).
- *Enterobacter* spp. showed high rates of resistance to ceftazidime (22.4%) and aztreonam (19.4%), indicating a high prevalence of strains with stably derepressed production of AmpC ß-lactamases (Table 1). In contrast, *Serratia* spp. were highly susceptible to cephalosporins and monobactam (90.7 - 99.6% susceptible), but showed the highest carbapenem resistance rates (0.3 - 0.4%).
- Reduced carbapenem susceptibility was detected in only four isolates among over 20,000 *E. coli* isolates evaluated (Table 2). Two of these isolates, both from New York, had the bla_{KPC-3}. They were isolated two years apart (2002 and 2004) and showed distinct molecular typing patterns (Table 3).
- Fourteen *Klebsiella* spp isolates were found to produce KPC-2 or KPC-3 enzyme, 12 from two medical centers located in New York City (10 *K. pneumoniae* and two *K. oxytoca*), and one each from medical centers located in Virginia and Arkansas (Table 2).
- bla_{VIM-1} was detected in nine *K. pneumoniae* isolates from one medical center located in Greece. Two major clusters were observed along with two unique isolates (Tables 2 and 3).
- Eleven *E. cloacae* isolates harboring bla_{IMP-1} were isolated from Turkey (18 from Ankara and one from Istanbul). Based on the molecular typing results, three distinct clones were identified in 2003 and 2004. The IMP-1 producing isolate from Istanbul was unrelated to any of the clones from Ankara (Tables 2 and 3).
- Two medical centers in Italy had single *E. cloacae* isolates carrying bla_{VIM-1}. These isolates showed distinct molecular typing patterns.
- Four *Enterobacter cloacae* isolates from two sites in the USA (Virginia [three isolates] and New York City [one isolate]) showed amplification products for bla_{KPC} primers (KPC-2/3), while one *E. cloacae* strain isolated in New York in 2001 was observed to harbor bla_{IMI-1} (Table 2).
- None of the *E. aerogenes* isolates with elevated carbapenem MIC values (n=21, 19 from European medical centers) were found to produce a carbapenemase.
- KPC-2/3-producing *K. pneumoniae* isolates with identical molecular typing patterns were identified in two distinct medical centers located in the New York City area, indicating inter-hospital transmission of resistance clones (Table 3).
- Two non-clonal *Citrobacter freundii* isolates from a New York medical center showed amplification product with bla_{KPC} primers (KPC-3; Tables 2 and 3).

Table 1. In vitro activity of select antimicrobial agents against five groups of Enterobacteriaceae isolated in North America, Latin America and Europe in the 2000 - 2004 period as part of the SENTRY Antimicrobial Surveillance Program.

Organism/antimicrobial agent (no. tested)	MIC (µg/ml): 50%	90%	Range	% by category: Susceptible	Resistant
E. coli (20,138)					
Imipenem	0.12	0.5	≤0.06->8	>99.9	<0.1[a]
Meropenem	≤0.06	≤0.06	≤0.06->8	>99.9	0.0
Ceftazidime	≤1	2	≤1->16	96.9	2.1(5.3)[b]
Ceftriaxone	≤0.25	≤0.25	≤0.25->32	96.6	2.6(4.5)[b]
Aztreonam	≤0.12	0.25	≤0.12->16	96.7	2.6(5.3)[b]
Piperacillin/Tazobactam	2	4	≤0.5->256	96.3	1.8
Gentamicin	≤2	≤2	≤2->8	93.2	6.0
Ciprofloxacin	≤0.03	4	≤0.03->4	86.5	13.4
Klebsiella spp. (8,977)					
Imipenem	0.12	0.5	≤0.06->8	99.7	0.2
Meropenem	≤0.06	≤0.06	≤0.06->16	99.6	0.2
Ceftazidime	1	>16	≤0.12->16	86.2	11.4(18.7)[b]
Ceftriaxone	≤0.25	>32	≤0.25->32	85.1	10.1(19.4)[b]
Aztreonam	≤0.12	>16	≤0.12->16	83.8	14.6(19.4)[b]
Piperacillin/Tazobactam	2	64	≤0.5->256	86.9	8.9
Gentamicin	≤2	>8	≤2->8	85.2	13.3
Ciprofloxacin	≤0.03	1	≤0.03->4	90.3	8.1
Enterobacter spp. (5,206)					
Imipenem	≤0.5	1	≤0.5->8	99.5	0.2
Meropenem	≤0.06	0.12	≤0.06->8	99.5	0.2
Ceftazidime	≤1	>16	≤1->16	73.4	22.4
Ceftriaxone	≤0.25	>32	≤0.25->32	75.8	13.9
Aztreonam	≤0.12	>16	≤0.12->16	75.1	19.4
Piperacillin/Tazobactam	2	64	≤0.5->256	77.7	10.0
Gentamicin	≤2	>8	≤2->8	88.4	10.1
Ciprofloxacin	≤0.03	4	≤0.03->4	87.2	10.9
Citrobacter spp. (1,061)					
Imipenem	≤0.5	1	≤0.5->8	99.9	0.1
Meropenem	≤0.06	≤0.06	≤0.06-4	100.0	0.0
Ceftazidime	≤2	>16	≤2->16	83.8	14.1
Ceftriaxone	≤0.25	32	≤0.25->32	85.2	6.1
Aztreonam	≤0.12	>16	≤0.12->16	84.5	12.0
Piperacillin/Tazobactam	2	32	≤1->256	86.8	5.3
Gentamicin	≤2	≤2	≤2->8	91.5	7.2
Ciprofloxacin	≤0.03	1	≤0.03->4	90.4	8.1
Serratia spp. (2,175)					
Imipenem	0.5	1	≤0.12->8	99.4	0.4
Meropenem	≤0.06	0.12	≤0.06->8	99.6	0.3
Ceftazidime	≤1	2	≤1->16	95.4	3.4
Ceftriaxone	≤0.25	8	≤0.25->32	90.7	4.7
Aztreonam	≤0.12	4	≤0.12->16	93.1	5.8
Piperacillin/Tazobactam	2	32	≤0.5->256	89.6	2.8
Gentamicin	≤2	8	≤2->8	89.2	8.8
Ciprofloxacin	0.12	1	≤0.03->4	91.3	5.3

a. Only one isolate resistant to imipenem.
b. Percentage of isolates with ESBL phenotype (MIC, ≥ 2 µg/ml).

- Two epidemiologically distinct SME-2-producing *Serratia marcescens* were isolated from a medical center in Texas, whereas one KPC-3-producing *S. marcescens* was recovered from a New York medical center (Table 3).
- $bla_{KPC-2/3}$ was identified in 10 isolates (one *E. coli*, three *E. cloacae*, two *K. oxytoca*, four *K. pneumoniae*) that had a negative disk approximation test for serine carbapenemases.

Table 2. Summary of the results on the evaluation of Enterobacteriaceae strains with decreased susceptibility to carbapenems.

Organism	No. tested	No. (%) of isolates with elevated CRB MIC	MIC range (µg/ml)[a]: Imipenem	Meropenem	No. of isolates with PCR+	Carbapenemase type	Organism (no.)	Country (state)
E. coli	20,138	4 (0.02)	2-8	4-8	2	KPC-3	*E. coli* (2)	USA (NY)
Klebsiella spp.	8,976	46 (0.51)	2->8	2->8	23	VIM-1	*K. pneumoniae* (9)	Greece
						KPC-2/3	*K. pneumoniae* (10)	USA (NY)[b]
							K. oxytoca (4)	USA (NY,VA,AR)
Enterobacter spp.	5,206	57 (1.09)	2->8	2->8	19	IMP-1	*E. cloacae* (11)	Turkey[b]
						VIM-1	*E. cloacae* (2)	Italy[b]
						KPC-2/3	*E. cloacae* (4)	USA (NY, VA)
						KPC-2	*E. hormaechei* (1)	USA (NY)
						IMI-1	*E. cloacae* (1)	USA (NY)
Citrobacter spp.	1,061	3 (0.28)	2-4	2-4	2	KPC-3	*C. freundii* (2)	USA (NY)
Serratia spp.	2,175	9 (0.41)	8->8	2->8	3	SME-2	*S. marcescens* (2)	USA (TX)
						KPC-3	*S. marcescens* (1)	USA (NY)

a. Range of imipenem and meropenem MIC values among the Enterobacteriaceae isolates with reduced susceptibility to carbapenems.
b. Strains in these groups were isolated from two medical centers.
Abbreviations = CRB, carbapenems (imipenem and meropenem)

Table 3. Epidemiological profiles of carbapenemase-producing Enterobacteriaceae isolates.

Carbapenemase	Location of medical center	Organism	Molecular typing (no. isolates)
Metallo-ß-lactamases			
VIM-1	Italy	*E. cloacae*	258.261.6 (1)
			105.225.4 (1)
	Greece	*K. pneumoniae*	258.211.6/D (5)
			105.736.5/C (1)
			258.258.4/E (2)
			105.444.1 (1)
IMP-1	Turkey (Ankara)	*E. cloacae*	258.250.1/B (4)
			255.187.2/A (4)
			258.213.6/C (2)
	Turkey (Istanbul)	*E. cloacae*	258.213.5 (1)
Serine carbapenemases			
KPC-2/3	New York (A)[a]	*C. freundii*	258.274.8 (1)
			258.274.7 (1)
		E. cloacae	105.539.8 (1)
		E. hormaechei	105.937.8 (1)
		E. coli	258.185.5(1)
			105.1177.6 (1)
		K. oxytoca	105.1784.3 (1)
			258.274.1 (1)
		K. pneumoniae	105.497.1/C (2)
			105.520.4/C (1)
			258.249.7 (1)
			105.203.3 (1)
			258.273.2 (1)
		S. marcescens	105.716.5 (1)
	New York (B)[a]	*K. pneumoniae*	105.497.1/C (2)
			105.520.4/C (2)
	Virginia	*E. cloacae*	105.226.3/A (2)
			258.274.5 (1)
		K. oxytoca	258.273.8 (1)
	Arkansas	*K. oxytoca*	258.277.1 (1)
IMI-1	New York (A)[a]	*E. cloacae*	258.69.3 (1)
SME-2	Texas	*S. marcescens*	258.249.6 (1)
			258.275.3 (1)

a. Designations A and B represent two different medical centers in New York City, USA.
Abbreviations: ND, not determined.

CONCLUSIONS

- Metallo-ß-lactamase (VIM-1 and IMP-1) producing *K. pneumoniae* and *E. cloacae* caused multiple epidemics in Greece and Turkey, respectively. However, these enzymes were not detected among Enterobacteriaceae isolates in North America or Latin America.
- Many families of serine carbapenemases were identified among multiple genera of enteric bacilli isolated in the USA, especially around the New York City area, but also in Virginia, Arkansas and Texas.
- This is the first report of *Citrobacter freundii* and *Serratia marcescens* harboring bla_{KPC-3}.
- There is an immediate need to contain the dissemination of carbapenemases, especially in the USA and Southeastern Europe, in order to preserve the activity of this important class of ß-lactams against Enterobacteriaceae and other Gram-negative bacilli.

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