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Evaluation of Clonality and Carbapenem Resistance Mechanisms among *Acinetobacter baumannii-Acinetobacter calcoaceticus* Complex and *Enterobacteriaceae* Isolates Collected in European and Mediterranean Countries and Detection of Two Novel β-Lactamases, GES-22 and VIM-35

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We evaluated doripenem-resistant *Acinetobacter baumannii-Acinetobacter calcoaceticus* complex (ACB; *n* = 411) and *Enterobacteriaceae* (*n* = 92) isolates collected from patients from 14 European and Mediterranean countries during 2009 to 2011 for the presence of carbapenemase-encoding genes and clonality. Following susceptibility testing, carbapenem-resistant (doripenem MIC, >2 μg/ml) isolates were screened for carbapenemases. New β-lactamase genes were expressed in a common background and susceptibility was tested. Class 1 integrons were sequenced. Clonality was evaluated by pulsed-field gel electrophoresis and multilocus sequence typing (Pasteur scheme). Relative expression of β-lactam intrinsic resistance mechanisms was determined for carbapenemase-negative *Enterobacteriaceae*. ACB and *Enterobacteriaceae* displayed 58.9 and 0.9% doripenem resistance, respectively. bla_{OXA-23}, bla_{OXA-58}, and $bla_{OXA-24/OXA-40}$ were detected among 277, 77, and 29 ACB, respectively (in 8, 6, and 5 countries). Ten Turkish isolates carried bla_{GES-11} or bla_{GES-22}. GES-22 (G243A and M169L mutations in GES-1) had an extended-spectrum β-lactamase profile. A total of 33 clusters of ≥2 ACB isolates were observed, and 227 isolates belonged to sequence type 2/international clone II. Other international clones were limited to Turkey and Israel. Doripenem-resistant *Enterobacteriaceae* increased significantly (0.7 to 1.6%), and 15 bla_{KPC-2}- and 22 bla_{KPC-3}-carrying isolates, mostly belonging to clonal complexes 11 and 258, were observed. *Enterobacteriaceae* isolates producing OXA-48 (*n* = 16; in Turkey and Italy), VIM-1 (*n* = 10; in Greece, Poland, and Spain), VIM-26 (*n* = 1; in Greece), and IMP-19, VIM-4, and the novel VIM-35 (*n* = 1 each from Poland) were detected. VIM-35 had one substitution compared to VIM-1 (A235T) and a similar susceptibility profile. One or more resistance mechanisms were identified in 4/6 carbapenemase-negative *Enterobacteriaceae*. This broad evaluation confirms results from country-specific surveys and shows a highly diverse population of carbapenemase-producing ACB and *Enterobacteriaceae* in Europe and Mediterranean countries.

Antimicrobial resistance in Gram-negative organisms continues to endanger patients hospitalized with infections caused by these pathogens (1). Among Gram-negative species, *Acinetobacter baumannii* is especially threatening, since this species is intrinsically resistant to various antimicrobial agents, is an important cause of nosocomial infections and outbreaks, and is capable of surviving up to 5 months in the environment (2, 3). The carbapenems are among the antimicrobial classes that remain active against *A. baumannii*; however, carbapenem resistance is increasing in many countries (3, 4). According to the most recent European Antimicrobial Resistance Surveillance Network (EARS-Net) report (5), carbapenem resistance rates in 2012 were greater than 50% in at least four European countries (Greece, Italy, Portugal, and Romania) and close to or greater than 50% in another three countries (Bulgaria, Cyprus, and Hungary). Although the resistance rates varied remarkably among other countries surveyed for the EARS-Net (5), the ability of *A. baumannii* to acquire resistance genes and the frequent exchange of patients among European countries could lead to changes in these scenarios in a reasonably short time period.

Carbapenem resistance in *A. baumannii* is mainly mediated by carbapenemases of the Ambler class D family, also known as oxacillinases. Although other mechanisms contribute to carbapenem resistance, the vast majority of carbapenem-resistant *A. baumannii* isolates harbor one or more of the following genes: bla_{OXA-23}, $bla_{OXA-24/OXA-40}$, and bla_{OXA-58} (6, 7). These acquired genes and bla_{OXA-51}, the intrinsic oxacillinase carried by *A. baumannii*, can be upregulated by insertion sequences (IS) located upstream of these genes, with IS*Aba-1* being the most commonly detected (7). Furthermore, carbapenemase-producing *A. baumannii* isolates are usually clonal, and a few international clones have been determined responsible for most intra- and interhospital dissemination of such isolates in European hospitals, as well as in other countries (4, 8).

Carbapenem resistance among the *Enterobacteriaceae* species is also worrisome, and carbapenem-resistant *Klebsiella pneumoniae* has gradually emerged as a cause of serious concern among infectious disease and clinical microbiology professionals worldwide.

Received 21 July 2014 Returned for modification 10 August 2014
Accepted 21 September 2014
Published ahead of print 29 September 2014
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doi:10.1128/AAC.03930-14

These isolates have been increasingly reported due to the worldwide dissemination of *K. pneumoniae* carbapenemase (KPC)-encoding genes (9, 10) and the endemic presence of metallo-β-lactamase-producing *K. pneumoniae* strains in certain countries (9, 11). KPC-producing isolates have been reported to be multidrug resistant (MDR) and, in certain instances, to be resistant to all clinically available antimicrobial agents, including polymyxins and tigecycline (12, 13).

In this study, we evaluated the presence of carbapenemase-encoding genes and the clonality among 411 *A. baumannii-Acinetobacter calcoaceticus* complex (herein named *A. baumanii* complex, or ACB) isolates and 92 *Enterobacteriaceae* isolates that displayed resistance to carbapenems (doripenem MIC, >2 μg/ml) according to the EUCAST interpretation criteria and were collected in 14 European and Mediterranean countries during the period from 2009 to 2011. In this process, we detected and characterized two new β-lactamase genes, bla_{GES-22} and bla_{VIM-35}, and evaluated other carbapenem resistance mechanisms among *Enterobacteriaceae* isolates that did not produce carbapenemases.

MATERIALS AND METHODS

Bacterial strains. A total of 697 *Acinetobacter baumannii* complex and 9,945 *Enterobacteriaceae* isolates were collected during 2009 to 2011 from 27 hospitals located in 14 European and Mediterranean countries. Isolates were consecutively collected, one per patient episode, and were considered the cause of infection by study participants. Isolates were identified in the participant hospitals, and identification was confirmed by using standard biochemical tests: the Vitek system (bioMérieux, Hazelwood, MO) or matrix-assisted laser desorption ionization–time-of-flight analysis (MALDI Biotyper; Bruker Daltonics, Billerica, MA) when necessary.

Susceptibility testing. Isolates were tested using the broth microdilution method described by the Clinical and Laboratory Standards Institute (CLSI) (14). Categorical interpretations for all antimicrobial agents were those found in CLSI standard M100-S24 (15) and on the EUCAST website (16), and quality control (QC) was performed with *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. All QC results were within the specified ranges published in CLSI documents (15). *Enterobacteriaceae* and *Acinetobacter* spp. isolates displaying doripenem MIC values of >2 μg/ml, which are resistant according to the EUCAST breakpoint criteria (16), were selected for further molecular analysis.

Molecular typing. All doripenem-resistant isolates from bacterial species that consisted of two or more isolates and were included in the study were epidemiologically typed by pulsed-field gel electrophoresis (PFGE). Genomic DNA was prepared in agarose blocks, digested with SpeI or ApaI (for *Enterobacteriaceae* and *A. baumannii* complex, respectively; New England BioLabs, Beverly, MA), and resolved in a CHEF-DR II apparatus (Bio-Rad, Richmond, CA). Results were analyzed by using GelCompar II software (Applied Maths, Kortrijk, Belgium). Percent similarities were identified on a dendrogram derived from the unweighted pair group method using arithmetic averages and based on Dice coefficients. Band position tolerance and optimization were set at 1.2% and 0.5%, respectively.

Multilocus sequence typing (MLST) was performed for selected isolates of *A. baumannii* and *K. pneumoniae* as previously described (8, 17).

Screening for acquired carbapenemases. Isolates were screened for the presence of bla_{KPC}, bla_{SME}, bla_{GES}, bla_{NMC-A}, bla_{IMI}, bla_{IMP}, bla_{VIM}, bla_{SPM-1}, bla_{GIM-1}, bla_{SIM-1}, bla_{AIM-1}, bla_{KHM-1}, bla_{NDM}, bla_{DIM-1}, and bla_{BIC-1} in four separate multiplex PCRs. *A. baumannii* complex isolates were also screened for bla_{OXA-23}-like, $bla_{OXA-24/OXA-40}$-like, bla_{OXA-51}-like (intrinsic in *A. baumannii*), and bla_{OXA-58}-like genes, as previously described (18). All PCR experiments included controls for all genes targeted and a blank template. Amplicons generated were sequenced on both strands; nucleotide and deduced amino acid sequences were analyzed using the Lasergene software package (DNASTAR, Madison, WI). Amino acid sequences were compared with those available through the internet using NCBI/BLAST.

Evaluation of intrinsic resistance mechanisms in carbapenemase-negative *Enterobacteriaceae*. The relative expression levels of genes encoding the chromosomal cephalosporinase (*Enterobacter cloacae* only), outer membrane proteins (OMP), and efflux system AcrAB-TolC were determined by quantitative real-time PCR (qRT-PCR) using DNA-free RNA preparations. *K. pneumoniae* OMP-encoding genes *ompK35*, *ompK36*, and *ompK37* and *E. cloacae ompC* and *ompF* were evaluated.

RNA extraction and treatment and relative quantification of target genes were performed as previously described (19). Endogenous reference genes (*rspL* for *E. cloacae* and *gyrA* for *K. pneumoniae*) and custom-designed primers showing efficiencies of >95.0% were used. Transcription levels were considered significantly different if at least a 10-fold difference was noted compared with the control isolates *E. cloacae* ATCC 700323 or *K. pneumoniae* ATCC 13883, which were considered the baselines in the experiments.

Characterization of new β-lactamase-encoding genes. Amplicons containing the open reading frame and promoter region of the new bla_{VIM} and bla_{VIM-1} were cloned into pPCRScriptCam SK+ (Stratagene, Santa Clara, CA). The colonies obtained after transformation in XL10-Gold Kan ultracompetent *E. coli* cells were selected on plates containing 30 μg/ml chloramphenicol. Due to the discontinuation of the cloning kit used above, genes encoding GES-11 and GES-22 were cloned using the cloning vector pCR-Blunt II-TOPO (Zero Blunt TOPO PCR cloning kit; Life Technologies) as recommended by the manufacturer, and colony selection was performed on plates containing 50 μg/ml of kanamycin and 0.5 μg/ml of ceftazidime. The presence and orientation of inserts were confirmed by PCR and sequencing. MIC testing was performed as described above.

Genetic location of novel β-lactamase-encoding genes. Agarose-embedded chromosomal DNA was subjected to ICeuI digestion and partial digestion with S1 nuclease. DNA digests were resolved by electrophoresis on the CHEF DRII system (Bio-Rad, Richmond, CA), followed by Southern blotting and hybridization with digoxigenin-labeled (Roche Diagnostics GmbH, Mannheim, Germany) bla_{GES}- or bla_{VIM}-specific probes.

Primers designed in the 5′ and 3′ conserved sequence (CS) regions of class 1 integrons (20) were used in combination with primers anchoring β-lactamase genes to determine the size and structure of the integron. Additional primers targeting the genes detected within the integron were used to complete sequencing. Amplicons were sequenced as described above.

RESULTS AND DISCUSSION

A total of 411 (58.9% overall) *A. baumannii* complex and 92 (0.9%) *Enterobacteriaceae* doripenem-resistant isolates (EUCAST criteria; MIC, >2 μg/ml) were selected for further analysis. These isolates were recovered from the bloodstream ($n = 186$), nosocomial respiratory tract infections ($n = 245$), or skin and skin structures ($n = 60$), and 12 were from other or unknown sources. Doripenem resistance was noted among 149 (56.4%), 99 (52.6%), and 163 (68.2%) of *A. baumannii* complex isolates from 2009, 2010, and 2011, respectively (Table 1), and no increasing trend was observed in the study period ($P = 0.02$; odds ratio and 95% confidence interval [OR and 95% CI], 1.53 and 95% CI 1.07 to 2.20). Doripenem-resistant *A. baumannii* isolates were present in France, Italy, Portugal, Spain, and Turkey in all years surveyed (Table 1) but, interestingly, they were present only in 2009 and 2011 in Israel and Poland (Table 1).

Elevated doripenem resistance rates (>50%) in *A. baumannii* complex isolates were observed in Greece (only surveyed during 2010 and 2011), Israel (2009 and 2011), Poland (2009), and in all years surveyed in Italy, Portugal, Spain, and Turkey (Table 1). Countries with low or null resistance rates included Belgium, Ger-

TABLE 1 *A. baumannii* complex and *Enterobacteriaceae* isolates identified as doripenem resistant during 2009 to 2011 from 14 European and Mediterranean countries

Country	No. (%) of doripenem-resistant isolates among surveyed strains					
	2009		2010		2011	
	A. baumannii complex	*Enterobacteriaceae*	*A. baumannii* complex	*Enterobacteriaceae*	*A. baumannii* complex	*Enterobacteriaceae*
All study countries	149 (56.4)	18 (0.7)	99 (52.6)	20 (0.6)	163 (68.2)	54 (1.6)
Belgium	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.6)
France	3 (8.1)	0 (0.0)	1 (6.7)	0 (0.0)	13 (41.9)	0 (0.0)
Germany	1 (8.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.3)
Greece	—[a]	—[a]	15 (83.3)	7 (6.5)	9 (90.0)	7 (6.3)
Ireland	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Israel	13 (68.4)	9 (6.3)	0 (0.0)	7 (5.3)	14 (93.3)	5 (3.9)
Italy	13 (100.0)	1 (0.4)	9 (75.0)	3 (1.3)	9 (75.0)	18 (4.6)
Poland	16 (66.6)	2 (7.4)	0 (0.0)	1 (2.1)	1 (33.3)	17 (45.6)
Portugal	29 (74.4)	0 (0.0)	26 (83.9)	0 (0.0)	30 (93.7)	0 (0.0)
Spain	10 (76.9)	3 (0.6)	2 (66.7)	0 (0.0)	7 (77.8)	0 (0.0)
Sweden	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (50.0)	0 (0.0)
Switzerland	1 (33.3)	0 (0.0)	—[b]	—[b]	—[b]	—[b]
Turkey	63 (70.8)	3 (1.1)	46 (78.0)	2 (0.7)	79 (96.4)	5 (1.0)
United Kingdom	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

[a] The medical site in Greece did not participate in the program in 2009.
[b] The medical site in Switzerland did not participate in the programs of 2010 and 2011.

many, Ireland, Sweden (50.0%, but only one isolate), Switzerland (surveyed only in 2009), and the United Kingdom (Table 1). Of note, a significant increase in doripenem resistance for *A. baumannii* complex isolates was observed in France during the period analyzed (from 8.1 to 41.9%; $P < 0.0001$; OR, 0.12, and 95% CI, 0.03 to 0.48) (Table 1).

The species distribution of the 92 *Enterobacteriaceae* isolates displaying elevated doripenem MIC values (>2 μg/ml) was as follows: 76 *K. pneumoniae*, 10 *E. cloacae*, 2 *Citrobacter freundii*, and 1 each of *Escherichia coli*, *Klebsiella oxytoca*, *Providencia stuartii*, and *Serratia marcescens*. These isolates were collected in seven countries that included Greece (which participated in 2010 and 2011 only), Israel, Italy, Poland, and Turkey (in all years surveyed), and in Belgium and Germany during 2011 only (Table 1). Overall, the doripenem resistance rates among *Enterobacteriaceae* isolates increased significantly in 2011 compared to the two initial years, and rates that were 0.7 and 0.6% in 2009 and 2010, respectively, were 1.6% in 2011 ($P < 0.0001$; OR, 0.43, and 95% CI, 0.26 to 0.69). This increase was mainly due to an elevated number of isolates from Italy and Poland and the emergence of resistance in Belgium and Germany (Table 1), countries considered to have low carbapenem resistance rates (9).

Epidemiology of and carbapenemase-encoding genes in the *A. baumannii* complex. The vast majority ($n = 391$; 95.1%) of *A. baumannii* complex isolates produced oxacillinases and genes encoding OXA-23, OXA-24/OXA-40, and OXA-58 were detected in several countries surveyed (Table 2). OXA-23 was the most prevalent carbapenemase, and the gene encoding this enzyme was detected among 285 isolates. A total of 277 isolates carried this gene alone, and two genetically identical isolates from Italy (2009) carried OXA-23- and OXA-58-encoding genes. OXA-23-producing isolates were observed in eight countries, including France (12 isolates), Israel (12), Italy (28, including 2 isolates coharboring bla_{OXA-58}), Poland (6), Portugal (79), Spain (1), Switzerland (1 [only tested in 2009]), and Turkey (148). An increase in OXA-23-producing *A. baumannii* complex isolates was noted in 2011, and this was mainly caused by an increase in doripenem-resistant isolates from Turkey (from 70.8% in 2009 to 96.4% in 2011) (Tables 1 and 2).

OXA-58 producers were observed among 76 *A. baumannii* complex isolates recovered in hospitals located in Greece, Italy, Poland, Spain, and Turkey (Table 2), and these isolates were detected in multiple study years in Greece, Spain, and Turkey. OXA-24/OXA-40-encoding genes were detected among 29 *A. baumannii* complex isolates from Israel, Poland, Portugal, Spain, and Sweden (Table 2). No trends in the prevalence of OXA-24/OXA-40- and OXA-58-producing isolates were noted during the study period. Among 411 isolates tested, only 1 was negative for the presence of bla_{OXA-51}, which is intrinsically detected in *A. baumannii sensu stricto*. This strain, which also carried $bla_{OXA-24/OXA-40}$, was identified as *Acinetobacter pittii* (formerly *Acinetobacter* genospecies 3) by *rpoB* sequencing (21).

In addition to the carbapenemase-hydrolyzing oxacillinases, 10 *A. baumannii* complex isolates from Turkey yielded positive results for generic bla_{GES} oligonucleotides targeting ESBL and carbapenemase variants. Eight isolates carried bla_{GES-11} and four of those recovered in 2011 also carried bla_{OXA-23} (Table 2). Two strains also recovered in 2011 carried a new variant, named GES-22 (GenBank accession number JX023441.1) and bla_{OXA-23}. The new gene had one amino acid substitution compared to GES-11 (M169L; 99.7% identity), showing the greatest homology, and two amino acid differences compared to GES-1 (G243A and M169L; 99.3% homology). GES-11 was initially described from an *A. baumannii* isolate recovered during 2008 in a French hospital, and this gene was carried in a class 1 integron that also harbored *aac*(6′)-Ib and *dfrA7* (22). Sequencing of the integron structure carrying bla_{GES-11} and bla_{GES-22} in isolates from Turkey evaluated in this study revealed the same genetic structure described earlier in France, suggesting a common source and genetic events leading

TABLE 2 Carbapenemases detected among 411 doripenem-resistant *A. baumannii* complex isolates

Country (no. of isolates tested)	No. of isolates with gene(s) encoding indicated carbapenemase in study year[a]												
	GES-11 (4)	GES-11 + OXA-23 (4)	GES-22 + OXA-23 (2)	OXA-23 (277)			OXA-24 (29)			OXA-58 (77)			OXA-23 + OXA-58 (2)
	2009	2011	2011	2009	2010	2011	2009	2010	2011	2009	2010	2011	2009
France (17)				3	1	8							
Germany (1)													
Greece (24)	—[b]			—			—			—	15	8	—
Israel (27)				6		6	5		7				
Italy (31)				9	8	9				2			2
Poland (17)				6			3		1	6			
Portugal (85)[c]				27	25	25	2	1	3				
Spain (19)													
Sweden (1)									1				
Switzerland (1)		—	—	1	—	—		—	—		—	—	
Turkey (188)	4	4	2	37	42	63				20	4	10	
Total (411)	4	4	2	89	76	112	14	2	13	33	20	24	2

[a] The total number of positive isolates for all countries is shown in parentheses for each of the carbapenemase/carbapenemase combinations. bla_{OXA-51} is intrinsically found in *A. baumannii* and was detected in all but one isolate from Portugal (2009); that isolate was identified as *Acinetobacter pittii* (formerly *Acinetobacter* genospecies 3) based on *rpoB* sequencing.

[b] —, no strains from that site were processed in that year.

to the amino acid change observed in GES-22. Additionally, bla_{GES-22} was genetically located in a 75-kb plasmid.

The biochemical characterization of GES-11 performed by Delbrück et al. (23) demonstrated that this enzyme is not a carbapenemase and that the substitution at position 243 does not cause conformational changes in the active binding site that would broaden the spectrum of hydrolysis compared to GES-1. The cloning of GES-11- and GES-22-encoding genes performed as part of this study confirmed that these genes do not encode carbapenem resistance in a similar *E. coli* background (see Table 5, below).

Genes encoding metallo-β-lactamases were not observed among the *A. baumannii* complex isolates analyzed.

A total of 186 unique PFGE patterns were detected among doripenem-resistant *A. baumannii* complex isolates, and 33 clusters (>2 samples with same pattern) were observed among isolates collected from 14 European and Mediterranean countries (Table 3). In the vast majority of the clusters (32/33), all or most isolates carried carbapenemase-encoding genes. Although clonal isolates were mostly limited to one hospital/city, two clusters were detected in more than one country (ACB62B/ACB85B in Greece and Italy and ACB138A/ACB75C in Italy and Portugal), and one was observed in two hospitals/cities from Turkey (ACB68A/ACB69F in Ankara and Istanbul) (Table 3). These clusters were detected in multiple years and encompassed large numbers of isolates (Table 3).

MLST was performed with one representative strain from each cluster identified by PFGE, and the results demonstrated that a total of 227 *A. baumannii* complex isolates belonged to sequence type 2 (ST2) or single-loci variants ST184 and ST97, which correspond to international clone II (Table 3) (8). This clone was found to be widespread in at least eight countries and corresponded to an elevated number of doripenem-resistant isolates producing carbapenemases. These results confirmed the findings of Karah et al. (4), who showed that clonal complex 2 (CC2, according to the Pasteur MLST scheme, or CC92 according to the Bartual scheme [24]) was the most prevalent CC among unique representative isolates submitted to two major MLST databases.

Seven isolates belonging to ST3, also known as international clone III, were detected in Israel, and two new STs were observed, ST157 (27 strains from Turkey belonging to PFGE pattern ACB68A/ACB69F) and ST158 (8 strains from Turkey producing GES-11). Twenty-four Turkish isolates belonged to ST15 and the single-locus variant type ST84. ST15 was associated with an outbreak in Leiden, Netherlands, during 2000 and was initially considered localized (8); however, more recent analysis showed that these STs were present in at least nine European countries, and also Argentina and Brazil (4). In summary, this population distribution of doripenem-resistant *A. baumannii* complex isolates confirms previous reports (4, 8) that the international clones II, III, and ST15 are often associated with MDR phenotypes and the presence of carbapenemases.

Epidemiology and carbapenem resistance mechanisms in *Enterobacteriaceae*. Among 92 doripenem-resistant *Enterobacteriaceae* isolates, 85 (92.4% of the doripenem-resistant isolates; 0.8% overall) were positive for carbapenemase-encoding genes. KPC-encoding genes were the most prevalent, and bla_{KPC-2} and bla_{KPC-3} were detected among 15 and 22 isolates, respectively, and these isolates comprised 36 *K. pneumoniae* and 1 *C. freundii* isolate (Table 4). KPC-3-producing isolates were detected in Israel (all three study years), Italy (2011 only), and Poland (2011 only), whereas isolates carrying bla_{KPC-2} were observed in smaller numbers in five surveyed countries (Germany, Greece, Israel, Italy, and Poland) (Table 4).

The majority of the KPC-producing *K. pneumoniae* isolates were clonal, regardless if they carried bla_{KPC-2} or bla_{KPC-3}, and at least one clonal group was noted in each country where more than one bla_{KPC}-carrying isolate was detected. In Greece, 10 out of 11 KPC-2-producing isolates belonged to two PFGE patterns (KPN62J and KPN62K) (Table 4), and 17 of the 18 KPC-3-producers from Israel belonged to a single clonal profile (KPN63C) (Table 4). Among 11 KPC-producing *K. pneumoniae* isolates from

TABLE 3 Clonal ACB complex isolates observed in European and Mediterranean countries during 2009 to 2011

Country (no. of ACB isolates tested/no. of PFGE patterns observed) and clonal pattern	MLST finding	No. of isolates by yr				Carbapenemase found	Similar hospital-specific clone (country of isolation)
		Total	2009	2010	2011		
France (17/11)							
ACB300A	ST2 (clone II)	4			4	None	
Greece (24/7)							
ACB62B (3 subtypes)	ST2 (clone II)	14		12	2	OXA-58	ACB85B (1 isolate from Italy, not listed)
ACB62G (1 subtype)	ST184 (single-locus variant of ST2, clone II)	5			5	OXA-58	
Israel (27/15)							
ACB63A (3 subtypes)	ST2 (clone II)	3	3			OXA-24	
ACB63B (2 subtypes)	ST2 (clone II)	4	4			OXA-23	
ACB63F (2 subtypes)	ST3 (clone III)	3	2		1	None or OXA-23	
ACB63I	ST2 (clone II)	3			3	OXA-23	
ACB63O (3 subtypes)	ST3 (clone III)	4	1		2	OXA-24	
Italy (31/8)							
ACB75B	ST2 (clone II)	3		3		OXA-23	
ACB75C (6 subtypes)	ST2 (clone II)	12		4	8	OXA-23	ACB138A (Portugal)
ACB86B (5 subtypes)	ST2 (clone II)	8	8			OXA-23	
Poland (17/10)							
ACB81A	ST2 (clone II)	3	3			OXA-58	
ACB81E	ST2 (clone II)	3	3			OXA-24	
Portugal (85/11)							
ACB138A (19 subtypes)	ST2 (clone II)	66	25	14	27	OXA-23	ACB75C (Italy)
ACB138B (4 subtypes)	ST2 (clone II)	5		5		OXA-23	
ACB138E (3 subtypes)	ST2 (clone II)	4		4		OXA-23	
Spain (19/7)							
ACB64A (3 subtypes)	ST2 (clone II)	6	5	1		OXA-58	
ACB64D	ST2 (clone II)	5			5	OXA-58	
ACB65A (3 subtypes)	ST2 (clone II)	4	3	1		OXA-24	
Turkey (188/58)							
ACB68A (3 subtypes)	ST157 (single-locus variant of ST10)	7	2	3	2	OXA-23 or OXA-58	ACB69F (Istanbul)
ACB68B	ST10	7	2		5	OXA-23	
ACB68C	ST15 (clone Leiden 2000)	4	4			OXA-23, OXA-23+ OXA-58 or none	
ACB68D (2 subtypes)	ST15 (clone Leiden 2000)	3	1	1	1	OXA-58	
ACB68E (3 subtypes)	ST84 (single-locus variant of ST15, clone Leiden 2000)	11	9	2		OXA-58	ACB69D (Istanbul)
ACB68G (7 subtypes)	ST2 (clone II)	15		13	3	OXA-23	
ACB68I	ST97 (single-locus variant of ST2, clone II)	4	3	1		OXA-23	
ACB68K	ST2 (clone II)	4		4		OXA-23	
ACB68L	ST2 (clone II)	5	5			OXA-23	
ACB68N (2 subtypes)	ST2 (clone II)	4			4	OXA-23	
ACB68AF	ST2 (clone II)	3			3	OXA-23	
ACB68AN (4 subtypes)	ST2 (clone II)	7			7	OXA-58	
ACB69C	ST158	4	4			GES-11	
ACB69D	ST15 (clone Leiden 2000)	6	6			OXA-58	ACB68E (Ankara, Turkey)
ACB69F (2 subtypes)	ST157 (single-locus variant of ST10)	20	10	9	1	OXA-23	ACB68A (Ankara, Turkey)
ACB69G	ST2 (clone II)	26	14	7	5	OXA-23	
ACB69I (3 subtypes)	ST2 (clone II)	7			7	OXA-23	

TABLE 4 Epidemiology and carbapenemase results for 92 doripenem-nonsusceptible *Enterobacteriaceae* strains collected from European and Mediterranean hospitals during 2009 to 2011

Country (no. of isolates)	City (no. of isolates)	Organism	PFGE result	MLST result	Carbapenemase	No. of isolates
Belgium (1)	Antwerp (1)	*C. freundii*	CF131A		None	1
Germany (1)	Leipzig (1)	*K. pneumoniae*	KPN95A	ST258 (CC11)	KPC-2	1
Greece (14)	Athens (14)	*K. pneumoniae*	KPN62J (2 subtypes)	ST258 (CC11)	KPC-2	3
		K. pneumoniae	KPN62K (3 subtypes)	ST258 (CC11)	KPC-2	7
		K. pneumoniae	KPN62L		KPC-2	1
		K. pneumoniae	KPN62M		VIM-1	1
		K. pneumoniae	KPN62N		VIM-26	1
		P. stuartii	NT		VIM-1	1
Israel (21)	Tel-Hashomer (21)	*C. freundii*	CF63A		KPC-2	1
		K. pneumoniae	KPN63C (4 subtypes)	ST258 or ST512 (CC11)	KPC-3	17
		K. pneumoniae	KPN63D		KPC-3	1
		K. pneumoniae	KPN63E		None	2
		K. pneumoniae	KPN63F	ST327	None	1
Italy (22)	Catania (1)	*K. pneumoniae*	KPN85D	ST147	VIM-1	1
	Genoa (16)	*E. cloacae*	ECL75A		VIM-1	1
		K. pneumoniae	KPN75B		KPC-2	1
		K. pneumoniae	KPN75C		KPC-2	1
		K. pneumoniae	KPN75D		KPC-2, OXA-48	1
		K. pneumoniae	KPN75E	ST258 (CC11)	KPC-2	1
		K. pneumoniae	KPN75F	ST101	KPC-2	3
		K. pneumoniae	KPN75G	ST512 (CC11)	KPC-3	8
	Rome (5)	*K. pneumoniae*	KPN86A	ST512 (CC11)	KPC-3	4
		K. pneumoniae	KPN86B		KPC-3	1
Poland (20)	Warsaw (20)	*E. cloacae*	ECL81A (2 subtypes)		VIM-1	2
		E. cloacae	ECL81B		VIM-4	1
		E. cloacae	ECL81C		None	1
		K. oxytoca	NT		VIM-35	1
		K. pneumoniae	KPN81A	ST258 (CC11)	KPC-2	5
		K. pneumoniae	KPN81B	ST416	KPC-2 or none	2
		K. pneumoniae	KPN81C (2 subtypes)	ST258 (CC11)	KPC-3	4
		K. pneumoniae	KPN81D		KPC-3	1
		K. pneumoniae	KPN81E		KPC-3	1
		K. pneumoniae	KPN81F		None	1
		S. marcescens	NT		IMP-19	1
Spain (3)	Madrid (3)	*E. cloacae*	ECL66C		VIM-1	2
		E. cloacae	ECL66D		VIM-1	1
Turkey (10)	Ankara (8)	*E. cloacae*	ECL68A		OXA-48	1
		E. cloacae	ECL68C		OXA-48	1
		E. coli	NT		OXA-48	1
		K. pneumoniae	KPN68I (2 subtypes)		OXA-48	2
		K. pneumoniae	KPN68K	ST258 (CC11)	OXA-48	1
		K. pneumoniae	KPN68N (2 subtypes)	ST11 (CC11)	OXA-48	2
	Istanbul (2)	*K. pneumoniae*	KPN69A	ST16	OXA-48	1
		K. pneumoniae	KPN69B		OXA-48	1

Poland, 9 belonged to three clones and the other 2 were genetically distinct. Similarly, clusters of KPC-producing isolates were observed in Genoa and Rome, but five genetically distinct isolates were also detected in these Italian cities (Table 4).

Among 11 KPC-producing *K. pneumoniae* isolates selected for MLST analysis, including representatives from all clusters observed, 9 belonged to CC11 (also known as CC258) that included the sequence types ST11, ST258, and ST512. These STs have been described in several European countries and have been associated with the dissemination of bla_{KPC-2} and bla_{KPC-3} (9).

Genes encoding OXA-48 and VIM-1 were detected among 11 and 10 isolates, respectively. OXA-48-producing strains were mainly from Turkey (7 *K. pneumoniae*, 2 *E. cloacae*, and 1 *E. coli* isolate) (Table 4), but one OXA-48-producing strain was observed in Genoa, Italy, and this isolate also carried bla_{KPC-2}. VIM-1-producing strains were detected in Greece (2 isolates, one each of *K.*

TABLE 5 Susceptibility results for clinical and recombinant isolates carrying genes encoding the new β-lactamase GES-22 and VIM-35[a]

Antimicrobial agent	MIC (μg/ml)							
	GES-22-producing *A. baumannii* clinical isolate	*E. coli* TOP10 pZERO Blunt (bla_{GES-22})	*E. coli* TOP10 pZERO Blunt (bla_{GES-11})	*E. coli* TOP10[b]	VIM-35-producing *K. oxytoca* clinical isolate	*E. coli* XLGold PCRScript (bla_{VIM-35})	*E. coli* XLGold PCRScript (bla_{VIM-1})	*E. coli* XLGold PCRScript
Doripenem	>8	≤0.06	≤0.06	≤0.06	>8	1	1	≤0.06
Imipenem	>32	0.25	0.25	0.25	>32	4	2	0.25
Meropenem	>32	≤0.06	≤0.06	≤0.06	16	0.25	0.25	≤0.06
Cefoxitin	>256	4	4	4	>256	>256	>256	4
Ceftriaxone	>256	16	256	≤0.25	64	8	32	≤0.25
Ceftazidime	>256	>256	>256	≤0.12	>256	128	64	≤0.12
Cefepime	>256	16	16	≤0.12	64	2	4	≤0.12
Aztreonam	>16	>16	>16	≤0.12	8	0.25	0.25	≤0.12
Ampicillin	>256	>256	>256	2	>256	>256	>256	4
Ampicillin-sulbactam	>32	32	32	8	>32	>32	>32	8
Piperacillin-tazobactam	>256	32	>256	8	64	16	>256	2
Amikacin	8	—[c]	—	—	16	—	—	—
Tobramycin	4	—	—	—	>16	—	—	—
Gentamicin	≤1	—	—	—	2	—	—	—
Ciprofloxacin	>4	—	—	—	>4	—	—	—
Tigecycline	0.5	—	—	—	1	—	—	—
Colistin	0.5	—	—	—	0.5	—	—	—

[a] The susceptibility profiles of recombinant strains carrying new enzymes were compared to those of recombinant strains carrying the GES-11 and VIM-1 genes that displayed the closest homology.
[b] Only recipient host was tested, since pZERO Blunt is a suicidal plasmid.
[c] —, not tested.

pneumoniae and *P. stuartii*), Italy (2 isolates, one each of *K. pneumoniae* and *E. cloacae*), Poland (2 isolates, both *E. cloacae*), and Spain (3 *E. cloacae*). Two VIM-1-producing *E. cloacae* strains from Poland were genetically related, and the other two from Spain had identical PFGE profiles.

Additionally, one *K. pneumoniae* isolate from Greece harbored bla_{VIM-26}, one *S. marcescens* isolate and one *E. cloacae* isolate from Poland carried the genes encoding IMP-19 and VIM-4, respectively, and a new VIM-variant was detected in a *K. oxytoca* from Poland (Table 4). This new variant, named VIM-35, displayed one amino acid substitution compared to VIM-1 (A235T; 99.6% homology). This gene was located in the chromosome of this isolate, and the integron carrying bla_{VIM-35} harbored *aacA*(6′)-*Ib* in the first position followed by the metallo-β-lactamase gene and the 3′ CS. A duplication of 155 bp of the bla_{VIM} 3′ end was detected between this gene and the integron at the 3′ CS, similar to sequences described for bla_{VIM-4}-carrying integrons among *P. aeruginosa* isolates from Poland (25) and other bla_{VIM}-carrying integrons from European countries (26–28). VIM-35, when expressed in a similar background, displayed similar MIC values as VIM-1, its closest ancestor (Table 5).

Five *K. pneumoniae*, one *E. cloacae* isolate, and one *C. freundii* isolate carried no carbapenemase genes, and these isolates were collected in Israel, Poland, and Belgium (Table 4). An analysis of the relative expression levels for the AcrAB-TolC efflux system and outer membrane proteins for these carbapenemase-negative *K. pneumoniae* and *E. cloacae* isolates and the *E. cloacae* chromosomal AmpC was performed. Among the *K. pneumoniae* strains, three isolates (from Israel and Poland) had decreased expression of *ompK36* that was significantly lower than the control strain (Table 6), and the isolates from Poland also had lower expression levels of *ompK35*. One strain from Israel had modestly elevated expression of *acrA* (4 times greater than the control). The *E. cloacae* strain from Poland had hyperexpression of AmpC and low transcription levels of *ompC*.

Among MDR organisms, *A. baumannii* and *K. pneumoniae*

TABLE 6 Chromosomal cephalosporinase (AmpC), efflux pumps, and outer membrane protein expression levels among carbapenemase-negative *Enterobacteriaceae* isolates collected from European and Mediterranean countries during 2009 to 2011[a]

Yr	Site code	Country	Organism	PFGE result	Relative expression[b]				
					acrA	*ampC*	*ompK35/ompF*	*ompK36/ompC*	*ompK37*
2009	081	Poland	*E. cloacae*	ECL81C	4.167	82.660	4.233	0.006	NT
2010	063	Israel	*K. pneumoniae*	KPN63F	4.369	2.203	0.970	1.292	0.635
2010	063	Israel	*K. pneumoniae*	KPN63F	1.886	0.953	0.718	0.011	0.612
2010	081	Poland	*K. pneumoniae*	KPN81F	1.562	1.216	0.468	1.033	0.736
2011	063	Israel	*K. pneumoniae*	KPN63E	0.999	1.108	6.478	0.020	0.826
2011	081	Poland	*K. pneumoniae*	KPN81B	6.587	1.800	0.193	0.000	0.472

[a] One *C. freundii* isolate from Belgium was not analyzed because it failed in assays designed with available DNA sequences for the intrinsic genes tested.
[b] Expression was normalized against that of a housekeeping gene (*rspL* for *E. cloacae* and *gyrA* for *K. pneumoniae*) and compared to expression of *E. cloacae* ATCC 700323 and *K. pneumoniae* ATCC 13883. Expression values considered significant are underlined. NT, not tested.

have been highlighted as the most challenging and to be of increasing importance for monitoring their prevalence (1). In this 3-year survey of 14 European and Mediterranean countries, we observed an overall high prevalence of doripenem-resistant *A. baumannii* (>58%), mostly producing OXA-type carbapenemases and belonging to the international clone II group. The prevalence of these isolates varied among the different countries, and a trend to increasing numbers was noted in at least one country. In a recent review of the epidemiology of carbapenemase-producing *Enterobacteriaceae* and *A. baumannii* in Mediterranean countries, KPC and OXA-48 for *Enterobacteriaceae* and all three families of acquired class D carbapenemases for *A. baumannii* were considered the most prevalent and widespread enzymes in this region (29). Local recent studies from other countries, such as France (30), the United Kingdom (31), and Spain (32), showed a scenario for *Enterobacteriaceae* similar to what we observed in this analysis, but the emergence and dissemination of NDM-producing isolates has been documented in various European countries (33).

Doripenem-resistant *Enterobacteriaceae* were still uncommon (<1%), but a trend for increasing prevalence and a higher number of countries where these isolates were noticed occurred in 2011 compared to the two previous years. The vast majority of doripenem-resistant isolates were KPC- or VIM-producing *K. pneumoniae*, and representatives of the isolates that were submitted for MLST analysis demonstrated that CC11 is widespread in Europe.

This broad investigation confirms individual findings previously published for each of the countries analyzed; however, the results of this study add the perspective of the overall prevalence in isolates collected according to common protocols and centrally tested for susceptibility and molecular methods. As with other surveys of this magnitude, only a small number of isolates per institution were analyzed yearly, which might impair the early detection of resistance mechanisms and limit the clinical information such as risk factors, outcome correlation, and results of infection control measures undertaken that might be available, but these restrictions do not undermine the importance of such initiatives.

ACKNOWLEDGMENTS

We thank the excellent technical support of A. J. Costello, S. J. Benning, and P. A. Clark that provided support in the MLST testing, preparation of the manuscript, and/or data analysis. We acknowledge the valuable contribution of the participants of the SENTRY Antimicrobial Surveillance Program in European and Mediterranean countries.

This work was supported by an educational/research grant from Janssen Research & Development.

M. Castanheira and/or R.N. Jones received research and educational grants in 2009 to 2011 from the following entities: American Proficiency Institute, Anacor, Astellas, AstraZeneca, Bayer, Cempra, Cerexa, Cosmo Technologies, Contrafect, Cubist, Daiichi, Dipexium, Enanta, Furiex, GlaxoSmithKline, Johnson & Johnson (Ortho McNeil), LegoChem Biosciences Inc., Meiji Seika Kaisha, Merck, Nabriva, Novartis, Pfizer (Wyeth), PPD Therapeutics, Rempex, Melinta (Rib-X) Pharmaceuticals, Seachaid, Shionogi, The Medicines Co., Theravance, ThermoFisher, Vertex Pharmaceuticals, and some other corporations. JMI employees are advisors/consultants for Astellas, Cubist, Pfizer, Cempra, Cerexa-Forest, Johnson & Johnson, and Theravance.

S.E.C., L.N.W., and L.M.D. declare no conflicts of interest. T.A.D. is an employee of Janssen Research & Development.

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