Genomic epidemiology of global VIM-producing Enterobacteriaceae

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Running Title: VIM-producing Enterobacteriaceae

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Synopsis (199 words)

Background: International data on the molecular epidemiology of Enterobacteriaceae with VIM carbapenemases are limited.

Methods: We performed short read (Illumina) whole genomic sequencing on a global collection of 89 VIM-producing clinical Enterobacteriaceae (2008-14).

Results: VIM-producing (11 varieties within 21 different integrons) isolates were mostly obtained from Europe. Certain integrons with *bla*$_{VIM}$ were specific to a country in different species and clonal complexes [CCs] (In*87*, In*624*, In*916*, and In*1323*) while others had spread globally among various Enterobacteriaceae species (In*110* and In*1209*). *Klebsiella pneumoniae* was the most common species (n=45); CC147 from Greece was the most prevalent clone and contained In*590*-like integrons with four different *bla*$_{VIMs}$. *Enterobacter cloacae* complex was the 2nd most common specie and mainly consisted of *E. hormaechei* (*E. xiangfangensis*, subsp. *steigerwaltii* and Hoffmann cluster III). CC200 (from Croatia, Turkey), CC114 (Croatia, Greece, Italy, USA), and CC78 (from Greece, Italy, Spain) containing *bla*$_{VIM-1}$ were the most common clones among *E. cloacae* complex.

Conclusions: This study highlights the importance of surveillance programs using the latest molecular techniques in providing insight into the characteristics and global distribution of Enterobacteriaceae with *bla*$_{VIMs}$.

Introduction

Carbapenems are often the last line of effective therapy available for the treatment of serious infections due to multidrug-resistant bacteria. The rapid evolution of carbapenem resistance in Enterobacteriaceae during the last decade is an emerging global threat.[1, 2] Enzymes that hydrolyze the carbapenems, known as carbapenemases, are the most important causes of carbapenem resistance. CPE have acquired multiple resistance genes making therapy of infections due to these bacteria challenging.[1, 2]

The most common carbapenemases among CPE are KPCs (Amber class A), IMPs, VIMs, NDMs (class B or metallo-β-lactamases), and OXA-48-like (class D) enzymes.[1] Metallo-β-lactamases (MBLs) hydrolyse all β-lactams except aztreonam although resistance levels may vary according to different subtypes. After the initial discovery of VIM-1 in Italy during 1997, bacteria with VIM enzymes have been detected worldwide.[1] VIMs are common among MBL-producing *Pseudomonas aeruginosa* but remain relatively rare among members of the Enterobacteriaceae.[3] VIM-producing Enterobacteriaceae are mainly found in Europe, especially Greece, Spain, Hungary, and Italy.[1, 4] The most common species associated with VIMs among the Enterobacteriaceae include *Klebsiella pneumoniae, Escherichia coli*, and *Enterobacter* spp.[2, 3] VIM genes are often situated within class 1 integrons harboured on broad-host range plasmids.[2, 3] These mobile genetic elements play an important role in the interspecies distribution of VIM types of carbapenemases.[5]

Comprehensive global data regarding the molecular epidemiology of CPE with bla_{VIM} are currently limited. We designed a study that utilized short read whole genome sequencing to describe the molecular characteristics and international distribution of bla_{VIM} among Enterobacteriaceae obtained from two global surveillance systems.

Methods

Bacterial isolates

We included 89 VIM-producing clinical, non-repeat Enterobacteriaceae collected from two global surveillance programs namely the Merck Study for Monitoring Antimicrobial Resistance Trends (SMART) [2008–2014] and the AstraZenaca's global surveillance study of antimicrobial resistance (2012–2013) [Dataset 1].

The SMART program included isolates from intra-abdominal and urinary tract infections from the following countries: Morocco, South Africa, Tunisia (Africa); China, Malaysia, Singapore, South Korea, Taiwan, Thailand, Vietnam (Asia); Czech Republic,

Estonia, France, Georgia, Greece, Germany, Hungary, Italy, Latvia, Lithuania, Portugal, Romania, Slovenia, Spain, Turkey, United Kingdom (Europe); Argentina, Brazil, Chile, Colombia, Dominican Republic, Ecuador, Guatemala, Mexico, Puerto Rico, Panama, Uruguay, Venezuela (Latin America); Jordan, Lebanon, Israel, Saudi Arabia, UAE (Middle East); Canada, United States (North America); and Australia, New Zeeland, Philippines, Japan (South Pacific).

The AstraZeneca program included isolates from skin and soft tissue and lower respiratory tract infections from the following countries: Egypt, Kenya, Nigeria, South Africa (Africa); China, South Korea, Taiwan, Thailand (Asia); Austria, Belgium, Bulgaria, Greece, Czech Republic, Denmark, France, Germany, Hungary, Italy, Macedonia, Portugal, Poland, Russia, Romania, Slovakia, Spain, Turkey, United Kingdom (Europe); Argentina, Brazil, Chile, Colombia, Mexico, Uruguay, Venezuela (Latin America); Lebanon, Israel, Syria, Kuwait (Middle East); United States (North America); and Australia, Philippines, Japan (South Pacific).

Both programs collected consecutive clinically relevant gram-negative aerobes in each institution. These isolates initially underwent micro-dilution panel susceptibility testing and molecular screening for bla_{VIM} as described previously.[6] Overall 107 366 isolates were obtained from 2008-14; of these 755 were positive for bla_{KPC}, 281 for $bla_{\mathrm{OX\text{-}48\text{-}like}}$, 271 for bla_{NDM}, 89 for bla_{VIM} and 38 for $bla_{\mathrm{IMP.}}$

Whole genome sequencing

We used the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, USA) to prepare libraries for sequencing. Samples were multiplexed and sequenced on an Illumina NextSeq500 for 300 cycles (151 bp paired-end).

Genomic analysis

Draft genomes were obtained using SPAdes version 3.8.1.[7] Species identification was performed using SILVA 16s rRNA gene database release 123.[8] In addition, we used

whole genome-based phylogenetic tree including type strains for identification of *Klebsiella* spp., *Enterobacter* spp.[9] and *Citrobacter* spp. (Dataset S2). Average nucleotide identity (ANI) was calculated using JSpecies.[10]

To define presence of genes and their alleles, we used SRST2[11] and BLAST+[12] in combination with following databases or typing schemes: NCBI BLAST database (http://blast.ncbi.nlm.nih.gov/Blast/), NCBI Beta-Lactamase Data Resources (http://www.ncbi.nlm.nih.gov/pathogens/beta-lactamase-data-resources/), ARG-ANNOT,[13] PlasmidFinder,[14] plasmid addiction systems,[15] and MLST (http://bigsdb.pasteur.fr/klebsiella/, http://pubmlst.org/ecloacae/, http://pubmlst.org/cfreundii/, http://mlst.ucc.ie/mlst/dbs/Ecoli/).

The goeBURST algorithm implemented in PHYLOViZ software[16] was used to demonstrate relationships between STs and to define the founder of a clonal complex (CC). We defined CCs at the single-locus variant level. Integrons were classified according to INTEGRALL (http://integrall.bio.ua.pt/) and promoters of gene cassettes were characterized according to a previous study.[17] For *Klebsiella* isolates, we performed *in silico* detection of K capsular type based on *wzi* alleles,[18] virulence genes (http://bigsdb.pasteur.fr/klebsiella), and promoters and coding sequences of *ompK35*/*K36*.[19, 20] For *E. coli* isolates, we performed *in silico* phylogenetic grouping.[21]

Phylogenetic analysis

We used a core genome SNP-based approach to create a phylogenetic tree for each Enterobacteriaceae genus. SNPs were identified using trimmed reads mapping to a genus specific reference genome (Dataset 2) followed by GATK Best Practices workflow[22] and SAMtools[23] (depth of sequencing >10 and Phred-score >20). Draft or complete genomes downloaded from the NCBI database (Dataset 2) were aligned against the reference genome of the genus using ProgressiveMauve to obtain pseudo-chromosomes that contained only SNPs.[24] The SNP-only core genome was identified as the common blocks of >500bp to all of the study isolates. Maximum-likelihood tree was build using RAxML[25] and visualized using

FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

Sequence data accession numbers

We deposited the sequencing data in the DDBJ and NCBI databases (accession no. DRA004879 and SRP046977). The sequences of new integrons described in this study ranged from accession no's LC169570 to LC169586.

Results and Discussion

Geographic distribution showed VIM-producing Enterobacteriaceae mostly in Europe.

The 89 VIM-producing Enterobacteriaceae were present in 17 countries, mostly from Europe (n=79) followed by Africa (n=4) (Fig. 1 and Dataset 1). The common sources were intra-abdominal specimens (n=59) and urines (n=28). The isolates include the following microorganisms: *Klebsiella pneumoniae* subsp. *pneumoniae* (n=45), *Klebsiella variicola* (n=2), *Enterobacter cloacae* complex (n=33), *Citrobacter* spp. (n=6), *E. coli* (n=1), *Proteus mirabilis* (n=1) and *Serratia marcescens* (n=1) (Fig 1, Table 1).

The 89 genomes were sequenced at an average depth of 167 (standard deviation [SD] 87.9) (Dataset 1). Assembled genomes had an average number of contigs of 101 (SD 50.4) and N50 value of 265,210 bp (SD 98,928 bp). We confirmed the presence of bla_{VIM} in the draft genomes of all the isolates.

The presence of resistance genes, antibiotic resistance profiles, plasmid replicons, and plasmid addiction systems are shown in Fig. S1. Table 1 shows the geographical distribution of the different species, types of carbapenemases and integrons. We identified 11 bla_{VIM} variants namely: $bla_{\mathrm{VIM-1}}$ (n=67), $bla_{\mathrm{VIM-2}}$ (n=2), $bla_{\mathrm{VIM-4}}$ (n=7), $bla_{\mathrm{VIM-5}}$ (n=2), $bla_{\mathrm{VIM-19}}$ (n=2), $bla_{\mathrm{VIM-23}}$ (n=1), $bla_{\mathrm{VIM-26}}$ (n=2), $bla_{\mathrm{VIM-27}}$ (n=1), $bla_{\mathrm{VIM-29}}$ (n=2), $bla_{\mathrm{VIM-31}}$ (n=1) and $bla_{\mathrm{VIM-33}}$ (n=2). VIM-1, 4 and 5 were present in different microorganisms (Table 1). The distribution of the different bla_{VIM} subtypes was similar to previously published data.[2, 26, 27] Our results show that VIM-1 has a global distribution, VIM-2 was present in Mexico and

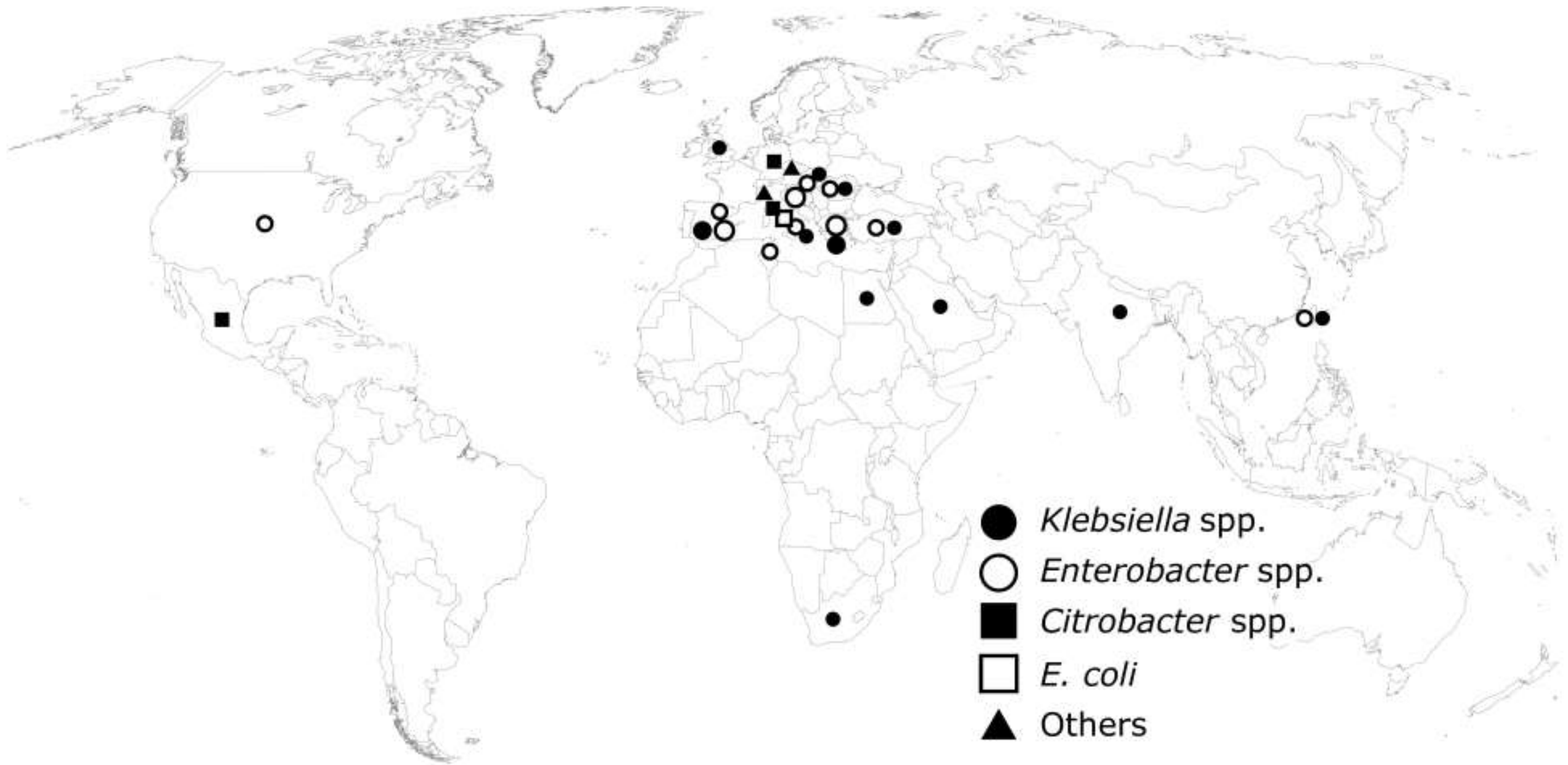


Fig. 1. Global distribution of VIM-producing Enterobacteriaceae isolates in this study.

Table 1. VIM subtypes and integrons of the Enterobacteriaceae isolates.

Carbapenemase (*n*)	Species, *n* (country)					Defined integron numbers (species, *n*)
	Klebsiella spp. (KP)	*E. cloacae* complex (Ecl)	*Citrobacter* spp. (CI)	*E. coli* (EC)	*P. mirabilis* (PM), *S. marcescens* (SM)	
VIM-1 (67)	Greece (14), Spain (12), Italy (4), South Africa (2), Egypt (1), Taiwan (1)	Greece (8), Croatia (7), Spain (6), Italy (4), Taiwan (1), Tunisia (1), USA (1)	Italy (2), Germany (1)	Italy (1)	PM, Italy (1)	In*916* [a] (KP, 4; Ecl, 2; CI, 1; PM 1), In*591*[b] (KP, 8), In*1209*[b] (KP, 5; Ecl 1), In*87*[a] (Ecl, 4; KP, 1), In*110*[b] (KP, 1; Ecl, 4; CI, 1), In*624*[a] (Ecl, 4; KP, 1), In*237* (Ecl, 2), In*1315* (Ecl, 1), In*1318* (Ecl, 1), In*1322* (CI, 1), In*3103* (Ecl, 1), In*4873* (Ecl, 1)
VIM-2 (2)			Mexico (1), Spain (1)			In*339* (CI, 1)
VIM-4 (7)	Hungary (2), Romania (1)	Romania (2), Hungary (1)			SM, Czech Republic (1)	In*1323*[a] (Ecl, 2; KP, 1), In*238* (SM, 1)

VIM-5 (2)	Turkey (1)	Turkey (1)		In*1316* (Ecl, 1)
VIM-19 (2)	Greece (2)			In*4863* (KP, 2)
VIM-23 (1)			Mexico (1)	In*1320* (CI, 1)
VIM-26 (2)	Greece (2)			In*1157* (KP, 2)
VIM-27 (1)	Greece (1)			Undefined
VIM-29 (2)	Saudi Arabia (1), UK (1)			Undefined
VIM-31 (1)		Turkey (1)		In*669* (KP, 1)
VIM-33 (2)	Greece (2)			In*1317* (KP, 2)

In*1315* to In*1318*, In*1320*, In*1322*, and In*1323* were novel integrons found in this study.

[a] The same integron was found in isolates from only one country: Greece (In*87*, In*237*), Italy (In*916*), Spain (In*624*), and Romania (In*1323*).

[b] The same integron was found in isolates from multiple countries: In*110*, Croatia (Ecl), South Africa (KP), Spain (Ecl), and Germany (CI); In*591*, Greece and Egypt (KP); In*1209*, Greece (KP) and USA (Ecl).

Spain, VIM-4 in Europe, VIM-5, 31 in Turkey, VIM-19, 26, 27, 33 was limited to Greece, VIM-23 in Mexico and VIM-29 was present in Saudi Arabia and the UK (Table 1). Enterobacteriaceae (most often *K. pneumoniae*) with *bla*$_{\text{VIM-1}}$ were previously responsible for nosocomial outbreaks throughout Greece, Italy during the early – mid 2000s[28, 29] and since then sporadic outbreaks had been described from different parts of the world.[1, 30] Apart from *bla*$_{\text{VIM-1}}$, Enterobacteriaceae with following *bla*$_{\text{VIMs}}$ has been reported: *bla*$_{\text{VIM-2}}$ in Austria,[31] Mexico[32] and Venezuela,[33] *bla*$_{\text{VIM-4}}$ in Czech Republic,[34] Egypt,[35] Hungary,[36] Italy[37] and Kuwait.[38] In addition, a recent global surveillance study from 2012–2014 reported Enterobacteriaceae with following *bla*$_{\text{VIMs}}$: *bla*$_{\text{VIM-5}}$ in Turkey and Nigeria, *bla*$_{\text{VIM-23}}$ in Mexico, *bla*$_{\text{VIM-26}}$ in Greece, *bla*$_{\text{VIM-32}}$ in the USA, and *bla*$_{\text{VIM-42}}$ from Italy.[39]

Characterization of Class 1 integrons identified 21 different integron types including 7 novel cassette combinations.

All of the *bla*$_{\text{VIMs}}$ were situated within class 1 integrons. We were unable to sequence the complete integron-associated gene cassettes in 30 isolates due to the limitations associated with short-read sequencing. We were able to partially characterize 27/30 additional integrons (Figures 2, 3 and Dataset 3).

We identified 21 different integron types containing *bla*$_{\text{VIM}}$ including 7 novel combinations (Table 2). In*110* and In*1209,* that contain *bla*$_{\text{VIM-1}}$ had international, intercontinental, and inter-genus distribution (In*110*, Croatia [*E. xiangfangensis*], South Africa [*K. pneumoniae*], Spain [*E. kobei*], and Germany [*C. freundii*]; In*1209*, Greece [*K. pneumoniae*] and the USA [*E. xiangfangensis*]). In*87*, In*624*, In*916*, and In*1323* were present in different species from the same country (Tables 1, 2). The international and inter-genus distribution of *bla*$_{\text{VIM-1}}$ was similar to integrons and their variants previously reported, including In*590*-like (In-e541-like) reported from Greece, In*416*-like from Greece, In*110* from Spain, Italy, and Latvia, In*476*-like (originally In*113*, corresponding to In*624* in this study) from Spain, and In*916* from Italy, France, and Spain.[28, 40]

Table 2. Details of class 1 integrons with *bla*$_{VIM}$.

Integron number						
Major type	Variant	*n*	Gene cassettes	Promoter type (*n*)	Downstream of gene cassettes (*n*)	Accession number of the In
In*87*		5	*bla*$_{VIM-1}$-*aacA27*	PcS (1), UD (4)	*qacEΔ1-sul1-orf5-orf6*-IS*26* (1), UD (4)	AY648125
In*110*		6	*bla*$_{VIM-1}$-*aacA4-aadA1*	PcH2 (6)	*qacEΔ1-sul1*-IS*CR1* (2), *qacEΔ1-sul1-orf5-orf6*-IS*6100* (1), *qacEΔ1-sul1-orf5*-Δ*tniB-tniA*-IRt (1), *qacEΔ1-sul1*-IS*CR1-sapA-orf2-qnrB2*-Δ*qacEΔ1-sul1-orf5-orf6*-IRt (1), UD (1)	LC169583
In*237*	In*237*[a]	2	*aacA4-bla*$_{VIM-1}$	PcS (1), UD (1)	*qacEΔ1-sul1-orf5*-IS*1326*-Δ*tniB-tniA*-IRt (1), UD (1)	LC169571
	In*238*[a]	1	*aacA4-bla*$_{VIM-4}$	PcS (1)	*qacEΔ1-sul1-orf5-orf6*-IS*6100* (1)	LC169580
In*339*		1	*bla*$_{VIM-2}$-*aacA7*	UD (1)	UD (1)	FJ627181
In*416*	In*416*	0[b]	*bla*$_{VIM-4}$-*aacA7-dfrA1*-Δ*aadA1-smr*	PcS	IS*Pa21*-like-*arsR*	AJ704863
	In*4863*	2	*bla*$_{VIM-19}$-*aacA7-dfrA1*-Δ*aadA1-smr*	PcH2 (1), UD (1)	IS*Pa21*-like-*arsR* (2)	LC169563
	In*4873*	1	*bla*$_{VIM-1}$-*aacA7-dfrA1*-Δ*aadA1-s*	PcS (1)	IS*Pa21*-like-*qacEΔ1-sul1-orf5*-ΔIS*1326*-IS*1353*-ΔIS*1326*-Δ*tni*	LC169572

			mr		*B*-Δ*tniA*-IS*26* (1)	
In*590*	In*590*	0[b]	*bla*$_{\text{VIM-1}}$-*aacA7-dfrA1-aadA1*[c]	PcS	*qacEΔ1-sul1-orf5*-IS*26*	AY339625
(In-e541)	In*591*	8	*bla*$_{\text{VIM-1}}$-*aacA7-dfrA1*-Δ*aadA1*	PcS (8)	*qacEΔ1-sul1*-Δ*orf5*-IS*26* (6), *qacEΔ1-sul1-orf5*-ΔIS*1326*-ΔIS*1353*-IS*26* (1), UD (1)	LC169574, LC169576, LC169577
	In*1157*	2	*bla*$_{\text{VIM-26}}$-*aacA7-dfrA1*-Δ*aadA1*	PcS (2)	*qacEΔ1-sul1*-Δ*orf5*-IS*26* (1), Δ*qacEΔ1*-IS*10* (1)	LC169582
	In*1209*	6	*bla*$_{\text{VIM-1}}$-*aacA7-dfrA1-aadA1*[c]	UD (6)	IS*1R* (5), IS*1R*-like (1)	LC169573
	In*1317*	2	*bla*$_{\text{VIM-33}}$-*aacA7-dfrA1*-Δ*aadA1*	PcS (2)	*qacEΔ1-sul1*-Δ*orf5*-IS*26* (2)	LC169581
In*624*		5	*bla*$_{\text{VIM-1}}$-*aacA4-dfrB1-aadA1*, *catB2*	PcH1$_{\text{TTN-10}}$ (2), UD (3)	*qacEΔ1-sul1-orf5*-ΔIS*1326*-IS*26* (2), UD (3)	GQ422827
In*669*		1	*bla*$_{\text{VIM-31}}$-*aacA4*	PcW$_{\text{TGN-10}}$ (1)	*qacEΔ1-sul1-orf5*-Δ*orf6*-IS*6100* (1)	JN982330
In*916*		8	*bla*$_{\text{VIM-1}}$-*aacA4-aphA15-aadA1-catB2*	PcS (1), UD (7)	*qacEΔ1-sul1-orf5*-Δ*tniB-tniA*-IS*26* (2), *qacEΔ1-sul1*-Δ*orf5-chrA-padR*-IS*6100* (2), UD (4)	KF856617
In*1315*		1	*bla*$_{\text{VIM-1}}$-*aacA7-smr*	UD (1)	IS*Pa21*-like-3'-CS[d] (1)	LC169570
In*1316*		1	*bla*$_{\text{VIM-5}}$-*gcuD-aacA4-bla*$_{\text{OXA-2}}$-*gcuD*	PcW$_{\text{TGN-10}}$ (1)	UD (1)	LC169578
In*1318*		1	*bla*$_{\text{VIM-1}}$, *aadA1*[e]	PcS (1)	*qacEΔ1-sul1-orf5*-IS*26* (1)	LC169584

In*1320*	1	bla_{VIM-23}-*gcu172-aacA7*	UD (1)	UD (1)	LC169586
In*1322*	1	bla_{VIM-1}-*aadA7*-Δ*gcuD*[f]	UD (1)	UD (1)	LC169574
In*1323*	3	bla_{VIM-4}-*aacA27*	PcW-P2 (1), UD (2)	*qacEΔ1-sul1-orf5*-Δ*tniB-tniA*-IRt (1), *qacEΔ1-sul1-orf5*-Δ*tniB*-Δ*tniA*-IS*26* (1), UD (1)	LC169579
In*3103*	1	bla_{VIM-1}-*aacA4-dfrB1-aadA1*	UD (1)	UD (1)	LC169588

UD, undetermined due to a contig break in 5'-conserved segment (CS) or 3'-CS; IRt, inverted repeat of Tn*402*-like transposon.

[a] These integrons lacked duplication of the Δbla_{VIM} regions which was present in the original sequences of In*237* (Genbank accession no. EF690695) and In*238* (EU581706).

[b] This type was not identified in this study but presented here for comparison.

[c] In*590* and In*1209* have a different *aadA1* allele (*aadA1a* and *aadA1b*, respectively).

[d] Contig break in the nucleotide position 123 of 3'-CS.

[e] Between bla_{VIM-1} and *aadA1*, putative group II intron reverse transcriptase, which has 93% nucleotide identity to the reverse transcriptase gene found in Genbank accession no. CP002811.1, was present disrupting the *attC* site.

[f] C to A mutation at nucleotide position 279 created premature stop codon.

Integrons with strong promoters (i.e. PcS and PcH2) were common whereas weak promoters (i.e. PcW and PcH1) were rare (Tables 2, S1). We were able to characterize the downstream structures in 16 bla_{VIM}-containing integrons (Tables 2, S2). The majority contained 3'-CS structures immediately downstream of the gene cassettes. Of these, variants of a typical class 1 integron structure, 3'CS-IS*1326*-Δ*tniB*-*tniA*-IRt,[41] with disruption by IS*26,* were prevalent. Non-3'-CS variants included IS*Pa21*-like or IS*1R*-like insertion sequences downstream in 4 integrons with bla_{VIM-1} and bla_{VIM-19} (Table 2).

***Klebsiella* spp. consisted mostly of *K. pneumoniae* subsp. *pneumoniae* with three dominant clonal complexes.**

The phylogenetic relationships of 47 *K. pneumoniae* and 2 *K. variicola* isolates are shown in Fig. 1. Genome analyses revealed that "*K. pneumoniae*" includes three distinct phylogroups of KpI (*K. pneumoniae*), KpII (*K. quasipneumoniae*), and KpIII (*K. variicola*).[42] *K. variicola* was previously identified among 11% and 24% of clinical "*K. pneumoniae*" isolates[43, 44] and patients with bloodstream infection due to *K. variicola* had higher mortality than those due to *K. pneumoniae*.[44]

K. pneumoniae subsp. *pneumoniae* from our study comprised of 15 CCs and 2 STs (Fig. 2). The most prevalent CCs (with ≥ 5 isolates) included CC147 [n=13] (from Italy and Greece) and CC11 [n=6] (from Spain and Romania); CC147 was dominated with ST147 and CC11 consisted only of ST11. CC147 accommodated 4 different integron types (the most common being In*590*-like) and were associated with the PcS strong promoter and the IS*26* insertion variant that formed part of the 3'CS downstream structures. CC147 with In*590*-like integrons is endemic in Greece and is currently emerging globally with different carbapenemase including KPCs, OXA-181 and NDMs.[28, 30, 45] ST11 is a successfully global, multidrug resistant clone and is a single-locus variant of ST258.[5] Some CCs in our study had an international distribution (i.e. present in ≥ 2 countries in different continents): CC17 (n=3) in South Africa and Greece, CC42 (n=3) in Greece and Egypt, and CC101 (n=3) in Saudi

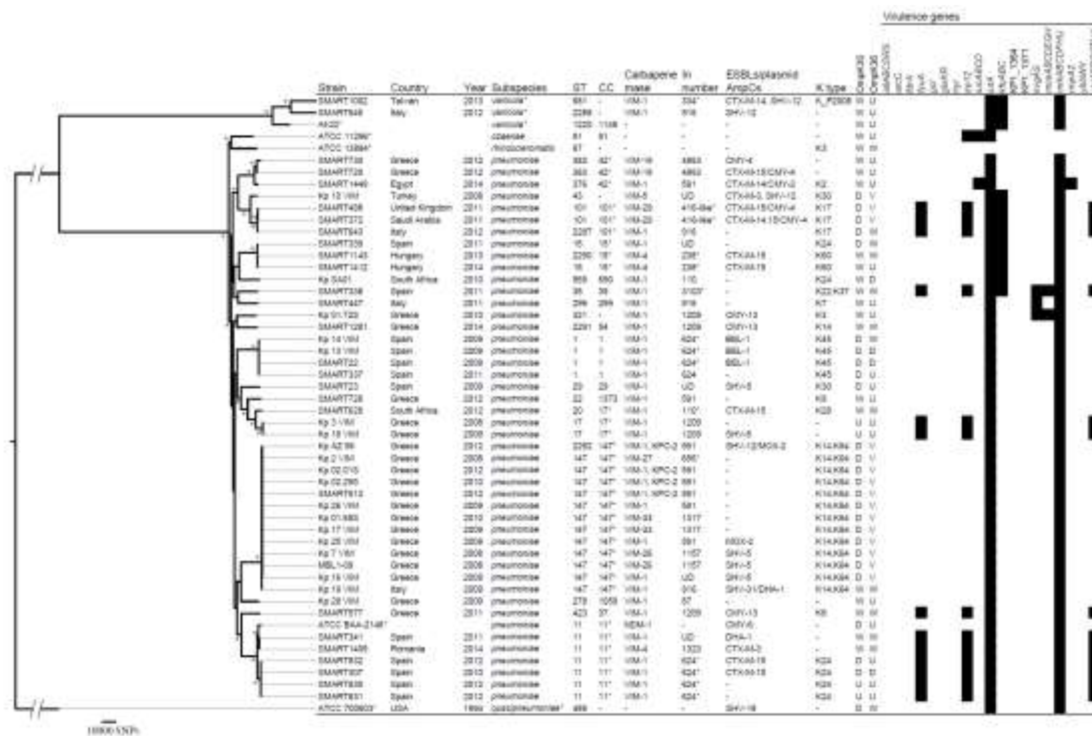


Fig. 2. Phylogenetic tree of VIM-producing *Klebsiella* spp. This maximum-likelihood phylogram is based on a 3,737,806 bp core genome and a total of 369,829 SNPs. The core genome was identified using *K. pnuemoniae* subsp. *pneumoniae* ATCC BAA-2146 as a reference genome. The tree included 47 study isolates and 5 reference strains (marked with asterisks). The tree is rooted by using the outgroup of *K. quasipneumoniae* ATCC700603 and asterisks indicates bootstrap support > 90% from 100 replicates. In the subspecies column, *K. variicola* and *K. quasipneumoniae* (marked with asterisks) are not subspecies of *K. pneumoniae* but distinct species. STs 2287 to 2292 were novel types found in this study. A CC marked with an asterisk distributed internationally. Integron numbers with asterisks were partially characterized (Dataset 3). OmpK35 and OmpK36 columns indicate predicted mutation of porins: W, wild; D, deficient (due to premature stop codon); V, variant associated with increased MIC of carbapenems; U, variant with unknown significance. Virulence genes of *clbA-R* (colibactin), *iroBCDN* (salmochelin), and *rmpA* were sought but not found. UD, undetermined.

Arabia, UK, and Italy.

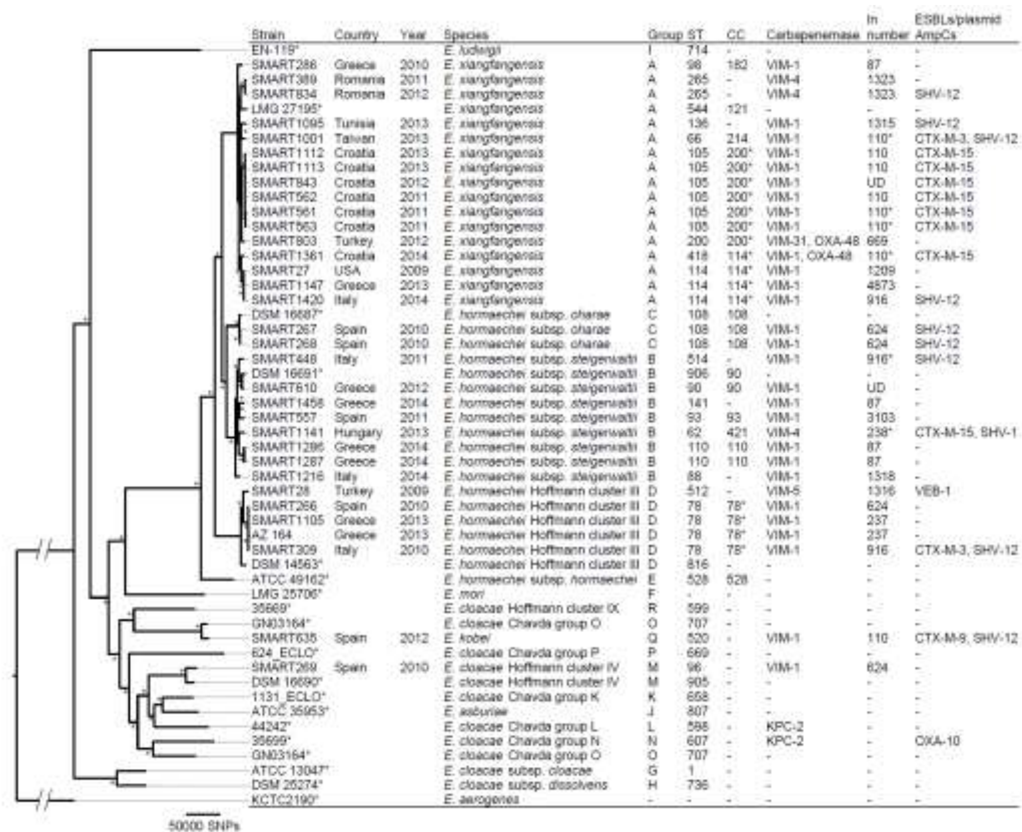
OmpK35 and OmpK36 deficiencies and variants are responsible for alterations in porins that contribute to increased MICs to the carbapenems.[30] The majority of the study isolates had OmpK35 deficiency due to premature stop codons and OmpK36 deficiency or variants (Fig. 2). Only 17% of the isolates had wild type OmpK35 and OmpK36.

Hypervirulent *K. pneumoniae* strains often possess siderophore clusters (i.e. yersiniabaction, aerobactin, colibactin, and salmochelin) as well as *rmpA* or *rmpA2*.[42] Yersiniabactin, which is encoded by a pathogenicity island that includes *ybt*, *irp12* and *fyuA* genes,[42] was present in isolates from this study belonging to CCs 11, 17, 35, 37, and 101 (Fig. 2).

***E. cloacae* complex consisted mostly of *E. hormaechei* with three dominant clonal complexes.**

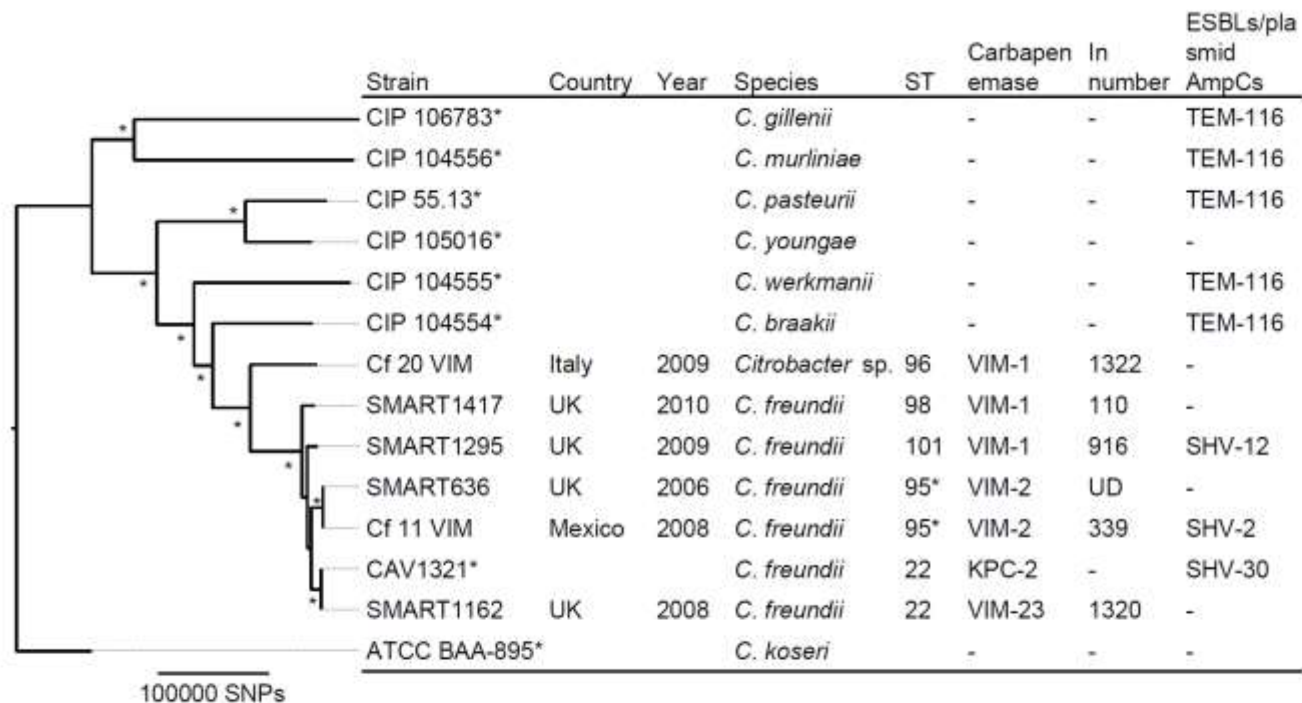
The latest WGS-based phylogenomic study revealed that *E. cloacae* complex is made up of 18 groups which are difficult to distinguish using phenotypic or conventional molecular methods.[9] This study proposed that *E. hormaechei* included 2 more subspecies of *E. xiangfangensis* and Hoffmann cluster III, in addition to the 3 original subspecies (*hormaechei*, *oharae*, and *steigerwaltii*) defined by Hoffmann *et al*.[46] *E. xiangfangensis* was the most common *Enterobacter* group associated with *bla*$_{KPC}$.[9] Other recent studies showed that *E. hormaechei* subsp. *steigerwaltii* and *E. hormaechei* Hoffmann cluster III are the most prevalent clinical species among *E. cloacae* complex.[47, 48]

E. cloacae complex (n=33) was the second most common microorganisms in our study and consisted mainly of *E. hormaechei*: *E. xiangfangensis* (n=16), subsp. *steigerwaltii* (n=8) and Hoffmann cluster III (n=5), and subsp. *oharae* (n=2) (Fig. 3). *In-silico* MLST analysis identified 11 CCs and 24 STs among *E. cloacae* complex (Fig. 3). *E. xiangfangensis* CC200 (with *bla*$_{VIM-1}$ from Croatia and Turkey), *E. xiangfangensis* CC114 (with *bla*$_{VIM-1}$ from Croatia, Greece, Italy, and USA) and *E. hormaechei* Hoffmann cluster III CC78 (with



Strain	Country	Year	Species	Group	ST	CC	Carbapenemase	In number	ESBLs/plasmid AmpCs
EN-119*			*E. ludwigii*	I	714	-	-	-	-
SMART286	Greece	2010	*E. xiangfangensis*	A	98	182	VIM-1	87	-
SMART389	Romania	2011	*E. xiangfangensis*	A	265	-	VIM-4	1323	-
SMART834	Romania	2012	*E. xiangfangensis*	A	265	-	VIM-4	1323	SHV-12
LMG 27195*			*E. xiangfangensis*	A	544	121	-	-	-
SMART1095	Tunisia	2013	*E. xiangfangensis*	A	136	-	VIM-1	1315	SHV-12
SMART1001	Taiwan	2013	*E. xiangfangensis*	A	66	214	VIM-1	110*	CTX-M-3, SHV-12
SMART1112	Croatia	2013	*E. xiangfangensis*	A	105	200*	VIM-1	110	CTX-M-15
SMART1113	Croatia	2013	*E. xiangfangensis*	A	105	200*	VIM-1	110	CTX-M-15
SMART843	Croatia	2012	*E. xiangfangensis*	A	105	200*	VIM-1	UD	CTX-M-15
SMART562	Croatia	2011	*E. xiangfangensis*	A	105	200*	VIM-1	110	CTX-M-15
SMART561	Croatia	2011	*E. xiangfangensis*	A	105	200*	VIM-1	110*	CTX-M-15
SMART563	Croatia	2011	*E. xiangfangensis*	A	105	200*	VIM-1	110*	CTX-M-15
SMART803	Turkey	2012	*E. xiangfangensis*	A	200	200*	VIM-31, OXA-48	669	-
SMART1361	Croatia	2014	*E. xiangfangensis*	A	418	114*	VIM-1, OXA-48	110*	CTX-M-15
SMART27	USA	2009	*E. xiangfangensis*	A	114	114*	VIM-1	1209	-
SMART1147	Greece	2013	*E. xiangfangensis*	A	114	114*	VIM-1	4873	-
SMART1420	Italy	2014	*E. xiangfangensis*	A	114	114*	VIM-1	916	SHV-12
DSM 16687*			*E. hormaechei* subsp. *oharae*	C	108	108	-	-	-
SMART267	Spain	2010	*E. hormaechei* subsp. *oharae*	C	108	108	VIM-1	624	SHV-12
SMART268	Spain	2010	*E. hormaechei* subsp. *oharae*	C	108	108	VIM-1	624	SHV-12
SMART448	Italy	2011	*E. hormaechei* subsp. *steigerwaltii*	B	514	-	VIM-1	916*	SHV-12
DSM 16691*			*E. hormaechei* subsp. *steigerwaltii*	B	906	90	-	-	-
SMART610	Greece	2012	*E. hormaechei* subsp. *steigerwaltii*	B	90	90	VIM-1	UD	-
SMART1458	Greece	2014	*E. hormaechei* subsp. *steigerwaltii*	B	141	-	VIM-1	87	-
SMART557	Spain	2011	*E. hormaechei* subsp. *steigerwaltii*	B	93	93	VIM-1	3103	-
SMART1141	Hungary	2013	*E. hormaechei* subsp. *steigerwaltii*	B	62	421	VIM-4	238*	CTX-M-15, SHV-1
SMART1286	Greece	2014	*E. hormaechei* subsp. *steigerwaltii*	B	110	110	VIM-1	87	-
SMART1287	Greece	2014	*E. hormaechei* subsp. *steigerwaltii*	B	110	110	VIM-1	87	-
SMART1216	Italy	2014	*E. hormaechei* subsp. *steigerwaltii*	B	88	-	VIM-1	1318	-
SMART28	Turkey	2009	*E. hormaechei* Hoffmann cluster III	D	512	-	VIM-5	1316	VEB-1
SMART266	Spain	2010	*E. hormaechei* Hoffmann cluster III	D	78	78*	VIM-1	624	-
SMART1105	Greece	2013	*E. hormaechei* Hoffmann cluster III	D	78	78*	VIM-1	237	-
AZ 164	Greece	2013	*E. hormaechei* Hoffmann cluster III	D	78	78*	VIM-1	237	-
SMART309	Italy	2010	*E. hormaechei* Hoffmann cluster III	D	78	78*	VIM-1	916	CTX-M-3, SHV-12
DSM 14563*			*E. hormaechei* Hoffmann cluster III	D	816	-	-	-	-
ATCC 49162*			*E. hormaechei* subsp. *hormaechei*	E	528	528	-	-	-
LMG 25706*			*E. mori*	F	-	-	-	-	-
35669*			*E. cloacae* Hoffmann cluster IX	R	599	-	-	-	-
GN03164*			*E. cloacae* Chavda group O	O	707	-	-	-	-
SMART635	Spain	2012	*E. kobei*	Q	520	-	VIM-1	110	CTX-M-9, SHV-12
624_ECLO*			*E. cloacae* Chavda group P	P	669	-	-	-	-
SMART269	Spain	2010	*E. cloacae* Hoffmann cluster IV	M	96	-	VIM-1	624	-
DSM 16690*			*E. cloacae* Hoffmann cluster IV	M	905	-	-	-	-
1131_ECLO*			*E. cloacae* Chavda group K	K	658	-	-	-	-
ATCC 35953*			*E. asburiae*	J	807	-	-	-	-
44242*			*E. cloacae* Chavda group L	L	598	-	KPC-2	-	-
35699*			*E. cloacae* Chavda group N	N	607	-	KPC-2	-	OXA-10
GN03164*			*E. cloacae* Chavda group O	O	707	-	-	-	-
ATCC 13047*			*E. cloacae* subsp. *cloacae*	G	1	-	-	-	-
DSM 26274*			*E. cloacae* subsp. *dissolvens*	H	736	-	-	-	-
KCTC2190*			*E. aerogenes*	-	-	-	-	-	-

Fig. 3. Phylogenetic tree of VIM-producing *Enterobacter* spp. This maximum-likelihood phylogram is based on a 1,738,728 bp core genome and a total of 511,679 SNPs. The core genome was identified using *E. cloacae* subsp. *cloacae* ATCC 13047 as a reference genome. The tree included 33 study isolates and 19 reference strains (marked with asterisks). The tree is rooted by using the outgroup of *E. aerogenes* KCTC 2190 and asterisks indicates bootstrap support > 90% from 100 replicates. Group column indicates *E. cloacae* complex groups defined by Chavda et al.[9] ST512, ST514, and ST520 were novel types found in this study. A CC marked with an asterisk distributed internationally. Integron numbers with asterisks were partially characterized (Dataset 3). UD, undetermined.



Strain	Country	Year	Species	ST	Carbapenemase	In number	ESBLs/plasmid AmpCs
CIP 106783*			*C. gillenii*		-	-	TEM-116
CIP 104556*			*C. murliniae*		-	-	TEM-116
CIP 55.13*			*C. pasteurii*		-	-	TEM-116
CIP 105016*			*C. youngae*		-	-	-
CIP 104555*			*C. werkmanii*		-	-	TEM-116
CIP 104554*			*C. braakii*		-	-	TEM-116
Cf 20 VIM	Italy	2009	*Citrobacter* sp.	96	VIM-1	1322	-
SMART1417	UK	2010	*C. freundii*	98	VIM-1	110	-
SMART1295	UK	2009	*C. freundii*	101	VIM-1	916	SHV-12
SMART636	UK	2006	*C. freundii*	95*	VIM-2	UD	-
Cf 11 VIM	Mexico	2008	*C. freundii*	95*	VIM-2	339	SHV-2
CAV1321*			*C. freundii*	22	KPC-2	-	SHV-30
SMART1162	UK	2008	*C. freundii*	22	VIM-23	1320	-
ATCC BAA-895*			*C. koseri*		-	-	-

Fig. 4. Phylogenetic tree of VIM-producing *Citrobacter* spp. This maximum-likelihood phylogram is based on a 2,406,029 bp core genome and a total of 594,405 SNPs. The core genome was identified using *C. freundii* CAV1321 as a reference genome. The tree included 6 study isolates and 8 reference strains (marked with asterisks). The tree is rooted by using the outgroup of *C. koseri* ATCC BAA-895 and asterisks indicates bootstrap support > 90% from 100 replicates. A ST marked with an asterisk distributed internationally. STs 95, 96, 98, and 101 were novel types found in this study. UD, undetermined.

bla_{VIM-1} from Greece, Italy, and Spain) were the most common CCs among *E. cloacae* complex. Previous molecular epidemiology studies have shown that CC200 (more specifically ST105) with bla_{VIM-1} are common in Croatia[49] while CC78 and CC114 are global clones associated with $bla_{CTX-M-15}$ or bla_{VIM-1} especially among European countries.[50] None of the study isolates belonged to ST171.

Citrobacter* spp. and *E. coli.

Citrobacter spp. isolates (n=6) included in our study belonged to ST22, ST95, ST96, ST98, and ST101 (Fig. 4). One isolate (Cf 20 VIM) were classified as *Citrobacter* spp. based on the phylogenetic tree constructed with type strains (Fig. 4).[51] The ANI values between this isolate and the three most closely related *Citrobacter* species (i.e. *C. freundii*, *C. braakii*, and *C. werkmanii*) were < 95 percentage (i.e. is the cut-off value of species definition) [Table S3]. ANI is a promising method of defining species using whole genome sequencing replacing DNA-DNA hybridization.[10]

The phylogenetic relationship of one *E. coli* isolate with bla_{VIM-1} belonged to phylogenetic group E and ST1955.

This study has some limitations. Our collection may not represent the global prevalence of VIM and integrons subtypes. We were unable to determine all of the integron structures due to the limitation of short-read sequencing. Long-read sequencing techniques including the detailed analysis of plasmids would provide more knowledge on location, mobile elements, and plasmid backbones of these carbapenemases.

Summary

To the best of our knowledge, this is the first study to elucidate the global epidemiology at a large scale of bla_{VIM}-containing Enterobacteriaceae using whole genome sequencing with comprehensive molecular analysis. The distribution of bla_{VIM}-containing integrons showed distinctive patterns: a) Certain integrons were present in specific countries

but in different species. i.e. In*87* with *bla*$_{VIM-1}$ from Greece; In*624* with *bla*$_{VIM-1}$ from Spain; In*916* with *bla*$_{VIM-1}$ from Italy; and In*1323* with *bla*$_{VIM-4}$ from Romania were present in different species from that country. This suggested the circulation of the same integron among different bacteria within the same country. b) The same integron was present globally in different species. We identified In*110* with *bla*$_{VIM-1}$ in *K. pneumoniae, E. xiangfangensis*, *E. kobei*, and *C. freundii* from Croatia, Germany, South Africa, and Spain. In*1209* with *bla*$_{VIM-1}$ was present in different *K. pneumoniae* CCs from Greece and *E. xiangfangensis* from the USA. c) The remaining *bla*$_{VIM}$ containing integrons were limited to one country within a single species.

The association of certain high-risk clones with specific integrons showed that *K. pneumoniae* CC147 from Greece was associated with In*590*-like integrons that only differ because of the VIM subtypes (i.e. In*591* with *bla*$_{VIM-1}$; In*1157* with *bla*$_{VIM-26;}$ and In*1317* with *bla*$_{VIM-33}$). This had previously been described.[28] *E. xiangfangensis* ST105 from Croatia was associated with In*110* containing *bla*$_{VIM-1}$.

This study highlights the importance of surveillance programs using the latest molecular techniques in providing insight into the characteristics, global distribution of CCs and their association with integrons containing *bla*$_{VIMs}$.

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Transparency declarations

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