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Genomic epidemiology of global VIM-producing Enterobacteriaceae

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

Methods: We performed short read (Illumina) WGS 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*$_{VIM}$s. *Enterobacter cloacae* complex was the second most common species and mainly consisted of *Enterobacter hormaechei* (*Enterobacter xiangfangensis*, subsp. *steigerwaltii* and Hoffmann cluster III). CC200 (from Croatia and Turkey), CC114 (Croatia, Greece, Italy and the USA) and CC78 (from Greece, Italy and Spain) containing *bla*$_{VIM-1}$ were the most common clones among the *E. cloacae* complex.

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

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 hydrolyse the carbapenems, known as carbapenemases, are the most important causes of carbapenem resistance. Carbapenemase-producing Enterobacteriaceae (CPE) have acquired multiple resistance genes making therapy for infections due to these bacteria challenging.[1,2]

The most common carbapenemases among CPE are KPCs (Amber class A), IMPs, VIMs, NDMs (class B lactamases or MBLs) and OXA-48-like (class D) enzymes.[1] 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, particularly 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 WGS 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 programmes namely the Merck Study for Monitoring Antimicrobial Resistance Trends (SMART) (2008–14) and the AstraZeneca global surveillance study of antimicrobial resistance (2012–13) (Dataset S1, available as Supplementary data at *JAC* Online).

The SMART programme included isolates from intra-abdominal and urinary tract infections from the following countries: Morocco, South Africa and Tunisia (Africa); China, Malaysia, Singapore, South Korea, Taiwan, Thailand and Vietnam (Asia); the Czech Republic, Estonia, France, Georgia, Greece, Germany, Hungary, Italy, Latvia, Lithuania, Portugal, Romania, Slovenia, Spain, Turkey and the UK (Europe); Argentina, Brazil, Chile, Colombia, Dominican Republic, Ecuador, Guatemala, Mexico, Puerto Rico, Panama, Uruguay and Venezuela (Latin America); Jordan, Lebanon, Israel, Saudi Arabia and UAE (Middle East); Canada and the USA (North America); and Australia, New Zealand, the Philippines and Japan (South Pacific).

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

Both programmes 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 107366 isolates were obtained from 2008 to 2014; of these 755 were positive for bla_{KPC}, 281 for $bla_{OX\text{-}48\text{-}like}$, 271 for bla_{NDM}, 89 for bla_{VIM} and 38 for bla_{IMP}.

WGS

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 a 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 S2) 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 S2) 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 >500 bp to all of the study isolates. Maximum-likelihood tree was built 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 number LC169570 to LC169586.

Results and discussion

Geographical 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 ($n = 4$) (Figure 1 and Dataset S1). 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* ($n = 1$) (Figure 1 and Table 1).

The 89 genomes were sequenced at an average depth of 167 [standard deviation (SD) 87.9] (Dataset S1). Assembled genomes had an average number of contigs of 101 (SD 50.4) and N50 value of 265210 bp (SD 98928 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 is shown in Figure S1. Table 1 shows the geographical distribution of the different species, types of carbapenemases and integrons. We identified 11 bla_{VIM} variants namely: $bla_{VIM\text{-}1}$ ($n = 67$), $bla_{VIM\text{-}2}$ ($n = 2$), $bla_{VIM\text{-}4}$ ($n = 7$), $bla_{VIM\text{-}5}$ ($n = 2$), $bla_{VIM\text{-}19}$ ($n = 2$), $bla_{VIM\text{-}23}$ ($n = 1$), $bla_{VIM\text{-}26}$ ($n = 2$), $bla_{VIM\text{-}27}$ ($n = 1$), $bla_{VIM\text{-}29}$ ($n = 2$), $bla_{VIM\text{-}31}$ ($n = 1$) and $bla_{VIM\text{-}33}$ ($n = 2$). 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 Spain, VIM-4 in Europe, VIM-5 and -31 in Turkey, VIM-19, -26, -27 and -33 were 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 $bla_{VIM\text{-}1}$ were previously responsible for nosocomial outbreaks throughout Greece and 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 $bla_{VIM\text{-}1}$, Enterobacteriaceae with the following bla_{VIM}s have been reported: $bla_{VIM\text{-}2}$ in Austria,[31] Mexico[32] and Venezuela[33]; and $bla_{VIM\text{-}4}$ in the Czech Republic,[34] Egypt,[35] Hungary,[36] Italy[37] and Kuwait.[38] In addition, a recent global

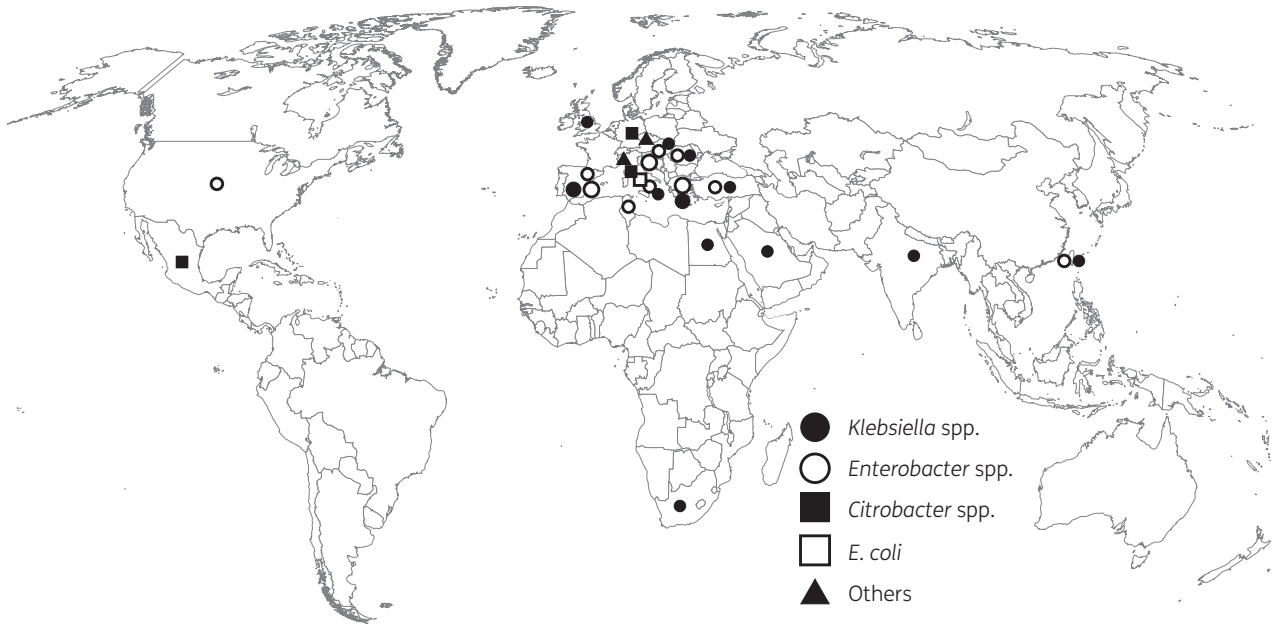


Figure 1. Global distribution of VIM-producing Enterobacteriaceae isolates in this study.

surveillance study from 2012 to 2014 reported Enterobacteriaceae with the following bla_{VIM}s: bla_{VIM-5} in Turkey and Nigeria; bla_{VIM-23} in Mexico; bla_{VIM-26} in Greece; bla_{VIM-32} in the USA; and bla_{VIM-42} from Italy.[39]

Characterization of class 1 integrons identified 21 different integron types, including seven novel cassette combinations

All of the bla_{VIM}s 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 characterize partially 27 of 30 additional integrons (Figures 2 and 3, and Dataset S3).

We identified 21 different integron types containing bla_{VIM}, including seven novel combinations (Table 2). In*110* and In*1209*, that contain bla_{VIM-1} had international, intercontinental and inter-genus distribution [In*110*, Croatia (*Enterobacter xiangfangensis*), South Africa (*K. pneumoniae*), Spain (*Enterobacter kobei*) and Germany (*Citrobacter 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 and 2). The international and inter-genus distribution of bla_{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]

Integrons with strong promoters (i.e. PcS and PcH2) were common whereas weak promoters (i.e. PcW and PcH1) were rare (Tables 2 and S1). We were able to characterize the downstream structures in 16 bla_{VIM}-containing integrons (Tables 2 and 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 ISs downstream in four integrons with bla_{VIM-1} and bla_{VIM-19} (Table 2).

Klebsiella spp. consisted mostly of K. pneumoniae subsp. pneumoniae with three dominant CCs

The phylogenetic relationships of 46 *K. pneumoniae* (including 1 reference strain) and 3 *K. variicola* isolates (including 1 reference strain) are shown in Figure 2. 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 23 different STs (Figure 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 by ST147 and CC11 consisted only of ST11. CC147 accommodated four 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 carbapenemases, including KPCs, OXA-181 and NDMs.[28,30,45] ST11 is a successful 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 at least two countries on different continents): CC17 ($n = 3$) in South Africa and Greece; CC42 ($n = 3$) in Greece and Egypt; and CC101 ($n = 3$) in Saudi Arabia, the UK and Italy.

OmpK35 and OmpK36 deficiencies and variants are responsible for alterations in porins that contribute to increased MICs of the carbapenems.[30] The majority of the study isolates had OmpK35

Table 1. VIM subtypes and integrons of the Enterobacteriaceae isolates

	Species, country (*n*)					
Carbapenemase (*n*)	*Klebsiella* spp. (KP)	*E. cloacae* complex (Ecl)	*Citrobacter* spp. (CI)	*E. coli* (EC)	*P. mirabilis* (PM), *S. marcescens* (SM)	Defined integron numbers (species, *n*)
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), the 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, the 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), the 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]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]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 the USA (Ecl).

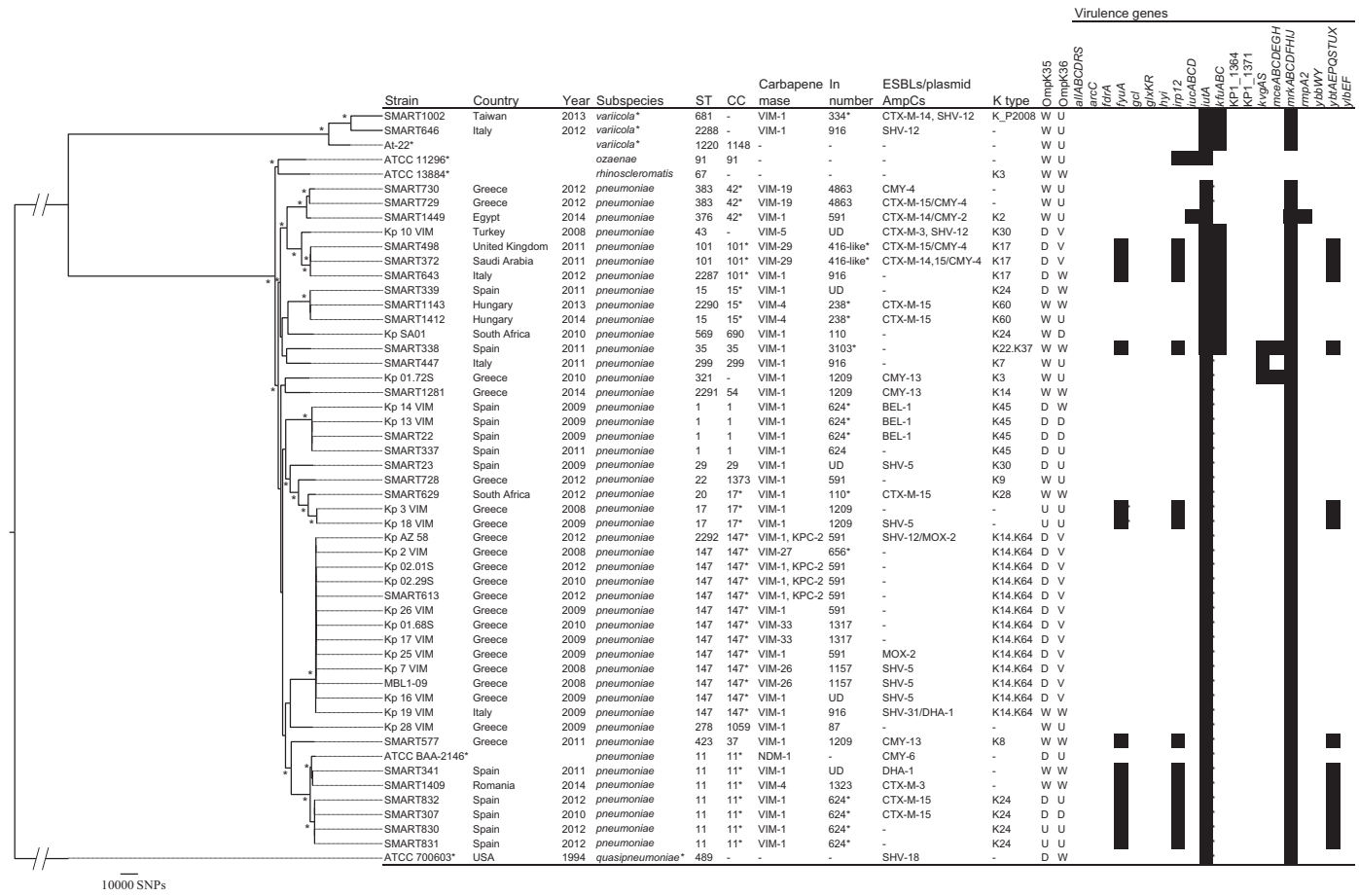


Figure 2. Phylogenetic tree of VIM-producing *Klebsiella* spp. This maximum-likelihood phylogram is based on a 3737806 bp core genome and a total of 369829 SNPs. Core genome was identified using *K. pneumoniae* subsp. *pneumoniae* ATCC BAA-2146 as a reference genome. Tree includes 47 study isolates and five reference strains (marked with asterisks). Tree is rooted by using the outgroup of *K. quasipneumoniae* ATCC 700603 and asterisks indicate 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 2287–2292 were novel types found in this study. A CC marked with an asterisk was distributed internationally. Integron numbers with asterisks were partially characterized (Dataset S3). 'OmpK35' and 'OmpK36' columns indicate predicted mutation of porins: W, WT; 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.

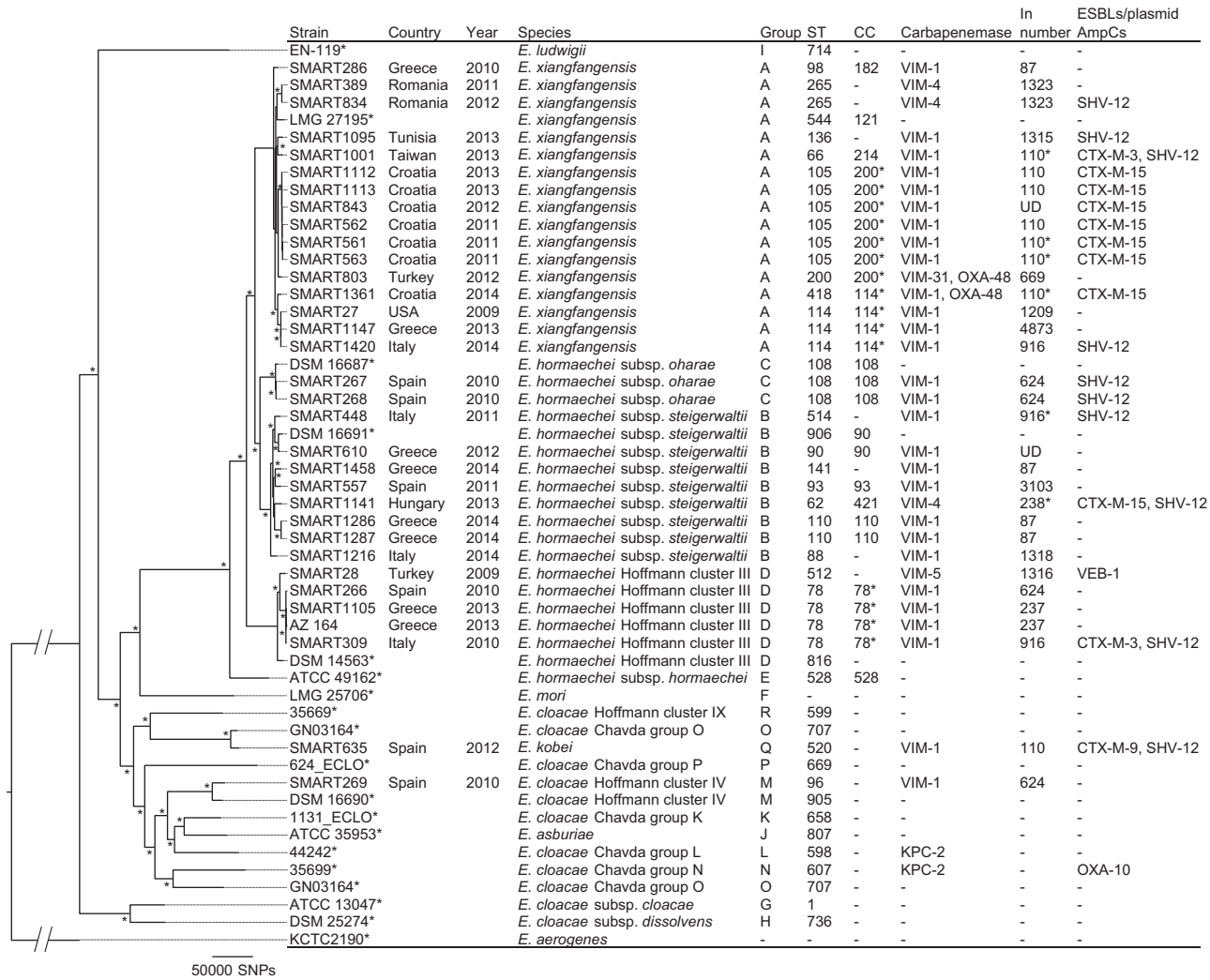
deficiency due to premature stop codons and OmpK36 deficiency or variants (Figure 2). Only 17% of the isolates had WT OmpK35 and OmpK36.

Hypervirulent *K. pneumoniae* strains often possess siderophore clusters (i.e. yersiniabactin, 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 (Figure 2).

E. cloacae complex consisted mostly of Enterobacter hormaechei with three dominant CCs

The latest WGS-based phylogenomic study revealed that the *E. cloacae* complex is made up of 18 groups, which are difficult to distinguish using phenotypic or conventional molecular methods.[9] That study proposed that *E. hormaechei* included two more subspecies of *E. xiangfangensis* and Hoffmann cluster III, in addition to the three 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 the *E. cloacae* complex.[47,48]

The *E. cloacae* complex ($n = 33$) was the second most common microorganism 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* ($n = 2$) (Figure 3). *In silico* MLST analysis identified 11 CCs and 24 STs among the *E. cloacae* complex (Figure 3). *E. xiangfangensis* CC200 (with bla_{VIM-1} from Croatia and Turkey), *E. xiangfangensis* CC114 (with bla_{VIM-1} from Croatia, Greece, Italy and the USA) and *E. hormaechei* Hoffmann cluster III CC78 (with bla_{VIM-1} from Greece, Italy and Spain) were the most common CCs among the *E. cloacae* complex. Previous molecular epidemiology studies have shown that CC200 (more specifically ST105) with bla_{VIM-1} are common in Croatia,[49]



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-12
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 25274*			*E. cloacae* subsp. *dissolvens*	H	736	-	-	-	-
KCTC2190*			*E. aerogenes*	-	-	-	-	-	-

Figure 3. Phylogenetic tree of VIM-producing *Enterobacter* spp. This maximum-likelihood phylogram is based on a 1738728 bp core genome and a total of 511679 SNPs. Core genome was identified using *E. cloacae* subsp. *cloacae* ATCC 13047 as a reference genome. Tree includes 33 study isolates and 19 reference strains (marked with asterisks). Tree is rooted by using the outgroup of *E. aerogenes* KCTC 2190 and asterisks indicate 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. CC marked with an asterisk was distributed internationally. Integron numbers with asterisks were partially characterized (Dataset S3). UD, undetermined.

while CC78 and CC114 are global clones associated with $bla_{\text{CTX-M-15}}$ or $bla_{\text{VIM-1}}$ particularly 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 (Figure 4). One isolate (Cf 20 VIM) was classified as *Citrobacter* spp. based on the phylogenetic tree constructed with type strains (Figure 4).[51] The ANI values between this isolate and the three most closely related *Citrobacter* species (i.e. *C. freundii*, *Citrobacter braakii* and *Citrobacter werkmanii*) were <95% (i.e. is the cut-off value of species definition) (Table S3). ANI is a promising method of defining species using WGS replacing DNA–DNA hybridization.[10]

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

This study has some limitations. Our collection may not represent the global prevalence of VIM and integron 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 on a large scale of bla_{VIM}-containing Enterobacteriaceae using WGS with comprehensive molecular analysis. The distribution of bla_{VIM}-containing integrons showed

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 integron
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-smr*	PcS (1)	IS*Pa21*-like-*qacEΔ1-sul1-orf5*-ΔIS*1326*-IS*1353*-ΔIS*1326*-Δ*tniB*-Δ*tniA*-IS*26* (1)	LC169572
In*590* (In-e541)	In*590*	0[b]	bla_{VIM-1}-*aacA7-dfrA1-aadA1*[c]	PcS	*qacEΔ1-sul1-orf5*-IS*26*	AY339625
	In*591*	8	bla_{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_{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_{VIM-1}-*aacA7-dfrA1-aadA1*[c]	UD (6)	IS*1R* (5), IS*1R*-like (1)	LC169573
	In*1317*	2	bla_{VIM-33}-*aacA7-dfrA1*-Δ*aadA1*	PcS (2)	*qacEΔ1-sul1*-Δ*orf5*-IS*26* (2)	LC169581
In*624*		5	bla_{VIM-1}-*aacA4-dfrB1-aadA1*, *catB2*	PcH1$_{TTN-10}$ (2), UD (3)	*qacEΔ1-sul1-orf5*-ΔIS*1326*-IS*26* (2), UD (3)	GQ422827
In*669*		1	bla_{VIM-31}-*aacA4*	PcW$_{TGN-10}$ (1)	*qacEΔ1-sul1-orf5*-Δ*orf6*-IS*6100* (1)	JN982330
In*916*		8	bla_{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_{VIM-1}-*aacA7-smr*	UD (1)	IS*Pa21*-like-3′-CS[d] (1)	LC169570
In*1316*		1	bla_{VIM-5}-*gcuD-aacA4-bla*$_{OXA-2}$-*gcuD*	PcW$_{TGN-10}$ (1)	UD (1)	LC169578
In*1318*		1	bla_{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′-CS or 3′-CS; IRt, inverted repeat of Tn*402*-like transposon.
[a]These integrons lacked the 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 is 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.

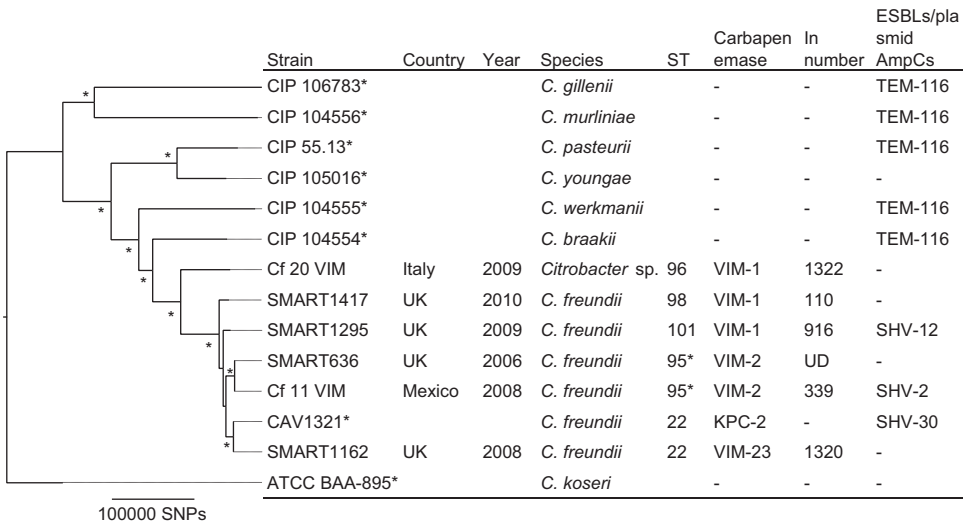


Figure 4. Phylogenetic tree of VIM-producing *Citrobacter* spp. This maximum-likelihood phylogram is based on a 2406029 bp core genome and a total of 594405 SNPs. Core genome was identified using *C. freundii* CAV1321 as a reference genome. Tree includes six study isolates and eight reference strains (marked with asterisks). Tree is rooted by using the outgroup of *Citrobacter koseri* ATCC BAA-895 and asterisks indicate bootstrap support >90% from 100 replicates. An ST marked with an asterisk was distributed internationally. STs 95, 96, 98 and 101 were novel types found in this study. UD, undetermined.

distinctive patterns. (i) 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. (ii) 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. (iii) 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 programmes using the latest molecular techniques in providing insight into the characteristics, global distribution of CCs and their association with integrons containing bla_{VIM}s.

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Transparency declarations

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Supplementary data

Datasets S1 to S3, Figure S1 and Tables S1 to S3 are available as Supplementary data at *JAC* Online.

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