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Molecular characterization of VIM-producing *Klebsiella pneumoniae* from Scandinavia reveals genetic relatedness with international clonal complexes encoding transferable multidrug resistance

Ø. Samuelsen[1], M. A. Toleman[2], V. Hasseltvedt[3], K. Fuursted[4], T. M. Leegaard[5], T. R. Walsh[2], A. Sundsfjord[1,6] and C. G. Giske[7]

1) *Reference Centre for Detection of Antimicrobial Resistance, Department of Microbiology and Infection Control, University Hospital of North Norway, Tromsø, Norway,* 2) *Department of Medical Microbiology, School of Medicine, Cardiff University, Cardiff, UK,* 3) *Department of Medical Microbiology, Innlandet Hospital Trust, Lillehammer, Norway,* 4) *Department of Clinical Microbiology, Århus University Hospital, Århus, Denmark,* 5) *Institute of Medical Microbiology, Oslo University Hospital, Rikshospitalet, Oslo,* 6) *Research Group for Host–Microbe Interactions, Department of Medical Biology, Faculty of Health Sciences, University of Tromsø, Tromsø, Norway and* 7) *Department of Clinical Microbiology, Karolinska-Institutet-MTC, Karolinska University Hospital, Solna, Stockholm, Sweden*

Abstract

VIM-producing *Klebsiella pneumoniae* (VPKP) has been identified as a source of hospital outbreaks and is prevalent particularly in the Mediterranean region. In this study we have characterized eight VPKP isolates identified in Scandinavia during 2005–2008. With the exception of one isolate, all were from patients with recent history of hospitalization abroad (Greece, *n* = 6; Turkey, *n* = 1). Multilocus sequence typing (MLST) resulted in five sequence types (STs), ST36 (*n* = 1), ST147 (*n* = 4), ST272 (*n* = 1), ST273 (*n* = 1) and ST383 (*n* = 1), which except for ST272 were part of putative international clonal complexes. All were multidrug resistant due to the presence of other resistance determinants, including extended-spectrum β-lactamases (CTX-M-3, SHV-5 and SHV-12), 16S rRNA methylases (ArmA) and plasmid-mediated quinolone resistance determinants (QnrS). One isolate harboured a novel VIM-variant (VIM-26) while VIM-1 and VIM-19 were detected in six and one isolate, respectively. Two different genetic structures surrounding the bla_{VIM} gene were identified in four isolates. In two isolates bla_{VIM-1} and bla_{VIM-26} were located in an integron similar to In-e541 (*int*I1;$bla_{VIM-1/-26}$;*aacA7*;*dhfrI*;*aadA1*;3′CS) while in the other two isolates bla_{VIM-1} was located in an integron lacking 3′CS but with an IS*26* element in the 3′end (*int*I1;bla_{VIM-1};*aac(6′)-Ib*;IS26), as identified in the IncN plasmid pKOX105. The bla_{VIM}-genes were located on transferable plasmids ranging from ~40 to ~240 kb and associated with Tn*21* in four isolates. PCR-based replicon typing indicated association of bla_{VIM} with IncN (*n* = 3) and A/C (*n* = 1) broad-host-range plasmids but also with unknown replicons (*n* = 4). In conclusion, Scandinavian VPKP is associated with importation and genetically related to international clones encoding transferable plasmid-mediated multidrug resistance.

Keywords: Integrons, *Klebsiella pneumoniae*, metallo-beta-lactamase, MLST, molecular epidemiology, plasmid-replicon typing, Scandinavia
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Corresponding author: Ø. Samuelsen, Reference Centre for Detection of Antimicrobial Resistance, Department of Microbiology and Infection Control, University Hospital of North Norway, N-9038 Tromsø, Norway
E-mail: orjan.samuelsen@unn.no

Introduction

The increased prevalence of acquired carbapenem-hydrolysing β-lactamases in Gram-negative bacteria is of great concern due to their ability to hydrolyse virtually all β-lactam antibiotics, as well as their genetic association with transferable multidrug resistance [1]. As a consequence,

infections with carbapenemase-producing bacteria have become a serious challenge for infection control and antibiotic therapy. Acquired metallo-β-lactamases (MBLs) are a diverse family of carbapenemases [1]. Among *Klebsiella pneumoniae* the VIM-group and particularly VIM-1 are dominating [1], although NDM-1 has been on the rise among this species recently [2]. VIM-producing *K. pneumoniae* (VPKP) has been associated with hospital outbreaks in several countries [3–6], and has become endemic in hospitals in certain countries such as Greece [5]. Worryingly, community-onset infections by VPKP have recently been described [7], as well as isolates producing both VIM and KPC-type carbapenemases [8].

The bla_{VIM}-genes are located in class 1 integrons as gene cassettes and have been identified on plasmids with different replicon types [9], increasing the possibility of dissemination and linkage to other antibiotic resistance genes. As a consequence, VPKP isolates often harbour other acquired resistance genes (i.e. genes encoding aminoglycoside modifying enzymes, 16S rRNA methylases, plasmid-mediated quinolone resistance, trimethoprim resistance and other β-lactamases). Molecular typing of VPKP isolates has shown that in general the dissemination is multiclonal with some clonal outbreaks. This is in contrast to what is observed for KPC-producing *K. pneumonia*, where the majority of isolates belong to multilocus sequence type (ST) 258 or related STs [10].

In Scandinavia the emergence of carbapenemase-producing Gram-negative bacteria has been mainly associated with the importation of isolates [11,12]. Here we report the characterization of VPKP isolates identified in Scandinavia from 2005 to 2008. (Part of this study was presented at the 20th European Congress of Clinical Microbiology and Infectious Diseases, Vienna, Austria.)

Materials and Methods

Bacterial isolates and clinical data

Eight clinical isolates of *Klebsiella pneumoniae* from the period 2005–2008 were included in this study (Table 1). Seven isolates had been referred to the national reference centres in Norway (n = 2) or Sweden (n = 5) based on non-susceptibility to carbapenems. One locally identified isolate from Denmark was also included. Clinical information was collected retrospectively, including data on recent hospitalization abroad. Bacterial identification was performed using VITEK2 (bioMérieux, Marcy l'Etoile, France).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using Etest according to the manufacturer's instructions (bioMérieux) and interpreted according to clinical breakpoints from the European Committee for Antimicrobial Susceptibility Testing (EUCAST, http://www.eucast.org/clinical_breakpoints).

Detection of resistance genes and sequencing of genetic structure surrounding the VIM-genes

PCR was performed for the detection of MBL-genes (bla_{IMP}, bla_{VIM}, bla_{SPM} and bla_{GIM}) [13], *Klebsiella pneumoniae* carbapenemase (KPC) [11], ESBL-genes (bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$) [11], plasmid-mediated quinolone resistance genes (*qnrA*, *qnrB* and *qnrS*) [14], and 16S rRNA methylases (*armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD* and *npmA*) [15,16]. Positive PCR products were purified using Exo-SAP-IT according to the manufacturer's instructions (GE Healthcare Bio-Sciences, Uppsala, Sweden) and sequenced using BigDye 3.1 technology (Applied Biosystems, Foster City, CA, USA).

The genetic structure harbouring the bla_{VIM} genes was identified by PCR amplification of integrons using previously described primers for *int*I1, *qac*EΔ1 and IS*26* [12,17] and in-house designed primers. PCR products were purified from agarose gels using a QIAquick gel extraction kit (QIAgen, Hilden, Germany) and subsequently sequenced. Sequence analysis and alignments were performed using Lasergene 8 (DNAStar, Madison, WI, USA) and compared with sequences deposited in the GenBank database (http://www.ncbi.nlm.nih.gov).

Multilocus sequence typing (MLST) and pulsed field gel electrophoresis (PFGE)

PFGE was performed using *Xba*I-digested genomic DNA as described previously [11] and MLST was performed

TABLE 1. VIM-producing *K. pneumoniae* from Scandinavia

Isolate	Year of isolation	Specimen	Place of isolation	Hospitalization abroad, country	MLST sequence type (ST)
ÖN-2211	2005	Sputum	Stockholm, Sweden	Greece	ST147
T14789	2005	Urine	Århus, Denmark	Greece	ST147
AO-15200	2006	Wound secretion	Stockholm, Sweden	Greece	ST147
U-60687	2006	Urine	Stockholm, Sweden	Greece	ST36
K45-67	2007	Pus, catheter	Oslo, Norway	Turkey	ST272
K46-62	2007	Urine	Kongsvinger, Norway	No	ST273
HY-1491/7	2007	Faeces (screening)	Stockholm, Sweden	Greece	ST147
B-22365	2008	Blood	Stockholm, Sweden	Greece	ST383

according to the *K. pneumoniae* MLST website (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html). eBURST (http://eburst.mlst.net) and minimum spanning tree analysis (BioNumerics, Applied Maths, Sint-Martens-Latem, Belgium) was used to analyse the MLST findings.

S1 nuclease digestion and in-gel hybridization

The genetic localization of bla_{VIM} in seven out of eight isolates was analysed by S1 nuclease treatment of total genomic DNA followed by PFGE and in-gel hybridization with a bla_{VIM}-probe as previously described [12].

Transfer studies and PCR-based replicon typing (PBRT)

Conjugative transfer was performed by broth-mating at donor:recipient ratios of 1:1 or 1:9 using the rifampicin resistant *Escherichia coli* J53-2 as recipient. Transconjugants were selected on Luria–Bertani (LB) agar supplemented with 100 mg/L rifampicin and 2–4 mg/L ceftazidime or 1 mg/L ertapenem. Plasmids were isolated using a Nucelobond Xtra Midi kit (Macherey-Nagel, Düren, Germany) from conjugation negative isolates and used for transformation experiments. Transformation was performed by electroporation of plasmid DNA into electrocompetent *E. coli* TOP'10 cells (Invitrogen, Carlsbad, CA, USA). Transformants were selected on LB agar supplemented with 4 mg/L ceftazidime, 1 mg/L ertapenem or 100 mg/L ampicillin. PBRT was performed using the protocol described by Carattoli *et al.* [18].

GenBank accession numbers

The nucleotide sequences for the integrons of strains K45-67, K46-62, HY-1491/7 and U-60687 have been deposited in GenBank with accession numbers FR748150, FR748151, FR748152 and FR748153, respectively.

Results and Discussion

Molecular epidemiology

With one exception, all isolates were derived from patients who had undergone recent medical treatment abroad either in Greece (n = 6) or Turkey (n = 1), and were thus likely to be imported cases (Table 1). Isolate K46-62 was recovered from an 83-year-old female with no history of international travel. A strong association with importation has also been observed with the emergence of other carbapenemase-producing Gram-negative clinical isolates in Scandinavia [11,12]. PFGE typing indicated a possible clonal relationship between four isolates (ÖN-2211, T14789, AO-15200 and HY-1491/7) with a Dice coefficient between 72% and 86% (data not shown). All four isolates were identified as MLST sequence type (ST) 147 (Table 1). Isolate K46-62 was typed to ST273, a single locus variant of ST147. K45-67, B-22365 and U-60687 were typed to ST272, ST383 and ST36, respectively. eBurst and minimum spanning tree analysis showed that with the exception of ST272, which is a singleton in the MLST database, the other STs are part of putative international clonal complexes (data not shown). Both ST147 and ST36 have previously been associated with CTX-M-15-producing isolates from Hungary, [19] Spain, [20,21] and Tunisia, [22] while ST383 has been associated with an isolate co-producing VIM-4, KPC-2 and CMY-4 from Greece [8].

Antimicrobial susceptibility

All isolates expressed high-level resistance to penicillins, cephalosporins and aztreonam, with the exception of one isolate that was susceptible to aztreonam (Table 2). Carbapenem MICs were variable, with two isolates categorized as

TABLE 2. Antimicrobial susceptibility of VIM-producing *K. pneumoniae* from Scandinavia and transconjugants (TC) and transformants (TF)

	Antimicrobial susceptibility, MIC (mg/L)													
Isolate	**AMC**	**TZP**	**CAZ**	**FEP**	**ATM**	**MEM**	**IPM**	**ETP**	**GEN**	**TOB**	**AMK**	**CIP**	**TIG**	**COL**
ÖN-2211	>256	>256	>256	128	32	32	32	8	>256	>256	>256	4	1	0.125
ÖN-2211-TC	32	>256	>256	128	32	1	4	1	>256	>256	>256	0.032	0.25	0.125
T14789	64	>256	>256	>256	8	>32	>32	>32	2	8	16	>32	2	0.125
T14789-TC	32	64	>256	32	1	0.5	2	0.25	1	4	8	0.032	0.25	0.125
AO-15200	32	>256	>256	128	256	32	32	8	2	16	16	>32	4	0.5
AO-15200-TF	64	>256	>256	64	0.125	1	4	0.5	1	8	8	0.008	0.5	0.125
U-60687	32	256	>256	256	256	>32	32	16	64	256	32	>32	0.5	0.125
U-60687-TF	64	>256	>256	16	16	1	8	2	1	8	8	0.008	0.5	0.125
K45-67	64	>256	>256	64	128	2	8	2	2	4	2	>32	1	0.5
K45-67-TC	32	128	>256	64	>256	1	4	0.5	2	4	1.5	1	0.25	0.125
K46-62	32	256	>256	64	0.25	8	8	8	2	4	2	1	0.5	0.25
K46-62-TC	64	>256	>256	128	0.125	2	4	1	4	8	2	2	0.25	0.125
HY-1491/7	32	>256	>256	>256	256	>32	16	>32	2	16	16	>32	1	0.25
HY-1491/7-TF	64	>256	>256	64	0.125	0.5	4	0.25	2	8	8	0.008	0.25	0.125
B-22365	32	>256	>256	64	256	2	2	4	64	32	16	>32	8	0.5
B-22365-TF	64	128	>256	16	0.125	0.5	4	0.25	1	4	8	0.006	0.25	0.125
E. coli J53-2	8	2	1	0.5	0.5	0.0625	0.5	0.016	0.5	1	2	0.032	0.25	0.25
E. coli TOP'10	4	2	1	0.5	0.125	0.032	0.5	0.016	0.5	1	2	0.032	0.25	0.125

TABLE 3. Genotypic characteristics of VIM-producing *K. pneumoniae* from Scandinavia and transconjugants (TC) and transformants (TF)

	β-lactamases	16S rRNA methylases/PMQR	Tn*21*-PCR and Tn*21*-bla_{VIM} linkage PCR	Tn402-PCR	VIM plasmid size	PBRT
ÖN-2211	VIM-1, CTX-M-3, SHV-11, TEM-1	ArmA	+ (ND)	–	~60 kb	IncN
ÖN-2211-TC	VIM-1, CTX-M-3, TEM-1	ArmA	–	–	ND	IncN
T14789	VIM-1, SHV-11, TEM-1	–	+ (linked to bla_{VIM}—1 kb)	–	~160 kb	A/C
T14789-TC	VIM-1	–	–	–	ND	–
AO-15200	VIM-1, SHV-5	–	+ (linked to bla_{VIM}—1 kb)	–	~240 kb	FIIAs
AO-15200-TF	VIM-1	–	–	–	ND	–
U-60687	VIM-26, SHV-5	–	+ (linked to bla_{VIM}—1 kb)	–	~40 kb	A/C, FIIAs
U-60687-TF	VIM-26	–	–	–	ND	A/C
K45-67	VIM-1, SHV-11, SHV-12	QnrS	+ (no linkage)	–	~50 kb	IncN
K45-67-TC	VIM-1, SHV-12	QnrS	–	–	ND	IncN
K46-62	VIM-1, SHV-1, SHV-12, TEM-1	QnrS	+ (no linkage)	–	~50 kb	IncN
K46-62-TC	VIM-1, SHV-12	QnrS	–	–	ND	IncN
HY-1491/7	VIM-1, SHV-11	–	+ (linked to bla_{VIM}—1 kb)	–	~220 kb	–
HY-1491/7-TF	VIM-1	–	–	–	ND	–
B-22365	VIM-19, CTX-M-gr.1, SHV-1	–	ND	ND	ND	A/C
B-22365-TF	VIM-19	–	ND	ND	ND	–

PBRT, PCR-based replicon typing; ND, not determined.

susceptible to meropenem. All isolates were intermediate susceptible or resistant to ciprofloxacin and at least one aminoglycoside. Two isolates were resistant to tigecycline and one was intermediate susceptible. Colistin was the only antibiotic that retained susceptibility in all the isolates. Several of the transformants and transconjugants in this study were fully susceptible to both meropenem and ertapenem (Table 2), which have recently been suggested as the two most sensitive antibiotics for detecting carbapenemases in *K. pneumoniae* [23]. Low carbapenem MIC values for VIM-producing clinical *E. coli* isolates have also been documented [24], although such isolates have so far been described very infrequently and in low numbers compared with VPKP [25]. According to these data, one explanation could be that the MIC levels in MBL-positive *E. coli* are very low for the carbapenems, making it very difficult to identify these carbapenemase producers unless epidemiological cut-off (ECOFF) values from EUCAST are used [23].

Resistance determinants

bla_{VIM-1} was identified in six isolates, bla_{VIM-19} and a novel variant, bla_{VIM-26}, were detected in one isolate each (Table 3). The novel variant, VIM-26 (isolate U-60687) harbours one amino acid mutation at position 224 (His224Leu, BBL numbering scheme), compared with VIM-1. Residue 224 is located close to the active site of VIM enzymes and has been shown to play a role in inhibitor interactions [26] and suggested to affect the affinity of substrates [27]. Leu224 has previously been found in VIM-5 [28] and VIM-13 [29]. Both VIM-5 and VIM-13 show reduced catalytic efficiency towards cephalosporins with a bulky positively charged substituent (e.g. ceftazidime and cefepime) [28,29]. However, mutagenesis studies on VIM-13 did not reveal a significant effect of Leu224 with respect to the susceptibility to ceftazidime and cefepime [29]. Further, the VIM-26 transformant (U-60687-TF, Tables 2 and 3) showed an elevated MIC of aztreonam although it was negative for CTX-M, SHV and TEM ESBLs. However, the presence of Leu224 in VIM-5 and VIM-13 did not seem to have an effect on aztreonam [28,29]. Thus, the elevated MIC is likely to be due to the presence of other ESBLs. However, the significance of the His224Leu mutation in VIM-26 needs to be determined by kinetic experiments. bla_{VIM-19}, identified in isolate B-22365, has previously only been identified in Algeria [30], France (patient transferred from Algeria to France) [31] and Greece [32]. Characterization of VIM-19 has suggested increased carbapenemase activity compared with VIM-1 [31]. However, comparison of B-22365 and the transformant with the other VPKP isolates in this study did not reveal any increased MIC towards the carbapenems.

Screening for other resistance genes in the isolates revealed the presence of genes encoding extended-spectrum *β*-lactamases (ESBLs) in five isolates ($bla_{CTX-M-3}$, $bla_{CTX-M-group\ 1}$, bla_{SHV-5} and bla_{SHV-12}, Table 3). Bla_{SHV-1} (n = 2), bla_{SHV-11} (n = 3) and bla_{TEM-1} (n = 3) were also detected. The 16S rRNA methylase gene *armA* was identified in one isolate that showed high-level aminoglycoside resistance and the plasmid-mediated quinolone resistance determinant *qnrS* was detected in two isolates. All isolates were negative for bla_{KPC}.

Genetic context of bla_{VIM} and plasmid analysis

The bla_{VIM} gene was successfully transferred from all isolates either by conjugation or transformation into recipient *E. coli*, suggesting plasmid localization (Table 3). S1 nuclease PFGE and in-gel hybridization of seven isolates (data not shown) showed that bla_{VIM} was located on plasmids ranging from ~40 to ~240 kb (Table 3). Co-transfer of bla_{VIM} with other resistance determinants such as $bla_{CTX-M-3}$, $bla_{CTX-M-gr.1}$,

bla$_{SHV-12}$, *bla*$_{TEM-1}$, *qnrS* and *armA* was observed. The co-transfer of *bla*$_{VIM-1}$, *bla*$_{CTX-M-3}$ and *armA* as observed in isolate ÖN-2211, resulting in resistance to all β-lactams and aminoglycosides, is worrying as dissemination of such multidrug resistance plasmids will have serious consequences for treatment options. Linkage of *bla*$_{VIM}$ to Tn*402* has been previously reported in Scandinavia for *Pseudomonas aeruginosa* [12]; however, none of the *K. pneumoniae* integrons possessed the *tni*A-C gene locus to the right-hand side of the integron. Tn*21*-like transposons have been linked with MBLs previously [33], and in the case of these isolates all demonstrated Tn*21* to the left-hand side of the class 1 integron, apart from isolates K45-67 and K46-62. These data suggest that whilst the transposons are carried on different plasmids that vary in size and type, the genetic structure of Tn*21* linked to *bla*$_{VIM}$ is largely conserved. Interestingly, although we believe Tn*21* to be functional in each of these cases, chromosomal location of *bla*$_{VIM}$ could not be demonstrated; however, the fact that this structure has appeared on different plasmid scaffolds suggests recent mobility.

Two different integron structures containing *bla*$_{VIM}$ were identified in four isolates. In isolates U-60687 and HY-1491/7, *bla*$_{VIM-26}$ and *bla*$_{VIM-1}$, respectively were located in an integron similar to In-e541 [34], where *bla*$_{VIM}$ is located as the first gene cassette followed by *aacA7*, *dhfrI*, *aadA1*, and the 3′conserved segment (3′CS) [*intI1*;*bla*$_{VIM-1/-26}$;*aacA7*;*dhfrI*;*aadA1*;3′CS]. However, the *aadA1* gene was truncated in HY-1491/7, as observed in VIM-1-producing *K. pneumoniae* identified in France [6]. In-e541 has previously been identified in different species, including *Escherichia coli* [34], *K. pneumoniae* [35] and *Acinetobacter baumannii*. PCR-based replicon typing (PBRT) [18] suggested that In-e541 was located on an A/C plasmid in isolate U-60687 and on an unknown replicon type in isolate HY-1491/7. This is in contrast to previous reports where In-e541 has been associated with IncN plasmids [35].

In isolates K45-67 and K46-62, *bla*$_{VIM-1}$ along with *aac(6′)-Ib* was located in an integron lacking the 3′CS but instead having an IS*26* element in the 3′end (*intI1*;*bla*$_{VIM-1}$;*aac(6′)-Ib*;IS*26*). An identical structure has been identified in the IncN plasmid pKOX105, shown to harbour *bla*$_{VIM-1}$, *qnrS1* and *bla*$_{SHV-12}$ [36]. Interestingly, K45-67, K46-62 and the respective transconjugants were positive for IncN, *qnrS* and *bla*$_{SHV-12}$, suggesting a similar plasmid. In the other isolates PCR amplification of the genetic context of *bla*$_{VIM}$ was unsuccessful despite repeated attempts and the use of different primer combinations.

In conclusion, the study shows that the emergence of VPKP in Scandinavia is associated with importation of international clones harbouring multiple resistance determinants on plasmids, including plasmids with a broad-host range, supporting further spread.

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Transparency Declaration

No conflicts of interest to declare.

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