

Epidemiology and molecular characterisation of metallo- β -lactamase-producing Enterobacteriaceae in a university hospital Intensive Care Unit in Greece

Evangelos Koratzanis, Maria Souli, Irene Galani, Zoi Chryssouli, Apostolos Armaganidis, Helen Giamarellou

▶ To cite this version:

Evangelos Koratzanis, Maria Souli, Irene Galani, Zoi Chryssouli, Apostolos Armaganidis, et al.. Epidemiology and molecular characterisation of metallo- β -lactamase-producing Enterobacteriaceae in a university hospital Intensive Care Unit in Greece. International Journal of Antimicrobial Agents, 2011, 38 (5), pp.390. 10.1016/j.ijantimicag.2011.06.014 . hal-00736740

HAL Id: hal-00736740 https://hal.science/hal-00736740

Submitted on 29 Sep 2012

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: Epidemiology and molecular characterisation of metallo- β -lactamase-producing Enterobacteriaceae in a university hospital Intensive Care Unit in Greece

Authors: Evangelos Koratzanis, Maria Souli, Irene Galani, Zoi Chryssouli, Apostolos Armaganidis, Helen Giamarellou



PII: DOI: Reference:	S0924-8579(11)00291-3 doi:10.1016/j.ijantimicag.2011.06.014 ANTAGE 3657										
To appear in:	International	Journal	of	Antimicrobial	Agents						
Received date: Revised date: Accepted date:	12-4-2011 1-6-2011 20-6-2011										

Please cite this article as: Koratzanis E, Souli M, Galani I, Chryssouli Z, Armaganidis A, Giamarellou H, Epidemiology and molecular characterisation of metallo- β -lactamase-producing Enterobacteriaceae in a university hospital Intensive Care Unit in Greece, *International Journal of Antimicrobial Agents* (2010), doi:10.1016/j.ijantimicag.2011.06.014

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Epidemiology and molecular characterisation of metallo-βlactamase-producing Enterobacteriaceae in a university hospital Intensive Care Unit in Greece

Evangelos Koratzanis ^{a,1}, Maria Souli ^{a,1,*}, Irene Galani ^a, Zoi Chryssouli ^a, Apostolos Armaganidis ^b, Helen Giamarellou ^c

 ^a 4th Department of Internal Medicine, Athens University School of Medicine, University General Hospital ATTIKON, 1 Rimini Str., 124 62 Chaidari, Greece
^b 2nd Department of Critical Care Medicine, Athens University School of Medicine, University General Hospital ATTIKON, 1 Rimini Str., 124 62 Chaidari, Greece
^c 6th Department of Internal Medicine, Diagnostic and Therapeutic Center of Athens 'Hygeia', 4 Erythrou Stavrou Str. and Kifissias Av., Maroussi, Greece

ARTICLE INFO

Article history:

Received 12 April 2011

Accepted 20 June 2011

Keywords:

VIM

Klebsiella pneumoniae

Carbapenems

Integron

* Corresponding author. Tel.: +30 210 583 1643; fax: +30 210 532 6446.

E-mail address: msouli@med.uoa.gr (M. Souli).

¹ These two authors contributed equally to this work.

کر

2

ABSTRACT

The molecular epidemiology of VIM-producing Enterobacteriaceae isolated at the beginning of an epidemic in the Intensive Care Unit (ICU) of a university hospital in Athens, Greece, was studied. All Gram-negative organisms isolated from March 2004 to November 2005 positive for metallo- β -lactamase (MBL) production were submitted to polymerase chain reaction (PCR) and sequencing, to repetitive sequence-based PCR (Rep-PCR) for molecular typing, and to S1 nuclease digestion for plasmid DNA characterisation. Conjugation experiments and isoelectric focusing were performed to identify co-existing β-lactamases. Among 23 patients, 12 suffered one or more clinical infections. Eighty-two isolates representing one isolate per clone, source and ICU patient were studied, including Klebsiella pneumoniae (77), Enterobacter cloacae (2), Citrobacter freundii (1) and Pseudomonas aeruginosa (2). High clonal diversity was detected among the K. pneumoniae, with 10 distinct clones identified. Conjugation was successful in 54.5% of K. pneumoniae, and five different sized plasmids were detected. All K. pneumoniae and both E. cloacae isolates shared the same *bla*_{VIM-1}-containing class 1 integron structure also carrying *aacA7*, dhfrl and aadA1 gene cassettes. The C. freundii isolate carried a different integron that included *bla*_{VIM-1} and *aac*(6')-*Ilc*. Both *P. aeruginosa* isolates were positive for bla_{VIM-2}. It was not possible to identify specific clones with the potential to cause clinical infections. In conclusion, a multiclonal cluster of MBL-producers was responsible for the first cases of colonisation and/or infection in the ICU. A single integron structure, common in Greek hospitals, efficiently disseminated between clones and species, suggesting that the epidemic was mainly the result of successful horizontal transfer of mobile genetic material rather than the result of horizontal transfer of one or a few clones.

3

1. Introduction

During the past decade, the acquired metallo- β -lactamases (MBLs) have emerged as a threatening new mechanism of broad-spectrum β -lactam resistance, first in *Pseudomonas aeruginosa* and later in other Gram-negative nosocomial pathogens. These enzymes efficiently hydrolyse all β -lactams (except aztreonam) and are not susceptible to β -lactamase inhibitors. Before the emergence of the NDM-1 enzyme, the most common and widespread acquired MBLs were those of the IMP and VIM types, which show a worldwide distribution and for which several allelic variants are known (http://www.lahey.org/Studies/). Since the first description of a VIM enzyme isolated in 1997 [1], VIM-producing *P. aeruginosa* strains have become endemic in many tertiary care hospitals in Greece. Over the past years, studies have reported the dissemination of VIM-type MBLs in Enterobacteriaceae in Greece and worldwide, suggesting the ongoing spread of these resistance determinants [2,3].

The respective genes are carried on mobile elements (known as gene cassettes) into class 1 integrons. These integrons are predominantly isolated from antibiotic-resistant Gram-negative organisms and usually contain various gene cassettes inserted between two conserved regions. The 5'-conserved segment (5'-CS) contains: an integrase gene, *intl*, which encodes a site-specific recombinase; an adjacent site, *attl*, which is recognised by the integrase and acts as a receptor site for gene cassettes; and a common promoter region, Pant (P1) and/or P2, from which integrated gene cassettes are co-transcribed [4]. The 3'-conserved segment (3'-CS), located downstream of the integrated gene cassettes, typically contains three genes: *gacE1*, which confers resistance to quaternary ammonium compounds; *sul1*, which

confers resistance to sulfonamides; and an open-reading frame of unknown function [4]. Horizontal transfer of integrons through plasmids and transposons has been found to play an important role in the dissemination of antimicrobial resistance genes [5].

In this report, the epidemiology and molecular characteristics of *bla*_{VIM}-carrying Enterobacteriaceae isolated from patients admitted to the Intensive Care Unit (ICU) of a tertiary care university hospital in Athens, Greece, at the beginning of an epidemic were studied.

2. Material and methods

2.1. Setting and patients

This study was conducted at University General Hospital 'Attikon' (Athens, Greece) from March 2004 to November 2005. The ICU of the hospital first operated in November 2003. During the study period, there were 320 beds in use in Attikon Hospital; 6 of these beds belonged to the general (surgical and medical) ICU. All ICU patients were routinely screened biweekly for colonisation with antibiotic-resistant bacteria by surveillance cultures from the rectum, bronchial secretions, urine and fluids from draining sites, as previously described [6]. Specimens were cultured on selective agar plates containing ceftazidime (8 μ g/mL), ciprofloxacin (1 μ g/mL), imipenem (1 μ g/mL) or piperacillin/tazobactam (TZP) (16/4 μ g/mL). Patients who were colonised and/or infected with MBL-producing Enterobacteriaceae were included in this study and their medical records were reviewed for clinical and epidemiological data. For the diagnosis of specific types of infection or colonisation,

the criteria proposed by Horan et al. [7] were used. Bacteria isolated from rectal swabs were characterised as colonisers in all cases.

2.2. Bacterial strains

All MBL-producing Gram-negative bacteria from all available sources characterised either as colonisers or as causing clinical infection from patients hospitalised in the ICU were further studied, but only one isolate per clone, source and patient was included in the final analysis.

2.3. Identification and antimicrobial susceptibility

Species identification of isolated bacteria was performed by an automated identification system (API ID32GN and ID32E; bioMérieux, Marcy-L'Étoile, France). Antibiotic susceptibility testing was performed by a disk diffusion method on Mueller– Hinton II agar (Becton Dickinson Microbiology Systems, Cockeysville, MD) and the strains were further examined by the double-disk synergy test for the presence of extended-spectrum β -lactamases according to Clinical and Laboratory Standards Institute (CLSI) guidelines [8]. The ethylene diamine tetra-acetic acid (EDTA)– imipenem disk synergy test was performed as a screening test for MBL production [9]. Minimum inhibitory concentrations (MICs) of imipenem, meropenem, doripenem, tigecycline and colistin were determined by Etest (AB BIODISK, Solna, Sweden) following the manufacturer's instructions, and those of ceftazidime, aztreonam, cefepime, TZP, ciprofloxacin, amikacin and gentamicin were determined by the microdilution method [10]. Interpretation of results was done in accordance with the CLSI 2009 proposed breakpoints [8], with the exception of colistin and tigecycline for

which breakpoints are not available from the CLSI and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints were applied (http://www.eucast.org).

2.4. Molecular typing

Molecular typing was performed by repetitive sequence-based polymerase chain reaction (Rep-PCR) using the primers REP-1 (5'-III, GCG CCG ICA TCA GGC-3') and REP-2 (5'-ACG TCT TAT CAG GCC TAC-3'). Patterns were considered different if they differed by more than one amplification band, regardless of the band intensity [11].

2.5. Detection and identification of the blavim gene

Whole-cell DNA extracts were prepared by boiling the cells for 5 min. The primers VIM-B (5'-ATG GTG TTT GGT CGC ATA TC-3') and VIM-F (5'-TGG GCC ATT CAG CCA GA TC-3') were used to amplify a 510-bp (nucleotides 152–661) internal fragment of the *bla*_{VIM} gene (GenBank accession no. AF191564) [12]. The amplified DNA was purified using a NucleoSpin Extract Kit (Macherey-Nagel GmbH, Düren, Germany), was sequenced on both DNA strands by Eurofins MWG (Ebersberg, Germany) and was aligned with the gene sequences available from GenBank (http://www.ncbi.nlm.nih.gov/genbank).

2.6. Detection and characterisation of blavim-carrying integrons

Template DNA was isolated using a NucleoSpin Tissue kit (Macherey-Nagel GmbH). The presence of class 1 integrons was investigated using PCR amplification with

primers for 5'-CS and 3'- CS. These primers amplify the region between the 5'-CS and 3'-CS segments, generating products of variable sizes depending on the number and length of the inserted gene cassettes. Mapping of class 1 integrons was performed by PCR with primers INT-F or 5'CS and 3'CS in combination with primers VIM-R and VIM-F, designed on the basis of the conserved regions of *bla*_{VIM} genes [13]. The amplified DNA was purified, cloned to pCR2.1 vector (TA Cloning Kit; Invitrogen, Carlsbad, CA) and sequenced as mentioned above.

2.7. Transfer of resistance and plasmid content

Conjugation experiments were performed with at least one strain of clonal types A, B, C, E, F, G and H. Rifampicin-resistant Escherichia coli K12 RC85 R- was used as the recipient strain. Liquid mating was performed in brain-heart infusion broth and was incubated overnight at 37 °C [14]. Transconjugants were selected on MacConkey agar plates containing both rifampicin (128 µg/mL) and ceftazidime (16 μ g/mL) or with each antibiotic alone for counterselection. Transfer frequencies were expressed as the ratio of the number of transconjugants to the number of recipients. Donor strains and transconjugants were tested for their susceptibility pattern as described above. Five transconjugants with a MBL phenotype per each conjugation experiment were further analysed. The presence of *blavim* and the detection and characterisation of integrons in the transconjugants was performed as described above. Plasmid size was determined in *E. coli* transconjugants (or wild-type strains, in the absence of transfer) by the technique described by Barton et al. [15]. Plasmids of transconjugants and of parent isolates with a single plasmid present were purified using a commercial kit (QIAGEN Inc., Valencia, CA). Purified plasmid preparations were then digested with *Pst*, *Sac* and *Bgl* (New England Biolabs GmbH, Frankfurt

8

am Main, Germany) according to the manufacturer's instructions and the generated fragments were separated in 0.7% agarose gels for restriction fragment length polymorphism (RFLP) analysis.

2.8. S1 nuclease digestion

To detect the existence of large plasmids in *K. pneumoniae* strains, S1 nucleasedigested (Promega, Southampton, UK) genomic DNA was examined by pulsed-field gel electrophoresis (PFGE), run in a Gene Navigator System (GE Healthcare Bio-Sciences, Uppsala, Sweden) at 15 °C, with a pulse time of 5–25 s for 6 h (block 1) and 30–45 s for 18 h (block 2), at 6 V/cm on a 120° angle in 0.5× Tris–borate–EDTA (TBE) buffer [15]. Each band was considered a unit length linear plasmid and the size was estimated in comparison with the Lambda and the Yeast Chromosome PFG markers (New England Biolabs, Boston, MA).

2.9. Isoelectric focusing (IEF) of β -lactamases

Crude β-lactamase extracts of parent isolates and their *E. coli* transconjugants were prepared by ultrasonic treatment of bacterial cells. IEF was performed on PhastGelTM polyacrylamide gel (pH 3–9) (Amersham Biosciences, Uppsala, Sweden) using a PhastSystem instrument (Pharmacia Biotech, Uppsala, Sweden) according to the method of Mathew et al. [16]. β-Lactamase activity was visualised by staining the gel with nitrocefin (Oxoid Ltd., Basingstoke, UK). The isoelectric points (pl values) were determined by comparison with the known β-lactamases TEM-1 (pl 5.4), TEM-28 (pl 6.1), SHV-1 (pl 7.6), SHV-5 (pl 8.2) and CTX-M-15 (pl 8.9).

3. Results

A total of 240 patients were admitted to the ICU of 'Attikon' Hospital from March 2004 to November 2005, with a mean duration of hospitalisation of 13.5 days. Surveillance and/or routine cultures detected 23 patients who were either only colonised (n = 11) or were infected (n = 12) by MBL-producing Enterobacteriaceae. During the study period, the overall prevalence rate of MBL-producers in the ICU was 9.6 single patient isolates per 100 admissions. In total, 82 Gram-negative isolates identified as MBL-producers were studied, including *Klebsiella pneumoniae* (n = 77), *Enterobacter cloacae* (n = 2), *P. aeruginosa* (n = 2) and *Citrobacter freundii* (n = 1). This number represented a mean of 3.6 organisms per patient (range 1–7 organisms per patient). Ten different clones, designated A to J, were detected by Rep-PCR (Table 1) among the *K. pneumoniae* isolates. Three clones (A, C and E) with 14 (18.2%), 22 (28.6%) and 13 (16.9%) representatives, respectively, predominated. Among *E. cloacae* and *P. aeruginosa* isolates, two genetically distinct clones were identified.

Antimicrobial susceptibilities of the Enterobacteriaceae (n = 80) included in the study are presented in Table 1. The carbapenem MICs ranged from 0.5 µg/mL to >32 µg/mL for imipenem and from ≤ 0.125 µg/mL to >32 µg/mL for meropenem and doripenem. All strains were resistant to most broad-spectrum cephalosporins, inhibitor-protected penicillins and ciprofloxacin. Aztreonam susceptibility was seen in 15.8 % of isolates. Gentamicin was the most active agent followed by colistin, with 89.6% and 66.2% of isolates being susceptible, respectively. Tigecycline showed activity against 62.3% of the isolates (MICs for 50% and 90% of the isolates of 1 µg/mL and 3 µg/mL, respectively). The two *P. aeruginosa* isolates were highly

resistant to carbapenems (MICs > $32 \mu g/mL$) and were resistant to all studied antimicrobials except colistin (one isolate was susceptible) (data not shown). According to the new CLSI breakpoints for Enterobacteriaceae, available since 2010, susceptibility to doripenem, imipenem and meropenem was 6.6%, 10.4% and 3.9%, respectively, whilst susceptibility to aztreonam remained unchanged (15.8%) [17].

All MBL-producing isolates were positive by PCR for the presence of a blaving gene. Enterobacteriaceae harboured blavIM-1, whilst P. aeruginosa isolates harboured bla_{VIM-2}. The clinical, epidemiological and molecular characteristics of these bacteria are presented in Table 2. PCR mapping indicated that *blavim-1* was part of a class 1 integron in all cases. DNA sequencing showed a gene cassette array that included (from 5' to 3') *bla*_{VIM-1}, *aacA7*, *dhfrl* and *aadA1* in all *K*. *pneumoniae* isolates. Conjugation experiments with at least one isolate from each main clonal type showed that *bla*_{VIM-1} was freely transferred from 6 (54.5%) of 11 K. pneumoniae isolates to E. coli recipient strain. Four of those six isolates also harboured blaSHV-5like, which was co-transferred to the recipient strain in only one case (Table 2). With the exception of the isolate belonging to clone J, all K. pneumoniae strains contained at least one, and up to four, plasmids with sizes ranging from 20 kb to 220 kb as shown by the S1 nuclease-digested genomic DNA. Restriction endonuclease digestion of plasmid DNA isolated from transconjugants and from parent isolates with single plasmid content exhibited the presence of five different *bla*_{VIM-1}-containing plasmids, with sizes of 200, 70, 65, 55 and 50 kb, the first of which harboured also a *bla*_{SHV-5}-like gene.

Both *E. cloacae* included in the study harboured a similar size plasmid of 90 kb and a different sized plasmid of 280 kb or 200 kb. The *bla*_{VIM-1}-containing integron was similar to those described in *K. pneumoniae* strains (Table 2).

The *C. freundii* isolate harboured a plasmid of 40 kb and an integron that included bla_{VIM-1} and aac(6')-*llc* and was identical to In87 (GenBank accession no. AY648125) previously reported from our group in an *E. cloacae* clinical isolate [19]. There was no apparent epidemiological link between these two patients since the first was transferred from a regional hospital with *E. cloacae* bacteraemia and was hospitalised in the medical ward of Attikon Hospital in 2003 and the second (patient No. 17 of this cohort) was hospitalised in 2004 in the ICU.

Among the 23 affected patients, 12 suffered one or more clinical infections by MBLproducing Enterobacteriaceae during the study period. A total of nine episodes of bloodstream infection (BSI), four ventilator-associated pneumonias and three soft tissue infections were detected in this cohort. Colonisation in one or more sites was detected in all of the patients with or without clinical infection. The gastrointestinal tract was the most common site of colonisation (21/23; 91.3%) followed by the respiratory tract (9/23; 39.1%). In two patients (Nos. 8 and 15), a bla_{VIM-2} -positive *P*. *aeruginosa* was identified in bronchial secretions and in urine, respectively (data not shown). Apparently, there was no evidence of horizontal transfer of the bla_{VIM} gene from MBL-producing non-fermenters to Enterobacteriaceae, as they did not share the same allelic variant of the gene.

4. Discussion

The ICU of 'Attikon' University Hospital started operating in late 2003. In 2002, the first outbreak of VIM-1-producing K. pneumoniae, involving the ICUs of three tertiary care hospitals in Athens, was detected [20]. The blavIM-1 resistance determinant was first introduced in the ICU of 'Attikon' University Hospital with the transfer of colonised patients from other hospitals. Soon it became apparent that it was mainly responsible for carbapenem resistance in Enterobacteriaceae in this institution. The data available in the National Surveillance System for Antibiotic Resistance database (http://www.mednet.gr/whonet/top.htm) suggest that this was true on a nationwide scale, as the emergence of carbapenem resistance in K. pneumoniae in Greece (between 2002 and 2003) coincided with the dissemination of blavIM-1 to most major Greek hospitals [3]. The present study was conducted to answer questions regarding the epidemiology of *bla*_{VIM-1}-positive Enterobacteriaceae. Was the outbreak in our institution clonal or was it due to horizontal gene transfer between various clones? Could it be due to the dissemination of a conjugative plasmid between various species? Was it possible that MBL-producing non-fermenters, which preceded the emergence of MBL-producing Enterobacteriaceae, were the reservoir of the gene? What was the role of colonisation by MBL-producers for the development of clinical infection? Is there a difference between various clones in their potential for causing clinical infection?

A complex multiclonal situation was detected in VIM-producing Enterobacteriaceae from patients hospitalised in the ICU of our institution. In total, 77 *K. pneumoniae* isolates represented ten different genetic clones, with 49 (63.6%) belonging to three major clonal types. A single patient could harbour more than one, and up to four,

13

unrelated clonal types, whilst different patients shared some common clones of K. pneumoniae even if there was no apparent time link between them (i.e. patients No. 1, 2 and 4 sharing clonal type E), suggesting the possibility of an environmental reservoir or a common source outside the ICU (another ward or hospital). Most identified clonal types were responsible for both colonisation and infection (i.e. clones E and J in patient No. 1 or clone C in patient No. 14 were faecal colonisers and the cause of BSIs). The opposite was also true; some patients were colonised by clonal types different than those causing clinical infections (i.e. patient No. 13 was colonised by clones H and I but the BSI was caused by clone F). Finally, some patients were colonised by more than one clone or more than one species in the gastrointestinal tract but never suffered a clinical infection (i.e. patient No. 17). We could not identify specific clones with the potential to cause clinical infections, neither could we identify an association between any one site of infection (i.e. bacteraemia) with a particular clone. Furthermore, the exact role of previous colonisation for the development of clinical infection was not elucidated by the present study and remains an 'open' question for future research.

The multiclonal nature of the cluster of MBL-producers in our institution is in contrast to earlier studies describing either clonal outbreaks in single hospitals [18,21,22] or the occurrence of a limited number of strains [20]. This study is representative of the situation depicted in recent publications as a result of the interhospital and intrahospital dissemination of several distinct clones [23]. Other European studies described mostly clonal outbreaks [24] although in some institutions a more complex epidemiology was observed [25].

Interestingly, unrelated clones of K. pneumoniae as well as both clones of E. cloacae shared the same integron structure that has been previously described in distinct clones of K. pneumoniae (GenBank accession no. AY340637) [20,23] as well as in E. coli (GenBank accession no. AY339625) [13], Providencia stuartii [26], Proteus mirabilis [21], Serratia liquefaciens, Klebsiella oxytoca [27] and Acinetobacter baumannii (GenBank accession no. DQ112355) [28] isolated in different hospitals in Greece. This integron (previously designated as In-e541) has mainly been found in a variety of IncN plasmids and rarely was chromosomally located [18]. Insertion sequence IS26 has been proposed to be responsible for its mobilisation [29]. More recently, similar integron structures containing bla_{VIM-4} or bla_{VIM-19} as the first gene cassette have been identified in Enterobacteriaceae in Greece [30,31]. It is evident that there is an extremely efficient interspecies transfer of integron-carrying elements that plays a dominant role in the development of multidrug resistance in Enterobacteriaceae, independent of species or origin [32]. This is also confirmed by the recent publication of an outbreak strain of K. pneumoniae in a hospital in Genoa, Italy, that harboured the integron carrying *bla*_{VIM-1} and *aac*(6')-*Ilc* [24], which was first identified in E. cloacae from our group [19] and in C. freundii in the present study. The authors from Italy highlighted the possibility of a common origin of those integrons, although epidemiological links between the source patient and Greece or Greek nationals could not be ascertained.

In the present study, we described the multiclonal nature of the VIM outbreak in 'Attikon' University Hospital and the dissemination of a single integron structure between various clones and species of enterobacteria. Among ten distinct clones of *K. pneumoniae*, we could not identify any one that exhibited a specific association

with clinical infections. These observations contribute to the knowledge of the epidemiology of VIM-producing Enterobacteriaceae that have now become endemic in major hospitals in Greece.

Funding

This study was supported by a grant from the National and Kapodistrian University of Athens (grant no. 70/4/9149).

Competing interests

None declared.

Ethical approval

Not required.

References

- [1] Lauretti L, Riccio ML, Mazzariol A, Cornaglia G, Amicosante G, Fontana R, et al. Cloning and characterization of *bla*_{VIM}, a new integron-borne metallo-β-lactamase gene from a *Pseudomonas aeruginosa* clinical isolate. Antimicrob Agents Chemother 1999;43:1584–90.
- [2] Walsh TR. Clinically significant carbapenemases: an update. Curr Opin Infect Dis 2008;21:367–71.
- [3] Vatopoulos A. High rates of metallo-β-lactamase-producing *Klebsiella pneumoniae* in Greece—a review of the current evidence. Euro Surveill 2008;13:pii:8023.
- [4] Hall RM, Collis CM. Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. Mol Microbiol 1995;15:593–600.
- [5] Recchia GD, Hall RM. Gene cassettes—a new class of mobile element. Microbiology 1995;141:3015–27.
- [6] Souli M, Kontopidou FV, Papadomichelakis E, Galani I, Armaganidis A, Giamarellou H. Clinical experience of serious infections caused by Enterobacteriaceae producing VIM-1 metallo-β-lactamase in a Greek University Hospital. Clin Infect Dis 2008;46:847–54.
- [7] Horan TC, Andrus M, Dudeck MA. CDC/NHSN surveillance definition of health care-associated infection and criteria for specific types of infections in the acute care setting. Am J Infect Control 2008;36:309–32.
- [8] Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; nineteenth informational supplement.
 Document M100-S19. Wayne, PA: CLSI; 2009.

- [9] Lee K, Chong Y, Shin HB, Kim YA, Yong D, Yum JH. Modified Hodge and EDTAdisk synergy tests to screen metallo-β-lactamase-producing strains of *Pseudomonas* and *Acinetobacter* species. Clin Microbiol Infect 2001;7:88–91.
- [10] Clinical and Laboratory Standards Institute. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard. 8th ed. Document M07-A8. Wayne, PA: CLSI; 2009.
- [11] Versalovic J, Koeuth T, Lupski JR. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res 1991;19:6823–31.
- [12] Poirel L, Naas T, Nicolas D, Collet L, Bellais S, Cavallo JD, et al. Characterization of VIM-2, a carbapenem hydrolyzing metallo-β-lactamase and its plasmid- and integron-borne gene from a *Pseudomonas aeruginosa* clinical isolate in France. Antimicrob Agents Chemother 2000;44:891–7.
- [13] Miriagou V, Tzelepi E, Gianneli D, Tzouvelekis LS. *Escherichia coli* with a self-transferable, multiresistant plasmid coding for the metallo-β-lactamase VIM 1. Antimicrob Agents Chemother 2003;47:395–7.
- [14] Rice LB, Bonomo RA. Genetic and biochemical mechanisms of bacterial resistance to antimicrobial agents. In: Lorian V, editor. *Antibiotics in laboratory medicine*. 4th ed. Baltimore, MD: Williams & Wilkins; 1996. p. 453–501.
- [15] Barton BM, Harding GP, Zuccarelli AJ. A general method for detecting and sizing large plasmids. Anal Biochem 1995;226:235–40.
- [16] Mathew M, Harris AM, Marshall MJ, Ross GW. The use of analytical isoelectric focusing for the detection and identification of β-lactamases. J Gen Microbiol 1975;88:169–78.

- [17] Clinical and Laboratory Standards Institute. *Performance standards for* antimicrobial susceptibility testing; twenty-first informational supplement.
 Document M100-S21. Wayne, PA: CLSI; 2011.
- [18] Vourli S, Tsorlini H, Katsifa H, Polemis M, Tzouvelekis LS, Kontodimou A, et al. Emergence of *Proteus mirabilis* carrying the *bla* metallo-β-lactamase gene. Clin Microbiol Infect 2006;12:691–4.
- [19] Giakkoupi P, Xanthaki A, Kanelopoulou M, Vlahaki A, Miriagou V, Kontou S, et al. VIM-1 metallo-β-lactamase-producing *Klebsiella pneumoniae* strains in Greek hospitals. J Clin Microbiol 2003;41:3893–6.
- [20] Scoulica EV, Neonakis IK, Gikas AI, Tselentis YJ. Spread of *bla*_{VIM-1}producing *E. coli* in a university hospital in Greece. Genetic analysis of the integron carrying the *bla*_{VIM-1} metallo-β-lactamase gene. Diagn Microbiol Infect Dis 2004;48:167–72.
- [21] Galani I, Souli M, Chryssouli Z, Orlandou K, Giamarellou H. Characterization of a new integron containing *bla*_{VIM-1} and *aac(6')-IIc* in an *Enterobacter cloacae* clinical isolate from Greece. J Antimicrob Chemother 2005;55:634–8.
- [22] Ikonomidis A, Tokatlidou D, Kristo I, Sofianou D, Tsakris A, Mantzana P, et al. Outbreaks in distinct regions due to a single *Klebsiella pneumoniae* clone carrying a *bla*_{VIM-1} metallo-β-lactamase gene. J Clin Microbiol 2005;43:5344–7.
- [23] Psichogiou M, Tassios PT, Avlamis A, Stefanou I, Kosmidis C, Platsouka E, et al. Ongoing epidemic of *bla*_{VIM-1}-positive *Klebsiella pneumoniae* in Athens, Greece: a prospective survey. J Antimicrob Chemother 2008;61:59–63.
- [24] Cagnacci S, Gualco L, Roveta S, Mannelli S, Borgianni L, Docquier JD, et al.Bloodstream infections caused by multidrug-resistant *Klebsiella pneumoniae*

producing the carbapenem-hydrolysing VIM-1 metallo- β -lactamase: first Italian outbreak. J Antimicrob Chemother 2008;61:296–300.

- [25] Tato M, Coque TM, Ruíz-Garbajosa P, Pintado V, Cobo J, Sader HS, et al. Complex clonal and plasmid epidemiology in the first outbreak of Enterobacteriaceae infection involving VIM-1 metallo-β-lactamase in Spain: toward endemicity? Clin Infect Dis 2007;45:1171–8.
- [26] Miriagou V, Tzouvelekis LS, Flevari K, Tsakiri M, Douzinas EE. Providencia stuartii with VIM-1 metallo-β-lactamase. J Antimicrob Chemother 2007;60:183–4.
- [27] Miriagou V, Douzinas EE, Papagiannitsis CC, Piperaki E, Legakis NJ, Tzouvelekis LS. Emergence of Serratia liquefaciens and Klebsiella oxytoca with metallo-β-lactamase-encoding IncW plasmids: further spread of the bla_{VIM-1}carrying integron In-e541. Int J Antimicrob Agents 2008;32:540–1.
- [28] Tsakris A, Ikonomidis A, Pournaras S, Tzouvelekis LS, Sofianou D, Legakis NJ, et al. VIM-1 metallo-β-lactamase in *Acinetobacter baumannii*. Emerg Infect Dis 2006;12:981–3.
- [29] Miriagou V, Carattoli A, Tzelepi E, Villa L, Tzouvelekis LS. IS26-associated In4-type integrons forming multiresistance loci in enterobacterial plasmids. Antimicrob Agents Chemother 2005;49:3541–3.
- [30] Pournaras S, Poulou A, Voulgari E, Vrioni G, Kristo I, Tsakris A. Detection of the new metallo-β-lactamase VIM-19 along with KPC-2, CMY-2 and CTX-M-15 in *Klebsiella pneumoniae*. J Antimicrob Chemother 2010;65:1604–7.
- [31] Ikonomidis A, Spanakis N, Poulou A, Pournaras S, Markou F, Tsakris A. Emergence of carbapenem-resistant *Enterobacter cloacae* carrying VIM-4 metallo-β-lactamase and SHV-2a extended-spectrum β-lactamase in a conjugative plasmid. Microb Drug Resist 2007;13:221–6.

[32] Leverstein-van Hall MA, Blok HEM, Donders ART, Paauw A, Fluit AC, Verhoef J. Multidrug resistance among Enterobacteriaceae is strongly associated with the presence of integrons and is independent of species or isolate origin. J Infect Dis 2003;187:251–9.

S

21

Table 1

Susceptibility phenotype of the studied metallo-β-lactamase-producing Gram-negative isolates, grouped according to species and

clonal type, as determined by repetitive sequence-based polymerase chain reaction (Rep-PCR)^a

Species (Rep-	No. of	ESBL	MIC range (μg/mL) (% susceptible)							
PCR clone)	isolates	phenotype	IPM ^b	MER ^b	DOR b,c	ATM	AMK	GEN	TIG ^b	COL ^b
		(%)								
Kpn-A	14	11 (78.6)	1 to >32	2 to >32	4 to >32	≤0.125 to	8–32	0.5 to	0.25–4	1 to >32
			(14.3)	(14.3)		>32	(71.4)	>32	(21.4)	(50.0)
						(21.4)		(92.9)		
Kpn-B	7	6 (85.7)	4 to >32	8 to >32	4 to >32	0.5 to >32	8–32	1–4	0.5–2	0.125 to
			(16.7)	(0)		(16.7)	(83.3)	(100)	(66.7)	>32
										(66.7)
Kpn-C	22	18 (81.8)	1 to >32	≤0.125 to	≤0.125	≤0.125 to	8 to	0.5 to	0.38–4	0.125 to
			(4.8)	>32 (9.5)	to >32	>32	>32	>32	(47.6)	>32
						(18.2)	(52.4)	(85.7)		(66.7)
Kpn-D	1	1	32	>32	>32	>32	16	2	2	8
Kpn-E	13	12 (92.3)	2 to >32	1 to >32	0.125 to	≤0.125 to	16 to	1 to	0.38 to	0.5 to
			(23.1)	(23.1)	>32	>32 (7.7)	>32	>32	>256	>32
							(23.1)	(76.9)	(76.9)	(76.9)

Kpn-F	5	4 (80)	0.5–8	1–32 (60)	≤0.125–	1 to >32	8–16	0.5–2	0.5–6	0.125 to
			(60)		16	(80)	(100)	(100)	(80)	>32
										(60)
Kpn-G	3	3 (100)	2 to >32	2 to >32	4 to >32	>32 (33.3)	16 to	2 to	0.125–1	0.125 to
			(33.3)	(33.3)			>32	>32	(100)	>32
							(33.3)	(33.3)		(33.3)
Kpn-H	9	9 (100)	1 to >32	4 to >32	2 to >32	>32 (0)	8–32	0.5–4	0.25–1	0.125 to
			(33.3)	(22.2)			(88.9)	(100)	(100)	>32
										(77.8)
Kpn-I	1	1	0.5	8	16	>32	16	1	1	2
Kpn-J	2	0	8–16 (0)	2–8 (50)	2–16	≤0.125	4 (100)	1 (100)	0.25–	0.5–4
						(100)			0.38	(50.0)
									(100)	
Ecl-1	1	0	8	>32	8	>32	8	1	3	0.125
Ecl-2	1	0	8	8	1.5	>32	>32	4	0.5	0.125
Cfr	1	0	1	4	1	32	16	1	1	0.25
Total	80		0.5 to	0.125 to	≤0.125	≤0.125 to	4 to	0.5 to	0.125 to	0.125 to
			>32	>32	to >32	>32	>32	>32	>256	>32
			(20.8)	(19.7)		(15.8)	(63.6)	(89.6)	(62.3)	(66.2)
MIC ₅₀ /MIC ₉₀ (μg/mL)	80		16/>32	>32/>32	32/>32	>32/>32	16/32	2/8	1/3	2/>32

ESBL, extended-spectrum β-lactamase; MIC, minimum inhibitory concentration; IPM, imipenem; MER, meropenem; DOR, doripenem; ATM, aztreonam; AMK, amikacin; GEN, gentamicin; TIG, tigecycline; COL, colistin; Kpn, *Klebsiella pneumoniae*; Ecl, *Enterobacter cloacae*; Cfr, *Citrobacter freundii*; MIC_{50/90}, MICs for 50% and 90% of the isolates.

^a MICs of piperacillin/tazobactam, ceftazidime, cefepime and ciprofloxacin were $\geq 64/4$, ≥ 16 and $\geq 2 \mu g/mL$, respectively, against all tested isolates.

^b MIC determined by Etest.

^c Breakpoints not available in Clinical and Laboratory Standards Institute (CLSI) document M100-S19 [8].



Table 2

Clinical significance and epidemiological and molecular characteristics of the metallo-β-lactamase (MBL)-producing

Enterobacteriaceae recovered from 23 patients during the study period

Patient	Date	Species	Clonal	Source	Clinical	Other bla	Plasmid size (kb)	Conjugation	Integron
No.	(admission-		type		significance	genes			gene
	discharge or								cassette
	death)								array (5'→3')
1	17/06/05–	Kpn	E	Blood	CRBSI	SHV-5-	190+170+100	N/D	bla _{∨IM-1} ,
	31/10/05					like, SHV-			aacA7,
				CVC		1-like			dhfrl,
									aadA1
				Bronchial	Carriage				
				secretions					
				Faeces	Carriage				
			н	Faeces	Carriage	SHV-5-	N/D	N/D	N/D
						like, SHV-			
						1-like			
			J	Blood	BSI	SHV-1-like	None	N/D	bla _{∨IM-1} ,
									aacA7,
				Faeces	Carriage		190+90		dhfrl,
					-				aadA1

2	08/04/04—	Kpn	С	Faeces	Carriage	SHV-5-	N/D	N/D	bla _{VIM-1} ,
	13/05/04					like, SHV-			aacA7,
						1-like			dhfrl,
				Pus	SSI	SHV-1-like			aadA1
			Е	Bronchial	Carriage	SHV-5-	N/D	N/D	N/D
				secretion		like, SHV-			
						1-like			
3	19/11/04—	Kpn	С	Faeces	Carriage	SHV-5-	N/D	N/D	N/D
	29/12/04			Drainage	Carriage	like, SHV-			
						1-like			
4	10/11/04—	Kpn	Е	Blood	BSI	SHV-5-	200+180+100+20	N/D	bla _{∨IM-1} ,
	16/11/04			Faeces	Carriage	like, SHV-			aacA7,
						1-like			dhfrl,
									aadA1
5	11/03/04—	Kpn	A	Pus	STI	SHV-5-	200	N/D	N/D
	12/05/04					like, SHV-			
						1-like			
			Е	Faeces	Carriage	SHV-5-	N/D	N/D	N/D
				CVC	Carriage	like, SHV-			
						1-like			

			G	Pus	STI	SHV-5-	55	N/D	bla _{∨IM-1} ,
						like, SHV-			aacA7,
						1-like,			dhfrl,
						TEM-1-			aadA1
						like			
			Н	Bronchial	VAP	SHV-1-like	N/D	N/D	<i>bla</i> _{∨IM-1} ,
				secretions	N.O				aacA7,
									dhfrl,
									aadA1
6	06/04/04—	Kpn	А	Blood	BSI	SHV-1-like	190+40	N/D	<i>bla</i> _{∨IM-1} ,
	10/05/04								aacA7,
									dhfrl,
									aadA1
			Н	Bronchial	VAP	SHV-5-	N/D	N/D	N/D
				secretions		like, SHV-			
						1-like			
7	23/06/04–	Kpn	В	Drainage	Carriage	SHV-5-	N/D	N/D	<i>bla</i> _{∨IM-1} ,
	29/07/04					like, SHV-			aacA7,
						1-like			dhfrl,
									aadA1
8	26/02/05-	Kpn	А	Bronchial	Carriage	SHV-5-	200	Successful	N/D
	01/06/05			secretion		like, SHV-		(SHV-5 co-	
						1-like		transferred)	

				Faeces	Carriage		200+70	Successful	
			В	Faeces	Carriage	SHV-1-like	125+70	Successful	bla _{∨IM-1} , aacA7, dhfrl, aadA1
			D	Faeces	Carriage	SHV-5- like, SHV- 1-like	70	N/D	bla _{∨IM-1} , aacA7, dhfrl, aadA1
		Ecl	1	Faeces	Carriage	AmpC	280+90	N/D	bla _{∨IM-1} , aacA7, dhfrl, aadA1 ª
9	20/12/04– 08/02/05	Kpn	c	Faeces	Carriage	SHV-5- like, SHV- 1-like	N/D	N/D	N/D
10	23/08/04– 07/02/05	Kpn	A	Blood CVC Bronchial secretions	CRBSI Carriage	SHV-5- like, SHV- 1-like	65	N/D	bla _{∨IM-1} , aacA7, dhfrl, aadA1
			В	Faeces	Carriage	SHV-5- like, SHV- 1-like	N/D	N/D	N/D

			С	Faeces	Carriage	SHV-1-like	N/D	N/D	N/D
				Bronchial	Carriage				
				secretions					
			F	CVC	Carriage	SHV-1-like	N/D	N/D	bla _{∨IM-1} ,
									aacA7,
									dhfrl,
									aadA1
11	06/10/04—	Kpn	С	Faeces	Carriage	SHV-5-	N/D	N/D	N/D
	02/11/04					like, SHV-			
						1-like			
12	18/06/04—	Kpn	В	Faeces	Carriage	SHV-5-	55	N/D	N/D
	22/07/04		С	Faeces	Carriage	like, SHV-	50	N/D	N/D
						1-like			
13	05/04/04–	Kpn	F	Blood	BSI	SHV-5-	180+55	N/D	bla _{∨IM-1} ,
	24/05/04					like, SHV-			aacA7,
						1-like			dhfrl,
									aadA1
			Н	Faeces	Carriage	SHV-5-	N/D	N/D	N/D
						like, SHV-			
						1-like			

			I	CVC	Carriage	SHV-5-	55	N/D	bla _{∨IM-1} ,
						like, SHV-			aacA7,
						1-like			dhfrl,
									aadA1
14	09/11/04-	Kpn	С	Blood	CRBSI	SHV-5-	N/D	N/D	<i>bla</i> _{∨IM-1} ,
	17/01/05			CVC		like, SHV-			aacA7,
				Drainage	Carriage	1-like			dhfrl,
				Faeces	Carriage				aadA1
			н	Faeces	Carriage	SHV-5-	N/D	N/D	N/D
						like, SHV-			
						1-like			
15	13/05/05–	Kpn	А	Urine	Carriage	SHV-5-	N/D	N/D	N/D
	27/08/05					like, SHV-			
						1-like			
			E	Faeces	Carriage	SHV-1-like	N/D	Successful	<i>bla</i> _{∨IM-1} ,
				Bronchial	Carriage	SHV-5-	200+100	N/S	aacA7,
				secretions		like, SHV-			dhfrl,
						1-like			aadA1
		Ecl	2	Faeces	Carriage	AmpC	200+90	N/D	<i>bla</i> _{∨IM-1} ,
									aacA7,
									dhfrl,
									aadA1 ^ª

16	05/08/04-	Kpn	А	Bronchial	Carriage	SHV-5-	N/D	N/D	N/D
	10/11/04			secretions		like, SHV-			
						1-like			
			С	Faeces	Carriage	SHV-5-	N/D	N/D	N/D
						like, SHV-			
						1-like			
17	09/03/04-	Kpn	Е	Faeces	Carriage	SHV-5-	N/D	N/D	N/D
	17/06/04					like, SHV-			
						1-like			
			F	CVC	Carriage	SHV-5-	N/D	N/D	N/D
						like, SHV-			
						1-like			
			G	Faeces	Carriage	SHV-5-	55	Successful	<i>bla</i> _{∨IM-1} ,
						like, SHV-			aacA7,
						1-like			dhfrl,
									aadA1
		Cfr		Faeces	Carriage	AmpC	40	N/S	bla _{∨IM-1} ,
									aac(6')-IIc
18	20/05/04-	Kpn	А	Urine	Carriage	SHV-5-	N/D	N/D	N/D
	28/09/04					like, SHV-			
						1-like			
			В	Pus	STI	SHV-5-	55	N/S	N/D

				Faeces	Carriage	like, SHV-	N/D		
						1-like			
			F	CVC	Carriage	SHV-5-	170+90+60	N/S	N/D
						like, SHV-			
						1-like			
19	03/06/04-	Kpn	В	Urine	Carriage	SHV-5-	N/D	N/D	N/D
	22/10/04					like, SHV-			
						1-like			
			С	Bronchial	Carriage	SHV-5-	50	Successful	N/D
				secretions		like, SHV-			
				Faeces	Carriage	1-like	N/D		
20	19/02/05–	Kpn	A	Bronchial	Carriage	SHV-1-like	N/D	N/D	N/D
	22/06/05			secretions					
				Faeces	Carriage				
21	14/04/04—	Kpn	С	Faeces	Carriage	SHV-5-	N/D	N/D	N/D
	03/08/04					like, SHV-			
						1-like			
			Е	Urine	Carriage	SHV-5-	220+200+100+60	N/D	N/D
						like, SHV-			
						1-like			
			Н	Blood	CRBSI	SHV-5-	180+80+50	N/S	N/D
				CVC		like, SHV-	160+80+50		

				Bronchial	Carriage	1-like	N/D		
				secretion					
				Faeces	Carriage		N/D		
22	22/10/04-	Kpn	А	Urine	Carriage	SHV-5-	N/D	N/D	N/D
	07/12/04			Faeces	Carriage	like, SHV-			
						1-like			
			С	Blood	BSI	SHV-5-	55	N/D	bla _{∨IM-1} ,
				Urine	Carriage	like, SHV-	N/D		aacA7,
						1-like			dhfrl,
									aadA1
			F	Bronchial	VAP	SHV-5-	N/D	N/D	N/D
				secretions		like, SHV-			
						1-like			
23	21/03/05–	Kpn	С	Bronchial	VAP	SHV-5-	55	N/D	N/D
	08/06/05			secretions		like, SHV-			
						1-like			
				Urine	Carriage	SHV-1-like	N/D		
				Faeces	Carriage	SHV-5-	N/D		
						like, SHV-			
						1-like			
			G	Urine	Carriage	SHV-5-	55	N/D	N/D
						like, SHV-			
						1-like			

Kpn, *Klebsiella pneumoniae*; Ecl, *Enterobacter cloacae*; Cfr, *Citrobacter freundii*; CRBSI, catheter-related bloodstream infection; CVC, central venous catheter; BSI, bloodstream infection; SSI, surgical site infection; STI, soft tissue infection; VAP, ventilator-associated pneumonia; N/D, not done; N/S, not successful.

^a Based on mapping by polymerase chain reaction (PCR) using primers based on the sequence of In-e541 [18] (sequencing of the whole integron was not performed).

10