Ongoing epidemic of \textit{bla}_{VIM-1}-positive \textit{Klebsiella pneumoniae} in Athens, Greece: a prospective survey

M. Psichogiou\textsuperscript{1}, P. T. Tassios\textsuperscript{2}, A. Avlamis\textsuperscript{3}, I. Stefanou\textsuperscript{3}, C. Kosmidis\textsuperscript{1}, E. Platsouka\textsuperscript{4}, O. Paniara\textsuperscript{4}, A. Xanthaki\textsuperscript{5}, M. Toutouza\textsuperscript{5}, G. L. Daikos\textsuperscript{1} and L. S. Tzouvelekis\textsuperscript{2}\textsuperscript{*}

\textsuperscript{1}First Department of Propaedeutic Medicine, Medical School, University of Athens, Athens, Greece; \textsuperscript{2}Department of Microbiology, Medical School, University of Athens, Athens, Greece; \textsuperscript{3}Department of Microbiology, Laikon General Hospital, Athens, Greece; \textsuperscript{4}Department of Microbiology, Evangelismos General Hospital, Athens, Greece; \textsuperscript{5}Department of Microbiology, Hippokration General Hospital, Athens, Greece

Received 25 July 2007; returned 29 August 2007; revised 15 October 2007; accepted 19 October 2007

\textbf{Objectives:} To determine the current frequency and study the characteristics of \textit{VIM-1}-producing \textit{Klebsiella pneumoniae} isolates from bloodstream infections in Greek hospitals.

\textbf{Methods:} All blood isolates of \textit{K. pneumoniae} were prospectively collected during 2004–06 in three teaching hospitals located in Athens. MICs of antibiotics were determined by the Etest. Extended-spectrum- (ESBL) and metallo-\textit{\beta}-lactamase (MBL) production was examined by clavulanate- and EDTA-based techniques, respectively. Isolates were typed by PFGE of \textit{XbaI}-digested genomic DNA. Detection of \textit{bla}_{VIM-1} and mapping of the VIM-1-encoding integrons were performed by PCR and sequencing. \textit{\beta}-Lactamase activities were analysed by IEF and imipenem hydrolysis was assessed by spectrophotometry. VIM-1-encoding plasmids were transferred to \textit{Escherichia coli} by conjugation and transformation and characterized by Inc/rep typing and RFLP.

\textbf{Results:} Sixty-seven (37.6\%) of 178 \textit{K. pneumoniae} blood isolates were \textit{bla}_{VIM-1}-positive (VPKP); 77.8\% of these were from ICUs. All VPKP isolates were multidrug-resistant. The MICs of carbapenems for VPKP varied from the susceptible range to high-level resistance overlapping with those of MBL-negative isolates. The EDTA-imipenem synergy methods had reduced sensitivity in detecting VPKP isolates when the MICs were in the susceptible range. ESBL production was common among VPKP isolates \textit{(n} = 45, 67.2\%) as indicated by resistance to aztreonam and confirmed by a clavulanate-based double-disc synergy test. The responsible ESBL was always an SHV-5-type enzyme as indicated by IEF. PFGE identified eight clusters (A–H) of VPKP isolates with related (>80\%) patterns, as well as four unique types. Both inter-hospital spread of several clones and genotypic similarities among susceptible, ESBL-positive and VPKP isolates were also observed. Location of \textit{bla}_{VIM-1} and expression of VIM-1 were studied in 12 isolates representing the eight PFGE clusters. In all isolates, \textit{bla}_{VIM-1} was part of a class 1 integron that also carried \textit{aacA4}, \textit{dhfrI}, \textit{aadA} and \textit{sulI}. In eight isolates (clusters C, D, G and H), the \textit{bla}_{VIM-1} integron was located in transferable IncN plasmids. A cluster F isolate carried a VIM-1-encoding, self-transferable plasmid that was not typeable by Inc/rep typing. VIM-1-encoding replicons were not identified in three isolates (PFGE clusters A, B and E). VPKP isolates exhibited differences in imipenem-hydrolysing activities which, however, were not correlated with the respective carbapenem MICs.

\textbf{Conclusions:} A multiclonal epidemic of \textit{bla}_{VIM-1}-carrying \textit{K. pneumoniae} is under way in the major hospitals in Greece. Microorganisms producing both VIM-1 and SHV-5 constitute the prevalent multidrug-resistant population of \textit{K. pneumoniae} in this setting.

\textbf{Keywords:} \textit{K. pneumoniae}, carbapenem resistance, metallo-\textit{\beta}-lactamases

\textsuperscript{*}Corresponding author. Tel: +30-210-7462152; Fax: +30-210-7462010; E-mail: ltzouvel@cc.uoa.gr
Introduction

Carbapenems are considered as a reliable choice for the treatment of infections caused by *Klebsiella pneumoniae* producing various types of extended-spectrum β-lactamases (ESBLs) and cephalosporinases. A recent development, however, is the emergence of strains producing metallo-β-lactamases (MBLs) hydrolysing carbapenems. The main foci of MBL-producing Gram-negative microorganisms, including *K. pneumoniae*, seem to be in Southern Europe and the Far East. 1 *bla*$_{\text{VIM}}$-positive *K. pneumoniae* (VPKP) strains have been isolated with increasing frequency in Greek hospitals since 2000$^{2-5}$ (see also www.rivm.nl/earss/database/). Yet, the extent of their spread is unclear. Previous studies covered limited time periods and were based on phenotypic criteria, mainly ‘decreased susceptibility’ to imipenem and/or results of various imipenem-EDTA synergy methods. We present here the results of a 1 year prospective study aiming at (i) the determination of the actual prevalence of VPKP in three of the major hospitals in Athens, (ii) the genotypic and phenotypic characterization of the VPKP strains, and (iii) the evaluation of MBL detection methods as applied to this bacterial population.

Materials and methods

**Bacterial isolates**

A total of 178 consecutive blood isolates of *K. pneumoniae* were collected from an equal number of patients in three hospitals in Athens, I (500 beds), II (1000 beds), and III (400 beds), during February 2004–March 2006 (hospital I) and from February 2005 to March 2006 in hospitals II and III. Species identification was confirmed with the API 20E (bioMérieux, Marcy l’Etoile, France). March 2006 in hospitals II and III. Species identification was confirmed with the API 20E (bioMérieux, Marcy l’Etoile, France).

**Susceptibility and imipenem-EDTA synergy testing**

MICs of β-lactams were determined by the Etest (AB Biodisk, Solna, Sweden). Susceptibility to other antibiotics was assessed by a disc diffusion method. All isolates were examined blind by two MBL screening tests based on imipenem-EDTA synergy; (i) the double disc test (MBL-DDT), 6 and (ii) the combined disc test (MBL-CDT). 7 In the MBL-DDT, a clear and reproducible synergy image was interpreted as a positive result. Isolates exhibiting an increase of ≥5 mm in the inhibition zone of imipenem in the MBL-CDT were characterized as MBL-positive. Selected isolates with varying imipenem MICs were also examined by the MBL-Etest (AB Biodisk). Isolates were screened for ESBL production by the double-disc synergy test (DDST) using amoxicillin/clavulinate combined with cefazidime, cefotaxime, aztreonam and ceftazidime discs.

**β-Lactamase studies**

β-Lactamase-containing extracts were prepared by ultrasonic treatment of cell suspensions. IEF of β-lactamases was performed in polyacrylamide gels containing ampholines (pH range 3.5–9.5) using nitrocefin as a chromogenic substrate. To facilitate the detection of MBLs, a ZnCl$_2$ solution (1 mM) was used to overlay the gels before application of nitrocefin. Imipenem hydrolytic activity of the β-lactamase extracts was determined by spectrophotometry as described previously 8 and expressed as units (U) (1 U was the amount of enzyme hydrolysing 1 nmol of substrate per min per mg of protein).

**Transfer of resistance and plasmid characterization**

Transfer of MBL-encoding plasmids to susceptible *Escherichia coli* recipient cells was carried out by either conjugation in mixed broth cultures 9 or transformation. Transferable plasmids were purified using a Nucleobond BAC100 kit (Macherey-Nagel, Duren, Germany). Replicon typing was performed by a PCR-based method. 9 For comparison of RFLPs, plasmids were digested with *PstI* and fragments were separated by electrophoresis in 0.8% agarose gels.

**Detection of bla*$_{\text{VIM}}$* and characterization of integrons**

Total bacterial DNA was extracted with a QIamp DNA mini kit (Qiagen GmbH, Hilden, Germany) and used as template in PCR assays for detection of *bla*$_{\text{VIM}},$  MBL and *spm* MBL genes with consensus primers. 8 PCR mapping of *bla*$_{\text{VIM}}$-carrying class 1 integrons was carried out as described previously. 10 Nucleotide sequences of selected PCR products were determined on both strands using an automated sequencer (Avant 3100; Applied Biosystems).

**Strain typing**

*K. pneumoniae* isolates were typed by PFGE. 2 Agarose-embedded genomic DNA was digested with *XbaI* and the resulting fragments were separated by PFGE using a CHEF DR-III apparatus (Bio-Rad, Athens, Greece). Electrophoresis was at 6.0 V/cm for 22 h with a pulsing time linearly ramped from 5 to 40 s. Un-weighted pair group method with arithmetic means (UPGMA) dendrograms were constructed with the GelCompar I software (Applied-Maths, Sint-Martens-Latem, Belgium), using the Dice similarity coefficient, with optimization and position tolerance settings of 1.0% and 0.5%, respectively.

**Statistical analysis**

Data were analysed by the χ² test using the SPSS v. 12.0 software.

**Results and discussion**

**VPKP isolation frequency**

Hospital I contributed 90, hospital II 62 and hospital III 26 *K. pneumoniae* isolates. Ninety-six isolates (53.9%) were from medical ward patients, 54 (30.3%) from ICUs and 28 (15.7%) from surgical wards. All isolates were studied for carriage of MBL genes irrespective of their resistance phenotype. Sixty-seven (37.6%) isolates were positive for *bla*$_{\text{VIM}}$ by PCR and DNA sequencing. Other types of MBL genes were not detected. Isolation rates of VPKP were similar in hospitals II and III (53.2% and 53.8%, respectively), whereas in hospital I the rate was lower (22.2%). The highest frequency of VPKP was observed in ICUs (77.8%), whereas it was significantly lower in the surgical and medical wards (28.5% and 17.7%, respectively; *P* < 0.001).

**Resistance phenotypes and MBL detection**

Imipenem MICs for the 67 VPKP isolates ranged from 0.125 to 32 mg/L. MIC$_{50}$ and MIC$_{90}$ were 1 and 16 mg/L, respectively. Consequently, 48 (71.6%) VPKP isolates would have been classified as phenotypically fully susceptible to imipenem according to the current breakpoints. For 31 (46.3%) VPKP isolates, imipenem...
MICs were ≤0.5 mg/L. For 35 (52.2%) of the VPKP isolates, meropenem MICs (range 0.03–32, MIC₉₀ = 1 and MIC₉₀ = 32 mg/L) were lower than those of imipenem by one to three doubling dilutions. All VPKP isolates were resistant to amoxicillin/clavulanate, ticarcillin/clavulanate, piperacillin, cefoxitin, cefotaxime, ceftiraxone and ceftazidime. One and two isolates were susceptible to piperacillin/tazobactam and cefepime, respectively. Forty-five (67.2%) of the VPKP isolates exhibited either resistance or decreased susceptibility to aztreonam, a β-lactam that is not inactivated by VIM-1. These isolates were all ESBL-positive as indicated by the synergy between aztreonam and clavulanate in the respective DDST. High resistance rates were also observed for tobramycin (100%), gentamicin (47.8%), amikacin (86.6%), co-trimoxazole (100%), tetracycline (85.1%) and ciprofloxacin (86.6%) (Table 1). Thus, the VPKP isolates were invariably multidrug-resistant. None of the 111 blaVIM-1-negative isolates exhibited either resistance or decreased susceptibility to carbapenems (imipenem MICs ranged from 0.064 to 0.5 mg/L). Nevertheless, 18 (16.2%) of the latter isolates were ESBL-positive and displayed a multidrug resistance phenotype including newer β-lactams (third-generation cephalosporins and aztreonam) and aminoglycosides. Fluoroquinolone resistance was also common in this group and the respective rate (83.3%) was comparable with that of the VIM producers (P > 0.05). The remaining 93 isolates were susceptible to newer β-lactams. Also, resistance rates to aminoglycosides, such as gentamicin, as well as co-trimoxazole and ciprofloxacin in the latter group (5.4%, 27% and 20.6%, respectively) were significantly lower than in the isolates possessing VIM and/or ESBL (P < 0.001).

High sensitivity scores were observed for both the EDTA-based techniques for MBL detection. The MBL-DDT correctly identified 63 of the VPKP isolates (94% sensitivity). The MBL-CDT using imipenem discs was positive for 62 VPKP isolates (92.5% sensitivity). False positives were not observed by either method. Four VPKP isolates with imipenem MICs ranging from 0.125 to 0.5 mg/L were misidentified by both these methods. Preliminary experiments with the MBL-Etest showed that this method performed poorly even with VPKP isolates with high-level resistance to imipenem (data not shown). This was probably due to the relatively high drug content of the strips.

**Molecular typing**

A total of 94 *K. pneumoniae* isolates were studied by PFGE. These included 61 VPKP and 33 MBL-negative isolates (18 ESBL producers and 15 randomly selected isolates negative for ESBL). Fifty-seven of the VPKP isolates were distributed into 8 PFGE clusters (A–H), each grouping together 2–14 isolates with chromosomal fingerprints displaying >80% similarity. The remaining four VPKP isolates displayed unique PFGE patterns (Figure 1 and Table 1). Three of the less populous clusters, B, G and H, were each associated with a single hospital. The remaining five clusters were composed of isolates from either two (clusters A and F) or all three hospitals (clusters C, D and E) (Figure 1 and Table 1). There were certain associations between PFGE types and antibiotic resistance phenotypes of the VPKP isolates. Clusters A, D and H included isolates with increased resistance to carbapenems, whereas most isolates belonging to clusters B, E, F and G exhibited low carbapenem MICs (Table 1). In contrast, isolates in PFGE cluster C exhibited a wider variety of carbapenem MICs, though they were uniformly resistant to the other β-lactams tested. VPKP isolates that were also positive for ESBL production were distributed in all clusters except G. Interestingly, 16 of the 18 ESBL-positive isolates lacking *blaVIM-1*-clustered together with VPKP isolates, mainly in clusters F (9 isolates) and E (6 isolates). Also, three of the MBL- and ESBL-negative isolates belonged to clusters E (two isolates) and C (Figure 1).

**Expression of VIM-1 and location of *blaVIM-1***

Twelve VPKP isolates representing the main PFGE clusters (one from each of the clusters A, B, E, F and G, two from each of the clusters D and H and three from cluster C) were studied. IEF of

---

**Table 1.** Characteristics of 67 *blaVIM-1*-positive *K. pneumoniae* isolates

<table>
<thead>
<tr>
<th>PFGE type (n)</th>
<th>Hospital (n)</th>
<th>Range of imipenem MICs (mg/L)</th>
<th>Positive by EDTA synergy tests (n)</th>
<th>ESBL producers (n)</th>
<th>Resistant to non-β-lactams (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (2)</td>
<td>I (1), II (1)</td>
<td>2–32</td>
<td>2</td>
<td>1</td>
<td>0 2 2 2 2 2</td>
</tr>
<tr>
<td>B (2)</td>
<td>I (2)</td>
<td>0.5</td>
<td>2</td>
<td>1</td>
<td>0 2 2 2 2 2</td>
</tr>
<tr>
<td>C (14)</td>
<td>I (9), II (4), III (1)</td>
<td>0.25–32</td>
<td>13</td>
<td>6</td>
<td>1 14 13 14 14 14 14</td>
</tr>
<tr>
<td>D (9)</td>
<td>II (7), III (2)</td>
<td>4–32</td>
<td>9</td>
<td>8</td>
<td>8 9 8 9 9 9 9 9</td>
</tr>
<tr>
<td>E (10)</td>
<td>I (2), II (7), III (1)</td>
<td>0.25–1</td>
<td>9</td>
<td>9</td>
<td>2 10 8 10 10 5</td>
</tr>
<tr>
<td>F (6)</td>
<td>I (2), II (4)</td>
<td>0.25–1</td>
<td>6</td>
<td>4</td>
<td>6 6 6 6 4 6 4 6</td>
</tr>
<tr>
<td>G (5)</td>
<td>II (5)</td>
<td>0.25–1</td>
<td>5</td>
<td>0</td>
<td>3 5 5 5 0 4 0 4</td>
</tr>
<tr>
<td>H (9)</td>
<td>III (9)</td>
<td>4–32</td>
<td>9</td>
<td>9</td>
<td>9 9 9 9 9 9 9 9</td>
</tr>
<tr>
<td>a (4)</td>
<td>I (2), II (1), III (1)</td>
<td>0.125–0.5</td>
<td>3</td>
<td>2</td>
<td>1 4 4 4 3 4</td>
</tr>
<tr>
<td>ND (6)</td>
<td>I (2), II (4)</td>
<td>0.25–16</td>
<td>5</td>
<td>5</td>
<td>2 6 1 6 5 5</td>
</tr>
<tr>
<td>Total (67)</td>
<td>I (20), II (33), III (14)</td>
<td>0.125–32</td>
<td>63</td>
<td>45</td>
<td>32 67 58 67 58 57</td>
</tr>
</tbody>
</table>

*Number of isolates.

*Positive by at least one EDTA-based synergy method.

*GEN, gentamicin; TOB, tobramycin; AMK, amikacin; SXT, co-trimoxazole; CIP, ciprofloxacin; TET, tetracycline.

*Isolates exhibiting unique PFGE patterns.

*Not determined. Isolates were not available for PFGE typing.
β-lactamase-containing extracts confirmed production of VIM-1 in all 12 VPKP isolates. These experiments also showed that the eight ESBL-positive and aztreonam-resistant isolates included in this group produced a β-lactamase with an isoelectric point of 8.2, consistent with an SHV-5-type enzyme. Differences were observed among imipenem hydrolytic activities ranging from 30 to 50 U. However, these values did not correlate with the respective carbapenem MICs.
PCR mapping and sequencing showed that the variable region of the \textit{blaVIM-1} -carrying integrons from all 12 VPKP isolates contained \textit{blaVIM-1}, \textit{aacA4}, \textit{dhfr1}, \textit{aadA} and \textit{sulI} similarly to the In-e541 integron (AY339625 in GenBank) commonly found among Enterobacteriaceae in this setting.\textsuperscript{4,8,10} VIM-1-encoding plasmids were transferred to \textit{E. coli} by conjugation from eight VPKP isolates belonging to PFGE clusters C, D, F and H. Transformation experiments revealed an additional VIM-1-encoding plasmid from an isolate of cluster G. \textit{blaVIM-1} -carrying replicons were not identified from the remaining three isolates (clusters A, B and E). Eight of the plasmids transferring VIM-1, although varied in size (50–70 kb) and exhibiting RFLP differences (data not shown), belonged to IncN. The self-transferable plasmid from the cluster F isolate was \textasciitilde{}150 kb in size and not typeable by \textit{Inc} \textit{rep} typing. None of the \textit{blaVIM-1} -carrying plasmids identified here encoded production of SHV-5.

Conclusions

In contrast to previous studies, describing either clonal outbreaks in single hospitals or occurrence of a limited number of VPKP strains, this work documents the inter- and intra-hospital dissemination of several distinct clones. It also shows that VPKP strains are currently prevalent in the major hospitals in Athens. They constitute the majority of multidrug-resistant \textit{K. pneumoniae}, whereas isolates with only an ESBL phenotype, which were predominant in this setting up to 2001, are observed less frequently nowadays. Results from previous studies on acquired genes in enterobacteria in Athens hospitals,\textsuperscript{11} as well as the data available in the National Surveillance System for Antibiotic Resistance database (www.mednet.gr/whonet/top.htm) suggest that this development must have taken place during a relatively short time period, probably between 2002 and 2003. Apart from the intensive use of carbapenems, the dissemination of VPKP isolates is probably associated with ineffective infection control practices. Evidence in support of this in the present study includes the persistence for at least one year, as well as the inter-hospital spread of several strains. Additionally, identification of multiple VPKP clones underscores the transfer potential of \textit{blaVIM-1} that is disseminated mostly via self-transmissible IncN plasmids as also observed in previous studies.\textsuperscript{4,8–10} A strong association of VIM-1 and SHV-5 production was noticeable; this has also been described previously\textsuperscript{2,4,12} and is due to acquisition of distinct plasmids. It is possible that some of the VPKP strains may have been derived from endemic SHV-5-producing progenitors. However, the presence of clonally related isolates producing none, one or both VIM-1 and SHV-5 indicates that frequent events of acquisition and loss of \textit{bla} genes probably operate alongside the more forbidding gradual accumulation of resistance.

Imipenem MICs for the previously described VPKP in Greece ranged from 1 to 64 mg/L.\textsuperscript{2–5} The present findings not only extend the lower limit to 0.125 mg/L, but also show that an imipenem MIC \textasciitilde{}1 mg/L is common. The wide range of carbapenem MICs among VPKP, including genotypically related isolates, may arise from differences in the outer membrane permeability,\textsuperscript{7} whereas the diversity in the levels of VIM-1 production probably plays a lesser role. The low carbapenem MICs of a significant proportion of the VPKP isolates makes their direct phenotypic identification difficult. Based on the present findings, the use of either the MBL-DDT or the MBL-CDT is proposed for all newer \textbeta{}-lactam-resistant \textit{K. pneumoniae} isolated in this setting.

Acknowledgements

We thank Georgia Diamantopoulou for expert assistance with PFGE.

Funding

This study was supported in part by a research grant from the ‘Kapodistrias’ program of the University of Athens.

Transparency declarations

None to declare.

References


