Carbapenemase-producing *Enterobacteriaceae* and *Pseudomonas* spp. are increasingly reported in many countries all over the world. Due to the resistance of those bacteria to almost all antibiotics (e.g. beta-lactams, aminoglycosides, fluoroquinolones), treatment options are seriously limited. In the Czech Republic, the incidence of carbapenemase-producing *Enterobacteriaceae* seems to be low, restricted to only three cases detected between 2009 and 2010. Here, we describe molecular typing of 15 carbapenemase-producing *Klebsiella pneumoniae* isolates identified in the Czech Republic during 2011. Five VIM-1-producing isolates belonging to sequence type (ST) 11 and one VIM-4-producing isolate of ST1029 have been detected. *bla*$_{VIM}$ and *bla*$_{VIM}$ as a part of class 1 integrons were chromosomally located or carried by a plasmid belonging to A/C replicon type (*bla*$_{VIM}$). KPC-3-producing isolates of ST512, recovered from six patients, caused an outbreak. Three more isolates producing KPC-2 enzyme belonged to ST258. Both *bla*$_{KPC}$ genes were part of the Tn$_{4401a}$ transposon carried on plasmids of the pKpQIL type. The isolates were resistant to all antibiotics tested except colistine and/or gentamicin. Four of these 15 strains were recovered from patients repatriated to the Czech Republic from Greece and Italy. This is the first report of outbreaks caused by carbapenemase-producing *Enterobacteriaceae* in the Czech Republic.

**Introduction**

Spread of carbapenemase-producing *Enterobacteriaceae* and *Pseudomonas* spp. has been observed in many countries across the world [1-3]. Carbapenemase producers are usually resistant to almost all of the effective antibiotics (such as beta-lactams, aminoglycosides, fluoroquinolones). Therapy of infections caused by such bacteria is limited to few choices (such as colistine and/or a combination therapy) with unpredictable effect [4]. Therefore, prevention of their spread in healthcare settings and in the community is a big challenge for medicine today.

In the Czech Republic, occurrence of carbapenemase-producing bacteria seemed to be rare with only sporadic cases of carbapenemase-producing *Klebsiella pneumoniae* (VIM-1, KPC-2), *Serratia marcescens* (VIM-1) and metallo-beta-lactamase-producing *Pseudomonas aeruginosa* (VIM-2, IMP-7) [1,5-7]. In 2011, however, the incidence of such bacteria increased, especially in *K. pneumoniae* and *P. aeruginosa*. The aim of this study was to analyse carbapenemase-producing *K. pneumoniae* isolates recovered from Czech hospitals in 2011.

**Methods**

**Bacterial isolates, identification and susceptibility testing**

In 2011, a total of 102 *Enterobacteriaceae* isolates, non-susceptible to carbapenems according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [8], were sent to the Czech national reference laboratory (NRL) for Antibiotics from local microbiology laboratories, for verification of carbapenemase production. All isolates were tested for carbapenem production by MALDI-TOF mass spectrometry (MS) meropenem hydrolysis assay [9,10]. Phenotypic identification of carbapenemases was performed by an inhibitor-based method [11]. Species identification was performed using a MALDI Biotyper Version 3.0 (Bruker Daltonik GmbH., Bremen, Germany). Minimum inhibitory concentrations (MICs) to 12 antibiotics (piperacillin, piperacillin/tazobactam, cefotaxime, ceftazidime, cefepime, meropenem, ciprofloxacin, gentamicin, amikacin, colistine, chloramphenicol, trimethoprim/sulfamethoxazole) were determined according to the EUCAST recommendations [12].
Typhing

All isolates were typed by pulsed-field gel electrophoresis (PFGE) [13] using the restriction enzyme XbaI; the results were interpreted according to Tenover et al. [14]. All isolates were also subjected to multilocus sequence typing (MLST) as described previously [15]. The database available at www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html was used for assigning sequence types (STs).

Beta-lactamase identification, bla gene environment mapping

Detection of bla genes, encoding important carbapenemase types, was performed by PCR using specific primers for blaOXA-48, blaIMP, blaNDM, blaVIM and blaKPC [2,16-18]. The gene environment of blaKPC was determined by PCR mapping as proposed by Naas et al. [17]. Mapping of the VIM-encoding integrons was performed by PCR [16]. For detection of blaCMY-type genes, a PCR assay was employed [19]. PCR products were sequenced on both strands.

Conjugation and transformation

To check transferability of the resistance genes on a conjugal plasmid, conjugal transfer was carried out by broth mating, using rifampin-resistant *Escherichia coli* A15 as previously described [20]. Transconjugants were selected with 50 mg/L ampicillin and 60 mg/L rifampin. Transformation experiments were performed with plasmid extracts, purified using a Qiagen Plasmid Maxi Kit (Qiagen GmbH, Hilden, Germany), and *E. coli* DH5alpha chemically competent cells as a recipient. Transformants were selected with 50 mg/L ampicillin.

Plasmid analysis

Plasmid content was visualised after S1 linearisation followed by PFGE separation [21]. Localisation of blaVIM, blaKPC and blaCMY genes was analysed by hybridisation. The bla-specific probes were prepared from PCR amplicons using a BrightStar Psoralen-Biotin kit (Applied Biosystems, Prague, Czech Republic). DNA after S1 linearisation and PFGE separation was transferred on BrightStar-Plus Positively Charged Nylon Membrane (Applied Biosystems, Prague, Czech Republic) according to manufacturer recommendations, and hybridised for 24 h at 42 °C. Detection of membranes was performed by BrightStar BioDetect Kit (Applied Biosystems, Prague, Czech Republic). PCR-based replicon typing (PBRT) of plasmids was performed as proposed by Carattoli et al. [22], using total DNA from transconjugants/transformants or from clinical isolates that were non-successful in conjugation and transformation experiments. IncF plasmids were further characterised by replica sequence typing (RST) [23]. Plasmids carrying blaKPC were identified by PCR mapping as proposed by Baraniak et al. [24].

Results

MALDI-TOF MS meropenem hydrolysis assay confirmed carbapenemase activity in 15 of the 102 isolates analysed. Ethylene-diamine tetra-acetic acid (EDTA)-meropenem combined disk test confirmed metallo-beta-lactamase production in six of the isolates. The respective aminophenylboronic acid–meropenem test was positive for KPC production in the remaining nine isolates. All of the suspected isolates based on the phenotypic tests were positive in MALDI-TOF MS meropenem hydrolysis assay.

VIM-producing isolates

Five of the six VIM-1-producing *K. pneumoniae* were isolated from one hospital (A5) in Prague (Table 1). In all five isolates, MICs of meropenem were in the susceptible category according to the EUCAST criteria, ranging from 1 to 2 mg/L. The five isolates were resistant to all antibiotics tested, except colistin. The variable region of their class 1 integron containing blaVIM-1 gene is described in Table 1. Neither transconjugants nor transformants were obtained from any of the five isolates detected in hospital A5. A blaVIM-1 specific probe hybridised strongly with a band corresponding to the chromosomal material, which confirmed the chromosomal location of the blaVIM-1-containing integron. All isolates belonged to ST11, which is a common clone of *K. pneumoniae* that possesses extended spectrum (ESBL) and AmpC-beta-lactamases [25,26].

### Table 1

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Isolation date</th>
<th>Hospital</th>
<th>ST</th>
<th>Conjugation</th>
<th>Replicon type</th>
<th>Gene cassettes</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>V554</td>
<td>1 Sep</td>
<td>A5</td>
<td>11</td>
<td>-</td>
<td>-</td>
<td>aac(6')-Ib, blaVIM-1</td>
<td></td>
</tr>
<tr>
<td>V555</td>
<td>24 Aug</td>
<td>A5</td>
<td>11</td>
<td>-</td>
<td>-</td>
<td>aac(6')-Ib, blaVIM-1</td>
<td></td>
</tr>
<tr>
<td>V564</td>
<td>26 May</td>
<td>A5</td>
<td>11</td>
<td>-</td>
<td>-</td>
<td>aac(6')-Ib, blaVIM-1</td>
<td></td>
</tr>
<tr>
<td>V602</td>
<td>10 Oct</td>
<td>A5</td>
<td>11</td>
<td>-</td>
<td>-</td>
<td>aac(6')-Ib, blaVIM-1</td>
<td></td>
</tr>
<tr>
<td>V633</td>
<td>21 Oct</td>
<td>A5</td>
<td>11</td>
<td>-</td>
<td>-</td>
<td>aac(6')-Ib, blaVIM-1</td>
<td></td>
</tr>
<tr>
<td>V624</td>
<td>17 Oct</td>
<td>NJ</td>
<td>1029</td>
<td>+</td>
<td>A/C</td>
<td>blaVIM-4</td>
<td>Import from Greece</td>
</tr>
</tbody>
</table>

ST: sequence type.
The sixth VIM-4-producing strain was detected in October 2011 in a patient admitted to the hospital in the Czech Republic after the medically assisted repatriation from a hospital in Northern Greece. Carbapenem-resistant *K. pneumoniae* (isolate no. V624; Table 1) was isolated from blood immediately after the admission to the hospital. The isolate belonged to ST1029, a novel sequence type, which is a single locus variant (SLV) of ST383 and was first reported in Greece in 2009 [19]. The strain produced VIM-4 and CMY-4 beta-lactamasases as described in ST383 by Papagiannitsis et al. [19]. However, no production of KPC enzyme was identified in our strain, contrary to the Greek strain. The class 1 integron consisting of a sole *bla*<sub>VIM-4</sub> gene cassette was harboured by a conjugative plasmid of A/C replicon type. A similar plasmid harbouring *bla*<sub>VIM-1</sub> was described by Samuelsen et al. in a patient repatriated from Greece in 2005 [27]. Immediately after the isolation of the carbapenem-resistant *K. pneumoniae* isolate, recommended isolation precautions were set up in the hospital and no transfer of the strain to another patient was found.

### Table 2

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Isolation date</th>
<th>Hospital</th>
<th>ST</th>
<th>KPC type</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>V514</td>
<td>13 Jul</td>
<td>A41</td>
<td>ST512</td>
<td>KPC-3</td>
<td>Import from Italy, index case</td>
</tr>
<tr>
<td>V556</td>
<td>18 Aug</td>
<td>A41</td>
<td>ST512</td>
<td>KPC-3</td>
<td></td>
</tr>
<tr>
<td>V557</td>
<td>18 Aug</td>
<td>A41</td>
<td>ST512</td>
<td>KPC-3</td>
<td></td>
</tr>
<tr>
<td>V646</td>
<td>14 Nov</td>
<td>A41</td>
<td>ST512</td>
<td>KPC-3</td>
<td></td>
</tr>
<tr>
<td>V719</td>
<td>28 Dec</td>
<td>A41</td>
<td>ST512</td>
<td>KPC-3</td>
<td></td>
</tr>
<tr>
<td>V597</td>
<td>4 Oct</td>
<td>A6</td>
<td>ST258</td>
<td>KPC-2</td>
<td>Import from Greece, index case</td>
</tr>
<tr>
<td>V640</td>
<td>7 Nov</td>
<td>A6</td>
<td>ST258</td>
<td>KPC-2</td>
<td></td>
</tr>
<tr>
<td>V601</td>
<td>21 Oct</td>
<td>A51</td>
<td>ST258</td>
<td>KPC-2</td>
<td></td>
</tr>
</tbody>
</table>

ST: sequence type.

KPC-producing isolates

In the Czech Republic, the first KPC-producing *K. pneumoniae* isolate was obtained from a patient repatriated from a hospital in Italy to hospital A41 in Prague in July 2011. A carbapenem-resistant isolate producing KPC-3 was cultivated from a urine sample (isolate no. V514; Table 2). From August till December, five more KPC-3-producing *K. pneumoniae* strains were identified in different patients. Their molecular and epidemiological characteristics are summarised in Table 2. Three of these patients were hospitalised on the same ward as, but without direct contact to, the index case, while the remaining two patients were hospitalised in the same time period but in different hospital wards (Figure 1).

Another KPC-producing isolate was recovered from a patient repatriated from a hospital on Greece to hospital A6 in Prague (isolate no. V597; Table 2). The strain was recovered from a blood sample. A second patient (isolate no. V640; Table 2) hospitalised in the same room as the previous one, was colonised with a strain of the same PFGE pattern and ST.

### Figure 1

Timeline of successive hospital outbreaks of KPC-producing *Klebsiella pneumoniae* isolates in Hospital A41, Czech Republic, 2011 (n=6)

- Patient 1 (isolate no. V514)
- Patient 2 (isolate no. V556)
- Patient 3 (isolate no. V557)
- Patient 4 (isolate no. V646)
- Patient 5 (isolate no. V719)
- Patient 6 (isolate no. V640)

First isolation of carbapenemase-producing isolate

Hospitalisation in the same hospital ward

Month 2011
The last case was detected in hospital A51 in Prague. This strain (isolate no. V601; Table 2) was obtained from the respiratory tract of a patient repatriated from a hospital on Crete (Greece). No spread to other patients was detected. No difference was detected in the PFGE patterns of ST258 and ST512 isolates.

According to the EUCAST criteria, the detected KPC-producing isolates were susceptible only to gentamicin (Table 3). MICs of colistin, which is sometimes the drug of the last choice in carbapenemase-producing Enterobacteriaceae infections, were in the resistant category (8–16 mg/L). Plasmid profiling with S1 linearisation of all clinical isolates showed a common profile with plasmids approximately 40, 110 and 200 kb in size [24]. All KPC-producing isolates harboured blaKPC-positive plasmids of similar size (approximately 110 kb). Those blaKPC-encoded plasmids were negative for all replicon sequences included in the PBRT panel. However, by the RST method, the KPC-encoding plasmids were positive for the FII replicon. Using PCR-based mapping, the plasmids were identified as the pKpQIL type [24]. Both blaKPC2 and blaKPC3 were part of the transposon Tn4401, isoform a. No transconjugants were obtained from KPC producers. KPC-encoding plasmids were only transferred by transformation of plasmid DNA obtained from isolate V597. MICs of the transformant are shown in Table 3.

All of the patients repatriated to the Czech Republic had been hospitalised in intensive care units in the countries they were repatriated from. In two hospitals in the Czech Republic (A6 and A51), isolation precautions were set up immediately after the identification of carbapenem-resistant K. pneumoniae isolate.

### Discussion

Carbapenemase-producing enterobacteria seem to be uncommon in the Czech Republic with only three reported cases in the period of 2009 and 2010 and six cases in 2012 [1,5,7]. In 2011, two outbreaks and a few cases of VIM- and KPC-producing K. pneumoniae were reported. The K. pneumoniae species was the only member of the Enterobacteriaceae family found to produce carbapenemases in that year in the Czech Republic. We believe that the situation is not underestimated because, since the mandatory official guideline was issued by the Ministry of Health in 2012, all carbapenem-resistant enterobacteria have been sent to the NRL for Antibiotics for confirmation of carbapenemase production and epidemiological typing.

The situation of VIM-1-producing K. pneumoniae in the hospital A5 seems to have been endemic. Even if no epidemiological connection among the isolates could be found (such as hospitalisation on the same ward, use of the same medical procedure or the same medical personnel), most of them were recovered the same time period between May and October 2011 (Table 1, Figure 2). Therefore, the occurrence of these isolates could be considered as an outbreak, but we were not able to identify an index case nor reservoir of the strains. Therefore, our hypothesis was based on molecular typing of the isolates only.

The increasing incidence of KPC-producing K. pneumoniae observed in the Czech Republic in 2011 was initially caused by the repatriation of infected patients from Italy (KPC-3, ST512) and Greece (KPC-2, ST258), followed by an outbreak with an ST512 strain in Hospital A41. All isolates showed identical PFGE.
patterns and belonged to ST512, supporting the theory of an outbreak.

This ST is a single locus variant of a widely spread KPC-2-producing ST258 clone. ST512 was first reported from Israel among the isolates producing KPC-3 carbapenemase [28]. All KPC-producing isolates detected in this study were resistant to colistin. Resistance to this drug in KPC-producing *K. pneumoniae* isolates is being described more and more frequently [29,30]. Treatment options for infections caused by carbapenemase-producing *Enterobacteriaceae* are seriously limited until new classes of antibiotics are found; therefore it is necessary to understand the epidemiological principles of the spread of such bacteria and to set up efficient infection control measurements.

It can be assumed that the repatriated patients acquired the carbapenemase-producing *Enterobacteriaceae* in the foreign countries, since their transport was organised through specialised medical assistance and they were admitted to a Czech hospital without delay. Molecular typing data also confirm this theory. In all of the described patients, screening (such as rectal swab, sputum, urine, wound swab etc.) for identification of carbapenemase-producing *Enterobacteriaceae* was performed in the intensive care units abroad in a way corresponding to what is recommended in the national guidelines issued by the NRL for Antibiotics [31].

Until mid-2012, there was no official document in the Czech Republic on isolation precautions for patients colonised or infected by carbapenemase-producing *Enterobacteriaceae*. However, recommendations regarding diagnostic procedures, screening, and specific hygienic measurements were available from the NRL for Antibiotics [31]. Recently, an official guideline for the management of imported cases of carbapenemase-producing *Enterobacteriaceae* including infection control procedures has been approved and published through a bulletin of the Ministry of Health of the Czech Republic [32]. In this document, screening procedures on medical wards with confirmed occurrence of carbapenemase-producing *Enterobacteriaceae* are described in detail. The recommended screening is based on rectal swabs collected from patients hospitalised on the same ward or in possible contact with an infected or colonised patient. Other tissues sampled for standard screening in intensive care units (such as sputum, urine, different swabs) should also be tested for carbapenemase-producing *Enterobacteriaceae*. For patients with suspected or proven carbapenemase-producing *Enterobacteriaceae*, strict isolation procedures have to be set up.

In 2012 and 2013, there has not been a further increase in the occurrence of carbapenemase-producing *Enterobacteriaceae* in the Czech Republic, and only one outbreak (five patients) and four sporadic cases have been noted until mid-2013 (data not shown). An almost similar number of carbapenem-non-susceptible isolates has been sent for confirmation of carbapenemase production from routine laboratories in 2012 as in 2011. Only two imported cases of VIM-1-producing *K. pneumoniae* and NDM-4-producing *Enterobacter cloacae* have been detected [33]. This situation signals that the proposed preventive recommendations have been able to stabilise or even decrease the incidence of carbapenemase-producing *Enterobacteriaceae* in our country.

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Authors’ contributions
J.Hrabak and C.C.Papagiannitsis performed molecular typing and prepared the manuscript. V.Studentova was responsible for performing some typing methods. V.Jakubu, M.Frídrichová, H.Zemlicková collected the isolates and the data about the patients from local laboratories and performed phenotypic tests for the detection of resistance mechanisms and determined MICs.

Conflict of interest
None declared.

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