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SHORT REPORT
Outbreak of KPC-3-producing ST15 and ST348 *Klebsiella pneumoniae* in a Portuguese hospital

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SUMMARY

To date, only a few sporadic cases of infections due to *Klebsiella pneumoniae* carbapenemase (KPC) producers have been reported in Portugal. Here, we report for the first time an outbreak of *K. pneumoniae* KPC-3 producers in a tertiary-care hospital during 2013. Twenty-seven ertapenem-resistant *K. pneumoniae* were identified in patients at a tertiary-care hospital during 2013 isolated predominantly from urine (48·1%) and blood (25·9%) cultures. All isolates were highly resistant to β-lactam antibiotics and most showed intermediate resistance to imipenem. The more frequent β-lactamases were TEM- (77·7%), CTX-M- (70·3%) and KPC-type (66·6%). KPC-3 was identified by sequencing. The bla_{KPC-3} gene was associated with an IncF plasmid, and efficiently transferred to *E. coli* J53. Pulsed-field gel electrophoresis typing revealed three clusters of isolates which were further characterized by multi-locus sequence typing as ST11, ST15 and ST348. Ertapenem-resistant ST15 was already in circulation in the hospital, related to expression of OmpK36 modified porin, but the other two sequence types had not been previously found in the hospital. We conclude that the IncF plasmid mediated transfer of KPC-3 in the outbreak and that implementation of carbapenemase gene screening in isolates from patients on admission to hospital is advisable in order to control dissemination of these antimicrobial resistance elements.

Key words: Antimicrobial resistance, β-lactamase KPC-3, carbapenemase, epidemiology, outbreak.

Carbapenems are antibiotics of choice for treatment of bacterial infections due to multi-drug resistant and/or extended-spectrum β-lactamase (ESBL)-producing organisms. However, carbapenem-resistant strains are increasingly reported and have emerged as a major cause of nosocomial infections worldwide [1]. Therefore, the detection and surveillance of such organisms is of major importance for selection of appropriate therapeutic schemes and improvement of infection control measures.

In Enterobacteriaceae, carbapenem resistance arises either from the acquisition of carbapenemase genes that encode for carbapenem-hydolysing enzymes or by decrease in antibiotic uptake due to a deficiency of porin expression [2]. In the carbapenemases, the *Klebsiella pneumoniae* carbapenemase (KPC) is one of the most important resistance mechanisms in

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clinical isolates. Since their first identification in the United States in 1996, KPCs have been increasingly identified worldwide [1], but their dissemination in Portugal has been barely reported. The first identification in Portugal of an *Escherichia coli* KPC-2 producer from an environmental origin was reported in 2012 [3], and served to alert the hospital community in the country to this threat. Moreover, a recent survey of Enterobacteriaceae collected from 2006 to 2013 from 13 hospitals, detected 165 ertapenem non-susceptible isolates, but only 35 were carbapenemase producers. Of these, 22 were *K. pneumoniae* KPC-3 producers belonging to ten different sequence types (STs), indicating that the appearance of KPC of clinical origin was generally sporadic [4].

In the University Hospital of Coimbra, a ST15 *K. pneumoniae* ertapenem-resistant clone had been identified in 2010 but carbapenem resistance was associated with a porin modification and not a carbapenemase [2]. Herein, we report the first outbreak of KPC-3 carbapenemase-producing ertapenem-resistant *K. pneumoniae* isolates recovered from patients attending a tertiary-care hospital, and the genetic characterization of the isolates.

A collection of 27 ertapenem-resistant *K. pneumoniae* isolates recovered from patients admitted to the University Hospital of Coimbra from January to December in 2013 was characterized by phenotype and genotype. The antimicrobial susceptibility of isolates was performed with the Vitek 2 Advanced Expert system (bioMérieux, Portugal) and by Kirby–Bauer disk diffusion standard method using amoxicillin plus clavulanic acid (30 μg), cefoxitin (30 μg), ceftazidime (30 μg), cefotaxime (30 μg), aztreonam (30 μg), imipenem (10 μg) and ertapenem (10 μg) (Oxoid, UK). Interpretation was performed according to the European Committee for Antimicrobial Susceptibility Testing (EUCAST) breakpoint guidelines [5]. Phenotypic confirmation of ESBLs was performed using the double-disk synergy test [5]. Additionally, the disc approximation test was used to detect inducible AmpC using imipenem and cefoxitin as inducers against cefotaxime or ceftazidime.

The presence of KPC carbapenemase, ESBL (TEM, SHV and CTX-M types) and inducible AmpC (DHA-1) was screened by polymerase chain reaction (PCR). Detection of TEM, SHV and CTX-M genes was performed as previously described [6–8]. The amplification of bla_{KPC} was performed with an initial step of 94 °C for 10 min, followed by 30 cycles at 94 °C for 45 s, 62 °C for 45 s and 72 °C for 90 s and a final extension cycle at 72 °C for 10 min. Detection of DHA-1 was performed with an initial step at 94 °C for 3 min, followed by 34 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and one final cycle at 72 °C for 7 min. All PCRs were performed in the Bio-Rad MJ Mini Thermo Cycler (Bio-Rad Laboratories, USA) in a final volume of 20 μl, containing 10 μl master mix 2× Dynazyme F-508 (Finnzymes, Thermo Fisher Scientific Inc., Finland), 0·5 μl (10 μM) of each primer and 1 μl DNA template. Positive and negative controls were included in each amplification reaction.

Selected β-lactamase genes (according to sequence typing, collection time and ward) were sequenced at Stab Vida (Portugal), after PCR product purification using 10 μl of the amplified PCR product and 4 μl ExoSAP-IT (USB Corporation, USA), incubated at 37 °C for 15 min, followed by enzyme inactivation step at 80 °C for 15 min. Gene sequences were assigned using the BLAST nucleotide sequence search tool.

Conjugation assays were performed to assess the potential of transfer of KPC genes. All isolates of *K. pneumoniae* carrying bla_{KPC} were used as donor strains and *E. coli* J53 (sodium azide resistant) as the recipient. Transconjugants were selected on trypticase soy agar (Oxoid) plates containing 100 μg/ml of sodium azide and 1·25 μg/ml cefotaxime. Transconjugants were characterized by antimicrobial susceptibility and PCR for specific β-lactamases.

Plasmids were extracted from whole bacterial cells using the Qiagen plasmid Midi Purification kit (Qiagen, Portugal) and the Kado & Liu method [9]. Incompatibility (Inc) groups of plasmids were identified according to the PCR-based replicon typing (PBRT) protocol [10].

Sixteen positive KPC and four non-KPC isolates were investigated for clonality using pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST). PFGE analysis was based on the PulseNet 1-day standardized PFGE protocol [11]. *K. pneumoniae* plugs were digested with *Xba*I (New England Biolabs, Portugal) restriction enzyme according to the manufacturer's instructions. Electrophoresis in agarose gel Seakem LE 1% (Cambrex Bio Science, USA) was performed with the CHEF-DR III System (Bio-Rad) in total volume of 3 l 0·5× TBE buffer previously cooled to 11 °C in the electrophoresis chamber. The programme was at 11 °C, 6 V/cm, 120°, 18·5 h, with switch times of 6–36 s. Gels were stained with ethidium bromide and visualized under UV light.

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Restriction patterns were interpreted according to Tenover *et al*. criteria [12].

MLST was performed on six isolates (four KPC, two non-KPC) by sequencing of seven housekeeping genes (*rphoB, gapA, mdh, pgi, phoE, infB, tonB*) and STs were assigned through the *K. pneumoniae* MLST database (http://bigsdb.web.pasteur.fr/klebsiella/klebsiella.html).

Twenty-seven ertapenem-resistant *K. pneumoniae* isolates were recovered from individual patients admitted to the University Hospital of Coimbra in 2013. The majority of isolates were recovered from urine ($n = 13$, 48·1%) and blood ($n = 7$, 25·9%); other sources were sputum ($n = 2$, 7·4%), and one isolate each from a bronchial aspirate, abscess, and wound with two isolates of unknown origin. Patients' age ranged from 23 to 82 years (average 52 years), and the majority were males ($n = 17$, 62·9%). The majority of the isolates were recovered from inpatients of the Surgery, Gastroenterology and Emergency wards (four from each ward, 14·8%), followed by the Renal Transplant and Cardiology/Cardiothoracic Surgery wards (three from each ward, 11·1%). The high proportion of isolates from urine and blood samples and the average age of the patients underlines the clinical importance of *K. pneumoniae* in urinary tract and bloodstream infections particularly in populations who have undergone catheterization or other invasive procedures.

All isolates were highly resistant to β-lactam antibiotics tested (cephalosporins, aztreonam and ertapenem). Fifteen isolates were classified as intermediate and one isolate as resistant to imipenem. This finding is consistent with the extensive use of β-lactam antibiotics in clinical practice worldwide where third-generation cephalosporins such as cefotaxime and ceftazidime, are increasingly used to treat infections due to β-lactamase producers, especially penicillinases, while the carbapenems (imipenem, meropenem, ertapenem, doripenem), are often reserved for severe infections due to ESBL-producing organisms.

The increasing global spread of carbapenemase-producing bacteria is of great concern. Here, we have shown a high prevalence of KPC-mediated carbapenem resistance in nosocomial isolates of ertapenem-resistant *K. pneumoniae* recovered in a short period of time. KPC was detected in 18/27 (66·6%) isolates and confirmed by sequencing to be KPC-3. Other β-lactamase families identified included TEM (21/27, 77·7%), SHV (11/27, 40·7%), and CTX-M types (19/27, 70·3%) and DHA-1 (2/27, 7·4%). TEM and SHV ESBLs are widely distributed among Enterobacteriaceae and are derived from the parental TEM-1&2 and SHV-1. TEM-1&2 are narrow spectrum enzymes, not ESBLs, and are the most common plasmid-mediated β-lactamase associated with ampicillin resistance in Gram-negative bacilli, mainly *E. coli*, while SHV-1 is produced by most *K. pneumoniae* [13]. The high prevalence of CTX-M-producing *K. pneumoniae* correlates with the global dissemination of CTX-M enzymes [14], in particular CTX-M-15, already found in this hospital both in *E. coli* and *K. pneumoniae* [15]. The phenotypic expression of production of ESBLs was corroborated by PCR but these genes, with one exception of CTX-M in a transconjugate, were not sequenced because it was not the focus of this study.

Although KPC has been increasingly identified in several countries, including in Europe [1], epidemiological reports from Portugal are scarce, the first identification of this enzyme in this country proved to be a KPC-2 *E. coli* producer isolated from an aquatic environment in 2011 [3]. Furthermore, a recent surveillance study of carbapenemases in Portugal reported KPC-3 in 22 *K. pneumoniae* collected from 2006 until early 2013 from diverse hospitals, scattered in time and geographical location, with ten different sequence types [4] and thus were sporadic cases. Of particular importance is the location of resistance genes on conjugative plasmids which facilitates their horizontal dissemination. In the present study, we identified four incompatibility groups of plasmids with IncF replicon type being the most prevalent (56%) followed by IncN (7·4%), IncHI1 (3·7%) and IncHI2 (3·7%). IncF is commonly detected in Enterobacteriaceae and has been associated with the dissemination of clinically important genes such those coding for CTX-M-15 and KPC enzymes [16]. All 18 KPC-3 producers were positive for IncF plasmid, which was successfully transferred from all isolates through conjugation to *E. coli* J53, and confirmed by antibiogram and PCR. By contrast, only one $bla_{\text{CTX-M-15}}$ determinant was successfully transferred which might suggest the presence of other plasmid types and/or chromosomal location for CTX-M genes. The PBRT protocol used here does not cover all plasmid types occurring in Enterobacteriaceae, and the CTX-M-15 gene, for example, has been found in non-self-transferable plasmids or integrated into the *K. pneumoniae* chromosome [16].

TEM-type determinants from all isolates proved to be transferable by conjugation while the SHV-type gene

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Table 1. *Sources of KPC- and non-KPC-producing* K. pneumoniae *and molecular typing*

Isolate	Collection date	Hospital ward	Sample	KPC	MLST	PFGE*
8Kp	January	Internal Medicine	Urine	Positive	ST15	3
17Kp	May	Surgery	Urine	Negative	ST11	5
39Kp	September	Cardiothoracic Surgery	Sputum	Negative	ST15	7
26Kp	October	Internal medicine	Urine	Positive	ST348	1
28Kp	November	Gastroenterology	Blood	Positive	ST348	2
13Kp	December	Emergency	Urine	Positive	ST15	1

KPC, *Klebsiella penumoniae* carbapenemase; MLST, multi-locus sequence typing; PFGE, pulsed-field gel electrophoresis.
*Arbitrary cluster profile.

was only transferred from one organism. The AmpC DHA-1 was not transferable. TEM-type enzymes are most often located on plasmids, while in *Klebsiella* spp. SHV-type may have a chromosomal origin. Plasmid extraction was unsuccessful with both the commercial kit and the standard Kado & Liu methodology [9], suggesting that these plasmids, whose presence was confirmed by the PBRT protocol, might be of high molecular size, which is frequent in *Klebsiella* spp., or exist as a low copy number.

Global spread of KPC strains has been linked to specific clones, particularly ST258 [17]. In this study, PFGE analysis revealed three clusters of KPC-producing isolates while all non-KPC were singletons (Table 1). MLST of selected KPC producers identified them as ST15 or ST348. Moreover, ST15 was also identified in non-KPC isolates together with ST11 (Table 1). PFGE profiles do not always accord with MLST, and for some species is often usually a more discriminatory method. This was illustrated here where isolates of identical sequence type showed heterogeneity of DNA patterns by PFGE. Indeed, a ST15 *K. pneumoniae* ertapenem-resistant lineage had been previously found in this hospital but the resistance was associated with modifications in the OmpK36-modified porin and not with carbapenemase production [2], while ST11 and ST348 were identified for the first time in this hospital, the latter being only reported previously in Tanzania, Southern Africa. KPC-producing *K. pneumoniae* ST15 was first detected in January 2013, while ST348 first appeared in October and November which coincided with a marked increase of KPC-3-producing isolates. It is possible that the resistant ST15 clone was already circulating in the hospital and acquired an IncF plasmid carrying the KPC-3 gene, while the novel ST348 clone harbouring $bla_{\text{KPC-3}}$ was imported into the hospital or possibly acquired the gene from ST15. Further characterization of the genetic environment of $bla_{\text{KPC-3}}$ may clarify this hypothesis. Moreover, being a tertiary-care hospital, the University Hospital of Coimbra receives many patients from other district hospitals that might originally have introduced a KPC-3 producer in the facility. However, the successful conjugation of $bla_{\text{KPC-3}}$ carried on an IncF replicon simultaneously with the transfer of TEM-type observed in this work, is supported by other studies where all transconjugants harboured $bla_{\text{KPC-3}}$ and $bla_{\text{TEM-1}}$ in IncF plasmids, although of different sequence types and from other hospitals [4]. Interestingly, we did not find the ST258 clone often associated with the dissemination of KPC, an observation in accordance with a previous report [4].

In conclusion, to our knowledge this is the first outbreak report associated with KPC-3 in Portugal, and confirms that the dispersion of KPC is not linked to a single clonal lineage, but is associated with highly successful conjugative IncF plasmids, since the determinant was present in a lineage already present in the hospital (ST15, previously KPC-negative), and in the uncommon ST348. Preliminary experiments suggest a potential for dissemination to other species (data not shown) which is worrisome and indicates that KPC-3 may spread fast in the clinical setting, both within and between hospitals. This reinforces the importance of continuous surveillance of antimicrobial resistance combined with screening for carbapenemase-producing isolates from patients on admission to hospital, and improvement of infection control.

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DECLARATION OF INTEREST

None.

REFERENCES

1. **Munoz-Price LS, *et al.*** Clinical epidemiology of the global expansion of *Klebsiella pneumoniae* carbapenemases. *Lancet Infectious Diseases* 2013; **13**: 785–796.
2. **Novais A, *et al.*** Spread of an OmpK36-modified ST15 *Klebsiella pneumoniae* variant during an outbreak involving multiple carbapenem-resistant *Enterobacteriaceae* species and clones. *European Journal of Clinical Microbiology and Infectious Diseases* 2012; **31**: 3057–3063.
3. **Poirel L, *et al.*** Environmental KPC-producing *Escherichia coli* isolates in Portugal. *Antimicrobial Agents and Chemotherapy* 2012; **56**: 1662–1663.
4. **Manageiro V, *et al.*** Predominance of KPC-3 in a survey for carbapenemase-producing *Enterobacteriaceae* in Portugal. *Antimicrobial Agents and Chemotherapy* 2015; **59**: 3588–3592.
5. **EUCAST.** European Committee on Antimicrobial Susceptibility Testing Breakpoint tables for interpretation of MICs and zone diameters European Committee on Antimicrobial Susceptibility Testing Breakpoint tables for interpretation of MICs and zone diameters 2014, pp. 1–77 (http://www.eucast.org/clinical_breakpoints). Accessed 15 January 2015.
6. **Mendonça N, *et al.*** Spread of extended-spectrum beta-lactamase CTX-M-producing *Escherichia coli* clinical isolates in community and nosocomial environments in Portugal. *Antimicrobial Agents and Chemotherapy* 2007; **51**: 1946–1955.
7. **Yigit H, *et al.*** Novel carbapenem-hydrolyzing b-lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy* 2001; **45**: 1151–1161.
8. **Dannelle C, *et al.*** Development of a set of multiplex PCR assays for the detection of genes encoding important beta-lactamases in *Enterobacteriaceae*. *Journal of Antimicrobial Chemotherapy* 2010, **65**: 490–495.
9. **Kado CI, Liu ST.** Rapid procedure for detection and isolation of large and small plasmids. *Journal of Bacteriology* 1981; **145**: 1365–73.
10. **Carattoli A.** Plasmids and the spread of resistance. *International Journal of Medical Microbiology* 2013; **303**: 298–304.
11. **Ribot EM, *et al.*** Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. *Foodborne Pathogens and Disease* 2006; **3**: 59–67.
12. **Tenover FC, *et al.*** Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *Journal of Clinical Microbiology* 1995; **33**: 2233–2239.
13. **Paterson DL, Bonomo RA.** Extended-spectrum beta-lactamases: a clinical update. *Clinical Microbiology Reviews* 2005; **18**: 657–86.
14. **D'Andrea MM, *et al.*** CTX-M-type beta-lactamases: a successful story of antibiotic resistance. *International Journal of Medical Microbiology* 2013; **303**: 305–317.
15. **Calhau V, *et al.*** Molecular characterization of *Klebsiella pneumoniae* isolated from renal transplanted patients: virulence markers, extended-spectrum beta-lactamases, and genetic relatedness. *Diagnostic Microbiology and Infectious Disease* 2014; **79**: 393–395.
16. **Coelho A, *et al.*** Characterisation of the CTX-M-15-encoding gene in *Klebsiella pneumoniae* strains from the Barcelona metropolitan area: plasmid diversity and chromosomal integration. *International Journal of Antimicrobial Agents* 2010; **36**: 73–78.
17. **Kitchel B, *et al.*** Molecular epidemiology of KPC-producing *Klebsiella pneumoniae* isolates in the United States: clonal expansion of multilocus sequence type 258. *Antimicrobial Agents and Chemotherapy* 2009; **53**: 3365–3370.