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ORIGINAL ARTICLE

Tracking KPC-3-producing ST-258 *Klebsiella pneumoniae* outbreak in a third-level hospital in Granada (Andalusia, Spain) by risk factors and molecular characteristics

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Abstract

The objective of this study was to determine clinical-epidemiological characteristics of the patients and the genetic characteristics of carbapenemase KPC-3-producing *Klebsiella pneumoniae* isolates belonging to sequence type ST258. The eligible study population was all patients with isolates detected between October 2015 and March 2017. Clinical–epidemiological and microbiological data were gathered on risk factors associated with infection by this clone. Antimicrobial susceptibility was determined using MicroScan system and diffusion in agar. Genes encoding carbapenemases were detected using PCR and Sanger sequencing. The sequence type was assigned by MLST, and the genetic relationship among clinical isolates was determined by pulsed field electrophoresis and by analysis of the genetic environment. The study included 23 individuals with isolates of KPC-3/ST258; the mean age was 77 year, and mean stay pre-isolation was 32 days; 81% received empirical antimicrobial treatment. Isolates were only susceptible to gentamicin (CIM ≤ 2 mg/L), tigecycline (CIM ≤ 1 mg/L), and colistin (CIM ≤ 2 mg/L). The isolates belonged to ST258, with five pulse types or subgroups. All isolates showed amplification of KPC, which was identified as KPC-3 variant. Gene $_{blaKPC-3}$ was flanked by insertion sequences *Kpn6* and *Kpn7* within *Tn4401* transposon isoform a. We report, for the first time in Spain, an 18-month outbreak by KPC-3-producing ST258 *K. pneumoniae*. Its acquisition was associated with a history of antimicrobial therapy, with three treatment options, and with high mortality. The detection of different pulse types is attributable to different introductions of the clone in our setting, supporting the need for multi-resistant isolate surveillance studies.

Keywords Carbapenemase · *Klebsiella pneumoniae* · Emerging infection

Introduction

Acquisition of carbapenemases-encoding genes by *Enterobacteriales*, especially by clinical isolates of *Klebsiella pneumoniae*, poses an increasing threat to public health worldwide [1]. Carbapenemase production one of the most frequent carbapenem-resistance mechanisms. These enzymes can hydrolyze beta-lactam antibiotics, including carbapenems, reducing therapeutic options to treatments of which there is little experience (e.g., ceftazidime/avibactam) or that can be toxic (e.g., colistin). Carbapenemases are usually linked to high-risk clones, whose predominant prototype worldwide is clonal complex 258, which permit their transfer and dissemination in the environment, producing infections with high mortality and morbidity rates [1–6].

In Spain, the first isolates of carbapenemase-producing enterobacteriaceae (CPE) were detected in 2005, with a type other than carbapenemase KPC. Since then there has been a 16-fold increase in the number of isolates of these microorganisms and a fivefold increase in the number of hospitals reporting their presence [4]. Spanish isolates have largely been producers of OXA-48, VIM-1, KPC-2, IMP, and NDM-1 types of carbapenemases, and the most frequent ST associations have been ST11, 15, 405, 16, 147, 340, 437,

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101, 464, 846, 13, 384, 388, 512, and 1235 [4, 7, 8]. KPC-3 has been detected in isolates from patients from Spanish hospitals; however, there has been no report on the presence of outbreaks related to clone ST258 in Spain, limiting our knowledge of their clinical and epidemiological impact [9]. In October 2015, our group described the only case reported to date in Spain, in a patient with urinary tract infection [10]. The objective of this study was to determine clinical-epidemiological characteristics of the patients and genetic characteristics of the isolates of *K. pneumoniae* with KPC-3 belonging to clone ST258 (*K. pneumoniae* KPC-3/ST258) from a third level hospital.

Materials and methods

Study population

The study included consecutive patients with *K. pneumoniae* KPC-3/ST258 isolates detected between October 2015 and March 2017 in the *Virgen de las Nieves* University Hospital of Granada, with a catchment population of 440,000 individuals in the province of Granada (Andalusia region, Southern Spain). Isolates were identified using the MicroScan system (Beckman Coulter, USA) and mass spectrometry (Maldi-Tof®, Bruker Daltonik GmbH, Germany). Resistance was characterized using the MicroScan system with subsequent carbapenemase determination, when applicable, by the NG-Test (Carba, NG Biotech, France). Results were confirmed by the Andalusian Molecular Typing Laboratory of the Spanish PIRASOA Program. Clinical-epidemiological data of the patients and microbiological data of their infection were gathered from their computerized clinical records, followed by analysis of the risk factors for acquisition of infection by this microorganism.

Clinical-epidemiological data

Data were gathered on community/hospital acquisition, total hospital stay, and hospital stay before the detection of *K. pneumoniae* KPC-3/ST258. In our hospital, carriers of these isolates were investigated with a rectal smear whenever possible.

We specified whether the occupation of the patient's hospital room was single, double, or triple at the time of detection. A close follow-up of patients was conducted, identifying the different hospital rooms occupied to analyze the transmission of isolates during the outbreak and recording the time intervals between analysis request and detection of the carbapenemase-producing bacteria and between receipt of the microbiological result and the implementation of preventive measures.

We recorded whether patients had previously presented isolates of *K. pneumoniae* KPC-3/ST258 and whether concomitant intestinal colonization and persistence of colonization after 30 days had been evaluated. Accordingly, systematic surveillance samples were taken from the rectal smear of patients and their contacts, which were incubated for 48 h in selective media for carbapenemases-producing bacteria (CHROMID® CARBA SMART, BioMerieux, Spain), identifying colonies by Maldi-TOF followed by carbapenemase determination (NG-Test Carba, NG Biotech, France). We recorded deaths during hospitalization and the time interval between *K. pneumoniae* KPC-3/ST258 detection and death.

Among possible risk factors for infection by *K. pneumoniae* KPC-3/ST258, we recorded whether patients had undergone urological manipulation during the previous week and whether they were carriers of a permanent urinary catheter or had been provisionally catheterized to gather urine samples. We specified the time spent with the same vesical catheter, the use of interventionist procedures (surgical or endoscopic techniques, presence of central, nasogastric, or PEG catheter), the length of stay in the intensive care unit, the use of mechanical ventilation (invasive or non-invasive), the receipt of antibiotherapy for more than 48 h in the previous week, the infection focus, qSOFA/SOFA scale sepsis scores, and the Charlson severity index. Data were also collected on the following factors related to immune suppression: presence of neutropenia, neoplasia, chemotherapy during previous month, transplantation, and corticoid treatment (> 10 mg/day prednisone or equivalent for > 2 weeks) [11]. We identified the presence of renal insufficiency by glomerular filtration (GF) estimation from the serum creatinine concentration, age, and sex by the CKD-EPI equation expressed in mL/min/1.73 m^2. Based on the GF estimation, renal insufficiency was classified as mild, moderate, or severe, and the need for renal replacement therapy was evaluated [12]. The presence of the following associated diseases was recorded, including diabetes mellitus, heart failure, ischemic heart disease, cerebrovascular disease, previous cognitive impairment, or HIV-positive serology.

Interventions of infection control

Control measures implemented after detection of the isolate were: contact precautions, including a single room for the exclusive use of the patient, special hand hygiene measures, information sheet for the patient, appropriate use of invasive devices, room cleaning, elimination of residues, and the study of contacts with the patient.

Microbiological study

Isolates were referred to the reference center of Andalusia (PIRASOA) for microbiological characterizations. The

antibiotic susceptibility study was performed in 41 isolates from clinical samples with indications of infection and/or colonization, including 1 or 2 per patient: 11 (26.8%) from urine, 21 (51.2%) from rectal smear (for colonization studies of patients and contacts), 4 (9.7%) from blood culture, 3 (7.3%) from abdominal fluid, and 2 (4.8%) from other matrices. The susceptibility study used NegCombo 44 panels of the MicroScan system® (Beckman-Coulter Inc., Alcobendas, Madrid, Spain), and diffusion in agar with ertapenem, meropenem, and imipenem disks, applying EUCAST 2018 cutoff points for clinical categories [13]. Imipenem hydrolysis was studied with the β-Carba Test (BioRad, Alcobendas) and meropenem activity inhibition (by dipicolinic acid, boronic acid, and cloxacillin) using disk diffusion (Rosco, Barcelona, Spain) [13]. Carbapenemase gene detection used PCR with specific primers for KPC, NDM, VIM, IMP, and OXA-48 groups (Supplementary Table), followed by Sanger sequencing [14].

We selected one isolate per patient for molecular typing, except for the two morphotypes from one biopsy sample (total of 24), performing massive sequencing using Illumina MiSeq 300-bp (WGS) with a mean coverage of 75, de novo assembling the result with CLC Workbench 9.01.1 (Qiagen, Las Rozas, Madrid, Spain). Resistance determinants were established with Resfinder and the comprehensive antibiotic resistance database (CARD). The genetic environment of bla_{KPC-3} was studied by analyzing mobile elements (35,639 bp, 30% coverage) at the site of the *bla* gene with the online MARA application (http://mara.spokade.com), determining the isoform according to the sequence before the gene [15].

Molecular typing

ST was assigned by MLST (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html). The relationship among isolates was analyzed by pulsed-field gel electrophoresis (PFGE) (http://www.pulsenetinternational.org/protocols/pfge.asp), using the *XbaI* restriction enzyme. Dendograms were generated with the Fingerprinting 3.0 application (Applied Mathematics), using the Dice index and 1% band position tolerance to compare genetic profiles. Isolates with a difference of < 3 bands (i.e., similarity > 94%) were assigned to the same pulse type.

Results

Between October 2015 and March 2017, 23 patients with isolates of *K. pneumoniae* KPC-3/ST258 were treated in our hospital: 17 (74%) were infected and 6 (26%) colonized at intestinal level.

Two of the colonized individuals were excluded from the clinical-epidemiological analysis due to incomplete clinical records. This analysis therefore included 17 infected and 4 colonized patients. Table 1 exhibits their clinical characteristics. All patients were diagnosed for the first time in our hospital. Among the 17 patients, 10 (58.8%) had urinary tract infection (alone in 7 and with sepsis in 3), 3 (17.6%) had pneumonia, and 4 (23.5%) had peritonitis associated with surgical wound infection. All patients were treated with antimicrobials before detection of the *K. pneumoniae* KPC-3/ST258 isolate: 39.1% with meropenem, 30.4% with ceftriaxone, 17.4% with moxifloxacin, 17.4% with ertapenem, 17.4% with piperacillin/tazobactam, 13.0% with ciprofloxacin, 8.6% with levofloxacin, 8.6% with amoxicillin/clavulanic acid, 8.6% with amikacin, 8.6% with ceftazidime, 4.3% with gentamicin, and 4.3% with cefepime.

The mean time interval between culture request and detection of *K. pneumoniae* KPC-3/ST258 was 2.57 ± 1.86 days. The mean delay between detection and implementation of preventive measures was 2.19 ± 1.77 days.

Out of the 12 patients (70.6%) dying in hospital, 11 died within 30 days after detection (5 in the first 15 days and 6

Table 1 Clinical and epidemiological characteristics of 21 individuals with KPC-3-producing ST258 *K.pneumoniae* in the Virgen de las Nieves Hospital of Granada

Characteristics	
Female sex	14 (66.7)
Mean age[a]	77.19 (11.2)
Charlson score[a]	7 (2.8)
Comorbidities	
Renal insufficiency	15 (71.4)
Cerebrovascular event	10 (47.6)
Ischemic heart disease	3 (14.3)
Heart failure	6 (28.6)
Diabetes	9 (42.9)
Cognitive impairment	9(42.9)
Use of > 10 mg corticosteroids for > 15 days	8(38.1)
Severe neutropenia	1 (4.8)
Solid organ transplantation	1 (4.8)
Median hospital stay (days) pre-isolation[a]	31.67 (29.5)
Presence of invasive devices	
Vesical catheter	16 (76.2)
Central venous catheter	4 (19.0)
Nasogastric catheter	5 (23.8)
Endoscopic gastrostomy catheter	1 (4.8)
Mechanical ventilation	1 (4.8)
Surgery	7 (33.3)
Mean no days receiving antibiotics before KPC-3 ST258 isolation[a]	17 (15.9)
Associated mortality	12 (57.2)

[a]Mean (SD)

in the second). Out of the four patients with bacteremia by *K. pneumoniae* KPC-3/ST258, all treated with tigecycline, two (50%) died in hospital due to this microorganism; the bacteremia was catheter-associated in the two non-survivors and derived from a urinary focus in the two survivors (currently alive).

At the time of the diagnosis, 11 (52.4%) of the patients were in single rooms, 7 (33.3%) in double rooms, and 3 (14.3%) in rooms with triple occupation. Ten (47.6%) of the patients had shared the same room; and 16 (76.2%) had been on the same floor and/or been treated by the same health staff. Intestinal colonization was investigated in 15 patients and was positive in all cases; follow-up of 7 of these patients showed persistence of the colonization for at least 30 days in 6 of them. In 16 (77.7%) of the patients, the isolate was detected in more than one clinical sample.

Microbiological characteristics of isolates

Resistance to amoxicillin/clavulanic acid, piperacillin/tazobactam, cefotaxime, ceftazidime, cefepime, ertapenem, meropenem, ciprofloxacin, levofloxacin, amikacin, and tobramycin was observed for all isolates from all 23 patients studied (one isolate per patient except for the recovery of two morphotypes from a single biopsy sample). Intermediate susceptibility to imipenem (8 mg/L) and cefoxitin (16 mg/L) was found in 16 (66.7%) of the 24 isolates studied. Susceptibility to fosfomycin (≤ 32 mg/L) was observed in 2 (8.3%) of the isolates and to trimetoprim-sulfametoxazol (≤ 2/38 mg/L) in 3 (12.5%). We highlight the susceptibility to gentamicin (≤ 2 mg/L), tigecycline (≤ 1 mg/L), and colistin (≤ 2 mg/L) found for all isolates.

We selected one isolate per patient for molecular typing, except for the two morphotypes from one biopsy sample. All isolates belonged to ST258. Five pulse types (A–E) were differentiated by *XbaI* PFGE (Fig. 1 and Supplementary Figure). An initial pulse type (A) was found for seven isolates, a second pulse type (B) for fourteen isolates; and three subsequent pulse types (C–E) for one isolate each. The two isolates recovered from the single biopsy sample belonged to the same pulse type, with only one band of difference. KPC-3 and TEM-1 were detected in all isolates. Gene bla_{KPC-3} was flanked by insertion sequences *Kpn6* and *Kpn7* within *Tn4401* transposon isoform a (Fig. 2).

The time course distribution of patients is depicted in Fig. 3, highlighting that three patients with different pulse

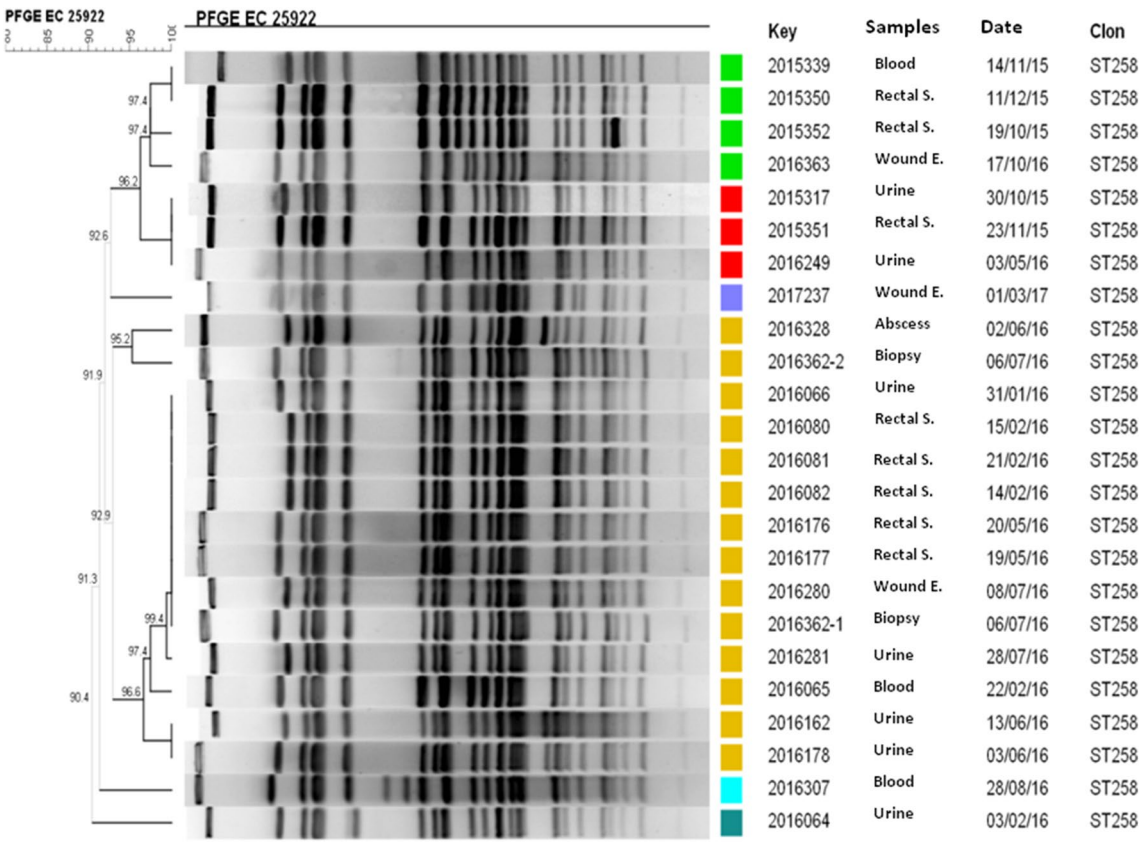

Fig. 1 Dendograms of the genetic profiles of isolates and their sequence type. Each pulse type is separated by a discontinuous line and with a color square. (Color figure online)

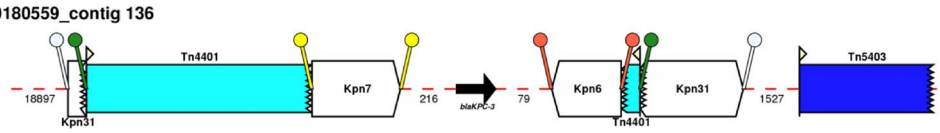

Fig. 2 Graphic representation of the genetic environment of KPC-3

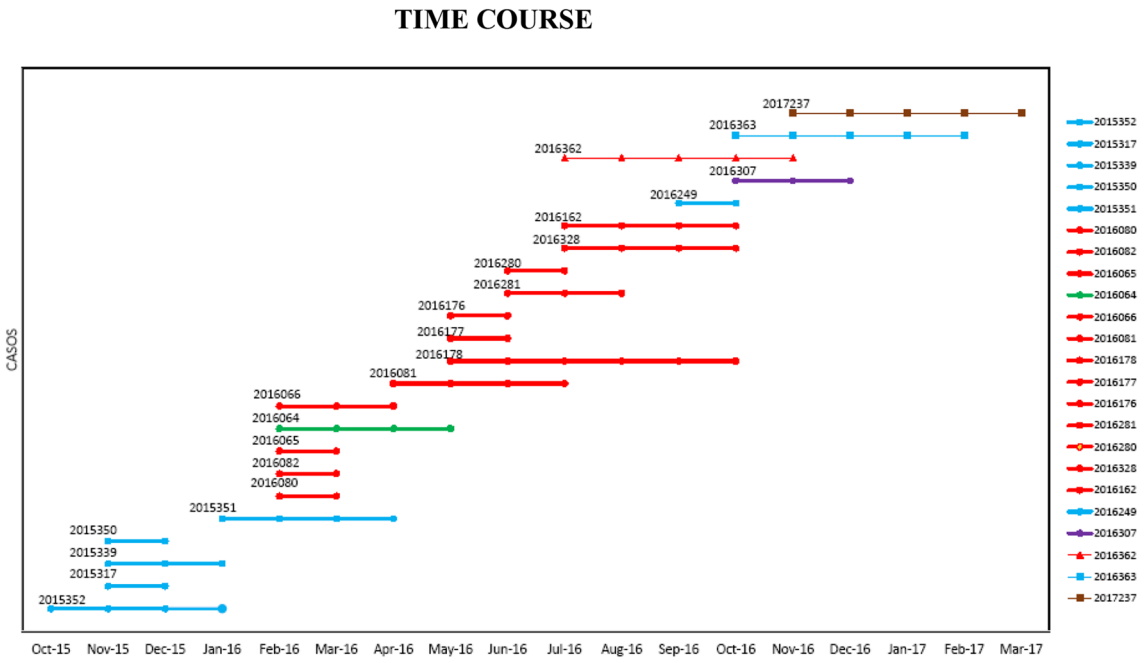

Fig. 3 Distribution of patients during the study period

types had been in other health centers (2016064-green line-; 2016307-purple line), but one (2017237-brown line) had not, although the stay of this patient in our center was > 4 months.

Discussion

We present the first report in our country of an outbreak caused by the international clone of *K. pneumoniae* ST258/KPC-3, which had first been detected in Spain in 2016 [10]. Relevant pre-detection factors in this first patient had included catheterization during two previous hospital stays and antibiotic treatment with non-carbapenem fluoroquinolones and beta-lactams. Our laboratory publishes annual surveillance reports on the susceptibility of bacteria to antibiotics (https://www.huvn.es/asistencia_sanitaria/microbiologia/mapa_microbiologico), and this was the first outbreak of infection by *K. pneumoniae* detected in our setting.

In the present study, most of the patients had a prolonged hospital stay, comorbidities, and a history of antimicrobial treatment, which have previously been identified as risk factors for the acquisition of infections by *K. pneumoniae* with KPC, especially treatment with third- or fourth-generation cephalosporins [16]. Few data have been reported on its association with surgical procedures, which was found in around one-third of our patients, suggesting the need for further investigation of this risk factor, especially in surgical patients requiring prolonged hospitalization [17].

Although we did not conduct a wide-ranging systematic search for carriers, all known contacts of patients positive for *K. pneumoniae* KPC-3 ST258 were also positive, consistent with previous reports on the transmission of this infection in outbreaks [18].

As previously reported, the isolates showed high co-resistance percentages, leaving colistin, gentamycin, ceftazidime/avibactam, and tigecycline as the sole therapeutic options in many cases [4, 19].

With regard to the bla_{KPC} gene, our isolates showed only the presence of KPC-3 in clone ST258. The bla_{KPC} gene has been detected in multiple clinical isolates and 23 variants have been reported, most frequently KPC-2 in Colombia, Brazil, Argentina, Ecuador, and Venezuela [17, 20] and KPC-3 in Europe [21]. However, although the bla_{KPC} gene has been more frequently isolated in *K. pneumoniae* ST 258, it has also been found in other species of

Gram-negative bacilli [22]. Studies in Spain have reported KPC-3 dissemination in *K. pneumoniae*, especially by clones ST512, ST348, and ST388 [8], although its isolation in Spain has been infrequent, unlike other CPEs [23]. Before the present outbreak, an outbreak by clone *K. pneumoniae* ST512/KPC-3 was reported in a neighboring area, originating with a patient from Italy [8].

KPC-3 has also been associated with other microorganisms, such as *E. cloacae* ST114 in an outbreak produced in a burns unit in the USA, whose transmission was related to mobile *Tn4401b* transposon [23]. Studies of the genetic environment of *blaKPC* indicate that its mobility and dissemination are due to *Tn4401* transposon, related to *Tn3*. *Tn4401* is an element of 10 Kb, delimited by two inverted repeated sequences of 39 pb, and it houses transposase and resolvase genes and two insertion sequences, ISKpn6 and ISKpn7, besides the blaKPC-2 gene [19]. Seven isoforms (a–g) of *Tn*4401 have been reported, differentiated by polymorphisms located above the bla_{KPC} gene [22]. Because this region includes the *blaKPC* promoter, variations affect the expression of messenger RNA of *blaKPC*. This transposon has been identified in isolates from different geographical locations and different STs in enterobacteria and *Pseudomonas* spp. [24]. In the present study, sequences Kpn6 and Kpn7 were found within *Tn* 4401 transposon isoform a. Isoform "b" has been more frequently observed in Venezuela, USA, Colombia, and Brazil [19], although outbreaks in the United Kingdom have been associated with *Tn4401a* in isolates of bla_{KPC} [25]. The carbapenemase bla_{KPC3} gene has been associated with *Tn4401a* in Italy, the USA, Poland, and Spain [7, 26, 27]. This is an important finding, because isoform a is usually linked to bla_{KPC-2} and only rarely to bla_{KPC-3} [7].

K. pneumoniae ST258 emerged as a serious clinical challenge in the USA mid-way through 2000 and has been the most frequently detected ST in that country, as in some Latin American and European countries [26, 28]. Likewise, ST258 was the predominant clone in the present study, whereas a study of 83 hospitals in Spain during 2013 found the most prevalent STs to be ST11 and ST405 for *K. pneumoniae* [4]. We highlight that outbreaks produced by carbapenemase-producing *K. pneumoniae* have been reported in Spain since 2007, and two ST variants (ST437 and ST340) have been associated with ST11 in the Spanish cities of Madrid, Malaga, and Alicante [4, 6, 8, 29–36] (Fig. 4).

The question arises whether these entities have recently emerged after the acquisition of resistance determinants or whether they were already a majority group, with multiple episodes of resistance mechanism acquisition serving as vehicle for their dissemination.

Analysis of the characteristics of the two patients who died from bacteremia revealed the involvement of bacteremia by *K. pneumoniae* KPC/ST258 in their prognosis (morbidity and mortality). The antimicrobial regimen was similar for all four patients with bacteremia, who all received tigecycline. The worst prognosis was for patients with catheter-associated bacteremia.

The specificity of the clonality results allowed us to establish a spatiotemporal sequence (Fig. 3) and identify possible transmission mechanisms between patients. In the majority of cases, colonized/infected patients shared the same physical space or the same health care professionals, demonstrating that preventive measures put in place were

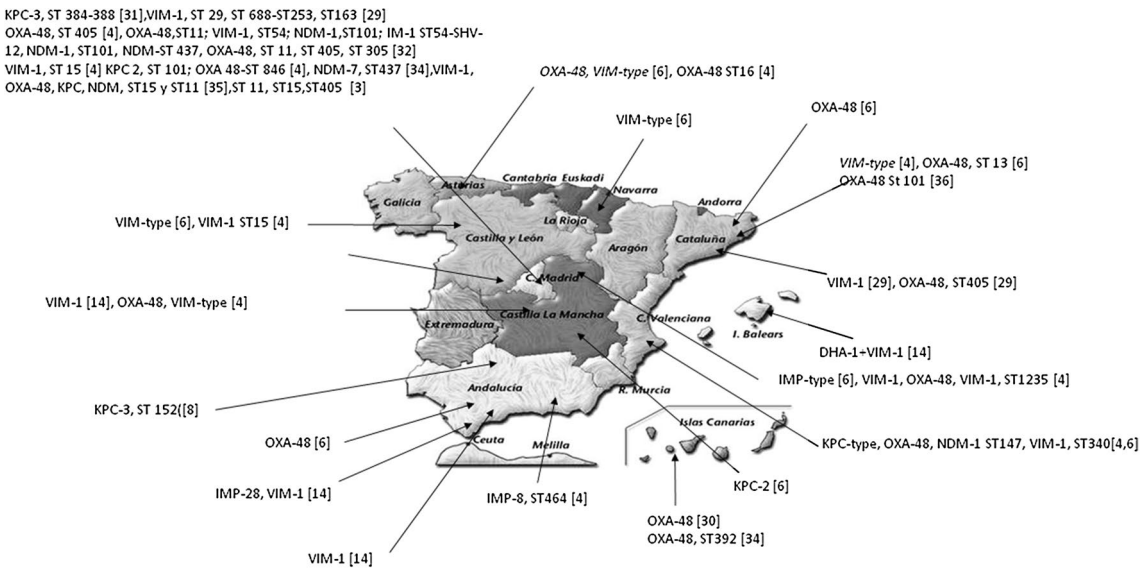

Fig. 4 Sequence types of carbapenemase-producing *K. pneumoniae* reported in Spain

belated or inadequate. Various pulse types or sub-lineages appeared sequentially over time. This may be explained by the transfer of colonized patients from other centers and/or by their development during transmission in our center. Several of these patients had been in other health centers and another had a prolonged hospital stay.

Finally, although the use of carbapenems was not controlled in departments where cases were detected, the planning and design of actions to control and prevent infections by multi-resistant bacteria should also consider the impact of prevalent or emergent clones on the evolution of the resistance of these species. These actions can also be useful to develop diagnostic, therapeutic, and vaccine methods to combat infections by multi-resistant *K. pneumonie* [25].

One limitation of our study is its retrospective character, which limited the gathering of data on these patients.

In conclusion, we report the presence for 18 months in a third-level Spanish hospital of KPC-3-producing *K. pneumoniae* of clone ST258, whose acquisition was associated with a history of antimicrobial treatment and invasive devices. The mortality rate was high in these patients, and the isolates were only susceptible to colistin, tigecycline, and gentamicin. These findings support the need for local, regional, and national collaboration to continue with antimicrobial resistance surveillance schemes and to strengthen programs for the rational use of antibiotics and the control of infections worldwide.

Funding None.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval The study protocol was carried out in accordance with the Declaration of Helsinki. This was a non-interventional study with no additional investigation to routine procedures. Biological material was only used for standard infection diagnostics following physicians' prescriptions. No additional sampling or modification of the routine sampling protocol was performed. Data analyses were carried out using an anonymous database. For these reasons, ethics committee approval was considered unnecessary according to national guidelines. The Clinical Microbiology Clinical Management Unit of the University Hospital Virgen de las Nieves of Granada (Spain) granted permission to access and use the data.

Informed consent The study protocol was carried out in accordance with the Helsinki Declaration. Data analyses were performed using an anonymous database. Therefore, approval was considered unnecessary according to the guidelines of our country (Law on Data Protection -Organic Law 15/1999 of 13 December on the protection of data of a personal nature, https://www.boe.es/buscar/doc.php?id=BOE-A-1999-23750).

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