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The spread of KPC-producing Enterobacteriaceae in Spain: WGS analysis of the emerging high-risk clones of *Klebsiella pneumoniae* ST11/KPC-2, ST101/KPC-2 and ST512/KPC-3

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Objectives: We analysed the microbiological traits and population structure of KPC-producing Enterobacteriaceae isolates collected in Spain between 2012 and 2014. We also performed a comparative WGS analysis of the three major KPC-producing *Klebsiella pneumoniae* clones detected.

Methods: Carbapenemase and ESBL genes were sequenced. The Institut Pasteur MLST scheme was used. WGS data were used to construct phylogenetic trees, to identify the determinants of resistance and to *de novo* assemble the genome of one representative isolate of each of the three major *K. pneumoniae* clones.

Results: Of the 2443 carbapenemase-producing Enterobacteriaceae isolates identified during the study period, 111 (4.5%) produced KPC. Of these, 81 (73.0%) were *K. pneumoniae* and 13 (11.7%) were *Enterobacter cloacae*. Three major epidemic clones of *K. pneumoniae* were identified: ST11/KPC-2, ST101/KPC-2 and ST512/KPC-3. ST11/KPC-2 differed from ST101/KPC-2 and ST512/KPC-3 by 27819 and 6924 SNPs, respectively. ST101/KPC-2 differed from ST512/KPC-3 by 28345 SNPs. Nine acquired resistance genes were found in ST11/KPC-2, 11 in ST512/KPC-3 and 13 in ST101/KPC-2. ST101/KPC-2 had the highest number of virulence genes (20). An 11 bp deletion at the end of the *mgrB* sequence was the cause of colistin resistance in ST512/KPC-3.

Conclusions: KPC-producing Enterobacteriaceae are increasing in Spain. Most KPC-producing *K. pneumoniae* isolates belonged to only five clones: ST11 and ST512 caused interregional spread, ST101 caused regional spread and ST1961 and ST678 produced independent hospital outbreaks. ST101/KPC-2 had the highest number of resistance and virulence genes. ST101/KPC-2 and ST512/KPC-3 were recently implicated in the spread of KPC in Italy.

Introduction

The spread of carbapenemase-producing Enterobacteriaceae (CPE) has posed a challenge for health services worldwide.[1] Plasmid-mediated carbapenemases detected in Enterobacteriaceae are usually KPC, VIM, NDM, IMP or OXA-48 types.[2–4] KPC carbapenemases have proliferated worldwide and have become endemic in hospitals in the north-east USA, Israel, Greece and Italy.[2,4,5–8] Cases in which KPCs are involved have also been reported in other European countries; some of these cases are linked to people who have travelled to endemic areas.[2,9,10] The spread of KPC enzymes is a concern because of the limited number of therapeutic options available and the high rate of associated morbidity.[11]

Recent studies in Spain have revealed an increase in the prevalence of CPE isolates,[9,12] mainly due to OXA-48 and VIM-1 producers.[9] A smaller percentage of isolates (2.1%–3.4%) was found to produce KPC.[9,12] Nevertheless, over the last few years, large KPC-associated nosocomial outbreaks have been reported.[13,14]

WGS can be applied to the study of MDR epidemic clones in a way that can improve our understanding of their evolution.[15,16]

The aims of this study were: (i) to characterize the microbiological traits, population structure and evolutionary trends of KPC-producing Enterobacteriaceae in Spain; and (ii) to carry out

a comparative WGS analysis of the three major KPC-producing epidemic clones in *Klebsiella pneumoniae*.

Materials and methods

Study design and bacterial isolates

Since 2009, our public health and reference institute has been operating an active and unrestricted national antibiotic resistance surveillance programme.[12] All Enterobacteriaceae isolates non-susceptible to carbapenems detected under the surveillance programme between January 2012 and December 2014 were included in this study. Only the first isolate per patient was analysed.

Enterobacteriaceae isolates were identified using standard microbiological methods and the API 20E identification system (bioMérieux, France). When necessary, species identification was confirmed by 16S ribosomal DNA sequencing.

Antibiotic susceptibility and phenotypic characterization of carbapenemase production

Following EUCAST guidelines, the disc diffusion and microdilution methods were used to test antibiotic susceptibility.[17,18] EUCAST screening cut-off values were used to identify CPE.[19] All of the isolates were analysed with both the Carba NP method[20] and a modified Hodge test. Carbapenemase activity was inhibited with EDTA, phenylboronic acid and cloxacillin, according to EUCAST guidelines.[19]

Characterization of resistance mechanisms and genetic environment

Genes encoding carbapenemases[5] and ESBLs[21] were detected using PCR and DNA sequencing assays.

The genetic environment of bla_{KPC} was studied by long-PCR mapping as previously described.[22] All PCR products were sequenced and compared with sequences available in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/).

Molecular epidemiology

Carbapenemase-producing *K. pneumoniae* and *Escherichia coli* isolates were typed using the MLST schemes of the Institut Pasteur (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html) and the University of Warwick (Warwick Medical School, Coventry, UK; http://mlst.warwick.ac.uk).

WGS, SNP tree and core genome MLST analysis

WGS was performed on the genomes of one representative isolate of each of the three major *K. pneumoniae* clones that had been revealed by MLST.

DNA was extracted using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany). Genomic DNA paired-end libraries were generated using the Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA, USA). These libraries were sequenced using an Illumina MiSeq next-generation sequencer with 2×300 bp paired-end reads (Illumina). The short reads acquired were subsequently assembled *de novo* into contigs using Velvet[23] under parameters optimized to give the highest N50 value. The draft genomes were ordered using the Artemis Comparison Tool[24] and Abacas[25] and then uploaded to the Rapid Annotation using Subsystem Technology (RAST) server for subsystem classification and functional annotation.[26]

Antimicrobial resistance genes were analysed using the ResFinder tool (https://cge.cbs.dtu.dk/services/ResFinder/) using an identity threshold of 98%. In order to reconstruct the plasmids of each genome, the recently developed methodology Plasmid Constellation Networks (PLACNET) was used.[27]

Virulence genes were identified using the BIGSdb-Kp database (Institut Pasteur; http://bigsdb.web.pasteur.fr).[28] For comparison purposes, the whole-genome sequences of three previously described KPC-producing *K. pneumoniae* strains were included in the analysis of resistance and virulence genes. These were: KPN1H1/ST258, ST512-K30BO and HS11286/ST11 (GenBank accession numbers NZ_CP008827.1, HS11286 and CAJM00000000.2, respectively).[29–31] These strains had recently been correlated with outbreaks in countries outside Spain.

Sequence reads were mapped to the chromosome of *K. pneumoniae* NTUH-K2044[32] (GenBank accession no. NC_012731.1). Subsequently, the software package Burrows-Wheeler Aligner (BWA) was used to identify SNPs.[33] Sequence reads from other bla_{KPC}-carrying *K. pneumoniae* isolates were included in order to establish phylogenetic relationships (GenBank accession numbers NZ_CP008827.1, CAJM00000000.2, AFXH01000001.1, AFQL01000001.1, AJJI01000001.1, AKAF01000001.1, AKAJ01000001.1, AKAN01000001.1, AMLO01000001.1, ATAK01000001.1, CANR01000001.1, NZ_JH930438.1, CP003200.1, CP003785.1, AFQK01000001.1, CP002910.1, NZ_JH930400.1, NZ_KI517376.1, CP000647.1, NZ_JH930419.1, NZ_JH930422.1, LGAB01000001.1 and CAJM02000001.1).

Sequence reads mapped to an average of 92% of the reference genome, with a mean depth of 75x in mapped regions across sequenced isolates. SNPs were identified as described by Harris *et al.*[34] and pairwise comparisons were carried out. SNPs called in integrative and conjugative elements and phage sequences that had been identified using a prophage locus prediction tool[35] were excluded. Sequences were suspected of having resulted from homologous recombination when an elevated SNP density was observed using Gubbins software.[36]

A maximum-likelihood phylogenetic tree was reconstructed as previously described.[16,37] A set of 694 genes was considered as the core genome MLST (cgMLST)[28] and cgMLST-based minimum spanning trees were calculated and drawn using Ridom SeqSphere+ software (Ridom, Munster, Germany).

Results and discussion

Bacterial isolates and carbapenemase types

Between January 2012 and December 2014, 2443 CPE isolates were identified, of which 111 (4.5%) were KPC-producing Enterobacteriaceae: 8 of these were isolated in 2012 (3.4% of the 237 CPE isolates studied), 34 in 2013 (4.4% of 775) and 69 in 2014 (4.8% of 1431). The 111 KPC isolates were collected in 29 Spanish hospitals located in 13 geographical areas.

Of the bacterial species carrying bla_{KPC}, the most prevalent was KPC-2-producing *K. pneumoniae* (59.5%) (Table 1). In Spain,

Table 1. Bacterial species carrying bla_{KPC} genes in Spain according to the Spanish Antibiotic Resistance Surveillance Program (2012–14)

Species	Number of isolates (%)	Carbapenemase types (*n*)
Klebsiella pneumoniae	81 (73.0)	KPC-2 (66)[a], KPC-3 (15)
Enterobacter cloacae	13 (11.7)	KPC-2 (12), KPC-3 (1)
Citrobacter freundii	9 (8.1)	KPC-2 (9)
Escherichia coli	4 (3.6)	KPC-2 (4)
Klebsiella oxytoca	2 (1.8)	KPC-2 (2)[b]
Serratia marcescens	1 (0.9)	KPC-2 (1)
Enterobacter aerogenes	1 (0.9)	KPC-2 (1)

[a]Two isolates coproduced both KPC-2 and OXA-48 carbapenemases.
[b]One isolate coproduced both KPC-2 and VIM-1 carbapenemases.

OXA-48 is the most common carbapenemase identified in Enterobacteriaceae;[9,38] however, the number of isolates producing KPC enzymes has significantly increased over the last few years.[9]

Population structure of KPC-producing Enterobacteriaceae isolates

The 81 KPC-producing *K. pneumoniae* isolates were grouped into three primary STs: ST11 (46 isolates), ST101 (13 isolates) and ST512 (10 isolates) (Table 2). ST11 and ST512 were involved in the interregional spread of *K. pneumoniae* and ST101 in the regional spread. ST101 predominated in 2012 (57.1%), ST512 appeared in 2013 and ST11 widely disseminated in 2013 and 2014.

The KPC-3-producing ST512 caused a severe outbreak in Cordoba,[13] Andalucía, Spain, whose index case was a patient transferred from Italy. ST512 is one of the most frequent STs of KPC-producing *K. pneumoniae* isolates in Italy.[4,39,40] ST11 is one of the STs most commonly associated with the production of various types of carbapenemases.[4,9] Both ST11 and ST512 are single-locus variants of the globally disseminated high-risk clone ST258,[41] which was not detected in this study. However, ST258 was shown to be a hybrid derivative of ST11, in which a substantial part of the genome has been replaced.[42] ST101 is an emerging clone that is unrelated to ST258, previously described in carbapenem-resistant *K. pneumoniae* isolates producing OXA-48,[43] KPC-2[12,40,44] and CTX-M-15 plus OmpK35 porin loss.[45]

The four KPC-producing *E. coli* isolates were unrelated and belonged to four different STs: ST131, ST90, ST355 and ST569. The high-risk *E. coli* clone ST131 had previously been described as producing, albeit infrequently, different types of carbapenemases.[9,46]

The genetic relationship between *Enterobacter cloacae* and *Citrobacter freundii* isolates, as determined by PFGE after digestion of chromosomal DNA with XbaI, is shown in Figure S1 (available as Supplementary data at *JAC* Online).

Table 2. Distribution of *K. pneumoniae* MLST clones producing KPC carbapenemases in Spain according to the Spanish Antibiotic Resistance Surveillance Program (2012–14)

ST	Carbapenemase	Number of cases	Number of hospitals	Provinces
11	KPC-2	44	13	Madrid, Toledo, Ciudad Real, Guadalajara and Cuenca
	KPC-3	2	2	Madrid
101	KPC-2	13	5	Madrid
512	KPC-3	10	7	Jaén, Málaga, Badajoz, Murcia, Toledo and Madrid
1961	KPC-2	5	1	Pontevedra
678	KPC-3	3	1	Madrid
359	KPC-2	1	1	Madrid
565	KPC-2	1	1	Madrid
1962	KPC-2	1	1	Madrid
1963	KPC-2	1	1	Almeria

Analysis of the major KPC-producing K. pneumoniae clones

ST11, ST101 and ST1961 isolates produced KPC-2, whereas ST512 and ST678 isolates produced KPC-3 (Table 2). Isolates of the ST101/KPC-2, ST1961/KPC-2 and ST678/KPC-3 clones coproduced the ESBLs CTX-M-15, CTX-M-14 and SHV-41, respectively. ESBL genes were not found in isolates of ST11/KPC-2 and ST512/KPC-3 clones. KPC-producing *K. pneumoniae* generally coproduce ESBLs with low frequency,[46] but this assertion is mainly based on observations on clonal complex 258. New KPC-producing clones that also produce ESBLs are emerging.[47]

The ST11/KPC-2 isolates were resistant to almost all of the antibiotics tested, but were fully susceptible to tigecycline, colistin, tetracycline and fosfomycin; 88.9% were susceptible to amikacin and 22.2% to gentamicin and tobramycin. All ST512/KPC-3 isolates were susceptible to tigecycline and gentamicin, while only 20% were susceptible to colistin. ST101 isolates were fully susceptible to tigecycline and colistin; one isolate (7.7%) was also susceptible to co-trimoxazole.

WGS of ST11/KPC-2, ST101/KPC-2 and ST512/KPC-3 clones

According to WGS data, ST11/KPC-2 differed from ST101/KPC-2 and ST512/KPC-3 by 27 819 and 6924 SNPs, respectively. ST101/KPC-2 differed from ST512/KPC-3 by 28 345 SNPs.

The metrics of all assemblies and the contig annotation results obtained with RAST are laid out in Table S1. Using this approach, the presence of 103, 109 and 128 genes related to antibiotic and toxic compound resistance was detected in ST11/KPC-2, ST101/KPC-2 and ST512/KPC-3 clones, respectively.

To show to what extent ST11/KPC-2, ST101/KPC-2 and ST512/KPC-3 clones are genetically related to previously sequenced KPC-producing *K. pneumoniae* isolates from other countries, we identified 83 521 high-quality SNPs with respect to the whole-genome sequence of *K. pneumoniae* strain NTUH-K2044[32] that were used to construct a maximum-likelihood phylogenetic tree (Figure 1a). The ST11/KPC-2 and ST512/KPC-3 isolates were grouped together with isolates belonging to ST258. In a pairwise comparison, the Italian isolate ST512-K30BO[30] was shown to be closely related to the Spanish ST512/KPC-3 representative isolate, since their sequences differed from each other by 75 SNPs (Figure 1a) and only had two allelic mismatches (Figure 1b).

ST101/KPC-2 was not grouped with the other KPC isolates analysed. There were ≥435 allelic mismatches between the sequences of ST101/KPC-2 and those of the other *K. pneumoniae* isolates. The main cluster was formed by ST258 and ST512 isolates (Figure 1b), which had an average of 10 allelic mismatches. There were 50 allelic mismatches between this cluster and ST11 isolates (Figure 1b), showing that ST512 and ST258 are more closely related to each other than ST11 as previously described by Bialek-Davenet *et al.*[28]

Profile of antibiotic resistance genes of ST11/KPC-2, ST101/KPC-2 and ST512/KPC-3 by WGS

The genetic profile of antibiotic resistance genes (resistome) differed in the ST11/KPC-2, ST101/KPC-2 and ST512/KPC-3 representative isolates, although all of them carried many accessory resistance genes: 9 in ST11/KPC-2, 11 in ST512/KPC-3 and 13 in ST101/KPC-2 (Figure 2). Only ST101/KPC-2 produced an ESBL

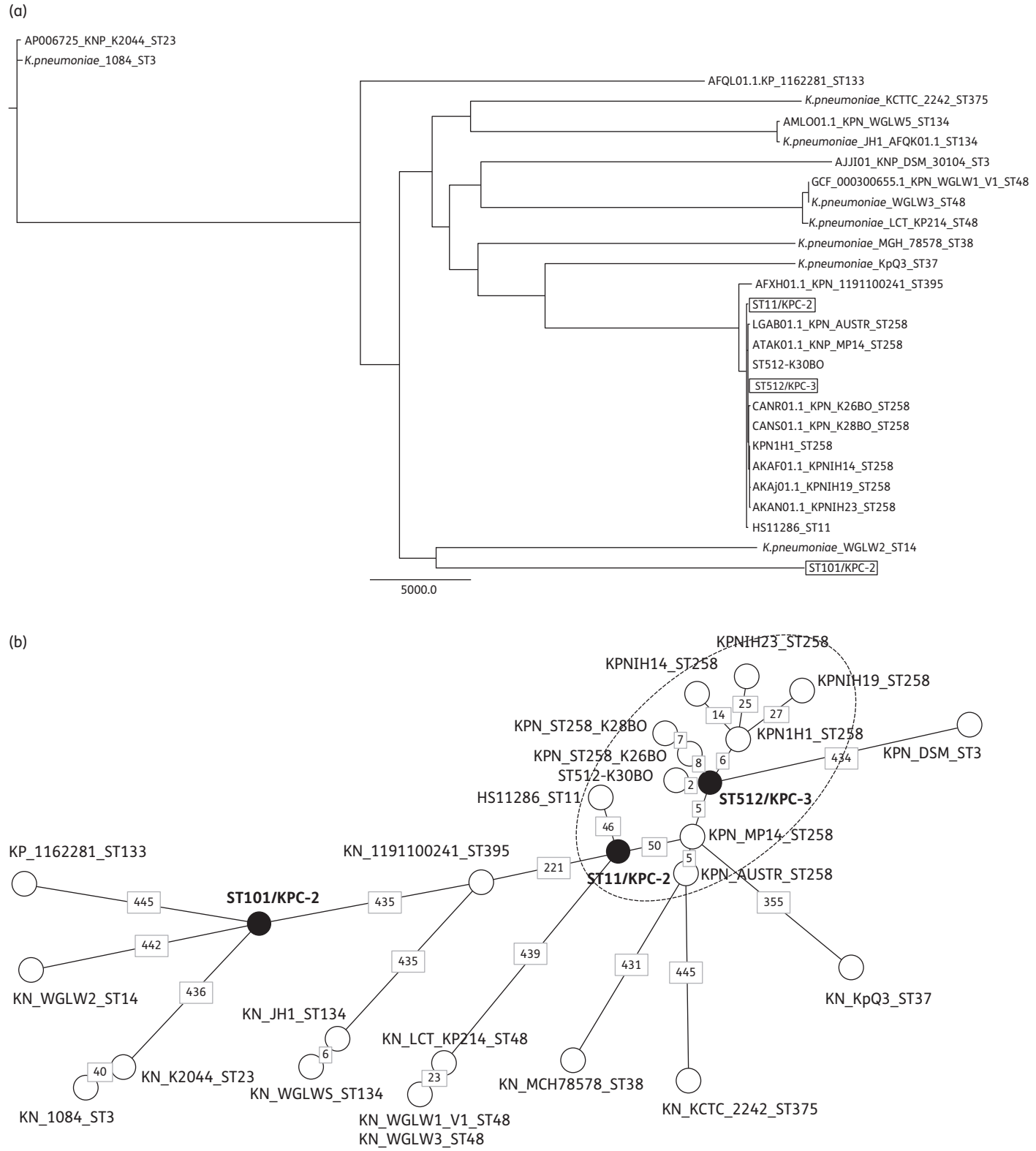

Figure 1. Population structure of *K. pneumoniae* expressing bla_{KPC} genes. (a) Maximum-likelihood tree showing the relationship between the isolates sequenced in this study and similar strains whose sequences were obtained from the GenBank database. Branch length is indicative of the number of SNPs. (b) Minimum spanning tree of *K. pneumoniae* genomes as determined on the basis of the 694 core genome multilocus ST. Identification of isolates includes the ST; Spanish sequenced isolates are highlighted in boxes (a) or black circles (b).

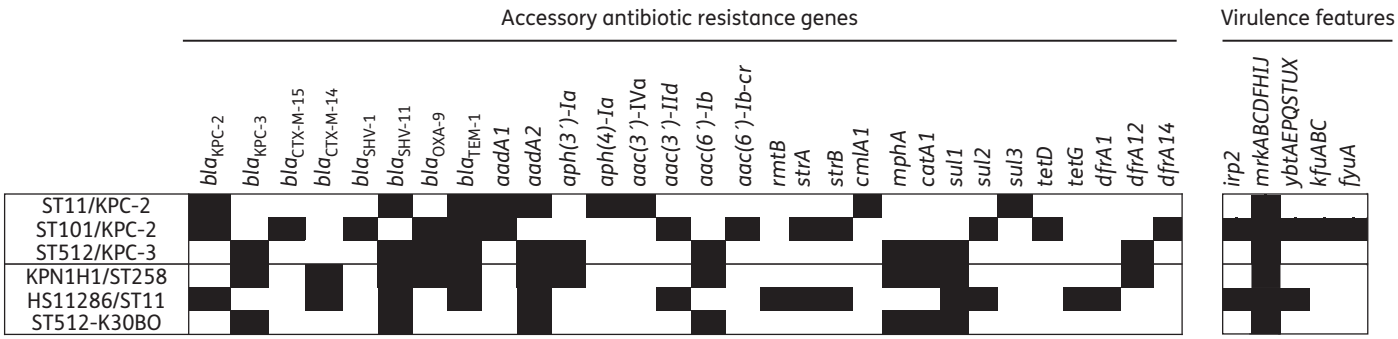

Figure 2. Distribution of multidrug resistance loci and virulence genes in the genomes of bla_{KPC} *K. pneumoniae* clones sequenced in Spain in comparison with strains isolated in other countries. The names of genes responsible for drug resistance (resistome) and virulence are found at the top of a heat map in which black rectangles indicate the presence of a gene and white rectangles indicate its absence. KPN1H1/ST258 was detected during an outbreak in the USA,[29] ST512-K30BO was isolated during an outbreak in Italy[30] and HS11286/ST11 was described in China.[31]

($bla_{CTX-M-15}$). A good correlation was observed between the genetic and phenotypic profiles of resistance for β-lactam and aminoglycoside antibiotics.

These results were compared with the previously sequenced genomes of KPC-producing *K. pneumoniae* (Figure 2).[29–31] The ST101/KPC-2 Spanish representative isolate had the highest number of resistance genes ($n=13$), while the Italian ST512-K30BO[30] isolate had the lowest number ($n=7$). Remarkably, the resistance gene profile of the ST512/KPC-3 Spanish representative isolate differed from the ST512 *K. pneumoniae* isolate detected during an outbreak in Italy;[30] however, it was very similar to the KPN1H1/ST258 isolate detected during an outbreak in the USA.[29]

The following topoisomerase mutations were identified: *gyrA* S83I, found in both ST11/KPC-2 and ST512/KPC-3; *gyrA* S83Y and D87G, found in ST101/KPC-2 only; and *parC* S80I, observed in all three isolates. An OqxAB efflux pump was also detected. Only ST101/KPC-2 had the *aac(6′)-Ib-cr* gene implicated in aminoglycoside and ciprofloxacin resistance.

All three Spanish representative isolates had the WT *ompK35* gene (GenBank accession no. AJ011501). ST101/KPC-2 had an *ompK36* gene with a 9 nt deletion at positions Δnt549–557 and an additional deletion at position 713. The latter deletion caused a frameshift that introduced a premature TGA stop codon after nine amino acids. The 9 nt deletion corresponded to a loss of Leu184, Ser185 and Pro186. These amino acids form part of the L4 loop, which is found on the cell surface.[48] ST11/KPC-2 and ST512/KPC-3 had the WT *ompK36* gene (GenBank accession no. NC_016845.1). Rapid characterization of *ompK36* genotypes by WGS might be a useful method for predicting the efficacy of combination therapies that include carbapenems.[49]

The colistin-resistant ST512/KPC-3 isolate had an *mgrB* coding sequence with a deletion of 11 bp (Δnt109–119) that resulted in a frameshift leading to a premature termination codon (TAA) associated with colistin resistance.[50] The clonal spread of a colistin-resistant ST512 strain with the same deletion in *mgrB* has been reported in a recent Italian study.[39] The colistin-susceptible isolates ST11/KPC-2 and ST101/KPC-2 had a WT *mgrB* gene (GenBank accession no. KF852760.1). The plasmid-carried colistin resistance gene *mcr-1* was not detected in any of these isolates.

Genetic environment of the bla_{KPC} genes

In the ST512 clone, the bla_{KPC-3} gene was carried by the classical transposon Tn*4401* 'isoform' (Figure S2). This transposon contains a 100 bp deletion between the sequences of *istB* and bla_{KPC}. The sequence detected was *tnpR-tnpA*-IS*Kpn7*(*istA-istB*)-bla_{KPC-3}-IS*Kpn6-tnpA*-IRrTn*4401-tnpR-tnpA* (total size 20338 bp), which was identical to that of the pNYC plasmid (GenBank accession no. EU176011).[51]

The genetic environment of bla_{KPC-2} in ST11 and ST101 was indistinguishable between them, but different from bla_{KPC-3} of ST512; it had IS*Kpn8* and a truncated bla_{TEM} gene that was 570 bp in length. The sequence was *tnpA-tnpR*-IS*Kpn8*-(trun)bla_{TEM}-bla_{KPC-2}-IS*Kpn6-tnpA-korC-klcA* (total size 7704 bp) (Figure S2). This sequence was very similar to Variant 1 described in the pKP048 plasmid from a Chinese *K. pneumoniae* isolate[52] (accession number not provided). A similar genetic environment for bla_{KPC-2} has been described in other countries such as Argentina.[53]

Reconstructing the bla_{KPC} plasmids of ST11/KPC-2, ST101/KPC-2 and ST512/KPC-3 using the WGS data

In ST512/KPC-3 and ST101/KPC-2 isolates, the bla_{KPC} genes were located in constructed IncF plasmids in which the RepFIA and FrepB replicons were detected. In the case of the ST11/KPC-2 isolate, the bla_{KPC-2} gene was detected in a non-typeable plasmid. The predominant bla_{KPC} plasmids described in the literature are IncF plasmids, mainly associated with ST258.[4] As shown in this and previous studies, IncF is also associated with the KPC-3-producing clone ST512 of *K. pneumoniae*.[44] In addition, bla_{KPC-2} has been found in plasmids of different incompatibility groups (IncP, IncR and IncA/C2) and non-typeable plasmids.[14,54,55]

All three sequenced strains had the IncW plasmid containing the resistance genes *aad*, *sul* and *dfrA*. In the ST101/KPC-2 isolate, $bla_{CTX-M-15}$ appeared exclusively in the chromosome and was directly linked to an upstream IS*Ecp1*-like element, which was known to affect expression and mobilization of this gene. Chromosomally encoded CTX-M alleles have been previously reported,[56–58] including ST101 *K. pneumoniae*.[59] An IncFII plasmid harbouring bla_{KPC-2} and $bla_{CTX-M-1-group}$ has recently been detected in an Italian ST101 isolate.[44]

Profile of the virulence genes of ST11/KPC-2, ST101/KPC-2 and ST512/KPC-3 by WGS

Five virulence genome features were detected out of the nine documented in the BIGSdb-Kp database[28] (Figure 2). These were the siderophore gene *irp2*, the yersiniabactin siderophore cluster (*ybtAEPQSTUX*), the mannose-resistant *Klebsiella*-like (type III) fimbriae cluster (*mrkABCDFHIJ*), the ferric uptake system (*kfuABC*) and the yersiniabactin receptor gene *fyuA*. Considering all together the different genes included in these virulence genome features, a total of 21 virulence genes were detected: 20 in ST101/KPC-2, 8 in ST512/KPC-3 and 7 in ST11/KPC-2. ST512/KPC-3 and ST11/KPC-2 only had virulence genes from the fimbriae cluster (Figure 2). The Spanish ST101/KPC-2 clone had the highest number of virulence genes, even when compared with the three KPC-producing isolates that had previously been sequenced (Figure 2).

The yersiniabactin siderophore cluster, only detected in the Spanish ST101/KPC-2 isolate in this study, has already been associated with ST258 isolates.[60] The presence of yersiniabactin in *K. pneumoniae* KPC producers is worrisome, because it is strongly linked with colonization of the respiratory tract in humans.[60] In our study, three out of the seven (42.9%) respiratory tract infections were caused by the ST101/KPC-2 *K. pneumoniae* clone.

Analysis of the capsular polysaccharide *cps* locus, which contained *galF*, *orf2*, *wzi*, *wza*, *wzb* and *wzc*, showed that each sequenced isolate had a different *wzi* allele. According to the BIGSdb-Kp database, these *wzi* alleles corresponded to a different K type: K13 corresponded to ST11/KPC-2, K17 corresponded to ST101/KPC-2 and no K type was associated with ST512/KPC-3. The *cps* gene cluster detected in ST512/KPC-3 is identical to the gene cluster $cps_{BO\text{-}4}$ already described in other Italian isolates of ST512; this CPS type is different from any other studied.[61]

The plasmid-associated heavy metal resistance clusters *pcoABCDERS* and *silCERS* (responsible for copper and silver resistance, respectively) were only detected in the ST512/KPC-3 isolate and in the previously reported KPN1H1/ST258 clone.[29] These clusters had the following allele assignments: 13,2,1,2,1,1,2 and 3,3,2,2, respectively; both clusters were widely distributed.[28]

Conclusions

KPC-producing isolates are increasing and spreading throughout many geographical areas of Spain, mainly due to the non-ST258 high-risk clones of *K. pneumoniae*: ST11/KPC-2, ST101/KPC-2 and ST512/KPC-3. The ST101/KPC-2 clone was highly resistant and hypervirulent. Of concern is the detection in Spain of *K. pneumoniae* clones similar to those recently implicated in the spread of KPC isolates in Italy.

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Transparency declarations

None to declare.

Supplementary data

Table S1 and Figures S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

References

1 Nordmann P, Poirel L. The difficult-to-control spread of carbapenemase producers among Enterobacteriaceae worldwide. *Clin Microbiol Infect* 2014; **20**: 821–30.

2 Cantón R, Akóva M, Carmeli Y *et al*. Rapid evolution and spread of carbapenemases among Enterobacteriaceae in Europe. *Clin Microbiol Infect* 2012; **18**: 413–31.

3 Oteo J, Miró E, Pérez-Vázquez M *et al*. Evolution of carbapenemase-producing Enterobacteriaceae at the global and national level: what

should be expected in the future? *Enferm Infecc Microbiol Clin* 2014; **32** Suppl 4: 17–23.

4 Pitout JD, Nordmann P, Poirel L. Carbapenemase-producing *Klebsiella pneumoniae*: a key pathogen set for global nosocomial dominance. *Antimicrob Agents Chemother* 2015; **59**: 5873–84.

5 Glasner C, Albiger B, Buist G *et al*. Carbapenemase-producing Enterobacteriaceae in Europe: a survey among national experts from 39 countries, February 2013. *Euro Surveill* 2013; **18**: pii=20525.

6 Giakkoupi P, Papagiannitsis CC, Miriagou V *et al*. An update of the evolving epidemic of bla_{KPC-2}-carrying *Klebsiella pneumoniae* in Greece (2009–10). *J Antimicrob Chemother* 2011; **66**: 1510–3.

7 Monaco M, Giani T, Raffone M *et al*. Colistin resistance superimposed to endemic carbapenem-resistant *Klebsiella pneumoniae*: a rapidly evolving problem in Italy, November 2013 to April 2014. *Euro Surveill* 2014; **19**: pii=20939.

8 Adler A, Hussein O, Ben-David D *et al*. Persistence of *Klebsiella pneumoniae* ST258 as the predominant clone of carbapenemase-producing Enterobacteriaceae in post-acute-care hospitals in Israel, 2008–13. *J Antimicrob Chemother* 2015; **70**: 89–92.

9 Oteo J, Ortega A, Bartolomé R *et al*. Prospective multicenter study of carbapenemase-producing Enterobacteriaceae from 83 hospitals in Spain reveals high in vitro susceptibility to colistin and meropenem. *Antimicrob Agents Chemother* 2015; **59**: 3406–12.

10 Baraniak A, Izdebski R, Herda M *et al*. Emergence of *Klebsiella pneumoniae* ST258 with KPC-2 in Poland. *Antimicrob Agents Chemother* 2009; **53**: 4565–7.

11 Tzouvelekis LS, Markogiannakis A, Piperaki E *et al*. Treating infections caused by carbapenemase-producing Enterobacteriaceae. *Clin Microbiol Infect* 2014; **20**: 862–72.

12 Oteo J, Saez D, Bautista V *et al*. Carbapenemase-producing Enterobacteriaceae in Spain in 2012. *Antimicrob Agents Chemother* 2013; **57**: 6344–7.

13 López-Cerero L, Egea P, Gracia-Ahufinger I *et al*. Characterisation of the first ongoing outbreak due to KPC-3-producing *Klebsiella pneumoniae* (ST512) in Spain. *Int J Antimicrob Agents* 2014; **44**: 538–40.

14 Ruiz-Garbajosa P, Curiao T, Tato M *et al*. Multiclonal dispersal of KPC genes following the emergence of non-ST258 KPC-producing *Klebsiella pneumoniae* clones in Madrid, Spain. *J Antimicrob Chemother* 2013; **68**: 2487–92.

15 Parkhill J, Wren BW. Bacterial epidemiology and biology: lessons from genome sequencing. *Genome Biol* 2011; **12**: 230.

16 Pérez-Vázquez M, Oteo J, García-Cobos S *et al*. Phylogeny, resistome and mobile genetic elements of emergent OXA-48 and OXA-245 *Klebsiella pneumoniae* clones circulating in Spain. *J Antimicrob Chemother* 2016; **71**: 887–96.

17 Matuschek E, Brown DF, Kahlmeter G. Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. *Clin Microbiol Infect* 2014; **20**: O255–66.

18 International Organization for Standardization. 2006. *Clinical Laboratory Testing and In Vitro Diagnostic Test Systems—Susceptibility Testing of Infectious Agents and Evaluation of Performance of Antimicrobial Susceptibility Test Devices—Part 1: Reference Method for Testing the In Vitro Activity of Antimicrobial Agents Against Rapidly Growing Aerobic Bacteria Involved in Infectious Diseases*. ISO 20776-1:2006. International Organization for Standardization, Geneva, Switzerland. http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=41630.

19 *EUCAST Guidelines for Detection of Resistance Mechanisms and Specific Resistances of Clinical and/or Epidemiological Importance*. Version 1.0, December 2013. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Resistance_mechanisms/EUCAST_detection_of_resistance_mechanisms_v1.0_20131211.pdf.

20 Nordmann P, Poirel L, Dortet L. Rapid detection of carbapenemase-producing Enterobacteriaceae. *Emerg Infect Dis* 2012; **18**: 1503–7.

21 Oteo J, Navarro C, Cercenado E *et al*. Spread of *Escherichia coli* strains with high-level cefotaxime and ceftazidime resistance between the community, long-term care facilities, and hospital institutions. *J Clin Microbiol* 2006; **44**: 2359–66.

22 Curiao T, Morosini MI, Ruiz-Garbajosa P *et al*. Emergence of bla_{KPC-3}-Tn*4401*a associated with a pKPN3/4-like plasmid within ST384 and ST388 *Klebsiella pneumoniae* clones in Spain. *J Antimicrob Chemother* 2010; **65**: 1608–14.

23 Zerbino DR, Birney E. Velvet: algorithms for *de novo* short read assembly using de Bruijn graphs. *Genome Res* 2008; **18**: 821–9.

24 Carver TJ, Rutherford KM, Berriman M *et al*. ACT: the Artemis Comparison Tool. *Bioinformatics* 2005; **21**: 3422–3.

25 Assefa S, Keane TM, Otto TD *et al*. ABACAS: algorithm-based automatic contiguation of assembled sequences. *Bioinformatics* 2009; **25**: 1968–9.

26 Overbeek R, Olson R, Pusch GD *et al*. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Res* 2014; **42**: D206–14.

27 Lanza VF, de Toro M, Garcillán-Barcia MP *et al*. Plasmid flux in *Escherichia coli* ST131 sublineages, analyzed by plasmid constellation network (PLACNET), a new method for plasmid reconstruction from whole genome sequences. *PLoS Genet* 2014; **10**: e1004766.

28 Bialek-Davenet S, Criscuolo A, Ailloud F *et al*. Genomic definition of hypervirulent and multidrug-resistant *Klebsiella pneumoniae* clonal groups. *Emerg Infect Dis* 2014; **20**: 1812–20.

29 Snitkin ES, Zelazny AM, Thomas PJ *et al*. Tracking a hospital outbreak of carbapenem-resistant *Klebsiella pneumoniae* with whole-genome sequencing. *Sci Transl Med* 2012; **4**: 148ra116.

30 Comandatore F, Gaibani P, Ambretti S *et al*. Draft genome of *Klebsiella pneumoniae* sequence type 512, a multidrug-resistant strain isolated during a recent KPC outbreak in Italy. *Genome Announc* 2013; **1**: e00035-12.

31 Liu P, Li P, Jiang X *et al*. Complete genome sequence of *Klebsiella pneumoniae* subsp. *pneumoniae* HS11286, a multidrug-resistant strain isolated from human sputum. *J Bacteriol* 2012; **194**: 1841–2.

32 Wu KM, Li LH, Yan JJ *et al*. Genome sequencing and comparative analysis of *Klebsiella pneumoniae* NTUH-K2044, a strain causing liver abscess and meningitis. *J Bacteriol* 2009; **191**: 4492–501.

33 Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 2009; **25**: 1754–60.

34 Harris SR, Feil EJ, Holden MT *et al*. Evolution of MRSA during hospital transmission and intercontinental spread. *Science* 2010; **327**: 469–74.

35 Bose M, Barber R. Prophage Finder: a prophage loci prediction tool for prokaryotic genome sequences. *In Silico Biol* 2006; **6**: 223–7.

36 Croucher NJ, Page AJ, Connor TR *et al*. Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. *Nucleic Acids Res* 2014; **43**: e15.

37 Stamatakis A. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 2006; **22**: 2688–90.

38 Albiger B, Glasner C, Struelens MJ *et al*. Carbapenemase-producing Enterobacteriaceae in Europe: assessment by national experts from 38 countries, May 2015. *Euro Surveill* 2015; **20**: pii=30062.

39 Giani T, Arena F, Vaggelli G *et al*. Large nosocomial outbreak of colistin-resistant, carbapenemase-producing *Klebsiella pneumoniae* traced to

clonal expansion of an *mgrB* deletion mutant. *J Clin Microbiol* 2015; **53**: 3341–4.

40 Giani T, Pini B, Arena F *et al*. Epidemic diffusion of KPC carbapenemase-producing *Klebsiella pneumoniae* in Italy: results of the first countrywide survey, 15 May to 30 June 2011. *Euro Surveill* 2013; **18**: pii=20489.

41 Munoz-Price LS, Poirel L, Bonomo RA *et al*. Clinical epidemiology of the global expansion of *Klebsiella pneumoniae* carbapenemases. *Lancet Infect Dis* 2013; **13**: 785–96.

42 Chen L, Mathema B, Pitout JD *et al*. Epidemic *Klebsiella pneumoniae* ST258 is a hybrid strain. *MBio* 2014; **5**: e01355–14.

43 Pitart C, Solé M, Roca I *et al*. First outbreak of a plasmid-mediated carbapenem-hydrolyzing OXA-48 β-lactamase in *Klebsiella pneumoniae* in Spain. *Antimicrob Agents Chemother* 2011; **55**: 4398–401.

44 Del Franco M, Paone L, Novati R *et al*. Molecular epidemiology of carbapenem resistant Enterobacteriaceae in Valle d'Aosta region, Italy, shows the emergence of KPC-2 producing *Klebsiella pneumoniae* clonal complex 101 (ST101 and ST1789). *BMC Microbiol* 2015; **15**: 260.

45 Poulou A, Voulgari E, Vrioni G *et al*. Outbreak caused by an ertapenem-resistant, CTX-M-15-producing *Klebsiella pneumoniae* sequence type 101 clone carrying an OmpK36 porin variant. *J Clin Microbiol* 2013; **51**: 3176–82.

46 Mathers AJ, Peirano G, Pitout JD. The role of epidemic resistance plasmids and international high-risk clones in the spread of multidrug-resistant Enterobacteriaceae. *Clin Microbiol Rev* 2015; **28**: 565–91.

47 Markovska R, Stoeva T, Schneider I *et al*. Clonal dissemination of multilocus sequence type ST15 KPC-2-producing *Klebsiella pneumoniae* in Bulgaria. *APMIS* 2015; **123**: 887–94.

48 Albertí S, Rodríquez-Quiñones F, Schirmer T *et al*. A porin from *Klebsiella pneumoniae*: sequence homology, three-dimensional model, and complement binding. *Infect Immun* 1995; **63**: 903–10.

49 Clancy CJ, Hao B, Shields RK *et al*. Doripenem, gentamicin, and colistin, alone and in combinations, against gentamicin-susceptible, KPC-producing *Klebsiella pneumoniae* strains with various ompK36 genotypes. *Antimicrob Agents Chemother* 2014; **58**: 3521–52.

50 Cannatelli A, Giani T, D'Andrea MM *et al*. MgrB inactivation is a common mechanism of colistin resistance in KPC-producing *Klebsiella pneumoniae* of clinical origin. *Antimicrob Agents Chemother* 2014; **58**: 5696–703.

51 Naas T, Cuzon G, Villegas MV *et al*. Genetic structures at the origin of acquisition of the β-lactamase bla_{KPC} gene. *Antimicrob Agents Chemother* 2008; **52**: 1257–63.

52 Shen P, Wei Z, Jiang Y *et al*. Novel genetic environment of the carbapenem-hydrolyzing β-lactamase KPC-2 among Enterobacteriaceae in China. *Antimicrob Agents Chemother* 2009; **53**: 4333–8.

53 Gomez SA, Pasteran FG, Faccone D *et al*. Clonal dissemination of *Klebsiella pneumoniae* ST258 harbouring KPC-2 in Argentina. *Clin Microbiol Infect* 2011; **17**: 1520–4.

54 Pecora ND, Li N, Allard M *et al*. Genomically informed surveillance for carbapenem-resistant Enterobacteriaceae in a health care system. *MBio* 2015; **6**: e01030.

55 Li G, Zhang Y, Bi D *et al*. First report of a clinical, multidrug-resistant Enterobacteriaceae isolate coharboring fosfomycin resistance gene *fosA3* and carbapenemase gene bla_{KPC-2} on the same transposon, Tn1721. *Antimicrob Agents Chemother* 2015; **59**: 338–43.

56 Coelho A, González-López JJ, Miró E *et al*. Characterisation of the CTX-M-15-encoding gene in *Klebsiella pneumoniae* strains from the Barcelona metropolitan area: plasmid diversity and chromosomal integration. *Int J Antimicrob Agents* 2010; **36**: 73–8.

57 Fabre L, Delauné A, Espié E *et al*. Chromosomal integration of the extended-spectrum β-lactamase gene $bla_{CTX-M-15}$ in *Salmonella enterica* serotype Concord isolates from internationally adopted children. *Antimicrob Agents Chemother* 2009; **53**: 1808–16.

58 Song W, Kim J, Bae IK *et al*. Chromosome-encoded AmpC and CTX-M extended-spectrum β-lactamases in clinical isolates of *Proteus mirabilis* from Korea. *Antimicrob Agents Chemother* 2011; **55**: 1414–9.

59 Mshana SE, Fritzenwanker M, Falgenhauer L *et al*. Molecular epidemiology and characterization of an outbreak causing *Klebsiella pneumoniae* clone carrying chromosomally located $bla_{CTX-M-15}$ at a German university-hospital. *BMC Microbiol* 2015; **15**: 122.

60 Bachman MA, Oyler JE, Burns SH *et al*. *Klebsiella pneumoniae* yersiniabactin promotes respiratory tract infection through evasion of lipocalin 2. *Infect Immun* 2011; **79**: 3309–16.

61 D'Andrea MM, Amisano F, Giani T *et al*. Diversity of capsular polysaccharide gene clusters in Kpc-producing *Klebsiella pneumoniae* clinical isolates of sequence type 258 involved in the Italian epidemic. *PLoS One* 2014; **9**: e96827.