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Epidemiology of carbapenemase-producing *Klebsiella pneumoniae* in northern Portugal: Predominance of KPC-2 and OXA-48

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ABSTRACT

Objectives: To provide, for the first time, data on the molecular epidemiology of carbapenemase-producing *Klebsiella pneumoniae* clinical isolates from the northern region of Portugal (Trás-os-Montes and Alto Douro).

Methods: A total of 106 carbapenemase-producing *K. pneumoniae* isolates recovered from clinical samples and rectal swabs between January 2018 and March 2019 were included in this study. All isolates were characterized by antimicrobial susceptibility, identification of resistance determinants, pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and plasmid analysis.

Results: The most common carbapenemase identified was KPC-2 (91%), followed by OXA-48 (9%). The bla_{KPC-2} gene was carried onto IncN (60%) and IncF (40%) plasmid types, whereas the bla_{OXA-48} gene was mainly located on the IncL (90%) incompatibility group. Molecular characterization distributed the 106 isolates into 29 PFGE types and 21 sequence types (STs), but three clones included 50% of the isolates: PFGE A-ST147-KPC-2 (29%), B-ST15-KPC-2 (15%), and C-ST11-OXA-48 (6%). Antimicrobial resistance rates were the following: ciprofloxacin (76%), trimethoprim–sulfamethoxazole (75%), tobramycin (62%), gentamicin (34%), amikacin (25%), tigecycline (21%), fosfomycin (10%), and colistin (7%). None of the colistin-resistant isolates harboured *mcr* genes. All isolates remained susceptible to ceftazidime/avibactam, but 10% presented elevated MICs (3 and 4 mg/L).

Conclusions: KPC-2 was the predominant carbapenemase among *K. pneumoniae* isolates currently circulating at this hospital from northern Portugal, followed by OXA-48. These data contrast with those obtained from the rest of the country, where KPC-3 predominates. This study showed a polyclonal structure of KPC-2-producing *K. pneumoniae* isolates with a predominance of the ST147 and ST15 clones.

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1. Introduction

Klebsiella pneumoniae is responsible for a wide range of infections, including urinary tract infections, pneumonia, septicaemia, and liver abscesses. Over the last few decades, there has been a concerning rise in the spread of *K. pneumoniae* strains producing carbapenemases, those enzymes conferring resistance to almost all available β-lactams, including carbapenems [1]. Moreover, carbapenem-resistant isolates are frequently resistant to many other antibiotics, leaving limited treatment options, and therefore are associated with high morbidity and mortality [2].

A large variety of carbapenemases have been identified in *K. pneumoniae* and are classified into Ambler class A (KPC, GES, IMI, NMC, SME), class B (NDM, VIM, IMP, GIM, SIM, SPM), and class D (OXA-48-like) [3].

In Portugal, after the isolation of the first carbapenemase-producing clinical isolate of *K. pneumoniae* at a Lisbon hospital in 2009 [4], single cases and sporadic outbreaks have been reported between 2010 and 2013 [5–7]. Since then, the country faced an

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increasing trend of carbapenem resistance among *K. pneumoniae* from 1.8% in 2014 to 8.6% in 2017, as reported by the European Centre for Disease Prevention and Control (ECDC) [8]. Among the few studies reporting the molecular epidemiology of carbapenemase-producing *K. pneumoniae* in Portugal, KPC-3 was found to be the main enzyme in hospitals in different parts of the country [9,10], as well as among patients from long-term care facilities and nursing homes [11]. However, none of the studies included isolates from the region of Trás-os-Montes and Alto Douro, in northern Portugal.

Therefore, the aim of the present study was to provide information on the molecular epidemiology of carbapenemase-producing *K. pneumoniae* isolates currently circulating in that geographical region.

2. Methods

2.1. Bacterial isolates

A total of 106 carbapenemase-producing *K. pneumoniae* isolates recovered between January 2018 and March 2019 at Centro Hospitalar de Trás-os-Montes and Alto Douro (CHTMAD), a tertiary and University hospital in Vila Real, northern Portugal, were included in the present study. All isolates were recovered from single patients and most were from infections: urine ($n = 52$), blood ($n = 15$), bronchial secretions ($n = 11$), sputum or exudate ($n = 6$), catheter ($n = 4$), drainage liquid ($n = 3$), biopsy ($n = 1$), and wound ($n = 1$). Five isolates were recovered from rectal swabs, and for eight isolates there was no information concerning the biological sample.

2.2. Molecular analysis

Identification of carbapenemases was performed by PCR amplification using specific primers [12], followed by sequencing of the amplicons. Similarly, screening of *mcr* genes (*mcr-1* to *mcr-9*) was performed by using previously published primers [13].

The clonal relationship of the isolates was evaluated by pulsed-field gel electrophoresis (PFGE), as described previously [14]. Multilocus sequence typing (MLST) was performed for a representative strain of each PFGE type and sequence types (STs) were assigned using the MLST database for *K. pneumoniae* (https://cge.cbs.dtu.dk/services/MLST-2.0/).

2.3. Conjugation experiments and plasmid analysis

Conjugation experiments were performed using the azide-resistant *Escherichia coli* J53 as the recipient strain. *E. coli* J53 and

Table 1
Characteristics of the 106 carbapenemase-producing *Klebsiella pneumoniae* isolates.

PFGE type	ST	No. of isolates	Carbapenemase	Plasmid type	AMC	TEM	CZD	CTX	CEF	FOX	ATM	ETP	IPM	MEM	AKN	GMI	TOB	FOS	SXT	CIP	TIG
A (30%)	147	31	KPC-2	IncN(29), IncF(2)	R	S	R	R	R	R	R(30)	R	R	R	S(30)	S(30)	R(22)	S(30)	R(28)	R	S(21)
		1	OXA-48	IncL	R	R	S	S	S	S	S	R	S	S	S	S	S	S	S	R	S
B (17%)	15	16	KPC-2	IncN(3), IncF(13)	R	S	R	R	R	R(14)	R	R	R	R	R(8)	R(11)	R(11)	S	R	R	S(14)
		2	OXA-48	IncL(1), IncN(1)	R	R	R	R	R	R	R	R	R	R	S	R	R	S	R	R	S
C (6%)	11	6	OXA-48	IncL	R	R	R	R	R	S(4)	R	R	R(4)	R(4)	S	S	R(5)	S(5)	R	R	S(5)
D (6%)	SLV517	6	KPC-2	IncN	R	S	R	R	R	R(5)	R	R	R	R	S	S	S	S	S	S	S
E (5%)	348	5	KPC-2	IncF(4), IncN(1)	R	S(4)	R	R	R	R(4)	R	R	R	R	R(3)	R(4)	R(4)	S	R(4)	R(3)	S(1)
F	277	4	KPC-2	IncN(1), IncF(3)	R	S	R	R	R	S	R(3)	R	S(2)	S(2)	S	S	S	S	S	S	S
G	34	3	KPC-2	IncN	R	S	R	R	R	R	R	R	R	R	S	S	S	S	S(2)	S	S
		1	KPC-2, GES-5	IncN	R	S	R	R	R	S	R	R	R	R	S	S	R	S	S	R	S
H	307	3	KPC-2	IncN	R	S	R	R	R	R	R	R	R	R	S(2)	S(2)	R	S	R	R	R(2)
I	280	3	KPC-2	IncF	R	S	R	R	R	R(2)	R	R	R	R	R	R	R	S	R	R	R(2)
J	45	2	KPC-2	IncN(1), IncF(1)	R	S	R	R	R	S	R	R	R	R	R	R	R	S	R	S	S(1)
K	3155	2	KPC-2	IncN(1), IncF(1)	R	S	R	R	R	S	R	R	R	S	S	S	S	S	S	R	S
L	147	2	KPC-2	IncF	R	S	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S
M	15	2	KPC-2	IncN(1), IncF(1)	R	S	R	R	R	S(1)	R	R	R	R	S(1)	S(1)	R	S	R	R	S
N	461	2	KPC-2	IncN	R	S	R	R	R	S(1)	R	R	R	R	S	R	R	S	R	S(1)	R
O	348	1	KPC-2	IncF	R	S	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
P	150	1	OXA-48	IncL	R	R	R	R	R	S	R	R	R	R	S	S	S	S	R	R	S
Q	17	1	KPC-2	IncF	R	S	R	R	R	S	R	R	R	R	S	S	S	S	S	S	S
R	643	1	KPC-2	IncF	R	S	R	R	R	R	R	R	R	R	R	R	R	S	R	S	S
S	35	1	KPC-2	IncN	R	S	R	R	R	R	R	R	R	R	S	S	S	S	S	S	S
T	359	1	KPC-2	IncN	R	S	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S
U	1916	1	KPC-2	IncF	R	S	R	R	R	R	R	R	R	R	R	R	R	S	R	S	S
V	15	1	KPC-2	IncF	R	S	R	R	R	R	R	R	R	R	S	S	S	S	R	R	S
W	517	1	KPC-2	IncF	R	S	R	R	R	S	R	R	R	R	S	S	S	S	S	S	S
X	307	1	KPC-2	IncN	R	S	R	R	R	R	R	R	R	R	S	S	S	S	S	R	S
Y	35	1	KPC-2	IncF	R	S	R	R	R	S	R	R	R	R	S	S	S	S	S	S	S
Z	147	1	KPC-2	IncN	R	S	R	R	R	S	R	R	R	R	S	S	S	S	S	R	S
AA	13	1	KPC-2	IncF	R	S	R	R	R	R	R	R	R	R	R	R	R	S	R	S	S
AB	433	1	KPC-2	IncN	R	S	R	R	R	R	R	R	R	R	S	R	R	S	R	R	S
AC	280	1	KPC-2	IncF	R	S	R	R	R	S	R	R	R	R	S	R	R	S	R	R	S

Numbers within parentheses indicate the number of isolates that are resistant or susceptible to the antibiotic, if not all.
Resistance was in grey shade for better visualisation relatively to susceptibility with no grey shade.
AKN, amikacin; AMC, amoxicillin/clavulanic acid; ATM, aztreonam; CEF, cefepime; CIP, ciprofloxacin; COL, colistin; CTX, cefotaxime; CZD, ceftazidime; ETP, ertapenem; FOS, fosfomycin; FOX, cefoxitin; GMI, gentamicin; IPM, imipenem; MEM, meropenem; SXT, trimethoprim-sulfamethoxazole; TEM, temocillin; TIG, tigecycline; TOB, tobramycin; PFGE, pulsed-field gel electrophoresis; R, resistant; S, susceptible; SLV, single locus variant; ST, sequence type determined by multilocus sequence typing.

bla$_{KPC-2}$- or *bla*$_{OXA-48}$-carrying donors were separately inoculated overnight into Luria-Bertani (LB) broth (5 mL) and incubated. The samples were subsequently mixed at a ratio of 10:1 (donor/recipient) for 5 h and 100 μL of this mix was deposited onto 22 μm filters and incubated overnight at 37 °C onto LB agar plates. After the incubation, filters were resuspended in NaCl 0.85% and 100 μL of this mixture was plated onto LB agar plates supplemented with ticarcillin (100 μg/mL) and sodium azide (100 μg/mL). Susceptibility testing was performed for all *E. coli* transconjugants, and positivity for *bla*$_{KPC-2}$ or *bla*$_{OXA-48}$ was checked by PCR.

Plasmids were classified according to their incompatibility group using the PCR-based replicon typing (PBRT) method as described previously [15].

2.4. Susceptibility testing

Antimicrobial susceptibility testing was performed using the disc diffusion method on Mueller-Hinton (MH) agar plates (Becton, Dickinson & Co, Franklin Lakes, NJ) for amoxicillin, amoxicillin/clavulanic acid, temocillin, ceftazidime, cefotaxime, cefepime, cefoxitin, aztreonam, ertapenem, imipenem, amikacin, gentamicin, tobramycin, fosfomycin, trimethoprim–sulfamethoxazole (SXT), ciprofloxacin, and tigecycline, following European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations. Colistin resistance was evaluated using the rapid polymyxin NP test [16], and the minimum inhibitory concentration (MIC) was determined by broth microdilution in cation-adjusted MH broth.

3. Results

Among the 106 carbapenemase-producing isolates, the large majority produced KPC-2 ($n = 96$; 91%), whereas 10 isolates (9%) produced OXA-48 (Table 1). Interestingly, one isolate co-harboured two carbapenemase genes (*bla*$_{KPC-2}$ and *bla*$_{GES-5}$). Mating-out assays followed by PBRT revealed that the *bla*$_{KPC-2}$ gene was carried onto IncN (58/96; 60%) and IncF (38/96; 40%) plasmid types, whereas the *bla*$_{OXA-48}$ gene was located on plasmids belonging to the IncL (9/10; 90%), and IncN ($n = 1$) incompatibility groups.

PFGE analysis of the 106 isolates revealed 29 different macro-restriction patters (Table 1), of which five included over 60% of the isolates: PFGE A ($n = 32$; 30%), B ($n = 18$; 17%), C ($n = 6$; 6%), D ($n = 6$; 6%), and E ($n = 5$; 5%). The 10 isolates producing OXA-48 belonged to 4 different PFGE types (A, B, C, and P), although the majority were included in PFGE C (6/10). The 96 isolates producing KPC-2 were distributed into a higher diversity of PFGE types ($n = 27$). Using MLST we found that the 29 PFGE types corresponded to 21 STs (Table 1). Despite the high diversity of backgrounds, three clones included 50% of the isolates: PFGE A-ST147-KPC-2 ($n = 31$; 29%), B-ST15-KPC-2 ($n = 16$; 15%), and C-ST11-OXA-48 ($n = 6$; 6%).

Antimicrobial susceptibility testing performed on those 106 carbapenemase-producing *K. pneumoniae* isolates showed that they were all resistant to ertapenem. However, seven isolates remained susceptible to meropenem (MIC $\leq$ 1 mg/L), of which five also remained susceptible to imipenem (MIC $\leq$ 2 mg/L). Resistance rates within that strain collection were as follows: ciprofloxacin (76%), SXT (75%), tobramycin (62%), gentamicin (34%), amikacin (25%), tigecycline (21%), and fosfomycin (10%). Moreover, seven (7%) colistin-resistant isolates were recovered (all producing KPC-2), presenting MIC values ranging from 4 to >128 mg/L, but none harboured the plasmid-borne colistin resistance genes *mcr-1* to *mcr-9*. Of note, 18 isolates (16%) showed a multidrug resistance pattern, being resistant to fluoroquinolones, aminoglycosides, and SXT, whereas five isolates (5%) showed additional resistance to tigecycline. Although all isolates remained susceptible to ceftazidime/avibactam (MIC values ranging from 0.025 to 4 mg/L), eleven (10%) had MICs $\geq$ 3 mg/L.

4. Discussion

Here, we provide the first molecular epidemiological data of clinical carbapenemase-producing *K. pneumoniae* isolates from the Trás-os-Montes and Alto Douro region, northern Portugal. The study demonstrates that this region has a different distribution of carbapenemases in *K. pneumoniae* with respect to the rest of the country. A clear predominance (91%) of KPC-2 producers was noticed, as well as the occurrence of OXA-48, in contrast to previous national studies that reported the dominance of KPC-3 [9–11,17], and the occurrence of OXA-181 [17].

In Portugal, the *bla*$_{KPC-2}$ β-lactamase gene was found so far in a single *E. coli* isolate recovered from the aquatic environment in the northern region of the country [18]. We report here the first clinical *K. pneumoniae* KPC-2 producers in Portugal. KPC-2 is widely spread among *K. pneumoniae* in many European countries, such as Spain [19], Italy [20], France [21], and Greece [22]. It is also the most common carbapenemase in China [23], and is endemic in Brazil [24], a country with important demographic exchanges with Portugal.

This study also reveals the emergence of OXA-48-producing *K. pneumoniae* isolates in Portugal, mostly with a ST11 background. The two first cases of OXA-48-producing Enterobacteriaceae in Portugal were found in *Enterobacter cloacae* and *E. coli* isolates recovered from the same patient admitted at a hospital in Lisbon in 2013 [25]. In addition, an OXA-48-producing *E. coli* was recovered in 2016 from a patient repatriated to France from Portugal where he had been hospitalized for 2 months [26], showing that the *bla*$_{OXA-48}$ was presumably already circulating in Portuguese hospitals at that time. However, to the best of our knowledge, we report here the first cases of OXA-48-producing *K. pneumoniae* isolated in Portugal. Currently, in the Mediterranean area, OXA-48 producing *K. pneumoniae* isolates are endemic in Turkey and Malta, diffused inter-regionally in Spain, France, Tunisia, Morocco, Lebanon, and Libya, regionally spread in Italy and Croatia, and found as sporadic outbreaks in Israel, Slovenia, and Albania [27]. A recent study that characterized a collection of carbapenemase-producing *K. pneumoniae* over time (2013–2018) in a hospital located in Lisbon revealed the emergence of an OXA-48-like β-lactamase (OXA-181) in 2016, with an increasing trend since then [17]. Therefore, the detection of OXA-48-producers in 2018 in our study, in a different region of Portugal, indicates that the prevalence of Ambler class D carbapenemases is on the rise in the country.

Interestingly, we detected one isolate co-producing KPC-2 and GES-5. This latter carbapenemase has been detected for the first time in Portugal in 2013 in a *K. pneumoniae* environmental isolate [28], subsequently in a single clinical *K. pneumoniae* strain [10], and more recently (2018) in combination with KPC-3 among seven *K. pneumoniae* isolates at a Lisbon hospital [17].

Of note, the carbapenemase-producing *K. pneumoniae* isolates from CHTMAD collected during 15 months belonged to a very high diversity of clones ($n = 29$) and the *bla*$_{KPC-2}$ gene was carried onto different plasmid types (IncN and IncF. Despite the high variability of lineages, ST147 ($n = 34$; 32%) and ST15 ($n = 19$; 18%) backgrounds producing KPC-2 were clearly predominant among the collection. ST147, belonging to Clonal Complex 258, was already one of the major backgrounds among clinical KPC-3 *K. pneumoniae* isolates collected during a national survey [9], and was also a successful clone among extended-spectrum β-lactamase-producing *K. pneumoniae* circulating in hospitals located in the northern and central regions of the country [29]. ST15 had been previously found in a Portuguese hospital in 2010 during an outbreak of carbapenem-resistant *K. pneumoniae* isolates with modifications in the OmpK36 porin [30] and later in the same hospital during an outbreak of KPC-3-producing *K. pneumoniae* [7]. Moreover, both backgrounds

were recovered among *K. pneumoniae* from patients from long-term care facilities and nursing homes in northern Portugal [11] and from hospitalized patients in different hospitals [11].

Notably, a considerable proportion of carbapenemase-producing isolates was multidrug resistant and 7% of the isolates showed resistance to colistin, reducing the treatment options considerably, but none harboured the plasmid-encoded *mcr* genes. Only two isolates exhibited in vitro resistance to fosfomycin, and all isolates remained susceptible to ceftazidime/avibactam, regardless of the clonal background and the type of carbapenemase produced (KPC-2, OXA-48, or GES-5), therefore highlighting that this novel β-lactam/β-lactamase inhibitor combination is a very potent therapeutic option for treating serious infections caused by most carbapenemase-producing *K. pneumoniae* in Portugal. Nevertheless, monitoring ceftazidime/avibactam resistance is mandatory since several isolates with reduced susceptibility (MICs of 3 and 4 mg/L) have been found. In addition, it is important to highlight that we recently reported the emergence of the NDM-1 carbapenemase that was identified in two enterobacterial species recovered in Lisbon, namely *Morganella morganii* and *Proteus mirabilis* isolates [31]. Those two isolates exhibited high-level resistance to the ceftazidime–avibactam combination, as expected for all metallo-β-lactamase producers, owing to the lack of inhibitory effect of avibactam towards such enzymes.

In summary, KPC-2 was found to be the predominant carbapenemase among *K. pneumoniae* isolates currently circulating at this hospital from northern Portugal, followed by OXA-48. These data actually contrast with those obtained from the rest of the country, where KPC-3 predominates. Nevertheless, there is no significant difference between KPC-2 and KPC-3 in terms of the β-lactam hydrolysis spectrum, therefore both enzymes do represent a similar threat.

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Competing interests

None declared.

Ethical approval

Not required.

Authors' contributions

EL performed the investigation; MJS and EC were responsible for acquisition of data; HL was responsible for supervision and reviewing of the manuscript. LP was responsible for the conceptualization, supervision and reviewing of the manuscript. MAS was responsible for the conceptualization, funding acquisition, supervision, and writing the original draft; all authors read and approved the final manuscript.

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