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High Prevalence of *bla*$_{NDM}$ Among Carbapenem Non-Susceptible *Klebsiella pneumoniae* in a Tunisian Hospital First Report of *bla*$_{NDM-9}$, *bla*$_{KPC-20}$, and *bla*$_{KPC-26}$ Genes

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Abstract

Fifty-four carbapenem non-susceptible *Klebsiella pneumoniae* (CNSKP) isolates were collected from a Tunisian hospital over a period of 13 consecutive months. Carbapenemase production and the prevalence of carbapenemase-encoding genes were investigated using combined-disk test (CDT), modified Carba NP (mCarba NP) test, and UV-spectrophotometry method complemented by PCR experiments and sequencing. Carbapenemase production was detected by the mCarba NP test and CDT in 92.59% and 96.29% of the 54 CNSKP isolates, respectively; while imipenem hydrolysis was detected using UV-spectrophotometry in the crude extracts of 44 isolates. *bla*$_{NDM,}$ *bla*$_{OXA-48-like}$, and *bla*$_{KPC}$ carbapenemase-encoding genes were found in 48, 31, and 22 isolates, respectively. Remarkably, *bla*$_{NDM-9,}$ *bla*$_{KPC-20}$, and *bla*$_{KPC-26}$ genes were reported. The co-occurrence of carbapenemase-encoding genes in a single isolate was detected in 62.96% of the isolates. The analysis of clonal relationships between the isolates by pulsed field gel electrophoresis revealed that the majority of them were genetically unrelated. Our investigation provides molecular data on enzymatic mechanism of carbapenem non-susceptibility among 54 CNSKP showing the dominance of *bla*$_{NDM,}$ and comprises the first identification of *bla*$_{NDM-9,}$ *bla*$_{KPC-20}$, and *bla*$_{KPC-26}$ genes in a Tunisia hospital.

Introduction

Carbapenemases represent the most potent and versatile family of β-lactamases, with a typical broad spectrum of nearly all β-lactam substrates, including carbapenems [1]. Three major classes of carbapenemases are currently found. Classes A and D possess a serine-based hydrolytic mechanism, while class B enzymes are metallo-β-lactamases (MBLs) requiring zinc for their activity [2]. The class A carbapenemases, mostly inhibited by clavulanic acid and tazobactam, include members of the IMI/NMC-A, SME, GES, and KPC-type enzymes [1, 2]. The carbapenem-hydrolyzing class D β-lactamases (CHDL), poorly inhibited by clavulanic acid and tazobactam, are oxacillinases (OXA). The majority of these enzymes, except OXA-48, are frequently detected in *Acinetobacter baumannii* [3]. The class B carbapenemases, which evade all approved β-lactamase inhibitors, are clinically the most relevant. Almost twenty types of MBLs have been described until now [2, 4]. However, the most common ones are IMP, VIM, and NDM-type enzymes that have been found in *Pseudomonas aeruginosa*, *A. baumannii* and members of *Enterobacteriaceae* [1, 2].

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Klebsiella pneumoniae is generally an opportunistic pathogen and the causative agent of a wide spectrum of serious nosocomial infections. The emergence and spread of carbapenem-resistant *K. pneumoniae* (CRKP) strains worldwide is increasing the scarcity of effective treatments resulting in high morbidity and mortality [5]. In 2017, the World Health Organization published its first global priority list of antibiotic-resistant pathogens for which innovative new treatments are urgently needed and ranked CRKP in the highest priority category (https://www.who.int/news/item/27-02-2017).

The major mechanism underlying carbapenem resistance in CRKP is associated with carbapenemase production. Currently, KPCs are commonly reported [1, 2]. Additional types of carbapenemases have been described in different geographic areas, as they are found across various *K. pneumoniae* clones [2, 3]. Thus, NDM-1 is predominantly detected in South Asian countries, especially in India and China, and is sporadically described in other countries, while *K. pneumoniae* strains harboring VIM carbapenemase are, for example, endemic in Greece [1, 2]. OXA-48, originating from Turkey, is rapidly spreading into other European countries, North Africa, and the Middle East [3].

In Tunisia, data on molecular characterization of carbapenem-hydrolyzing β-lactamases have been reported since the second half of 2000s. Indeed, the first case of carbapenemase (MBL VIM-4) was described in *K. pneumoniae* strains isolated in a Tunisian University Hospital, in 2006 [6]. Since then, there have been increasing reports on the emergence and spread of carbapenemase-producing bacteria, especially *A. baumannii* (such as GES- and OXA-type carbapenemases), and *Enterobacteriaceae* (such as OXA-, NDM-, KPC, and VIM-types) [7–13]. OXA-48-like enzymes are by far the most frequently reported carbapenemases in *K. pneumoniae* isolated in Tunisian hospitals [14–16].

The present study was undertaken to provide molecular and epidemiological data on carbapenem resistance among *K. pneumoniae* strains isolated in the Regional Hospital of Ben Arous (Tunisia) during a 13-month period ending in March 2019. Therefore, we aimed to identify the carbapenemase-encoding genes, to explore the enzymatic basis of carbapenem resistance, and to investigate the genetic relatedness of the isolates.

Materials and Methods

Bacterial Isolates and Susceptibility Testing

Regional Hospital of Ben Arous is one of the two largest health care centers of Ben Arous governorate. The hospital includes a polyvalent intensive care unit, with 10 beds. The *K. pneumoniae* isolates included in our study were recovered from different clinical specimens, and then identified phenotypically in the Clinical Biology Laboratory of the hospital from March 2018 to March 2019. Standard microbiological methods (including Gram stain preparation and growing bacterial cultures on differential media) and routine biochemical tests (API 20E strip test, bioMérieux SA, France) were used for the identification of bacteria.

Antibiotic susceptibility testing was performed, in the Clinical Laboratory and also in the Laboratory of Biochemistry (LR01ES05), by using the antibiotic-disc diffusion method on Mueller–Hinton agar (MH) (Condalab, Madrid, Spain), following the guidelines of the Antibiogram Committee of the French Society of Microbiology and the European Committee on Antimicrobial Susceptibility Testing (CA-SFM/EUCAST) (https://www.sfmmicrobiologie.org). Eighteen β-lactam and 9 non β-lactam antimicrobial agents (Bio-Rad, Hercules, CA, USA) were tested. The isolate was considered as multidrug-resistant (MDR) when it was non-susceptible (resistant or intermediate) to at least one agent in three or more antibiotic categories [17]. Among 133 *K. pneumoniae* isolates resistant to at least one β-lactam antimicrobial agent, a total of 54 non-duplicate isolates non-susceptible to carbapenems (imipenem, ertapenem, meropenem, and doripenem) were included in the present study. Samples received were accompanied with minimal patient's information; gender, age, sampling data and isolation site. Twenty-eight isolates (51.85%) were collected from males. The mean age was 46.5 years old (range: 4 days–89 years). More than half of the 54 CNSKP isolates were from inpatients (32 isolates, 59.25%) being predominantly (24, 44.44%) isolated from intensive care unit, followed by pediatric ward (7, 12.96%) and internal medicine department (1, 1.85%). Among the 54 CNSKP, isolates were mainly recovered from urine (24, 44.44%), followed by rectal swab (17, 31.48%) and blood (6, 11.11%). The other isolates were from catheters (4, 7.4%), distal protected aspirate (2, 3.7%), and bronchial aspiration (1, 1.85%).

The minimum inhibitory concentrations (MICs) of carbapenems were determined by E-test method according to the manufacturer's guidelines (bioMérieux SA, France). Results were interpreted according the recommendations of CA-SFM/EUCAST.

The control isolates used in this study were strains carrying NDM-1 (*A. baumannii* H446), OXA-48 (*K. pneumoniae* 1193), KPC-3 (*K. pneumoniae* C4420), VIM-1/KPC-3 (*E. coli* C7114), GES-14 (*A. baumannii* 8098), TEM-1 (*K. pneumoniae* PEP-108), SHV-11 (*K. pneumoniae* PEP-041), CTX-M-15 (*Enterobacter cloacae* COL20140080), CTX-M-1 (*E. coli* COL20140062), OXA-1 (*E. coli* CP 40), CMY-2 (*Citrobacter freundii* COL20140084), and *E.coli* ATCC 25922 strain.

Modified Carba NP Test

Modified Carba NP (mCarba NP) test was performed as described by Kumar et al. [18] Bacterial crude extract (80 µg of proteins), prepared as reported previously [19], was added to a reaction mixture containing 3 mg/mL imipenem (Sigma Aldrich, St. Louis, MO, USA), 0.1 mM $ZnSO_4$, and 0.5% phenol red as pH indicator in the presence or absence of β-lactamase inhibitor (4 mg/mL tazobactam sodium salt or 3 mM EDTA). Positive result interpreted as a color change from red to yellow or orange [18].

Combined-disc Test

Combined-disc test (CDT) was performed as described previously with minor modifications [20]. Tested isolate was inoculated on MH agar plate. Thereafter, sets of four carbapenem discs each containing imipenem, meropenem, ertapenem, or doripenem and one disc of temocillin were placed onto MH agar. Immediately afterward, 20 µL of a β-lactamase inhibitor solution: 60 mg/mL phenylboronic acid (PBA) (Sigma-Aldrich, USA), 100 mg/mL dipicolonic acid (DPA) (Sigma-Aldrich, USA) or 75 mg/mL cloxacillin were dispensed onto one of the carbapenem discs in each set. Then, the plates were incubated at 37 °C for 18–24 h. The diameter of the inhibition zone (IZ) around each carbapenem/β-lactamase inhibitor disc was measured and compared to that of the carbapenem alone disc. Results were interpreted as described previously [20].

UV-Spectrophotometric Assay

The enzyme extract (80 µg of proteins) was tested for its hydrolytic activity against 50 µM imipenem by UV-spectrophotometry (Varian Cary 50 Bio UV/VIS spectrophotometer, CA, USA) ($\Delta\varepsilon^{297} = -9000\ M^{-1}\ cm^{-1}$) [19]. The reaction was performed at room temperature in 2 mL of 25 mM sodium phosphate buffer (pH 7) supplemented with 50 µM $ZnSO_4$ when testing MBLs or 50 mM $NaHCO_3$ when testing OXA enzymes [21]. Initial rate (IR) of imipenem hydrolysis was expressed as absorbance units/min (Au/min).

PCR Amplification and Sequencing of Carbapenemase-Encoding Genes

Genes encoding carbapenemases and other β-lactamases were screened by polymerase chain reaction with primers (Eurogentec, Belgium) listed in Table S1 (available as Supplementary data at Curr Microbiol Online) using a DNA template extracted by boiling and freeze-thawing processes [18]. PCR amplification was performed as reported elsewhere [22–29] in a thermal cycler (Bio-Rad, CA, USA) and Taq Master Mix kit with Standard Buffer according to the supplier's instructions (BioLabs, MA, USA). PCR products were purified with Gel Extraction Kit and PCR Clean-up (NucleoSpin, Macherey–Nagel™ Germany) and sequenced on both strands using NovaSeq 6000 Illumina technology (Nextera XT DNA Library Preparation Kit, Illumina, CA, USA) by the GIGA-Genomics platform Uliege, Belgium. The nucleotide sequences were analyzed using software available at the National Center of Biotechnology Information. (http://www.ncbi.nlm.nih.gov/Blast.cgi).

Pulsed Field Gel Electrophoresis Typing

The clonal relatedness among the 54 CNSKP isolates was investigated by pulse field gel electrophoresis (PFGE) of their *Xba*I-digested genomic DNA (Promega, Madison, USA) as described elsewhere [30]. The PFGE patterns were compared using the unweighted pair group method with arithmetic averages clustering (UPGMA) method using the Dice similarity coefficient and the BioNumerics software (version 7.6) (Applied Maths, Belgium). *Salmonella enterica* subsp. *enterica* serotype Braenderup strain (ATCC® BAA 664™) was used as a control strain.

Results

Phenotypic Antimicrobial Resistance Profiles

Results of antibiotic susceptibility test (Fig. S1) showed that among 54 CNSKP isolates, identified phenotypically, 41 (75.92%) were resistant to ertapenem, followed by doripenem (18 isolates, 33.33%), imipenem (15, 27.77%), and meropenem (12, 22.22%), whereas 44.44% (24 isolates), 12.96% (7), 11.11% (6), and 9.25% (5) of the isolates showed reduced susceptibility to doripenem, meropenem, imipinem, and ertapenem, respectively (Fig. 1). Twenty-eight percent of the isolates were non-susceptible to the four tested carbapenems. The E-test method revealed that 75.92% (41 isolates), 33.33% (18 isolates), 25.92% (14 isolates), and 18.51% (10 isolates) of the isolates were resistant to ertapenem, doripenem, imipinem, and meropenem, respectively (Table 1). Moreover, 9.25% (5 isolates), 44.44% (24 isolates), 12.96% (7 isolates), and 16.66% (9 isolates) of the isolates showed reduced susceptibility to ertapenem, doripenem, imipenem, and meropenem, respectively. Almost one third of the isolates (17, 31.48%) were resistant to two carbapenems and 11.11% (6 isolates) were resistant to three carbapenems, while non-susceptibility to at least two carbapenems was detected in more than half of the isolates (38, 70.37%).

Antimicrobial susceptibility testing revealed that all CNSKP isolates were non-susceptible to nine or more antibiotics. Multidrug-resistance (MDR), defined as

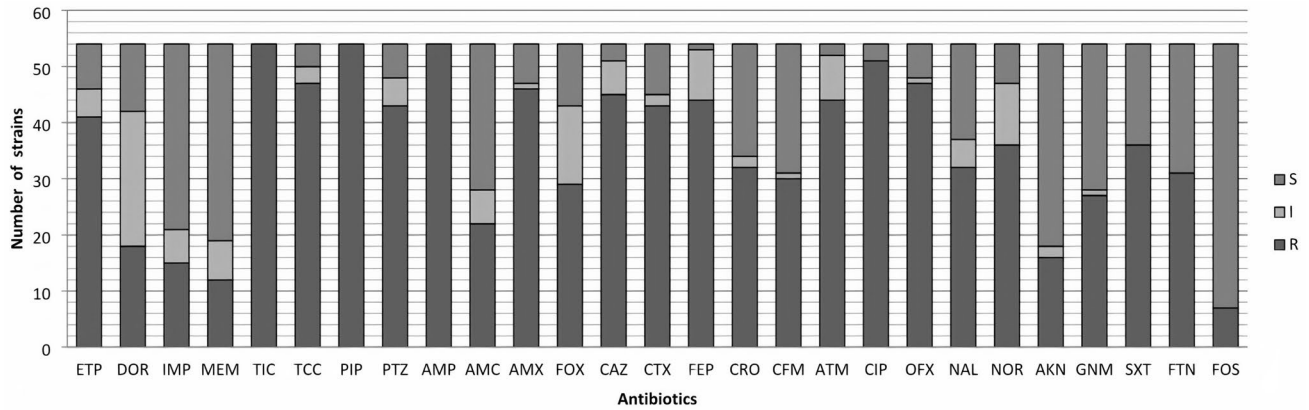

Fig. 1 Distribution of antibiotic susceptibility among the 54 CNSKP as determined by disc diffusion assay and classified according to CA-SFM/EUCAST guidelines. *S* susceptible, *I* intermediate, *R* resistant, *ETP* ertapenem, *DOR* doripenem, *IMP* imipenem, *MEM* meropenem, *TIC* ticarcillin, *TCC* ticarcillin-clavulanate, *PIP* piperacillin, *PTZ* piperacillin-tazobactam, *AMP* ampicillin, *AMC* amoxicilin-clavulanate, *AMX* amoxicillin, *FOX* cefoxitin, *CAZ* ceftazidime, *CTX* cefotaxime, *FEP* cefepim, *CRO* ceftriaxon, *CFM* cefixim, *ATM* aztreonam, *CIP* ciprofloxacin, *OFX* ofloxacin, *NAL* nalidixicacid, *NOR* norfloxacin, *AKN* amikacin, *GNM* gentamicin, *SXT* trimethoprim/sulfamethoxazole, *FTN* nitrofuran, *FOS* fosfomycin

non-susceptibility to at least one agent in three or more antibiotic categories, was observed in 43 (79.64%) of 54 the isolates of the isolates.

Carbapenemase Detection using Biochemical and Phenotypic Methods

Overall, the majority of the strains were distinctly positive or negative by the biochemical mCarba NP test and the phenotypic CDT. The mCarba NP test gave positive results for fifty isolates (92.59%) (Table 1). Through the addition of EDTA and tazobactam, the test allowed the detection of class B MBL and class A carbapenemases in 12 (22.22%) and two (3.7%) isolates, respectively. However, the lack of inhibition by both β-lactamase inhibitors was observed in 36 isolates (66.66%).

Regarding the combined-disc test (Fig. 2), 52 out of 54 isolates (96.29%) exhibited positive results (Table 1). The test identified 50 MBL producers (92.59%), showing an enhancement in inhibition zone by more than 5 mm (range 5–32 mm) around the combined carbapenem/DPA disc compared to carbapenem alone disc. Twenty-eight (51.85%) isolates were picked up as class A carbapenemase-producers since disc containing carbapenem/PBA resulted in increased inhibition zone by more than 4 mm (range 4–32 mm) without synergy with cloxacillin. Based on the concomitant absence of synergy with any inhibitor and inhibition-zone diameter ≤ 10 with the temocillin disc, six (11.11%) isolates were presumptively OXA-48 positive. An enhancement in inhibition zone ≥ 5 mm in presence of cloxacillin was observed with eight isolates indicating the production of class C extended-spectrum AmpC β-lactamase. The combined-disc test failed to detect carbapenemase production in 2 out of 54 isolates (KP12 and KP16).

Imipenem hydrolysis was detected in the enzyme extracts of 44 isolates (81.48%) using UV-spectrophotometry (Table 1). Strong imipenem hydrolysis was recorded for three (5.55%) of the 54 isolates, while moderate imipenemase activity (IRs ranging from 0.01 to 0.055 Au/min) was observed in 34 of the 54 isolates (62.96%).

Carbapenemase Identification using PCR and Gene Sequencing

PCR results showed that 96.29% (52/54) of the CNSKP isolates harbored at least one carbapenemase gene (Table 1). The Ambler class B bla_{NDM} gene was the most frequently detected in our study. It was present in 88.88% of the isolates. Sequence analysis of 35 randomly selected amplicons indicated that the predominantly identified bla_{NDM} variant was bla_{NDM-1} (97.14%). One isolate (KP26) harbored the variant bla_{NDM-9}. bla_{NDM-1} and bla_{NDM-9} genes had sequence similarity of 99% to bla_{NDM-1} from *K. pneumoniae* KP53 strain (GenBank accession number OL348378.1) and 89% to bla_{NDM-9} from *K. pneumoniae* 13–14 strain (GenBank accession number MN175386.1), respectively. Ambler class A bla_{KPC} genes were detected in 22 out of 54 isolates. Sequencing of half of them revealed the prevalence of bla_{KPC-2} (81.81%). Besides, bla_{KPC-20} and bla_{KPC-26} were found in KP28 and KP14, respectively. bla_{KPC-2}, bla_{KPC-20}, and bla_{KPC-26} genes showed sequence similarity of 99% to bla_{KPC-2} in *K. pneumoniae* SCPM-O-B-8919 strain (accession number CP094994.1), bla_{KPC-20} in *E. coli* 15-50575E strain (GenBank accession number MF772496.1), and bla_{KPC-26} in *K. pneumoniae* BT_746

Table 1 Phenotypic and molecular features of the 54 CNSKP

Strain	Unit	Sample	MIC (μg/mL)				mCarba NP test result	CDT result	IR of imipenem hydrolysis measured by UV-spectrophotometry[a]		Carbapenemase genes	Other β-lactamase genes	Pulsotype
			ETP	IMP	MEM	DOR			+50 μM $ZnSO_4$	+50 mM $NaHCO_3$			
KP1	IC	RS	4	0.19	0.032	0.047	B	B	0.02	0.028	bla_{NDM}	$bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}15}$	P27
KP2	PED	Blood	0.047	0.125	0.023	4	A	A	0.02	0.016	$bla_{KPC\text{-}2}$	$bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}15}$, bla_{SHV}, $bla_{OXA\text{-}1}$	P19
KP3	PED	Urine	3	0.125	0.032	0.032	B	B	0.017	0.022	bla_{NDM}	$bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}15}$, bla_{SHV}	P37
KP4	OUT	Urine	8	0.19	0.032	0.064	B	B	0.018	0.029	bla_{NDM}	bla_{SHV}	P9
KP5	OUT	Urine	6	0.047	0.006	0.008	Und	A + B	0	0	$bla_{KPC\text{-}2}$, $bla_{NDM\text{-}1}$	$bla_{CTX\text{-}M\text{-}15}$, bla_{SHV}	P33
KP6	IC	RS	24	3	3	12	NI	A + B	0.025	0	bla_{NDM}	$bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}15}$, bla_{SHV}, $bla_{OXA\text{-}1}$, bla_{TEM}	P10
KP7	IC	RS	0.004	0.094	0.016	6	NI	B	0	0	$bla_{NDM\text{-}1}$	$bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}15}$, bla_{SHV}, bla_{TEM}	P41
KP8	IC	DPA	4	0.5	3	1	Und	A + B	0.036	0.047	bla_{KPC}, $bla_{NDM\text{-}1}$, $bla_{OXA\text{-}48}$	$bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}15}$, bla_{SHV}, $bla_{OXA\text{-}1}$, bla_{TEM}	P22
KP9	OUT	Urine	4	0.125	0.016	0.032	Und	A + B	0	0	bla_{KPC}, bla_{NDM}	bla_{SHV}	P5
KP10	PED	Blood	8	0.094	0.016	0.016	B	B	0	0.035	bla_{NDM}	bla_{SHV}	P30
KP11	IC	RS	3	0.125	0.016	6	B	B	0.02	0.039	bla_{NDM}	$bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}15}$, bla_{SHV}, $bla_{OXA\text{-}1}$, bla_{TEM}	P44
KP12	OUT	Urine	8	0.19	0.047	3	NI	ND	0	0	ND	$bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}15}$, bla_{SHV}, bla_{TEM}	P26
KP13	PED	Blood	3	0.125	0.032	4	Und	A + B	0.018	0.023	$bla_{OXA\text{-}48}$	$bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}15}$, bla_{SHV}, bla_{TEM}	P34
KP14	OUT	Urine	0.008	0.19	0.016	4	A	A	0.027	0.034	$bla_{KPC\text{-}26}$	bla_{SHV}	NT
KP15	OUT	Urine	1	0.125	0.023	4	B	B	0.03	0	bla_{NDM}	$bla_{CTX\text{-}M\text{-}15}$, bla_{SHV}	P39
KP16	OUT	Urine	0.75	0.125	0.008	0.012	NI	ND	0	0	ND	$bla_{CTX\text{-}M\text{-}1}$, bla_{SHV}	P45
KP17	OUT	Urine	6	14	0.047	12	B	B	0	0	bla_{NDM}	bla_{SHV}	P42
KP18	IC	RS	6	3	12	2	Und	B + D	0.055	0.055	$bla_{NDM\text{-}1}$, $bla_{OXA\text{-}48}$	$bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}15}$, bla_{SHV}, bla_{TEM}	P16
KP19	OUT	Urine	0.006	0.19	0.047	6	Und	A + B	0	0	$bla_{KPC\text{-}2}$, bla_{NDM}	$bla_{CTX\text{-}M\text{-}15}$,$bla_{SHV}$, bla_{TEM}	P32
KP20	IC	RS	8	3	4	4	Und	A + B + D	0.038	0.14	bla_{KPC}, bla_{NDM}, $bla_{OXA\text{-}48}$	$bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}15}$, bla_{SHV}, $bla_{OXA\text{-}1}$, bla_{TEM}	P17
KP21	IM	Blood	3	0.094	0.012	4	Und	A + B	0	0	$bla_{KPC\text{-}2}$, bla_{NDM}, $bla_{OXA\text{-}48}$	$bla_{CTX\text{-}M\text{-}15}$, bla_{SHV}, bla_{TEM}	P40
KP22	OUT	Urine	3	0.38	0.032	4	Und	A + B	0.034	0.024	bla_{KPC},bla_{NDM}	$bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}15}$, bla_{SHV}, $bla_{OXA\text{-}1}$, bla_{TEM}	P43
KP23	PED	Urine	3	0.094	0.008	8	Und	B + AmpC	0	0.032	bla_{KPC}, $bla_{NDM\text{-}1}$, $bla_{OXA\text{-}48}$	$bla_{CTX\text{-}M\text{-}15}$, bla_{SHV}, bla_{TEM}, $bla_{CMY\text{-}2}$	P35
KP24	OUT	Urine	8	0.19	1	0.125	Und	B	0.01	0	$bla_{NDM\text{-}1}$, $bla_{OXA\text{-}48}$	$bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}15}$, bla_{SHV}, bla_{TEM}	P36

Table 1 (continued)

Strain	Unit	Sample	MIC (µg/mL)				mCarba NP test result	CDT result	IR of imipenem hydrolysis measured by UV-spectrophotometry[a]		Carbapenemase genes	Other β-lactamase genes	Pulsotype
			ETP	IMP	MEM	DOR			+50 µM $ZnSO_4$	+50 mM $NaHCO_3$			
KP 25	OUT	Urine	0.004	0.125	0.016	6	B	B	0	0.02	bla_{NDM-1}	bla_{SHV}	P14
KP26	IC	RS	12	12	0.016	0.032	Und	A + B + AmpC	0.051	0.15	bla_{NDM-9}, bla_{OXA-48}	$bla_{CTX-M-15}$, bla_{CMY-2}	P11
KP27	IC	Catheters	6	12	4	12	Und	A + B	0.034	0.055	bla_{OXA-48}	bla_{TEM}	P1c
KP28	IC	Catheters	12	24	12	12	Und	A + B + AmpC	0.027	0.096	bla_{KPC-20},bla_{NDM-1}, bla_{OXA-48}	$bla_{CTX-M-1}$, $bla_{CTX-M-15}$, bla_{SHV}, bla_{OXA-1}, bla_{TEM}, bla_{CMY-2}	P1a
KP29	IC	RS	8	12	12	12	Und	B	0.018	0.028	bla_{NDM-1}, bla_{OXA-48}	$bla_{CTX-M-1}$, $bla_{CTX-M-15}$, bla_{SHV}, bla_{TEM}	P1b
KP 30	OUT	RS	12	12	12	12	Und	A + B + D	0.034	0.042	bla_{NDM-1}, bla_{OXA-48}	$bla_{CTX-M-1}$, $bla_{CTX-M-15}$, bla_{SHV}, bla_{TEM}	NT
KP31	IC	Blood	8	3	8	4	Und	A + B	0.018	0.025	bla_{KPC}, bla_{NDM-1}, bla_{OXA-48}	$bla_{CTX-M-1}$, $bla_{CTX-M-15}$, bla_{SHV}, bla_{TEM}	P15
KP32	OUT	Urine	4	0.125	0.016	4	Und	B	0.014	0.019	bla_{NDM-1}, bla_{OXA-48}	$bla_{CTX-M-15}$, bla_{SHV}	P1d
KP33	IC	Urine	0.002	0.094	0.016	12	B	B	0.018	0.016	bla_{NDM-1}	$bla_{CTX-M-15}$, bla_{SHV}, bla_{TEM}	P4
KP34	IC	RS	8	24	12	12	Und	A + B + D	0.027	0.073	bla_{KPC}, bla_{NDM-1}, bla_{OXA-48}	$bla_{CTX-M-15}$, bla_{SHV}, bla_{TEM}	P6a
KP35	PED	BA	12	0.125	0.023	3	Und	A + B	0.018	0	bla_{KPC-2}, bla_{NDM-1}, bla_{OXA-48}	$bla_{CTX-M-15}$, bla_{SHV}, bla_{TEM}	P6b
KP36	IC	RS	4	12	0.032	12	Und	A + B + D + AmpC	0.018	0.073	bla_{KPC}, bla_{NDM-1}, bla_{OXA-48}	$bla_{CTX-M-1}$, $bla_{CTX-M-15}$, bla_{SHV}, bla_{OXA-1}, bla_{TEM}	P21
KP37	IC	RS	12	24	12	24	Und	A + B + D	0.032	0.029	bla_{KPC}, bla_{NDM-1}, bla_{OXA-48}	$bla_{CTX-M-1}$, $bla_{CTX-M-15}$, bla_{SHV}, bla_{TEM}	P7
KP 38	OUT	Urine	0.012	0.19	0.032	3	Und	B	0.021	0.021	bla_{NDM-1}, bla_{OXA-48}	$bla_{CTX-M-1}$, bla_{SHV}, bla_{TEM}	P29
KP39	OUT	Urine	0.002	0.064	3	12	Und	B + AmpC	0.016	0.014	bla_{NDM-1}, bla_{OXA-48}	$bla_{CTX-M-1}$, $bla_{CTX-M-15}$, bla_{SHV}, bla_{OXA-1}, bla_{TEM}	P13
KP40	IC	RS	8	3	12	12	Und	A + B	0.017	0.067	bla_{KPC-2}, bla_{NDM-1}, bla_{OXA-48}	$bla_{CTX-M-1}$, $bla_{CTX-M-15}$, bla_{SHV}, bla_{TEM}	P8c
KP41	IC	RS	6	12	3	12	Und	A + B + AmpC	0.035	0.09	bla_{KPC-2}, bla_{NDM-1}, bla_{OXA-48}	$bla_{CTX-M-1}$, $bla_{CTX-M-15}$, bla_{SHV}, bla_{TEM}	P8a
KP42	OUT	Urine	32	32	32	32	Und	A + B	0.015	0.11	bla_{KPC-2}, bla_{NDM-1}, bla_{OXA-48}	$bla_{CTX-M-1}$, $bla_{CTX-M-15}$, bla_{SHV}, bla_{TEM}	P8b
KP 43	PED	Blood	1	0.094	0.023	3	Und	B	0.033	0.037	bla_{NDM-1}, bla_{OXA-48}	$bla_{CTX-M-1}$, $bla_{CTX-M-15}$, bla_{SHV}, bla_{TEM}	P31
KP44	OUT	Urine	8	0.19	0.032	12	Und	A + B	0	0.026	bla_{KPC-2}, bla_{NDM-1}, bla_{OXA-48}	$bla_{CTX-M-1}$, $bla_{CTX-M-15}$, bla_{SHV}, bla_{TEM}	P28
KP45	IC	Catheters	8	0.25	0.047	6	Und	B	0	0	bla_{NDM-1}, bla_{OXA-48}	$bla_{CTX-M-1}$, $bla_{CTX-M-15}$, bla_{SHV}, bla_{TEM}	P3
KP46	IC	RS	8	12	12	32	Und	A + B	0.016	0.034	bla_{NDM-1}, bla_{OXA-48}	$bla_{CTX-M-15}$, bla_{SHV}, bla_{TEM}	P23

Table 1 (continued)

Strain	Unit	Sample	MIC (μg/mL)				mCarba NP test result	CDT result	IR of imipenem hydrolysis measured by UV-spectrophotometry[a]		Carbapenemase genes	Other β-lactamase genes	Pulsotype
			ETP	IMP	MEM	DOR			+50 μM $ZnSO_4$	+50 mM $NaHCO_3$			
KP47	IC	DPA	8	12	12	12	Und	A + B	0.005	0.026	bla_{KPC}, bla_{NDM-1}	bla_{SHV}, bla_{TEM}	P2
KP48	OUT	Urine	1	0.125	3	3	Und	A + B	0.045	0.02	bla_{KPC}, bla_{NDM-1}, bla_{OXA-48}	$bla_{CTX-M-15}$, bla_{SHV}, bla_{TEM}	NT
KP49	OUT	Urine	8	3	0.016	6	B	B + AmpC	0	0.036	bla_{NDM-1}	$bla_{CTX-M-15}$, bla_{SHV}, bla_{TEM}	P12
KP50	OUT	Urine	32	3	0.032	6	B	B	0	0.056	bla_{NDM-1}	$bla_{CTX-M-15}$, bla_{SHV}, bla_{TEM}	P18
KP51	IC	Catheters	4	12	4	12	B	A + B	0	0.036	bla_{NDM-1}	$bla_{CTX-M-1}$, $bla_{CTX-M-15}$, bla_{SHV}, bla_{TEM}, bla_{CMY-2}	P24
KP52	IC	RS	3	0.064	0.064	3	Und	B + AmpC	0	0.09	bla_{NDM-1},bla_{OXA-48}	$bla_{CTX-M-1}$, $bla_{CTX-M-15}$, bla_{SHV}, bla_{TEM}, bla_{CMY-2}	P38
KP53	OUT	Urine	3	0.5	0.023	4	Und	B	0	0.09	bla_{NDM-1}, bla_{OXA-48}	$bla_{CTX-M-15}$, bla_{SHV}, bla_{TEM}	P20
KP54	IC	RS	1	0.125	0.032	0.047	Und	B	0	0	bla_{NDM-1}, bla_{OXA-48}	$bla_{CTX-M-1}$, $bla_{CTX-M-15}$, bla_{SHV}, bla_{TEM}	P25

[a]Initial rate (IR) of imipenem hydrolysis was expressed as absorbance units/min (Au/min) and was arbitrarily considered as strong (IR ≥ 0.1 Au/min), moderate (0.01Au/min ≤ IR < 0.1 U/mg), or weak (IR < 0.01 Au/min)

IC intensive care, *PED* pediatric, *OUT* outpatients, *IM* internal medicine, *RS* rectal swab, *DPA* distal protected aspirate, *BA* bronchial aspiration, *MIC* minimum inhibitory concentration, *ETP* ertapenem, *IMP* imipenem, *MEM* meropenem, *DOR* doripenem, *A* class A carbapenemase, *B* class B carbapenemase, *D* class D carbapenemase, *AmpC* a class C β-lactamase, *Und* undetermined (i.e., neither A nor B, it could be imipenem-hydrolyzing class D and/or C β-lactamase), *NI* non-interpretable, *ND* not detected, *NT* non typable

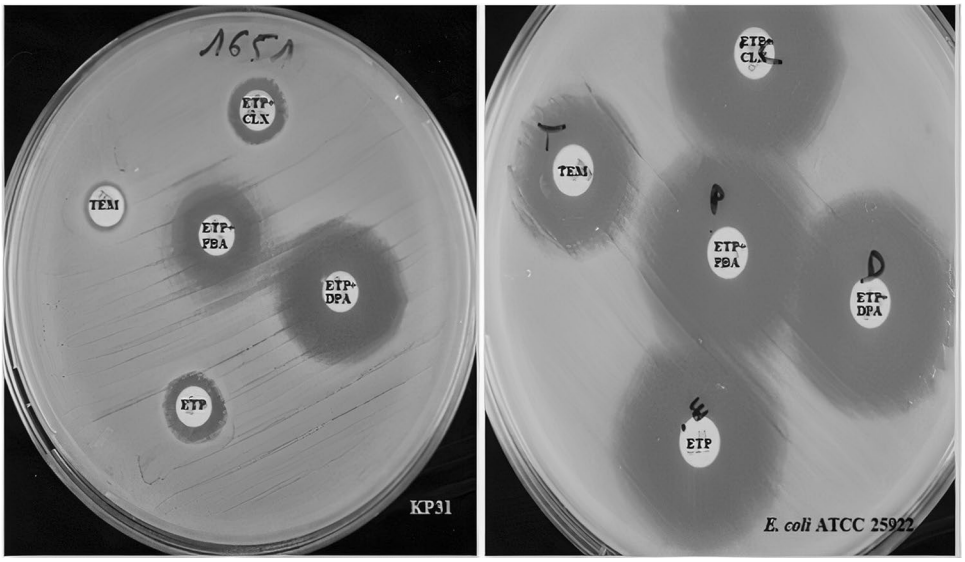

Fig. 2 A representative result of combined-disc test for carbapenemase differentiation in KP31 isolate and *E. coli* 25922 strain (negative control) using discs of ertapenem alone, ertapenem with phenylboronic acid (PBA), ertapenem with dipicolonic acid (DPA), or ertapenem with cloxacillin, and temocillin (TMO). An increase in inhibition zone by more than 4 mm around the combined ertapenem/PBA disc or more than 5 mm around the combined ertapenem/DPA disc, compared to ertapenem alone, indicates the production of classes A and B β-lactamases, respectively, in KP31 isolate

strain (GenBankaccession number NG_051469.1), respectively. PCR experiments showed that the second most frequent carbapenemase-encoding gene was the presumptive Ambler class D $bla_{\text{OXA-48-like}}$ gene (57.4%). All the 54 isolates were PCR-negative for bla_{IMP}, bla_{VIM}, bla_{GIM}, and bla_{GES} genes.

Considering the co-occurrence of *bla* genes in a single isolate, more than half (62.96%) of CNSKP isolates co-harbored more than one carbapenemase-encoding gene. Thus, the combination of bla_{NDM}, bla_{KPC}, and $bla_{\text{OXA-48-like}}$ genes showed the highest prevalence (27.77%), followed by the association of bla_{NDM} and $bla_{\text{OXA-48-like}}$ (25.92%). The combination of bla_{NDM} and bla_{KPC} genes was present in 9.25% of the isolates. However, the remaining 18 isolates showed the presence of only one gene per isolate.

Among the 54 CNSKP isolates, 85.18% harbored ESBL-encoding genes $bla_{\text{CTX-M-15}}$ (44/54) and $bla_{\text{CTX-M-1}}$ (31/54). Other *bla* genes were also detected among the CNSKP studied, mainly bla_{SHV} (94.44%) followed by bla_{TEM} (74.07%). Additionally, the Ambler class C $bla_{\text{CMY-2}}$ gene was detected in five isolates.

PFGE Molecular Typing

Fifty-one PFGE patterns of CNSPK were obtained, while three isolates were non typable (KP14, KP30, and KP48) (Fig. 3). Analysis of the 51 PFGE patterns revealed genetic diversity with 45 different pulsotypes. Three minor clusters were identified. Four isolates (KP27, KP28, KP29, and KP32), which harbored $bla_{\text{OXA-48-like}}$ gene, formed the cluster A with a genetic similarity of about 93%. Two (KP29 and KP32) of them carried also bla_{KPC} and bla_{NDM}. The two other minor clusters B and C included two (KP34 and KP35) and three (KP40, KP41, and KP42) isolates, respectively, with genetic relatedness of about 82%. Isolates of cluster C harbored the bla_{KPC}, $bla_{\text{NDM-1}}$, $bla_{\text{OXA-48-like}}$, $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-15}}$, bla_{SHV}, and bla_{TEM} genes.

Discussion

We report here the first study on CNSKP in the Regional Hospital of Ben Arous. More than half of the isolates were from inpatients occurring predominantly in intensive care unit. Remarkably, almost one third of the isolates were recovered from rectal swab. Although the gastrointestinal carriage of *K. pneumoniae* strains is generally asymptomatic, genomic studies confirmed that gut colonization is an established risk factor for subsequent extraintestinal infections, especially in intensive care patients [31, 32]. Nevertheless, the mechanisms allowing *K. pneumoniae* strain to establish within intestinal microbiota and progress to cause disease remain poorly elucidated. It is noteworthy that 21.87% of the inpatient CNSKP isolates were isolated from pediatric wards. Indeed, given the limited treatment options for pediatric patients, CNSKP isolated from neonates, infants, and children is a serious threat.

Our investigation revealed a high incidence (40.6%) of CNSKP among 133 β-lactam resistant *K. pneumoniae* isolated during a 13-month period. They displayed high genetic diversity through PFGE. According to reports on CRKP in two Tunisian hospitals, 13.2% and 15.8% of the isolated *K. pneumoniae* were resistant to carbapenems [15, 16]. Qin X et al. [33] described a higher prevalence (54%) of CRKP among 68 *K. pneumoniae* isolated in ICU of a Chinese hospital.

Our study revealed that the non-susceptibility rate to ertapenem (85.17%) was the highest, followed by doripenem

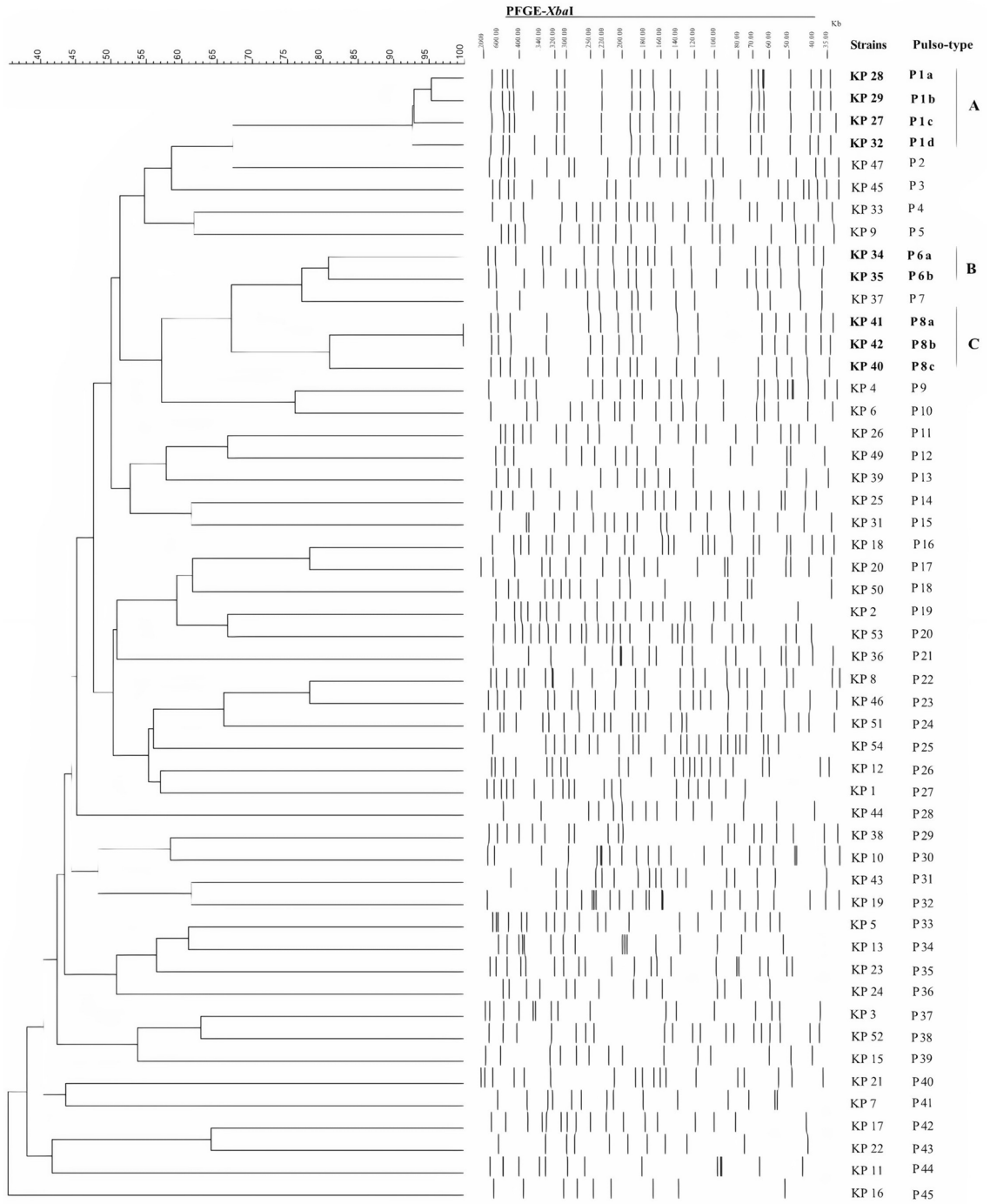

Fig. 3 *Xba*I PFGE patterns of 51 CNSKP isolates. The dendrogram was generated by Dice similarity coefficient (optimization 1.0%, band matching tolerance 1.0%, tolerance change 1.0%) and an unweighted pair group method using arithmetic averages (UPGMA). A, B, and C represent PFGE clusters with > 80% similarity. Forty-five isolates displayed different pulsotypes. Three strains (KP14, KP30, and KP48) were untypable by *Xba*I

(77.77%), imipenem (38.88%), and meropenem (35.18%). Our findings are in line with those described in previous Tunisian investigations. Thus, in a study conducted on *Enterobacteriaceae* isolated from 2014 to 2016 at the Military Hospital of Tunis, all 98 CRKP were resistant to ertapenem and 58.18% of them were imipenem resistant [11]. Also, another Tunisian work reported that 20.83% and 8.33% of 24 colistin-resistant *K. pneumoniae* strains were resistant to ertapenem and imipenem, respectively [34]. In a Libyan study, 94% and 70% of 47 *K. pneumoniae* were

resistant to ertapenem and meropenem, respectively [35]. Moreover, 100%, 93.39%, and 95.28% of 106 carbapenemase-producing *K. pneumoniae* strains, isolated at a Portuguese hospital, were resistant to ertapenem, meropenem, and imipenem, respectively [36].

It is worrying that more than three-quarters (79.62%) of the 54 CNSKP isolates were MDR bacteria. In agreement with our findings, all the 29 ertapenem-resistant *K. pneumoniae* (100%) isolated in a university hospital in Tunisia were MDR strains [16]. High MDR prevalence was also described among ertapenem-resistant *K. pneumoniae* isolated in Libyan, Italian, Moroccan, and Egyptian hospitals, respectively [35, 37–39]. Moreover, according to another study carried in Greece, 65% of 300 CNSKP were MDR [40].

Carbapenemase production was detected by the mCarba NP test and CDT in 92.59%, and 96.29% of the 54 CNSKP isolates, respectively. Carbepenemsase genes bla_{NDM}, $bla_{KPC,}$ and $bla_{OXA-48-like}$ were identified in all CNSKP isolates, except two (KP12 and KP16). CDT was positive for 100%, 95.45% (21/22), and 19.35% (6/31) of CNSKP isolates harboring bla_{NDM}, bla_{KPC}, and $bla_{OXA48-like}$ genes, respectively, while the mCarba NP test was positive for 25% (12/48) and 9.09% (2/22) of the isolates carrying bla_{NDM} and bla_{KPC} genes, respectively. Imipenem hydrolysis was detected using UV-spectrophotometry in 81.48% of the isolates. Nevertheless, overall there is no clear relationship between imipenem hydrolysis and PCR results. Previous studies evaluating phenotypic and biochemical carbapenemase detection tests versus gold-standard PCR showed their high sensitivity and specificity only when one carbapenemase type was produced [18, 41, 42].

In the present study, 96.26% of CNSKP isolates harbored carbapenemase genes being predominantly bla_{NDM} (88.88%), followed by $bla_{OXA-48-like}$ (57.4%), and bla_{KPC} (40.74%). To the best of our knowledge, our results described for the first time in Tunisia the presence of bla_{NDM-9}, bla_{KPC-20}, and bla_{KPC-26}. Our findings are in disagreement with previous Tunisian studies stating the prevalence of $bla_{OXA-48-like}$ over bla_{NDM}-gene among *K. pneumoniae* isolates. Thus, $bla_{OXA-48-like}$ /bla_{NDM} were detected in 80.61% (79/98) / 13.6% (13/98), 78.23% (133/170)/ 8.23% (14/170), and 68.96% (20/29) / 24.13% (7/29) of CRPK isolated in Tunsian hospitals [11, 16, 43]. However, according to the same studies, no bla_{KPC} gene was detected among the CRKP isolates. Recently, the first clinical cases of KPC-2-producing *K. pneumoniae* were described in Algeria with a prevalence of 21.4%, whereas 78.6% of the isolates were positive for bla_{OXA-48} [44]. Among 81clinical CRPK isolated in Qatar, bla_{NDM-1}, bla_{OXA-48}, and bla_{KPC-2} were detected in 27.2%, 19.8%, and 3.7% of the isolates, respectively [45]. According to a Spanish study, bla_{OXA-48} and bla_{KPC} were mainly detected among *K. pneumoniae* isolates [46]. Only bla_{KPC} genes were identified among 18 CRKP isolated in an Italian hospital [37]. Moreover, moderate to high prevalence of bla_{KPC} was reported in Canada [47], Iran [48], and China [49]. Nevertheless, no bla_{KPC} gene was detected according to Moroccan and Egyptian studies [38, 39].

Remarkably, the present study pointed out the co-occurrence of carbapenemase genes. More than half (62.96%) of CNSKP isolates harbored more than one carbapenemase-encoding gene. The combination of bla_{NDM}, bla_{KPC}, and $bla_{OXA-48-like}$ showed the highest prevalence (27.77%) among the 54 CNSKP. No previous study described similar combination in Tunisia among clinical *K. pneumoniae* strains. Our results also revealed high prevalence (25.92%) of the association bla_{NDM-1} and $bla_{OXA-48-like;}$ while 9.25% of the CNSKP strains co-harbored bla_{NDM-1} and bla_{KPC} genes. On the other hand, a clonal spread of colistin-resistant *K. pneumoniae* co-harboring bla_{KPC} and bla_{VIM} genes has been reported in neonates in a Tunisian hospital [50].

Conclusion

In conclusion, our study highlights the high prevalence of bla_{NDM} among 54 CNSKP isolates. To our knowledge, our results described for the first time in Tunisia the presence of bla_{NDM-9}, bla_{KPC-20}, and bla_{KPC-26}. Worryingly, the co-occurrence of bla_{NDM}, bla_{KPC}, and $bla_{OXA-48-like}$ was detected in nearly one-third of the isolates. Further genomic sequencing and sequence analysis of the region surrounding the resistance gene are necessary to perform in order to elucidate the regulation of resistance expression and to provide more details about the molecular epidemiology of bla_{NDM}, bla_{KPC}, and $bla_{OXA-48-like}$ in the CNSKP. Compared to the phenotypic CD test, the mCarba NP test had limitations to identify the type of carbapenemase in CNSKP isolates showing a complex carbapenem resistance gene profile, whose identification would be challenging at the phenotypic level. Molecular assays offer rapidity and sensitivity to detect the presence of carbapenemase-encoding genes; however, their high cost may hamper their application in clinical laboratories. Furthermore, implementation of government policies to reduce the overuse and misuse of antimicrobial agents, effective infection prevention and control practices, and CNSKP screening upon hospital admission may help to decrease the resistance rates of CNSKP.

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Author Contributions EZ performed experiments and drafted the manuscript; PSM and MG supervised PCR and sequencing assays; AR helped with PFGE technical assistance; NB-S provided bacterial samples and clinical data; KB-M contributed to the manuscript revision; SR supervised the work and edited the manuscript. All authors read and approved the final manuscript.

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Data Availability All data supporting the findings of this study are available within the article.

Code Availability Not applicable.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical Approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the Regional Hospital of Ben Arous**.** Approval number 13/2022.

Consent to Participate Applicable.

Consent for Publication Applicable.

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