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Outbreak of colistin-resistant carbapenemase-producing *Klebsiella pneumoniae* in Tunisia☆

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ABSTRACT

Objectives: Mechanisms of colistin and carbapenem resistance among a collection of *Klebsiella pneumoniae* isolates recovered in a university hospital in Tunisia were studied.
Methods: In vitro antimicrobial susceptibility testing, S1 nuclease pulsed-field gel electrophoresis (S1-PFGE), Southern blotting and PCR-based replicon typing (PBRT) were performed. Extended-spectrum β-lactamases (ESBLs), carbapenemases, AmpC-type enzymes and *mgrB* genes were detected by PCR and sequencing. Clonality of isolates was assessed by PFGE and multilocus sequence typing (MLST).
Results: Of 940 Enterobacteriaceae isolates recovered from June 2015 to March 2016 in Tahar Sfar Hospital (Mahdia, Tunisia), 220 were identified as *K. pneumoniae*, among which 29 were carbapenem-resistant. Carbapenem resistance was mostly due to expression of bla_{OXA-48} or $bla_{OXA-204}$ in combination with bla_{CMY-4}. Seven isolates carried bla_{NDM-1}, of which two also harboured bla_{OXA-48}, together with bla_{CMY-16} in one of them. All but two isolates also harboured $bla_{CTX-M-15}$. All 20 bla_{OXA-48} genes were part of transposon Tn*1999* on an IncL plasmid, whereas $bla_{OXA-204}$ was found on transposon Tn*2016* on an IncA/C plasmid. Finally, all bla_{NDM-1} genes were located within a Tn*125* transposon on an IncFIIk plasmid. Interestingly, 7 (24.1%) of 29 carbapenem-resistant isolates were resistant to colistin, of which 6 were assigned to ST101, had similar PFGE profiles and presented the same 2-kb insertion in the *mgrB* gene.
Conclusions: This study reports, for the first time in Tunisia, the full molecular characterisation of colistin resistance in *K. pneumoniae*. There is an urgent need for control measures and prudent use of colistin in treatment of infections with carbapenemase-producing *K. pneumoniae*.

1. Introduction

Enterobacteriaceae are a large family of Gram-negative bacteria that includes human pathogens such as *Klebsiella pneumoniae*, a bacterial species continuously acquiring resistance determinants in healthcare settings worldwide. Until recently, carbapenems were one of the favoured antibiotics used to manage human infections due to multidrug-resistant (MDR) *K. pneumoniae*. However, carbapenemase-producing *K. pneumoniae* isolates emerged a decade ago and disseminated worldwide, principally producing the OXA-48-like, NDM-type and KPC-type enzymes [1].

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To tackle the issue of carbapenem-resistant isolates, colistin was considered one of the last-resort molecules. Thus, colistin regained strong interest in human medicine following a long period of rare use because of its nephrotoxicity and neurotoxicity. Unfortunately, the first report of colistin- and carbapenem-resistant *K. pneumoniae* occurred shortly after the re-introduction of colistin, and the increased reliance on colistin has resulted in the emergence of colistin-resistant isolates in many areas [2,3].

Colistin resistance in *K. pneumoniae* can be mediated by alterations in the two-component systems PhoPQ and PmrAB, but the most frequently encountered resistance mechanism is inactivation of the *mgrB* gene owing to insertions/mutations in the promoter or coding sequence. Whereas *mgrB* mutations are selected by the use of colistin, resistant isolates of epidemic clones such as ST258 or ST11 can then further spread without colistin exposure and cause major outbreaks [4]. In contrast, the presence of the recently identified plasmid-mediated *mcr-1* gene is still rare in *K. pneumoniae* [2,5].

In Tunisia, OXA-48 is considered as endemic; moreover, very recent reports also demonstrated the presence of NDM-1- and KPC-2-producing *K. pneumoniae* in hospital settings [6–9]. Colistin is often prescribed in Tunisian hospitals against these MDR pathogens. However, there is a lack of information about the prevalence and mechanisms of colistin resistance in countries such as Tunisia, largely due to the methods used for the determination of colistin susceptibility, which are generally not adequate. Thus, the aim of this study was to determine the prevalence and mechanisms of colistin and carbapenem resistance among a collection of carbapenemase-resistant *K. pneumoniae* isolates recovered in Tahar Sfar University Hospital (Mahdia, Tunisia) over a 10-month period.

2. Materials and methods

2.1. Bacterial isolates

From June 2015 to March 2016, a total of 940 Enterobacteriaceae isolates were collected from different wards of Tahar Sfar University Hospital, a 800-bed university hospital located in the city of Mahdia in the Centre-East of Tunisia. All isolates were identified using the API20E galleries (bioMérieux, Marcy-l'Étoile, France). Screening for carbapenem resistance was performed by the disk diffusion method, and all isolates presenting a non-susceptible phenotype to ertapenem were retained for further studies.

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed on Muller–Hinton agar plates (bioMérieux, Craponne, France) using the standard disk diffusion procedure as described by the Comité de l'antibiogramme de la Société Française de Microbiologie (CA-SFM; http://www.sfm-microbiologie.org). A total of 16 β-lactam (amoxicillin, piperacillin, ticarcillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, ticarcillin/clavulanic acid, cefalotin, cefuroxime, cefotaxime, ceftiofur, ceftazidime, cefoxitin, cefepime, cefquinome, aztreonam and ertapenem) and 16 non-β-lactam (tetracycline, kanamycin, tobramycin, gentamicin, amikacin, apramycin, netilmicin, streptomycin, florfenicol, chloramphenicol, colistin, sulfonamides, trimethoprim, nalidixic acid, ofloxacin and enrofloxacin) antibiotics were tested. *Escherichia coli* ATCC 25922 was used as a quality control strain. Minimum inhibitory concentrations (MICs) of ertapenem, imipenem, meropenem and tigecycline were determined using Etest strips (bioMérieux, Craponne, France) according to the manufacturer's instructions. Results were interpreted according to European Committee on Antimicrobial susceptibility testing (EUCAST) 2015 breakpoints (http://www.eucast.org). The MIC of colistin was determined by broth microdilution in 96-well plates (Thermo Fisher Scientific, Waltham, MA) according to EUCAST recommendations. Isolates presenting an MIC >2 μg/mL were considered resistant.

Metallo-β-lactamase (MBL) production was tested by the modified Hodge test, the Carba NP test and the ethylene diamine tetra-acetic acid (EDTA) double-disk synergy test.

2.3. Screening for β-lactamases and mgrB genes

PCR experiments were performed to screen for genes encoding extended-spectrum β-lactamases (ESBLs), carbapenemases and AmpC-type enzymes (*bla*$_{CTX-M}$, *bla*$_{KPC}$, *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{NDM}$, *bla*$_{OXA-48}$, *bla*$_{CMY}$ and *bla*$_{DHA}$) as described previously [10–12]. The *mgrB* gene was amplified with specific external primers [13]. All positive amplicons were sequenced by GENEWIZ (London, UK).

2.4. Molecular characterisation of plasmids

Plasmids were typed in donor cells using a PCR-based replicon typing (PBRT) kit (Diatheva, Fano, Italy) [14]. Plasmids carrying carbapenemase genes were determined by S1 nuclease pulsed-field gel electrophoresis (S1-PFGE) followed by Southern blotting using the appropriate digoxigenin (DIG)-labelled probes. Detection was performed using the DIG DNA Labelling and Detection Kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions.

2.5. Genetic environment of carbapenemase genes

PCR mapping was performed on all isolates to assess the genetic environment of the carbapenemase genes. Insertion sequence IS*1999* upstream the *bla*$_{OXA-48}$ gene was detected to confirm the presence of transposon Tn*1999*, and insertion of IS*1R* into IS*1999* was screened to characterise the Tn*1999* element [15–17]. The presence of IS*Ecp1* upstream the *bla*$_{OXA-204}$ gene was detected to assess the presence of the Tn*2016* transposon [18]. Localisation of the *bla*$_{NDM-1}$ gene on transposon Tn*125* was assessed by PCR, and all genes of Tn*125* were detected using the primers described in Table 1.

2.6. Clonality of the isolates

The clonality of the *K. pneumoniae* isolates was investigated by PFGE using the *Xba*I restriction endonuclease (Promega, Charbonnières-les-Bains, France). Computer analysis of the PFGE banding

Table 1
Oligonucleotides designed for PCR mapping of the sequences surrounding *bla*$_{NDM-1}$.

Primer	Sequence 5′ → 3′	Gene	Amplicon size (bp)
IS*Aba125*For	AAGAAGGCTTTTCAGCCAGA	Tn*125*	9834
IS*Aba125*Rev	AATGCTGATCTGCCTGATTT		
ble$_{MBL}$For	GCGATTTCGATGTGACAGAG	*ble*$_{MBL}$	332
ble$_{MBL}$Rev	TCAGTCGGGGTTCTGGAT		
*iso*For	ACTATGCCGGGTTGGTGTT	*iso*	446
*iso*Rev	GGCAACATTCGTCGGATTTA		
*tat*For	ATCGATTGTTCCGACAGAGG	*tat*	829
*tat*Rev	ACGAGGTCTTCGTCCTGTCC		
*dct*For	GTTCGTAGGGATGCAGTTGG	*dct*	252
*dct*Rev	GCTACCCTATGCCGGTGAG		
groES-For	TTTTCCACGTCAATCAACCA	*groES*	312
groES-Rev	CGAACAGGTCGTCTTCCTTC		
groEL-For	GTCAACGTGCTCGCCAAC	*groEL*	1503
groEL-Rev	CATCGCTTCGGTGGTGAT		
IS*cr21*For	CGCTGCTGTACGCGCTAGT	IS*cr21*	1374
IS*cr21*Rev	CGCCGTGTTTCTCGAAGT		

Table 2
Patient demographics and epidemiological data of carbapenemase-producing *Klebsiella pneumoniae*.

Isolate	Date	Age (years)	Sex	Ward	MLST	Additional resistance	Treatment	Discharge disposition
P1	9 June 2015	30	F	ICU	ST147	KAN, GEN, TOB, AMK, CHL, TET, SUL, TMP, NAL, ENR	Cefotaxime, tazobactam, amikacin	Alive
P2	8 July 2015	60	M	Surgery	ST101	STR, KAN, GEN, TOB, TET, SUL, TMP, NAL, ENR	Amoxicillin/clavulanic acid, ciprofloxacin, ofloxacin, imipenem, pristinamycin	Alive
P3	27 July 2015	72	F	ICU	ST147	SUL, TMP, NAL, ENR	Imipenem, tigecycline, amikacin	Alive
P4	13 August 2015	69	F	ICU	ST147	KAN, TOB, TET, COL, SUL, TMP, NAL, ENR	Amoxicillin/clavulanic acid, piperacillin, imipenem, tigecycline, amikacin, colistin	Dead
P5	14 August 2015	N/A	F	Urology	ST101	STR, KAN, GEN, TOB, TET, SUL, TMP, NAL, ENR	N/A	N/A
P6	15 August 2015	90	M	ICU	ST101	STR, KAN, GEN, TOB, TET, SUL, TMP, NAL, ENR	N/A	Dead
P7	4 September 2015	45	F	Gynaecology	ST147	KAN, GEN, TOB, TET, SUL, TMP, NAL, ENR	Amoxicillin/clavulanic acid	Alive
P8-1	23 October 2015	55	F	Urology	ST147	SUL, TMP, NAL, ENR	Ertapenem, fosfomycin, amikacin, colistin	Alive
P8-2	12 November 2015	55	F	Surgery	ST147	GEN, TOB, TET, SUL, TMP, NAL, ENR	Ertapenem, fosfomycin, amikacin, colistin	Alive
P9	7 October 2015	20	M	ICU	ST147	KAN, GEN, TOB, TET, SUL, TMP, NAL, ENR	N/A	Dead
P10-1	3 November 2015	75	F	ICU	ST147	KAN, GEN, TOB, AMK, CHL, TET, SUL, TMP, NAL, ENR	Amoxicillin/clavulanic acid, tazobactam, tigecycline	Dead
P10-2	2 December 2015	75	F	ICU	ST101	KAN, GEN, TOB, AMK, CHL, TET, SUL, TMP, NAL, ENR	Amoxicillin/clavulanic acid, tazobactam, tigecycline	Dead
P11	4 November 2015	76	M	ICU	ST101	STR, KAN, GEN, TOB, TET, SUL, TMP, NAL, ENR	N/A	Dead
P12	8 November 2015	85	F	ICU	ST101	STR, KAN, GEN, TOB, TET, SUL, TMP, NAL, ENR	N/A	Dead
P13-1	9 November 2015	60	F	ICU	ST101	STR, KAN, GEN, TOB, TET, SUL, TMP, NAL, ENR	Amoxicillin/clavulanic acid, colistin	Alive
P13-2	17 December 2015	60	F	ICU	ST101	STR, KAN, GEN, TOB, TET, COL, TMP, NAL, ENR	Tigecycline, piperacillin, amikacin	Alive
P13-3	6 January 2016	60	F	ICU	ST101	STR, KAN, GEN, TOB, TET, COL, TMP, NAL, ENR	Fosfomycin	Alive
P14	26 December 2015	N/A	M	Urology	ST101	STR, KAN, GEN, TOB, TET, COL, TMP, NAL, ENR	N/A	N/A
P15	31 December 2015	50	F	Orthopaedics	ST101	STR, KAN, GEN, TOB, TET, TMP, NAL, ENR	Amoxicillin/clavulanic acid, gentamicin, ciprofloxacin, imipenem, amikacin, tigecycline	Alive
P16	21 January 2016	NA	F	Orthopaedics	ST101	STR, KAN, GEN, TOB, TET, SUL, TMP, NAL, ENR	N/A	N/A
P17	22 January 2016	79	M	Urology	ST101	STR, KAN, GEN, TOB, TET, COL, TMP, NAL, ENR	Cefotaxime, ciprofloxacin	Alive
P18	8 February 2016	72	M	Surgery	ST147	KAN, TOB, TET, SUL, TMP, NAL, ENR	Amoxicillin/clavulanic acid, fosfomycin, ceftazidime	Alive
P19	12 February 2016	27	M	ICU	ST101	STR, KAN, GEN, TOB, TET, COL, TMP, NAL, ENR	Ciprofloxacin, imipenem, colistin	Alive
P20	15 February 2016	72	M	ICU	ST392	GEN, TOB, TET, SUL, TMP, NAL, ENR	Tigecycline, imipenem, colistin	Alive
P21	26 February 2016	N/A	M	ICU	ST147	STR, KAN, GEN, TOB, TET, TMP, NAL, ENR	N/A	N/A
P22	29 February 2016	NA	F	ICU	ST101	STR, KAN, GEN, TOB, TET, SUL, TMP, NAL, ENR	N/A	N/A
P23	8 March 2016	58	F	General medicine	ST101	STR, KAN, GEN, TOB, TET, COL, TMP, NAL, ENR	Cefotaxime, levofloxacin, imipenem, amikacin, teicoplanin	Alive
P24	15 March 2016	68	F	ICU	ST147	SUL, TMP, NAL, ENR	Ertapenem	Alive
P25	22 March 2016	73	M	Surgery	ST101	STR, KAN, GEN, TOB, TET, TMP, NAL, ENR	Amoxicillin/clavulanic acid, fusidic acid, tigecycline	Alive

MLST, multilocus sequence typing; ICU, intensive care unit; KAN, kanamycin; GEN, gentamicin; TOB, tobramycin; AMK, amikacin; CHL, chloramphenicol; TET, tetracycline; SUL, sulfonamides; TMP, trimethoprim; NAL, nalidixic acid; ENR, enrofloxacin; STR, streptomycin; COL, colistin; N/A, not assigned.

patterns was performed using the BioNumerics fingerprinting software package (Applied Maths, Sint-Martens-Latem, Belgium; http://www.applied-maths.com/bionumerics). Dice similarity indices were used to generate a dendrogram describing the relationships among PFGE profiles. Tolerance was set at 1% and optimisation at 0.5%.

The *K. pneumoniae* genetic background was determined by multilocus sequence typing (MSLT) through amplification of internal fragments of seven housekeeping genes (*rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB* and *tonB*) as described previously [19]. The allelic profiles of the gene sequences and the sequence types (STs) were determined via the electronic database at the *K. pneumoniae* MLST website (http://pubmlst.org/).

3. Results

3.1. Epidemiology of carbapenem-resistant K. pneumoniae isolates

Of the 940 Enterobacteriaceae isolates recovered, 220 (23.4%) were identified as *K. pneumoniae*, among which 29 (13.2%) were defined as carbapenem-resistant. The distribution of carbapenem-resistant *K. pneumoniae* isolates among the different hospital wards was as follows: intensive care unit (ICU) ($n = 17$); urology ($n = 4$); surgery ($n = 4$); orthopaedics ($n = 2$); gynaecology ($n = 1$); and general medicine ($n = 1$) (Table 2).

3.2. Antimicrobial susceptibility testing and phenotypic detection of β-lactamases

The 29 *K. pneumoniae* isolates exhibiting resistance to ertapenem by disk diffusion presented MICs ranging from 3 μg/mL to >32 μg/mL (Table 3). These isolates showed resistance to all β-lactams tested. In addition, seven of them were positive by phenotypic screening for MBL.

All isolates were MDR since they presented resistance to at least three antibiotic families (Table 3). The most frequently encountered resistance was to trimethoprim (100%), quinolones and fluoroquinolones (100%), tetracycline (89.7%), aminoglycosides (10.3–82.8% depending on the molecule) and sulfamethoxazole (69.0%). In addition, 7 (24.1%) of the 29 *K. pneumoniae* isolates were resistant to colistin with MICs ranging from 12 to 24 μg/mL (Table 3).

3.3. Molecular characterisation of β-lactamases

Resistance to carbapenems was due essentially to expression of the $bla_{\text{OXA-48-like}}$ gene alone (22/29; 75.9%), with 18 (81.8%) of the 22 isolates presenting the $bla_{\text{OXA-48}}$ gene and the remaining 4 isolates (18.2%) harbouring the $bla_{\text{OXA-204}}$ gene in systematic combination with the $bla_{\text{CMY-4}}$ gene (Table 3). The seven isolates that tested positive for the presence of a MBL were NDM-1 producers; two isolates (6.9%) co-produced $bla_{\text{OXA-48}}$ and $bla_{\text{NDM-1}}$ genes. One of these NDM-1- and OXA-48-producing isolates displayed an additional $bla_{\text{CMY-16}}$ gene. All but two *K. pneumoniae* isolates harboured the $bla_{\text{CTX-M-15}}$ ESBL gene (Table 3).

3.4. Plasmid characterisation

All 20 $bla_{\text{OXA-48}}$ genes were identified on an IncL plasmid. PCR mapping showed that the $bla_{\text{OXA-48}}$ gene was systematically part of the composite transposon Tn*1999*. Tn*1999.1* and the variant Tn*1999.2*, presenting an IS*1R* disruption of the upstream IS*1999* element, were detected in 2 and 18 cases, respectively. In contrast, the $bla_{\text{OXA-204}}$ gene proved to be located on an IncA/C plasmid. The genetic environment of the $bla_{\text{OXA-204}}$ gene carried by four isolates (P3, P8-1, P8-2 and P21) had an overall backbone similar to the Tn*2016* transposon with the presence of the IS*Ecp1* element upstream the $bla_{\text{OXA-204}}$ gene (Table 3). Finally, the $bla_{\text{NDM-1}}$ gene

Table 3
Molecular characteristics of carbapenemase-producing *Klebsiella pneumoniae*.

Isolate	MLST	Carbapenemase gene	Carbapenemase-carrying plasmid	Associated β-lactamase	Tn*1999*-type	MIC (μg/mL)					*mgrB* genotype
						IPM	ETP	MEM	TIG	COL	
P1	ST147	$bla_{\text{NDM-1}}$	IncFIIk	$bla_{\text{CTX-M-15}}$	–	24	24	32	6	<0.5	Wild-type
P2	ST101	$bla_{\text{NDM-1}}$,$bla_{\text{OXA-48}}$	IncFIIk, IncL	$bla_{\text{CTX-M-15}}$, $bla_{\text{CMY-16}}$	Tn*1999.2*	>32	>32	>32	4	<0.5	Wild-type
P3	ST147	$bla_{\text{OXA-204}}$	IncA/C	$bla_{\text{CMY-4}}$	–	4	>32	>32	0.75	<0.5	Wild-type
P4	ST147	$bla_{\text{NDM-1}}$	IncFIIk	$bla_{\text{CTX-M-15}}$	–	8	24	32	2	16	IS*Kpn14* insertion
P5	ST101	$bla_{\text{OXA-48}}$	IncL	$bla_{\text{CTX-M-15}}$	Tn*1999.2*	2	>32	16	0.5	<0.5	Wild-type
P6	ST101	$bla_{\text{OXA-48}}$	IncL	$bla_{\text{CTX-M-15}}$	Tn*1999.2*	2	32	16	0.5	<0.5	Wild-type
P7	ST147	$bla_{\text{NDM-1}}$	IncFIIk	$bla_{\text{CTX-M-15}}$	–	24	24	32	1.5	<0.5	Wild-type
P8-1	ST147	$bla_{\text{OXA-204}}$	IncA/C	$bla_{\text{CMY-4}}$	–	2	>32	>32	0.75	<0.5	Wild-type
P8-2	ST147	$bla_{\text{OXA-204}}$	IncA/C	$bla_{\text{CTX-M-15}}$, $bla_{\text{CMY-4}}$	–	8	>32	>32	0.5	<0.5	Wild-type
P9	ST147	$bla_{\text{NDM-1}}$	IncFIIk	$bla_{\text{CTX-M-15}}$	–	24	24	>32	1.5	<0.5	Wild-type
P10-1	ST147	$bla_{\text{NDM-1}}$	IncFIIk	$bla_{\text{CTX-M-15}}$	–	32	>32	>32	1.5	<0.5	Wild-type
P10-2	ST101	$bla_{\text{OXA-48}}$	IncL	$bla_{\text{CTX-M-15}}$	Tn*1999.2*	4	16	16	0.5	<0.5	Wild-type
P11	ST101	$bla_{\text{OXA-48}}$	IncL	$bla_{\text{CTX-M-15}}$	Tn*1999.2*	4	32	16	0.5	<0.5	Wild-type
P12	ST101	$bla_{\text{OXA-48}}$	IncL	$bla_{\text{CTX-M-15}}$	Tn*1999.2*	2	32	16	0.5	<0.5	Wild-type
P13-1	ST101	$bla_{\text{OXA-48}}$	IncL	$bla_{\text{CTX-M-15}}$	Tn*1999.2*	2	>32	16	0.75	<0.5	Wild-type
P13-2	ST101	$bla_{\text{OXA-48}}$	IncL	$bla_{\text{CTX-M-15}}$	Tn*1999.2*	2	>32	16	0.75	16	2-kb insertion
P13-3	ST101	$bla_{\text{OXA-48}}$	IncL	$bla_{\text{CTX-M-15}}$	Tn*1999.2*	2	>32	16	0.75	16	2-kb insertion
P14	ST101	$bla_{\text{OXA-48}}$	IncL	$bla_{\text{CTX-M-15}}$	Tn*1999.2*	4	16	16	1	16	2-kb insertion
P15	ST101	$bla_{\text{OXA-48}}$	IncL	$bla_{\text{CTX-M-15}}$	Tn*1999.2*	2	32	16	0.5	<0.5	Wild-type
P16	ST101	$bla_{\text{OXA-48}}$	IncL	$bla_{\text{CTX-M-15}}$	Tn*1999.2*	2	32	16	0.38	<0.5	Wild-type
P17	ST101	$bla_{\text{OXA-48}}$	IncL	$bla_{\text{CTX-M-15}}$	Tn*1999.2*	2	>32	16	1.5	16	2-kb insertion
P18	ST147	$bla_{\text{NDM-1}}$, $bla_{\text{OXA-48}}$	IncFIIk, IncL	$bla_{\text{CTX-M-15}}$	Tn*1999.1*	2	4	4	2	<0.5	Wild-type
P19	ST101	$bla_{\text{OXA-48}}$	IncL	$bla_{\text{CTX-M-15}}$	Tn*1999.2*	2	>32	16	1	12	2-kb insertion
P20	ST392	$bla_{\text{OXA-48}}$	IncL	$bla_{\text{CTX-M-15}}$	Tn*1999.2*	1.5	3	1	4	<0.5	Wild-type
P21	ST147	$bla_{\text{OXA-204}}$	IncA/C	$bla_{\text{CTX-M-15}}$, $bla_{\text{CMY-4}}$	–	8	>32	>32	0.75	1	Wild-type
P22	ST101	$bla_{\text{OXA-48}}$	IncL	$bla_{\text{CTX-M-15}}$	Tn*1999.1*	4	32	16	0.5	>0.5	Wild-type
P23	ST101	$bla_{\text{OXA-48}}$	IncL	$bla_{\text{CTX-M-15}}$	Tn*1999.2*	3	>32	16	0.75	24	2-kb insertion
P24	ST147	$bla_{\text{OXA-48}}$	IncL	$bla_{\text{CTX-M-15}}$	Tn*1999.2*	32	>32	32	1.5	>0.5	Wild-type
P25	ST101	$bla_{\text{OXA-48}}$	IncL	$bla_{\text{CTX-M-15}}$	Tn*1999.2*					<0.5	Wild-type

MLST, multilocus sequence typing; MIC, minimum inhibitory concentration; IPM, imipenem; ETP, ertapenem; MEM, meropenem; TIG, tigecycline; COL, colistin.

was carried on an IncFIIk plasmid in all NDM-1-positive isolates. In all these strains, bla_{NDM-1} was located within a Tn*125* transposon comprising the 3′ part of the upstream IS*Aba125* element and the bla_{NDM-1}, ble_{MBL}, *iso*, *tat*, *dct*, *groES* and *groEL* genes, which was then truncated after the *groEL* gene.

3.5. Analysis of mgrB gene sequences in colistin-resistant isolates

The entire *mgrB* gene of the 29 carbapenem-resistant isolates was amplified by PCR and was sequenced, including the seven colistin-resistant isolates and 22 colistin-susceptible isolates investigated for control purposes. Sequence analysis of the amplicons showed that the seven colistin-resistant strains had a larger amplicon size compared with the wild-type gene present in the susceptible isolates (Table 3). In one strain (P4), analysis revealed that the truncation resulted from a 772-bp insertion corresponding to the IS*Kpn14* sequence. This insertion occurred in the putative *mgrB* promoter region, 11 nucleotides upstream the start codon of the *mgrB* gene (Table 3). In the six other colistin-resistant isolates, *mgrB* was truncated by the same 2-kb sequence between nucleotides 123 and 124 of the *mgrB* coding sequence. The inserted sequence at the origin of the truncation of the *mgrB* gene is identical to *K. pneumoniae* sequences present in published complete genome but falls in non-annotated regions and does not match with any identified IS sequences (Table 3).

3.6. Clonality of the isolates

According to the criteria of Tenover et al. [20], *Xba*I PFGE profiles revealed the presence of two major clusters, namely clusters A and B (Fig. 1). These two PFGE clusters almost perfectly matched with the identification of ST147 and ST101, respectively (Fig. 1). Only one isolate belonging to cluster A was identified as ST392, which is closely related to ST147 since it differs by only three point mutations in the *tonB* allele (allele 40 in ST392 compared with allele 38 in ST147). ST147 was found in 11 isolates recovered from four wards, i.e. ICU ($n = 7$), surgery ($n = 2$), gynaecology ($n = 1$) and urology ($n = 1$), whereas ST392 was found in one patient hospitalised in the ICU ward (P20). ST101 corresponded to the dominant cluster B ($n = 17$). These isolates originated from five different wards, i.e. ICU ($n = 9$), urology ($n = 3$), surgery ($n = 2$), orthopaedics ($n = 2$) and general medicine ($n = 1$).

Six of the seven NDM-producers were assigned to ST147, whereas the remaining isolate was assigned to ST101. The opposite situation was described for the colistin-resistant isolates, where six were assigned to ST101 and presented the abovementioned identical insertion in the *mgrB* gene whereas the remaining isolate was assigned to ST147 and displayed a different insertion (IS*Kpn14*) in the promoter region of *mgrB*. Interestingly, the first colistin-resistant isolate identified originated from patient P13, a 60-year-old woman admitted in the ICU ward in August 2015 for herpetic encephalitis. Three successive OXA-48- and CTX-M-15-producing *K. pneumoniae* isolates were collected from this patient (P13-1, P13-2 and P13-3) in November 2015, December 2015 and January 2016, respectively. P13-1 was susceptible to colistin and the patient was thus treated with colistin plus amoxicillin/clavulanic acid. The patient rapidly developed renal failure, probably associated with colistin therapy, and a colistin-resistant isolate (P13-2) was identified from a surgical site, presenting an *mgrB* gene inactivated by the insertion of a 2-kb sequence between nucleotides 123 and 124. The patient finally recovered after successive treatments with tigecycline/amikacin and fosfomycin.

4. Discussion

This study provides a molecular insight in the epidemiology of carbapenem-resistant *K. pneumoniae* during a 10-month period spanning 2015–2016 at Tahar Sfar University Hospital in the city of Mahdia, Tunisia, including the report of an outbreak of colistin-resistant OXA-48-producing ST101 *K. pneumoniae*. The importance

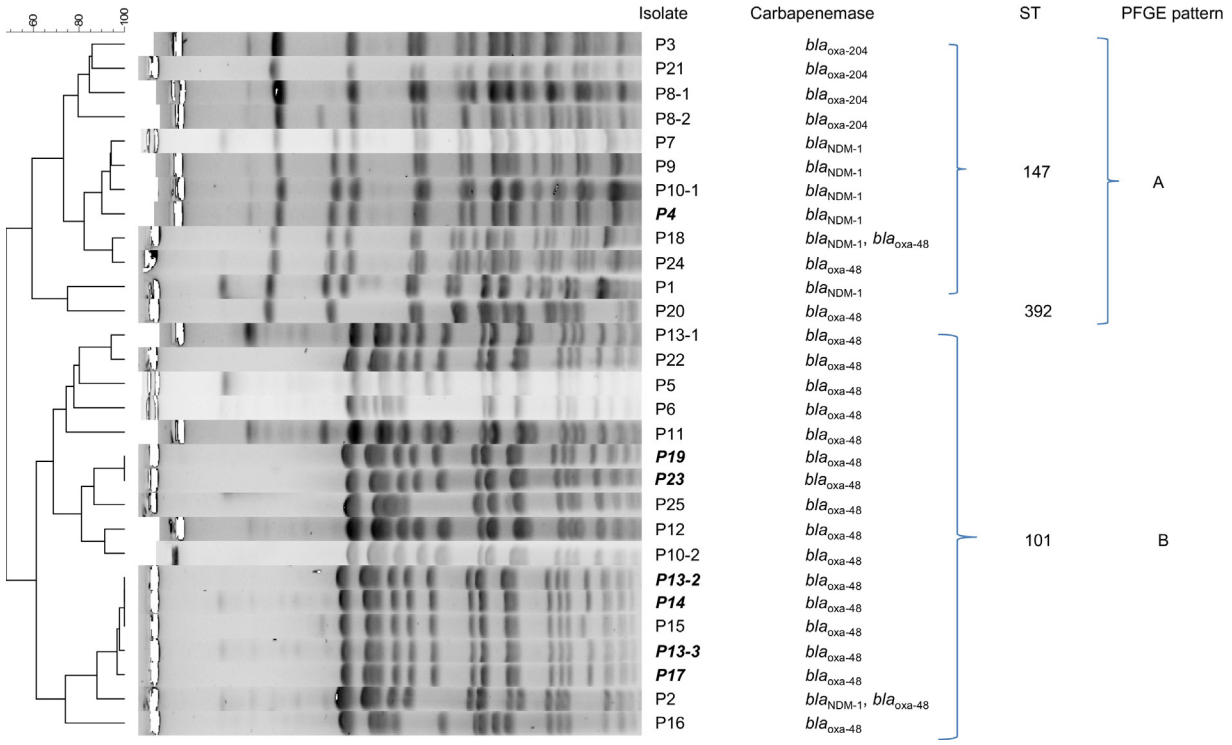

Fig. 1. Clonal relationship of carbapenemase-producing *Klebsiella pneumoniae* isolates from hospitalised patients. Strain references presented in bold character correspond to the seven colistin-resistant isolates. ST, sequence type; PFGE, pulsed-field gel electrophoresis.

of this study relies on the highlight of a dramatic evolution towards pan-resistance over the last 10 years in this hospital. First, a retrospective study on the molecular features of ESBL-producing *K. pneumoniae* isolates has shown the large dissemination of the $bla_{CTX\text{-}M\text{-}15}$ ESBL gene carried by IncFIIk plasmids among several STs (including, but not only, ST101) between 2009 and 2011 in this setting [21]. Second, the first cases of carbapenem-resistant ST101 and ST147 *K. pneumoniae* isolates, producing OXA-48 and its derivative OXA-204 enzymes, respectively, were isolated in December 2012 and May 2013 in this hospital [22]. Third, two of the first autochthonous Tunisian cases of NDM-1-producing ST147 *K. pneumoniae* isolates were again detected in 2015 in this hospital [6]. Here we report the first characterisation of MDR carbapenem-resistant *K. pneumoniae* isolates presenting resistance to colistin, which were also collected in 2015.

4.1. Carbapenem resistance in K. pneumoniae isolates

A 13.2% (29/220) prevalence of carbapenem-resistant *K. pneumoniae* isolates was observed in Tahar Sfar University Hospital, only 4 years after the very first detection of an OXA-48-producing *K. pneumoniae* isolate in December 2012. This indicates a rapid increase of these MDR pathogens in a very short period of time. Moreover, this rise was not only the result of nosocomial dissemination of a unique *K. pneumoniae* clone. Indeed, two major clusters (A and B) comprising 17 and 12 isolates, respectively, were identified as belonging to ST101 (cluster A) and ST147 and ST392 (cluster B). ST101 is the most predominant *K. pneumoniae* clone in several North African countries, including Tunisia [23,24]. ST147, which has caused outbreaks in Europe [25–27], is also emerging as one of the main carbapenem-resistant *K. pneumoniae* clones in Tunisia after the recent description as an OXA-204- and NDM-1-producer [6,23]. ST147 was also recently associated with an infection caused by a *K. pneumoniae* isolate co-producing OXA-48 and VEB-8 β-lactamases in another Tunisian hospital [28]. Of note, ST147 has been also reported worldwide spreading different carbapenemase genes. ST392 is reported here for the first time in Tunisia, but this ST closely related to ST101 is known to produce OXA-48-like enzymes in Europe [29], NDM-1 in Columbia [30] and KPC-2 in China where it is considered as an emergent clone [31]. In all, several carbapenemase-encoding genes were detected. The $bla_{OXA\text{-}48}$ gene carried by IncL plasmids was the most prevalent (18/29), followed by $bla_{NDM\text{-}1}$ (7/29) and $bla_{OXA\text{-}204}$ (4/29) on IncFIIK and IncA/C plasmids, respectively. Two isolates co-harboured $bla_{NDM\text{-}1}$ and $bla_{OXA\text{-}48}$, an accumulation of carbapenem resistance determinants that has recently been reported in Tunisia [6]. Interestingly, $bla_{NDM\text{-}1}$ was found in a ST101 isolate, which is only the second report of this combination after the recent study published by Czobor et al. [32]. Of note, 27/29 isolates additionally carried the $bla_{CTX\text{-}M\text{-}15}$ gene and 4 presented $bla_{CMY\text{-}4}$, thereby highlighting the very frequent combination of several β-lactamases in a single isolate.

4.2. Clonal transmission of a colistin-resistant clone

In this study, 24.1% (7/29) of the carbapenem-resistant *K. pneumoniae* isolates were resistant to colistin, with MICs ranging from 12 to 24 μg/mL. To date, in Tunisia only two studies have reported colistin-resistant *K. pneumoniae* strains [8,9]. In the first study, 3 (15.8%) of 19 clinical OXA-48-producing *K. pneumoniae* isolates showed colistin resistance [9]. In the second study, the prevalence of colistin-resistant *K. pneumoniae* in 2014 among all *K. pneumoniae* strains was 2.96% [8]. The proportion described in the current study is much higher than in other Tunisian hospitals and is also higher than that reported in neighbouring countries such as France (3.6%) [33] or Spain (20%) [34], but much lower than in Italy (43%) where several outbreaks occurred [35].

In Tunisia, the molecular determinants conferring resistance to colistin in *K. pneumoniae* in clinics have never been investigated. Among the seven colistin-resistant isolates, none carried the plasmid-mediated *mcr-1* gene, which has been recently reported to confer resistance to colistin worldwide, including in the animal sector in Tunisia [36]. Of note, *mcr-1* has rarely been reported in *K. pneumoniae* compared with *E. coli* so far. Here, interruption of the *mgrB* gene was responsible for colistin resistance in all isolates, as reported for *K. pneumoniae* in other studies. Indeed, whereas a unique isolate belonged to ST147 and presented an IS*Kpn14* insertion in the promoter region of the *mgrB* gene, the six other colistin-resistant isolates belonged to ST101 and all shared the same 2-kb insertion in the *mgrB* gene. ST101 colistin-resistant *K. pneumoniae* clones have recently been described in France but were from unrelated single colistin-resistant isolates, as proved by different PFGE profiles [33]. Here, the P13 clinical case clearly suggests the in vivo selection of colistin-resistant mutants following colistin therapy, which was followed by the detection of P13-2 and P13-3 colistin-resistant isolates. Subsequently, two other isolates (P14 and P17) presenting identical PFGE profiles were identified in the urology ward, strongly suggesting clonal transmission of the same colistin-resistant ST101 *K. pneumoniae* clone from patient to patient in this hospital. This outbreak of colistin-resistant carbapenem-resistant *K. pneumoniae* is reminiscent of others that have recently been reported, mostly associated with the epidemic ST258 *K. pneumoniae* clone or closely related clones, such as ST512 [4,37–39]. On the other hand, patient P19 presented a close but non-identical PFGE profile. Since this patient had been treated with colistin, we cannot exclude that colistin resistance was selected separately. The last isolate (P23), which has an identical PFGE profile as the P19 isolate, originated from the general medicine ward. It may be hypothesised that this patient had not been treated with colistin and thus likely acquired the colistin-resistant *K. pneumoniae* isolate from another unidentified source.

5. Conclusions

This study is the first in Tunisia reporting the molecular mechanisms of colistin resistance in clinical *K. pneumoniae* isolates. It also shows that colistin-resistant *K. pneumoniae* isolates circulate at a high rate (24.1% of the carbapenem-resistant isolates) in Tahar Sfar University Hospital, a striking situation that most probably occurred in very recent years. This resistance was obviously selected by the use of colistin and was found in the currently predominant *K. pneumoniae* clones circulating in Tunisia, i.e. ST101 and ST147, including through an outbreak most probably spreading colistin resistance from patient to patient in the same hospital. The occurrence of colistin resistance in successful carbapenemase-producing *K. pneumoniae* lineages in this country is of major concern. In particular, NDM-1-producing ST147 *K. pneumoniae* emerged recently in Tunisia, and the acquisition of colistin resistance in such an isolate also having a MIC of 2 μg/mL to tigecycline highlights the risk of pandrug-resistant *K. pneumoniae* in this setting. This is particularly worrying considering that last-resort molecules such as ceftazidime/avibactam would be useless against these colistin-resistant NDM-producers. Altogether, these data emphasise the need for systematic detection of colistin resistance in the hospital laboratory using reliable methods (including broth microdilution or polymyxin NP tests) in order to prevent the dissemination of such isolates. Prudent use of colistin in the treatment of carbapenem-resistant *K. pneumoniae* is also urgently required.

Competing interests

None declared.

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Ethical approval

Not required.

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