

Original Article

## Prevalence of carbapenemases among Gram-negative bacteria in Tunisia: first report of KPC-2 producing *Acinetobacter baumannii*

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### Abstract

**Introduction:** The rapid evolution of the antibacterial resistance problem worldwide, including the Mediterranean countries, constitutes a real threat to public health. This study aims to characterize carbapenemase encoding genes among Gram-negative bacteria collected from some Tunisian hospitals.

**Methodology:** Twenty-two clinical carbapenem-resistant Gram-negative bacteria were recovered, and identified by the matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) method. Antibiotic resistance was tested by disk diffusion method on Muller-Hinton Agar. The minimum inhibitory concentration (MIC) for imipenem was revealed by the E-test method. Carbapenemase encoding genes were screened by polymerase chain reaction (PCR). Genetic relatedness was performed by the pulsed field gel electrophoresis (PFGE) method.

**Results:** Our isolates, identified as *K. pneumoniae* (n = 7), *P. mirabilis* (n = 1), *A. baumannii* (n = 13), and *P. aeruginosa* (n = 1), presented high MIC values for imipenem. Enterobacterales were resistant to carbapenems due to OXA-48 production. Only, four *K. pneumoniae* harbored the *bla<sub>NDM-1</sub>* gene. VIM-2 production was detected in *P. aeruginosa*. However, OXA-23 production was observed in *A. baumannii* isolates, one of which co-produced the KPC-2 enzyme that was identified for the first time in Tunisia in this species. A high genetic diversity was demonstrated by pulsed-field gel electrophoresis in *K. pneumoniae* and *A. baumannii* after *Xba*I and *Apa*I digestion respectively.

**Conclusions:** Our findings highlight the spread of various unrelated clones of carbapenemase-producers in some Tunisian hospitals as well as the spread of several carbapenemase types.

**Key words:** Gram-negative bacteria; OXA-23; NDM-1; VIM-2; KPC-2; Tunisia.

*J Infect Dev Ctries* 2023; 17(11):1591-1597. doi:10.3855/jidc.17978

(Received 22 January 2023 – Accepted 26 June 2023)

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### Introduction

The discovery of antibiotics and their clinical use since the 1940s have transformed modern medicine by reducing the morbidity and mortality rates related to severe infections significantly [1]. However, the large-scale antibiotic consumption and the inappropriate level

of prescribing antibiotics led to the emergence and the dissemination of multidrug resistant bacteria that became a global crisis [2]. The widespread existence of multidrug-resistant bacterial infections around the world is an alarming risk and poses a real threat. Multidrug-resistant bacterial infections have not only

led to the increase of mortality and morbidity rates, but also the cost of treatment [3]. Furthermore, the most worrisome infections that occurred in hospitals and the community, were caused by multidrug-resistant Gram-negative species, specifically *Acinetobacter baumannii*, Enterobacterales and *Pseudomonas aeruginosa* [2]. These species are opportunistic pathogenic bacteria and responsible for several serious infections notably pneumonia, bloodstream and urinary tract infections, mainly in immunocompromised patients [4].

Carbapenems are considered as one of the last resort antibiotics used for the treatment of severe infections owing to their broad antibacterial spectrum of activity towards almost all  $\beta$ -lactams [5]. Despite its effectiveness, the crisis has been dramatically worsened by the emergence and spread of carbapenem resistant isolates which are often linked to carbapenemase production [6]. In fact, a wide variety of these enzymes has been described throughout the world, noting a different prevalence in each region, and recording epidemic, endemic or sporadic transmission of each enzyme [7]. The most frequently reported carbapenemase types include; *Klebsiella pneumoniae* carbapenemase (KPC), New Delhi metallo- $\beta$ -lactamase (NDM), Verona integron-encoded metallo- $\beta$ -lactamase (VIM), imipenem metallo- $\beta$ -lactamase (IMP), and Oxacillinase (OXA-48) [7]. In several Mediterranean countries including Tunisia, resistance to carbapenems was closely related mainly by the production of OXA-48 enzyme, in addition to the detection of other carbapenemase types, including metallo-beta-lactamases [8,9]. Nevertheless, class A carbapenemases are still uncommon in our country. The, KPC enzyme has been recently detected only among *E. coli* and *K. pneumoniae* clinical isolates [10,11].

This work aimed to identify carbapenemase encoding genes among multidrug-resistant Gram-negative bacteria isolated from patients hospitalized in two northern Tunisian hospitals. This study reported for the first time KPC-2 producing *A. baumannii* in Tunisia and also demonstrated the detection of several carbapenemases, including NDM-1 and OXA-48 among Enterobacterales, and VIM-2 and OXA-23 among non-fermentative Gram-negative bacteria.

**Methodology**

*Bacterial isolates*

Twenty-two carbapenem resistant Gram-negative isolates were recovered from several sources (urine, bone, pus, fistula, sepsis) from patients hospitalized in different departments in two northern Tunisian hospitals: Mohamed Kassab Orthopedics Institute and Ben Arous Regional Hospital, as described in Table 1 and Table 2. The isolates were then purified by streaking the entire surface of the brain heart infusion agar plate. They were identified using biochemical tests (API 20E, Biomérieux, Marcy-l'Etoile, France) and by mass spectrometry using the matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer (Microflex, Bruker Daltonic, Bremen, Germany).

*Phenotypic characterization of antibiotic resistance*

Resistance to antibiotics were investigated using the disk diffusion method on Mueller-Hinton agar medium. A total of fifteen antibiotics were tested for both fermentative and non-fermentative Gram-negative bacteria and nine similar antibiotics were used [piperacillin-tazobactam (TZP), cefepime (FEP), imipenem (IMP), ciprofloxacin (CIP), doxycycline

**Table 1.** Phenotypic and molecular characterization of carbapenem-resistant fermenter Gram-negative bacteria.

Isolates	Pulsotypes	Hospitals	Wards	Origins	Sampling dates	Phenotypic resistance	MIC (mg/L) (IMP)	Carbapenemases type
Kp 13	B1	OIMK	Traumatology	Urine	30/10/2017	AX; AMC; TZP; FEP; CRO; CIP; ETP; GEN; F; KM; DO	6	OXA-48
Kp 15	A1	OIMK	Septic	Bone	24/06/2017	AX; AMC; TZP; FEP; CRO; CIP; ETP; IMP; GEN; AK; F; K; SXT; DO	> 32	OXA-48; NDM-1
Kp 16	C	OIMK	Septic	Pus	20/02/2017	AX; AMC; TZP; FEP; CRO; CIP; ETP; GEN; KF; SXT; DO	1,5	OXA-48
Kp 17	E	OIMK	Septic	Fistula	25/05/2018	AX; AMC; TZP; FEP; CRO; CIP; ETP; IMP; GEN; FF; F; KM; DO	> 32	OXA-48
Kp 26	A2	RHB	Traumatology	Urine	12/04/2018	AX; AMC; TZP; FEP; CRO; CIP; ETP; IMP; GEN; F; KM; SXT; DO	> 32	OXA-48; NDM-1
Kp 34	D	RHB	Traumatology	Urine	08/04/2018	AX; AMC; TZP; FEP; CRO; CIP; ETP; IMP; F; KM; SXT; DO	> 32	OXA-48; NDM-1
Kp 35	B2	RHB	Traumatology	Urine	10/04/2018	AX; AMC; TZP; FEP; CRO; CIP; ETP; IMP; F; KM; SXT; DO	> 32	OXA-48; NDM-1
Pm 14	-	OIMK	Septic	Sepsis	15/02/2017	AX; AMC; TZP; FEP; CRO; CIP; ETP; IMP; GEN; F; KM; DO	> 32	OXA-48

Kp: *Klebsiella pneumoniae*; Pm: *Proteus mirabilis* OIMK: Orthopedic Institute Mohamed KASSAB; RHB: Regional Hospital of Ben Arous; OXA-48: Oxacillinase; NDM: New Delhi metallo- $\beta$ -lactamase; AX: ampicillin; AMC: amoxicillin + clavulanic acid; TZP: piperacillin + tazobactam; FEP: cefepime; CRO: ceftriaxone; CIP: ciprofloxacin; ETP: ertapenem; IMP: imipenem; GEN: gentamicin; F: nitrofurantoin; KM: kanamycin; DO: doripenem.

(DO), fosfomycine (FF), nitrofurantoin (F), trimethoprim-sulfamethoxazole (SXT), amikacin (AK)] while six different antibiotics [amoxicillin (AX), amoxicillin + clavulanic acid (AMC), cephalothin (KF), ceftriaxone (CRO), ertapenem (ETP), gentamicin (GEN)] and [ticarcillin (TIC), ticarcillin + clavulanic acid (TCC), meropenem (MEM), ceftazidime (CAZ), rifampicin (RA), tobramycin (TOB)] were tested for fermentative and non-fermentative Gram-negative bacteria respectively. Besides, the minimum inhibitory concentrations (MIC) of imipenem were investigated by the E-test method. The results were analyzed according to the guidelines of the Antibiogram Committee of the French Society of Microbiology (CA-SFM / EUCAST 2018, <http://www.sfm-microbiologie.org/>).

*Molecular characterization of carbapenem resistance genes*

Molecular characterization of carbapenemases encoding-genes was performed by polymerase chain reaction (PCR) using specific primers for the amplification of genes coding for class A (*bla<sub>KPC</sub>*, and *bla<sub>GES</sub>*) [12], class B (*bla<sub>NDM</sub>*, *bla<sub>VIM</sub>*, and *bla<sub>IMP</sub>*) [13]; and class D (*bla<sub>OXA-23</sub>*, *bla<sub>OXA-24</sub>* and *bla<sub>OXA-48</sub>*) as

previously described [14]. The amplicons were then sequenced to identify the corresponding variants.

*Screening for clonal relationship by pulsed-field gel electrophoresis*

To investigate the genetic relatedness among carbapenem resistant *K. pneumoniae* and *A. baumannii* isolates, we have carried out the pulsed-field gel electrophoresis (PFGE) using *XbaI* and *ApaI* restriction enzymes respectively. The restriction patterns obtained were analyzed by the FPQuest Version 5 software using UPGMA (Unweighted Pair Group Method with Arithmetic mean) algorithm.

**Results**

*Bacterial isolates: origin and susceptibility testing*

A total of 22 carbapenem-resistant Gram-negative clinical isolates were collected from two hospitals, Mohamed Kassab Orthopedics institute (n = 18) and Ben Arous Regional Hospital (n = 4) in northern Tunisia, and identified as follows: *K. pneumoniae* (n = 7); *P. mirabilis* (n = 1); *A. baumannii* (n = 13); and *P. aeruginosa* (n = 1). The majority of these isolates were recovered from septic wards (n = 10), followed by intensive care units (n = 7), and traumatology wards (n = 5) (Table 1 and Table 2). The results showed that all

**Table 2.** Phenotypic and molecular characterization of carbapenem-resistant non-fermentative Gram-negative bacteria.

Isolates	Pulsotypes	Hospitals	Wards	Origins	Sampling dates	Phenotypic resistance	MICs (mg/L) (IMP)	Carbapenemases types
Ab 1	J1	OIMK	ICU	Urine	08/06/2015	TIC; TCC; TZP; MEM; IMP; CIP; TOB; AK; DO; F; SXT; FF; CAZ; FEP	> 32	-
Ab 2	C1	OIMK	ICU	Blood	03/04/2017	TIC; TCC; TZP; MEM; IMP; CIP; TOB; AK; F; SXT; FF; CAZ; FEP	> 32	OXA-23
Ab 3	C2	OIMK	ICU	Blood	27/04/2017	TIC; TCC; TZP; MEM; IMP; CIP; AK; RA; F; SXT; CAZ; FEP	> 32	OXA-23
Ab 4	F	OIMK	ICU	Trachea	03/05/2017	TIC; TCC; TZP; MEM; IMP; CIP; TOB; RA; DO; F; SXT; FF; FEP	> 32	OXA-23
Ab 5	D	OIMK	Septic	Articular fluid	17/06/2017	TIC; TCC; TZP; MEM; IMP; CIP; TOB; AK; RA; F; SXT; FF; CAZ; FEP	> 32	OXA-23
Ab 6	B	OIMK	Septic	Prosthesis	03/08/2017	TIC; TCC; TZP; MEM; IMP; CIP; TOB; AK; F; SXT; CAZ; FEP	> 32	OXA-23
Ab 7	A1	OIMK	Septic	Blood	05/08/2017	TIC; TCC; TZP; MEM; IMP; CIP; TOB; AK; RA; F; SXT; CAZ; FEP	> 32	OXA-23
Ab 8	E1	OIMK	Septic	Skeleton	10/08/2017	TIC; TCC; TZP; MEM; IMP; CIP; TOB; AK; DO; F; SXT; CAZ; FEP	> 32	OXA-23
Ab 9	E2	OIMK	ICU	Blood	11/08/2017	TIC; TCC; TZP; MEM; IMP; CIP; TOB; AK; DO; F; SXT; FF; CAZ; FEP	> 32	OXA-23
Ab 10	J2	OIMK	ICU	Urine	29/08/2017	TIC; TCC; TZP; MEM; IMP; CIP; TOB; AK; DO; F; SXT; FF; CAZ; FEP	> 32	OXA-23
Ab 11	H	OIMK	Septic	Fistula	04/05/2017	TIC; TCC; TZP; MEM; IMP; CIP; TOB; RA; F; SXT; CAZ; FEP	> 32	OXA-23
Ab 12	A1	OIMK	Septic	Surgical wound	17/03/2018	TIC; TCC; TZP; MEM; IMP; CIP; TOB; AK; F; SXT; CAZ; FEP	> 32	OXA-23
Ab 24	I	RHB	Traumatology	Urine	07/04/2018	TIC; TCC; TZP; MEM; IMP; CIP; RA; F; FF; CAZ; FEP	> 32	OXA-23; KPC-2
Ps 20	-	OIMK	ICU	Trachea	12/05/2018	TIC; TCC; TZP; MEM; IMP; CIP; TOB; RA; DO; F; SXT; CAZ; FEP	> 32	VIM-2

Ab: *Acinetobacter baumannii*; Ps: *Pseudomonas aeruginosa* OIMK: Orthopedic Institute Mohamed KASSAB; RHB: Regional Hospital of Ben Arous ; ICU: intensive care Unit; TIC: ticarcillin; TCC: ticarcillin-clavulanic acid; TZP: piperacillin-tazobactam; MEM: meropenem; IMP: imipenem; CIP: ciprofloxacin; TOB: tobramycin; AK: amikacin; DO: doripenem; F: fosfomycin ; SXT: trimethoprim-sulfamethoxazole ; FF: fosfomycin ; CAZ: ceftazidime ; FEP: cefepime; OXA: Oxacillinase ; KPC: Klebsiella pneumoniae carbapenemase.

carbapenem-resistant isolates collected from intensive care units belonged to the non-fermentative Gram-negative bacteria group.

As described in Tables 1 and 2, the antibiotic susceptibility testing results showed that all isolates were resistant to almost all tested antibiotics, namely  $\beta$ -lactams, fluoroquinolones, aminoglycosides and tetracycline, which confirms the multidrug resistant pattern of these isolates. On the other hand, high MIC values of IMP were observed among the majority of our isolates (MIC > 32 mg/L), thus showing a high imipenem resistance level.

*Molecular characterization of carbapenem resistance encoding-genes*

PCR results showed that 12 *A. baumannii* isolates harbored *bla*<sub>OXA-23</sub> gene, and one of them harbored *bla*<sub>KPC-2</sub> (Table 1 and Table 2). However, *bla*<sub>VIM-2</sub> was detected among one *P. aeruginosa*. Furthermore, *bla*<sub>OXA-48</sub> was detected in all *K. pneumoniae* and one *Proteus mirabilis*, noting a co-production of this enzyme with NDM-1 metallo- $\beta$ -lactamase in 4 *K. pneumoniae* isolates. In addition, we noted the absence of other carbapenemase encoding genes, namely GES, OXA-24 and IMP.

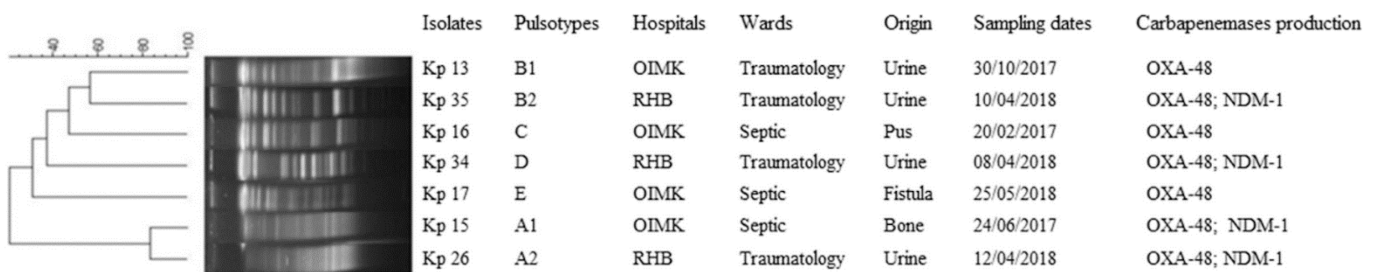
*Genetic relatedness of carbapenem-resistant isolates*

A high genetic diversity was observed among our carbapenem-resistant isolates. In fact, the analysis of PFGE patterns using the UPGMA algorithm showed a genetic relatedness less than 60%, along with seven different pulsotypes of *K. pneumoniae* (Figure 1), and twelve different pulsotypes of *A. baumannii* (Figure 2). Thus, only two *A. baumannii* isolates, Ab7 and Ab12, appeared to be clonal with 100% identity (Figure 2).

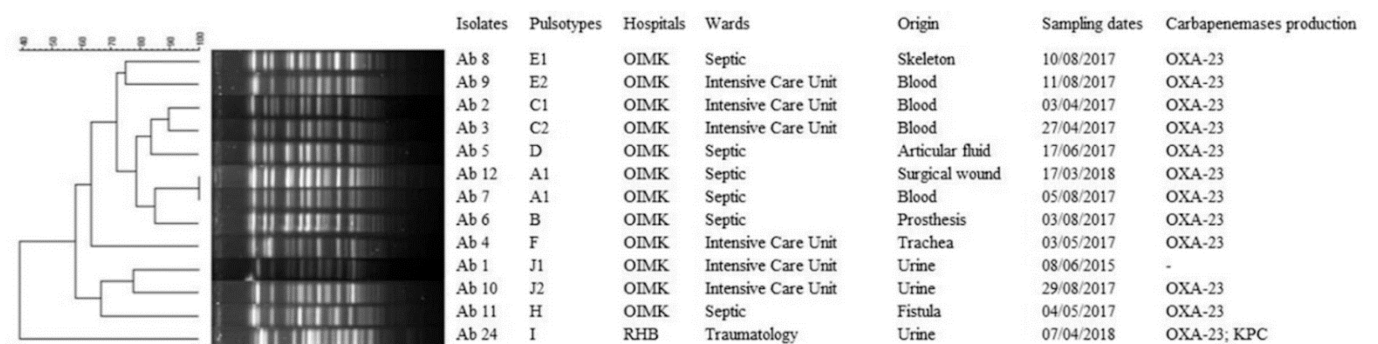
**Discussion**

The increasing prevalence of carbapenem resistant Gram-negative species is a growing problem in hospitals, and constitutes a real threat to public health worldwide [15,16]. Indeed, therapeutic options for carbapenem-resistant bacterial infections have become very limited, leading to prolonged hospitalization as well as the increasing level of mortality and morbidity [17]. A recent study has demonstrated that carbapenem resistant Gram-negative species differed by infection sites. Respiratory tract infections are commonly caused by *P. aeruginosa* and *A. baumannii*; while, the urinary tract infections are caused mainly by Enterobacterales, especially the most clinically relevant species, *E. coli* and *K. pneumoniae* [17]. In the present report, twenty-two carbapenem resistant Gram-negative species were detected and isolated from various infection sites,

**Figure 1.** Dendrogram generated by UPGMA algorithm FPQuest software showing the PFGE (Pulsed Field Gel Electrophoresis) patterns of the 7 *K. pneumoniae* genomic DNA after digestion by XbaI enzyme.



**Figure 2.** Dendrogram generated by UPGMA algorithm FPQuest software showing the PFGE (Pulsed Field Gel Electrophoresis) patterns of the 13 *A. Baumannii* genomic DNA after digestion by ApaI enzyme.



noting *A. baumannii*, *P. aeruginosa*, *K. pneumoniae* and *P. mirabilis*. Carbapenemase production constitutes the main mechanism conferring resistance to this class of antibiotics [6]. In fact, several carbapenemase variants have been identified in various species throughout the world (> 350) [35] recording a heterogeneous geographic distribution.

Indeed, several studies reported a high prevalence of KPC producing isolates in Greece, Italy, and Latin America, where they frequently belonged to the Enterobacterales family and most commonly *K. pneumoniae* [18-20]. In Tunisia, this enzyme is still uncommon. It was recently detected among *E. coli* and *K. pneumoniae* clinical isolates in the north of Tunisia [10,11]. Thus, the detection of KPC producing *A. baumannii* in this northern regional hospital caught our attention while it was not hitherto recorded in our country.

Herein, we revealed for the first time the occurrence of KPC-2 producing *A. baumannii* in Tunisia. Moreover, the presence of KPC enzymes in *A. baumannii* species was first described in Puerto Rico in 2009 [21]. To our knowledge, it was limited to this region [19] and it was scarcely reported in other countries such as Portugal where it was recently isolated [22]. Resistance to carbapenems among *A. baumannii* was mainly attributed to the OXA-23 carbapenemase production. This class D carbapenemase was detected for the first time in the world in a clinical *A. baumannii* isolated in 1985 in Scotland, and then it was globally disseminated [23,24]. In Tunisia, the OXA-23 positive carbapenem resistant *A. baumannii* was reported for the first time in 2008 in a coastal city [25]. To the best of our knowledge, it has been recently demonstrated that, the existence of *bla*<sub>OXA-23</sub>-like was closely related to carbapenem resistance phenotype. Indeed, a previous observational study revealed that carbapenem resistance is closely related to the expression of *bla*<sub>OXA-23</sub> as well as the contribution of the *bla*<sub>TEM</sub> gene [26]. In the present report, the *bla*<sub>OXA-23</sub> gene was detected in all our carbapenem-resistant *A. baumannii* except one that could be resistant to carbapenems through the production of the naturally occurring OXA-51-like enzyme or by porin loss [27,28]. This non-carbapenemase-mediated mechanism was frequently observed among non-fermentative Gram-negative bacteria, especially *P. aeruginosa*. Indeed, loss of function or decrease in expression of OprD porins contribute significantly to carbapenem resistance among this species [29]. In this study, the *P. aeruginosa* isolate was resistant to carbapenem by VIM-2 metallo-

$\beta$ -lactamase production. Since its first description in Tunisia, this enzyme was frequently reported in several Tunisian hospitals, and it was mainly detected in *P. aeruginosa* species [30,31].

Two carbapenemase types were detected among fermentative carbapenem-resistant Gram-negative isolates. NDM-1 metallo- $\beta$ -lactamase type was detected among the four *K. pneumoniae* in association with OXA-48 enzyme. This carbapenemase combination was described in Tunisia since 2013, the date of the first report of NDM-1 in Tunisia [32]. This was later disseminated in Tunisian hospitals and was described in various species, but it was not detected previously in these two hospitals. In addition, we have noted that all our Enterobacterales isolates harbored the OXA-48 encoding genes that was regarded as endemic in Mediterranean countries including Tunisia [8,33]. In spite of this, the detection of this enzyme in *P. mirabilis* was uncommon worldwide as well as in Tunisia. In fact, there is limited data describing OXA-48 producing *P. mirabilis* isolate. To our knowledge, only one OXA-48 producing *P. mirabilis* isolate was detected in Tunisia to date, and it was recently isolated from a southern hospital [34]. Additionally, our data reported for the first time the detection of OXA-48 producing *P. mirabilis* in a northern Tunisian hospital.

## Conclusions

Overall, our findings highlight the spread of various unrelated clones of carbapenemase-positive isolates in Tunisian hospitals, as well as the dissemination of several carbapenemases. Our study is the first description of KPC-2 producing *A. baumannii* in Tunisia and the emergence of a clinical *P. mirabilis* isolate harboring the *bla*<sub>OXA-48</sub> gene in a northern Tunisian hospital. Thus, controlling antibiotic resistance in our healthcare settings should be one of the top priorities to reduce the diffusion of resistance in bacteria, especially to the last resort antibiotics.

## Acknowledgements

The authors thank the staff of the laboratory of the regional hospital of Ben Arous as well as the laboratory of Mohamed Kassab Institute of Orthopedics for their helpful assistance to obtain the clinical isolates.

## Funding

This work was partly funded by IHU Méditerranée Infection, Aix-Marseille-University in France. This work was also supported by the Tunisian Ministry of Higher Education and Scientific Research and Campus France under the PHC-Utique project (Code 18G0819) which offered a scholarship

to Miss. Dhouha Miniaoui to attend the IHU Méditerranée Infection, Aix-Marseille-University in France.

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**Conflict of interests:** No conflict of interests is declared.