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Article *in* Microbial drug resistance (Larchmont, N.Y.) · January 2015

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MICROBIAL DRUG RESISTANCE
Volume 00, Number 00, 2015

DOI: 10.1089/mdr.2014.0235

Emergence of Carbapenem-Resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* Clinical Isolates Collected from Some Libyan Hospitals

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The aim of the present study was to investigate the molecular mechanism of carbapenem resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* clinical isolates recovered from Libyan hospitals between April 2013 and April 2014. In total, 49 strains (24 *P. aeruginosa* and 25 *A. baumannii*) were isolated, including 21 *P. aeruginosa* and 22 *A. baumannii* isolates (87.75%) resistant to imipenem (minimum inhibitory concentrations ≥ 16 μg/ml). The $bla_{\text{VIM-2}}$ gene was detected in 19 *P. aeruginosa* isolates. All imipenem-resistant *P. aeruginosa* isolates showed the presence of *OprD* mutations. Acquired OXA-carbapenemase-encoding genes were present in all *A. baumannii* isolates: $bla_{\text{OXA-23}}$ ($n=19$) and $bla_{\text{OXA-24}}$ ($n=3$). Finally, a total of 13 and 17 different sequence types were assigned to the 21 *P. aeruginosa* and the 22 *A. baumannii* carbapenem-resistant isolates, respectively. This study is the first report describing imipenem-resistant *P. aeruginosa* and *A. baumannii* isolated from patients in Libya. We report the first case of co-occurrence of $bla_{\text{VIM-2}}$ with *oprD* porin loss in identical isolates of *P. aeruginosa* in Libya and demonstrate that these *oprD* mutations can be used as a tool to study the clonality in *P. aeruginosa* isolates. We also report the first identification of multidrug-resistant *A. baumannii* isolates harboring $bla_{\text{OXA-23}}$-like, $bla_{\text{OXA-24}}$-like, and $bla_{\text{OXA-48}}$-like genes in Libya.

Introduction

The increase and spread of multidrug-resistant (MDR) gram-negative bacteria have become major concerns worldwide. *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are among the most common pathogens causing nosocomial infections.[12] The ability to develop multidrug resistance makes these infections difficult to treat, and they are associated with high mortality rates ranging from 18% to 61%.[29] Indeed, *P. aeruginosa* is characterized by an innate resistance to multiple classes of antimicrobials.

The broad-spectrum resistance of *P. aeruginosa* is mainly due to a combination of different factors: (1) low outer membrane permeability,[23] (2) the presence of the inducible AmpC chromosomal β-lactamase,[24] (3) synergistic action of several multidrug efflux systems,[27] and (4) the presence of transferable resistance determinants, in particular, carbapenem-hydrolyzing enzymes (mainly metallo-β-lactamases [MBLs])[19] and also aminoglycoside-hydrolyzing enzymes.

The main porin for uptake of carbapenems in *P. aeruginosa* is the outer membrane protein OprD. Inactivating mutations in the *oprD* gene is the most common molecular mechanism known to confer resistance to carbapenems.[15] Carbapenem resistance can also arise from MBL production, but this mechanism is a less commonly found mechanism than the mutation-driven resistance mechanisms.[6] The most common MBLs found in *P. aeruginosa* include VIM, IMP, GIM, FIM-1, and SPM. In particular, $bla_{\text{VIM-2}}$ has emerged as a dominant MBL variant in North Africa[8] and worldwide.[9] The VIM types have been identified in carbapenem-resistant isolates of

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P. aeruginosa from African countries (Tunisia, Algeria, and Egypt)[7,36,37,39] and from countries in the Mediterranean basin (Italy, France, Lebanon, Spain, and Greece).[1,10,26,34] MDR *A. baumannii* are also associated with a wide spectrum of infectious diseases, ranging from nosocomial and community-acquired infections to those acquired in natural disasters or wars.[33] The most important mechanism of carbapenem resistance in *A. baumannii* is the enzymatic hydrolysis mediated by carbapenem-hydrolyzing β-lactamases, belonging to class D ($bla_{\text{OXA-23}}$-like, $bla_{\text{OXA-24}}$-like, $bla_{\text{OXA-51}}$-like, $bla_{\text{OXA-58}}$-like, $bla_{\text{OXA-104}}$, $bla_{\text{OXA-143}}$, $bla_{\text{OXA-164}}$, and $bla_{\text{OXA-182}}$).[3] Carbapenemase-producing *A. baumannii* strains are increasingly reported in Europe, South America, Asia, Oceania, and Africa.[11,30]

In Libya, to the best of our knowledge, there are few reports of resistance to carbapenems in clinical isolates. As very little information is known regarding the antibiotic resistance in nonfermenting gram-negative bacteria in addition to the desperate need for insight into antibiotic resistance in Libyan hospitals, this study was undertaken to investigate the molecular mechanism of resistance to carbapenems in *P. aeruginosa* and *A. baumannii* clinical isolates resistant to imipenem. Few reports described carbapenem resistance in nonfermenters.[16,17] The occurrence of a novel MBL ($bla_{\text{TMB-1}}$) was reported from *Achromobacter xylosoxidans* isolated from Tripoli Central Hospital, Tripoli, Libya, reflecting the lack of hospital hygiene.[16] This study will hopefully provide useful insight into the problem of antibiotic resistance to provide a therapeutic alternative when clinicians are facing such MDR bacteria.

Materials and Methods

Bacterial isolates

A total of 49 nonreplicate clinical isolates were collected over 12 months between April 2013 and April 2014 from two hospitals (Burn and plastic Surgery Hospital [BPSH] and Benghazi Medical Center [BMC]) in two different cities in Libya (Tripoli and Benghazi). These strains were isolated from different pathological specimens, primarily wounds, recovered from hospitalized patients in various hospital departments, generally intensive care units and burns units. These patients were civilians severely burned in war in Libya. The isolates were identified using biochemical tests, Phoenix, and confirmed by matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS). Among this collection, 24 *P. aeruginosa* and 25 *A. baumannii* were identified.

Antibiotic susceptibility testing

Antibiotic susceptibility was determined on Mueller–Hinton agar using a standard disk diffusion procedure, as described by the Antibiogram Committee of the French Society for Microbiology (CA-SFM) (www.sfm-microbiologie.org/). Fifteen antibiotics were tested, including ticarcillin, ticarcillin–clavulanic acid, piperacillin, piperacillin–tazobactam, ceftazidime, cefotaxime, cefepime, aztreonam, amikacin, tobramycin, gentamicin, ciprofloxacin, meropenem, imipenem, and colistin (Bio-Rad). For all isolates, minimum inhibitory concentrations of imipenem were determined using an Etest® strip (AB BioMerieux). The results were interpreted according to the CA-SFM breakpoints.

Phenotypic detection of carbapenemases

Isolates were screened for carbapenemase production using the modified Hodge test (MHT), the modified Carba NP test (MCNP), and the EDTA test, as previously described.[13,14,22,38]

Molecular detection of carbapenemases

For *P. aeruginosa* strains, carbapenemase-encoding genes were detected using specific primers for bla_{IMP}, bla_{VIM}, bla_{KPC}, and bla_{NDM}. Concerning *A. baumannii* strains, carbapenemase-encoding genes were detected using specific primers for $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24}}$, $bla_{\text{OXA-48}}$, $bla_{\text{OXA-58}}$, and bla_{NDM}. Sequence analyses were performed using Big Dye® terminator chemistry on an automated ABI 3730 Sequencer (PE Applied Biosystems). All sequences obtained were analyzed using BlastN and BlastP to search the NCBI database (www.ncbi.nlm.nih.gov/blast).[36]

PCR amplification and sequencing of oprD

PCR amplification of *opr*D was performed on imipenem-resistant *P. aeruginosa* strains using specific primers. PCR products were fully sequenced as described above, and the resulting sequences were compared to the PAO1 reference strain sequence (GenBank accession number CAA7844).[36]

Molecular strain typing

The epidemiological relatedness of *P. aeruginosa* and *A. baumannii* was studied by multilocus sequence typing (MLST) as described.[3,36] Isolates were attributed to a sequence-type (ST) number according to the allelic profiles available in the Institute Pasteur's MLST website (www.pasteur.fr/mlst).

Results

A total of 24 *P. aeruginosa* and 25 *A. baumannii* were identified by the MALDI-TOF MS. These strains were isolated from different pathological specimens, including wounds (47.8%), tracheal suctioning (25.1%), and urinary tracts (15.9%), and recovered from hospitalized patients in various hospital units, including intensive care (44.9%), trauma (20.4%), surgery (14.4%), cardiology (7.8%), pediatrics (7%), and internal medicine (5.5%). The results of antibiotic susceptibility testing revealed that the isolates were resistant to almost all antibiotics, including β-lactams, aminoglycosides, and fluoroquinolones. All isolates were sensitive to colistin (Fig. 1). All imipenem-resistant *A. baumannii* and 19 imipenem-resistant *P. aeruginosa* were positive using the MHT and MCNP, suggesting carbapenemase production (Tables 1 and 2). In addition, the activity of β-lactamase was inhibited by the EDTA solution in 19 *P. aeruginosa*, showing the production of MBL (Table 1). The majority of these strains were isolated from wounds of patients hospitalized at burn units from BPSH in Tripoli. These patients were civilians severely burned during the revolution, in Libya, in 2013.

Acquired OXA-carbapenemase-encoding genes were present in all *A. baumannii* isolates, including $bla_{\text{OXA-23}}$ ($n=19$) and $bla_{\text{OXA-24}}$ ($n=3$). Among these strains, one

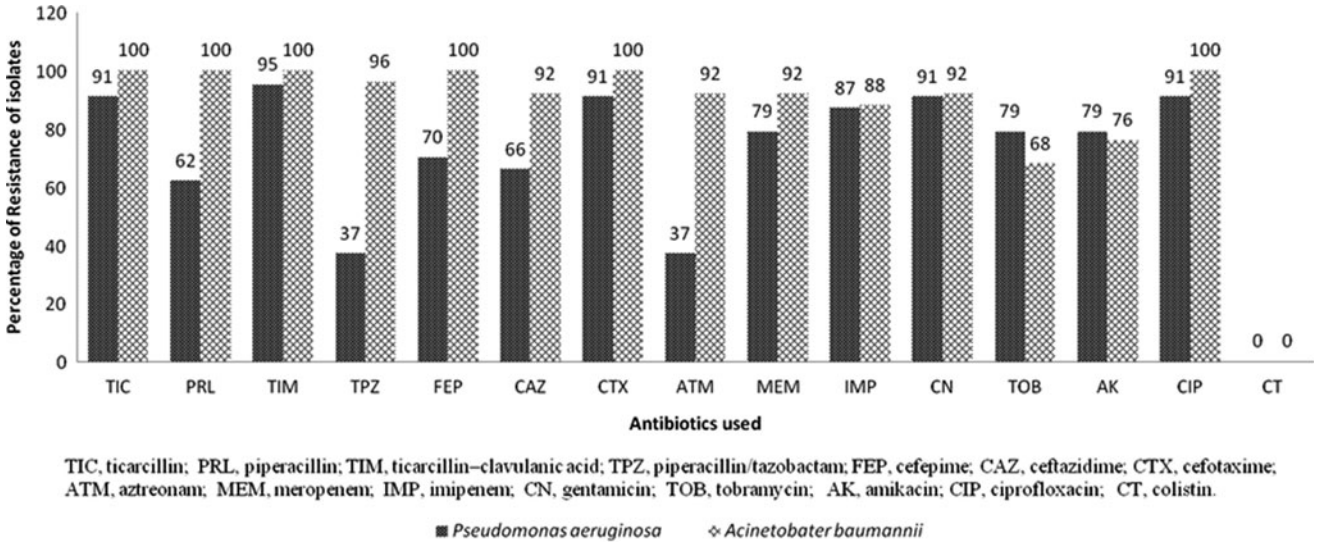

FIG. 1. Antibiotic susceptibility of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* clinical strains.

TABLE 1. PHENOTYPIC AND GENOTYPIC FEATURES OF THE 21 IMIPENEM-RESISTANT *PSEUDOMONAS AERUGINOSA* CLINICAL ISOLATES

Isolate	*Location*	*Date of isolation (mo-yr)*	*Ward*	*Type of swabs*	*IMP MIC (μg/ml)*	*Hodge test*	*EDTA test*	*Carba test*	*VIM-2*	OprD *mutational groups*	*ST*
Z22	Benghazi	11-2013	ICU	Tracheal	>32	+	+	+	+	G1	911
Z27	Benghazi	11-2013	ICU	Tracheal	>32	+	+	+	+	G1	911
Z26	Benghazi	11-2013	ICU	Tracheal	>32	+	+	+	+	G1	911
Z29	Benghazi	11-2013	ICU	Tracheal	>32	+	+	+	+	G1	911
Z24	Benghazi	11-2013	ICU	Tracheal	>32	+	+	+	+	G1	911
451	Tripoli	4-2013	Burn	Wound	>32	+	+	+	+	G2	227
761	Tripoli	4-2013	Burn	Wound	>32	−	−	−	−	G2	227
Z10	Tripoli	4-2013	Burn	Wound	>32	+	+	+	+	G2	227
139	Tripoli	4-2013	Burn	Wound	>32	+	+	+	+	G3	235
401	Tripoli	4-2013	Burn	Wound	>32	+	+	+	+	G3	235
101	Tripoli	4-2013	Burn	Wound	>32	+	+	+	+	G4	1584
222	Tripoli	4-2013	Burn	Wound	>32	+	+	+	+	G4	1584
759	Tripoli	4-2013	Burn	Wound	>32	+	+	+	+	G5	699
605	Tripoli	4-2013	Burn	Wound	>32	−	−	−	−	G6	539
151	Tripoli	4-2013	Burn	Wound	>32	+	+	+	+	G7	622
224	Tripoli	4-2013	Burn	Wound	>32	+	+	+	+	G8	660
52	Tripoli	4-2013	Burn	Wound	>32	+	+	+	+	G9	1924
762	Tripoli	4-2013	Burn	Wound	>32	+	+	+	+	G10	1925
Z9	Benghazi	11-2013	ICU	Tracheal	>32	+	+	+	+	G11	1926
Z30	Benghazi	11-2013	ICU	Tracheal	>32	+	+	+	+	G12	1927
760	Tripoli	4-2013	Burn	Wound	>32	+	+	+	+	G13	1928

ICU, intensive care unit; IMP, imipenem; mo, month; yr, year; ST, sequence type; MICs, minimum inhibitory concentrations; G1, deletion of G in nucleotide position 326 leading to a premature stop codon TGA in oprD resulting in a truncated polypeptide of 110 amino acid residues; G2, several mutation types leading to the premature stop codon TAA in oprD resulting in a truncated polypeptide of 219 amino acid residues; G3, insertion of G in nucleotide positions 19 and 36 leading to the premature stop codon TAG in oprD resulting in a truncated polypeptide of 17 amino acid residues; G4, several mutation types leading to the premature stop codon TGA in oprD resulting in a truncated polypeptide of 239 amino acid residues; G5, several mutation types leading to the premature stop codon TGA in oprD resulting in a truncated polypeptide of 349 amino acid residues; G6, insertion of C in nucleotide position 185 leading to the premature stop codon TGA in oprD resulting in a truncated polypeptide of 74 amino acid residues; G7, G to A substitution in nucleotide position 622 leading to the premature stop codon TAA in oprD resulting in a truncated polypeptide of 222 amino acid residues; G8, G to C substitution in nucleotide position 952 leading to the premature stop codon TGA in oprD resulting in a truncated polypeptide of 318 amino acid residues; G9, several mutation types leading to the premature stop codon TAA in oprD resulting in a truncated polypeptide of 220 amino acid residues; G10, insertion of C in nucleotide position 129 leading to the premature stop codon TGA in oprD resulting in a truncated polypeptide of 55 amino acid residues; G11, insertion of C in nucleotide position 1318 leading to the premature stop codon TAA in oprD resulting in a truncated polypeptide of 451 amino acid residues; G12, several mutation types leading to the premature stop codon TGA in oprD resulting in a truncated polypeptide of 438 amino acid residues; G13, C to T substitution in nucleotide position 355 leading to the premature stop codon TGA in oprD resulting in a truncated polypeptide of 119 amino acid residues.

Table 2. Phenotypic and Genotypic Features of the 22 Imipenem-Resistant *A. baumannii* Clinical Isolates

Isolate	*Location*	*Date of isolation (mo-yr)*	*Ward*	*Type of swabs*	*IMP MIC (μg/ml)*	*Hodge test*	*EDTA test*	*Carba test*	*Carbapenemase genes*	*ST*
466	Tripoli	4-2013	Burn	Wound	>32	+	−	+	OXA-23	588
631	Tripoli	4-2013	Burn	Wound	>32	+	−	+	OXA-23	1
410	Tripoli	4-2013	Burn	Wound	>32	+	−	+	OXA-23	589
60	Tripoli	4-2013	Burn	Wound	>32	+	−	+	OXA-23	590
436	Tripoli	4-2013	Burn	Wound	>32	+	−	+	OXA-23	591
459	Tripoli	4-2013	Burn	Wound	>32	+	−	+	OXA-23	588
363	Tripoli	4-2013	Burn	Wound	>32	+	−	+	OXA-23	1
Z1	Benghazi	11-2013	ICU	Tracheal	>32	+	−	+	OXA-23	2
Z47	Benghazi	11-2013	ICU	Tracheal	>32	+	−	+	OXA-23	1
Z64	Benghazi	11-2013	ICU	Tracheal	>32	+	−	+	OXA-24	592
Z74	Benghazi	11-2013	ICU	Tracheal	>32	+	−	+	OXA-23	593
Z6	Benghazi	11-2013	ICU	Tracheal	>32	+	−	+	OXA-23	594
Z40	Benghazi	11-2013	ICU	Tracheal	>32	+	−	+	OXA-23, OXA-48	595
Z45	Benghazi	11-2013	ICU	Tracheal	>32	+	−	+	OXA-23	596
Z46	Benghazi	11-2013	ICU	Tracheal	>32	+	−	+	OXA-24	2
Z14	Benghazi	11-2013	ICU	Tracheal	>32	+	−	+	OXA-24	2
Z39	Benghazi	11-2013	ICU	Tracheal	>32	+	−	+	OXA-23	81
Z68	Benghazi	11-2013	ICU	Tracheal	>32	+	−	+	OXA-23	597
78	Benghazi	11-2013	ICU	Tracheal	>32	+	−	+	OXA-23	598
79	Benghazi	11-2013	ICU	Tracheal	>32	+	−	+	OXA-23	599
121	Benghazi	11-2013	ICU	Tracheal	>16	+	−	+	OXA-23	600
7588	Benghazi	11-2013	ICU	Tracheal	>16	+	−	+	OXA-23	601

isolate coexpressed the $bla_{\text{OXA-23}}$ and the $bla_{\text{OXA-48}}$ genes (Table 2). This strain was isolated from intensive care units in BMC in Benghazi. None of the strains contained the bla_{NDM}, bla_{IMP}, bla_{VIM}, or bla_{KPC} gene. PCR followed by sequence analysis revealed a $bla_{\text{VIM-2}}$ gene in all MBL-positive *P. aeruginosa* isolates (Table 1). Because of various mutations, all carbapenem-resistant *P. aeruginosa* isolates had a modification in the amino-acid sequence of the OprD protein based on comparison to the sequence of the PAO1 reference strain. Indeed, all the isolates had modifications of

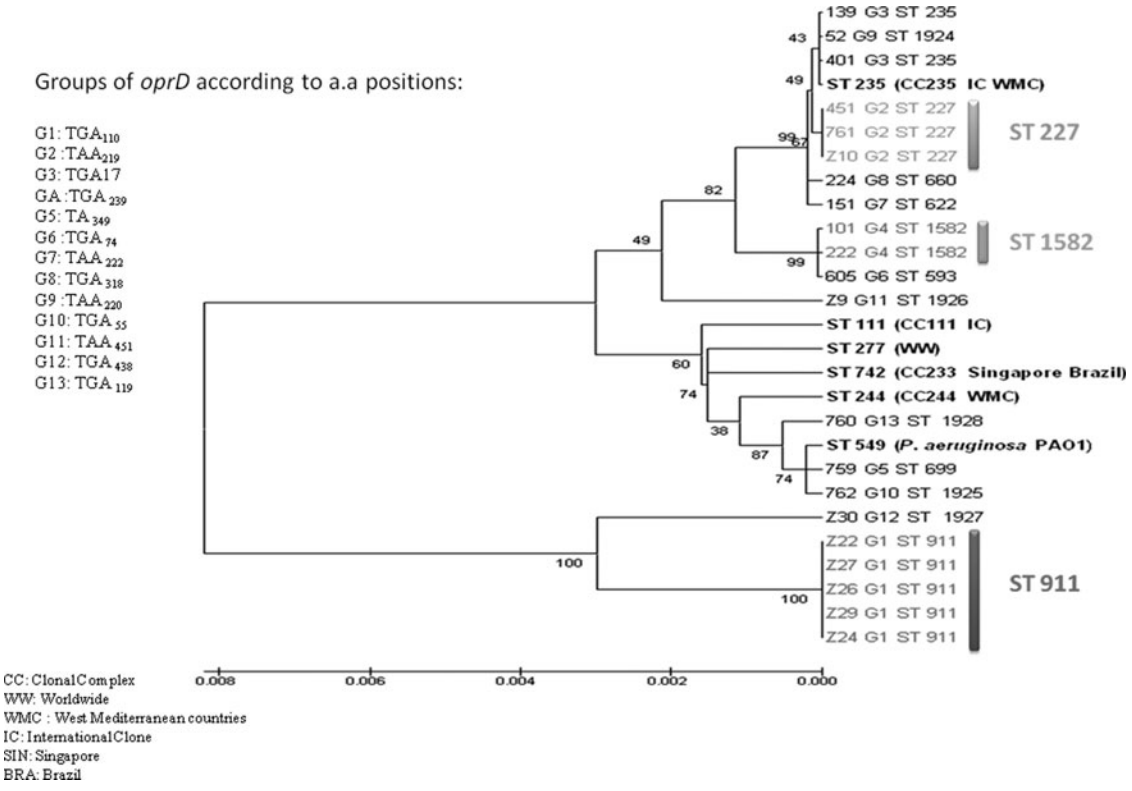

FIG. 2. Phylogenetic tree of the 21 imipenem-resistant *P. aeruginosa* clinical isolates based on the multilocus sequence typing (MLST) concatenated gene sequences of each isolate aligned with the PAO1 strain.

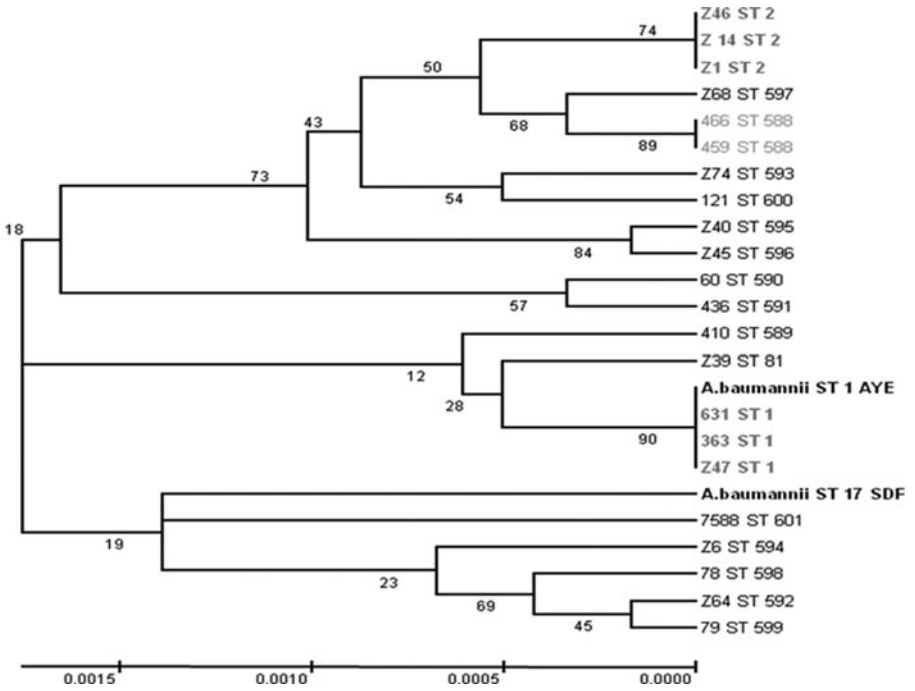

FIG. 3. Phylogenetic tree of the 22 imipenem-resistant *A. baumannii* clinical isolates based on the MLST concatenated gene sequences of each isolate aligned with reference strains SDF and AYE.

their *oprD* gene sequence with a stop codon. Based on the mutations in the *oprD* gene sequences, carbapenem-resistant *P. aeruginosa* isolates could be classified into 13 *oprD* mutational groups (Table 1). A total of 13 and 17 different STs were assigned to the investigated 21 *P. aeruginosa* and 22 *A. baumannii* imipenem-resistant strains, respectively (Figs. 2 and 3).

Discussion

In this study, we investigated the molecular mechanism of resistance to carbapenems in *P. aeruginosa* and *A. baumannii* clinical isolates recovered from Tripoli and Benghazi hospitals in Libya. All MBL-positive *P. aeruginosa* produced $bla_{\text{VIM-2}}$. These data confirm previous studies performed in the Mediterranean basin, which concluded that the main MBL produced by *P. aeruginosa* is VIM-2. Indeed, Sefraoui *et al.* detected the $bla_{\text{VIM-2}}$ gene in two strains of *P. aeruginosa* isolated from three hospitals in western Algeria in 2013.[36] Recently, Al Bayssari *et al.* reported the emergence of VIM-2 in a series of clinical isolates of carbapenem-resistant *P. aeruginosa* in Lebanon.[1] Additionally, in Tunisia, Hammami *et al.* demonstrated the incidence of the MBL VIM-2 as a gene cassette in class 1 integron in *P. aeruginosa* collected from different wards at Charles Nicolle hospital of Tunis.[20] In addition, data obtained in a previous study conducted in Egypt, a neighboring country of Libya, showed that the $bla_{\text{VIM-2}}$ gene was the most prevalent gene in *P. aeruginosa*.[39] These findings may reflect the current spread of MBLs in clinically relevant gram-negative strains throughout northern Africa and also show that the Libyan isolates share the same genetic pool with bacterial species worldwide. In the absence of MBL, mutational inactivation of the *oprD* gene is the major determinant of resistance to carbapenem, particularly to imipenem, in *P. aeruginosa* strains.[31] The present study is the first report of coexpressing VIM-2 and OprD porin loss in identical clinical isolates of *P. aeruginosa* in Libya.

These results indicated that the mutational inactivation of the *oprD* gene was the main mechanism for imipenem resistance in *P. aeruginosa* clinical isolates, as previously described in many studies.[1,36] Members of an identical clone maintained an identical sequence of the *oprD* gene, illustrating the stability of these clonal complexes, as previously demonstrated by Sefraoui *et al.*[36] In our study, MLST analysis revealed that the presence of multiple clones, with clones belonging to ST911 and to ST235 being the most frequent. Other MLST analysis studies reported ST235 in Mediterranean countries.[35] The results of this work are consistent with the studies of Nho *et al.*, who reported the dissemination of genetically unrelated isolates of *P. aeruginosa* carrying $bla_{\text{VIM-2}}$ in Korea.[28]

A PubMed search did not identify any published report describing the occurrence or spread of $bla_{\text{OXA-23}}$- and $bla_{\text{OXA-24}}$-producing *A. baumannii* in Libya. In this study, we report for the first time the presence of imipenem-resistant *A. baumannii* producing these oxacillinases in Libya. Several studies have shown the dissemination of carbapenem-resistant *A. baumannii* isolates in different geographic regions, including the neighboring countries of Libya, such as Tunisia and Egypt. In Tunisia, Mansour *et al.* reported the dissemination of the $bla_{\text{OXA-23}}$ gene in 99 clinical strains of *A. baumannii*.[25] In Egypt, Al-Agamy *et al.* reported that the *A. baumannii* carrying $bla_{\text{OXA-23}}$- and $bla_{\text{OXA-24}}$-like genes were found to be the most prevalent type of β-lactamase-encoding genes in *A. baumannii*.[2] In North Africa, the resistance rate of *A. baumannii* to imipenem was found to be 47.9% in Algeria,[4] 45% in Tunisia,[25] and 75% in Egypt.[2] In this study, the most prevalent CHDL-encoding gene in *A. baumannii* was $bla_{\text{OXA-23}}$, with a prevalence rate of 86.36% ($n = 19$), which is in agreement with previous

studies.[11,21,32] In this study, we also detected the co-occurrence of bla_{OXA-23} and bla_{OXA-48} in one *A. baumannii* isolate. Recently Evans *et al.*, reported that Gonçalves *et al.* described the first detection of OXA-48-like-producing *A. baumannii* in the fecal flora of nursing home residents in Northern Portugal.[18] This report coincided with our study; thus, this is the second description of OXA-48-producing *A. baumannii*. OXA-48 and its variants are widespread in *Klebsiella pneumoniae* and other *Enterobacteriaceae* and have now been reported in *A. baumannii* as well, and they represent one of the most concerning developments in carbapenem resistance in the last decade.[18] Finally, MLST analysis for *A. baumannii* isolates revealed also the presence of multiple clones in our study. The clones belonging to ST1 and ST2 were the most frequent and correspond to the most prevalent Mediterranean *A. baumannii* clone according to a study conducted in Spanish hospitals.[5]

In conclusion, this study described the first detection of VIM-2- and OXA-23/OXA-24/OXA-48-producing *P. aeruginosa* and *A. baumannii* in Libya. These findings are of great concern because the rapid dissemination of carbapenem-resistant strains represents a major therapeutic and epidemiological threat and requires the implementation of strict hygiene procedures and regular surveillance studies, especially in Libyan hospitals, where the adherence to internationally accepted infection control policies is not optimal.

Acknowledgments

The authors thank Linda Hadjadj for technical assistance. This work was partly funded by CNRS and IHU Méditerranée Infection. This work was supported by the Ministry of Higher Education, Scientific Research and Information and Communication Technologies of Tunisia offering a Scholarship to Miss. Najla Mathlouthi in Aix-Marseille-University, France.

Disclosure Statement

No competing financial interests exist.

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