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Spread of imipenem-resistant *Acinetobacter baumannii* co-expressing OXA-23 and GES-11 carbapenemases in Lebanon

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SUMMARY

Objectives: The acquisition of carbapenemases by *Acinetobacter baumannii* is reported increasingly worldwide, but data from Lebanon are limited. The aims of this study were to evaluate the prevalence of imipenem-resistant *A. baumannii* in Lebanon, identify resistance determinants, and detect clonal relatedness.

Methods: Imipenem-resistant *A. baumannii* were collected from nine Lebanese hospitals during 2012. Antimicrobial susceptibility, the cloxacillin effect, and ethylenediaminetetraacetic acid (EDTA) synergy were determined. Genes encoding carbapenemases and insertion sequence IS*Aba1* were screened via PCR sequencing. IS*Aba1* position relative to genes encoding Acinetobacter-derived cephalosporinases (ADCs) and OXA-23 was studied by PCR mapping. Clonal linkage was examined by enterobacterial repetitive intergenic consensus PCR (ERIC-PCR).

Results: Out of 724 *A. baumannii* isolated in 2012, 638 (88%) were imipenem-resistant. Of these, 142 were analyzed. Clavulanic acid–imipenem synergy suggested carbapenem-hydrolyzing extended-spectrum β-lactamase. A positive cloxacillin test indicated ADCs, while EDTA detection strips were negative. Genotyping indicated that 90% of isolates co-harbored $bla_{\text{OXA-23}}$ and $bla_{\text{GES-11}}$. The remaining strains had $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24}}$, $bla_{\text{GES-11}}$, or $bla_{\text{OXA-24}}$ with $bla_{\text{GES-11}}$. IS*Aba1* was located upstream of bla_{ADC} and $bla_{\text{OXA-23}}$ in 97% and 100% of isolates, respectively. ERIC-PCR fingerprinting revealed 18 pulsotypes spread via horizontal gene transfer and clonal dissemination.

Conclusion: This survey established baseline evidence of OXA-23 and GES-11-producing *A. baumannii* in Lebanon, indicating the need for further surveillance.

1. Introduction

Acinetobacter baumannii is a Gram-negative, non-fermenting opportunistic pathogen that is frequently associated with nosocomial epidemics in intensive care and burn therapy units, where it causes septicemia, pneumonia, and urinary tract infections with high mortality. The treatment of infections due to *A. baumannii* is a challenge because of resistance to the antimicrobial agents of last resort, β-lactams.[1] Resistance to this class of antibiotics in *A. baumannii* results mainly from the production of β-lactamases, and also from other non-enzymatic pathways, like changes in outer membrane proteins or over-expression of efflux pumps.[1,2] All A, B, C,

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and D Ambler classes of β-lactamase have been described in *A. baumannii*: (1) class A extended-spectrum β-lactamases (ESBLs), (2) class B metallo-β-lactamases, (3) class C Acinetobacter-derived cephalosporinases (ADCs), and (4) carbapenem-hydrolyzing class D β-lactamases (CHDLs).

Resistance to extended-spectrum cephalosporins is mainly attributed to the over-production of chromosomal ADCs or, less frequently, to the acquisition of an ESBL (PER, VEB, GES).[3–6] Resistance to carbapenems is mainly due to transferable class D oxacillinases of subfamilies OXA-23, OXA-24/33/40, OXA-58, and OXA-143, or to over-production of the intrinsic OXA-51-like enzymes.[7] Some carbapenem-resistant *A. baumannii* expressing class B enzymes (IMP, VIM, SIM, SPM, GIM, and NDM) and, more recently, acquired GES and KPC carbapenemases of class A, have been reported.[8–10] Among the GES carbapenemases, GES-11 was the first described conferring reduced susceptibility to carbapenems and initially detected in *A. baumannii*.[8] The presence of insertion sequence IS*Aba1* upstream from $bla_{\text{OXA-23}}$, $bla_{\text{OXA-58}}$, $bla_{\text{OXA-51}}$, and bla_{ADC} provides an efficient promoter for the expression of these genes, leading to higher β-lactam hydrolysis rates.[11–14]

While carbapenem resistance due to the production of CHDLs is the most widespread in *A. baumannii*, Ambler class B enzymes have been associated with carbapenem resistance in *A. baumannii* in several countries, including Italy, Portugal, Japan, and Brazil.[2,15] In addition, *A. baumannii* acquiring GES carbapenemases have been described in France,[8] Belgium,[16] and Turkey.[4] In Lebanon, in 2008, Zarrilli et al. reported a plasmid-borne $bla_{\text{OXA-58}}$ gene in *A. baumannii* strains selected in a Lebanese hospital.[17] Lately, in 2012, NDM-1-harboring *A. baumannii* strains were isolated in another Lebanese hospital from Syrian patients injured during the civil war.[18] However, no countrywide epidemiological survey has addressed the prevalence of carbapenem-resistant *A. baumannii* in Lebanon until now.

The aims of the current study were to evaluate the occurrence of carbapenem-resistant *A. baumannii* in nine Lebanese medical centers, and to describe the types of β-lactamases involved in such resistance, as well as to investigate clonal relatedness of incriminated strains.

2. Materials and methods

2.1. Bacterial strains

From January 1 to December 31, 2012, imipenem-resistant isolates of *A. baumannii* were collected from nine Lebanese hospitals located in diverse geographic areas: Beirut (Hotel Dieu de France, Saint-George Hospital), Mount Lebanon (Bellevue Medical Center), North Lebanon (Mounla Hospital), South Lebanon (Secours Populaire Libanais Hospital, Labib Medical Center), and Bekaa (Bekaa Hospital, Farhat Hospital, Chtaura Hospital). Selection criteria for strains were based upon the recommendations of the Clinical and Laboratory and Standards Institute (CLSI);[19] strains with an inhibition zone diameter of imipenem $\leq$13 mm were included and these were delivered to the central microbiology laboratory at the Faculty of Pharmacy, Saint-Joseph University, Beirut. The isolates were re-identified using the API 20 NE system (BioMérieux, Marcy l'Etoile, France) and confirmed using PCR to detect the intrinsic $bla_{\text{OXA-51-like}}$ gene.[20]

2.2. In vitro susceptibility testing

Antimicrobial susceptibility testing was performed by disk diffusion on Mueller–Hinton agar using amoxicillin/clavulanic acid (AUG), ceftazidime (CAZ), cefotaxime (CTX), cefepime (CPM), and imipenem (IMI) disks (Mast Diagnostics, Merseyside, UK), in accordance with the recommendations of the CLSI.[19]

2.3. Cloxacillin test and metallo-β-lactamase detection

Antibiotic susceptibility testing was also performed using Mueller–Hinton agar with cloxacillin (200 mg/l) to inhibit intrinsic ADCs. An increase in inhibition zone diameters of cephalosporins in the presence of cloxacillin was a qualitative indicator of ADC hyper-production. Also, the cloxacillin test was used to allow better detection of synergy between clavulanic acid and imipenem, indicating the production of an ESBL with carbapenem-hydrolyzing activity. Metallo-β-lactamases were screened for using the combined imipenem/imipenem+EDTA Etest (Liofilchem, Roseto degli Abruzzi, Italy). A ratio of the minimum inhibitory concentration of imipenem compared to that of imipenem in the presence of EDTA ($\text{MIC}_{\text{imipenem}}/\text{MIC}_{\text{imipenem+EDTA}}$) of $\geq$8 was considered a presumptive diagnosis of metallo-β-lactamase.[21]

2.4. Identification of carbapenem resistance genes

PCR sequencing experiments were used to detect carbapenemase genes $bla_{\text{oxa-23}}$, $bla_{\text{oxa-24}}$, $bla_{\text{oxa-40}}$, $bla_{\text{oxa-58}}$, bla_{KPC}, bla_{GES}, $bla_{\text{IMP-1}}$, $bla_{\text{IMP-2}}$, bla_{NDM}, and bla_{VIM}.[22] Isolates were also screened for insertion sequence IS*Aba1*.[14] PCR mapping using IS*Aba1* forward and OXA-23 reverse primers was done on $bla_{\text{oxa-23}}$-positive isolates. Also, IS*Aba1* forward and bla_{ADC} reverse primers were used to locate the position of IS*Aba1* relative to bla_{ADC}.

2.5. ERIC-PCR

The genetic relationship between the isolates was determined using enterobacterial repetitive intergenic consensus PCR (ERIC-PCR), as described by Ferreira et al.[23] The amplification conditions were as follows: an initial denaturation cycle at 95 °C for 7 min, 35 cycles at 95 °C for 1 min, 51 °C for 1 min, and 72 °C for 1 min and 30 s, and a final extension at 72 °C for 15 min. The amplified products were visualized on 1.5% agarose gel stained with 0.5 μg/ml of ethidium bromide. The banding patterns were converted by GelQuest/Sequentix software (Klein Raden, Germany) into a binary matrix, calculated using the Dice coefficient. A dendrogram was constructed via the unweighted pair-group method using arithmetic averages (UPGMAs). Only visible bands in the ERIC-PCR fingerprinting were used to construct the similarity matrix and the dendrogram. Isolates with more than 85% similarity were considered to be clonally related.

3. Results

Out of 723 *A. baumannii* isolated in 2012, 638 (88%) were imipenem-resistant. Of these, 142 non-duplicate imipenem-resistant *A. baumannii* isolates were received by the laboratory for analysis. These isolates were distributed as follows: 63 were from Saint-Georges Hospital, 36 from Labib Medical Center, 22 from Hotel Dieu de France, five from Bellevue Medical Center, five from Bekaa Hospital, five from Chtaura Hospital, four from Farhat Hospital, one from Mounla Hospital, and one from Secours Populaire Libanais Hospital.

3.1. Antimicrobial resistance pattern, cloxacillin effect, and EDTA synergy

All 142 isolates showed high resistance to the tested β-lactams (Table 1). Sixty-eight isolates yielded a positive cloxacillin test, indicating ADC hyper-production. Sixty-one showed synergy between clavulanic acid and imipenem, indicating the production

Table 1
Classification of studied imipenem-resistant *Acinetobacter baumannii* into seven groups based upon phenotypic and genotypic profiles

Group	Isolate characteristics	Number (%) of strains	Number of strains showing IMI–AUG synergy[a]	Inhibition zone diameters (mm)[b]				
				AUG	CTX	CPM	CAZ	IMI
1	OXA-23 + GES-11+ hyper-produced ADC	124 (87)	57	6	6	6–9 (10–14)	6–10 (6–15)	6–12
2	OXA-23	7 (5)	0	6	6	14	6	13
3	OXA-23 + GES-11	4 (3)	2	6	6	6–10	6–8	10–13
4	OXA-23+ hyper-produced ADC	4 (3)	0	14	8 (15)	20 (30)	23 (30)	6
5	OXA-24 + GES-11	1 (0.7)	1	6	6	11	6	12
6	OXA-24+ hyper-produced ADC	1 (0.7)	0	6	6	13 (16)	6	9
7	GES-11+ hyper-produced ADC	1 (0.7)	1	6	6	9 (12)	6	10

ADC, Acinetobacter-derived cephalosporinases; AUG, amoxicillin/clavulanic acid; CAZ, ceftazidime; CPM, cefepime; CTX, cefotaxime; IMI, imipenem.
[a] IMI–AUG synergy = synergy between amoxicillin/clavulanic acid disk and imipenem disk.
[b] Inhibition zone diameters between brackets are those increased in the presence of cloxacillin 200 mg/l.

of a class A ESBL with carbapenemase activity. Metallo-β-lactamase detection strips were negative for all isolates.

3.2. Molecular analysis

Genotyping allowed the detection of bla_{ADC} and bla_{OXA-51} genes. GES-type carbapenemase concomitant with OXA-23 enzyme was observed in the majority of the isolates (128 isolates, 90%), indicating that these two carbapenemases are the most prevalent mechanisms of resistance among our strains. Sequencing of the bla_{GES} variant revealed GES-11. Eleven strains harbored either OXA-23 or OXA-24 alone, or OXA-24 with GES-11. In one strain, GES-11 was the only detected enzyme with carbapenemase-hydrolyzing capacity. Tested metallo-β-lactamase-encoding genes were not detectable in any of the isolates.

Insertion sequence IS*Aba1* was identified in all isolates regardless of the type of carbapenem-hydrolyzing enzyme. Mapping of this element in OXA-23-producers revealed a band of size 1371 bp, indicating that IS*Aba1* lay upstream and adjacent to bla_{OXA-23} in all strains. Mapping of IS*Aba1* to bla_{ADC} revealed a band with size of 693 bp, showing that Is*Aba1* existed upstream and adjacent to ADC in 97% of the isolates.

Phenotypic results combined with detected carbapenemases were used to classify isolates into seven groups, shown in Table 1 in decreasing frequency of detection.

3.3. Results of ERIC-PCR

Fingerprinting by ERIC-PCR showed the existence of 18 pulsotypes of imipenem-resistant *A. baumannii* (Figure 1). Eighty-seven strains belonged to pulsotype 17 and originated from seven out of nine hospitals. A smaller portion of 25 isolates belonged to pulsotype 16 and were disseminated among seven hospitals. The remaining strains showed various fingerprinting patterns, with fewer than 10 strains in each pulsotype, indicating different clones. Strains from Saint-George Hospital showed the widest genotypic variation, with 13 out of the 18 pulsotypes. Interestingly, the single strain obtained from Secours Populaire Libanais Hospital was a unique pulsotype (pulsotype 7). In pulsotypes 1, 16, and 17, different carbapenemase content was noticeable among the related isolates. For instance, strains harboring bla_{OXA-23} or bla_{GES-11} alone belonged to pulsotype 17 together with those co-harboring bla_{OXA-23} and bla_{GES-11}.

4. Discussion

Resistance to carbapenems makes *A. baumannii* a challenging pathogen to cure due to significant compromise of treatment options.[1] In Lebanon, imipenem-resistant *A. baumannii* has been described in two reports related to isolated Lebanese healthcare centers,[17,18] however no epidemiological countywide evaluation has investigated the current status of carbapenem resistance in this species. This study was conducted in nine Lebanese centers all over the country in order to gain a wide overview of the status of imipenem-resistant *A. baumannii* over a 1-year period. The prevalence of imipenem resistance among *A. baumannii* in 2012 was 88%. This number is close to reported rates of 70% in Egypt,[24] 72% in Turkey,[25] 75% in Spain,[26] and up to 100% in Italy.[27]

The accumulation of diverse intrinsic and acquired antimicrobial resistance mechanisms to carbapenem accounts for these high rates of imipenem resistance in *A. baumannii* worldwide. The most prevalent mechanism of carbapenem resistance in *A. baumannii* is the enzymatic degradation by carbapenemases. In this study, we showed the presence of bla_{OXA-23} and bla_{GES-11} in the majority (90%) of *A. baumannii*, confirming the predominance of these genes among Lebanese isolates. The dissemination of OXA-23 in Lebanon is consistent with the worldwide epidemiology of OXA-23[15] and with reports from neighboring countries including Egypt,[24] Iraq,[28] Iran,[29] and the United Arab Emirates.[30] However, the co-existence of both OXA-type and GES-type carbapenemases in *A. baumannii* is not frequent, although previously reported in Kuwait[31] and recently in Tunisia.[32] In addition, another OXA family, OXA-24 (groups 5 and 6), was found in Lebanon, a carbapenemase that has been described in *A. baumannii* from close countries like Turkey, where it was responsible for a hospital outbreak in an intensive care ward.[33] The OXA-58 oxacillinase, previously reported in a Lebanese hospital,[17] was not detected among our isolates. This agrees with reports of a progressive change from bla_{OXA-58} to bla_{OXA-23} carriage worldwide.[34] In addition, a recent rise in metallo-β-lactamases in *A. baumannii* has been observed in some European countries.[34,35] However, even though NDM-1 has been detected recently in *A. baumannii* isolated from Syrian patients hospitalized in Lebanon,[18] this metallo-β-lactamase has apparently not spread to Lebanese patients according to the present findings.

Phenotypically, the presence of GES-11 independently from OXA-type carbapenemases did confer cephalosporin resistance as well as imipenem hydrolysis, like in the single isolate of group 7. In the absence of GES-11, as in groups 4 and 6, the inhibition zone diameters of cephalosporins were relatively higher than in group 7, while hydrolysis of imipenem was maintained. This corresponds to the properties of OXA-23, which exhibits mostly imipenem-hydrolyzing activity and does not act on extended-spectrum cephalosporins. Of note, only 62 out of the 130 GES-11-positive isolates did yield a positive synergy test between imipenem and clavulanic acid, indicating the pivotal role of molecular methods for the detection of GES-producing isolates.

In addition to the acquired carbapenemases, intrinsic ADCs play a major role in resistance of *A. baumannii* to β-lactams. In this

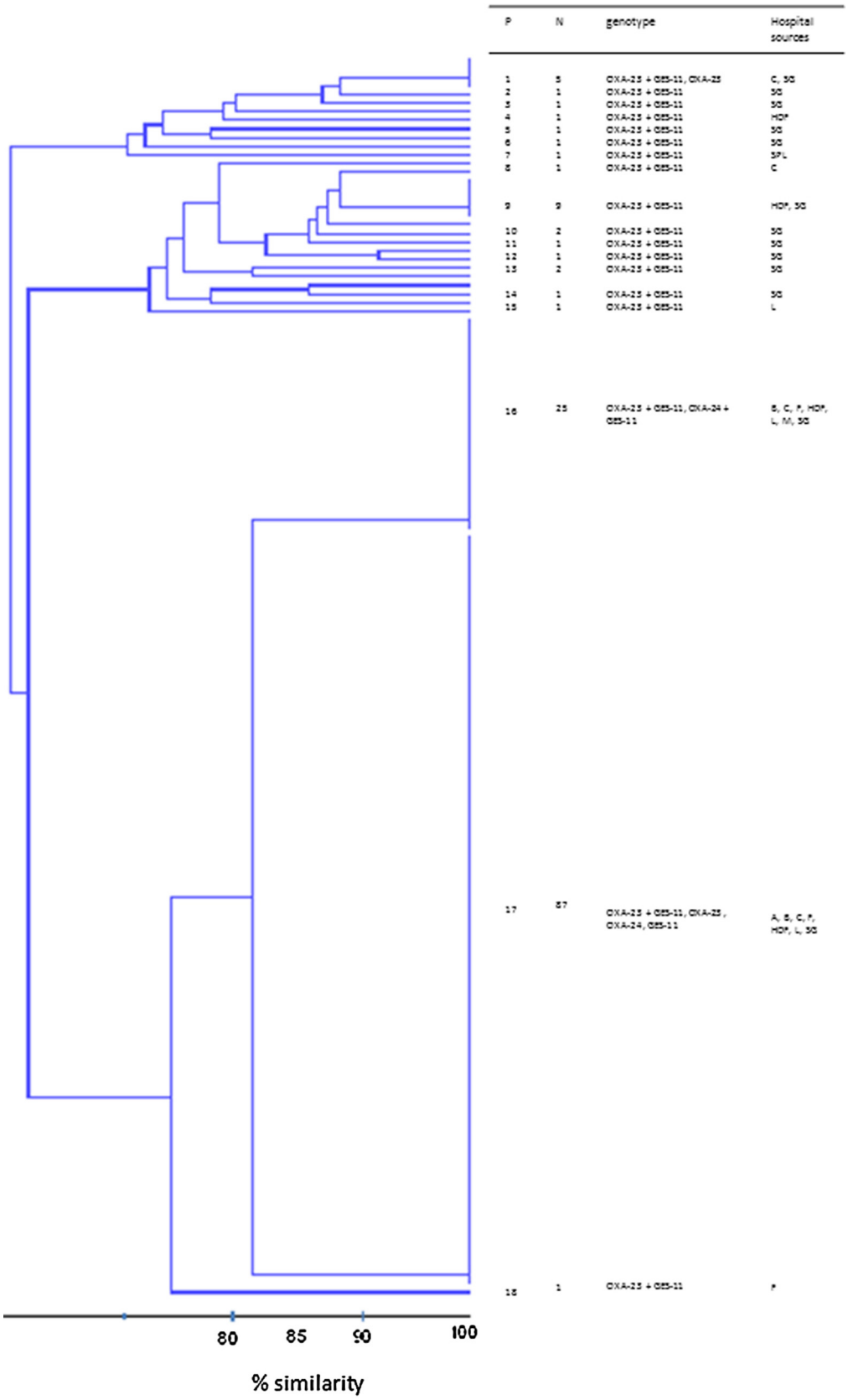

Figure 1. Dendrogram showing the genetic relationship of *Acinetobacter baumannii* by ERIC-PCR fingerprinting, with corresponding number of strains, genotypes, and hospital sources for each pulsotype.
A = Bellevue Medical Center, B = Bekaa Hospital, C = Chtaura Hospital, F = Farhat Hospital, HDF = Hotel Dieu de France, L = Labib Medical Center, M = Mounla Hospital, N = number of strains, P = pulsotype, SG = Saint-George Hospital, SPL = Secours Populaire Libanais.

study, the hyper-production of ADCs together with carbapenemase production reflected the multifactorial aspects of β-lactam resistance in *A. baumannii*. Groups 1, 4, 6, and 7 of the studied isolates (92%) showed phenotypic evidence of hyper-produced ADCs. In genotypic screening, 97% of the isolates revealed the presence of IS*Aba1* upstream from bla_{ADC}. A comparison of groups 1 and 3, which differ only in the extent of bla_{ADC} expression, showed that *A. baumannii* resistance to extended-spectrum cephalosporins can be due to over-expression of the resident ADCs, possibly with concomitant production of ESBLs.[36]

Insertion sequence IS*Aba1* was also adjacent to bla_{OXA-23}. The importance of this genetic element for carbapenem resistance in *A. baumannii* has been reported, where IS*Aba1* exists most commonly in association with bla_{OXA-23}, bla_{OXA-58}, $bla_{OXA-51-like}$, and bla_{ADC}, and constitutes a strong promoter for increased expression of the downstream gene.[1,24] In a previous study, intrinsic OXA-51 expression was reported to be increased by eight times following insertion of IS*Aba1*.[14]

ERIC-PCR, a method for rapid genetic fingerprinting, was applied to study clonal relationships between our isolates. Eighteen different clones of *A. baumannii* were identified that could not be correlated according to their phenotype or hospital source. Pulsotype 17 was predominant in various hospitals, indicating clonal diffusion, which may be dependent on the permanence of *A. baumannii* for long periods in the hospital environment and the transfer of patients among hospitals, which is a common practice in Lebanon. Nevertheless, the variability in pulsotypes also indicated probable horizontal transmission of resistance determinants among isolates from different sources. The results of our fingerprinting analysis revealed that strains with bla_{OXA-23} alone or bla_{GES-11} alone exhibited clonal relatedness to those co-harboring the two genes, as in pulsotypes 1 and 17. This may raise the possibility that these genes may be part of different mobile elements. It was not possible in this study to perform multilocus sequence typing (MLST). It would have been interesting to determine if the OXA-23 and GES-11 international clones are present in Lebanon.

In conclusion, we report the status of imipenem-resistant *A. baumannii* in Lebanon with the preponderance of OXA-23 and GES-11. Not only does this study represent the first countrywide assessment of carbapenem resistance in this organism, but it is also the first report of OXA-23, OXA-24, and GES-11 carbapenemases in Lebanon. These resistance traits appear to spread successfully via both bacterial epidemics and horizontal transfer. Given the versatile and adaptable genetic background of *A. baumannii* and the current population displacement and patient transfer due to civil wars in the region, carbapenem-resistant *A. baumannii* strains are expected to escalate. Continuous surveillance involving Lebanon and neighboring countries is, therefore, crucial to limit their propagation.

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