

Original Article

Increased *bla*_{OXA-23-like} prevalence in *Acinetobacter baumannii* at a tertiary care center in Lebanon (2007-2013)

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Abstract

Introduction: *Acinetobacter baumannii* has become one of the most feared organisms in hospital-acquired infections during the past decades. Their multi-drug resistant profiles have rendered many broad-spectrum antibiotics ineffective. The purpose of this retrospective study is to describe and compare molecular characteristics of *A. baumannii* isolated from patients at a tertiary care center in Lebanon from two outbreaks, the first in 2007-2008 as part of a case-controlled study involving *Acinetobacter baumannii* cases admitted to the ICU and the second in 2013. **Methodology:** A total of 148 *A. baumannii* clinical isolates were collected from various clinical specimens during 2007-2008 and 2013. All *A. baumannii* isolates were subjected to PCR amplification of *bla*_{OXA-23-like} and *bla*_{OXA-51-like} genes of carbapenem resistance. Random amplification of polymorphic DNA (RAPD) was also performed to assess their genomic relatedness. **Results:** There was an increase in the prevalence of *bla*_{OXA-23-like} and *bla*_{OXA-51-like} between the two time periods; however, only with 22% genomic relatedness between 2007-2008 and 2013 isolates. Taking 80% as margin of compatibility, 31 distinct clusters containing 2 to 11 strains were observed in both time periods. **Conclusion:** The presence of numerous clusters accompanied by a predominant increase in the prevalence of *bla*_{OXA-23-like} gene between 2007 and 2013 suggests a horizontal transmission of the gene within various strains of the species, constituting a primary factor in the continued increase of carbapenem resistance over the years. As such, infection control measures ought to be taken with the highest priority and compliance among all involved healthcare workers is of utmost importance.

Key words: *Acinetobacter baumannii*; carbapenem resistance; *bla*_{OXA-23-like}; horizontal gene transfer; outbreaks.

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Introduction

Acinetobacter baumannii is a species of Gram-negative, obligatory aerobic, oxidase-negative, and catalase-positive coccobacillus [1]. It is best known for its implication in healthcare settings; causing a variety of infections, including ventilator-associated pneumonia, central line-associated bloodstream infections, catheter-related urinary tract infections, and wound infections [2] especially in intensive care unit (ICU) patients with altered immunity [3]. Its evident association with medical equipment is explained by its biofilm-forming capabilities that allow it to colonize surfaces and protect it from desiccation [4-6].

Another attribute of *A. baumannii* is its high tendency to resist several classes of antimicrobials, which has been reported since the 1980s [2]; however, during the 1990s, *A. baumannii* was reported as resistant to imipenem, a carbapenem, which is a class of β -lactams often regarded as the last line of safe antimicrobials to be used when treating complicated bacterial infections [2]. There are several mechanisms that *A. baumannii* can use to achieve carbapenem resistance with enzymatic degradation via β -lactamases being the principal process [7]. OXA-type carbapenemases are Class D β -lactamases, according to the Ambler molecular classification [8], and the ones implicated in *A. baumannii* include OXA-23, OXA-24,

Table 1. Primer sequences used to amplify genes using PCR.

Primer	Sequence	Amplicon Size (bp)	Reference	
OXA-23F	5'-GATCGGATTGGAGAACCAGA-3'	501	[15]	
OXA-23R	5'-ATTCTGACCGCATTCCAT-3'			
OXA-51F	5'-TAATGCTTTGATCGGCCTTG-3'	353		
OXA-51R	5'-TGGATTGCACTTCATCTTGG-3'			
OXA-24F	5'-GGTTAGTTGGCCCCCTAAA-3'	246		
OXA-24R	5'-AGTTGAGCGAAAAGGGGATT-3'			
OXA-58F	5'-AAGTATTGGGGCTTGTGCTG-3'	599		
OXA-58R	5'-CCCCTCTGCGCTCTACATAC-3'			
OXA-143F	5'TGGCACTTTCAGCAGTTCCT-3'	149		[32]
OXA-143R	5'-TAATCTTGAGGGGCAACC-3'			

OXA-51, OXA-58, and OXA-143 [9-14] encoded by *bla*_{OXA-23-like}, *bla*_{OXA-24/40}, *bla*_{OXA-51-like}, *bla*_{OXA-58}, and *bla*_{OXA-143-like} genes, respectively [12,15].

Over the past few years, carbapenem resistance in *A. baumannii* has been rising at an alarming rate. In Lebanon, a retrospective nation-wide survey reported that *Acinetobacter spp.* susceptibility to imipenem decreased from 49.2% in 2011 to 15.1% in 2013 while the number of isolates identified increased from 242 to 1463 during the same time period [16]. At a major tertiary care center in Lebanon, carbapenem susceptibility has decreased from 38% in 2009 [17] to 10% by 2013 [18] and only 7% by 2016, with 97% of the *Acinetobacter spp.* recovered being *A. baumannii* [19]. The mortality rate of patients with nosocomial *Acinetobacter* infections at the tertiary care center between 2012 and 2014 was 58.6% (unpublished data from the Infection Control and Prevention Program). Although this does not represent attributable mortality, it highlights the importance of this pathogen in the critically ill patients. This retrospective study investigates the drastic change in resistance patterns of this pathogen at the molecular level.

Methodology

Sample Collection and DNA extraction

A total of 148 carbapenem resistant *A. baumannii* clinical isolates [18] were collected from two separate time periods at the tertiary care center: 93 isolates from

a 2007-2008 outbreak, within the context of a case-controlled study performed between 2007-2008 involving diagnosed *Acinetobacter baumannii* cases admitted to the ICU during the same time, and 55 isolates from a 2013 outbreak. *Acinetobacter baumannii* was identified using API 20NE (bioMérieux, Marcy l'Etoile, France) coupled with phenotypic characteristics such as colonial morphology, Gram staining, other microscopic features. Isolates were anonymized/de-identified and were stored in BBL Brucella broth (BD, Franklin Lakes, USA) with 15% glycerol at -80°C until usage. Isolates were then cultured in Difco LB broth (BD, Franklin Lakes, USA) and their DNA extracted using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) as per manufacturer's instructions.

PCR Amplification and Gel Electrophoresis

Polymerase chain reaction (PCR) was performed on the extracted DNA from all 148 isolates to detect the mostly encountered *bla*_{OXA-23-like} genes. In addition, PCR amplification of *bla*_{OXA-51-like}, *bla*_{OXA-24/40}, *bla*_{OXA-58}, and *bla*_{OXA-143-like} genes was performed on 68 of the 93 isolates from 2007-2008, due to resource constraints, and on all 55 isolates from 2013. Primer sequences of all genes are listed in Table 1. PCR amplification was achieved using the C1000™ Thermal Cycler (Bio-Rad, Hercules, USA) with cycling conditions, listed in Table 2, determined via the Protocol AutoWriter feature of the

Table 2. PCR cycling conditions of target *bla*_{OXA} genes.

Target Gene	Cycling Conditions
<i>bla</i> _{OXA-23-like}	Initial denaturation at 95°C for 3 minutes, followed by 39 cycles of denaturation at 95°C for 30 seconds, annealing at 55.7°C for 30 seconds, and elongation at 72°C for 36 seconds, then a final extension at 72°C for 5 minutes.
<i>bla</i> _{OXA-24/40}	Initial denaturation at 95°C for 3 minutes, followed by 39 cycles of denaturation at 95°C for 30 seconds, annealing at 55.9°C for 30 seconds, and elongation at 72°C for 30 seconds, then a final extension at 72°C for 5 minutes.
<i>bla</i> _{OXA-51-like}	Initial denaturation at 95°C for 3 minutes, followed by 39 cycles of denaturation at 95°C for 30 seconds, annealing at 56.8°C for 30 seconds, and elongation at 72°C for 30 seconds, then a final extension at 72°C for 5 minutes.
<i>bla</i> _{OXA-58}	Initial denaturation at 95°C for 3 minutes, followed by 39 cycles of denaturation at 95°C for 30 seconds, annealing at 56.4°C for 30 seconds, and elongation at 72°C for 36 seconds, then a final extension at 72°C for 5 minutes.
<i>bla</i> _{OXA-143-like}	Initial denaturation at 95°C for 3 minutes, followed by 39 cycles of denaturation at 95°C for 30 seconds, annealing at 58.2°C for 30 seconds, and elongation at 72°C for 30 seconds, then a final extension at 72°C for 5 minutes.

thermal cycler. PCR amplicons were then electrophoresed on 1.5% agarose gel, using Agarose-Molecular Biology Grade (GeneDireX, Taiwan). A GeneRuler 50bp DNA ladder (Thermo Scientific, Waltham, USA) was run in parallel with the samples to serve as size marker. The gel electrophoresis product was then visualized using Gel DocXR+ System (BioRad, Hercules, USA) and a digital record of them was saved using Quantity One 1-D Analysis Software (BioRad, Hercules, USA).

Random Amplification of Polymorphic DNA (RAPD)

RAPD was performed on the DNA extracts of all 148 isolates using Illustra Ready-To-Go RAPD Analysis Beads (GE Healthcare Bio-Sciences, Pittsburgh, USA) per manufacturer's instructions. Amplification products were electrophoresed on 2% agarose gel, using Agarose-Molecular Biology Grade (GeneDireX, Taiwan), with a ready-to-load 100bp DNA ladder (Solis BioDyne, Tartu, Estonia) as size marker. The resulting gel was visualized using the same transilluminator. Genomic typing analysis was performed using BioNumerics (Applied Maths NV, Sint-Martens-Latem, Belgium) to generate a dendrogram showing the extent of genomic relatedness between the isolates, taking 80% as margin of compatibility.

Results

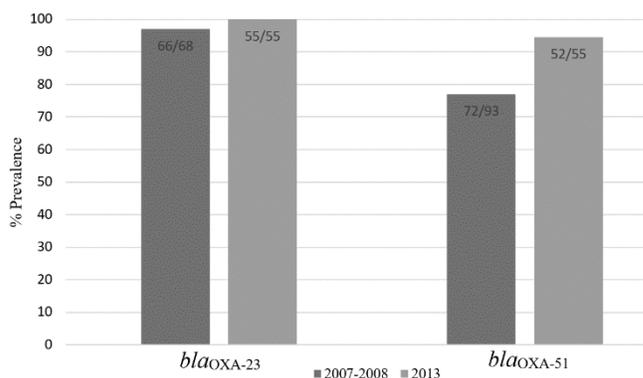
PCR Amplification and Gel Electrophoresis

Among the isolates collected during 2007-2008, 77.4% (72 of 93 isolates) were positive for *bla*_{OXA-23}-like genes, 97% (66 of 68 isolates) were positive for *bla*_{OXA-51}-like genes, 16.2% (11 of 68 isolates) were positive for *bla*_{OXA-24/40} genes, 27.9% (19 of 68 isolates) were positive for *bla*_{OXA-58}, and 22.1% (15 of 68 isolates) were positive for *bla*_{OXA-143}-like genes (Figure 1). As for the 55 isolates collected during the 2013 outbreak, 94.5% (52 of 55 isolates) were positive for *bla*_{OXA-23}-like genes, 100% were positive for *bla*_{OXA-51}-like genes, 3.6% (2 of 55 isolates) were positive for *bla*_{OXA-143}-like genes, and none of the isolates were positive for either *bla*_{OXA-24/40} or *bla*_{OXA-58} (Figure 1).

Random Amplification of Polymorphic DNA (RAPD)

The dendrogram generated from the RAPD data of the 2007-2008 isolates shows a heterogeneous distribution of samples with 1 major cluster having 11 isolates, 22 minor clusters having 2-4 isolates, and the rest as single strains (Supplementary Figure 1). In contrast, the dendrogram results of the 2013 outbreak isolates shows a more homogenous distribution with 4

Figure 1. Percent prevalence of *bla*_{OXA} carbapenem resistance-encoding genes among tested isolates for both 2007-2008 and 2013 time periods.



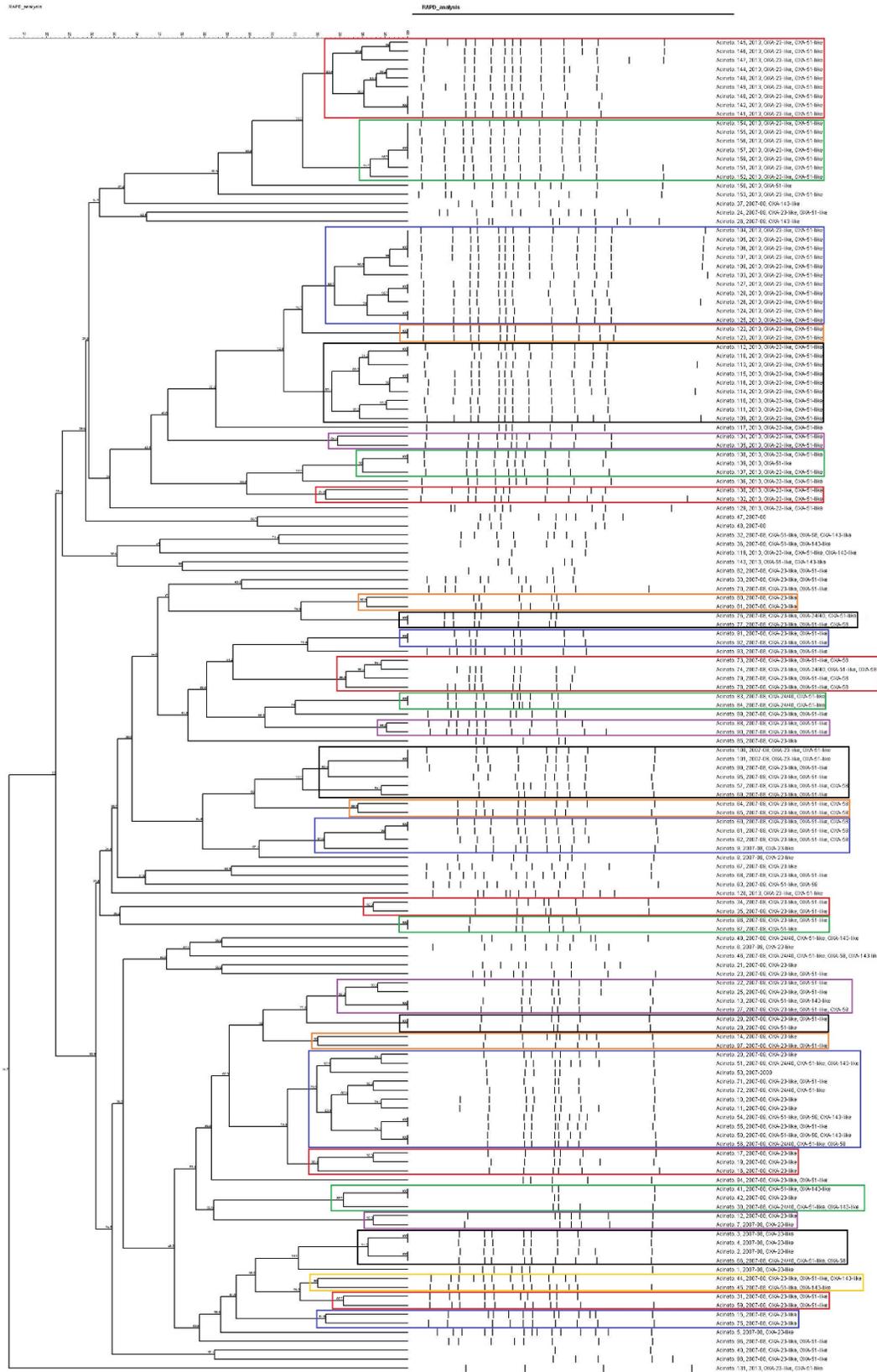
major clusters containing 7-11 isolates, 4 minor clusters containing 2-3 isolates, and the rest as single strains (Supplementary Figure 2).

When the RAPD results from both collections are combined to generate the final dendrogram, the resulting tree shows a distinct separation between the isolates collected from either time period, with minimal overlap. As such, the clusters for the 2007-2008 and 2013 isolates were observed to add up to 31 total clusters (Figure 2).

Discussion

The experimental results attained concerning the higher prevalence of carbapenemase-encoding genes for OXA-23-like and OXA-51-like in *A. baumannii* are in line with the observed increase in carbapenem resistance reported at AUBMC throughout the years. Concerning OXA-51-like, the increase to have 100% of the isolates carrying the gene from the 2013 collection adds further evidence to the possibility of using *bla*_{OXA-51}-like genes as a determinant when identifying *A. baumannii*, as first suggested by Turton, Woodford [20] and later adapted in several subsequent studies. However, the detection of OXA-51-like in non-*baumannii* *Acinetobacter* spp. [21,22] provides a discrepancy in the literature when it comes to confirming the identification of *A. baumannii* through OXA-51-like detection only. Further evidence that alludes to this point is the two isolates in this study that lacked *bla*_{OXA-51}-like genes, but were confirmed to be *A. baumannii* through API® 20NE, phenotypic characteristics, and their genomic relatedness to other isolates as part of our RAPD studies. As such, OXA-51-like may be of assistance when confirming the detections of *A. baumannii*, but it cannot be the only reference. Concerning OXA-23-like, the data reported here is similar to that of other reports published:

Figure 2. Dendrogram showing the genomic relatedness of the tested isolates from 2007-2008 and 2013. The colored boxes indicate the grouped clusters.



The first, encompassing *A. baumannii* isolates from several other hospitals across the country during 2012, in which 97.89% of them carried *bla*_{OXA-23} [7] and the second, including bacteria collected from another tertiary care center in Lebanon during 2013-2014, showing that 91.3% of the *A. baumannii* isolates harboring the *bla*_{OXA-23-like} gene [23]; however, these studies show higher numbers than a different report that also collected bacterial isolates from a number of hospitals in Lebanon, between 2013-2015, but only found 83.5% of *A. baumannii* carrying the OXA-23-like gene [24]. In other countries within the Middle East and the Mediterranean region, epidemiological data from Central Greece revealed similar results to our data with the presence of *bla*_{OXA-23-like} genes in 100% (87 of 87) *A. baumannii* isolates collected between 2012 and 2014 [25]. In Egypt, that percentage decreased to 50% as one study reported that 20 of 40 *A. baumannii* isolates collected in 2012 were positive for *bla*_{OXA-23-like} genes [26], while another report that screened isolates from Egypt and Saudi Arabia identified the *bla*_{OXA-23-like} in 69% (44 of 64) of their *A. baumannii* isolates [27]. More from Saudi Arabia, 83 *A. baumannii* isolates collected from the Eastern province between 2008 and 2012 showed *bla*_{OXA-23-like} in 58% (n=48) of the sample size [28]. In Kuwait, a study reported that 85% (28 of 53) *A. baumannii* isolates, collected between 2011 and 2012, carry *bla*_{OXA-23-like} [29]. While in Iran, several studies reported the prevalence of *bla*_{OXA-23-like} in *A. baumannii* isolates, including one by Bahador, Raoofian [30], that reported *bla*_{OXA-23-like} in 40% (34 of 85) *A. baumannii* isolates collected during 2011, and another by Farshadzadeh, Hashemi [31], that reported *bla*_{OXA-23-like} in 88.4% (61 of 69) *A. baumannii* isolates collected between 2012 and 2013. Such variation in prevalence data suggests that various strains circulating in different parts of the region have different frequencies in harboring the OXA-23-encoding gene. As for OXA-24/40, OXA-58, and OXA-143-like, their observed low prevalence in this study suggests that their role in conferring resistance to carbapenems among the tested isolates may be limited, especially with the marked decrease in their prevalence over the years opposite to the increase in carbapenem resistance among *A. baumannii* isolates. The low percentage of OXA-24/40 detected in this study from the 2013 isolates is similar to those from reports by Hammoudi, Moubareck [7] and Al Atrouni, Hamze [24] where their gene percent prevalence were 1.4% and 7.6%, respectively. The low percentages of OXA-58 and OXA-143-like detected in this study from the 2013 isolates is also similar to that detected by Al Atrouni,

Hamze [24] where each of them had a prevalence of 0.84%. Outside Lebanon, *bla*_{OXA-24/40} showed low prevalence in Egypt at 7.5% (3 of 40 isolates) [26] and in Kuwait at 6% (2 of 33 isolates) [29]. Similarly, *bla*_{OXA-58} was detected in 5% (2 of 40) isolates in Egypt [26], 3% (2 of 69) isolates in Iran [31], and 0% (0 of 33) isolates in Kuwait [29].

Moving to RAPD analysis, the results were confirmatory in their findings concerning the genomic assortment of the *A. baumannii* pool available in our tertiary care center. The fact that a total of 31 different clusters were observed over two time periods, 6 years apart, sheds light on the rapidly changing landscape and diversity of such a dangerous pathogen. When coupled with the PCR results, several conclusions can be drawn. First, having several clusters with predominance of a certain gene of resistance may suggest its horizontal transmission between the different isolates, as OXA-23 (previously named ARI 1) has been proven to be plasmid-encoded [11], thus propagating antibiotic resistance and further complicating treatment options. Another explanation to these findings might be the pre-existence of several different strains among the population that express a similar genetic profile as OXA-23-like is commonly encountered in *A. baumannii* isolates. Either way, the healthcare threat that *A. baumannii* poses remains despite the means to achieve it. Additionally, with the continued war-related conflict in the region and influx of patients from different parts of the neighboring countries into local hospitals, more diverse isolates of *A. baumannii* are expected to be encountered and end up circulating in different hospitals across the country due to their multidrug-resistant nature and difficulty in eradication. In addition to the extent of resistance in our isolates, the scarcity of new antimicrobial agents in the country poses a major problem against bringing the outbreaks to a halt. Therefore, it is imperative that our focus shifts towards prevention and aggressive infection control measures in order to contain the situation and improve patient outcome. At AUBMC, such preventive measures include on admission and weekly screening of all intensive care unit (ICU), and respiratory care unit (RCU) patients, as well as high-risk patients especially those transferred from other countries and other hospitals in Lebanon with high rates of resistant pathogens. Screening entails culturing swabs from the oropharynx, axillary, umbilicus, perianal region, and the rectum. All ICU patients are placed on isolation until the culture screening results are available. Contact isolation is applied on all colonized and infected patients on all wards until discharge. Also, colonized

and infected patients are bathed with chlorhexidine on a daily basis. These efforts of the infection control program and the increase in compliance among healthcare workers to hand hygiene led to the rapid containment of the 2013 outbreak as compared to the one in 2007-2008. In addition, molecular identification of isolates was used as means to track down the source of infection and control it using available resources. Future efforts should be directed towards early detection of similar outbreaks using molecular and clinical identifiers and promoting a culture of compliance to infection control measures.

Conclusion

The circulation of carbapenem-resistant *Acinetobacter baumannii* at a tertiary care center in Beirut, Lebanon is a matter of concern to healthcare providers as carbapenems are the last line of safe drugs to be used [2] before having to resort to toxic drugs such as colistin. This retrospective study portrays the diverse and shifting nature of *A. baumannii* by studying samples collected from two time periods separated by 6 years. It reveals that genes encoding OXA-23-like and OXA-51-like have increased in prevalence, while those encoding OXA-24/40, OXA-58, and OXA-143-like have decreased in prevalence and that the collected isolates experience a range of diverse genomic makeup. As such, infection control measures ought to be taken with the highest priority and compliance among all involved healthcare workers is of utmost importance.

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Conflict of interests: No conflict of interests is declared.

Annex – Supplementary Items

Supplementary Figure 1. Dendrogram showing the genomic relatedness of isolates collected in 2007-2008. The colored boxes indicate the grouped clusters.

