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Significant spread of extensively drug-resistant *Acinetobacter baumannii* genotypes of clonal complex 92 among intensive care unit patients in a university hospital in southern Iran

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Abstract

**Purpose.** Infections associated with *Acinetobacter baumannii* represent an increasing threat in healthcare settings. Therefore, we investigated the epidemiological relationship between clinical isolates of *A. baumannii* obtained from patients in a university hospital in Bandar Abbas in southern Iran.

**Methodology.** Sixty-four consecutive non-duplicate clinical isolates collected during 2014–2015 were subjected to susceptibility testing, clonal relationship analysis using PFGE, multilocus variable-number tandem-repeat analysis (MLVA) and multilocus sequence typing (MLST), and examined for the presence of carbapenemases and integrons.

**Results.** Almost all *A. baumannii* isolates were extensively drug-resistant (XDR; 98 %) and carried an OXA carbapenemase gene (*bla*<sub>OXA-23-like</sub>; 98 %) and class 1 integrons (48 %). PFGE and MLST analysis identified three major genotypes, all belonging to clonal complex 92 (CC92): sequence type 848 (ST848) (*n* = 23), ST451 (*n* = 16) and ST195 (*n* = 8). CC92 has previously been documented in the hospital setting in northern Iran, and ST195 has been reported in Arab States of the Persian Gulf. These data suggest national and global transmission of *A. baumannii* CC92.

**Conclusion.** This report demonstrates the occurrence and potential spread of closely related XDR genotypes of *A. baumannii* CC92 within a university hospital in southern Iran. These genotypes were found in the majority of the investigated isolates, showed high prevalence of *bla*<sub>OXA-23</sub> and integron class 1, and were associated with stay in the intensive care unit. Very few treatment options remain for healthcare-adapted XDR *A. baumannii*, and hence effective measures are desperately needed to reduce the spread of these strains and resultant infections in the healthcare setting.

INTRODUCTION

Species of the genus *Acinetobacter*, particularly *Acinetobacter baumannii*, have now emerged as among the most important nosocomial pathogens, being associated with a large spectrum of hospital-acquired infections, such as ventilator-associated pneumonia, surgical wound infections and catheter-related bloodstream infections [1]. *A. baumannii* constitutes an increasing threat to hospitalized patients worldwide due to its high predisposition to nosocomial transmission and failure to respond to antimicrobial treatment due to rapid acquisition of multidrug resistance [2]. This resistance includes non-susceptibility to carbapenems, and hence the bacteria are called carbapenem-resistant *A. baumannii* (CRAB). The appearance of CRAB has substantially reduced the numbers of antibiotics available for treatment, and patients with CRAB infections have a significantly higher risk of mortality [3]. The global occurrence of CRAB is an escalating public health challenge, especially in South and Southeast Asia, where such strains are becoming one of the predominant nosocomial pathogens [4]. Recently, the World Health Organization (WHO) included *A. baumannii* on the list of pathogens that should be given top priority in research and development of new antibiotics, because this species poses a particular threat to patients in the intensive care unit (ICU) setting [5].
Genes mediating antimicrobial resistance are often incorporated into mobile genetic elements and integrons to promote acquisition of resistance genes and dissemination to other bacteria [6–8]. With regard to A. baumannii, CRAB isolates are frequently associated with the presence of OXA-23, OXA-24 and OXA-58 type class D carbapenemases [9]. The OXA-23-like type was the first group of these beta-lactamases to be identified, interestingly coinciding with the introduction of carbapenems in 1985 [10]. Present in transposons, these genes have rapidly spread in successful clonal lineages of A. baumannii and presently represent the predominant mechanism of carbapenem resistance, having a huge impact on the ability to treat A. baumannii infections globally [11]. Characterization of the local molecular epidemiology of A. baumannii strains involved in hospital-associated infections is critical when determining the disease burden and magnitude of nosocomial transmission related to these bacteria. Such information is also essential to enable infection control professionals to develop and balance control strategies aimed at reducing the spread and disease burden of multidrug-resistant (MDR) A. baumannii in the hospital setting. Several valuable techniques have been utilized and have contributed to our understanding of the genotypic characterization of A. baumannii isolates in the healthcare setting: PFGE [9, 12, 13], multilocus sequence typing (MLST) [12, 14, 15] and multilocus variable-number tandem-repeat (VNTR) analysis (MLVA) [13, 16].

Investigations of the molecular epidemiology of clinical isolates of A. baumannii have indicated that the eight international clonal lineages (ICLs) dominate in outbreaks around the globe [12, 17, 18]. The most common lineages (ICL1–3) were initially reported in Europe and the United States, but later also in several countries in other parts of the world [15]. According to studies conducted in several European countries, the majority of CRAB outbreaks in that region have been associated with ICL2 isolates harbouring the oxacillinase blaOXA-23-like carbapenemase gene [2, 12]. Epidemiological investigations have described dissemination of A. baumannii isolates belonging to clonal complex 92 (CC92), corresponding to ICL2, in two hospitals in northern Iran [14, 16]. However, there is no available information indicating such spread in the hospital setting in southern Iran (i.e. 1300 km south-east of the location of the hospitals in the north). Therefore, the aim of the present study was to explore the genetic relatedness and distribution of carbapenemase resistance genes and integrons in A. baumannii isolates at a university hospital in southern Iran.

METHODS

Clinical sample collection and microbial identification

From the beginning of March 2014 to the end of February 2015, 64 consecutive non-duplicate A. baumannii isolates were obtained from various clinical specimens collected from affected patients at the 400-bed university hospital in Bandar Abbas, Iran. All isolates were identified to the species level by matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF MS) assay [19], using a score of ≥2.00 as the criterion for species identification, as stipulated by the manufacturer (Bruker Daltronics).

Antimicrobial susceptibility testing

The disc diffusion method was used for antimicrobial susceptibility testing of amikacin, cefepime, cefotaxime, ceftriaxone, cefotizidime, ciprofloxacin, doxycycline, ertapenem, gentamicin, imipenem, meropenem, piperacillin/tazobactam and ticarcillin according to the Clinical and Laboratory Standard Institute (CLSI) guidelines [20]. Escherichia coli (ATCC 25922) was used as a quality control strain. Susceptibility to colistin and tigecycline was not examined. All isolates were stored at −80 °C in trypticase soy broth containing 30 % (v/v) glycerol until used. The phenotype of A. baumannii was defined as MDR or extensively drug-resistant (XDR) according to the International Expert Proposal for Interim Standards Guidelines [21].

Identification of carbapenemase and integron genes

The blaOXA carbapenemase genes and integrons were detected by use of multiplex PCR as previously described [22, 23].

Multilocus variable-number tandem-repeat analysis

MLVA genotyping was carried out using the method previously developed by Porcel et al. [13], with the following exception: the three VNTR loci consisting of short 6–7 bp repeats (Abau0845, Abau2396 and Abau3468) were not investigated due to lack of access to a high-precision capillary electrophoresis instrument, which is necessary to obtain accurate results. The occurrence of the remaining VNTR loci (Abau0017, Abau0826, Abau1988, Abau2240, Abau3002, Abau3406 and Abau3530) was examined by PCR amplification of genomic DNA using primers targeting 5′ and 3′ ends of VNTR flanking regions. To calculate the number of repeats in each tandem allele, the size of the amplicon was subtracted from the size of the flanking region, and the difference was divided by the length of the repeating unit [13]. The polymorphism index of individual loci or combined VNTR loci was calculated by the Simpson diversity index using an online tool available at www.comparingpartitions.info/. Cluster analysis of the MLVA typing data was done using the MLVA plus online tools software (www.miru-vntrplus.org) [24]. Double locus variation was the maximum locus difference within a CC [25].

Pulsed-field gel electrophoresis

PFGE analysis was performed as previously described [26] but with some modifications. Bacterial DNA was digested with the restriction enzyme Apal (Thermo Scientific), and Salmonella enterica H9812 (control strain) was digested with XbaI (Thermo Scientific). The DNA fragments were separated using a CHEFF DRIII system for 24 h at 14 °C.
with a pulse angle of 120° from 5 to 30 s. Genetic similarity between isolates was calculated using GelCompar II 4.0 (Applied Maths) and the Dice coefficient, and the unweighted pair group method with arithmetic mean (UPGMA) with 1.3 % tolerance and 0.8 % optimization settings. Isolates with more than three-band differences in the PFGE pattern were defined as genetically unrelated. PFGE types were arbitrarily assigned letters (A, B, C, . . . ) and isolates with one- to three-band differences were assigned to PFGE subtypes (letter codes with numbers: A1, A2, A3, . . . ); this corresponded to a similarity coefficient of 90 % in the cluster analysis.

**Multilocus sequence typing**

Selected isolates from each PFGE type that included three or more isolates were further examined using the Oxford MLST scheme [27]. Sequence types (STs) were identified by amplification and sequencing of seven housekeeping genes (gltA, gyrB, gdhB, recA, cpn60, gpi and rpoD) according to conditions outlined at http://pubmlst.org/abaumannii/. CCs were assigned using the BURST algorithm and were defined as single-locus and double-locus variants (SLVs and DLVs) [12].

**RESULTS**

**Antimicrobial resistance in A. baumannii**

Sixty-four consecutive A. baumannii isolates were obtained in the studied hospital, 47 from male patients and 17 from female patients. The 64 isolates originated from the following wards: 49 (77 %) from the ICU, five (8 %) from internal medicine, three (5 %) from orthopaedics, two (3 %) from surgery, two (3 %) from the emergency department, and one (2 %) each from burns unit, cardiac care and thoracic care (Fig. 1). The 64 A. baumannii isolates were recovered from samples collected from trachea (33, 51.5 %), wounds (12, 19 %), sputum (seven, 11 %), urine (five, 8 %), ascites fluid, bronchoalveolar lavage or throat specimens (two, 3 %), and one abscess (1.5 %).

The carbapenemase blaOXA-23-like gene was identified in 98 % (63/64) of the isolates, whereas the blaOXA-24-like gene was present in only 3 % (2/64). One isolate carried both those genes. The blaOXA-58-like gene was not found in this cohort. Class 1 and class 2 integrons were detected in 48 % (31/64) and 6 % (4/64) of the isolates, respectively (Fig. 1). Nearly all of the 64 isolates, 97 % (62/64), exhibited an XDR phenotype, and two isolates were MDR. The lowest nonsusceptibility to antimicrobials among the isolates was noted for amikacin (48/64, 75 %), doxycycline (48/64, 75 %) and gentamicin (57/64, 89 %).

**Genetic relatedness of A. baumannii isolates from southern Iran**

DNA macro-restriction analysis revealed 22 different PFGE types, nine of which were singletons (Fig. 1). The three most prevalent PFGE types with the corresponding ST types were as follows: PFGE type D, ST848; PFGE type I, ST195; and PFGE type J, ST451, representing 16 % (10), 12 % (eight) and 11 % (seven) of the 64 isolates, respectively. MLST analysis identified three major ST types, all belonging to CC92 (corresponding to ICL2): ST848 (23 isolates; PFGE types A–E), ST451 (16 isolates; PFGE types F–K and V), and ST195 (eight isolates; PFGE type I). Thus CC92 comprised 73 % (47/64) of the isolates (Table 1). ST195 and ST451 were identified as SLVs of ST92, and ST848 as an SLV of ST451, which corroborates the results of the PFGE analysis (Fig. 1). Furthermore, two additional STs were identified: ST387 (eight isolates; PFGE types N, Q and R) and ST460 (four isolates; PFGE types S and T).

Among the VNTR loci, Abaum_0017 and Abaum_0826 proved to be the most discriminatory, containing 21 and 20 different alleles, respectively. Abaum_2240 contained only two different alleles and showed the lowest discriminatory power. The number of alleles in the remaining VNTR loci was as follows: Abaum_1988, 15; Abaum_3002, 7; Abaum_3406, 6; and Abaum_3530, 9. The locus Abaum_3406 was absent in 48 % (31) of the 64 isolates. Cluster analysis of the MLVA data revealed nine CCs and 28 singletons (Fig. 1). Agreement between the MLVA and PFGE or MLST typing was very low, with values of <0.1 using the adjusted Wallace coefficient (data not shown).

**DISCUSSION**

The aim of the present study was to examine the genetic relatedness of A. baumannii isolates collected at a university hospital in Bandar Abbas in southern Iran. Our findings showed a high prevalence of A. baumannii isolates that were XRD, which agrees with other investigations conducted in Iran [14, 28]. Resistance to carbapenems was common, and indeed 92 % of the isolates were resistant to all three of the tested carbapenems. In addition, we found a high prevalence (98 %) of the blaOXA-23-like gene across different genotypes. These results are consistent with observations reported from various parts of the world, and they explain the high risk of failure of carbapenem treatment in A. baumannii infections [14, 29–34].

Our study also highlights the significance and severity of extensive drug resistance in A. baumannii, because most of the antimicrobial-resistant isolates in this assessment originated from ICU patients. The presence of integrons is an important virulence factor mediating transmission of antimicrobial resistance genes. The high degree of mobility that characterizes the class 1 integrons is illustrated by our finding that indistinguishable PFGE types varied in their integron content. In short, we found a high prevalence of class I integrons and low prevalence of class 2 integrons in XDR phenotypes, which agrees with data obtained in other investigations [6, 28, 35].

A clear majority of the strains in our study belonged to CC92, corresponding to ICL2. CC92 is highly prevalent throughout Asia, and it is the CC with the widest global distribution [11]. Overall, our findings corroborate previous surveys conducted in Europe, where the majority of outbreaks have been associated with CRAB producing
Fig. 1. Dendrogram cluster analysis of the genetic similarity of 64 A. baumannii isolates using PFGE. The horizontal upper bar indicates genetic similarity (per cent). The dotted lines in the centre of the diagram represent digitalized transformation of the PFGE DNA pattern. The columns to the right present the following: isolate number, PFGE type, MLVA profile, CC based on MLVA profile, ST, integron content and hospital ward where isolates were obtained.
oxacillinase OXA-23 and belonging to ICL2 [12]. Similarly, in a recent study performed at a referral burns centre in Tehran, the majority of CRAB isolates assessed belonged to CC92 and carried class I integrons and OXA-23-like carbapenemase [16]. The most prevalent STs in the present investigation (ST848, ST451, ST195) have not been observed in previous studies at Iranian hospitals [28], whereas other STs belonging to CC92 (e.g. ST75 and ST118, both SLVs of ST92) have been found to be prevalent in the Iranian healthcare setting [16]. These data suggest that highly adapted hospital-associated A. baumannii genotypes belonging to CC92 have spread among Iranian hospitals and evolved locally, resulting in the emergence of separate SLVs or DLVs in CC92 [36]. In agreement with this, ST195 (CC92) with a comparable blaOXA-23-like gene and antimicrobial susceptibility pattern has been described in Arab States of the Persian Gulf [33]. It is possible that spread of that particular strain may be due to the proximity between those countries and individuals travelling to the United Arab Emirates, as well as the annual pilgrimages to Mecca, which represent plausible risk factors for dissemination and outbreaks of antimicrobial-resistant strains [33].

Our study shows that the various ST groups of A. baumannii comprise different MLVA types, as determined by the greater discriminatory power of the MLVA, which corroborates findings reported by other researchers [15, 28, 30–32]. The majority of the six VNTR markers we used in the MLVA have also been applied in epidemiological investigations conducted in other Iranian hospitals [16, 30], whereas no previous reports from Iran have included results obtained for Abaum_0017. Nevertheless, the marked discriminatory power of Abaum_0017 has been acknowledged, but there is also limited agreement with other typing methods, and hence this locus has been considered unsuitable for inclusion in a first-line MLVA scheme. In our study, the Abaum_3406 locus was present in approximately half of the isolates that were assessed, which supports recent data indicating a high degree of variability and polymorphism in Abaum_3406 [37]. Likewise, the poor amplification of this locus implies that it is not consistently present in A. baumannii isolates, suggesting that the distribution of VNTR loci differs between isolates from different geographical regions. The amplicons in the VNTR loci varied considerably in size in our study, and the same variation is apparent when comparing results obtained in other laboratories [16]. These discrepancies may be due to minor differences in the MLVA protocol employed, but may also be explained by the knowledge that individual VNTR loci can evolve rapidly. We used gel electrophoresis in our study, a method that inherently includes a manual reading and is thus associated with greater variation in the results compared to the high precision offered by capillary electrophoresis [38].

The present study has some limitations. First, no clinical data were available for the patients from whom the A. baumannii isolates were obtained. Second, the accuracy of the MLVA would probably have been improved by the utilization of a capillary electrophoresis system.

However, a strength of our investigation is that it is the first to address the molecular epidemiology of A. baumannii in the healthcare setting in southern Iran. Furthermore, we used three different techniques for epidemiological molecular typing of the isolates to facilitate interlaboratory comparison of the genetic relatedness of the isolates.

In conclusion, this study demonstrates the occurrence and potential spread of closely related XDR genotypes of A. baumannii CC92 with a high prevalence of blaOXA-23 and integron class 1 in a university hospital in southern Iran. Considering that very few options remain for treatment of healthcare-adapted A. baumannii, there is an urgent need for effective measures aimed at reducing the spread of these strains and resultant infections in the healthcare setting.

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### Conflicts of interest
The authors declare that there are no conflicts of interest.

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**Table 1. Distribution of 64 non-duplicate clinical isolates of A. baumannii according to CC, ST, MLST profile and PFGE type**

<table>
<thead>
<tr>
<th>CC (n=47)</th>
<th>ST (n)</th>
<th>MLST profile gldA, gyrB, gldH, recA, cpn60, gpi, rpoD</th>
<th>PFGE types (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>92</td>
<td>848 (23)</td>
<td>1, 15, 3, 2, 2, 142, 3</td>
<td>A (3), B (1), C (5), D (10), E (4)</td>
</tr>
<tr>
<td></td>
<td>451 (16)</td>
<td>1, 3, 3, 2, 2, 142, 3</td>
<td>F (3), G (1), H (2), I (7), K (2), V (1)</td>
</tr>
<tr>
<td></td>
<td>195 (8)</td>
<td>1, 3, 3, 2, 2, 96, 3</td>
<td>I (8)</td>
</tr>
<tr>
<td>S*</td>
<td>387 (8)</td>
<td>1, 15, 4, 11, 4, 140, 4</td>
<td>N (4), Q (3), R (1)</td>
</tr>
<tr>
<td>S*</td>
<td>460 (4)</td>
<td>65, 17, 107, 11, 1, 156, 6</td>
<td>S (2), T (2)</td>
</tr>
<tr>
<td>S*</td>
<td>387 (8)</td>
<td>1, 15, 4, 11, 4, 140, 4</td>
<td>N (4), Q (3), R (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>L (1), M (1), O (1), P (1), U (1)</td>
</tr>
</tbody>
</table>

The allelic MLST profile for ST92 (1, 3, 2, 2, 7, 3) is included for comparison.

*S, singleton*
Ethical statement
Hormozgan University of Medical Sciences approved the study (reference number: HUMS.rec.1394.32).

References


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