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Research paper

IS*Aba1*/$bla_{\text{OXA-23-like}}$ family is the predominant cause of carbapenem resistance in *Acinetobacter baumannii* and *Acinetobacter nosocomialis* in Iran

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1. Introduction

Acinetobacter species have become a menace due to hospital-acquired infections. (McConnell et al., 2013) Numerous outbreaks from five continents revealed this pathogen as the fifth problematic organism in healthcare-associated infections. (Vincent et al., 2009) Intrinsically antibiotic resistance genes, dozens of virulence factors and genome plasticity has made such organism that resists under tough conditions, e.g. hospital environments. Infections created by such resistant isolates are difficult to treat with routine antibiotics. (McConnell et al., 2013) Carbapenems, as the last resort of treatment in Acinetobacter infections, are prescribed solely or in combination with other antibacterial agents. (Maragakis and Perl, 2008) However, in the last decade, many outbreaks of carbapenem-resistant Acinetobacter (CRA) were reported around the world. (Poulikakos et al., 2014) It should be noted that most of our knowledge about Acinetobacter epidemiology is related to the most isolated species, *A. baumannii*, and, data about other Acinetobacter species other than *A. baumannii* is limited. (Weber et al., 2016).

Mechanisms involved in resistance to β-lactam antibiotics in CRA spp. were discussed in detail. (Evans and Amyes, 2014) Of the most reported, carbapenem resistance genes have been the production of carbapenem-hydrolyzing class D β-lactamase (bla_{CHDLs}). (Evans and Amyes, 2014) Of the four bla_{CHDL} gene clusters that have been described, $bla_{\text{OXA-23}}$, $bla_{\text{OXA-40}}$ and $bla_{\text{OXA-58}}$-like genes were reported to be acquired, whereas the $bla_{\text{OXA-51}}$ like gene cluster was reported to be intrinsic to Acinetobacter spp.. (Evans and Amyes, 2014) bla_{CHDLs} are weakly carbapenemases, whereas, upstream placement of insertion sequence (IS) elements to bla_{CHDLs}, mainly IS*Aba1*, overexpresses the downstream located bla_{CHDL}. (Jane F. Turton et al., 2006) Moreover, the role of metallo-β-lactamases (bla_{MBLs}) should also be stressed as carbapenemase in CRA isolates, but in less frequency. (Walsh et al., 2005) Other factors involved in carbapenems resistance in CRA isolates were ascribed to altered outer membrane proteins and the intervention of efflux pumps. (Catel-Ferreira et al., 2012).

Reports of CRA isolates from Iran that have been published over the past five years have been mono-centered. (Mohammadpour et al., 2018; Pourhajibagher et al., 2016) The aims of this study were to find not only the dominant reason behind carbapenem resistance in Acinetobacter spp. isolated in this country but also the clonal relationship and distribution of prevalent sequence types (STs) of isolates.

2. Material and methods

2.1. Bacterial isolates

During Oct 2012 to Mar 2013, 166 Acinetobacter spp. were collected for this study from referral centers of the seven provinces located in different parts of Iran, including Kurdistan (n = 30), Hamadan (n = 12), West Azerbaijan (n = 30), Tehran (n = 26), Khorasan Razavi (n = 30), Kerman (n = 13) and Fars (n = 25). Collection of the isolates in the university hospitals assigned to this study is part of normal laboratory procedure. One referral hospital center per province was considered. All of the centers were > 400 beds general hospitals and affiliated to the universities. The patient transfer did not occur frequently among participated centers. One isolate per patient was included and isolates from environmental wards of hospitals were

Abbreviations: CRA, Carbapenem-Resistant Acinetobacter; STs, Sequence Types; MIC, Minimum Inhibitory Concentration; EPIs, Efflux Pump Inhibitors; NMP, 1-(1-Naphthylmethyl)-Piperazine; PAβN, Phenyl-Arginine-β-Naphthylamide; CCCP, Carbonyl Cyanide 3-chlorophenylhydrazone; IS, Insertion Sequence; IC, International Clon; REP-PCR, Repetitive Extragenic Palindromic PCR; UPGMA, Unweighted Pair Group Method with Mathematical Averaging; MLST, Multi-Locus Sequence Typing; SLV, Single Locus Variants; DLV, Double Locus Variants; CC, Clonal Complex; CRAB, Carbapenem Resistant *A. baumannii*; CRAN, Carbapenem Resistant *A. nosocomialis*; MDRAB, Multidrug-Resistant *A. baumannii*; MDRAN, MDR *A. nosocomilais*; PFGE, Pulse Field Gel Electrophoresis

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excluded. In the cases that Acinetobacter bacterium was sequentially isolated from a patient during the hospital stay, the first Acinetobacter isolate was considered for our study. Sites of recovering isolates were respiratory tract ($n = 127$), bloodstream ($n = 22$), urinary tract ($n = 10$), cerebral spinal fluids ($n = 3$) and wounds ($n = 4$) (Supplementary Tables 1 and 2). Isolates were characterized by API20NE (bioMérieux, Marcy l'Etoile, France), the presence of $bla_{\text{OXA-51-like}}$ family (Jane F Turton et al., 2006) and a set of *gyrB* multiplex PCR to the species level detection. (Higgins et al., 2007).

2.2. Antibacterial susceptibility testing

All of the collected isolates were targeted to find susceptibility profile. Susceptibility testing by Kirby–Bauer disk diffusion method was done by Neo-Sensitab disks (Rosco, Taastrup, Denmark). Minimum inhibitory concentration (MIC) of imipenem and meropenem were performed by microdilution method in duplicate repeats. (CLSI, 2012) Results of susceptibility testing and MIC were interpreted according to the CLSI guidelines. (CLSI, 2014) Carbapenem resistant isolates were used to investigate the carbapenem resistance genes.

2.3. Detection of efflux pump phenotype

To find the effect of efflux pumps in carbapenem resistance, efflux pump inhibitors (EPIs), Verapamil, 1-(1-naphthylmethyl)- piperazine (NMP), phenyl-arginine-β-naphthylamide (PAβN), reserpine and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Sigma, St. Louis, MO) were added to the cations adjusted Mueller-Hinton broth medium (Himedia, India) at the final concentration of 100, 70, 50 and 5 μg/mL, respectively. (Deng et al., 2014) The MIC testing was repeated with aforementioned carbapenems. A decrease of at least 4-fold of the MIC of imipenem and meropenem was set as the criteria for phenotypic detection of efflux pumps. (Deng et al., 2014).

2.4. Molecular detection of carbapenemases, IS elements and carO

PCR method was used to detect the bla_{CHDLs} ($bla_{\text{OXA-23, 24/40, 51, 58 and 143}}$-like families), bla_{MBLs} ($bla_{\text{IMP, VIM, GIM, SIM, KPC, NDM, GES}}$) and IS*Aba1* genes, as previously described. (Dallenne et al., 2010; Poirel et al., 2011; Woodford et al., 2006) IS*Aba1,* IS*Aba2*, IS*Aba3*, IS*Aba4* and IS*18* primers were used to detect the presence of insertion sequence (IS) elements upstream or downstream of the bla_{CHDLs}. (Corvec et al., 2007; Segal et al., 2005) Furthermore, to assay the CarO disruption, a channel forming protein to enter imipenem, the complete gene was amplified. (Maria et al., 2005).

2.5. Clonality relationships

A two multiplex PCR-based assays were used to identify three major international clonal (IC) lineages of *A. baumannii* isolates, as described previously. (Turton et al., 2007) Furthermore, repetitive extragenic palindromic PCR (REP-PCR) was performed to evaluate the clonal relatedness of all isolates. (Salimizand et al., 2015) The pictures captured from electrophoresis were embedded in GelJ software (v. 1.3). In order to draw dendrograms unweighted pair group method with mathematical averaging (UPGMA), and Dice coefficient with a 1.5% band tolerance was used. > 95% similarity was considered to find clones. (Meshkat et al., 2017) A representative of each clone was subjected to Multi-locus sequence typing (MLST) to find the related ST. In the cases that two different *A. baumannii* and *A. nosocomialis* species came in the same REP-PCR generated clones, both of which were considered as the unrelated clones and were subjected to MLST typing. Furthermore, the same clones with different antibacterial susceptibility pattern considered to MLST typing. MLST based on Pasteur scheme was done (http://pubmlst.org/abaumannii/) and eBURST (version 3, http://eburst.mlst.net/) was used to find the relatedness of STs. Identical alleles at 6 and 5 of 7 loci were defined for determining single locus and double locus variants (SLV and DLV), respectively. If more than three STs had the same allele numbers in at least six loci, they were considered as a clonal complex (CC). (Diancourt et al., 2010).

3. Results

3.1. Clinical characteristics, susceptibility testing and efflux pump phenotype detection

A total of 166 Acinetobacter spp. isolates were collected during this study. Of which, 122 (73.5%) isolates were *A. baumannii* and the rest was *A. nosocomialis* (44/166, 26.5%). Other species of Acinetobacter genus were not detected (Supplementary Tables 1 and 2). Center from West Azerbaijan was only colonized by *A. baumannii*, whereas, both of aforementioned species were detected in all of the rest of centers.

Susceptibility testing by disk diffusion method showed high rates of resistance to β-lactams, fluoroquinolones and aminoglycosides. Antibacterial susceptibility by Kirby-Bauer testing in *A. baumannii* isolates were imipenem (89.3%), meropenem (87.7%), doripenem (91.8%), ampicillin/sulbactam (95.9%), ceftazidime (99.1%), cefotaxime (99.1%), ceftriaxone (99.1%) and cefepime (99.1%), ciprofloxacin (96.7%), levofloxacin (96.7%), gentamicin (93.4%), tobramycin (90.9%), amikacin (95.9%), tetracycline (92.6%), doxycycline (49.1%), minocycline (33.6%) and trimethoprim-sulfamethoxazole (93.4%) (Supplementary Table 1). Susceptibility testing for *A. nosocomialis* isolates in the same order of antibiotics that listed to *A. baumannii* isolates were 70.4%, 70.4%, 72.7%, 97.7%, 97.7%, 97.7%, 97.7%, 93.2%, 93.2%, 86.4%, 86.4%, 77.3%, 81.8%, 88.6%, 38.6%, 18.2% and 88.6% (Supplementary Table 2). Minocycline was the compound that had the best activity against both species, such that 76.4% of carbapenem resistant *A. baumannii* (CRAB) and 81.8% of carbapenem resistant *A. nosocomialis* (CRAN) were susceptible (Supplementary Tables 1 and 2). The rate of multidrug-resistant *A. baumannii* (MDRAB) and MDR *A. nosocomilais* (MDRAN) were 93% and 78%, respectively (Supplementary Tables 1 and 2).

MIC_{90} for imipenem and meropenem for both of Acinetobacter spp. was 32 μg/mL. MIC experiment revealed that *A. baumannii* isolates were more resistant to carbapenems than *A. nosocomialis* isolates (Table 1). Centers from Tehran and Kurdistan had the most and the least frequency of CRAB isolates, respectively (100% vs 61.9%). Furthermore, CRAN isolates were mainly isolated from three provinces, Tehran, Hamadan and Khorasan Razavi. Obviously, the frequency of CRAB isolates was more than CRAN isolates (90.1% vs 68.1%).

As the MIC for these two antibacterials did not decrease by more than four-fold, it can be deduced that carbapenem resistance was not affected by efflux pumps in our clinical isolates.

3.2. Molecular detection of carbapenemases, IS elements and carO gene

Among acquired bla_{MBLs} that have been investigated in this study, bla_{VIM} and bla_{GES} types were detected in four (1.7%) and six (2.5%) isolates from Khorasan Razavi and Fars, respectively (Supplementary Table 3). $bla_{\text{NDM, KPC, SIM, GIM, IMP and SPM}}$ were not found in any center. bla_{CHDLs} including $bla_{\text{OXA-51}}$, $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24/40}}$ and $bla_{\text{OXA-58}}$ were identified in 122 (100%), 111 (90.9%), 12 (9.8%) and two (1.6%) of *A. baumannii* and 44 (100%), 36 (81.8%), 6 (13.6%) and none of *A. nosocomialis* isolates, respectively. $bla_{\text{OXA-143}}$ was not found in any isolate.

IS*Aba1* was found in 121 (99.2%) *A. baumannii* as well as 40 (90.9%) *A. nosocomialis* isolates, and, the only $bla_{\text{OXA-23}}$ was found to be upstream adjacent to this element in both species. The IS*Aba1*/$bla_{\text{OXA-23}}$ arrangement was in 126 (87.5%) and 32 (72.7%) of *A. baumannii* and *A. nosocomialis* isolates, respectively (Supplementary Table 3). In the CRAB and CRAN isolates that IS*Aba1*/$bla_{\text{OXA-23}}$ was not detected, IS*Aba2*, IS*Aba3*, IS*Aba4* and IS18 were sought to find the adjacent placement to $bla_{\text{OXA-51}}$, $bla_{\text{OXA-24/40}}$ and $bla_{\text{OXA-58}}$, but, none of them

Table 1
Critical characteristics and MIC of imipenem and meropenem for *A. baumannii* and *A. nosocomialis* isolates (%).

Acinetobacter spp.	*N* = 166	IC1	IC2	UN IC	STs	$bla_{\text{OXA-like}}$ families			MBLs
						51	23	40	58	
A.baumannii	122	15 (12.3)	76 (62.3)	31 (25.5)	1, 2, 85, 94, 328, 570, 599, 642, 724, 1085, 1086	122 (100)	111 (90.9)	12 (9.8)	2 (1.6)	bla_{VIM}, bla_{GES}
A.nosocomialis	44	3 (6.8)	10 (22.7)	31 (70.5)	2, 25, 85, 113, 307, 570, 589, 642, 708, 734, 1085, 1086, 1087	44 (100)	36 (81.8)	6 (13.6)	0	bla_{GES}

Acinetobacter spp.	ISA*ba1*	IS*Aba1*/$bla_{\text{OXA-23}}$	Resistance profile							
			IMI				MEM			
			MIC_{90} (μgr/mL)	MIC ≤ 2	MIC = 4	MIC ≥ 8	MIC_{90} (μgr/mL)	MIC ≤ 2	MIC = 4	MIC ≥ 8
A.baumannii	111 (99.2)	106 (86.8)	32	10 (8.2)	2 (1.7)	110 (90.1)	32	11 (9)	4 (3.3)	107 (87.7)
A.nosocomialis	40 (90.9)	32 (72.7)	32	11 (25)	2 (4.5)	31 (70.4)	32	11 (25)	2 (4.5)	31 (70.4)

Abbreviation: IC, international clone; UN, Unknown; STs, sequence types; MBLs, metallo-β-lactamases; MIC, minimum inhibitory concentration; IMI, imipenem; MEM, meropenem.

were associated with investigated IS elements.

All of the isolates showed expected PCR product length of *carO* gene (885 bp).

3.3. Clonality relationships

IC multiplex revealed that 75 (61.5%) *A. baumannii* isolates belonged to IC2, while IC1 encompassed 15 *A. baumannii* isolates (12.3%). Distribution of clonal lineages was not related to a specific geographical region. But, in Kerman, Tehran and West Azerbaijan centers, IC2 lineage was more prevalent (61.5%, 80.8% and 86.7% respectively).

The REP-PCR result showed a highly divergent genomic content of *A. baumannii* and *A. nosocomialis* from different centers (Supplementary Figs. 1–7). According to the REP-PCR generated dendrogram of isolates which were bla_{GES} producer, four of isolates were clonally related. SHR-22, SHR-24, SHR-25 and SHR-58 were the strains with clonal relationship (Supplementary Table 3 and Supplementary Fig. 5).

MLST technique showed that our collection was composed of 18 STs. New alleles of *rpoB* (allele 146) and *cpn60* (allele 163) were discovered, both of which in *A. nosocomialis*, from Khorasan Razavi and Kurdistan provinces, respectively. Furthermore, three new sequence types including, ST1085 (3,3,2,1,7,4,4), ST1086 (56,3,55,2,9,1146) and ST1087 (163,3,2,2,51,1,4) were discovered. It should be noted that ST2 was the prominent ST that distributed in all studied provinces (Fig. 1). Most of the ST2 isolates belonged to IC2. Other SLVs of ST2 were ST570 and ST724. It should be noted that some STs were found in both species (e.g. ST2) while others were ascribed to specific species (Table 1).

Three clonal complexes (CCs) were found (Fig. 2). CC2 with 114 isolates contained three STs (ST2, ST570 and ST724) that ST2 found to be predicted founder. CC642 comprising 23 isolates in four STs (ST1, ST642, ST734 and ST589) that ST642 was the predicted founder. CC25 included six isolates composed of two STs (ST307 and ST25) without the predicted founder.

4. Discussion

This study was proposed to find the genetic context of Acinetobacter isolated from clinical specimens from seven disparate provinces of Iran.

Resistance to carbapenems was sought in all isolates. bla_{MBL} genes were limited to a small population of isolates restricted to two provinces, bla_{GES} for Fars and bla_{VIM} for Khorasan Razavi. These genes were previously reported from other centers and neighborhood countries as well. (Castanheira et al., 2014; Pourabbas et al., 2016; Sohrabi et al., 2012; Soleimanpour et al., 2015; Zowawi et al., 2015).

Interestingly, bla_{OXA}-types have followed a geographical pattern. $bla_{\text{OXA-58}}$ was only found in two (1.2%) isolates in the center located in West Azerbaijan province, North-west of Iran. This province is the neighboring of Turkey, a country with a high rate of $bla_{\text{OXA-58}}$, but low frequency of $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-24/40}}$. (Kulah et al., 2010) However, this type of oxacillinase was reported previously in an isolate from Tehran (Karmostaji et al., 2013), and it can be deduced that $bla_{\text{OXA-58}}$ spread sporadically. $bla_{\text{OXA-24/40}}$ was identified in six out of seven provinces with a higher frequency in the North-east of Iran. $bla_{\text{OXA-23}}$ was the most prevalent oxacillinase found in all centers (147, 88.5%), as endemic to the country. These results are in accordance with reports by the other investigators from different centers in Iran. (Karmostaji et al., 2013; Mohajeri et al., 2013; Shoja et al., 2013) In comparison to other countries in this region, it can be concluded that the Middle-East is blended by various bla_{OXA}-families. (Jane F. Turton et al., 2006).

It was identified that resistance to carbapenems was associated with $bla_{\text{OXA-23}}$ proceeded by IS*Aba1*, but not $bla_{\text{OXA-51, 23/40}}$ or $bla_{\text{OXA-58}}$. In previous works by Farsiani et al., Salimizand et al. and Peymani et al. IS*Aba1*/$bla_{\text{OXA-23}}$ genetic arrangements were reported from north-east and north-west of Iran, respectively. (Peymani et al., 2012; Salimizand et al., 2015; Soleimanpour et al., 2015) But, wide dissemination of this

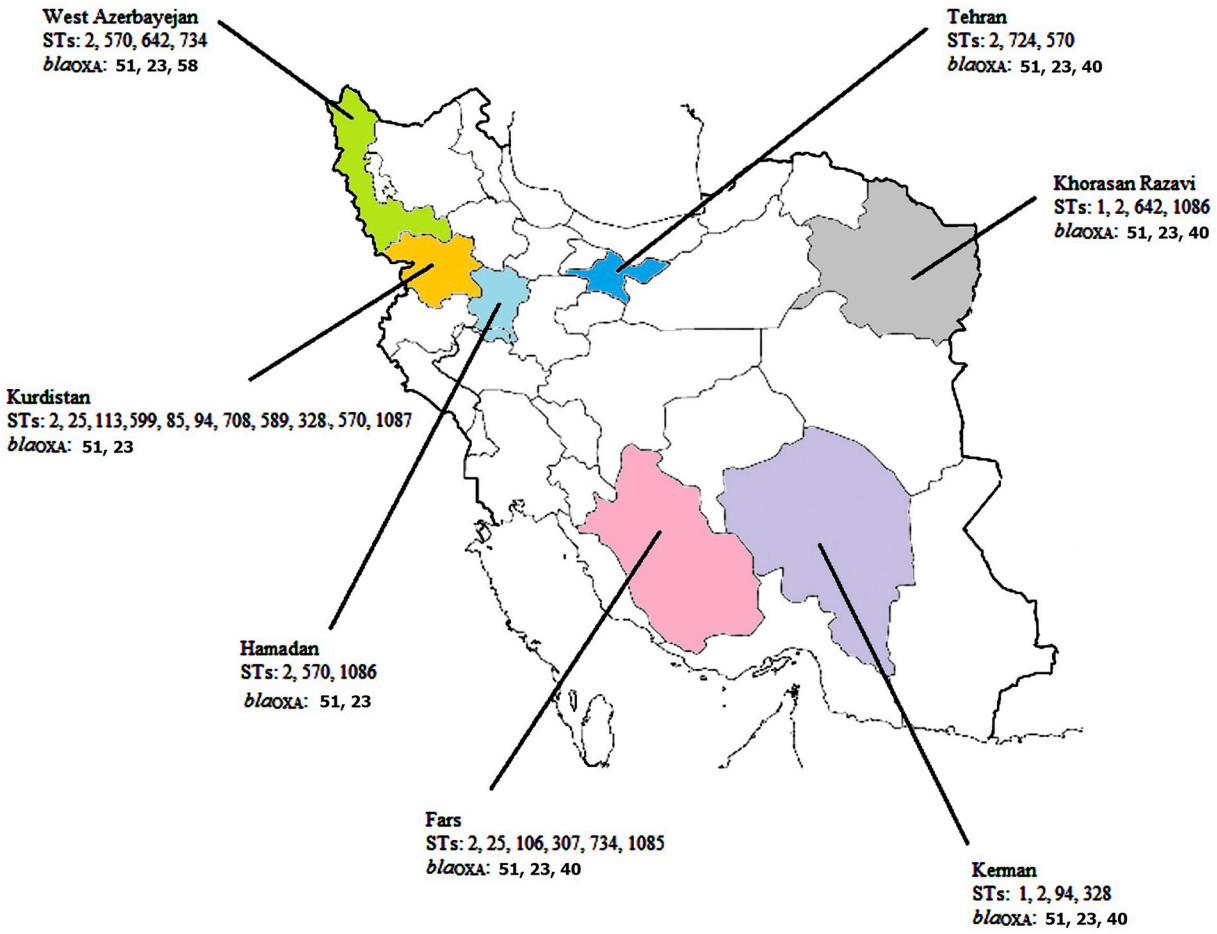

Fig. 1. Distribution of the STs and $bla_{\text{OXA-23-like}}$ families among participated provinces in Iran.

structure in this geographical area is reported in detail in this study. Worldwide dissemination of IS*Aba1*/$bla_{\text{OXA-23}}$ was frequently reported globally. (Higgins et al., 2010) Stable association of IS*Aba1* with $bla_{\text{OXA-23}}$-like, and not with other bla_{OXA}-like families in our isolates, shows that possessing only one IS*Aba1*/bla_{OXA}-type is enough to overcome antibiotic pressure by carbapenems, and, suggesting no needs to gain or evolve the other IS*Aba1*/bla_{OXA}-family forms. (Jane F. Turton et al., 2006) The role of other IS elements in *A. baumannii* for carbapenem resistance should be highlighted. (Evans and Amyes, 2014) However, upstream located IS*Aba1* does not always guarantee resistance to carbapenems and these constructions may be non-functional. (Higgins et al., 2012).

The phenotype of efflux pumps was not affected by EPIs. Hence, carbapenem resistance could be assigned to the IS*Aba1*/$bla_{\text{OXA-23}}$-like family in Iran. However, Mirshekar et al. reported disruptions in *carO* and *dacD* genes in a limited number of their isolates that were disrupted by IS*Aba1* and IS*Aba125*, respectively. (Mirshekar et al., 2018) The two other carbapenem outer membrane transporters, Omp33 and OprD, were not evaluated, which were the two limitations of this work.

REP-PCR results showed divergent clusters of Acinetobacter spp. This technique has been shown to be as powerful as pulse field gel electrophoresis (PFGE) to discriminate *Acinetobacter baumannii* isolates. (Bou et al., 2000) In terms of national distribution of clonal lineages, 73.8% (90 out of 122) of *A. baumannii* isolates belonged to IC1 and 2 in our centers, which shows a wide distribution of these two successful clones. These results are in accordance with previous reports from Iran. (Hojabri et al., 2014; Peymani et al., 2012) According to *A. baumannii* international clonal lineage typing, about half of our collection was IC2. This clone has frequently reported from most regions of the world, as well as Iran. (Farshadzadeh et al., 2015; Hojabri et al., 2014; Pournaras et al., 2014) MLST typing revealed IC2 isolates as ST2 and its SLVs, ST570 and ST724, founded CC2 (CC92 oxford scheme (Tomaschek et al., 2016)). This CC was isolated from different provinces (Farshadzadeh et al., 2015; Saffari et al., 2017), neighbor joining and Middle-east countries. (Al Atrouni et al., 2016; Ganjo et al., 2016; Khurshid et al., 2017; Saffari et al., 2017) Widespread geographical distribution of ST2 supporting this ST as a successful clone that spreads globally. *A. nosocomialis* isolates were also considered for MLST typing. Although most of them were ST2, however, it does not mean that they had a correlation with *A. baumannii* ST2 isolates. By using eBURST analysis of *A. baumannii* and *A. nosocomialis* together, as expected, separation of the species was not possible. Some of the other investigators addressed such experience. (Martins et al., 2013; Park et al., 2018; Wisplinghoff et al., 2018).

An interesting finding of our results was the epidemiology of Acinetobacter species. During six months of study from different centers, we found only *A. baumannii* and *A. nosocomialis* species. It may be the outcome of the detection method of Acinetobacter spp. that was

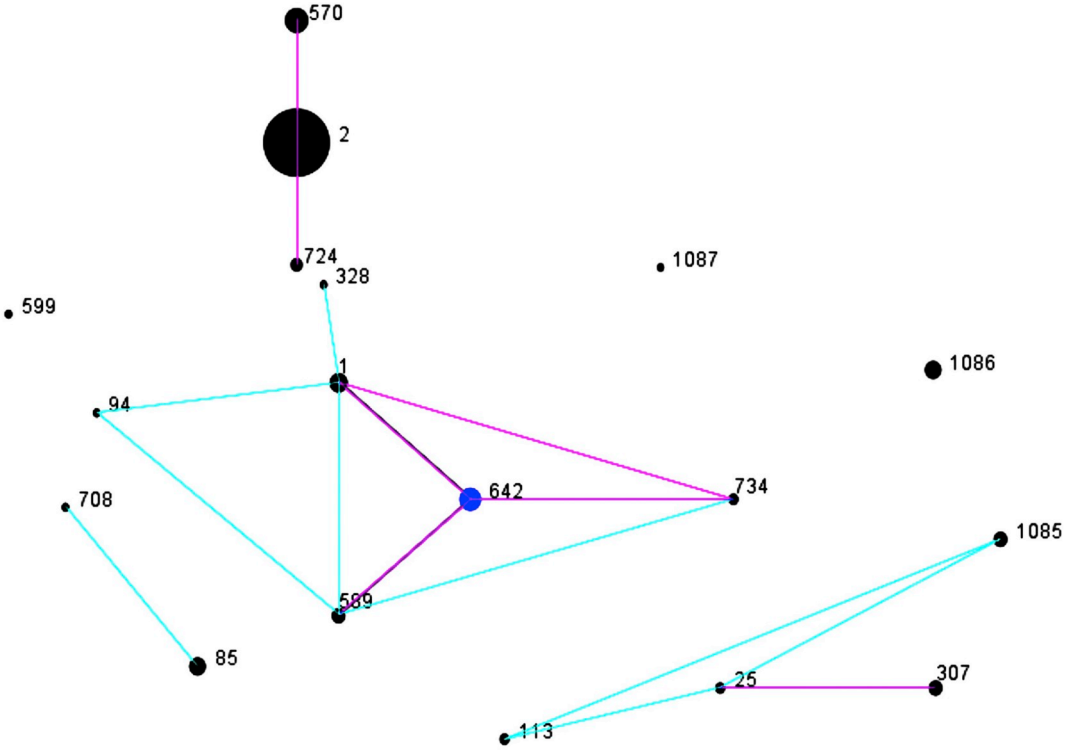

Fig. 2. eBURST generated diagram of 122 *A. baumannii* isolates from seven provinces of Iran. The pink line shows the SLVs and the green shows the DLVs. The size of each circle represents the number of STs in each clonal complex. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

based on *gyrB* multiplex PCR. Based on this method four Acinetobacter species, *A. baumannii*, *A. calcoaceticus*, *A. pittii* and *A. nosocomialis*, can be detected. But, however, we only encountered *A. baumannii* and *A. nosocomialis* species. The *rpoB* sequence-based method effectively discriminates all Acinetobacter species.

Acinetobacter species epidemiology varies greatly from reports around the world. Van den Broek et al. reported different Acinetobacter species with *A. baumannii* as the most prevalent species followed by *A. pittii, A. lwoffii, A. ursingii, A. johnsonii*, *A. junii* and *A. nosocomialis*, respectively (van den Broek et al., 2009). Other European studies revealed the low frequency of *A. nosocomialis*, while East of Asia reports signified higher prevalence of *A. nosocomialis* (Carretto et al., 2011; Park et al., 2018; van den Broek et al., 2009). Finally, a published paper by Pourabbas et al. from south of Iran enunciated that *A. baumannii* accounted for 93.5%, *A. nosocomialis* 5.2% and *A. junii* for 1.3% (Pourabbas et al., 2016).

5. Conclusion

In summary, this is the most comprehensive report of the molecular epidemiology of Acinetobacter spp. from seven Iranian provinces to find the main reason of carbapenem resistance. The ST2 and its SLVs are in circulation in all centers. Surveillance programs and controlled antibiotic regimens should be regarded to limit spreading CRA isolates. Further investigations will be required to identify transposons that carry IS*Aba1*/$bla_{\text{OXA-23}}$, and determine their location in either plasmids or chromosomes.

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Author contributions

Data curation: HS, Y.
Formal analysis: HS, ZM.
Investigation: HS, YA, ZM.
Methodology: HS, YA, ZM.
Project administration: HS, YA, ZM, HS.
Resources: ZM, HS.
Supervision: HS, ZM.
Validation: ZM, HS.
Visualization: HS, YA, ZM, HS.
Writing – original draft: HS.
Writing – review & editing: HS, YA, ZM, HS.

Disclosure

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