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RESEARCH ARTICLE

Emergence of colistin resistant *Acinetobacter baumannii* clonal complex 2 (CC2) among hospitalized patients in Iran

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ABSTRACT

Acinetobacter baumannii is a major causative agent of serious nosocomial infections. This study was carried out to investigate the molecular characterization of colistin resistant isolates of *A. baumannii* from hospitalized patients, based on multilocus sequence typing (MLST). A cross-sectional study was conducted to collect *A. baumannii* from clinical samples in Isfahan from 2021 to 2022. Isolates were identified as *A. baumannii* using biochemical tests and PCR of $bla_{\text{OXA-51}}$. Antibiotic susceptibility testing was carried out using the Kirby-Bauer method and minimum inhibitory concentration (MIC) values were determined for colistin. Additionally, MLST was performed according to the Pasteur scheme to assess the relationship between colistin resistant *A. baumannii*. A total of 70 non-repetitive *A. baumannii* isolates were obtained from different clinical samples. MIC results showed that seven *A. baumannii* isolates were resistant to colistin. The antibiotic susceptibility pattern revealed that all seven colistin resistant strains were resistant to all tested antibiotics. Based on MLST analysis, the colistin resistant isolates were assigned to five unique STs namely, ST2 (3; 42.9%) followed by ST78 (1; 14.3%), ST1077 (1; 14.3%), ST415 (1; 14.3%) and ST391 (1; 14.3%). Among them ST2, ST391 and ST415 belong to clonal complex 2. Colistin resistant *A. baumannii* ST2 is the main circulating clone in clinical settings in Iran, but additionally ST415, ST391, and ST1077 are found for the first time in our country. Intensive control procedures and strict adherence to surveillance programs are recommended to decrease the spread of carbapenem and colistin resistant *A. baumannii* strain.

KEYWORDS

Acinetobacter baumannii, colistin-resistant, multi-drug resistance, MLST

INTRODUCTION

Acinetobacter baumannii is a non-motile, oxidase-negative, catalase-positive, and non-fastidious, strictly aerobic, gram-negative coccobacillus. It might be a part of healthy people's pharyngeal and skin flora [1]. The ability of this organism to endure drying and resist numerous antibiotics are crucial factors in its characteristic to survive and proliferate in the hospital setting [2, 3]. As one of the pioneers in the challenge of antibiotic resistance, *A. baumannii* is regarded as a significant causative agent of nosocomial infections [4]. Patients hospitalized in the intensive care unit (ICU) are more likely to acquire nosocomial infections due to their extended stays, the use of invasive central venous or arterial lines, and urine catheterization [5]. Five hundred deaths are reportedly linked to *A. baumannii* infections annually in the USA, where 12,000 infections are estimated [6].

Resistance to three or more antibiotics from the carbapenem, aminoglycoside, third-generation cephalosporin, and fluoroquinolone groups is called multidrug-resistant (MDR).

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As a common MDR bacterium in the ICU and burn units, it has become an issue in hospitals [7].

Polymyxins are cyclic peptide-containing antibiotics with a generic structure that consists of five chemically distinct molecules (A–E). Gram-negative bacterial infections are treated with polymyxins B and E (colistin) [8]. Since the emergence of carbapenem-resistant *A. baumannii* (CRAB) isolates in recent years, colistin has become an important treatment option for a variety of illnesses [9]. Especially when it comes to *A. baumannii*, colistin shows great activity against gram-negative bacteria that are multidrug-resistant and extensively drug-resistant (XDR). However, due to colistin's severe neurotoxicity and nephrotoxicity, its use has been restricted [10]. Colistin works by adhering to lipopolysaccharide found in gram-negative bacteria's outer membrane. Although colistin resistance mechanisms are not fully known, adaptability and mutation are the two main functions of its resistance in bacteria [11]. According to a number of surveys, it is believed that more than half of all antibiotics recommended by specialists are unnecessary [12]. Additionally, inappropriate colistin prescription and usage, increase number of colistin resistant gram-negative bacteria [7, 13].

When estimating the disease burden and scope of nosocomial transmission associated with such bacteria, the molecular epidemiology of *A. baumannii* strains involved in hospital-related infections must be characterized [14]. The ability to establish and balance control methods to minimize the spread and illness burden of MDR *A. baumannii* in the hospital context is also made possible by such knowledge, which is crucial for infection control specialists. The genotypic characterization of *A. baumannii* isolates in the hospital setting has been studied using a number of useful approaches, including pulsed-field gelelectrophoresis (PFGE), multilocus sequence typing (MLST), and multilocus variable-number tandem-repeat (VNTR) analysis (MLVA) [15]. MLST is an effective method for analyzing the *A. baumannii* global epidemiology. The conserved areas of seven housekeeping genes have served as the foundation for this. The excellent reproducibility, portability, which enables cross-country comparisons, and simplicity of data interpretation in evolutionary terms are some benefits of MLST [16]. However, there is no available information indicating such a spread in the hospital setting in Iran. Therefore, this study is carried out to investigate the molecular characterization of colistin resistant isolates of *A. baumannii* from patients hospitalized in ICU based on MLST.

MATERIAL AND METHODS

A cross-sectional study, 70 clinical samples collected from hospitalize patients in Health center in Isfahan from 2021 to 2022. Initial identification was performed by culture and biochemical tests and then final confirmation were conducted using PCR of the *bla*OXA-51 gene which is inherent in *A. baumannii*. The *A. baumannii* strain ATCC19606 was used as the reference strain.

ANTIMICROBIAL SUSCEPTIBILITY TESTING AND PHENOTYPIC ASSAYS

To determine the antibiotic susceptibility profile of *A. baumannii* isolates, according to CLSI 2021 guidelines. Antibiotic susceptibility testing was performed using Kirby-Bauer method: gentamicin (10 mg), meropenem (10 mg), cefepime (30 mg), ceftazidime (30 mg), ceftriaxone (30 mg), piperacillin-tazobactam (100/10 mg), gentamicin (10 mg), amikacin (30 mg), ampicillin-sulbactam (10 mg), ciprofloxacin (5 mg), and trimethoprim-sulfamethoxazole (30 mg) disks (MAST, Merseyside, UK). Minimum inhibitory concentrations (MICs) of colistin were determined by broth microdilution according to the CLSI 2021. *Escherichia coli* ATCC 25922 were used as the control strain [17].

PHYLOGENETIC ANALYSIS AND MULTILOCUS SEQUENCE TYPING (MLST)

The Pasteur scheme was utilized for MLST, as previously mentioned [18]. Using the ABI 3730xl DNA Analyzer, internal segments of the seven housekeeping genes *fusA*, *gltA*, *pyrG*, *recA*, *cpn60*, *rplB*, and *rpoB* were amplified, purified, and sequenced (Life Technologies). http://pubmlst.org/abaumannii/ contains a description of the PCR conditions and primers. The allelic number and sequence types (STs) of each strain were determined by comparing the nucleotide sequences to the *A. baumannii* MLST database. Oligonucleotid primers for MLST are shown in Table 1.

RESULTS

A total of 70 non-repetitive *A. baumannii* isolates were obtained from different clinical sample of hospitalized patients. MIC results showed that seven *A. baumannii* isolates were resistant against colistin. Colistin resistant strain was isolated from urine, blood and wound samples in 57.1%, 28.6% and 14.3%, respectively. Antibiotic susceptibility pattern revealed that all seven colistin resistant strains were resistant to all tested antibiotics.

MLST analysis of colistin resistant isolates identified that the seven isolates were assigned to five unique STs, the most common were ST2 (three isolates; 42.9%) followed by, ST78 (one isolate; 14.3%), ST1077 (one isolate; 14.3%), ST415 (one isolate; 14.3%) and ST391 (one isolate; 14.3%).

Overall, using the goeBURST algorithm, altogether 3 STs were belonging to one clonal complex (CC) namely, CC2 (ST2, ST391 and ST415) and remaining 2 STs were classified as singleton. The distribution of STs and characteristics of the seven colistin resistant isolates are shown in Table 2, Figs 1 and 2. Moreover, phylogenic analysis of our STs and 500 STs of database are shown in Fig. 3.

Table 1. Primers used for MLST of *A. baumannii* complex (Pasteur scheme)

Genes	Oligonucleotid primers	Amplicon size	Annealing temperature	Reference
cpn-60	F: ACTGTACTTGCTCAAGC R: TTCAGCGATGATAAGAAGTGG	405bp	50 °C	[18]
fusA	F: ATCGGTATTTCTGCKCACATYGAT R: CCAACATACKYTGWACACCTTTGTT	633bp	50 °C	
gltA	F: AATTTACAGTGGCACATTAGGTCCC R: GCAGAGATACCAGCAGAGATACACG	483bp	50 °C	
pyrG	F: GGTGTTGTTTCATCACTAGGWAAAGG R: ATAAATGGTAAAGAYTCGATRTCACCMA	297bp	50 °C	
recA	F: CCTGAATCTTCYGGTAAAAC R: GTTTCTGGGCTGCCAAACATTAC	372bp	50 °C	
Rplb	F: GTAGAGCGTATTGAATACGATCCTAACC R: CACCACCACCRTGYGGGTGATC	330bp	50 °C	
rpoB	F: GGCGAAATGGC(AGT)GA(AG)AACCA R: GA(AG)TC(CT)TCGAAGTTGTAACC	456bp	50 °C	

F: Forward; R: Reverse.

Table 2. Distribution of 7 colistin resistant *A. baumannii* isolates according to MLST profile, clinical sample and antibiotic resistant pattern

Isolate	Allelic profile (*cpn-60, fusA, gltA, pyrG, recA, rplb, rpoB*)	ST	CC		Antibiotic resistant pattern
#238	25-3-6-2-28-1-29	78	–	Urine	SXT, CIP, TET, AN, GEN, CO, MEM, IMI, CTZ, PIP, SAM, CFP
#211	25-3-6-2 -28-1-2	1077	–	Urine	SXT, CIP, TET, AN, GEN, CO, MEM, IMI, CTZ, PIP, SAM, CFP
#204	2-2-2-2-68-2-2	415	CC2	Blood	SXT, CIP, TET, AN, GEN, CO, MEM, IMI, CTZ, PIP, SAM, CFP
#200	2-2-2-2-2-2-2	2	CC2	Blood	SXT, CIP, TET, AN, GEN, CO, MEM, IMI, CTZ, PIP, SAM, CFP
#165	2-2-2-2-2-2-2	2	CC2	Urine	SXT, CIP, TET, AN, GEN, CO, MEM, IMI, CTZ, PIP, SAM, CFP
#173	2-2-2-2-2-2-2	2	CC2	Urine	SXT, CIP, TET, AN, GEN, CO, MEM, IMI, CTZ, PIP, SAM, CFP
T	2-2-58-2-2-2-2	391	CC2	Wound	SXT, CIP, TET, AN, GEN, CO, MEM, IMI, CTZ, PIP, SAM, CFP

SXT: Trimethoprim-sulfamethoxazole; CIP: Ciprofloxacin; TET: Tetracycline; AN: Amikacin; GEN: Gentamicin; CO: Colistin; MEM: Meropenem; IMI: Imipenem; CTZ: Ceftazidime; PIP: Piperacillin; SAM: Ampicillin-sulbactam; CFP: Cefepime; CC: Clonal Complex; ST: Sequence type.

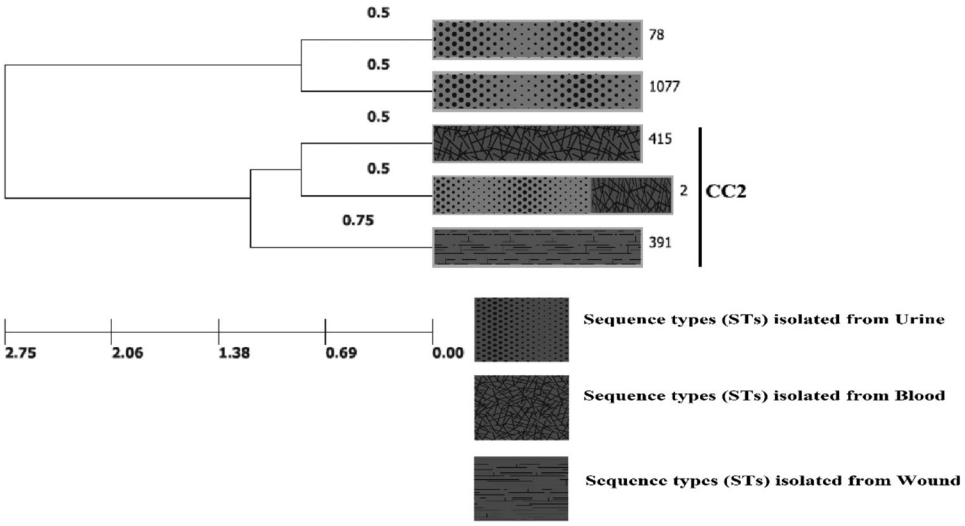

Fig. 1. Phylogenetic tree of colistin -resistant *Acinetobacter baumannii* strain; ST78, ST1077, ST2 (two isolates) from Urine; ST415 and ST2 from blood; ST391 from wound

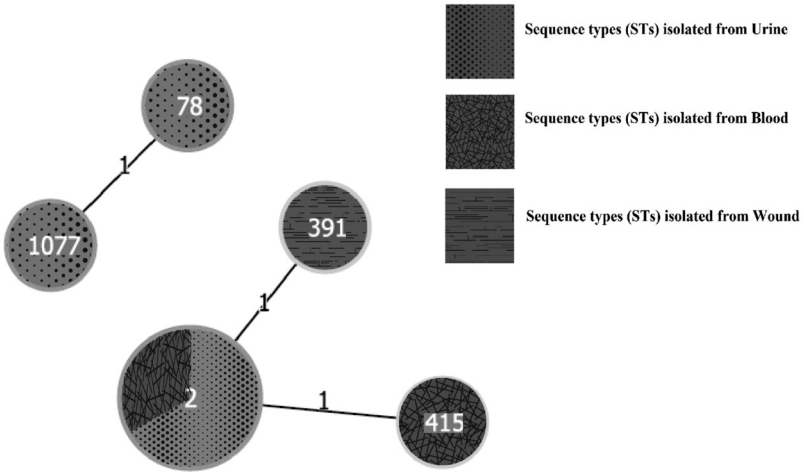

Fig. 2. Multilocus sequence typing (MLST) data set generated using PHYLOViZ, indicating the sequence types (STs) of colistin -resistant *Acinetobacter baumannii*; ST78, ST1077, ST2 (two isolates) from Urine; ST415 and ST2 from blood; ST391 from wound

DISCUSSION

Acinetobacter species, especially *A. baumannii*, have been identified as one of the most common gram-negative bacteria that cause hospital outbreaks, especially in immunocompromised patients in intensive care units [1, 19]. Because of the spread of MDR international clones in various countries, including Iran, *A. baumannii* has become a global health crisis [20, 21].

Recently, in some countries, colistin resistance in *A. baumannii* clinical isolates has been reported, and its incidence has continued to rise, posing a serious issue for public health systems and infection control [13]. Since currently colistin is the most active therapeutic alternative, the emergence of colistin resistant *A. baumannii* in Iran is troublesome [11, 22]. There are very limited therapeutic options available for colistin resistant infections [23].

According to the present research, seven colistin resistant *A. baumannii* isolates demonstrated a high level of coresistance to other antimicrobial agents such as trimethoprim-sulfamethoxazole, ciprofloxacin, tetracycline, amikacin, gentamicin, meropenem, imipenem, ceftazidime, piperacillin, ampicillin-sulbactam, and cefepime as reported previously [20, 24].

Accordingly, high resistance to carbapenems (imipenem and meropenem 100% (7/7)) was observed. Since carbapenem is a last-line antibiotic, high resistance rates can be regarded as a warning about multidrug-resistant *A. baumannii*, causing difficult-to-treat infections [21].

The fast spread of *A. baumannii* clinical isolates from effective clones with multi-drug and extensively-drug resistance patterns during various hospital outbreaks is a serious trend [25]. PFGE and MLST are typically employed to determine clonal relatedness amongst *A. baumannii* isolates; these approaches have suitable discriminatory power and are considered gold standard procedures [26, 27]. The biological properties of *A. baumannii* clonal groups, including virulence and drug resistance, often differ [28]. Due to clonal spread, it is necessary to identify potential reservoirs and transmission modes [29, 30].

The clonal distribution information is not complete, and there is little data published on the STs of Iran isolates. This research sheds light on the molecular epidemiology of colistin-resistant *A. baumannii* in Iran.

According to the MLST analysis, 42.8% of the colistin-resistant *A. baumannii* isolates were assigned to ST2, while one isolate was attributed to ST415 (SLV of ST2), ST391, ST78, and ST1077 (SLV of ST78). Based on the clusters identified in the phylogenetic tree, MLST analysis revealed a low level of genetic variation within the examined *A. baumannii* strains. Additionally, the findings shed light on the circulating colistin-resistant clones in the country.

The ST2 clone, constituting the major ST in the CC2 or international clone two, has also been defined as the most prevalent ST in a number of countries, such as Italy, Greece, Turkey, Lebanon, and Algeria, as shown in Iranian data [20, 31–35].

Indeed, even if various ST clones are defined in Iran, the ST2 clone seems to be the major ST found recently by Piran et al., Hojabri et al., and Rezaei et al. Further, ST2 has been broadly distributed in clinical settings among Iranian patients, which accounts for about 62%–90% of isolates [20, 26, 35].

Therefore, contrary to previous studies [26], in which CC92 represented the recently distributed clone in Iran, the present research showed that effective clone CC2, recovered from urine and blood samples, accounted for most nosocomial outbreaks of *A. baumannii* infections; it was related to MLST clonal complexes CC2/CC92 (Oxford/Pasteur).

To date, according to previous studies in Iran, different STs, including ST118, ST95, ST75, ST405, ST231, ST788, ST225, ST 227, ST133, ST669, ST102, ST404, ST787, ST138,

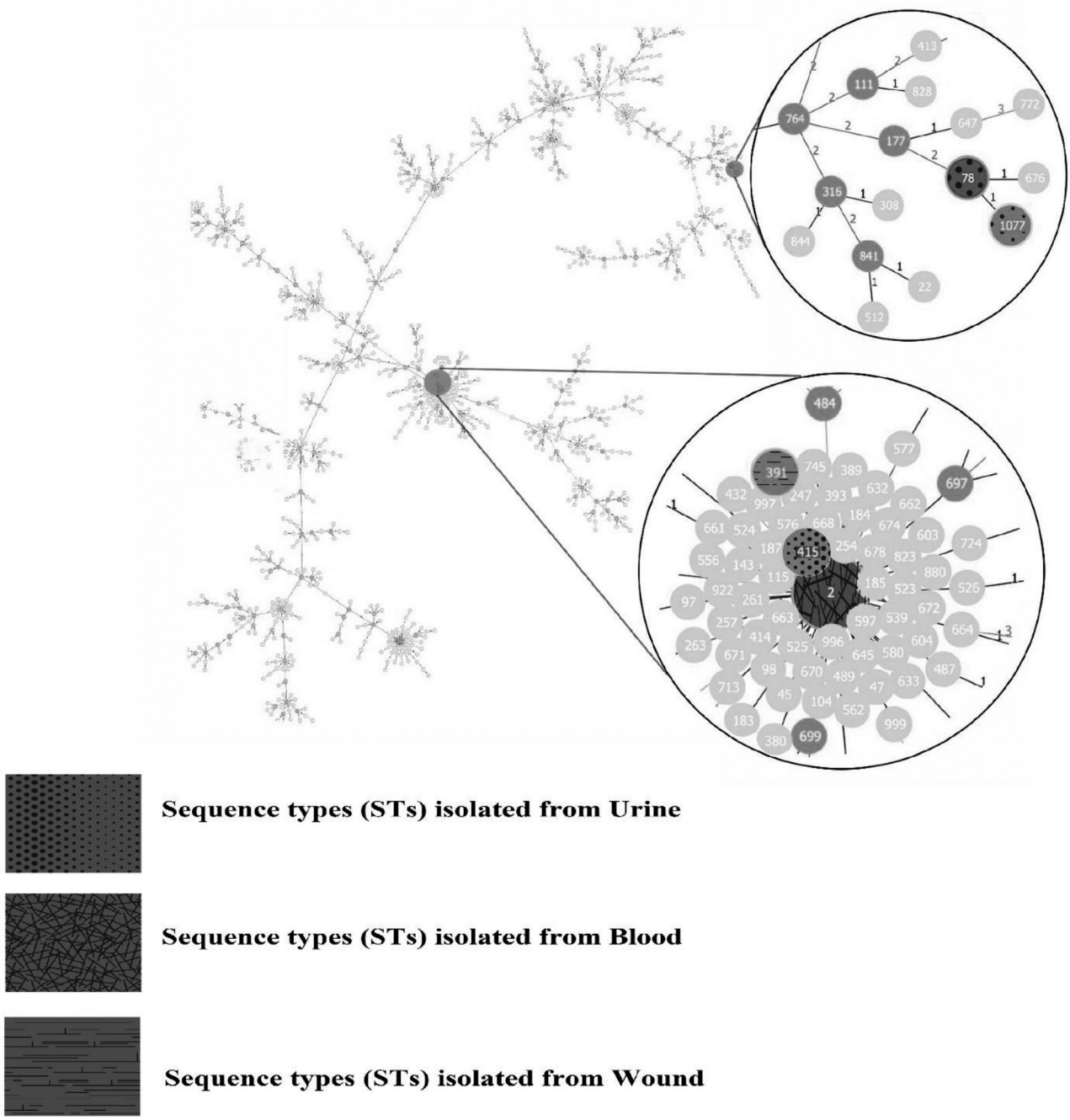

Fig. 3. Minimum Spanning Tree of 500 ST of the database and five identified ST generated by PHYLOViZ based on using the goeBURST full MST algorithm; ST78, ST1077, ST2 (two isolates) from Urine; ST415 and ST2 from blood; ST391 from wound

ST368, ST512, ST801, ST546, ST245, ST137, ST99, ST197, ST258, ST246 [36], ST848, ST451, ST195, ST387, ST460 [37], ST2, ST25, ST85, ST94, ST136, ST307, ST323 to ST328 [26], ST328, ST323, ST2, ST78, ST154, ST625, ST1035 [20], ST2, and ST307 [38], have been observed at Iranian hospitals, whereas ST1077, ST415, and ST391, not yet.

According to the eBURST analysis of the 4 STs, compared to the 500 profiles of the database, ST1077 was the SLV of ST78. Two other STs (ST415 and 391) were, on the other hand, found in this report, belonging to the same CC of ST2 (CC2). These results suggest that ST415 and 391 clones as colistin-resistant isolates are emerging within hospitals in Isfahan.

In South Korea, ST1077 has been identified in *A. baumannii* isolates in patients with bacteremia, as in the present study [39]. Contrary to the present work, Osman et al. reported ST415 in the sputum samples, which was an extensive-drug resistant and colistin-susceptible clone [34]. Moreover, ST415 has earlier been found in animal isolates in France. Additionally, Al-Hassan reported carbapenem-susceptible colistin-resistant *A. baumannii* isolates in clinical samples of ST391 isolated from Immunosuppression

patients [40]. According to Agodi et al. and Cafiso et al., ST415 isolates with colistin resistance were assigned to ST2, broadly consistent with the present research results [31, 41].

In conclusion, the present study showed the occurrence of colistin-resistant isolates in the center of Iran (Isfahan). Furthermore, it revealed that resistance to various antibiotics, particularly to colistin and carbapenems, in samples obtained from Isfahan is very high.

To the best of our knowledge, this is the first MLST analysis of colistin-resistant *A. baumannii* in the region. The MLST analysis indicates that colistin-resistant isolates belonging to ST2 are the main circulating clone. Moreover, although ST2 is endemic in Iran in clinical settings, ST415, ST391, and ST1077 are found for the first time.

Intensive control procedures and strict adherence to surveillance programs are recommended to decrease the spread of the carbapenem- and colistin-resistant *A. baumannii* strain.

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