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Genetic diversity and methicillin resistance of *Staphylococcus aureus* originating from buffaloes with mastitis in Iran

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ABSTRACT

The aims of the present study were to investigate the genetic diversity and methicillin resistance in *S. aureus* isolates recovered from mastitis-affected buffaloes. Five hundred seventy-eight milk samples were obtained from buffaloes with mastitis in three provinces, Iran. Ninety-one of the 578 tested samples contained *S. aureus* (15.74%), in two cases were methicillin resistant *S. aureus* (MRSA). Isolates were typed by *spa* typing, followed by MLST on some representative isolates and SCC*mec* typing for MRSA strains. The presence of genes encoding Panton–Valentine leukocidin (PVL) was also tested by PCR. Eight *spa* types were identified, with t3576 (n = 18), t7311 (n = 18) and t937 (n = 17) were the most common, followed by t304 (n = 11), t7308 (n = 9), t521 (n = 7), t267 (n = 6), and t527 (n = 5). MLST revealed four different sequence types (STs) including ST97 (related to t521 and t527 *spa* types), ST352 (related to t267), ST291 (related to t304 and t937) and ST522 (related to t7338, t7311 and t3576). Two MRSA were identified as t304-ST291-SCC*mec*IV and t7311-ST522-SCC*mec*IV. No PVL-positive *S. aureus* were found. A significant difference in geographical distribution of genotypes was observed, with some types being prevalent in all studied provinces ($P < 0.001$). The results demonstrated genetic diversity among the *S. aureus* strains involved in mastitis in buffaloes. This study also provides evidence of the presence of MRSA belonging to genotypes which have been earlier reported in human infections, emphasizing the need for their epidemiological monitoring.

1. Introduction

Mastitis is known to be the most economically common health problems affecting dairy cattle and buffaloes [1]. Various surveys throughout the world have indicated that *S. aureus* is one of the major mastitis causing organisms in dairy buffaloes [2,3]. A better knowledge about the genotypic variation among *S. aureus* isolates involved in mastitis might help to implement more effective strategies to decrease the spread of infection and could be useful in source tracking. Several molecular subtyping methods has been used to identify and compare *S. aureus* genotypes [4]. The *S. aureus* protein A (*spa*) gene has been considered as a highly effective and rapid typing tool for short-term epidemiological investigations that has significant advantages in terms of speed, ease of use, ease of interpretation, and standardization among laboratories [5]. Multilocus sequence typing (MLST) is also a common tool for epidemiological investigation of *S. aureus*, where the nucleotide sequence of seven housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqiL*) is analyzed [6].

Furthermore, *S. aureus* is well known to acquire antibiotic resistance determinants which may lead to complications in the treatment of its infections [7] and increase the cost of treatments [8]. In addition, the entrance of such antibiotic-resistant *S. aureus* strains in milk and milk-products mainly via the dairy animals and more specifically infected mammary glands, is another serious concern as it may pose a public health problem [9,10]. Of particular concern are methicillin-resistant *S. aureus* (MRSA) strains that display resistant to almost all available β-lactams mostly through the presence of protein PBP2 A (penicillin binding protein 2 A) encoded by the *mecA* gene located within the mobile genetic element called Staphylococcal Cassette Chromosome (SCC, SCC*mec* when harboring the *mecA* gene) [11] as especially in cattle *mec*C was also found to be prevalent [12]. So far, a total of 13 types of SCC*mec* (I-XIII) have been characterized based on their structural organization, different allotypes, and genetic content. Hospital acquired-MRSA (HA-MRSA) are predominantly related to SCC*mec* types I, II, and III, while community acquired-MRSA (CA-MRSA) are related to SCC*mec* types IV and V [13]. Moreover, the Panton-Valentine leukocidin (PVL) encoded by the *pvl* genes (*lukS-PV* and *lukF-PV*) is a virulence factor that is predominantly associated with CA-MRSA in many parts of the world [14]. There have been increasing numbers of reports on MRSA infection and colonization in both companion and

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food-chain animals evidencing MRSA as an important veterinary and zoonotic pathogen [15]. More recent report concern the detection of MRSA colonization in Buffaloes which may act as a risk factor for human's infection [16]. Also, the presence of MRSA in cows and buffaloes with mastitis, and in workers who were in contact with the animals provided the evidence for a public health burden of MRSA transmission [3].

There is a considerable population (about 459 thousand head) of buffaloes in Iran which is ranked 16th among 43 countries in the world [17], with over 80 percent of its population concentrated in the north (Mazandaran) and north-west (Azerbaijan provinces) and 18 percent in the south of the country [18]. However, information about the prevalence and molecular characteristics of *S. aureus*, especially MRSA in dairy buffaloes is lacking and concentrate exclusively on cows [4,19]. Therefore, the aims of this study were to investigate, for the first time, the prevalence and genetic characteristics of *S. aureus* isolates involved in etiology of mastitis in Iranian buffaloes and to identify their methicillin resistance.

2. Materials and methods

2.1. Sampling and isolation of S. aureus

A total of 578 milk samples were aseptically collected from buffaloes with clinical sign of mastitis in three different provinces of Iran, including two Azerbaijan provinces (East Azerbaijan: n = 157 sample, 34 herds; West Azerbaijan: n = 217 sample, 62 herds) and Mazandaran province (n = 204 sample, 47 herds) which are located in the north-western and central-northern of the country, respectively (Fig. 1). The farming system of buffaloes in these areas is mainly based on smallholders (with an average of eight animals); a few herds have between 20 and 50 buffaloes and some of them have > 50 buffaloes. Therefore, the studied herds were categorized as small (1 to 20), medium (21 to 50), and large (> 50). Also, the buffaloes are fed green forages indoors and outside are left grazing.

Milk sampling was carried out according to the previously described method [20]. In order to isolate the *S. aureus* isolates, primary culture of milk samples was performed on mannitol salt agar (MSA, Merck, Germany). Yellow colored colonies which were mannitol positive, suspected as *S. aureus* on MSA were selected and subcultured on sheep blood agar plates to make pure culture [4]. The strains were characterized as *S. aureus* by standard culture and biochemical tests (Gram stain, catalase, coagulase and DNase tests) as well as PCR amplification of the thermonuclease (*nuc*) gene specific for this species [21]. The retrieved *S. aureus* isolates were kept frozen at -20 °C in tryptic soy broth (TSB, Merck, Germany) containing 15% glycerol until molecular tests were carried out.

2.2. Phenotypic and genotypic characterization of MRSA

MRSA was detected by cefoxitin (30 µg) susceptibility tests following the agar disc diffusion method, according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [22]. Isolates showing inhibition zone diameter ≤ 21 mm were classified as resistant. The PCR method described by Murakami et al. [23] was performed for the detection of the *mecA* gene of MRSA using the primers 5'-AAA ATC GAT GGT AAA GGT TGG C-3', corresponding to nucleotides 1282–1303, and 5'-AGT TCT GCA GTA CCG GAT TTG C-3', corresponding to nucleotides 1793–1814. *S. aureus* ATCC 29213 (methicillin sensitive control strain) and ATCC 25923 (methicillin resistant control strain) were used as a control for disc diffusion and PCR assays.

2.3. spa typing, MLST and SCCmec typing

The isolates were subjected to PCR amplification of the polymorphic X region of the *spa* gene using the standard primers (spa-1113f: 5'-TAA AGA CGA TCC TTC GGT GAG C-3' and spa-1514r: 5'-CAG CAG TAG TGC CGT TTG CTT-3') and protocol available on Ridom Staph Type (www.ridom.com). The purified PCR products of the *spa* gene were sent for sequencing (SinaClon, Iran) and then, *spa* type was deduced by using the Ridom SpaServer database (http://www.spaserver.ridom.de/) [24] via DNAGear. The BURP (Based Upon Repeat Patterns) algorithm analysis was performed using the Ridom StaphType software (Ridom GmbH, Germany) to cluster related *spa* types. Analysis was done with two default parameters for cluster designation: (1) "exclude *spa* types that are shorter than 5 repeats", and (2) "cluster *spa* types into the same group if cost distances are less than or equal to 4 repeats". A cluster consisting of two or more related *spa* types was regarded as a clonal complex. A singleton was defined as a *spa* type that was not grouped into a clonal complex [25]. To determine the genetic relationship between the different *spa* types, the nucleotide sequences were also aligned using ClustalW and phylogenetic analysis was carried using MEGA 6 program in Neighbour-Joining method with bootstrap replicates 1000 [26].

MLST was performed on representative *S. aureus* isolates (1 for each *spa* types identified) as well as on two MRSA isolates as described by Enright et al. [6]. Sequence types (STs) were assigned using an established MLST database (http://www.mlst.net) and STs were analyzed for relatedness with eBURST v3 software. Isolates that shared six of seven MLST loci belonged to the same clonal complex (CC).

The MRSA isolates were subjected to multiplex PCR for subtyping of Staphylococcal cassette chromosome *mec* (SCC*mec*) as described previously [27].

2.4. PVL encoding gene detection

The presence of genes encoding the Panton-Valentine leukocidin (PVL, *lukS-lukF-PV*) was determined by PCR using the primers luk-PV-1 and luk-PV-2 as described by Lina et al [28].

2.5. Statistical analysis

Statistical analysis of genotype distribution among the studied provinces were assessed by either Chi squared test using SPSS Software version 22 (IBM SPSS Statistics for Windows, Armonk, NY, USA: IBM Corp.). A P value < 0.05 was considered statistically significant.

3. Results

A total of 91 *S. aureus* strains (15.74%) isolated from buffaloes with mastitis, 24 (15.3%), 30 (13.8%) and 37 (18.1%) of those from East Azerbaijan, West Azerbaijan and Mazandaran provinces, respectively. Table 1 summarizes the overall prevalence of *S. aureus* mastitis in buffaloes on the basis of herd size and sampling region.

The PCR for amplification of the X region of the *spa* gene produced a single amplicon in each isolate but of variable sizes ranging from approximately 280–450 bp. The analysis of the isolates by *spa*-based sequencing yielded eight different *spa* types (t521, t527, t267, t937, t304, t7311, t3576, and t7308) with five to twelve repeats (Table 2). The sequence of the representative strain of each *spa* type was submitted to the GenBank database and were assigned the following GenBank accession numbers, respectively: MG684887, MG684889, MG684884, MG684886, MG684888, MG684891, MG684885, and MG684890. *spa* typing pointed to the predominance of t3576 (n = 18), t7311 (n = 18) and t937 (n = 17), which together represented 58.2% of the isolates, followed by t304 (n = 11), t7308 (n = 9), t521 (n = 7), t267 (n = 6), and t527 (n = 5). *S. aureus* isolates belonging to *spa* types t304, t937 and t3576 were recovered from all studied provinces, whereas t527, t521 and 267 were found in Azerbaijan provinces and t7308 was found only in Mazandaran province with the prevalence rate of 24.3% within the province. We found 7 *spa* types among 30 isolates from West Azerbaijan province, 6 *spa* types out of 24 isolates from East Azerbaijan

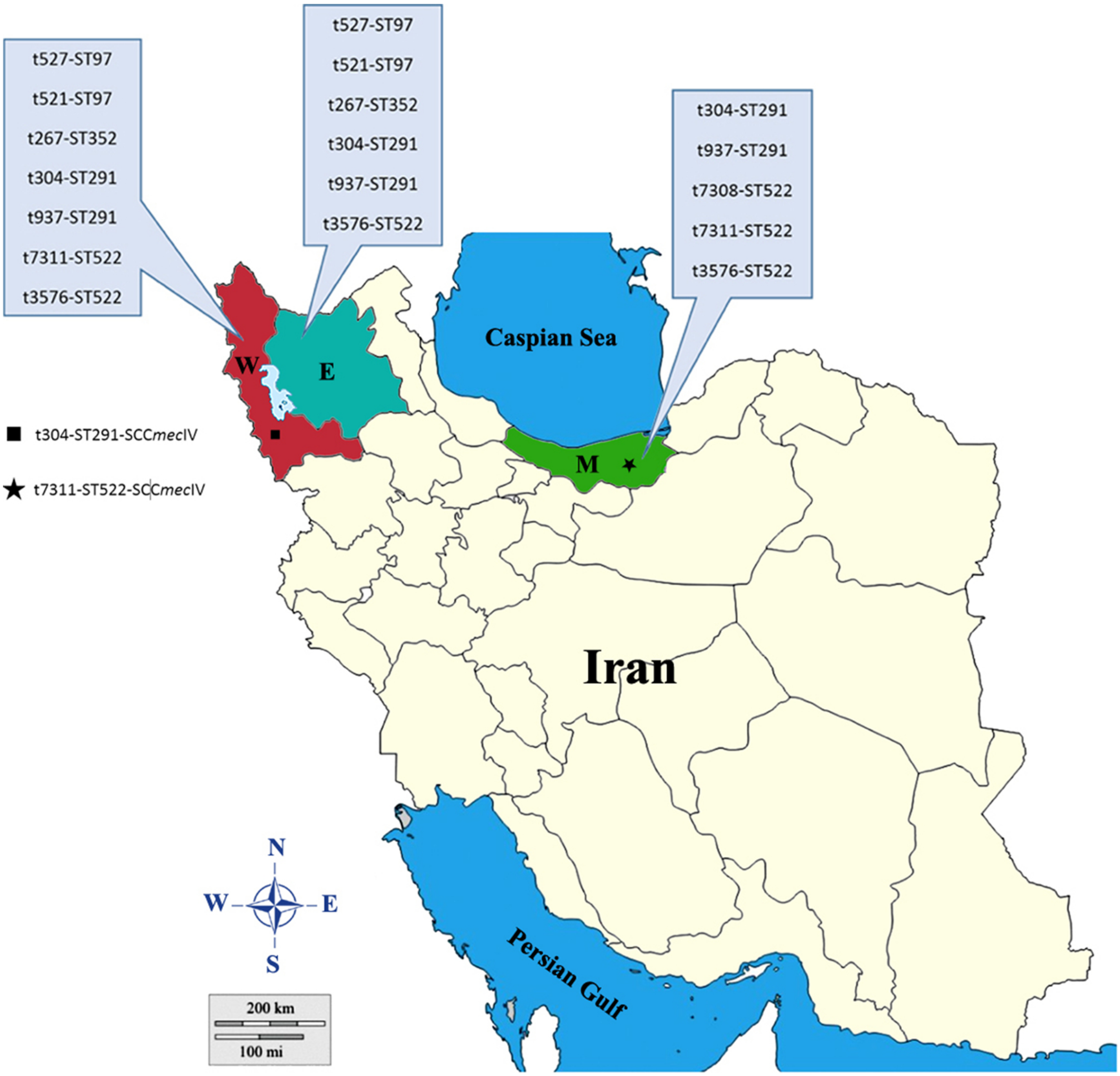

Fig. 1. Map of Iran showing the locations of the studied provinces, and the geographic clonal distribution.

Table 1
Summary of the overall prevalence of *S. aureus* mastitis in buffaloes on the basis of herd size and sampling region.

Province	Herd size	No. of herds sampled	No. of milks sampled	No. of *S. aureus*-positive samples [%]
East Azerbaijan				
	Small	25	79	12 [15.19%]
	Medium	8	67	10 [14.92%]
	Large	1	11	2 [18.18%]
West Azerbaijan				
	Small	45	128	19 [14.84%]
	Medium	15	72	8 [11.11%]
	Large	2	17	3 [17.65%]
Mazandaran				
	Small	34	121	21 [17.35%]
	Medium	11	59	11 [18.64%]
	Large	2	24	5 [20.83%]
Total		143	578	91 [15.74%]

province, 5 *spa* types out of 37 isolates from Mazandaran province (Table 2). Regarding the BURP analysis, the resulting *spa* types were clustered into 2 groups (no founder): group 1 consists of *spa* types t7308, t7311 and t3576 and group 2 consists of t527, t521 and t267. The *spa* types t304, and t937 were not assigned to any BURP group and were identified as singletons.

Phylogenetic analysis grouped eight different *spa* types into two major clusters. Of these, cluster I was further grouped into two sub-clusters SC1 (consisted of *spa* types t521, t527, and t267) and SC2 (contained *spa* types t937 and t304). Cluster II included *spa* types t7311, t3576 and t7308 (Fig. 2).

MLST analysis of isolates representative of each *spa* types as well as two MRSA isolates revealed four different sequence types (STs) including ST97 (related to t521 and t527 *spa* types), ST352 (related to t267), ST291 (related to t304 and t937) and ST522 (related to t7338, t7311 and t3576). Regarding the eBURST analysis, the resulting STs were clustered into 1 group (none predicted founder) consists of ST97 and ST352. The ST291 and ST522 were not assigned to any eBURST group and were identified as singletons.

Two isolates (2.2%) showed resistance to Cefoxitin and both carried the *mecA* gene. Of them, one isolate exhibited a composite profile of *spa* t304-ST291-SCC*mec* IV, while the other one was as *spa* t7311-ST522-SCC*mec* IV. No *S. aureus* isolate was positive for the lukS-lukF-PV genes. Statistical analysis of the geographical occurrence of different types revealed divergent distribution between studied provinces ($p < 0.001$).

Table 2
Molecular characteristics and distribution of *S. aureus* isolates from buffaloes with mastitis.

spa type[a]	*spa*-CC	*spa* repeat successions	MLST_ST	Province			No. of isolates[b]
				West Az.	East Az.	Mazandaran	
t527	no founder 2	07-23-12-21-17-34-34-34-34-34-33-34	97	2	3	–	5 (5.5)
t521	no founder 2	07-23-12-21-17-34-34-34-34-33-34	97	3	4	–	7 (7.7)
t267	no founder 2	07-23-12-21-17-34-34-34-33-34	352	3	3	–	6 (6.6)
t304	Singleton	11-10-21-17-34-24-34-22-25	291	6[c]	4	1	11 (12.1)
t937	Singleton	08-16-34-24-34-34-17-17	291	8	5	4	17 (18.7)
t7308	no founder 1	04-31-17-25-25-17-17	522	–	–	9	9 (9.9)
t7311	no founder 1	04-25-17-25-16-17	522	5	–	13[b]	18 (19.8)
t3576	no founder 1	04-31-17-25-17	522	3	5	10	18 (19.8)
Total				30	24	37	91

Abbreviations: MLST, multilocus sequence typing; CC, clonal complex; ST, sequence type.

[a] *spa* types marked in bold are present in all provinces studied.

[b] Values in the parenthesis is in percentage.

[c] One of these isolates was methicillin-resistant *S. aureus* (MRSA).

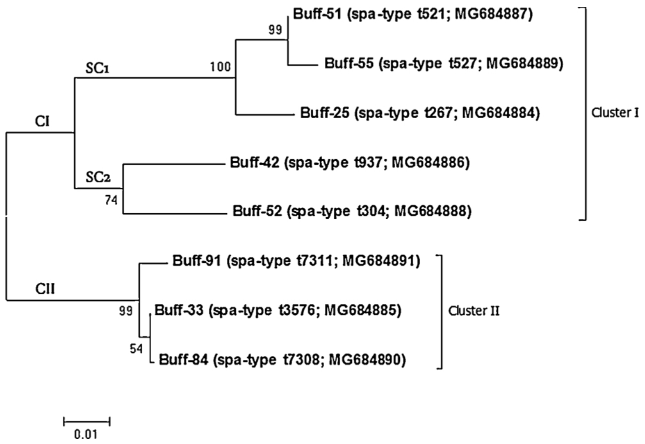

Fig. 2. Neighbor Joining tree constructed based on the nucleotide sequences of *spa* gene of eight *S. aureus* strains isolated from mastitis milk of Buffalo (representatives of eight *spa* types obtained by *spa* typing). Each strain is indicated by the *S. aureus* sample number followed by the *spa*-type and GenBank accession number given in parenthesis.

4. Discussion

The overall isolation frequency of *S. aureus* in milk from buffaloes with mastitis (15.74%) in the present study was lower than those previously reported from India [29], Greece [29], and Egypt [3,30,31]. This discrepances could be explained by application of various strategies of herd health management including treatment or culling of affected animals, implementation of biosecurity and hygienic practices which have been recommended as a means of preventing and controling udder health problems cause by *S. aureus* in Mediterranean buffaloes (MB) farms [32]. However, our present finding of the prevalence in East Azerbaijan province (15.3%) is comparable to that reported by other authors in Iran [33], who showed 15% prevalence of buffalo mastitis caused by *S. aureus* in Tabriz (the capital city of the East Azerbaijan province in northwest of the country). Such similarity could be arise from the same geographical and climatic conditions along with common herd management practices.

Our finding revealed a wide degree of *spa* gene (X region) polymorphism in a collection of 91 strains of *S. aureus* and the presence of 5–12 number of tandem repeats, which is in agreement with a previous study [34]. Overall, the analysis identified eight *spa* types (t521, t527, t267, t937, t304, t7311, t3576, and t7308) and four sequence types (ST97, ST352, ST29, and ST522) in isolates from buffaloes with mastitis in this study, with t3576-ST522, t7311-ST522 and t937-ST291 being the most prevalent (53 out of 91); of which, t3576-ST522 and t937-ST291 were isolated from all three provinces studied. Similarly, the predominance of some genotypes has previously been reported among *S. aureus* isolates recovered from bovine mastitis [35]. These suggest contagious transmission, a common source, or host adaptation of subset of the population of *S. aureus* strains as describe about bovine mastitis [4].

Among these *spa* types, t267 (belonging to ST747) was the only type that has earlier been reported from buffaloes with subclinical mastitis in Brazil [36]. This *spa* type (t267) along with t521 and t527 were detected only in Azerbaijan provinces and grouped into the same cluster (cluster 2) according to the BURP analysis as well as the same subcluster (SC1) by phylogenetic analysis. These types have previously mainly been found associated to bovine milk samples from quartes with intra-mammary infections (IMI) or from bulk tanks [37–39]. Intrestingly, *spa* types t7308, t7311, and t3576, which were commonly (71.1%) observed in Mazandaran province and grouped together in the same cluster by BURP and phylogenetic analysis, have frequently been isolated from mammary infections but also from nasal samples originating from the small ruminants [40,41]. Considering these reports, there is a possibility that *S. aureus* isolates causing buffalo mastitis in different areas may be acquired from different sources. More detailed studies are needed in order to draw any definitive conclusions regarding the source of *S. aureus* intramammary infections in buffaloes.

As results, *spa* types t304 and t937 that originated from all studied regions were defined as singletons by BURP and located in the formation of subcluster 2 (SC2). These *spa* types has previously been detected in the country, in wound and urinary tract infections (UTI) as well as adenoid tissue of patients admitted to hospitals of Isfahan (Center), Mashhad (Northeastern) and Tehran (North-central) Iran [42,43]. On the other hand, MRSA isolates represent 2.2% (2 isolates) of the total *S. aureus* isolated, which ascribed to t304-ST291-SCC*mec*IV (isolated from West Azerbaijan) and t7311-ST522-SCC*mec*IV (isolated from Mazandaran). It should be noted that phenotypic methicillin resistance in staphylococci is conferred not only by *mecA*, but also by homologous gene *mecC* and that MRSA harboring the later gene (*mecC*-MRSA) cause difficulties for both, detection and identification by the means of conventional phenotypic methods and DNA-based molecular assays [12,44]. Even thought, the genetic characteristics of the isolated MRSA strains were inconsistent with those from Egyptian buffaloes [31], MRSA strains with t304-*SCCmec*I and t304-*SCCmec*IV have previolsuly been documented in human clinical specimens in Iran [43,45]. This represents a worrisome situation with the possibility of zoonotic transmission and dissemination of t304-ST291 strains in the community. To our knowledge, there are no published reports on the occurrence of MRSA t7311-ST522 in animals possibly due to the fact that some specialized types appear to be limited to a specific geographic area.

None of the isolates in this study were found to be positive for *lukS-lukF-PV* genes, as reported in *S. aureus* strains isolated from bovine mastitis [46,47] and milk samples of different animal species [36]. In contrast, El-Ashker et al. [31] identified the leucocidin genes in some lineage of *S. aureus* isolates derived from cattle and buffaloes with mastitis in Dakahlia Governorate, Egypt.

Significant difference in distribution of genotypes was found between *S. aureus* isolates from West and East Azerbaijan provinces and those from Mazandaran province. This might be the result of differences in the breed of buffaloes and climatic condition of the study sites. Smith et al. [48] proposed that the site specificity of some sequence types might be due to localized selection pressures that drive the evolution of *S. aureus* in diverse hosts and environments.

5. Conclusion

In conclusion *S. aureus* isolates from buffaloes with mastitis showed genetic diversity, but the geographical distribution of genotypes was heterogeneous. The present study also revealed the presence of MRSA strains that are genetically similar to human-associated strains, represents a potential threat for public health and therefore deserve further investigation on MRSA contamiation sources.

Conflict of interest statement

The authors declare that they have no competing interests related to this study.

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