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Molecular Characterization of *Staphylococcus aureus* Isolated from Clinical Samples Based on *16srRNA, rpoB,* and *hsp70* Genes by MLSA

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ABSTRACT

Aims: *Staphylococcus aureus* is a Gram-positive bacterium with the capability of causing a variety of nosocomial and community-acquired infections. Evaluating the genetic structure, polymorphism, genotyping, and phylogeny of *S. aureus* isolates could contribute to the prevention and treatment of infections caused by this microorganism.

Materials & Methods: In this study, the polymorphisms of *16S rRNA*, *rpoB*, and *hsp70* genes were investigated in a total of 50 *S. aureus* isolates using *S. aureus* NCTC 8325 as the reference strain. Polymerase chain reaction (PCR) was used for the detection and amplification of the studied genes. The amplicons were then sequenced using a Sanger sequencing method. Moreover, phylogeny of the isolates was studied using Neighbor-joining and Maximum Parsimony methods for *16S rRNA*, *rpoB*, and *hsp70* genes individually and in combination.

Findings: After Sanger sequencing, data obtained by Sequencher and Mesquite software programs revealed several polymorphisms of *S. aureus* isolates 16S rRNA, rpoB, and hsp70 genes, respectively. These polymorphisms included transversion, transition, insertion, and deletion. Among the studied strains, 10 cases showed no polymorphism. Multi-locus sequence analysis (MLSA) showed several genetic diversities in *S. aureus* isolates.

Conclusion: It seems essential to rapidly and reliably identify the phylogenetic sources and characteristics of this microorganism and to have a better understanding of its molecular epidemiology in order for infection practical surveillance and control.

Keywords: *Staphylococcus aureus*, Polymorphism, Multi-locus sequence analysis (MLSA).

CITATION LINKS

[1] Colonization and infection of the skin by *S. aureus*: Immune system evasion and the response to cationic antimicrobial... [2] *Staphylococcus aureus* in the community: Colonization... [3] *Staphylococcus aureus* infections: Epidemiology, pathophysiology, clinical... [4] *Staphylococcus aureus* nasal colonization: An Update on mechanisms, epidemiology, risk factors and subsequent infections. Front Microbiol. 2018; 9:2419. [5] Methicillin resistance in *Staphylococcus aureus.* Pet-to-man travelling *Staphylococci*: Elsevier; 2018: 225-35. [6] Methicillin-resistant *Staphylococcus aureus*: An evolving... [7] The molecular evolution of... [8] Prokaryotic taxonomy in the sequencing era-the polyphasic approach... [9] Stepwise decrease in daptomycin susceptibility in clinical *Staphylococcus aureus* isolates associated with an initial mutation.. [10] Mutations in HSP70-2 gene change the susceptibility to clinical mastitis in Chinese Holstein. Gene. 2015; 559(1):62-72. [11] Molecular identification and genotyping of MRSA isolates. FEMS Immunol Med Microbiol. 2009; 57(2):104-15. [12] Molecular cloning of two new heat shock genes related to the hsp70 genes in *Staphylococcus aureus.* J Bacteriol. 1994; 176(15):4779-83. [13] Genotypic diversity of coagulase-negative... [14] Mechanisms of methicillin resistance in *Staphylococcus aureus*. Annu Rev Biochem. 2015; 84:577-601. [15] Focus: Infectious diseases: Vancomycin resistance in *Staphylococcus aureus.* Yale J Biol Med. 2017; 90(2):269-81. [16] Antibiotic resistance in *Staphylococcus aureus.* Current status... [17] Comparison of traditional and molecular methods of typing... [18] Spa typing and multilocus sequence typing show comparable performance in a macroepidemiologic study of *Staphylococcus aureus* in... [19] Multilocus sequence analysis (MLSA) in... [20] The role of universal stress proteins in Edwardsiella... [21] Genetic variation among *Staphylococcus aureus* strains from bovine milk and their... [22] Genetic variation in *Staphylococcus aureus* surface and immune evasion genes is lineage associated: Implications... [23] Phylogenetic relationships among *Staphylococcus* species and refinement of... [24] Comparison of complete *rpoB* gene sequence typing and multi-locus sequence typing for phylogenetic analysis of *Staphylococcus aureus.* J Gen ...

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Introduction

Staphylococcus aureus is a commensal Gram-positive organism present on the skin and mucosal surface with the capability of surviving on dry surfaces due to its thick peptidoglycan layer [1]. Risk factors of *S. aureus* infections include external devices, history of surgery, and extensive antibiotic use [2]. Patients at risk of *S. aureus* infections include neonates, children with poor sanitation, women during menstruation, and patients with intravascular catheters. This microorganism could mainly lead to the bacteremia, endocarditis, osteomyelitis, pneumonia, skin and soft tissue infections [3]. Although *S. aureus* is generally considered as an opportunistic pathogen, some clones may be more capable of causing invasive disease due to the presence of certain virulence factors facilitating access to normally sterile sites [4].

Recently, *S. aureus* has exhibited great resistance against multiple antimicrobial agents, which is of great concern. Methicillin-resistant *S. aureus* (MRSA) strains; including hospital-acquired MRSA (HA-MRSA), community-acquired MRSA (CA-MRSA), and livestock-associated MRSA (LA-MRSA) strains; are resistant to all β-lactam antibiotics through acquiring mobile genetic elements called staphylococcal cassette chromosome mec (SCCmec) [5-7]. In addition to acquiring antibacterial resistance, differences in staphylococcal pathogenicity, depending on different geographical regions and epigenetics, necessitate the investigation of the genomic structure, polymorphism, and phylogenetic relationships between different *S. aureus* clinical isolates.

Multi-locus sequence analysis (MLSA) is a powerful high-resolution method which provides data on genetic changes in housekeeping genes and could be served as a valid technique for the study of epidemiological relationships [8]. In fact, MLSA is able to compare the primary DNA sequences of multiple conserved protein-coding loci in order to assess the diversity and relationship between different isolates and to determine the sources and evolutionary alterations of different taxa.

Objectives: This study aimed to investigate the genetic diversity of *S. aureus* clinical strains isolated from different body sites. In this study, a MLSA protocol was developed based on genes coding for β-subunit bacterial RNA polymerase (*rpoB*) and heat-shock protein 70 (*hsp70*) as well as *16S rRNA* gene. These genes are essential for bacteria, and their polymorphism is highly important in determining bacterial genetic behaviors corresponding to different environmental factors [9-10].

Materials and Methods

***Staphylococcus aureus* strains and growth conditions:** A total of 50 S*. aureus* strains were isolated from patients with urinary, wound, blood, eye, synovial, and pharyngeal infections, referring to Shahid Rahimi hospital, Lorestan, Iran from March 2018 to June 2018. Patients received no antibiotic two weeks prior to sampling. In addition to observing the presence of Gram-positive cocci bacteria under microscope, catalase, coagulase, DNAse, and growth on mannitol salt agar tests were performed for the confirmation of *S. aureus* isolates. All of the *S. aureus* strains were grown on blood agar (Merck Millipore, Germany) and incubated at 37°C for 18 to 24 hrs. *S. aureus* ATCC 25923 was used as the standard strain.

Isolation of Genomic DNA: Chromosomal DNA was extracted from overnight cultures of *S. aureus* isolates grown on blood agar at 37°C for 18 to 24 hrs using the Qiagen DNA extraction kit according to the manufacturer's protocol (Hilden, Germany). Genomic samples with an OD260/OD280 ratio of ≥1.8 were considered as pure and

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employed for further analysis. Also, to confirm the quality of the extracted DNA, samples were analyzed by 1% agarose gel electrophoresis (Sigma, USA).

Polymerase Chain Reaction (PCR) and Sequencing: Amplification of *16SrRNA*, *rpoB*, and *hsp70* genes was performed in an automated thermal cycler (Bio-Rad, USA) in a total volume of 25 µL consisting of 2 µl of DNA template, 1 µL of the previously described primers (forward and reverse) (Table 1), 12.5 µL of Taq PCR Master Mix 2X (Fermentas, Lithuania), and 8.5 µL of DNase/RNAse free distilled water (Thermo Fisher Scientific). The PCR program included an initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing (annealing Tm for each primer is shown in Table 1) for 60 sec, extension at 72°C for 60 sec, and a final extension at 72°C for 5 min. Finally, amplicons in each reaction were analyzed on 1% agarose gel treated with safe stain (Thermo Fisher Scientific, USA) in 0.5X TBE after electrophoresis. Gels were visualized under the gel documentation system (Bio-Rad, UK).

The PCR positive products with the expected size were sequenced by Sanger method for five times (Forward and reverse). The sequences were then blasted against the *16S rRNA*, *rpoB,* and *hsp70* GenBank databases (NCBI).

Sequence data analyses: Chromatographs were annotated with the Sequencher DNA analyzer program Ver.5.1 (Gene Codes Corpora tion). After finding contigs, they were blasted to find any change compared to the reference sequence of *S. aureus* NCTC 8325 standard strain.

Phylogeny of the isolates was analyzed using the maximum parsimony or neighbor-joining method using the Mega software Ver.6.0. Standard strain of *S. aureus* NCTC 8325 was used as the reference strain.

Findings

Genetic variations in the studied genes: All MLSA loci of 50 *S. aureus* isolates were successfully amplified. Sequencing analysis of the PCR products created sequences in both directions. The collections of sequences were transferred from the Sequencher to Mesquite software Ver.3.6 for alignment in order to find any type of polymorphism in the studied genes. According to the results, among the 50 studied *S. aureus* strains, 21, 33, and 23 strains showed genetic variations caused by mutations including insertion, transversion, transition, and deletion in *16S rRNA*, *rpoB*, and *hsp70* genes, respectively. Among the studied isolates, 10 cases showed no genetic variation compared to the reference strain (Tables 2, 3, and 4).

Phylogenetic analysis of the strains using the Neighbor Joining method: Phylogenetic trees constructed based on *16s rRNA* gene using the neighbor joining method showed that the Strain 14 was different from the

Table 1) Characteristics of primers used in this study

Targeted Genes	Sequence (5′ → 3′)	Tm(°C)	Amplicon Size (bp)	Reference
16S rRNA	F AACTCTGTTATTAGGGAAGAACA R CCACCTCCTCCGGTTTGTCACC	55	689	(11)
hsp70	F CCIGAYGARGTIGTIGC R TCIGCRTCYTTIACCAT	46.6	610	(12)
rpoB	F AACCAATTCCGTATIGGTTT R CCGTCCCAAGTCATGAAAC	50.5	1183	(13)

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Table2) Polymorphisms in *16S rRNA* gene of 50 *S. aureus* clinical strains with different sources compared to the reference strain

Strain No.	Source	Nucleotide Change (Base Number/ Type of Mutation) in *hsp70* Gene
1	Urine	AAC→AGC (273/ transition)
2	Urine	TTT→TCT,TGT→TGTT(203, 297/transition/insertion)
3	Blood	-
4	Blood	-
5	Wound	TTT→TCT,CAC→CGC,AAT→CCC,TTC→TTT,AAT→GAAT,TAA→CAA (203,254,263,264,266,269 ,272,273,282/ transition, insertion)
6	Blood	ATA→AATA,TCA→TACA,CAA→ACAA (32,35,37/insertion)
7	Blood	AAT→ATT (264, transversion)
8	Blood	-
9	Urine	-
10	Eye	TTC→TTCC,CTA→CTTA (244, 260/ insertion)
11	Blood	-
12	Wound	AGA→AGCA (287/insertion)
13	Blood	-
14	Blood	TGT→TGTT (297/insertion)
15	Blood	-
16	Wound	-
17	Blood	TTA→TTT (4/transversion)
18	Throat	-
19	Urine	-
20	Wound	-
21	Blood	-
22	Blood	-
23	Blood	-
24	Wound	AAC→AGC (237/transition)
25	Blood	-
26	Blood	-
27	Blood	ACT→ATT (179/transition)
28	Blood	-
29	Blood	-
30	Urine	CTA→CAA,ACT→ATT,CTG→CAG,TTT→AAA (177, 179, 183, 266, 267, 268/ transversion, transition)
31	Blood	-
32	Urine	-
33	Blood	-
34	Blood	-
35	Blood	AAT→ATTT,ATA→ACA,ACC→A-C (273, 275, 276, 287/ transition, insertion, deletion)
36	Blood	-
37	Wound	CAA→CGA,TCA→TCC,ATA→TTT (232,271,274,277/transversion)
38	Blood	GTT→GTC (3/transition)
39	Blood	-
40	Blood	-
41	Urine	-
42	Blood	-
43	Wound	ACA→ACT,ACG→AAC,TTT→TCT,TCA→TCT,AAT→ATT,TCA→TTC,GTG→GAT (6, 238, 267, 271, 274, 277, 295, 296/ transversion, transition)
44	Synovial	-
45	Sputum	CCG→CCAG,CAA→CTAA,AAT→AAA (19,37,92/insertion)
46	Blood	GTG→GGG (295/tranversion)
47	Blood	ACC→AGG (278, 279/transversion)
48	Blood	ACC→ATC (278/transition)
49	Blood	GTT→TTT,ATT→ATAT,AAA→GGA,AGA→AAA ,AGT→AAT (1,8,38,39,206,221/transversion, insertion)
50	Urine	TGG→TTG (94/ transversion)

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Table3) Polymorphisms in *rpoB* gene of 50 *S. aureus* clinical strains with different sources compared to the reference strain

Strain No.	Source	Nucleotide Change (Base Number/ Type of Mutation) in *rpoB* Gene
1	Urine	TGT→TAT, CTG→CCG (127, 331/transition)
2	Urine	-
3	Blood	-
4	Blood	ATG→ATGG, CCA→CCCA, TTT→TTTT, AAT→ATT, TGT→TAT (5, 7, 14, 58, 619/transversion, transition, insertion)
5	Wound	CTG→CTGA, TAG→TAA, TTA→TTT, CGA→CAA, CAT→CCT, CGA→CCA, GTG→GGG, ACA→AAA, ATA→AAA, AGT→AAT, ATG→AGG, ATC→ACC, CGA→C-A, ATC→A-C (542,603,627,643,649,684,729,735,792,795,827,844,586,941) (transversion, transition, insertion, deletion)
6	Blood	TCT→TCCT, CAA→CAGA (16,936/ insertion)
7	Blood	CAC→CACC, TGC→TGCC, CAT→CATT, TAA→TAAA (476,555,570,582/ insertion)
8	Blood	-
9	Urine	ACC→ACCT (202/insertion)
10	Eye	TCA→TTA, ATA→AAA, CTC→CGC, AGA→AAA, ATC→ACC, ACA→AAA, GTG→GGG, TCT→T-T, AAA→AA-, CGG→CGGG, TCG→TTG, CTG→CGG, CAC→CCC, CAT→C-T, ACT→ATT, CCA→CCC (287,323,331,342,345,388,399,404,421,477,533,537,632,649,659,676/ transversion, transition, insertion, deletion)
11	Blood	AAT→AT,CCA→A,AGA→AGAT,TGG→TGGA,TCG→TCCC,TTC→TCC,CAC→CCC,CGA→CAA,AGA→AAA,AGT→AAT, CTT→CTTG, CAT→CAA, AGC→ACC, TAT→TTT, AGT→AAT, ATA→A-A, ATG→CAG, ATC→ACC, TGT→GG- (1,8,271,272,273,511,534,535,596, 632,643,714,716,726,749,753,757,795,821,826,827,844,947,950/ transversion, transition, insertion, deletion)
12	Wound	-
13	Blood	ATG→ATGG,TGT→TAT,CAT→CCT,AGA→AAA,TGC→TTGC (5,6,7,619,700,714,720/ transversion, transition, insertion)
14	Blood	-
15	Blood	TGC→GGT,AAA→--A (399,401,789,790/ transversion, deletion)
16	Wound	-
17	Blood	ATT→ATAT,TTA→TAA(710,713/transversion, insertion)
18	Throat	-
19	Urine	TTC→TCC,TCA→TAA,TTG→TGG,CAT→CCT (411,417,435,488/ transversion, transition)
20	Wound	CAA→ACAA,GCT→GGCT,CAA→AAA (78,720,739/ transversion, insertion)
21	Blood	TGT→TGTT,CAT→CGC,TAC→TTC,TGT→TAT,TTT→TTTT,CTT→CTTG (138,293,382,619,720,726,733/ transversion, transition, insertion)
22	Blood	CCA→CCCA,TTC→TTTC,AAA→AAAA,GTG→GGTC (628,660,705,720/ insertion)
23	Blood	-
24	Wound	TGT→TAT (127/ transition)
25	Blood	-
26	Blood	AAT→TAT,TAC→TTC (1,382/ transversion)
27	Blood	-
28	Blood	GAC→GTC,TGT→TTT (296,303/ transversion)
29	Blood	-
30	Urine	ATT→ATTT,TGT→TAT,TTT→GGG,GCC→GAC,TCC→TGC (111,619,869,870,871,884,959/ transversion, transition, insertion)
31	Blood	-
32	Urine	TAC→CCC,ATA→CAC (990,991,999,1000/ transversion, transition)
33	Blood	
34	Blood	TGC→TGCC,--- →TTA,CTT→CTTT,CAA→CCAA (99,271,272,273,914,944/ insertion, deletion)
35	Blood	TGT→TAT,TTG→TTTG (619,952/ transition, insertion)
36	Blood	GTA→GAA,TTC→TTTC,CTG→CCG,GGT→GGGT,TGT→TAT (246,286,331,398,619/ transversion, transition, insertion)
37	Wound	ATG→ATGG,AAT→A-T (5,23/ insertion, deletion)
38	Blood	-
39	Blood	ATG→ATGG,TTC→TTTC,TGT→TAT (5,14,619/ transition, insertion)
40	Blood	AAT→TAT,GCC→G-C,AAT→A-T,CAA→CACA (1,8,23,76/transversion, insertion, deletion)
41	Urine	TTC→TTT,TTC→TTA,ACA→AAA (663,767,988/ transversion, transition)
42	Blood	-
43	Wound	AAT→A-T,TAC→TTC,TCA→TCG (23,382,492/ transversion, transition, deletion)
44	Synovial	TTC→TATC,CTG→CCG,TGT→TAT (275,331,619/transition, insertion)
45	Sputum	TCG→TCCG,TGT→TAT (454,619/ transition, insertion)
46	Blood	-
47	Blood	TGT→TAT,TTC→TT- (619,663/ transition, deletion)
48	Blood	CTG→CCG,TGT→TAT (330,619/ transition)
49	Blood	-
50	Urine	-

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Table4) Polymorphisms in *hsp70* gene of 50 *S. aureus* clinical strains with different sources compared to the reference strain

Strain No.	Source	Nucleotide Change (Base Number/ Type of Mutation) in *hsp70* Gene
1	Urine	-
2	Urine	GTA→GAA (476/ tranversion)
3	Blood	-
4	Blood	-
5	Wound	-
6	Blood	ATA→ACA (427/transition)
7	Blood	-
8	Blood	-
9	Urine	-
10	Eye	-
11	Blood	-
12	Wound	GCT→GCTT (433/ insertion)
13	Blood	-
14	Blood	AGA→AAA,AGT→AAA,TCC→TTC,CTC→TTT,GCT→GCTT (212,222,223,243,253,255,491/ transversion, transition, deletion)
15	Blood	GGT→GCT,CGT→CCT,CTT→GAT (12,19,35/ transversion, insertion)
16	Wound	-
17	Blood	-
18	Throat	-
19	Urine	GGT→GCT,GGC→G-C (11,489/ transversion, deletion)
20	Wound	-
21	Blood	-
22	Blood	CCA→CCCA,CCT→C-T,TTT→-TT (156, 442, 492/insertion, deletion)
23	Blood	TTT→TAT,GCT→GCTT (264, 491/ transversion, insertion)
24	Wound	-
25	Blood	-
26	Blood	-
27	Blood	-
28	Blood	TCC→TCCC (14/ insertion)
29	Blood	-
30	Urine	-
31	Blood	-
32	Urine	-
33	Blood	-
34	Blood	-
35	Blood	CAG→CGG (157/ transition)
36	Blood	-
37	Wound	GCT→GCTT (491/insertion)
38	Blood	CTA→CGA,CTA→CAA (389, 443/ transversion)
39	Blood	ATA→ACA,ATG→AGG (250, 278/ transversion)
40	Blood	-
41	Urine	AGA→AGAA,CTC→CTTC,GCA→GCCA,CCG→CGG,GAA→GAA, CTG→CTTC,AGT→AGGT,GAG →GGAAG, GCT→GTT,AGA→AAA, CTA→CAA,GCT→GCTTT,ATA→AAA (218,245,260,269,281,307,321, 348,360,372,389 ,399,400,427/ transversion, transition, insertion)
42	Blood	-
43	Wound	CCT→CCCT (117/ insertion)
44	Synovial	GCT→GGCT (462/ insertion)
45	Sputum	-
46	Blood	GCG→GCCG,GAA→GAAA,ACG→ACGG (47,281,342/ insertion)
47	Blood	CTT→CTTT,TTA→TCA (491, 502/ transition, insertion)
48	Blood	CGG→CTG,CTT→C-T,TTA→TAA (423, 434, 452/ deletion)
49	Blood	CTC→CCTC,GCG→GGCG (56, 65/ insertion)
50	Urine	GTC→GGTC,CAG→CCG,CTA→CTTA,CGC→CCC,CCA→CGA (12, 62, 118, 392, 409/ transversion, insertion)

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Strains 23, 49, and 42 (>99% difference) as well as the Strains 5 and 6 (>73%). Moreover, Strain 48 showed a sequence different from the Strains 6, 14, 15, 19, 22, 23, 42, and 49 (>99% difference). Finally, the Strain 50 was different in sequence from the Strains 1, 2, 3, 4, 5, 6, 11, 16, 20, 25, 30, 33, 37, 38, 39, 40, 46, 48, and 49 (Fig. 1A). Phylogenetic trees based on *hsp70* gene showed that the Strains 5, 30, 43, and 49 were different from other strains in sequence (>99% difference). However, other strains were highly correlated, and the sequence similarity of the strains could be considered as an indicative of common ancestors (Fig. 2A). Based on the *rpoB* gene sequences, the Strain 11 showed a high correlation to the reference strain and Strains 2, 3, 5, 11, 24, 26, 37, 39, and 43, while it was different from other strains (>99% difference). Moreover, the Strain 15 was highly correlated with other strains, except for 19, 25, 29, 35, 41, 45, and 49 (>99% difference) (Fig. 3A).

Phylogenetic trees constructed based on the concatenation of *16s rRNA*, *rpoB*, and *hsp70* genes sequences by maximum parsimony and neighbor-joining methods showed that the Strain 5 was different from the Strains 6, 15, 19, 30, 32, 42, 46, 47, 48, and 40 with a bootstrap value of 99%. Moreover, the Strain 11 showed a phylogenetic difference with the Strains 1, 2, 3, 4, 5, 6, 15, 19, 30, 32, 42, 46, 47, 48, and 49 with a bootstrap value of 99%. However, other strains were highly similar in sequence and showed a close relationship to one other. Notably, the Strain 24 only showed similar genetic sequence to the reference strain and Strain 7 and showed 99% difference with other strains (Fig. 4A).

Phylogenetic analysis of the strains using the Maximum Parsimony method: According to the results of phylogenetic analysis based on *16s rRNA* gene using maximum parsimony method, all *S. aureus* isolates showed similar genetic sequences and were not phylogenetically different and were presented in a monophyletic manner. However, it must be noted that the Strains 13 and 39 showed a trivial difference with other isolates with a bootstrap value of 63% (Fig. 1B). Molecular analysis based on *hsp70* gene indicated that the Strains 2, 5, 27, and 30 were different from other strains with a bootstrap value of 62%, while the Strains 1 and 24 were different from other strains with a bootstrap value of 61% (Fig. 2B). Finally, Phylogenetic analysis based on *rpoB* gene using the maximum parsimony method revealed the differentiation of the Strains 5 and 11 with 99% bootstrap value as well as the differentiation of the Strain 33 with 53% bootstrap value compared to the other strains (Fig. 3B). Phylogenetic analysis based on *16srRNA, rpoB,* and *hsp70* genes in concatenation indicated that the Strains 5 and 11, 1 and 24, and 37 and 43 were different from other strains with the respective bootstrap values of 99, 58, and 51% (Fig. 4B).

Discussion

S. aureus is one of the most common causes of both endemic and epidemic hospital-acquired infections with a high morbidity and mortality rate [3]. The frequency of multidrug-resistant *staphylococci* strains is reportedly increasing worldwide, including isolates that are resistant to methicillin, aminoglycosides, macrolides, fluoroquinolones, lincosamides, or combinations of these antibiotics. Therefore, the severe consequences of infections caused by *S. aureus* strains and the incidence of antibiotic resistance have increased the importance of prevention [14-16]. The study of genomic structure, polymorphism, and phylogenetic relationships between *S. aureus* clinical isolates could facilitate infection control, prevention, and surveillance.

The typing of *S. aureus* strains may show

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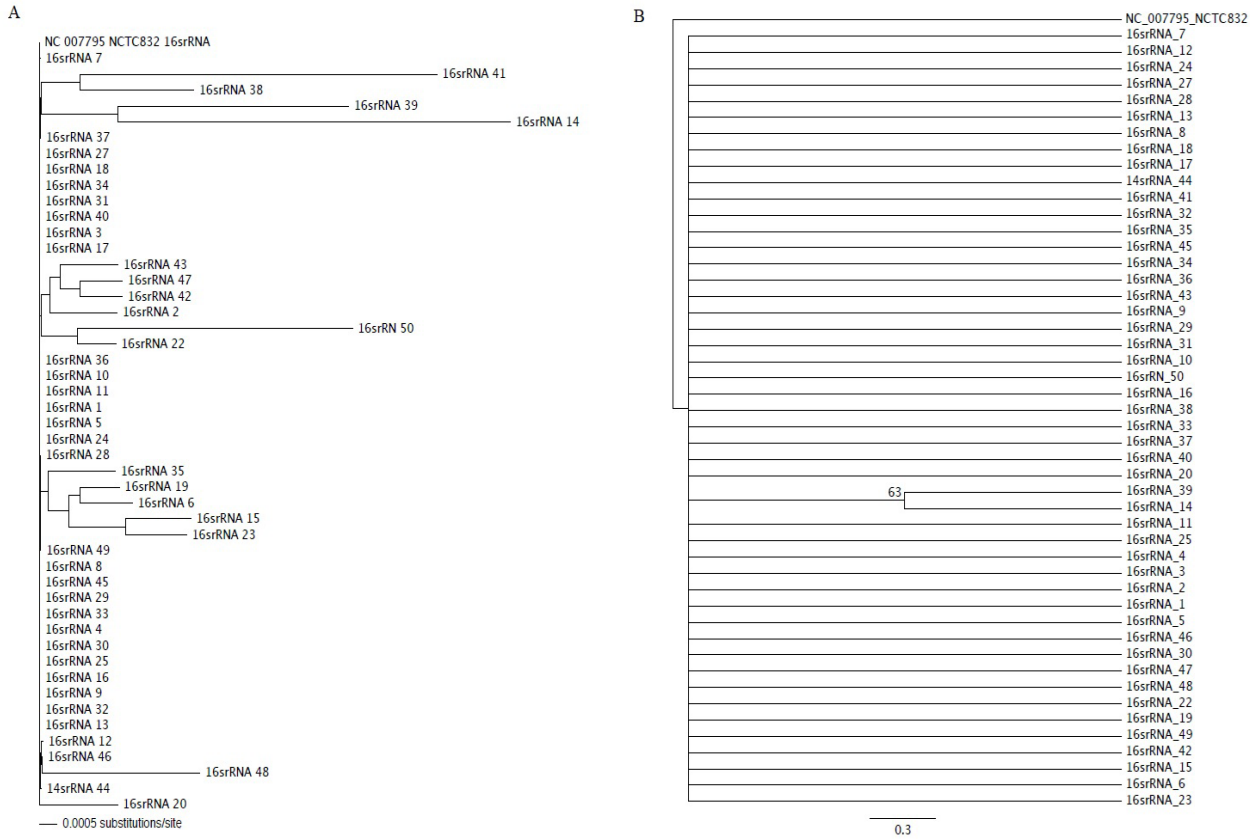

Fig. 1. A) Neighbour-joining and **B)** Maximum Parsimony trees based on the *16S rRNA* gene sequences of 50 *S. aureus* clinical isolates

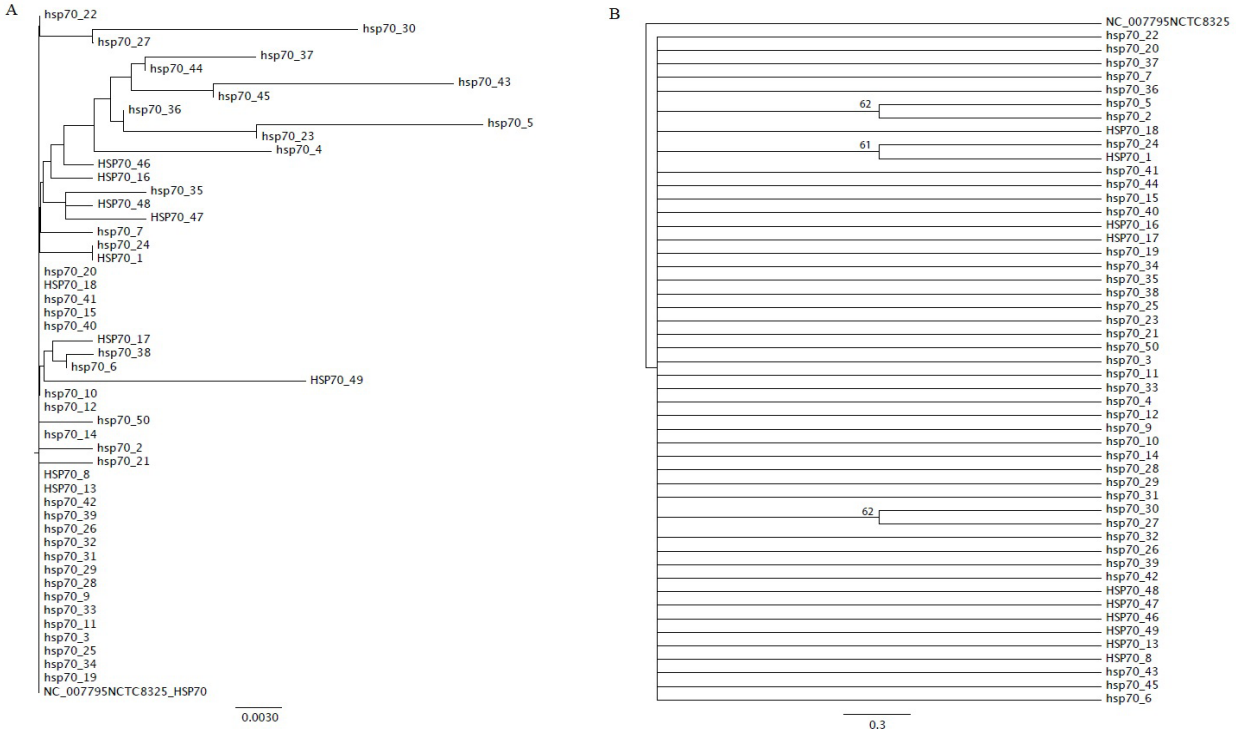

Fig. 2. A) Neighbour-joining and **B)** Maximum Parsimony trees based on the *hsp70* gene sequences of 50 *S. aureus* clinical isolates

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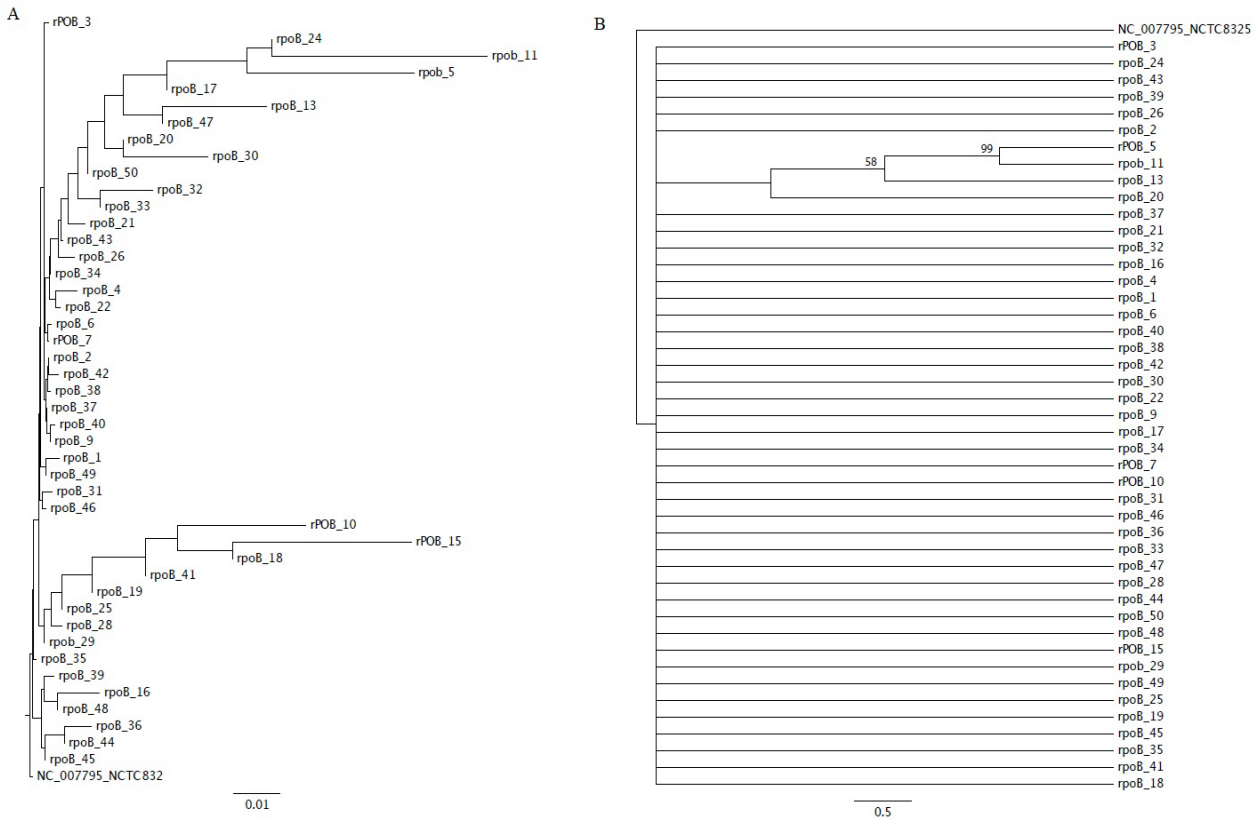

Fig. 3. A) Neighbour-joining and **B)** Maximum Parsimony trees based on the *rpoB* gene sequences of 50 *S. aureus* clinical isolates

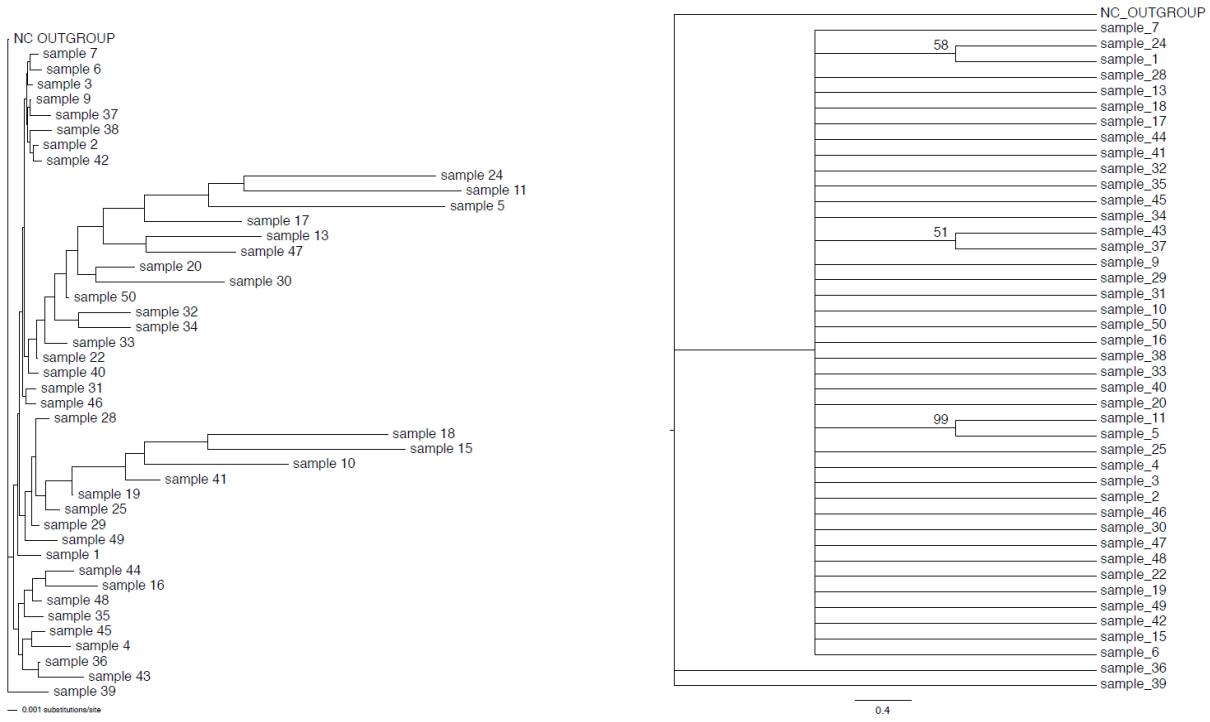

Fig. 4. A) Neighbour-joining and **B)** Maximum Parsimony trees based on the concatenated sequences of the *16S rRNA*, *rpoB*, and *hsp70* genes among the 50 *S. aureus* clinical isolates

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possible differences in their characteristics. Moreover, it could help us distinguish bacterial patterns regarding their hosts and sources. To date, several phenotypic and genotypic methods have been used for the typing of *S. aureus* strains, including biotyping, antibiotic susceptibility testing, pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and PCR-based techniques. Although PFGE has been introduced as an effective method for the typing of *S. aureus* strains, it is not cost-effective and requires technicality and effort. Therefore, in the current study, it was attempted to develop a MLSA method which is considered as an easy, useful, and affordable method [17-18]. Although the *16S rRNA* gene sequencing has remained as the primary choice for the bacterial identification and different molecular targets, different factors such as genetic variation, horizontal gene transfer, and recombination may challenge this approach. Therefore, in this study, a three-locus MLSA scheme was used to reliably identify and characterize *S. aureus* isolates. In fact, using MLSA ensures that recombination at one locus could be compensated by the indications of relatedness between the strains provided by the others [19]. Therefore, in the current study, the polymorphism and genetic diversity of *S. aureus* isolates was investigated based on *16S rRNA*, *hsp70*, and *rpoB* genes which are important in bacterial cell cycle and protein synthesis, thereby affecting the clinical course of infection [20]. Polymorphism could disrupt cell processes including replication, transcription, and translation, which could be horizontally transferred to the next bacterial generations, leading to the emergence or appearance of a single or multiple bacterial characteristics. In fact, any change in genomic organization leads to the emergence of novel genotypes which could affect the rate of infection in any given geographical region [21-22].

To the best of our knowledge, this was the first study using MLSA for *S. aureus* typing. However, similar studies have been conducted to determine the phylogenetic relationship between staphylococcal isolates. Lamers et al. (2012) used *16S rRNA*, *rpoB*, and *hsp60* genes to estimate the relationship between staphylococcal isolates using Bayesian partition modeling and maximum likelihood analysis. According to their phylogenetic estimates, they proposed a refined classification for *Staphylococcus,* in which species were classified into 15 cluster groups according to molecular data [23]. Similar to the current study results, they indicated that *16S rRNA*, *rpoB*, and *hsp60* genes were suitable for distinguishing *S. aureus* strains.

In another study by Seong et al. (2013), the complete *rpoB* and seven partial house-keeping genes sequences of 29 human and poultry isolated *S. aureus* strains were determined, and the phylogenetic analysis of these strains was conducted using the GenBank and EMBL databases. Their RS typing results showed the differentiation between the poultry and human isolated ST5 strains and the mutations related to the rifampin resistance in some human *S. aureus* strains [24], indicating that the study of polymorphism and genetic variations in both antibacterial resistant and sensitive strains could be very useful for comparing the pathogenicity and genomic organization in these isolates. Similar to the current study, they showed that *rpoB* gene could be used to assess the genetic relationship between *S. aureus* isolates.

Conclusion

Today, understanding the phylogeny of bacteria based on housekeeping genes is of great importance. In this study, the polymorphisms were determined, and a

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MLSA technique was developed for 50 *S. aureus* clinical isolates based on *16S rRNA*, *hsp70*, and *rpoB* genes. The MLSA method was clearly capable of discriminating between the *S. aureus* genotypes. Overall, there was a high genetic diversity in the three studied MLSA loci among the 50 *S. aureus* clinical isolates compared to the reference strain of *S. aureus* NCTC 8325. The use of multi-locus sequence analysis and the study of polymorphisms in *S. aureus* clinical isolates are proposed for infection control and surveillance.

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