Shiraz University

IJVR
ISSN: 1728-1997 (Print)
ISSN: 2252-0589 (Online)

Vol. 23 No. 3 Ser. No. 80 2022

IRANIAN JOURNAL OF VETERINARY RESEARCH

Original Article

Phylogenetic relationship, virulence factors, and biofilm formation ability of human, pet animals, and raw milk *Staphylococcus aureus* isolates

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10.22099/IJVR.2022.42752.6208

(Received 10 Jan 2022; revised version 22 Jun 2022; accepted 26 Jul 2022)

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Abstract

Background: Identification of genotypic characteristics and pathogenicity of *Staphylococcus aureus* isolates is very important in the epidemiological study of its related diseases. **Aims:** The present study was done to compare the *S. aureus* isolates from different sources on the basis of virulence gene properties, biofilm production ability, and phylogenetic variations. **Methods:** Seventy *S. aureus* isolates (including 25 human, 25 raw milk, and 20 pet animal isolates) were subjected to slime production ability testing, polymerase chain reaction (PCR) detection of 14 different virulence genes, and DNA fingerprinting using restriction fragment length polymorphism (RFLP) of *coa* gene PCR products. **Results:** Among 70 *S. aureus*, 64 (91.4%) isolates were slime producers on Congo red agar (CRA) medium. The *spa* and *icaD* virulence genes were present in all isolates and the *seD* and *etaA* genes were not detected in any of the isolates. In total, 22 different virulence gene patterns and nine distinct clusters of *coa*-PCR-RFLP were identified among isolates. **Conclusion:** According to the results, *S. aureus* strains of human origin showed a significant association with specific virulence gene profiles and genotypes. *seB* and *seC* were the most responsible genes for *S. aureus* enterotoxin among human and animal isolates, respectively. *Coa*-RFLP showed partially appropriate results in the classification and source detection of *S. aureus* isolates.

Key words: Biofilm, PCR-RFLP, *Staphylococcus aureus*, Virulence factors

Introduction

Staphylococcus aureus the Gram-positive bacterium is one of the most prevalent and important infectious agents in human and animals (Zimmerli *et al*., 2004). This bacterium carries a variety of virulence genes related to infectious diseases. More precisely, *S. aureus* harbors abundant numbers of toxins, enzymes, and other virulent factors including cell surface components such as protein A, fibronectin-binding protein, collagen binding protein, and clumping factor, and exoproteins such as enterotoxins, exfoliatins, toxic shock syndrome toxin, coagulase protein, and Panton-Valentine leucocidin (PVL) (Al-Mebairik *et al*., 2016; Cheung *et al*., 2021). The presence of these potent virulence agents allows the bacterium to show different forms and symptoms in the related infections. Along with this condition, the presence of this bacterium as a human microbial flora and the ability of *S. aureus* in transmission through food, as well as the possibility of spreading infections through animals doubles the importance of this microorganism. In addition, *S. aureus* has the capacity to adhere to devices such as medical catheters and it forms biofilm which can make the microorganism more resistant to antibiotics and it can cause the transmission of the bacterium (Halim *et al*., 2018). Transmission of resistant strains of this bacterium, especially methicillin resistant *S. aureus* (MRSA) strains

from companion animals to humans has been proven. Reports of identification of these strains have been obtained from different countries (Aires-de-Sousa, 2017). Nevertheless, what causes common infections between humans and animals as potential sources of the bacterium and incidence of the similar symptoms is the presence of specific virulence factors among the common strains of the bacterium that circulate regularly among humans, companion animals, foods, and food animals in different ways. Therefore, the present study was conducted to assess the possible associations between human and animal *S. aureus* isolates. As a result, the isolates of *S. aureus* obtained from humans, raw milk (dairy retail shops), and pet animals (dogs and cats) were compared for phenotypic and genotypic biofilm formation ability, their content of different virulent genes, and DNA fingerprinting using valuable *coa*-PCR-RFLP method.

Materials and Methods

Bacterial isolates and DNA extraction

In the present study, a total of 70 *S. aureus* isolates that had been previously isolated and stored at -20°C in the microbiology laboratory of the Faculty of Veterinary Medicine, Amol University of Special Modern Technologies were considered for investigation. The isolates were recovered from sampling process during the summer of 2020 from human cases referred to laboratories, pets referred to veterinary clinics, and raw milk retail shops in the city of Amol, northern Iran. Necessary ethical permission to conduct this study was obtained from Amol University of Special Modern Technologies Ethics Committee with the code of ethics IR.AUSMT.REC.1400.15. (Pahlavanzadeh *et al*., 2021). DNA extraction from *S. aureus* isolates was done using gram positive DNA extraction kit (Sinaclon, Tehran, Iran) according to the manufacturer's instructions. The extracted DNA and the isolates were stored at -20°C for use in other steps of the study.

Biofilm formation assay

Detection of biofilm formation was performed by culturing the *S. aureus* isolated on Congo red agar (CRA) containing 0.8 g of Congo red dye and 36 g saccharose (HiMedia, India) according to the previously described method (Zmantar *et al*., 2008). The isolates were incubated at 37°C for 24 h under aerobic condition and, finally, the results were interpreted as follows: very black, black, and almost black with a rough, dry, and crystalline consistency on CRA were considered to be strong biofilm-producing strains; reddish-black colonies were considered as moderate biofilm; red or pink colonies with a rough appearance indicate the formation of a weak biofilm, while very red, red, and smooth colonies were classified as non-biofilm-producing strains, as previously described (Freeman *et al*., 1989; Arciola *et al*., 2002).

Detection of biofilm and virulence genes

The isolates were examined for the presence of 14 virulence genes in order to determine the potential differences in the presence of the genes in *S. aureus* isolates obtained from different sources. *SeA*, *seB*, *seC*, *seD*, and *seE* related to *Staphylococcus* enterotoxins, *hlyA*, and *hlyB* related to heamolysin production, *etaA*, and *etaB* genes related to exfoliatin toxin, *icaA* and *icaD* for biofilm production, *pvl* gene related to panton-valentine leukocidin (pvl) toxin, *tsst-1* gene for toxic shock syndrome, and toxin-1 and *spa* gene for *S. aureus* immunoglobulin G-binding protein A were all subjected to detection. PCR was performed using specific primers (Table 1) in the final volume of 25 µL (including 12.5 µL of a PCR master mix (Sinaclon, Iran), 1 µL (0.5 µM) of both forward and reverse primers and 2 µL of each DNA) (Lina *et al*., 1999; Mehrotra *et al*., 2000; Jarraud *et al*., 2002; Vasudevan *et al*., 2003). Then the PCR products were evaluated and confirmed using electrophoresis in 1.5% agarose gel with the assistance of the 100 bp DNA marker (Sinaclon, Iran). Different virulent gene patterns were described according to the presence of the genes.

DNA fingerprinting and phylogenetic tree

The genetic similarity of the isolates was analyzed by *coa*-PCR-RFLP technique as previously described (Goh *et al*., 1992). In the first testing step, *coa* gene was partially amplified, using forward: 5´-ACC ACA AGG TAC TGA ATC AAC G-3´ and reverse: 5´- TGC TTT CGA TTG TTC GAT GC-3´ primers which produce 570-970 bp products. The PCR reaction mixture was prepared as stated in previous experiments. The program was run in the PCR machine as follows: initial denaturation was set at 94°C for 4 min, followed by 36 cycles of denaturation at 94°C for 40 s, annealing at 57°C for 40 s, extension at 72°C for 60 s, and a final extension step at 72°C for 7 min. Then PCR products were digested with *Alu*I enzyme (Fermentas, USA). For this purpose, 10.5 µL of distilled water, 2.5 µL of 10X restriction buffers, and 7 µL of the purified PCR products were mixed with 2 U of *Alu*I. The mixture was incubated at 37°C for 1 h. Finally, the product obtained by RFLP method was analyzed using electrophoresis in 2.5% agarose gel and the images of RFLP reactions were loaded in GelClust software for analysis. Genetic similarity was calculated using the Pearson correlation in which 2% of the optimization tolerance and 4% of the position tolerance shift were set. The dendrogram of the isolates was also created by the Dice correlation coefficient and the un-weighted pair group method with arithmetic averages (UPGMA).

Statistical analysis

The results of the study on different variables were analyzed using SPSS version 22 software (IBM Armonk, North Castle, NY, USA). Statistical analyses were carried out with a statistical significant $P<0.05$.

Table 1: Nucleotide sequences used as primers in PCR for identification of *S. aureus* virulence genes

Target gene	Sequence (5´ to 3´)	Annealing temperature (°C)	PCR product size (bp)	Reference
seA	F: GGTTATCAATGTGCGGGTGG R: CGGCACTTTTTTCTCTTCGG	52	102	Mehrotra *et al*. (2000)
seB	F: GTATGGTGGTGTAACTGAGC R: CCAAATAGTGACGAGTTAGG	49	164	Mehrotra *et al*. (2000)
seC	F: AGATGAAGTAGTTGATGTGTATGG R: CACACTTTTAGAATCAACCG	50	451	Mehrotra *et al*. (2000)
seD	F: CCAATAATAGGAGAAAATAAAAG R: ATTGGTATTTTTTTTCGTTC	45	278	Mehrotra *et al*. (2000)
seE	F: AGGTTTTTTCACAGGTCATCC R: CTTTTTTTTCTTCGGTCAATC	50	209	Mehrotra *et al*. (2000)
hlyA	F: CTGATTACTATCCAAGAAATTCGATTG R: CTTTCCAGCCTACTTTTTTATCAGT	52	210	Jarraud *et al*. (2002)
hlyB	F: GTGCACTTACTGACAATAGTGC R: GTTGATGAGTAGCTACCTTCAGT	52	310	Jarraud *et al*. (2002)
etaA	F: GCAGGTGTTGATTTAGCATT R: AGATGTCCCTATTTTTGCTG	48	93	Mehrotra *et al*. (2000)
etaB	F: ACAAGCAAAAGAATACAGCG R: GTTTTTGGCTGCTTCTCTTG	49	226	Mehrotra *et al*. (2000)
icaA	F: CCTAACTAACGAAAGGTAG R: AAGATATAGCGATAAGTGC	44	1315	Vasudevan *et al*. (2003)
icaD	F: AAACGTAAGAGAGGTGG R: GGCAATATGATCAAGATA	45	381	Vasudevan *et al*. (2003)
pvl	F: ATCATTAGGTAAAATGTCTGGACATGATCCA R: GCATCAAGTGTATTGGATAGCAAAAGC	56	433	Lina *et al*. (1999)
Tsst-1	F: ACCCCTGTTCCCTTATCATC R: TTTTCAGTATTTGTAACGCC	50	326	Mehrotra *et al*. (2000)
spa	F: TCAACAAAGAACAACAAAATGC R: GCTTTCGGTGCTTGAGATTC	51	226	Wada *et al*. (2010)

F: Forward, and R: Reverse

Table 2: Slime production ability of the *S. aureus* isolates

Source of sample	Biofilm formation status (%)			Total biofilm producers
	No biofilm	Poor/moderate biofilm	Strong biofilm	
Human (n=25)	2 (8)	13 (52)	10 (40)	23 (92)
Raw milk (n=25)	4 (16)	15 (60)	6 (24)	21 (84)
Pet (n=20)	0 (0)	14 (70)	6 (30)	20 (100)
Total (n=70)	6 (8.5)	42 (60)	22 (31.4)	64 (91.4)

Table 3: Distribution of virulence genes among *S. aureus* isolates

Origin of isolates	Virulence gene distribution (%)													
	seA	*seB*	*seC*	*seD*	*seE*	*hlyA*	*hlyB*	*etaA*	*etaB*	*icaA*	*icaD*	*pvl*	*tsst-1*	*spa*
Human (n=25)	2 (8)	20 (80)	9 (36)	0 (0)	1 (4)	25 (100)	3 (12)	0 (0)	2 (8)	25 (100)	25 (100)	0 (0)	6 (24)	25 (100)
Raw milk (n=25)	3 (12)	0 (0)	25 (100)	0 (0)	4 (16)	24 (96)	20 (80)	0 (0)	0 (0)	23 (92)	25 (100)	3 (12)	0 (0)	25 (100)
Total (n=70)	5 (7.1)	28 (40)	54 (77.1)	0 (0)	6 (8.5)	69 (98.5)	35 (50)	0 (0)	2 (2.8)	67 (95.7)	70 (100)	3 (4.2)	7 (10)	70 (100)

Results

Among 70 *S. aureus* isolates, 64 (91.4%) isolates demonstrated the ability to produce biofilms on the congo red agar medium. The highest number of strong biofilm producer isolates was observed in human origin. Detailed results of the slime production of the isolates have been shown in Table 2. The *spa* and *icaD* genes were detected among all 70 isolates and after them the most prevalent virulent genes were *hlyA* and *icaA* with

the frequency of 69 (98.5%) and 67 (95.7%), respectively. The *seD* and *etaA* genes were not identified in any of the isolates (Table 3). The prevalence of the virulence genes among the isolates showed 22 different virulence gene patterns in which the *seC/hlyA/hlyB/ icaA/icaD/spa* pattern were the most prevalent genes with the frequency of 19 (27.1%). The highest number of virulent gene patterns was observed in human isolates and only one virulence gene pattern was common among three different sources (Table 4). All isolates were typed by *coa*-PCR-RFLP (Figs. 1, 2 and 3). According to the analysis of *coa*-PCR-RFLP fingerprinting, nine distinct clusters (SID = 0.14 and Shannon Entropy = 2.0680) named R-1 to R-13, were identified, the most prevalent genotype of which was the R-8 cluster (Fig. 4). The genotypes R-2 and R-7 were only associated with human isolates and R-8 genotype was significantly associated with pet isolates ($P<0.05$). Human and raw milk isolates showed more common genotypic patterns in comparison with pet isolates (Table 5). Statistical analysis revealed that the difference between the isolates in biofilm production was not significant ($P>0.05$) but the production of biofilm in raw milk isolates was lower than that of the other two origins. A significant relationship was observed between the presence of the *seB* (higher occurrence) and *seC* (lower occurrence) genes and human isolates ($P<0.05$). All isolates with strong biofilm production had both *icaA* and *icaD* genes, but there were not any significant relationship between the biofilm formation and the presence of these two related genes. Prevalence of other virulent genes was not different significantly among isolates from the three various sources ($P>0.05$).

Discussion

S. aureus slime production has been considered as an important virulence aspect contributing to infections associated with medical devices and causing nosocomial infections (Halim *et al*., 2018). The ability to produce biofilms between pet and raw milk isolates makes them more important in human staphylococcal infections. It has already been shown that the presence of *icaA* and *icaD* genes is associated with biofilm production among isolates of catheter-related infections in humans (Arciola *et al*., 2001). In the present study, despite the fact that all strong biofilm producing isolates had both genes and human isolates showing more production of strong slime, there was no significant association between the presence of the genes and the quality of biofilm production. It seems that the other genes of *icaADBC* operon, *icaB*, and *icaC* could play a greater role in producing weak and moderate forms of the slime layer than what it was previously thought. Another remarkable point in the biofilm production by isolates was the high percentage of biofilm-producing isolates on the CRA medium compared to other similar studies (Ciftci *et al*., 2009; Taj *et al*., 2012; Halim *et al*., 2018). From this perspective, CRA medium showed good results in detecting biofilm-producing strains by identifying weak, moderate, and strong slime production ability of the isolates.

Staphylococcal enterotoxins (SEA to SEE) are known as the common causes of human disease. Cheraghi *et al*. (2017) in their study on methicillin resistant *S. aureus* (MRSA) isolated from human clinical samples indicated that the most frequent toxin genes among their isolates were *see* gene (40.5%) followed by *sea* (26.7%) and the

Table 4: Virulence gene profiles of the *S. aureus* isolates obtained from different sources

Virulence gene profiles	Number of isolates			
	Human	Pet	Raw milk	Total
seC/hlyA/icaD/spa	-	1	-	1
hlyA/icaA/icaD/spa	2	-	-	2
seB/hlyA/icaA/icaD/spa	8	-	-	8
seC/hlyA/icaA/icaD/spa	2	1	5	8
seC/hlyA/hlyB/icaD/spa	-	-	2	2
hlyA/hlyB/icaA/icaD/spa	1	-	-	1
seC/hlyB/icaA/icaD/pvl/spa	-	-	1	1
seA/seB/hlyA/icaA/icaD/spa	1	-	-	1
seC/seE/hlyA/icaA/icaD/spa	-	1	-	1
seB/seC/hlyA/icaA/icaD/spa	2	5	-	7
seB/hlyA/hlyB/icaA/icaD/spa	2	-	-	2
seC/hlyA/hlyB/icaA/icaD/spa	-	8	11	19
seB/hlyA/icaA/icaD/tsst-1/spa	1	-	-	1
seC/hlyA/hlyB/icaA/icaD/pvl/spa	-	-	2	2
seB/seC/hlyA/hlyB/icaA/icaD/spa	-	3	-	3
seC/seE/hlyA/hlyB/icaA/icaD/spa	-	-	1	1
seB/seC/hlyA/etaB/icaA/icaD/spa	1	-	-	1
seA/seB/hlyA/icaA/icaD/tsst-1/spa	1	-	-	1
seB/seC/hlyA/icaA/icaD/tsst-1/spa	3	-	-	3
seC/hlyA/hlyB/icaA/icaD/tsst-1/spa	-	1	-	1
seA/seC/seE/hlyA/hlyB/icaA/icaD/spa	-	-	3	3
seB/seC/seE/hlyA/etaB/icaA/icaD/tsst-1/spa	1	-	-	1
Number of patterns	12	7	7	22

Table 5: Distribution of the *coa*-RFLP fingerprinting genotypes

Biological origin of isolate/No.	Frequency of *coa*-PCR-RFLP patterns (%)								
	R1	R2	R3	R4	R5	R6	R7	R8	R9
Human/25	1 (4)	3 (12)	7 (28)	4 (14)	3 (12)	4 (16)	3 (12)	0 (0)	0 (0)
Pets/20	3 (15)	0 (0)	0 (0)	0 (0)	0 (0)	1 (5)	0 (0)	14 (70)	2 (10)
Raw milk/25	4 (16)	0 (0)	6 (24)	1 (24)	3 (12)	5 (20)	0 (0)	1 (4)	5 (20)
Total/70	8 (11.4)	3 (4.2)	13 (18.5)	5 (7.1)	6 (8.5)	10 (14.2)	3 (4.2)	15 (21.4)	7 (20)

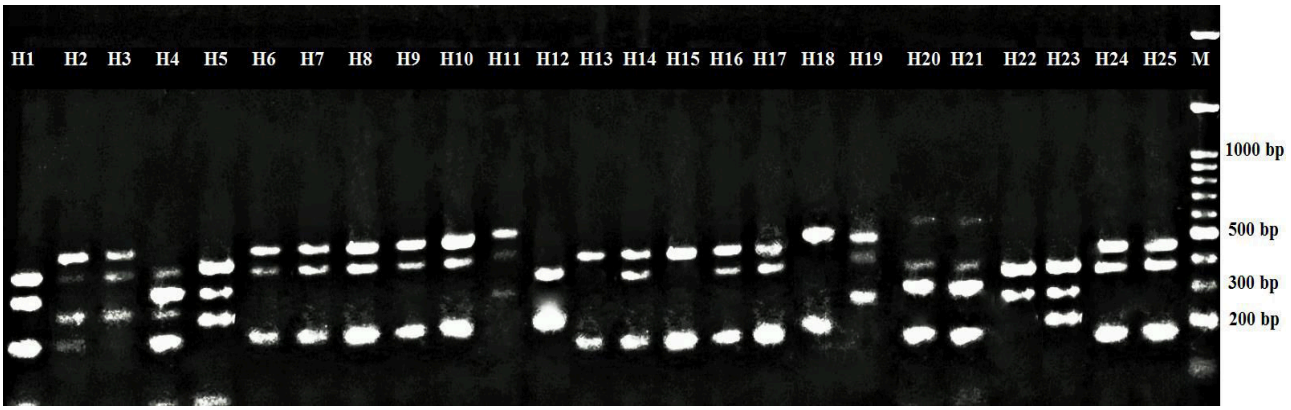

Fig. 1: *coa*-PCR-RFLP results of the *S. aureus* isolates obtained from human samples. Lane M: 100 bp DNA marker. Lanes H1-H25: *Alu*I digestion of *coa* gene of 25 human *S. aureus* isolates

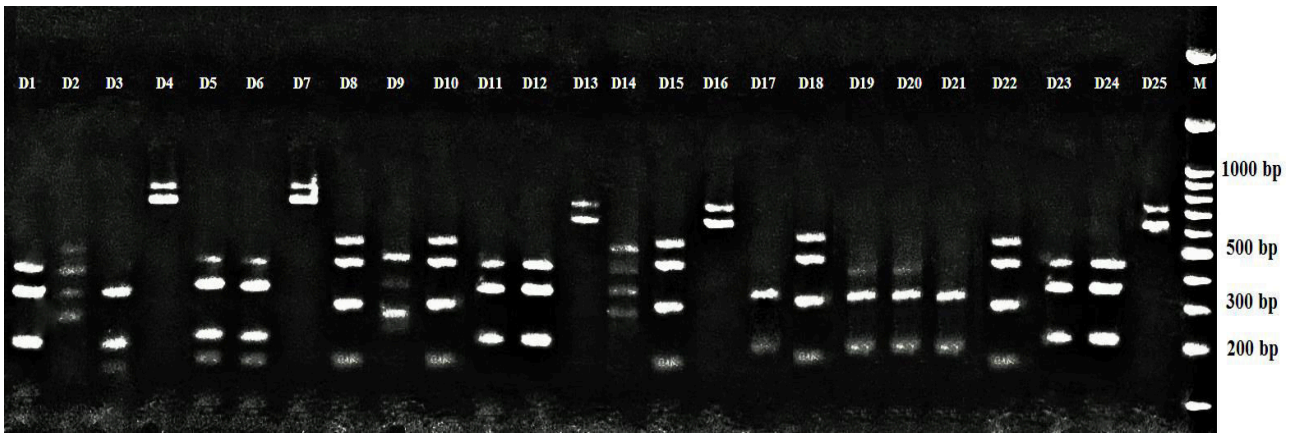

Fig. 2: *coa*-PCR-RFLP results of the *S. aureus* isolates obtained from raw milk samples. Lane M: 100 bp DNA marker. Lanes D1-D25: *Alu*I digestion of *coa* gene of 25 raw milk *S. aureus* isolates

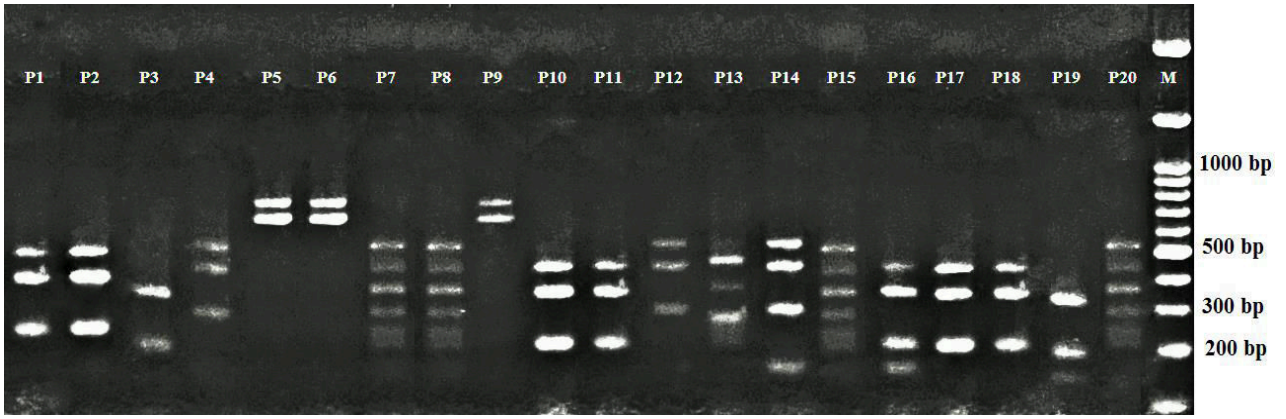

Fig. 3: *coa*-PCR-RFLP results of the *S. aureus* isolates obtained from pet animal samples. Lane M: 100 bp DNA marker. Lanes P1-P20: *Alu*I digestion of *coa* gene of 20 pet animal *S. aureus* isolates

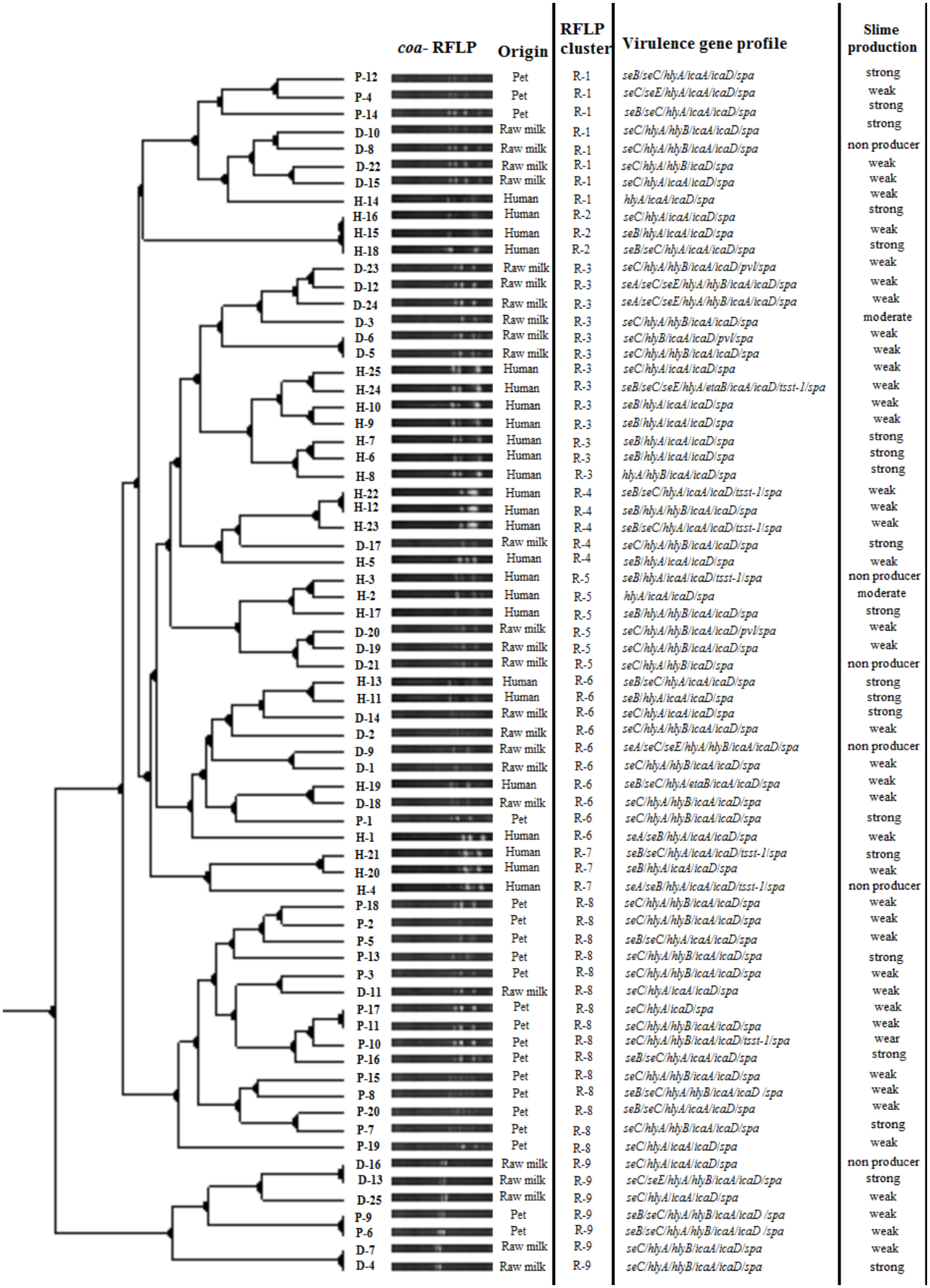

Fig. 4: Dendrogram of *coa*-PCR-RFLP results of the *S. aureus* isolates drawn using the GelClust software in comparison with virulence gene pattern and slime production status

presence of *seb* and *seh* were not obvious in their isolates. In another study, the prevalence of enterotoxigenic *S. aureus* isolates in pork samples was 82.61%, and of the various toxin genes *sei* was the major gene followed by *seg*, *seb*, *sej*, *sed*, *seh*, *sec*, and *sea* (Savariraj *et al*., 2019). In the present study, the most prevalent staphylococcal enterotoxin genes among human and animal isolates were significantly different (P<0.05), *seB* and *seC* respectively, and *seD* were not found among the isolates. Similar differences were observed with other genes, so that *hlyB* and *pvl* genes being more prevalent among animal isolates. On the other hand, this was the case with the *etaB* and *tsst* genes in reverse where the presence of these genes was higher in human isolates. In fact, according to the results, the predominant pattern of the presence of virulence genes was also different between animal and human isolates. Other similar studies previously showed massive presence of twenty-six genes in *S. aureus* isolates obtained from bovine mastitis in Italy which indicate the potential virulence properties of animal *S. aureus* strains (Magro *et al*., 2017).

Previous studies have shown that the *coa*-RFLP based genotyping method has shown acceptable results in comparison with even pulse field gel electrophoresis (PFGE) method for classification and origin expectation of *S. aureus* isolates from different sources. These studies reported various numbers of RFLP genotypes (patterns) which could partially identify the source of infection (Dendani *et al*., 2016; Mohajeri *et al*., 2016). Hakimi Alni *et al*. (2017) found that most of the isolates from pastry showed 860 bp DNA fragment in *coa* gene PCR and the majority of cheese isolates showed a 970 bp DNA fragment related to the *coa* gene. Nevertheless, it seems that the *coa* gene PCR products from *S. aureus* isolates, regardless of the origin of the samples, could be 570 to 900 bp long and this point cannot be significant. In fact, the results of the enzymatic digestion will help to distinguish the source of the samples (Tiwari *et al*., 2008; Mahmoudi *et al*., 2017). In our study, in line with others, the *Alu*I enzyme showed good enzymatic digestion on *coa* gene PCR products which was effective in better separation of bands and prediction of differences (Sohail and Latif, 2018; Aalaa'A and Abd Al-Abbas, 2019). As a final point, the results of the present study, in accordance with others, demonstrated that *S. aureus* isolates from human, pet, and raw milk samples are genetically varied, and that they can be typed by *coa*-PCR-RFLP technique.

In conclusion, the results of the present study indicate that *S. aureus* strains with biofilm formation ability and possible pathogenicity properties are commonly present in pet animals and raw milk samples. A lot of animal isolates carried important virulence genes which suggest a possible origin of transmission of virulence to human strains. *Coa*-PCR-RFLP fingerprinting of *S. aureus* isolates revealed appropriate separation and identification power which can be used for determining the origin of infections in humans and animals.

Acknowledgements

This study was supported by Islamic Azad University, Shiraz Branch, Shiraz, Iran and Amol University of Special Modern Technologies, Amol, Iran.

Conflict of interest

Authors have no conflict of interest.

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