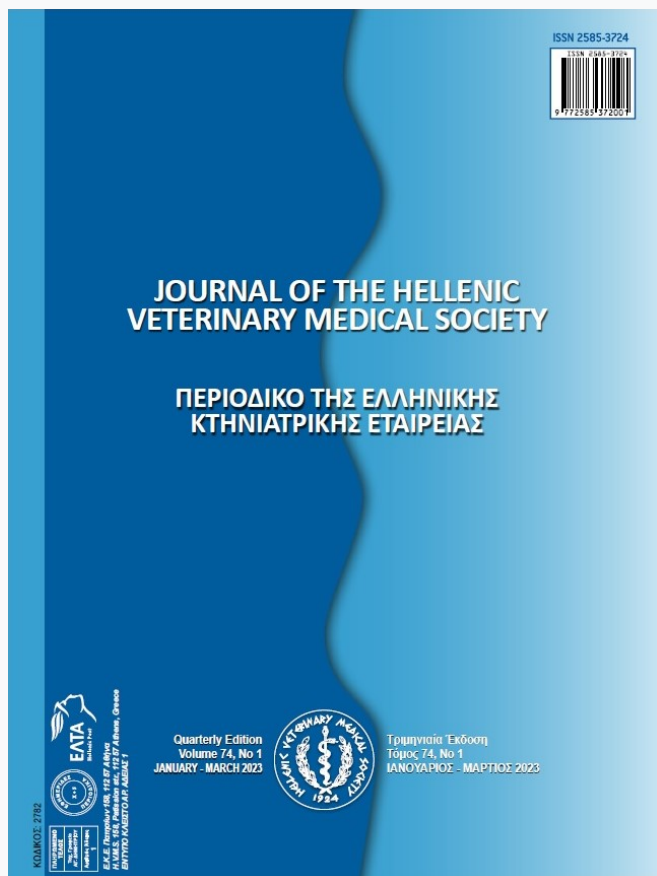


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Molecular typing of *Staphylococcus aureus* isolated from bovine and poultry based on PCR-RFLP of *spa* gene

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ABSTRACT: *Staphylococcus aureus* (*S. aureus*) is considered one of the most important pathogen in bovine and poultry. The aim of the present study was to assess the genotypic polymorphism among *S. aureus* isolate were recovered from bovine and poultry in a geographically restricted area by PCR-RFLP of the *spa* gene. Fifty-eight isolates of *S. aureus* were obtained from bovine mastitis (n=30) and poultry (n=28) during six months. Identification of the isolates was done using conventional methods then confirmed by species-specific PCR method. The *spa* typing was performed by amplification of the *spa* gene X region. Digestion of the amplified *spa* gene fragment with Bsp143I endonuclease produced 4 to 5 bands. Dendrogram obtained by RAPD results revealed eight RAPD clusters (named as A to H). Isolates with more than 70% similarity were clustered together as a same cluster. Cluster A contains most *S. aureus* isolates of bovine origin and cluster B contains most isolates of poultry origin. Only F and H cluster contained both poultry and bovine isolates (7/58, 12%).

Our results confirmed that RFLP of *spa* gene product using *Bsp143I* enzyme can be applied perfectly to differentiate bovine's and chicken's *S. aureus* isolates, also supporting the idea that *S. aureus* isolates with different genotype may constitute a distinct infection.

Keywords: PCR-RFLP, *Staphylococcus aureus*, Mastitis, *spa* gene, Bovine, Poultry.

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INTRODUCTION

S. aureus is able to cause a broad range of human and animal infections. This pathogen considered as the third most common pathogen causing food poisoning in the world (Arefi, Mohsenzadeh et al. 2014). It is considered the most important and prevalent contagious mammary pathogen in bovine mastitis worldwide with the ability to cause chronic infections, increase somatic cell, reduce milk quality and is extremely difficult to treat and eradicate. It causes clinical and subclinical intramammary infection with serious economic loss and herd management problems in dairy cows (DeGo, Van Dijk et al. 2002, Sahebkhari, Nochi et al. 2011).

In poultry, *S. aureus* has been described associated with a variety of different infections such as septic arthritis, osteomyelitis, subdermal abscesses, and gangrenous dermatitis that may result high mortality rate, decreased daily weight gain, decreased egg production and egg quality and lameness and has been found in the bones, joints, skin, sternal bursa, and yolk sac of the birds (Nazia, Durrani et al. 2015). Molecular typing of *S. aureus* can provide a good tool for epidemiological tracing. Several different molecular typing methods with specific advantages and disadvantages have been described for *S. aureus*. Pulsed-Field Gel Electrophoresis (PFGE) and Multilocus Sequence Typing (MLST) are the best methods for molecular typing but some limiting factors such as costs have caused these methods to be used only in certain laboratories (Sahebkhari, Nochi et al. 2011). Protein A is one of the main cell wall protein, unique in *S. aureus*. Polymorphism of the gene encoding this protein is one of the methods used for molecular typing named *spa* typing.

The polymorphic X region consists of a variable number of 21-bp to 27-bp repeats. These differences cause major polymorphisms that used in this typing method (Shakeri, Ghaemi et al. 2014). *spa* typing is highly reproducible and the interpretation of results is simple but require DNA sequencing which is expensive and time consuming in some undeveloped countries. Polymerase Chain Reaction Restriction

Fragment-Length Polymorphism (PCR-RFLP) of *spa* for *S. aureus* typing can replace the DNA sequencing. Different Enzymes, like HaeII and BspI43I have been used for digestion of the amplified PCR products (Wichelhaus, Hunfeld et al. 2001, Shakeri, Ghaemi et al. 2014). There is currently little information on genotyping of *S. aureus* isolated from bovine and poultry in Iran. The aim of the present study was to evaluate the discrimination ability of PCR-RFLP of *spa* gene and epidemiological comparison of *S. aureus* isolate recovered from bovine and poultry in a geographically restricted area.

MATERIALS AND METHODS

A total of 58 isolates of *S. aureus* was included in the present study. The samples were obtained from bovine mastitis (n=30) and poultry (n=28) during a six months period. Subclinical mastitis was diagnosed using California Mastitis Test (CMT). A total number of 30 bovine *S. aureus* isolates from more than 300 individual quarter milk from three different farms at Mashhad city (Khorasan province, Iran) were collected. Poultry isolates came from broilers suffering from femoral head necrosis (n=25), turkey poults with bumble foot abscess (n=2) and a duck with web foot necrosis (n=1).

All samples were cultured on blood agar, isolation and primary identification were done based on conventional methods, including Gram staining, colony morphology, production of coagulase, catalase, DNase and fermentation of mannitol. To confirm the identity of the *S. aureus* isolates, the *nucA* gene was amplified by PCR method using primers listed in Table 1. The confirmed *S. aureus* isolates were stored at -70 °C in brain heart broth plus 20% glycerol.

The whole genomic DNA from cultured strains was prepared using DNA extraction kit (GeneAll, Seoul, South Korea). The *spa* typing by amplification of the *spa* gene X region was performed according to the method described previously (Emaneini, Khoramrooz et al. 2011). Specific primers were used with product lengths varying between 1150-1500 bp (Table 1). PCR reactions were performed in a final volume of 50 µl

Table 1. The oligonucleotide primers used in this study.

Gene	Primers (5'-3')	Size of product (bp)	Reference
<i>nucA</i>	F- CTGGCATATGTATGGCAATTGTT R-TATTGACCTGAATCAGCGTTGCT	613	(Khoramian, Jabalameli et al. 2015)
<i>Spa</i>	F- ATCTGGTGGCGTAACACCTG R- CGCTGCACCTAACGCTAATG	-	(Emaneini, Khoramrooz et al. 2011)

containing 20 µl of Taq DNA polymerase 2x master mix red containing; 2 mM MgCl₂, Tris-HCl pH = 8.5, [NH₄]₂SO₄, 4 mM MgCl₂, 0.2% tween 20, 0.4 mM dNTPs, 0.2 units/µl ampliqon *Taq* DNA polymerase inert red dye and stabilizer (Ampliqon®, Denmark), 0.5 mg/mL of each primer and 5µl of DNA template. The PCR conditions consisted of a pre-denaturation step at 94 °C for 5 min, followed by 35 cycles of 40s at 94 °C, 55°C for 40s and 72 °C for 60 s. A final extension step was performed at 72 °C for 10 min. Primary analysis of amplified products performed by electrophoresis on 1% agarose gel. DNA bands were visualized by staining with ethidium bromide and photographed under UV illumination.

Different Enzymes, like HaeII and Bsp143I have been used for digestion of the amplified PCR products in RFLP method. In the present study, Bsp143I enzyme used for digestion of *spa* gene product as described by the instructions of Fermentas Co., Briefly, 10 µL (~ 0.1-0.5 µg of DNA) of PCR reaction mixture was added to 18 µL of nuclease-free water, 2 µL of 10X buffer x Bsp143I buffer, and 2 µL of Bsp143I

enzyme (Fermentas, Germany). The mixture was mixed gently and spin down for a few seconds and incubated at 37°C for four hours. Afterwards, Bsp143I was inactivated by incubation at 65°C for 20 minutes. The final products after Bsp143I digestion were analyzed by electrophoresis on 1.5% gel. Gel Compare II software was used in pattern analysis and isolates with more than 70 percent similarities were taken as different cluster.

RESULTS

Fifty-eight *S. aureus* strains that were positive in biochemical tests (*Mannitol salt agar*, *DNase test* and *coagulase*) were used and confirmed by the detection of a 613 bp-long fragment of *nuc* gene by PCR. The *spa* gene was amplified in all 58 isolates. Digestion of the amplified *spa* gene fragment with Bsp143I endonuclease revealed restriction patterns consisting of 4 to 5 bands. Dendrogram obtained by RAPD results showed that among the 58 *S. aureus* strains eight RAPD cluster were identified (A-H) (Figure 1). Cluster A was more common among isolates from bovine mastitis (11 of 30 samples). The majority (90%) of

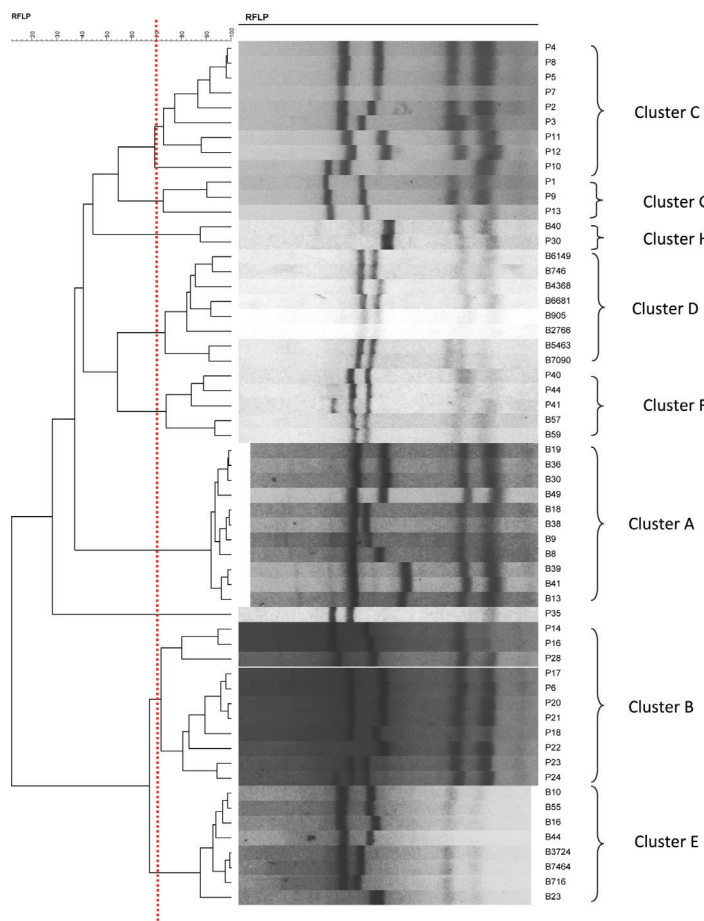


Figure 1. The strain discrimination threshold (dashed line) was set at a similarity value of 70%

Table 2. Different genotypes among the bovine *S. aureus* and poultry isolates

	Number of isolates	Bovine isolates	Poultry isolates
Cluster A	11	11	0
Cluster B	11	0	11
Cluster C	9	0	9
Cluster D	8	8	0
Cluster E	8	8	0
Cluster F	5	2	3
Cluster G	3	0	3
Cluster H	2	1	1
Uncluster	1	0	1
	58	30	28

bovine isolates clustered in cluster A, D and E and only three bovine isolates did not clustered into these clusters. All isolates in cluster D obtained from bovine in farm three. Cluster B contained most *S. aureus* isolates of poultry origin (11/28).82.1% of poultry-origin isolates cluster in B, C and G.Only F and H clusters contained both poultry and bovine isolates (7/58, 12%).Only one poultry strain was not clustered in any of the eight cluster (Table 2).

DISCUSSION

The epidemiology of infectious diseases relies on typing methods for the characterization and discrimination of pathogenic bacteria. These methods were used to determine the clonal relationships between strains and trace the geographic dissemination of bacterial clones.

Molecular methods such as multilocus sequence typing (MLST), *spa* typing and pulsed field gel electrophoresis (PFGE) are among the efficient genotyping methods that frequently used for *S. aureus* (Eslampour, Hovareshti et al. 2009, O'Hara, Suaya et al. 2016).The *Spa* typing and MLST are based on the DNA sequencing method and PFGE needs expensive equipment. An alternative to current techniques for use in research and clinical applications is PCR-RFLP of the *spa* gene.

In this study digestion of the *spa* gene with Bsp143I revealed restriction patterns consisting of 4 to 5 bands. Shakeri et al, (2013)using Hae II enzyme on *spa* gene showed that most isolates (69.7%)have three bands on agarose gel,26.4 and 3.9 percent of isolates showed 2 and 4fragments, respectively.

In the present study, nine cluster of *S. aureus* pattern were classified using dendrogram and the strain discrimination threshold of 70 percent.Shakeri et

al., (2013) diagnosed 3 clusters and 8 types among *S. aureus* isolates that were classified by PCR-RFLP method with HaeII enzyme, the *spa* gene length in the isolated cases were varied from 1150 to 1500 bp.

Ninety percent of bovine isolates in this study clustered in group A, D and E. 82.1 % of poultry host isolates clustered in group B, C and G. Only two clusters contained both poultry and bovine isolates, so digestion of the *spa* gene with Bsp143I may be used as a method to distinguish between *S. aureus* isolates. No epidemiological similarity between bovine and poultry isolates was found in this study. Saei et al., (2020) showed no clonal dissemination between cows and small ruminants in Iran (Saei and Panahi 2020).

Surprisingly, two clusters contained both poultry and bovine isolates that means we can consider other possibilities such as: 1) the method needs improving using proper restriction enzyme for these isolates,2) these isolates might have the same origin, 3) using other methods (sequencing analysis, multilocus enzyme electrophoresis, pulse field gel electrophoresis and so on) our next project is focusing on these isolates. Most of isolates in each farm had a specific cluster and all isolates in cluster D belonged to farm number three, so suggests that they were epidemiologically related or are due to transferring of pathogen between cows in each farm.Eslampour et al., (2009) typed *S. aureus* isolates using PFGE method and revealed seven main types that only one type was pre-dominant in each farm (Eslampour, Hovareshti et al. 2009)

In conclusion, results of present study demonstrated that RFLP of *spa* gene product using Bsp143I enzyme can be used perfectly for differentiating and typing of *S. aureus* isolates.Low epidemiological similarity between *S. aureus* isolates from bovine and

poultry isolates in Iran is also shown, supporting the idea that *S. aureus* isolates with different genotype may constitute a distinct infection.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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