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RESEARCH ARTICLE

Molecular Characteristics of *Staphylococcus aureus* Isolates from Buffaloes Milk [1]

Jalal SHAYEGH [1] Abolfazl BARZEGARI [2] Peyman MIKAILI [3]

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[1] Department of Veterinary Medicine, Shabestar branch, Islamic Azad University (IAU), Shabestar, IRAN
[2] Research Center for Pharmaceutical Nanotechnology, Tabriz University of Medical Sciences, Tabriz, IRAN
[3] Department of Pharmacology, School of Medicine, Urmia University of Medical Sciences, Urmia, IRAN

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Summary

The aim of the present study was to analyse the *aroA* and X region of *spa* genes on *Staphylococcus aureus* isolates collected from milks of native buffaloes in north-west of Iran. For this purpose, seventy five *S. aureus* isolates were examined by PCR-RFLP. Amplification of *AroA* gene revealed a single amplicon with a size of approximately 1,153 bp, but restriction profile analysis of this amplicon yielded three different genotypes including genotype A (9.3 %), B (88%) and N (2.7%). Amplification of X region of the *spa* gene showed an amplicon of 1110; 1130; 1160; 1190 bp in 19, 15, 12 and 29 isolates, respectively. *Hhal* digestion of this amplicons demonstrated that the isolates having the same PCR band size were in the same PCR-RFLP patterns and there was not variation into isolates belonged to the same PCR amplicon.

Keywords: *Staphylococcus aureus, aroA gene, spa gene, PCR-RFLP*

Manda Sütünden Izole Edilen *Staphylococcus aureus* Türlerinin Moleküler Karakterizasyonu

Özet

Bu çalışmanın amacı, İran'ın Kuzey Batısı'nda yerli mandaların sütlerinden izole edilen *Staphylococcus aureus* izolatlarında *spa* genlerinin *aroA* ve X bölgelerini analiz etmektir. Bu amaçla, yetmiş beş *S. aureus* izolatı PCR-RFLP yöntemi ile incelendi. *AroA* gen amplifikasyonu, yaklaşık olarak 1,153 bp büyüklüğünde olan tek bir amplikon oluşturmasına rağmen bu amplikon restriksiyon profil analizi ile genotip A (9.3%), B (88%) ve N (2.7%) olmak üzere üç farklı genotipi oluşturduğu belirlendi. *spa* geninin X bölgesinin amplifikasyonu 19, 15, 12 ve 29 izolatta sırasıyla 1110, 1130, 1160 ve 1190 bp büyüklüğünde fragman ürettiği gözlemlendi. Bu amplikonun *Hhal* enzimi ile kesimi, aynı PCR bant büyüklüğüne sahip izolatların aynı PCR-RFLP profillerinde olduğunu ve aynı PCR amplikonuna ait izolatlar içinde varyasyon olmadığını gösterdi.

Anahtar sözcükler: *Staphylococcus aureus, aroA gen, spa geni, PCR-RFLP*

INTRODUCTION

Buffaloes account for about 5 percent of the milk production in the world but this rate is greater in the Near East and Asia. Buffaloe milk is also considered as an important milk source in Iran [1]. Clinical and subclinical mastitis are associated in decrease of milk production [2].

Among pathogenic bacteria wich have been isolated as etiological agents of mastitis in ruminants, *Staphylococcus aureus* has been mentioned as the main one. The prevalence of subclinical and clinical bovine, caprine and ovine mastitis caused by *S. aureus* ranges from 5 to 50%, 5.6 to 17%, 0.22 to 11% in different countries, respectively [3]. However, only a few studies on staphylococcal mastitis concerning buffalo infections have been published. Staphylococcal mastitis is important in ruminants of Iran and mastitis caused by this organism is a major cause of economical losses in the Iranian dairy industry [4]. *S. aureus* is also one of the main pathogens isolated from buffaloes in this country [5]. Some studies confirm this subject in other countries in Asia [6,7].

İletişim (Correspondence)
+98 914 3008812
jalal_shayeghi@yahoo.co.in

Different molecular methods such as PCR - restriction fragment length polymorphism (PCR-RFLP) analysis of the virulence genes [8-10], multilocus sequence typing [11], pulsed -field gel electrophoresis [12-14], multilocus enzyme electrophoresis [15,16] have been used molecular characterization of the *S. aureus* isolates. Molecular characterization of *S. aureus* isolated from bovine milk samples was studied by many researchers [17]. However, a few papers studied molecular profile of *S. aureus* isolated from buffaloes in worldwide [3,6].

In contrast of some molecular studies on bovine milk isolates of *S. aureus* in Iran [4,18], there are not similar studies in buffalo isolates. The aim of the present study was to characterize analysis of the *aroA* and *spa* gene of *S. aureus* isolates collected from native buffaloes in villages around of Tabriz city, Capital of East Azerbaijan province in Iran.

MATERIAL and METHODS

Samples

Seventy five *S. aureus* isolates were isolated from milk of buffaloes in Tabriz, Northwest of Iran from the microbiology laboratory of Islamic Azad University, Tabriz branch. All isolates had been confirmed by standard biochemical tests [4] and stored in brain heart infusion (BHI) with 30% glycerol at -70°C, previously.

DNA Extraction

A colony of Brain heart infusion (BHI) agar was transferred to a clean microtube and it was added 500 µl lysis buffer (pH 8) including (containing 5 mol NaCl, 1 mol Tris-base, 0.5 mol EDTA and CTAB 2%). Then incubated in 60-65°C for 2 h and centrifuged at 12.000 x g for 5 min. The pellet was resuspended in $CHCl_3$-isoamyl alcohol (24:1), and centrifuged for five min at 12.000 x g. Pellet was incubate with RNAase in 37°C for 30 min, then was resuspended in cold isopropanol and transfer to -20°C for 15 min. In the next step, ethanol 70% was added to supernatant and centrifuged for one min at 12.000 x g. Finally, 50 µl TE-buffer was add to pellet and stored as DNA template.

Confirmation Isolates by PCR

All isolates were confirmed as *S. aureus* by the detection of *nuc* gene by PCR described by Brakstad et al.[19].

Molecular Typing by aroA Gene Restriction Profile Analysis

The *aroA* gene was amplified by PCR using the special primers [8]. PCR products were digested with 50 U *Taq*I (Fermentas) at 65°C for 3 h. All digested fragments were assessed by electrophoretic separation in 2% (w/v) agarose gels.

Molecular Typing by X Region of the spa Gene Restriction Profile Analysis

Amplification of the repeat region of the *S. aureus* protein A gene (*spa*) was performed by using a specific primer set as described previously [18,19]. PCR products were digested with 50 U *Hha*I (Fermentas) at 37°C for 3 h. All digested fragments were assessed by electrophoretic separation in 2% (w/v) agarose gels.

RESULTS

According to amplification of the *nuc* gene, all 75 isolates confirmed as *S. aureus*.

AroA Gene Restriction Profile Analysis

AroA gene amplification revealed a single amplicon with an expected size of approximately 1.153 bp, but restriction profile analysis of mentioned gene by U *Taq*I enzyme indicated three differentiated profile. Sixty-six of the 75 isolates (88%) shows a profile known as genotype B and 7 isolates (9.3%) as genotype A. Two isolates (2.7%) have 4 detectable bands and were considered a different genotype showed in this study *(Fig. 1)*.

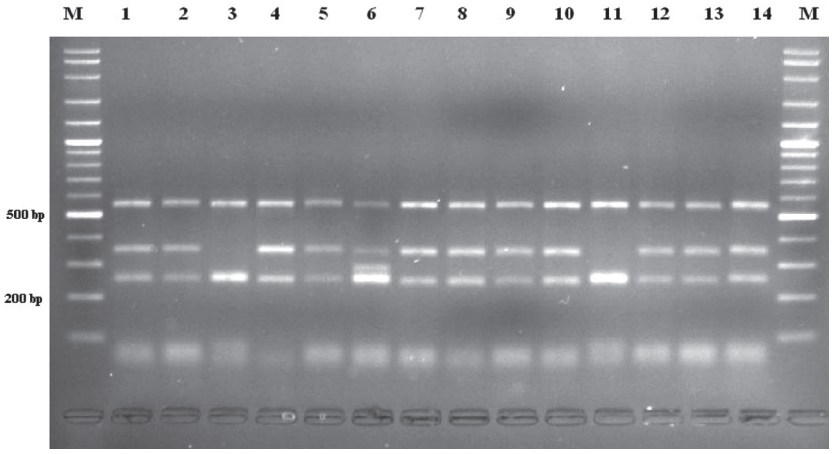

Fig 1. PCR-amplified *aro*A gene digested with the *Taq*I. **Lane M:** DNA marker, **Lanes 1,2,4,5,7** to **10,12** to**14** Genotype B, **Lanes 3,11** Genotype A, **Lane 6** new genotype in this study (N)

Şekil 1. PCR ile amplifiye edilen *aro*A gen amplikonunun *Taq*I ile kesimi. **Sıra M:** DNA marker, **Sıralar 1,2,4,5,7** 'den **10,12**'den**14**'e Genotip B, **Sıralar 3,11** Genotip A, **Sıra 6** bu çalışmadaki yeni genotip (N)

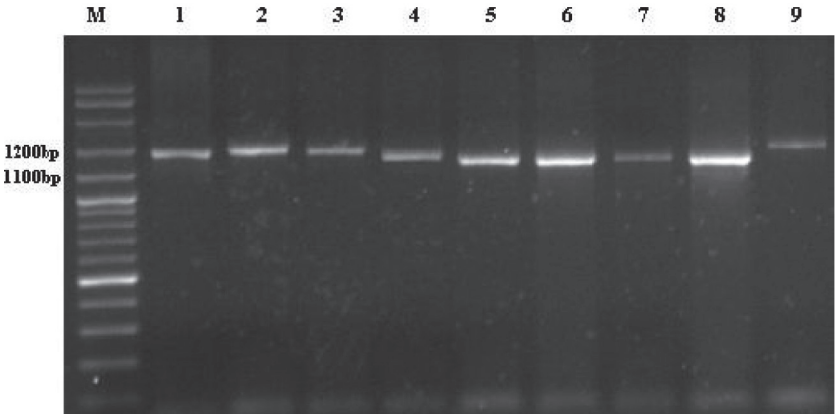

Fig 2. Agarose gel electrophoresis of PCR products obtained from amplification of the *spa* gene. **Lane M:** DNA marker, **Lanes 1,2,3:** 1160, **Lanes 4,7:** 1130, **Lanes 5,6,8:** 1110 and **Lane 9:** 1190 bp

Şekil 2. *spa* geninin amplifikasyonu sonuzu elde edilen PCR ürünlerinin agaroz jel elektroforezi. **Sıra M:** DNA marker, **Sıralar 1,2,3:** 1160, **Sıralar 4,7:** 1130, **Sıralar 5,6,8:** 1110 ve **Sıra 9:** 1190 bp

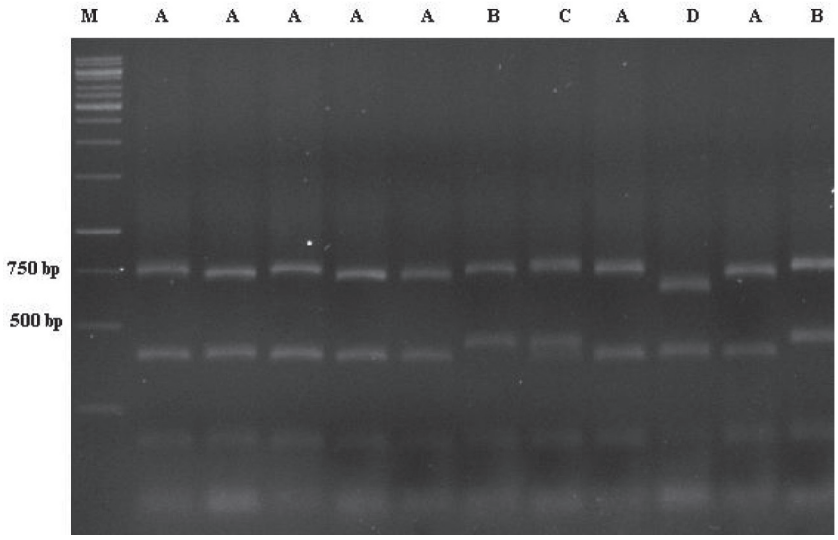

Fig 3. PCR-amplified *spa* gene digested with the *Hha*I. **Lane M:** DNA marker, **Lane A:** pattern produces by 1190 bp band size, **Lane B:** pattern produces by 1130 bp band size, **Lane C:** pattern produces by 1160 bp band size and **Lane D:** pattern produces by 1110 bp band size

Şekil 3. PCR ile amplifiye edilen *spa* gen amplikonunun *Hha*I ile kesimi. **Sıra M:** DNA marker, **Sıra A:** 1190 bp bant büyüklüğü üreten profil, **Sıra B:** 1130 bp bant büyüklüğü üreten profil, **Sıra C:** 1160 bp bant büyüklüğü üreten profil ve **Sıra D:** 1110 bp bant büyüklüğü üreten profil

Analysis of x Region of the spa Gene

All of the 75 *S. aureus* isolates were PCR positive for X region of spa gene. Amplification of the spa gene showed an amplicon of 1110; 1130; 1160; 1190 bp in 19, 15, 12 and 29 isolates, respectively *(Fig. 2)*.

PCR-RFLP of X region of the spa gene demonstrated 4 profiles. Isolates with same PCR band size were same into the PCR-RFLP patterns and there was not variation into isolates belonged to same PCR amplicon *(Fig. 3)*.

DISCUSSION

This paper is the first report describing molecular characteristic of *S. aureus* isolated from buffalo's milk. Studies of the molecular epidemiology of *S. aureus* collected from cows have been already published in the literature worldwide [17] and in Iran [4,18], but there is a few published studies of *S. aureus* isolated from buffalo [3,22].

Similar to *S. aureus* isolated from other source amplicon size of *aroA* gene is a 1,153-bp fragment and this confirmed *aroA* gene is specific for *S. aureus*, hence it could be used as a powerful tool for the identification of *S. aureus* isolates in buffaloes, too.

AroA gene restriction profile studies showed none of *S. aureus* isolated from cattle were genotype C or D in previous [4,8,23]. Our study confirms this again in buffaloes once. It may suggest that genotype C or D have link to isolates from human origins. Marcos et al.[8] reported these two genotype especially D (44%) from human isolates. In animal, instead of genotype C or D, there are different RFLP patterns with various bands named N reported up to 50% in *S. aureus* with bovine origin [4,23]. In this study, we reported 2.7% these ones in buffalo's isolates. High prevalence of genotype B in animal origin isolates in the some studies, included present study, showed this genotype may has been link to animal source [4,8]. In contrast, Marcos et al.[8] reported low prevalence of genotype B in human isolates.

Koreen, et al.[24] suggested a new typing method for *S. aureus* based on sequencing of X region of staphylococcal protein A *(spa)* gene instead of expensive, time-consuming methods such as pulsed-field gel electrophoresis (PFGE), multilocus enzyme electrophoresis (MLEE), multilocus sequence typing (MLST). The X region of *spa* gene is very highly polymorphism that reflex epidemiological variation

among different *S. aureus* isolates. As a substitution for Koreen, et al.[24] method, Wichelhaus et al.[20] designed X region of *spa* gene based on PCR-RFLP, described previously. We used the same method in this study.

The ampilcon size of X region of *spa* gene in different *S. aureus* has varieties because of polymorphism of this region of *spa* gene. The ampilcon size of X region of *spa* gene in this study has not report in previous papers and it may be belonged to new *spa* type. However, the PCR-RFLP of the mentioned region of each different amplicon size have not produced variation in internal of each groups and this suggest sequence based *spa* typing for determination of new spa type of *S. aureus* from buffaloes origin. On the other hand, *spa* gene variation may be related with the geographical and host factors similar to that of *aroA* gene.

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