Jundishapur Journal of Microbiology. 2013 Aug; 6(6): e7934. DOI: 10.5812/jjm.7934

Published Online 2013 August 01. **Research Article**

Evaluation of Protein A Gene Tandem Repeat Polymorphism of *Staphylococcus aureus* Isolated From Bovine Mastitis in Tabriz

Mohammad Ali Hosseinpour Feizi [1], Gholamreza Zarrini [1,*], Mehdi Tahmasebi [1]

[1] Department of Biology, Faculty of Natural science, University of Tabriz, Tabriz, IR Iran

*Corresponding author: Gholamreza Zarrini, Department of Biology, Faculty of Natural science, University of Tabriz, Tabriz, IR Iran. Tel: +98-4113392346, E-mail: Zarrini@tabrizu.ac.ir.

Received: August 26, 2012; **Revised:** January 11, 2013; **Accepted:** February 18, 2013

Background: *Staphylococcus aureus* is responsible for about one third of mastitis cases in dairy cattle and it is also the main pathogen of contagious mastitis.

Objectives: Staphylococcal protein A (SPA) is one of the virulence factors of *S. aureus* which were encoded by spa gene. Different strains of *S. aureus* are varying in dissemination ability and power to infect mammary glands. The *spa* gene region Xr polymorphic sequence can be used for typing.

Materials and Methods: Twenty *S. aureus* cultures were isolated from bovine raw milk and analyzed for the number of repeats in region Xr of the *spa* gene by PCR.

Results: A number of 7-11 repeats in the *spa* gene Xr region were determined. Strains with 10 repeats were 65%, therefore they had the highest percentage in isolates. Seven repeats strains were 20% and each of the strains with 8, 9 and 11 repeats had the frequency of 5%. *S. aureus* strains antibiotic resistance was 35%, 5%, 45% and 40% for Tetracycline, Amoxicillin, Gentamicin and Erythromycin respectively. All strains were susceptible to Methicillin and Vancomycin.

Conclusions: Results of the current study indicated that 80% of strains had more than 7 repeats in the Xr region of the *spa* gene and these data were consistent with the previous findings. Significance and impact of the study: Evaluation of *spa* gene polymorphism can be useful in epidemiological studies on *S. aureus* distribution and its control.

Keywords: Staphylococcus aureus; spa Gene; Polymorphism; Bovine Mastitis

1. Background

Mastitis is the most common disease in dairy cows (1). *Staphylococcus aureus* is responsible for about one third of clinical and subclinical mastitis in dairy cattle which leads to dairy industries economic losses all over the world (2) and it is also the main pathogen of contagious mastitis that transfers during milking between animals (1). Different strains of *S. aureus* are varying in dissemination ability and power to infect mammary glands. To infect mammary glands the bacteria must attach to cells and act against host nonspecific defense mechanisms such as phagocytosis. *S. aureus* produce many virulence factors including coagulase, protein A, leucocid in and several toxins that have a critical role in mammary gland infection (3).

Protein A is a cell wall component of *S. aureus* which inhibits bacterial cell opsonophagocytosis and it can induce complement activation, hypersensitivity and histamine release from basophils (4). Protein A belongs to a group of virulence factors produced during the first phase of infection. The main characteristic of protein A is the ability of specific binding to Fc fragment of immunoglobulins in particular IgG (5). Staphylococcal protein A consists of five repetitive domains which bind to Fc fragment of IgG, and protrude from bacterial cell surface (6). The hydrophobic C-terminal end of the protein includes a sequence required for cell membrane binding and Region X, part of which comprises tandemly repeated octapeptide units, is responsible for the molecule spanning, and the peptidoglycan of the cell wall (4, 7).

Protein A is encoded by an approximately 2 kb *spa* gene composed of different regions. The Fc binding region comprises five 160 bp repeats. The gene encoding protein A includes a polymorphic Xr region of a variable number (3-15) of 24-bp tandemly repeats (9). The number and sequence of repeats differ between bacterial strains. The number of repeats is associated with the dissemination ability of *S. aureus* and strains with more than seven repeats in the Xr region of the *spa* gene are epidemic by high probability strains with seven or less repeats (10).

2. Objectives

The current study aimed to evaluate *spa* gene tandem repeat polymorphism of the *S. aureus* isolated from raw

Implication for health policy/practice/research/medical education:
The current study attempted to evaluate if any differences in the number of repeat units within the X region could be observed among strains isolated from bovine mastitis, that the results can be used for epidemiological studies.

milk samples in Tabriz, Iran, and determine the frequency of different genotypes.

3. Materials and Methods

3.1. Bacterial Isolates

Two hundred and twenty of raw milk samples were collected from cows with clinical and subclinical mastitis symptoms in three herds and some local areas around Tabriz. Sample collection and transfer to laboratory were performed under sterile conditions. Isolates were characterized on the basis of colony morphology on the Mannitol salt agar (MSA) (Himedia, India) and Baird parker agar (BPA) (Liofilchem, Italy) media, Gram staining and coagulase production. Coagulase positive isolates were confirmed as *S. aureus.*

3.2. Xr Region of spa Gene Amplification by PCR

Bacterial DNA extraction was performed by boiling extraction method (12). In practice after an overnight culture on Tripton soy agar (Liofilchem, Italy) two or three colonies were suspended in 50 µl of distilled water and then were boiled at 95°C for 5 min. Then the suspension was centrifuged at 13000 RPM for 1 min and subsequently supernatant that contained bacterial DNA was used as a template in PCR. The Xr region of the *spa* gene was amplified by using Spa F: 5'-AGA CGA TCC TTC AGT GAG C-3' and Spa R: 5'-GCT TTT GCA ATG TCA TTT ACT G-3' oligonucleotide primers (12). The polymerase chain reaction was carried out in 0.2 ml tubes and the reaction was performed in a final volume of 25 µl of mixture containing MgCl2 (2 mM), PCR buffer (1X), 0.2 mM dNTPs, 1.5 µl of each respective primers (1 µM), 1.5 µl of Taq DNA polymerase (2.5 U), 5 µl of DNA template (25 - 50 ng/mL) and 13.5 µl of distilled water.

The PCR was performed with a thermal cycler Senso Quest and for the Xr region amplification, PCR cycles were as follows: 35 cycles of denaturation at 94°C for 30 seconds, primer annealing at 62°C for 30 seconds and extension at 72°C for 35 seconds. An initial preheating step was performed at 94°C for 5 min and the final extension step was elongated to 5 min at 72°C. Amplified PCR products were analyzed by electrophoresis through a 3% agarose gel and ethidium bromide post run staining for 10 min, subsequently visualized in documentation system. To estimate the number of repetitive units in Xr regiona 50 bp DNA step size marker was used and a negative control containing all reagents except DNA was included in the experiment. The bacterial samples were divided into two groups with regard to the number of repeats in the Xr region of the spa gene: I- number of repeats ≤ 7 and II- number of repeats > 7 (1).

3.3. Antibiotic Susceptibility Assay

The isolates were tested for antibiotic susceptibility by standard disc diffusion method on Muller-Hinton agar (Merck, Germany) according to the Clinical and Laboratory Standards Institute (CLSI) recommendations (15). Susceptibility to amoxicillin, gentamicin, tetracycline, erythromycin, Methicillin and Vancomycin was determined. Samples were divided into three groups of susceptible, semi susceptible and resistant according to National Committee for Clinical Laboratory Standards (NCCLS) guidelines (14).

4. Results

Among the 220 raw milk samples, 20 were identified as *Staphylococcus* spp. Positive samples grew as yellow and dark colonies on selective media MSA and BPA respectively. Furthermore all of the positive samples were Gram positive Staphylococci in Gram staining and produced coagulase. The PCR amplification of the Xr region of the gene encoding protein A yielded a single amplicon with distinct size for each sample. Totally, five individual amplicons were observed. According to the size of the PCR products 7 - 11 repeats in the Xr region of the *spa* gene were suggested. The strains with ten repeats had a frequency of 65% that included the highest number of strains. Twenty percent of strains had 7 repeats and the strains with 8, 9 and 11 repeats just included 5% of strains (Table 1). Percentage of group I strains (number of repeats equal or less than 7) was lower than group II (number of repeats more than 7). All of the samples were tested for their resistance to four common antibiotics. Resistance to gentamicin, erythromycin, tetracycline and amoxicillin was observed in 45%, 40%, 35% and 5% of *S. aureus* isolates respectively. All strains were susceptible to Methicillin and Vancomycin (Table 1).

Table 1. Distribution of the Number of Repeats in the Xr Region of the spa Gene and Antibiotic Resistance

Number of Repeats in Xr Region	Number and Percentage of Repeats in Strains	Percentage of Antibiotic Resistance					
		Gentamicin	Erythromycin	Tetracyclin	Amoxicillin	Methicillin	Vancomycin
7	4 (20%)	50	75	25	0	0	0
8	1 (%5)	100	0	0	0	0	0
9	1 (%5)	0	0	0	0	0	0
10	13 (%65)	46	38	46	8	0	0

11	1(%5)	0	0	0	0	0	0

5. Discussion

S. aureus is mostly found on mammalian skin and mucous membranes. It is a major causative pathogen of clinical and subclinical mastitis of dairy cattle and secretes into the milk during milking. Therefore milk and in particular raw milk is considered as important vehicles of bacteria to infect human and other healthy animals. Protein A is an important virulence factor because it binds to the Fc fragment of IgG and protects bacteria during the early stages of infection. Several studies have tried to discriminate between epidemic and nonepidemic strains and much of them have reported that strains with more than seven repeats in the Xr region of the protein A tend to be epidemic, while the presence of seven or fewer repeats was indicative of nonepidemic strains (9).

It is conceivable that a longer Xr region would allow a more favorable exposition of the Fc binding region of protein A at the cell surface, thereby facilitating colonization and infection of the skin, mammary glands, and other sites may be important for epidemic dissemination (9). Previous studies have shown that among strains isolated from cows that were affected by mastitis, a highly variable number of repeats were observed but the frequency of strains with more than 7 repeats was higher (11). Also many of the researches have shown that strains with 7 - 11 repeats are dominant and the frequency of these strains was several times more than other strains (1, 5, 8, 13) however few studies have not confirmed these findings (16). El-Sayed et al. characterized nineteen *S. aureus* strains isolated from birds and found only one with less than seven repeats (17).

One study found that the number of repeats in human isolates was more than those of bovine isolates and bovine isolates were scattered in a number of repeats and vice versa (18). Moreover Kurlenda et al. showed that the majority of MSSA strains had more than 7 repetitive units whereas in the case of MRSA strains no isolates with less than 7 repeats were described (5). Amplification of the Xr region of the *spa* gene yielded a single amplicon for each isolate in the current study. *S. aureus* strains isolated from bovine raw milk had 7 - 11 repeats and isolates with less than 7 repeats were not observed. Consistent with previous findings the current study results showed that 80% of strains had more than 7 repeats in the Xr region of the *spa* gene (1). Because the number of repetitions 2-6 and 12-15 marginal polymorphisms that evolved through duplications and deletions (6) were not observed, it can be concluded that polymorphisms in the intermediate range (7, 9, 10, 12, 15) are directly selected.

Virulence factors are largely host specific so that the contribution of each factor in various diseases and hosts are quantitatively different. The importance and function of some virulence factors differ among strains likely reflecting host-specific adaptation and cross strain inferences should be viewed as presumptive. Similarly, human and bovine isolates have different patterns of *spa* gene polymorphism. In general, it can be concluded that 7 or more repeats in the Xr region of the protein A gene is one of the factors affecting pathogenesis and spread of bacteria. Antimicrobial susceptibility testing results showed that 45% of samples were sensitive or semi-sensitive to all four antibiotics.

Acknowledgements

The authors wish to thank the staff of Shirin Asal dairy Company for their kind assistance in the sample collection.

Authors' Contribution

None declared.

Financial Disclosure

None declared.

Funding/Support

None declared.

References

1. Kuźma K, Malinowski E, Lassa H, Kłossowska A. Analysis of protein A gene polymorphism in Staphylococcus aureus isolates from bovine mastitis. *Bull Vet Inst Pulawy.* 2005;**49**:41-44.
2. Sakwinska O, Morisset D, Madec JY, Waldvogel A, Moreillon P, Haenni M. Link between genotype and antimicrobial resistance in bovine mastitis-related Staphylococcus aureus strains, determined by comparing Swiss and French isolates from the Rhone Valley. *Appl Environ Microbiol.* 2011;**77**(10):3428-32.
3. Lowy FD. Staphylococcus aureus infections. *N Engl J Med.* 1998;**339**(8):520-32.
4. Patel AH, Foster TJ, Pattee PA. Physical and genetic mapping of the protein A gene in the chromosome of Staphylococcus aureus 8325-4. *J Gen Microbiol.* 1989;**135**(7):1799-807.
5. Kurlenda J, Grinholc M, Szweda P. Lack of correlation between X region spa polymorphism and virulence of methicillin resistant and methicillin sensitive Staphylococcus aureus strains. *Acta Biochim Pol.* 2010;**57**(1):135-8.
6. Uhlen M, Guss B, Nilsson B, Gatenbeck S, Philipson L, Lindberg M. Complete sequence of the staphylococcal gene encoding protein A. A gene evolved through multiple duplications. *J Biol Chem.* 1984;**259**(3):1695-702.
7. Guss B, Uhlen M, Nilsson B, Lindberg M, Sjoquist J, Sjodahl J. Region X, the cell-wall-attachment part of staphylococcal protein A. *Eur J Biochem.* 1984;**138**(2):413-20.
8. Ciftci A, Onuk EE, Findik A, Yildirim T, Sogut MU. Molecular typing of Staphylococcus aureus strains from ovine mastitis by pulsed-field gel electrophoresis and polymerase chain reaction based on coagulase and protein A gene polymorphisms. *J Vet Diagn Invest.* 2009;**21**(6):849-53.
9. Frenay HM, Bunschoten AE, Schouls LM, van Leeuwen WJ, Vandenbroucke-Grauls CM, Verhoef J, et al. Molecular typing of methicillin-resistant Staphylococcus aureus on the basis of protein A gene polymorphism. *Eur J Clin Microbiol Infect Dis.* 1996;**15**(1):60-4.

10. Frenay HM, Theelen JP, Schouls LM, Vandenbroucke-Grauls CM, Verhoef J, van Leeuwen WJ, et al. Discrimination of epidemic and nonepidemic methicillin-resistant Staphylococcus aureus strains on the basis of protein A gene polymorphism. *J Clin Microbiol.* 1994;**32**(3):846-7.
11. Jakubczak A, Szweda P, Lukaszewska K, Kur J. Molecular typing of Staphylococcus aureus isolated from cows with mastitis in the east of Poland on the basis of polymorphism of genes coding protein A and coagulase. *Pol J Vet Sci.* 2007;**10**(4):199-205.
12. Mehndiratta PL, Bhalla P, Ahmed A, Sharma YD. Molecular typing of methicillin-resistant Staphylococcus aureus strains by PCR-RFLP of SPA gene: a reference laboratory perspective. *Indian J Med Microbiol.* 2009;**27**(2):116-22.
13. Singh RS, Kumar R, Yadav BR. Distribution of pathogenic factors in Staphylococcus aureus strains isolated from intramammary infections in cattle and buffaloes. *Indian J Biotechnol.* 2011;**10**(4):410-416.
14. National Committee for Clinical Laboratory Standards . *Performance standards for antimicrobial disk susceptibility tests, M2-A5.* 1994.
15. Disk Diffusion Method for Antimicrobial Susceptibility Testing - Version. 2009; Available from: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/Disk_method_description_v1.0.pdf.
16. El-Sayed A, Alber J, Lammler C, Jager S, Woter W, Vázquez HC. Comparative study on genotypic properties of Staphylococcus aureus isolated from clinical and subclinical mastitis in Mexico. *Veterinria Mexico.* 2006;**37**(2):165.
17. El-Sayed A, Alber J, Lammler C, Bonner B, Huhn A, Kaleta EF, et al. PCR-based detection of genes encoding virulence determinants in Staphylococcus aureus from birds. *J Vet Med B Infect Dis Vet Public Health.* 2005;**52**(1):38-44.
18. Indarjulianto S. Protein-A gene polymorphism of Staphylococcus aureus isolated from bovine and humans. *Indonesian J Biotechnol.* 2000;**2000**(12):1-10.