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Full Article

Identification of Toxic Shock Syndrome Toxin-1 (TSST-1) gene in *Staphylococcus aureus* isolated from bovine mastitis milk

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ABSTRACT

Staphylococcus aureus is a major causative pathogen of clinical and subclinical mastitis of dairy domestic ruminants. This agent produces a variety of extracellular toxins and virulence factors including Toxic Shock Syndrome Toxin-1 (TSST-1) which is the major cause of Toxic Shock Syndrome (TSS). In this study 58 *S. aureus* isolates obtained from 9 dairy herds in East and West Azerbaijan provinces of Iran. The tested isolates were identified on the basis of the cultural and biochemical properties as well as by amplification of the *aroA* gene, specific to *S. aureus*. Isolates were also analyzed for the presence of the TSST-1 encoding gene (*tst*) using Polymerase Chain Reaction (PCR). *tst* gene 350 bp was detected in 9 (15.5%) of the total number of 58 isolates. The present study revealed that the PCR amplification of the *aroA* gene could be used as a powerful tool for identification of *S. aureus* from the cases of bovine mastitis. Results also showed that the strain of *S. aureus* which caused mastitis can potentially produce staphylococcal toxic shock syndrome toxin-1. Overall, our results suggest that it is of special importance to follow the presence of TSST-1 producing *S. aureus* in foodstuffs, especially for protecting the consumers from toxic shock syndrome.

Keywords: *S. aureus*, *aroA*, TSST-1, PCR, Mastitis

INTRODUCTION

Inflammation of the bovine udder is usually caused by bacterial infection; however it may be the result of sterile inflammation due to chemical, physical or mechanical trauma (Zadoks *et al* 2002). *S. aureus* is a worldwide pathogen causing many serious diseases in humans and animals. It is the most common etiological agent of clinical and subclinical bovine mastitis (Watts 1988). Mastitis caused by *S. aureus* is a disease of major economic importance to the dairy industry causing reduced milk quality and leading to a loss in production and increased use of drug and veterinary services (Beck *et al* 1992). Furthermore *S. aureus* is one of the most important reasons for bacterial food poisoning. Milk and milk products are common vehicles of Staphylococcal food poisoning (De Buyser *et al* 2001, Jorgensen *et al* 2005). The importance to

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evaluate *S. aureus* pathogenic activity assessing the combination of virulence genes has been emphasized both in human and in veterinary medicine (Zecconi *et al* 2006, Piccinini *et al* 2008). *S. aureus* isolates produce several virulence factors that can contribute in different ways to their pathogenicity (Dinges *et al* 2000, Peacock *et al* 2002). Though the udder pathogenicity of *S. aureus* has not yet been thoroughly explained, its super antigenic toxins, such as enterotoxins (SEs) and TSST-1, appear to be of primary importance (Ferens *et al* 1998). All these toxins are secreted proteins that interact with antigen-presenting cells to induce cellular proliferation and high-level cytokine expression (Marrack & Kappler 1990, Schlievert 1993).

Toxic shock syndrome was first described by Todd *et al* (1978) as a severe acute illness in young children. Although Staphylococci are easy to cultivate and are not difficult to identify, there is still a need for a rapid and sensitive DNA-based assay which is specific for *S. aureus*. Amplification of conserved gene sequences such as *aroA* by PCR (Marcos *et al* 1999) is used for the identification of *S. aureus*.

The *S. aureus* which cause mastitis can potentially produce staphylococcal toxic shock syndrome toxin-1. As there are limited data on the distribution of (*tst*) gene in *S. aureus* isolates, so it has special importance to follow the presence of TSST-1 producing *S. aureus* with different sources.

In this study, 58 *S. aureus* isolates from 370 bovine mastitis milk samples from nine different farms in Tabriz and Urmia regions, Iran were identified on the basis of cultural and biochemical properties as well as amplification of the *aroA* gene specific to *S. aureus* and analyzed for the presence of the TSST-1 encoding gene (*tst*) using polymerase chain reaction.

MATERIALS AND METHODS

Sampling. A total of 58 isolates of *S. aureus* in a period of time 9 month were obtained from 370 bovine mastitic milk collected from nine dairy farms from east and west Azerbaijan provinces of Iran, following the recommendations of the National Mastitis Council methods (National Mastitis Council 1987). All the lactating cows were chosen based on clinical and subclinical mastitis and checked for udder health comprising clinical examination of udder, teats and visual milk inspection. Cows were in first to eighth lactation and were milked twice daily by machine. Teats were washed and dried with a clean towel and sprayed with 70% ethyl alcohol. The first few streams of milk were discarded and 10 ml of milk samples were collected from each quarter in a sterile tube.

Isolation and identification of *S. aureus*. These isolates were cultured on mannitol salt agar (MSA, Merck, Germany). Mannitol-positive colonies suspected as *S. aureus* cultured on sheep blood agar plates and were presumptively identified as *S. aureus* according to the following scheme: Gram-positive cocci, haemolytic on sheep blood agar, catalase positive and coagulase-positive. All coagulase positive isolates were further identified using standard microbiological techniques (Quinn *et al* 1998). The confirmed *S. aureus* isolates were stored at −20°C in tryptic soy broth (TSB, Merck, Germany) plus 15% glycerol until molecular tests were carried out.

Molecular analysis of *S. aureus* isolates

DNA Extraction. DNA extraction was carried out using from 24 hours cultures of the bacteria in 10 ml of brain infusion broth (Merck, Germany) using genomic DNA purification kit (Fermentas, Germany) according to manufacturer's instructions for staphylococci.

PCR amplification of the *aroA* gene. *S. aureus* isolates were evaluated by PCR for the presence of the *aroA* gene, as described by Marcos et al (1999).The PCR was performed in a 25-µl reaction mixture containing 12.5µl of 2X master mix (0.04 U/µl Taq DNA polymerase, reaction buffer, 3 mM $MgCl_2$ 0.4 mM of each dNTP), 0.4 µM of each primer, and 2 µl of template DNA. For the negative control, sterile water was added instead of nucleic acids. As positive control, we used *S. aureus* ATCC 29213. The molecular amplification was conducted for the detection of the *aroA* gene by using species specific primers and thermal profile which is shown in table 1.

Table 1. Oligonucleotide primers and PCR programs for amplification of *aroA* and *tst* gene.

Primers	Sequence (5′–3′)	PCR program[a]	Size (bp)	Reference
aroA		1	1,153	Marcos et al. (1999)
FA1	AAGGGCGAAATAGAAGTGCCGGGC			
RA2	CACAAGCAACTGCAAGCAT			
tst		2	350	Akineden et al. (2001)
TSST-1	ATGGCAGCATCAGCTTGATA			
TSST-2	TTTCCAATAACCACCCGTTT			

[a1] Thirty-two times (92°C, 1 min; 63°C, 1 min; 72°C, 1.5 min)
[a2] Thirty times (94°C, 2 min; 55°C, 2 min; 72°C, 1 min)

Analysis of the PCR products for *aroA* was performed by agarose gel electrophoresis using a 1.2% gel and 0.5 µg/ml ethidium bromide in 0.5X TBE electrophoresis buffer at 80 V for 1 h and photographed under UV light. A single PCR product 1,153-bp was obtained from all *S. aureus* DNA extracts. The size of the PCR product was determined by comparison to the ΦX174 DNA/*Hae*III markers (Fermentas, Germany).

PCR amplification of the *tst* gene. To amplify the *tst* gene The PCR method described by Akineden et al (2001) were used. The PCR reaction mixture (total volume 25 µl) included 12.5µl of 2× master mix (CinnaGen, Iran), the primers (final concentration of each primer 0.4 µM), and 2 µl of extracted DNA. For the negative control, sterile water was added instead of nucleic acids. As positive control, we used *S. aureus* positive reaction.

The amplification of *tst* gene was subjected with the specific primer pairs in the DNA thermal cycling (Corbett Research CP2-2003, Australia) with the program shown in Table 1. The PCR product samples were located onto a 1.2% agarose gel .Electrophoresis was performed for 60 min at 80V, while staining the amplicons with 0.5 µg/ml ethidium bromide, revealed a single amplicon with an expected size of approximately 350 bp. The size of the PCR product was determined by comparison to the 100 bp DNA ladder markers (Fermentas, Germany).

RESULTS

A total of 58 *S. aureus* isolates, which were identified using standard biochemical tests, were subjected to molecular identification using amplification of the *aroA* gene specific to *S. aureus*. Nucleotide primers, FA1 and RA2, from *S. aureus aroA* gene successfully primed the synthesis of an expected 1,153-bp DNA fragment in all *S. aureus* tested (Figure 1).

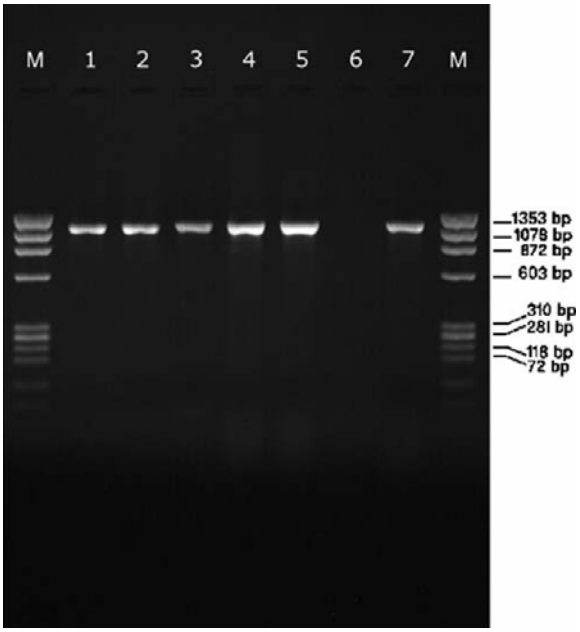

Figure 1. Agarose gel electrophoresis of PCR products obtained from amplification of the *aroA* gene. Lane M: ΦX174 DNA/*Hae*III marker. Lanes 1–5: PCR products with the expected size of approximately 1,153 bp. Lane 6: Negative control (reaction mixture without DNA). Lane 7: Positive control (*S. aureus* ATCC 29213)

All *S. aureus* isolates also analyzed for the presence of the *tst* gene by PCR. 9 (15.5%) isolates were positive for gene and revealed a single amplicon with an expected size of approximately 350 bp DNA fragment (Figur 2).

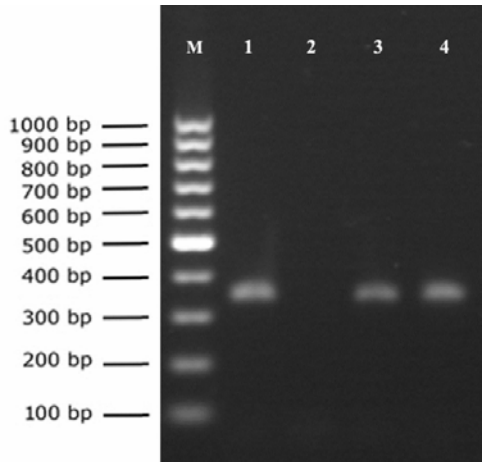

Figure 2. Agarose gel electrophoresis of PCR products obtained from amplification of the *tst* gene. Lane M: 100bp DNA ladder marker. Lane 1: Positive control. Lane 2: Negative control (reaction mixture without DNA). Lanes 3-4: PCR products with the expected size of approximately 350 bp.

DISCUSSION

The fact that bovine mastitis covers approximately 30% of all cows' diseases provides evidence for the potential of its economic significance in dairy cattle industry (De Graves and Fetrow, 1993). Because *S. aureus* is the most frequently isolated agent in mastitis cases all over the world, it is important to reveal subtypes and virulence factors of the agent to develop effective control strategies against mastitis caused by *S. aureus*. Furthermore *S. aureus* is a major foodborne pathogen throughout the world. Enterotoxins and Toxic Shock Syndrome Toxin-1 are important virulence factors and as pyrogenic toxin superantigens have profound effects on the host. So circulation of TSST-1 producing *S. aureus* among people and food chain is a worrying issue. While the conventional methods have some drawbacks, recently developed molecular methods have proved to be efficient. This study therefore aimed to investigate the presence of TSST-1 gene by PCR in *S. aureus* isolates obtained from cows with mastitis.

In this study, by amplification of the *aroA* gene, which is specific to *S. aureus*, 58 isolates were identified as *S. aureus*. As with a previous study by Marcos *et al* (1999), a 1,153-bp fragment was amplified from all *S. aureus*. Marcos *et al* (1999) showed that PCR amplification of the *aroA* gene is specific for *S. aureus* and could be used as a powerful tool for the identification of this microorganism. According to the results of the present study, the *aroA* based identification approach can be used as a complementary molecular method to the phenotypic diagnostic test which is in agreement with Marcos *et al* (1999), which they analyzed 44 *S. aureus* isolates from milk from both cows and ewes with clinical acute mastitis. In the current study all *aroA*-positive strains were tested for *tst* genes by PCR, 9 (15.5%) isolates were positive for TSST-1 gene. Tsen *et al* (1998) in Taiwan, also employing a PCR assay (using the TSST-1 specific primers of the present study), identified three (4.8%) of 62 strains of *S. aureus* from clinical sources as *tst*-carrying strains. Generally staphylococcal enterotoxins and TSST-1 may act as super antigens for cells of the bovine immune system. According to (Ferens *et al* 1998) the super antigenic toxins seem to induce immunosuppression in dairy animals that promotes the persistence of bacteria in cattle and contributes to chronic mastitis. The super antigenic toxins play an important role in modulating the host immune response and may therefore contribute to maintain a suitable environment for colonization (Omoe *et al* 2003). Accordingly, these super antigens have been suggested to enhance the persistence of bovine mastitis. However, as their role as virulence factors in bovine mastitis is still purely speculative and the importance of toxin formation by *S. aureus* for udder pathogenesis remains unclear (Akineden *et al* 2001; Schuberth *et al* 2001).

It has been recognized for many years that TSST-1 from *S. aureus* is the major cause of TSS in humans (Bergdoll 1981). Matsunaga *et al* (1993) concluded that all *S. aureus* isolates from peracute bovine mastitis cases produced TSST-1 and correlated the results with clinical presentation. Smyth *et al* (2005) published a high prevalence (19.2%, 46.2% and 60.9%) of the *tst* gene in bovine, goat and sheep isolates. To the

knowledge of the authors peer reviewed literature reporting the status of TSS in Iran and the occurrence of TSST-1 in clinical strains of *S. aureus* are extremely lacking. However, *S. aureus* contamination of food especially TSST -1 producing isolates in our country is quite remarkable (Eshraghi *et al*., 2009). This is the first time that PCR detection of the TSST-1 encoding gene from mastitic cow milk has been reported from Iran. The importance of this toxin on animal health, which causes toxic shock syndrome particularly in humans, was not explained completely in our country, therefore, large-scale studies are required to determine the presence and role of TSST-1 in *S. aureus* isolates originating from cattle milk. The detection of tsst-1 producing gene, in the isolates of bovine milk origin made it obligatory that raw milk consumption and its processing be considered very carefully.

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