



DETECTION OF TOXIC SHOCK TOXIN (*TST*) GENE IN *STAPHYLOCOCCUS AUREUS* ISOLATED FROM BOVINE MILK SAMPLES

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Summary

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Staphylococcus aureus is a major causative pathogen of clinical and subclinical mastitis in dairy cattle all over the world. 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 the present study, 76 *S. aureus* isolates have been obtained from milk samples collected from 7 dairy herds in Hamedan province of Iran. The isolates were identified based on the biochemical and molecular methods using PCR amplification of the *femA* gene. The staphylococcal isolates were also examined for the presence of TSST-1 (*tst*) encoding gene. This gene was detected in only one *S. aureus* isolate (1.3%). The results revealed that *S. aureus* strains causing bovine mastitis may potentially produce staphylococcal toxic shock syndrome toxin-1, indicating that it is very important to follow the presence of TSST-1 producing *S. aureus* isolates in foodstuffs to protect consumers against the risk of toxic shock syndrome.

Key words: mastitis, *Staphylococcus aureus*, toxic shock toxin (*tst*)

INTRODUCTION

Bovine mastitis is one of the most economically important diseases of dairy cattle which is associated with financial implications and affects both cattle and human health. This disease can have an infectious or noninfectious etiology. Among infectious organisms, bacteria are the most commonly encountered causes of the dis-

ease. Of these bacteria, *Staphylococcus aureus* has frequently been isolated from bovine mastitis (Watts, 1988) and it can directly be transferred by infected milk and/or dairy products or indirectly through environmental contamination of milk during handling and processing (Jorgensen *et al.*, 2005). Protein toxins

and other virulence factors produced by *S. aureus* strains isolated from subclinical and clinical mastitis cases are thought to contribute to the pathogenicity of the organism (Zschokck, 2000). TSST-1 is one of virulence factors which consists of a single-chain polypeptide with a molecular weight of about 22 KDa and an isoelectric point of 7.2. The toxin belongs to the enterotoxins, a larger family of pyrogenic exotoxins produced by the bacterium (Marrack & Kappler, 1990). TSST-1 commonly causes the toxic shock syndrome in humans and animals. TSST-1 has many biologic properties in common with other pyrogenic exotoxins including the ability to induce IL-1, TNF- α , and fever, to enhance lethal endotoxin shock, and stimulate nonspecific T cell proliferation (Takeuchi, 1998). After entering into the bloodstream, the toxin affects the immune system by binding to the proteins of the class-II major histocompatibility complex and the activation of specific T-cell types, leading to a massive release of several cytokines followed by systemic toxicity and suppression of the adaptive immune response, hence the typical clinical signs – multiple organ dysfunction syndrome and eventually fatal shock (Fueyo *et al.*, 2005; Omoe *et al.*, 2005; Vasconcelos *et al.*, 2010). Circulation of these strains in the food chains and human populations, particularly in immunocompromised patients, will increase the risk of developing the disease (Adesiyun *et al.*, 1992; Blaiotta *et al.*, 2006). Therefore, it is important to reveal subtypes and virulence factors of the circulating staphylococcal strains to develop effective control strategies against the disease.

Consequently, the present study was conducted to investigate staphylococcal infections in bovine milk samples from dairy farms of Hamedan province of Iran

followed by possible detection of *tst* gene in the isolated *S. aureus* strains.

MATERIALS AND METHODS

Sampling

A total of 415 milk samples were collected from 7 dairy farms in Hamedan province of Iran. These milk samples were collected in two seasons, warm-dry (n=234) and cold-wet (n=181) during 11 months from March 2013 to January 2014. Selection of lactating cows was carried out based on the abnormal mammary gland, last two-month history of mastitis and drop in milk production. Teats were washed and dried with a clean towel and sprayed with 2% povidone-iodine solution. The first few streams of milk were discarded and 50 mL of milk samples were collected from quarters in a sterile tube and immediately transferred to the microbiology laboratory of Faculty of Veterinary Medicine.

Microscopic somatic cell count (SCC) and California mastitis test (CMT) were carried out on the collected milk samples for direct and indirect estimation of the number of somatic cells in order to identify possible bacterial mastitis.

Bacterial isolates and culture methods

Bacterial strains were isolated using direct cultivation of the milk samples on 5% sheep blood agar followed by diagnostic biochemical tests and media including Gram staining, catalase, coagulase, mannitol salt agar, and DNase agar (Merck).

Extraction of DNA samples

DNA was extracted from each of the isolated bacterial strains identified as *S. aureus* by biochemical tests and media in the previous step using a previously de-

scribed protocol (Reisch, 2000). Briefly, about 3 mL of an overnight Nutrient broth culture of each of the bacterial isolates was transferred into a microtube and the bacterial cells were precipitated at 8000 rpm for 3 min. Afterward, 200 µL of a lysis buffer (1% Triton X-100, 0.5% Tween 20, 10 mM Tris-HCl, 1 mM EDTA, pH=8.0) was added to the pellets and microtubes were incubated in a boiling water bath (100 °C) for 10 min followed by centrifugation at 10000 rpm for 2 min. The supernatants were transferred into clean microtubes and 3–5 µL of each sample was used as template DNA in PCR assays.

Multiplex PCR

Biochemically characterised *S. aureus* isolates (n=76) were also examined by multiplex PCR assays targeting two genes, *femA* and *tst*, to genetically detect *S. aureus* strains and TSST-1 encoding DNA, respectively using oligonucleotide primers previously described (Mehrotra, 2000). *S. aureus femA* gene encodes an essential factor for methicillin resistance and is universally present in all *S. aureus* isolates. The *femA* gene product, a 48 kDa protein, has been implicated in cell wall and metabolism and is found in large amounts in actively growing cultures (Mehrotra, 2000). The sequences of primers are presented in Table 1.

The PCR mastermix (25 µL) contained 3–5 µL of template DNA, 2.5 µL of 10× PCR buffer, 0.75 µL of 50 mM MgCl₂, 0.5 µL of 10 mM deoxyribonucleoside triphosphates, 0.25 µL of 5 U/µL of Taq DNA polymerase, 10 pmol of each of the pairs of primers. The reaction was performed using the following thermal cycling programme; pre-denaturing at 94 °C for 5 min; denaturing at 94 °C for 2 min, annealing at 57 °C for 2 min, extension at 72 °C for 1 min, (35 repeats); final extension at 72 °C for 7 min (Mehrotra, 2000). *S. aureus* strains ATCC 33591 and ATCC 13566 were used as positive controls for *femA* and *tst* genes, respectively. The products of PCR were analysed by electrophoresis on 2% agarose gel in T.A.E. buffer containing ethidium bromide (0.5 µg/mL).

Statistical analysis

The data were compared using Student's *t*-test by SAS software (v. 8.2) and P<0.05 was considered to be statistically significant.

RESULTS

The milk samples containing more than 300,000 cell/mL were considered as positive cases of bovine mastitis in SCC test. A score of one or more was also considered as a positive result in CMT. The

Table 1. Characteristics of the primers used to detect *femA* and *tst* genes

Primer	Oligonucleotide sequence	Target	Product (bp)
GFEMAR-1	5' AAAAAAGCACATAACAAGCG 3'	<i>femA</i>	132
GFEMAR-2	5' GATAAAGAAGAAACCAGCAG 3'		
GTSSTR-1	5' ACCCCTGTTCCCTTATCATC 3'	<i>tst</i>	326
GTSSTR-2	5' TTTTCAGTATTTGTAACGCC 3'		

Table 2. Distribution of *S. aureus* isolates for the two sampling seasons

Season	Number of samples	Number of isolates	CMT positive number (%)	SCC positive number (%)
Warm-dry	234	51	53 (22.64)	69 (29.45)
Cold-wet	181	25	61 (30.26)	63 (31.50)
Total	415	76	114 (27.47)	132 (31.81)

Table 3. Characteristics of culture-positive and culture-negative samples within CMT and SCC groups

<i>S. aureus</i>	CMT positive	CMT negative	SCC positive	SCC negative
Culture-positive	31	45	56	20
Culture-negative	83	256	76	263
Total	114	301	132	283

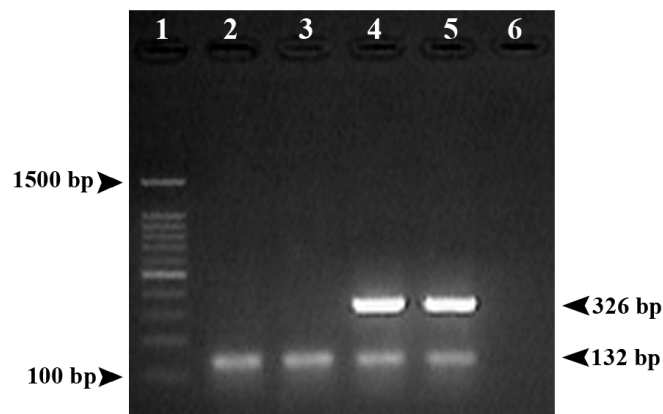


Fig. 1. Agarose gel electrophoresis of the products of multiplex PCR. Lane 1: DNA ladder (100 bp), lane 2: *S. aureus* ATCC 33591 – a positive control for *femA* gene but doesn't have the *tst* gene, lane 3: a *femA*- positive *S. aureus* isolate, lane 4: *S. aureus* ATCC 13566 – a positive control for both *femA* and *tst* genes, lane 5: the only *tst* gene positive *S. aureus* isolate, lane 6: negative control.

results showed that 114 (27.47%) and 132 (31.81%) samples were positive in CMT and SCC, respectively. Table 2 shows the distribution of samples in each season. There was no statistically significant difference between the results of CMT and SCC for collected milk samples in the two seasons. However, the correlation between the results of the two methods was high ($r=0.948$, $P<0.05$).

Seventy six strains (18.31%) were isolated and identified as *S. aureus* from 415 milk samples using biochemical tests and media. The isolated bacteria were also confirmed to be *S. aureus* by PCR using amplification of the *femA* gene which is a specific target for identifying *S. aureus* species. The size of the PCR product for this gene was 132 bp and a DNA fragment with the same size was observed for each of the isolates. The detailed characteristics

of *S. aureus* culture positive milk samples in each group of CMT and SCC positive are given in Table 3. Moreover, *S. aureus* isolates were also simultaneously analysed for the presence of *tst* gene by a multiplex PCR and one isolate (1.31%) was only positive and revealed a single amplicon with an expected size of approximately 326 bp (Fig. 1). This isolate was positive in both CMT (+3) and SCC (1250000 cell/mL) and had been isolated in warm-dry season. The statistical analysis indicated that the difference between the numbers of isolated *S. aureus* strains in warm-dry (51) and cold-wet (25) seasons was statistically significant ($P < 0.05$). Meanwhile, isolation of *S. aureus* strains was relevant to the subclinical mastitis cases with a positive reaction in the CMT and SCC test in the present study. From the total number of 76 *S. aureus* strains, 31 strains were isolated from cases with a CMT score of +2 or +3, and 56 strains were isolated from milk samples which contained higher than 300,000 cell/mL in SCC test (some isolates belonged to both CMT and SCC positive groups).

DISCUSSION

Staphylococcus aureus is a major food-borne pathogen throughout the world and considerably affects human and animal health. The bacterium has several virulence factors including different exotoxins which play important roles in the pathogenesis of the bacterial infection. Although there are no conclusive studies on the relationship between kind of mastitis and toxin production, Matsunaga *et al.* (1993) suggested that toxin production could be more frequent in high pathogenic strains than in strains with low pathogenicity. One of the important virulence factors of *S. aureus* is toxic shock syn-

drome toxin-1 which like staphylococcal superantigenic enterotoxins has profound effects on the host. Although *S. aureus* TSST-1 has been recognised as the major cause of TSS in humans (Bergdoll, 1981) and it has been found that strains with the ability to produce TSST-1 could stimulate T cells of ruminants (Yokomizo *et al.*, 1995), the complete role of TSST-1 in the pathogenesis of mastitis is still unknown. However, it is evident that mastitis can be caused by *S. aureus* strains which lack the capacity to secrete TSST-1. TSST-1 exhibits various biological activities. It is capable of acting like superantigens for cells of bovine immune system and may potentially contribute to the pathological mechanisms of bovine mastitis caused by *S. aureus* strains which produce this toxin (Yokomizo *et al.*, 1995). Superantigenic toxins seem to induce immunosuppression in dairy animals that promotes the persistence of bacteria in cattle and contributes to chronic mastitis (Ferens *et al.*, 1998; Omoe *et al.*, 2003). Accordingly, these superantigens have been suggested to enhance the persistence of bovine mastitis. However, 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; Schubert *et al.*, 2001). Thus, circulation of TSST-1 producing *S. aureus* among human and animal populations, and food chains is a worrying issue.

Consequently, the present study was conducted to investigate subclinical mastitis cases, with *S. aureus* as the causative agent, in apparently healthy cattle and to seek the presence of TSST-1 gene in *S. aureus* isolates obtained from the milk samples using PCR. Our results showed that the numbers of *S. aureus* isolates in warm-dry (21.79%) and cold-wet (13.81%)

seasons were statistically significant different ($P < 0.05$). On the other hand, some milk samples were CMT and SCC positive, while their *S. aureus* culture was negative. This may come from infections with other causative agents. However, a statistically significant difference was not observed between the results of CMT and SCC in the two seasons ($P > 0.05$), suggesting that although these parameters are applicable to distinguish subclinical mastitis, they may not be suitable for presumptive detection of bacterial infections (e.g. *S. aureus*) in such milk samples. The results also showed that only one *S. aureus* isolate (1.32%) had the *tst* gene indicating very low frequency of this gene among staphylococcal agents of mastitis in dairy cattle herds in the investigated area. However, the results proved the presence of this gene in a *S. aureus* isolate originating from an animal source and this is very important, particularly from human health point of view. The results are in agreement with other studies which also did not report high frequency of this gene. Takeuchi *et al.* (1996) performed a study on 125 bovine bulk milk samples and they detected the TSST-1 gene in 10 (8%) of the samples using PCR. Tsen *et al.* (1998) employed a PCR assay and identified that 3 (4.8%) out of 62 strains of *S. aureus* from clinical sources were *tst*-carrying strains in Taiwan. In a research carried out by Farahmand-Azar *et al.* (2013), the TSST-1 gene was detected in 9 (15.51%) out of 58 *S. aureus* strains isolated from milk samples of bovine mastitis cases. Momtaz *et al.* (2010) found 3 TSST-1 positive *S. aureus* isolates (3.48%) in milk samples with high CMT scores. In another study, Larsen *et al.* (2000) found that out of 414 *S. aureus* strains isolated from bovine mastitis cases in Denmark, only one isolate had the TSST-1 gene. Islam *et al.*

(2007) also recognised one TSST-1 positive bacterium (3.33%) from thirty *S. aureus* strains which had been isolated from clinical cases. However, some studies did not report any TSST-1 positive strains from milk and food samples (Tsen *et al.*, 1998; El-Ghodban *et al.*, 2006; Peles *et al.*, 2007). The detection of TSST-1 encoding gene in a *S. aureus* isolate of bovine milk origin in the present study makes it obligatory to consider raw milk consumption and its processing very carefully. As the importance of the presence of TSST-1 and its effects on different hosts have not been completely explained, more studies are required to determine the presence and possible roles of TSST-1 in the pathogenesis of *S. aureus* isolates originating from cattle milk.

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