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Short communication

Molecular typing of *Staphylococcus aureus* isolated from bovine mastitis based on polymorphism of the coagulase gene in the north west of Iran

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ABSTRACT

The objective was to investigate the genotypic characteristics and distribution of *Staphylococcus aureus* in 9 dairy herds of Tabriz and Urmia regions which are located in east and west Azerbaijan provinces, respectively, Iran. In this study 58 *S. aureus* isolates were recovered from 370 milk samples of cows with clinical and subclinical mastitis. *S. aureus* 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* and then were analyzed using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) of the coagulase gene (*coa*). Amplification of the variable region of the *coa* gene from these isolates produced five different PCR products ranging in size from approximately 490 bp to 850 bp. To obtain RFLP patterns of the PCR products they were subjected to digestion with restriction endonuclease *Hae*III and the fragments separated by gel electrophoresis. Nine *coa* gene RFLP patterns, numbered I–IX, were observed, with 23 isolates (39.66%) assigned to RFLP pattern I and 14 isolates (24.14%) assigned to RFLP pattern III. Five out of nine patterns were found in both regions and four of nine patterns were only found in one region. The results demonstrated that several variants of the *coa* gene are present in the studied regions, but only a few of them were predominant, suggesting contagious transmission, a common source, or host adaptation of subset of the population of *S. aureus* strains. This study also indicated that genetic heterogeneity among *S. aureus* isolates recovered from bovine mastitis may be exists within and among herds in different regions.

1. Introduction

Mastitis caused by *Staphylococcus 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). Currently used comprehensive mastitis control protocols (National Mastitis Council, 1996) have decreased the incidence of bovine mastitis, but *S. aureus* still remains one of the most significant organisms associated with clinical and subclinical bovine mastitis in the worldwide. The main reservoir of *S. aureus* seems to be the infected quarter, and the transmission between cows usually occurs during milking (Akineden et al., 2001). In the last few years, numerous molecular techniques such as pulsed-field gel electrophoresis (PFGE) (Zadoks et al., 2002; Melles et al., 2007), multilocus enzyme electrophoresis (MLEE) (Kapur et al., 1995; Enright et al., 2000), multilocus sequence typing (MLST) (Enright et al., 2000), *spa* typing (Strommenger et al., 2008) and coagulase gene typing (Ishino et al., 2007; Reinoso et al., 2008) have been used to identify and compare *S. aureus* genotypes. Coagulase gene (*coa*) typing has been considered as a simple, accurate, sufficiently reproducible, specific, easy to

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interpret and discriminatory method for typing *S. aureus* isolated from distinct sources (Goh et al., 1992; Raimundo et al., 1999; Shopsin et al., 2000; Schlegelova et al., 2003).

Since the mastitis caused by *S. aureus* is a major cause of economical losses in Iranian dairy industry and the scarcity of data on the distribution and genotypic characteristics of this organism in the east and west Azerbaijan provinces of Iran, in the present study 58 *S. aureus* isolates from 370 bovine mastitis milk samples from 9 different herds in Tabriz and Urmia were genotyped by PCR-RFLP analysis of *coa* gene.

2. Materials and methods

2.1. Sampling, isolation and identification of S. aureus

During a time span of 9 months (September 2007 to June 2008) a total of 370 milk samples were collected according to the recommendations of the National Mastitis Council methods (National Mastitis Council, 1987) from cows at mid-lactation. Cows were chosen based on clinical mastitis (recognized by observation and palpation of the udder, presence of clots in the milk and inflammation in the infected quarter) and subclinical (mammary gland without clinical abnormalities and apparently normal milk that were bacteriological positive and with positive CMT) at nine Holstein dairy herds with open management included 300–500 milking cows located in Tabriz ($n = 5$) and Urmia ($n = 4$) regions which are located in east and west Azerbaijan provinces, respectively, Iran. In herds cows were housed in free stall barns. Cows were in first to 8th lactation and were milked twice daily by machine milking. Cows were fed ad libitum by a total mixed ratio that had been formulated to meet the nutritional requirements of a 650-kg cow, yielding 15–40 kg of milk/day with about 3.5% milk fat and 3.4% protein. All cows were subjected to post-milking teat disinfection, those were dried off approximately 2-month before expected calving and all quarters of cows were infused with an antibiotic preparation approved for use in non-lactating cows following the last milking of lactation. Teats were washed thoroughly and dried with a single-use paper towel. The first three streams of milk from each teat were discarded. The teat end and orifice was sanitized with cotton swabs soaked in 10% ethyl alcohol and approximately 10 ml foremilk sample were collected from each quarter of cow in a sterile tube held horizontally. The selection of the herds was carried out to achieve representative distribution across the regions concerning size and location of the herds. Herds in Tabriz region were designated by a T letter (first letter of Tabriz) and the number of herds indicated with a numeric suffix. The similar nomenclature was used for the herds in Urmia region.

A total of 58 isolates of *S. aureus* from Tabriz ($n = 32$) and Urmia ($n = 26$) regions were isolated from these bovine mastitis milk samples. In order to isolate the *S. aureus* isolates, primary culture of milk samples was performed on mannitol salt agar (MSA, Merck, Germany). Yellow colored colonies which were mannitol positive, suspected as *S. aureus* on MSA were selected and subcultured on sheep blood agar plates to make pure culture. Gram stain, culture characteristics, catalase test, and coagulase test were used for the presumptive identification of all isolates (National Mastitis Council, 1999). All coagulase-positive isolates were further identified using standard microbiological techniques (Quinn et al., 1998) and kept frozen at −20 °C in tryptic soy broth (TSB, Merck, Germany) containing 15% glycerol until molecular tests were carried out.

2.2. DNA extraction

S. aureus DNA was extracted from overnight cultures in 10 ml of brain infusion broth (Merck, Germany) by genomic DNA purification Kit (Fermentas, Germany) with some modifications.

2.3. Amplification of the aroA gene

S. aureus species confirmation was also performed by polymerase chain reaction amplification of the *aroA* gene, as described by Marcos et al. (1999) with some modifications. The amplification of *aroA* gene with a pair of primer, FA1 (5′-AAG GGC GAA ATA GAA GTG CCG GGC-3′) and RA2 (5′-CAC AAG CAA CTG CAA GCA T-3′), revealed a single amplicon with an expected size of approximately 1153 bp.

2.4. Coagulase gene typing

Coagulase gene typing was carried out by a modification of the procedure described by Hookey et al. (1998). The method is based on heterogeneity of a region containing 81 bp tandem repeats at the 3′ end of the coagulase gene.

PCR: The 3′ end region of the *coa* gene was amplified with use of the primer pairs previously reported by Hookey et al. (1998): 5′-ATA GAG ATG CTG GTA CAG G-3′ (bases 1513–1531); and 5′-GCT TCC GAT TGT TCG ATG C-3′ (bases 2188–2168). The PCR reaction was carried out by using CinnaGen PCR master kit (CinnaGen, Iran) in 25 μl mixtures containing 12.5 μl of 2X master mix, 0.5 μM of each primer and 4 μl of extracted DNA. For the negative control, sterile water was added instead of nucleic acids. *S. aureus* ATCC 29213 was used as positive control. Amplification was performed in CORBETT thermocycler (Model CP2-003, Australia) by using the following program: 45 s at 94 °C, followed by 30 cycles of 20 s at 94 °C, 15 s at 57 °C, and 15 s at 70 °C with a final step at 72 °C for 2 min (Hookey et al., 1999). A single PCR product was obtained for each isolate, and that band size differed between isolates. Amplified products were separated by 1.2% agarose gel electrophoresis at 80 V for 1 h and photographed under UV illuminator.

DNA restriction endonuclease analysis of the PCR-amplified coagulase genes: After amplification of the variable region of the coagulase gene, 7–10 μl of PCR products were incubated with 6 U of *Alu*I or *Hae*III restriction enzymes (Fermentas, Germany) at 37 °C for 2 h and 30 min. Eight microliters of the digested PCR products were electrophoresed on 1.2% agarose gel, and visualized under UV illumination.