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Detection of biofilm related genes, classical enterotoxin genes and *agr* typing among *Staphylococcus aureus* isolated from bovine with subclinical mastitis in southwest of Iran

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ABSTRACT

Staphylococcus aureus by producing biofilm and facilitating chronic infection is a common cause of mastitis in cows and thereby can cause food poisoning by production of enterotoxins in milk. The *agr* typing method is an important tool for epidemiological investigation about *S. aureus*. The aims of the present study were to detect biofilm related genes, 5 classical enterotoxin genes and the *agr* types among *S. aureus* isolates. The ability of *S. aureus* isolates to produce biofilm was evaluated by modified CRA plate. Six biofilm related adhesion genes (*ica*D, *ica*A, *fnb*A, *bap*, *clf*A and *cna*), five classical enterotoxin genes (*sea, seb, sec, sed* and *see*) and *tst*-1 gene were detected by PCR methods. Multiplex-PCR was used to determination of the *agr* groups. 55 out of 80(68.8%) *S. aureus* isolates were biofilm producer. The *ica*D gene was detected in 70 (87.5%) of isolates. The prevalence rates of *fnb*A, *ica*A, *clf*A, *cna* and *bap* were 72.5, 56.25, 50, 22.5, and 5% respectively. The *agr* group I and III were detected in 57.5% 25% of studied isolates. The *sea*, *sed* and *tst*-1 genes were found in 10%, 7.5% and 1.25% of isolates respectively. The majority of *S. aureus* were able to produce biofilm. Significant associations were observed between presence of the *ica*D, *ica*A, *fnb*A, *clf*A and the *cna* genes as well as biofilm formation. The present study revealed that isolates with the *agr* type III are more potent for biofilm production. Our data supported a possible link between the *agr* types and certain SE genes.

1. Introduction

Staphylococcus aureus is one of the main common causes of mastitis in cows [1] which is contagious and can transmit from infected to non-infected cows at milking in poor hygienic condition [2]. Mastitis is generally classified as clinical or subclinical depending on the degree of inflammation in the mammary gland [3] and contribute to reduced milk production, economic loses and increase management costs. Mastitis also by influencing milk composition, negatively affects milk quality and consumer demand [4]. In spite of decrees in serious mastitis cases, the number of subclinical and serious mastitis cases is still high and difficult to treatment [5]. Many virulence factors have been detected in *S. aureus* strains isolated from cows with subclinical mastitis [6]. Biofilm formation by *S. aureus* is an important factor in mastitis pathogenesis [7]. The ability of biofilm production of *S. aureus* facilitates adherence and colonization of microorganism on the mammary epithelium cells, evasion from host immunological

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response and resistance against antibiotics [8,9]. Two step is needed for biofilm formation, in the first step *S. aureus* attaches to the surface with capsular antigen that known as capsular polysaccharide/adhesion (PS/A), then in the next step by multiplication of bacteria, multilayered biofilm is formed which is related to the production of polysaccharide intracellular adhesion (PIA) [9]. The intracellular adhesion (*ica*) locus is an operon presents in *S. aureus* and is responsible for synthesis of PIA and PS/A. Among *ica* locus, the *ica*A and *ica*D genes play an important role in the biofilm formation rather than other genes [3].

In *S. aureus* isolates from human sources a series of adhesion molecules have been identified which mediate the initial bacteria adhesion to the host tissue. Such adhesions interact and bind to variety of relevant mammalian extracellular proteins, which called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) and are present in microbial surface protein [10]. Several studies have shown that fibronectin binding proteins (FnbA, FnbB), three fibrinogen binding proteins (ClfA, ClfB and Efb) and a collagen binding proteins (Cna) can bind to a variety of mammalian extracellular proteins [11]. Biofilm associated protein (Bap) is one of the adhesion protein of MSCRAMMs group that has an important role in the primary attachment and cell accumulation in *S. aureus* strains isolated from bovine mastitis [12]. Although many studies have been reported the role of the *bap*, *ica*A and *ica*D genes in biofilm formation [7,10,11,13], but little information exist about the function of *fnb*A, *clf*A and *cna* genes in *S. aureus* causing bovine mastitis. Biofilm formation is also associated with reduced susceptibility to antibiotics by reduced dissemination of antibiotics to the multilayered biofilm and also decreased biological activity of bacteria [14].

In the cases of subclinical mastitis, *S. aureus* can access to milk by direct excretion from udders during milking [15]. *S. aureus* is considered the third most common cause of food born disease. This organism can multiply in milk and produce enterotoxins that cause food poisoning if ingested [16]. Staphylococcal enterotoxins (SE) are resistant to heat and cannot be destroyed by cooking. Although this organism dies by pasteurization, but biological activity of toxin remains and can cause food poisoning [17]. Traditionally five classical antigenic SE types (SEA, SEB, SEC, SED and SEE) have been recognized, but in recent years many new types of SE including SEG, SHE, SEI, SEJ, SEK, SEL, SEM, SEM, SEN, SED, SEP, SEQ, SER and SEU have been reported [18].

For epidemiological studies, several typing methods have been used to investigate the genetic relatedness and heterogeneity of *S. aureus*, including pulsed field gel electrophoresis (PFGE); multilocus sequence typing (MLST), polymorphism of protein A gene (*spa* typing) and accessory gene regulator gene (*agr* typing) [19]. The *agr* locus plays an important role in controlling expression of the majority of virulence factors in *S. aureus* [20]. The *agr* locus comprises two divergent operons, RNAII and RNAIII which expressed from promoters P2 and P3 respectively. The P2 operones includes four genes: *agr*D and *agr*B which encode the precursor of the autoinducing peptide (AIP) and processing or secretion of AgrD to form the mature AIP respectively. The two other genes *agr*C and *agr*A encodes the membrane sensor (AgrC), and the response regulator (AgrA). Briefly by increasing bacterial cell density, the concentration of AIP would be increased, then interacts with AgrC in the membrane and activate the AgrA. AgrA is a DNA-binding protein that activates promoter P2 and promoter P3. Promoter P3 initiates transcription of δ-hemolysin and an effector called RNAIII [21,22]. So far based on the amino acid sequence polymorphism of the agr-encoded autoinducing peptide (*agr*D) and its corresponding receptor (Agr C), four major agr groups (I-IV) have been identified [20,23]. Many studies have shown that biofilm formation in *S. aureus* isolated from bovine mastitis with *agr* type I is higher than those with other *agr* types [19,24]. Moreover Francois et al. introduced the *agr* typing as an important tool to deciphering of important epidemiological information about *S. aureus* in clinical isolates [25].

Considering important role of *S. aureus* in bovine mastitis, the aims of the present study were to determine the: 1- ability of *S. aureus* to produce biofilm, 2- detection of important genes involved in biofilm formation, 3- detection of 5 classical enterotoxin genes and 4- determine the *agr* types in these isolates in southwest of Iran.

2. Materials and methods

2.1. Bacterial isolation and identification

This study was carried out in 8 dairy herd (1 large, 9 medium size) in rural area of Kohgiluyeh and Boyerahmad province; southwest of Iran between 2014 and 2015. The milk of 502 cows with positive California Mastitis Test − (CMT) and subclinical mastitis were selected for microbial evaluation. For isolation of *S. aureus*, 10 CC of milk sample were centrifuged at 6000rpg for ten min, after discarding the supernatant, the sediment were inoculated on Mannitol salt agar for primary isolation [26]. Suspected colonies were selected and subcultured on blood agar medium for pure culture. *S. aureus* was identified at the species level using standard biochemical methods including Gram stain, catalase test, tube coagulase, DNase and fermentation of mannitol [27]. Detection of the *nuc*A gene by PCR method was used for confirmation of isolates as described previously by Sahebekhtiari et al. [28].

2.2. Biofilm assay

The ability of *S. aureus* isolates to produce biofilm was evaluated by modified CRA (Congo Red Agar) plates according to Kaiser et al. method [29]. Briefly CRA medium was prepared by adding 0.8 g of Congo red (Sigma-Aldrich, Germany), 15 g NaCl and 36 g of Saccharose (Sigma-Aldrich, Germany) to 1 liter of Brain Heart Infusion Agar (BHI Agar) (Himedia, India). Then 2% glucose and 0.5 μg/ml Vancomycin were added to the autoclaved BHI Agar at 50 °C. A 10 μl of aliquot of a bacterial suspension with 10^8 CFU/mL was inoculated in a spot and was incubated at 35 °C under aerobic condition. The result was checked by two observers independently and the experiments with CRA were performed at least three times. Black and brown colonies were considered as biofilm producing strains while red and dark red colonies classified as non-biofilm producing strains.

2.3. PCR for detection of adhesion genes

Total Chromosomal DNA was extracted by bioloing methods as described previously by Fatholahzadeh et al. [30]. Six adhesion genes (*ica*D, *ica*A, *fnb*A, *bap*, *clf*A and *cna*) that are related to biofilm formation were detected by PCR method in 5 different reactions. The primer sequences for amplification of these genes has been described previously [11,31] which are presented in Table 1. The PCR reaction for one of each gene was carried out in 25 μl volumes containing 12.5 μl master mix (Amplicon, Denmark), 25 ρmol of each primer and 5 μl of template DNA. PCR conditions included initial denaturation (94 °C for 5 min), followed by 32 cycles of denaturation (94 °C for 30 s), annealing 30 s at 49 °C for *ica*A and *ica*D, at 60 °C for *clf*A and *bap*, at 52 °C for *fnb*A, and 54 °C for *cna* and *tst-1*, along with a final extension at 72 °C for 10 min were performed in a thermocycler (BioRad, T100, USA). PCR products were analyzed by electrophoresis in 1.5% agarose gel stained with ethidium bromide (0.5 μg/μl), then was visualized under UV light