PREVALENCE OF COAGULASE NEGATIVE *STAPHYLOCOCCUS* INCLUDING METHICILLIN RESISTANT STRAINS IN BUFFALO SUBCLINICAL MASTITIS IN NORTHWEST OF IRAN

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ABSTRACT

Staphylococcal mastitis is a worrisome dairy problem worldwide. The emergence of coagulase negative staphylococcus (CNS) as the predominant agent of subclinical mastitis is reported frequently.

The aim of the present research was to evaluate the frequency of individual CNS in buffalo subclinical mastitis and to screen the existence of methicillin resistant gene (*mecA*) among them. A total of 279 bubaline subclinical mastitis milk samples were analysed for the presence of *Staphylococcus* spp. phenotypically. The isolates were categorized as coagulase positive or negative. A 23S rRNA-PCR reaction was applied for molecular identification of the isolates. PCR-RFLP patterns generated following the amplification and digestion of a 933 bp fragment of the *gap* gene with *Alu*I restriction endonuclease was used for the identification of CNS species. Finally, *mecA* gene was searched in a PCR reaction among all the isolates. The results represented 61.29% staphylococcal subclinical mastitis with the proportion of 66.08% and 33.91% CNS and *Staphylococcus aureus*, respectively. The frequency of CNS were as 30.40% *Staphylococcus epidermidis*, 26.31% *Staphylococcus simulans*, and 9.35% *Staphylococcus xylosus*. *mecA* gene was detected in 19.29% of the all staphylococcal isolates including 9 *S. aureus*, 15 *S. epidermidis*, 7 *S. simulans*, and 2 *S. xylosus* with no statistical significance among the isolates. These findings manifested the significant role of CNS in buffalo subclinical mastitis in the northwest of Iran. The reservoir status of CNS for *mecA* highlights the need for continuous monitoring programs in order to prevent or diminish the spreading risk of *mecA*-harbouring strains to human.

Keywords: *Bubalus bubalis*, buffaloes, subclinical mastitis, CNS, *mecA* gene, Iran

INTRODUCTION

Mastitis is considered as the most deleterious disease in dairy industry, imposed a plethora of economic burdens. *Staphylococcus* spp. is incriminated as an important etiological agent

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of intramammary infections (IMI) (Bochniarz *et al*., 2013). *S. aureus* is well-known for its relevance in mastitis worldwide. However, the prevalence of coagulase negative staphylococci (CNS)-originated mastitis escalates in various parts of the world, recently (Bochniarz *et al*., 2013; Hosseinzadeh and Dastmalchi, 2014; Rahman *et al*., 2016; Coimbra-e-Souza *et al*., 2017; Pizauro *et al*., 2017). Although CNS commonly yields a mild inflammation in mammary glands leading to subclinical mastitis, clinical mastitis is also reported (Kaliwal *et al*., 2011; Hosseinzadeh and Dastmalchi, 2014). A key problem associated with CNS mastitis is elevation of somatic cell count (SCC) in milk. SCC is a reliable parameter to determine the relationship between IMI and alterations in milk characteristics and therefore evaluating the quality of milk (Aulrich and Barth, 2008). Approximately 15 CNS species are generally involved in mastitis. In order to determine their respective significance and to apply effective controlling procedures of mastitis, it is needed to identify the individual CNS species (Bergeron *et al*., 2011).

Because of the climate and geographical conditions in northwest of Iran and the local welcome for buffalo milk, rearing of buffalo in the form of small-scale dairy farms has a long traditional background in the region. Buffalo milk is never pasteurized and the dairy products including cream, butter, ice cream and yogurt are prepared from warm milk without boiling. Therefore, the magnitude of CNS mastitis is due to not only economic impediments but also hygienic and sanitation issue regarding the microbial load of milk (Schukken *et al*., 2009). Moreover, acquisition of *mecA* gene, a common feature among *Staphylococcus* spp., encodes a low affinity protein, PBP2a. This confers resistance against beta lactam antibiotics. Transmission and propagation of staphylococcal harbouring *mecA* gene from animal reservoirs to human may jeopardize remedy protocols in human health sector (Garza-Gonzalez *et al*., 2010).

The aim of the current investigation was to assess the role and *mecA* carrier state of CNS species in buffalo subclinical mastitis in the northwest of Iran.

MATERIALS AND METHODS

In a cross-sectional study, from May 2015 to May 2018, a total of 279 subclinical buffalo mastitis milk samples were collected aseptically from all over west Azerbaijan province in the northwest of Iran. Subclinical mastitis was identified by the California mastitis test (CMT). Sampling was in accordance with the procedures of the National Mastitis Council (1999). The samples were stored in a cool box and transferred to the microbiology laboratory within maximum five hours and analysed for bacteriological identification. Initial cultivation of the samples was undertaken on blood agar (Merck, Germany) containing 5 to 7% ovine blood. After a 24 to 48 h incubation period in 37^{o}C, the isolates were identified based on colony morphology, gram staining, and catalase test. Gram positive, catalase positive cocci suspected to *Staphylococcus* spp. were further characterized as coagulase positive or negative by the coagulase tube test (Quinn *et al*., 1998).

Genomic repertoire of each isolate was extracted using gram positive DNA extraction kit according to the manufacture's recommendation (SinaClon, Iran). Molecular identification of the isolates was performed in a genus-specific (23S rRNA gene) PCR reaction using the primer pair and thermal condition described by Forsman *et al*.

(1997). PCR-restriction fragment polymorphism (PCR-RFLP) of the *gap* gene was applied for classification of staphylococcal isolates to species level. A 933 bp fragment of the *gap* gene was amplified in a PCR reaction based on the method introduced by Yugueros *et al*. (2000). Fast digest *Alu*I restriction endonuclease was used to digest 10 µL of PCR products under the conditions described by the supplier (SinaClon, Iran). The RFLP patterns of the PCR products were visualized on 1.1% agarose gel (SinaClon, Iran) following electrophoresis at 80 V for 3 h. The known PCR-RFLP patterns of staphylococcal species were used to identify the isolates in species level (Yugueros *et al*., 2000; Yugueros *et al*., 2001). Finally, *mecA* gene was probed in all *Staphylococcus* species based on the PCR protocol represented elsewhere (Geha *et al*., 1994). The statistical relationship between the frequency of *mecA* gene and *Staphylococcus* species were analysed in Chi-square test using SPSS software (version 22). A P-value ≤0.05 was regarded as significant.

RESULTS AND DISCUSSION

An important issue of subclinical mastitis is due to reduction in milk quantity and quality. This, in addition to lack of any clinical symptoms which postpones identification and subsequent curing of the infection, constitutes great economic losses to dairy husbandry. *Staphylococcus* spp. are among the most prevalent bacteria causing IMIs in domestic animals (Hashemi *et al*., 2011). Despite some literature which have investigated staphylococcal clinical and subclinical mastitis in Iran (Hashemi *et al*., 2011; Hosseinzadeh and Dastmalchi Saei 2014; Rahman *et al*., 2016), there is still scant information about the role of the bacterium in IMI in buffalo in the northwest of Iran. In the present study a total of 171 (61.29%) staphylococcal strains were isolated from mastitis milk samples in phenotypic method, among which 113 (66.08%) and 58 (33.91%) were CNS and coagulase positive staphylococcus (CPS), respectively. DNA bands in the range of 100 to 300 bp were generated in 23S rRNA gene amplification in all of the isolates, confirming them as *Staphylococcus* spp. molecularly (Figure 1). The most prevalent bacteria isolated from bubaline subclinical mastitis were CNS (Ali *et al*., 2008; Medeiros *et al*., 2013; Pizauro *et al*., 2017). Besides, CNS were stated as the predominant bacteria isolated from dairy buffalo milk in Italy (Moroni *et al*., 2006). The frequency of staphylococcal species isolated from bovine clinical and subclinical and ovine subclinical mastitis in the studied region were 71.5% and 36.48%, respectively (Hosseinzadeh and Dastmalchi Saei, 2014; Rahman *et al*., 2016). Hosseinzadeh and Dastmalchi Saei (2014) segregated the staphylococcal species frequency as 17.7% (0% CPS, 100% CNS) and 82.3% (5.37% CPS, 94.62% CNS) in clinical and subclinical mastitis, respectively. While this proportion was 11.2% CPS versus 88.80% CNS in ovine subclinical mastitis (Rahman *et al*., 2016). In another study undertaken in Iran, the prevalence of staphylococcal subclinical mastitis in goats was 20% with the frequency of 12.17% CPS and 7.82% CNS (Ebrahimi *et al*., 2010). Other studies have reported CNS as the predominant causal organism associated with subclinical mastitis in domestic animals from various countries with different prevalence rates (Dhakal *et al*., 2007; Thorberg *et al*., 2009; Raspanti *et al*., 2016). Climate conditions, management strategies, nutritional status and breed may influence the distribution of CNS mastitis among different studies (Beheshti *et*

al., 2010). It is documented that in the herds with high milk production rate and use of successful preventive practices of major mastitis pathogens, the frequency of CNS is high. CNS are part of skin microbiota and also known as environmental staphylococcus. The use of dried manure solids or organic materials as bedding in the studied herds may be a risk factor for CNS IMI (Ferguson *et al*., 2007). Another possible explanation for colonization of CNS in teat canals and udder tissue is due to loosing of teat sphincter as a consequence of late lactation or high parity (Krishnamoorthy *et al*., 2016). Moreover, the high prevalence of CNS implies that current mastitis management procedures including teat disinfection and dry animal therapy is not sufficient in controlling CNS IMIs (Hosseinzadeh and Dastmalchi, 2014).

Glyceraldehyde-3-phospahte de-hydro-ge-nase (*gap*) gene is exploited as a dependable housekeeping gene used for molecular differentiation of staphylococcus species. This is based on RFLP patterns render by restriction endonucleases (Yugueros *et al*., 2000; Yugueros *et al*., 2001). It is proved that *Alu*I restriction endonuclease has the most discriminatory power for PCR-RFLP analysis of the *gap* gene (Bal *et al*., 2010). A single 933 bp amplicon of the *gap* gene was produced in all of the isolates (Figure 2) and incised using *Alu*I enzyme. Comparing with available patterns (Yugueros *et al*., 2000; Yugueros *et al*., 2001), Four RFLP patterns generated among the 171 staphylococcal isolates represented *Staphylococcus aureus* (58 isolates, 33.1%), *Staphylococcus epidermidis* (52 isolates, 30.40%), *Staphylococcus simulans* (45 isolates, 26.31%), and *Staphylococcus xylosus* (16 isolates, 9.35%) (Figure 3). *S. simulans* and *S. xylosus* are related to environmental CNS species (Piessens *et al*., 2011), while *S. epidermidis* is a normal microbiota of human skin (Nagase *et al*., 2002). Considering similar pulsed-field gel electrophoresis types recognized in *S. epidermidis* originated from milk and dairymen, it is speculated that the bacterium may be transferred from human to cows (Thorberg *et al*., 2006). An extensive variation is being reported regarding CNS IMI in domestic animals. Our results are in accordance with previous study introduced *S. simulans* and *S. epidermidis* among the most common CNS isolated from bubaline mastitis (Ali *et al*., 2008). The distribution of CNS subclinical mastitis in buffalo reported by Pizauro *et al*. (2017) was 26.25% with the highest proportion of *S. chromogenes* (55.95%), *S. agnetis* (11.90%), and *S. epidermidis* (7.14%) among CNS isolates. Besides other CNS species including *S. caprae*, *S. equorum*, *S. haemolyticus*, *S. hominis*, *S. pasteuri*, *S. sciuri*, *S. saprophyticus*, and *S. warneri* were also detected (Pizauro *et al*., 2017). In a study undertaken in Nepal, CNS including *S. epidermidis* (11.1%) and *S. albus* (33.3%) were the most common bacteria isolated from buffalo subclinical mastitis (Dhakal *et al*., 2007). *S. chromogenes* was the mere CNS isolated from buffalo subclinical mastitis reported elsewhere (Coimbra-e-Souza *et al*., 2017). The most frequent CNS species involved in ovine subclinical mastitis in the studied region were *S. epidermidis*, *S. xylosus* and *S. chromogenes* (Rahman *et al*., 2016). In comparison, *S. haemolyticus* and *S. chromogenes* devoted the highest prevalence among the CNS species involved in bovine mastitis in the northwest of Iran (Hosseinzadeh and Dastmalchi, 2014). It is revealed that virulence content and genetic antimicrobial resistant determinants of CNS species may affect and alter pathogenicity, propagation, and sustainability of the isolates. Hence, analysing the genetic components of CNS species is pertinent in elucidation of the frequent distribution of some

Figure 1. Agarose gel electrophoresis of PCR products generated from 23S rRNA gene amplification in staphylococcal species.
M: 100 bp DNA plus Ladder (SinaClon, Iran), Lane 1: negative control,
Lane 2: positive control (*S. aureus* ATCC 29213), Lane 3-10: Different staphylococcal species.

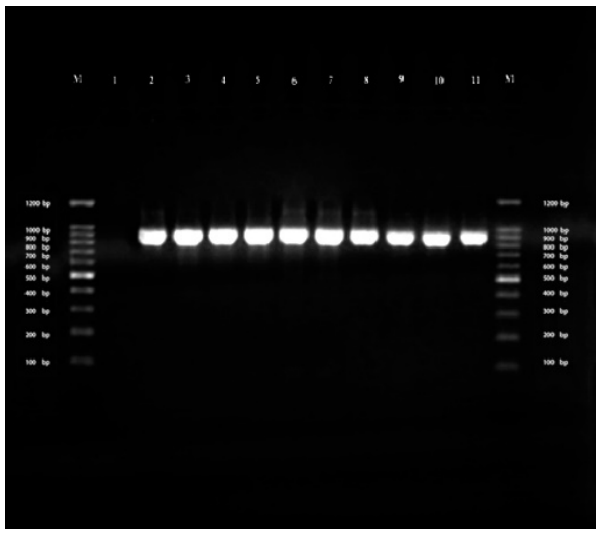

Figure 2. Agarose gel electrophoresis of PCR products generated from *gap* gene amplification in staphylococcal species.
M: 100 bp DNA plus Ladder (SinaClon, Iran), Lane 1: negative control,
Lane 2: positive control (*S. aureus* ATCC 29213),
Lane 3-10: showing amplified *gap* gene in isolates (933 bp).

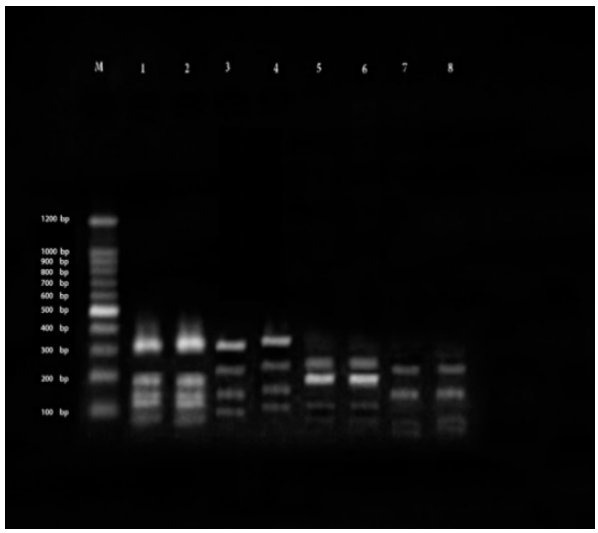

Figure 3. Agarose gel electrophoresis of RFLP patterns generated from digestion of *gap* gene band with *Alu*I endonuclease.
M: 100 bp DNA plus Ladder (SinaClon, Iran), Lane 1 and 2: pattern for *S. aureus*.,
Lane 3 and 4: pattern for *S. simulans*, Lane 5 and 6: pattern for *S. xylosus*,
Lane 7 and 8: pattern for *S. epidermidis*.

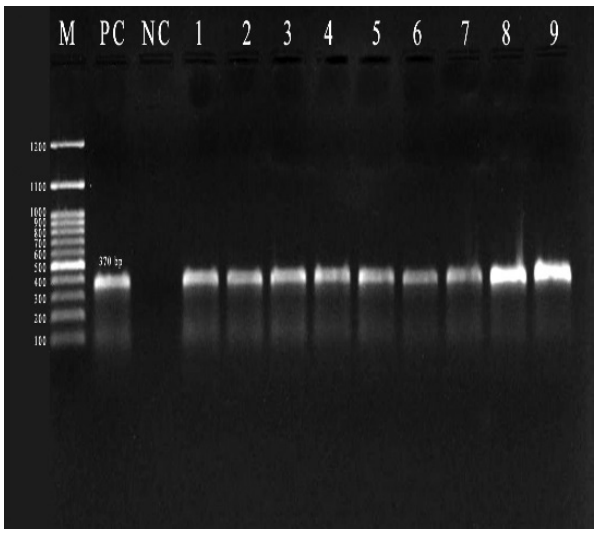

Figure 4. Agarose gel electrophoresis of PCR products generated from *mecA* gene amplification in staphylococcal species.
M: 100 bp DNA plus Ladder (SinaClon, Iran),
PC: positive control (*S. aureus* ATCC 79231), NC: negative control,
Lanes 1-9: showing amplified *mecA* gene in isolates (370 bp).

individual species in different regions (El-Jakee *et al*., 2013). In addition, high tolerance against teat anti-disinfectants used post milking (Quirk *et al*., 2012), proteolytic and hemolytic activities (Bochniarz and Wawron, 2012), and capability to escape from immune defenses (Chaffer *et al*., 1999) are versatile abilities help particular CNS species to colonize in teat canals. Beta lactam antibiotics are frequently used as the first choice in treatment and prevention of mastitis in Iran (Hashemi *et al*., 2011; Hosseinzadeh and Dastmalchi, 2014; Rahman *et al*., 2016).

Despite the less common consumption of antibiotics in buffalo husbandry in Iran, they are in close contact with cows. It is regularly proved that overuse and/or misuse of antibiotics, and incomplete therapeutic period of remedy lead to the development of antimicrobial resistance (Timofte *et al*., 2014). *mecA* gene, responsible for methicillin resistance, is located on a genetic element with the ability to transfer among staphylococcal species. Therefore, dissemination of *mecA* gene may happen not only between species but also from animals to human through close contact or food chain. This is considered as a serious public health hazard (Hanssen *et al*., 2004). Several studies have represented frequent infections with methicillin-resistant *S. aureus* (MRSA) and CNS (MR-CNS) in human in recent years (Bouza *et al*., 2004; Cuevas *et al*., 2004; Garza-Gonzalez *et al*., 2010). In the current survey, *mecA*-based PCR revealed approximately high prevalence of the gene among the isolates. *mecA*-harbouring isolates were detected by the generation of a 370 bp band in 15.51% (9) *S. aureus* and 21.23% CNS (15 *S. epidermidis*, 7 *S. simulans*, and 2 *S. xylosus*) species (Figure 4). Additionally, no statistical association was observed between the prevalence of *mecA* gene and staphylococcal isolates (P=0.072). Despite the rare data regarding the prevalence of MR-CNS in buffalo, Coimbra-e-Souza *et al*. (2017) reported 0% frequency of the gene in CNS isolated from buffalo mastitis milk in Brazil. The prevalence of MR-CNS isolated from bovine mastitis milk samples was 4.6% and 16.7% reported from Iran and Argentina, respectively (Dastmalchi Saei and Hossein-zadeh 2014; Raspanti *et al*., 2016). This implies that CNS with animal origin may be a methicillin resistance reservoir for human. In addition to methicillin resistant *S. aureus*, presence of methicillin resistant *S. epidermidis* (MRSE) is of great concern as these species are important human pathogens. A clonal transmission of a bovine MRSE is proved (Gindonis *et al*., 2013). Besides, propagation of methicillin resistant harbouring isolates through the milking machine and/or milkers' hands within the herds should also be considered. Phenotypic and genotypic analysis of antimicrobial resistant profile of the isolates is recommended in order to apply effective prevention and controlling strategies of mastitis. This also reduces the menace imposed for human health.

CONCLUSION

Collectively, it was revealed that CNS plays an important role in bubaline subclinical mastitis in the northwest of Iran. Identification of MR staphylococci is of great concern due to the probable transmission of the isolates to human. Continuous surveillance programs must be developed in order to monitor the frequency of bacterial, including CNS, mastitis and distribution of methicillin resistant determinant among the isolates in buffalo and other domestic animals in the region.

ACKNOWLEDGENT

The authors would like to express their great attitude toward Islamic Azad University, Sanandaj Branch.

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