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Full Article

A study of coagulase-negative staphylococci (CoNS) isolated from bovine mastitis for the presence of penicillin and methicillin resistance-encoding genes in the north west of Iran

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ABSTRACT

Coagulase-negative staphylococci (CoNS) are often associated with bovine mastitis and may be resistant to antimicrobial therapy. The aim of the current study was to investigate the presence of *blaZ* (responsible for penicillin resistance) and *mecA* (responsible for methicillin resistance) genes among 108 CoNS belonging to 9 different species isolated from bovine mastitis in seven dairy herds (H1-H7). Of 108 CoNS isolates, 44 were *Staphylococcus haemolyticus*, 17 *S. chromogenes*, 11 *S. epidermidis*, 11 *S. warneri*, 11 *S. cohnii*, 6 *S. simulans*, 4 *S. hominis*, 3 *S. capitis*, and 1 *S. xylosus*. The *blaZ* was detected in 65.7% (n=71) of all *Staphylococcus* spp. isolates. Five isolates were positive for the presence of *mecA* gene (4.6%), including 2 *S. hominis*, 1 *S. haemolyticus*, 1 *S. epidermidis*, and 1 *S. warneri*. All *mecA*-carrying CoNS were also positive for the *blaZ* gene and were recovered from two studied herds (H3 and H6). Some variations were also observed in distribution of both *blaZ* and *mecA* genes between CoNS species. This study demonstrates that CoNS from bovine mastitis can be reservoirs of *blaZ* gene. This study also provides evidence of the presence of methicillin resistant CoNS (MR-CoNS) and emphasizes the need for their epidemiological monitoring, in order to prevent the risk of spread to human through direct contact and/or consumption of contaminated food.

Keywords: Coagulase-negative staphylococci, bovine mastitis, *blaZ*, *mecA*, PCR, Iran

INTRODUCTION

Coagulase-negative staphylococci (CoNS) have become the predominant group of bacteria associated with bovine intra-mammary infections (IMI) worldwide and are regarded as emerging mastitis pathogens (Pyorala & Taponen 2009). So far more than 15 CoNS species have been identified in association with bovine IMI, but the prevalent species are *Staphylococcus haemolyticus*, *Staphylococcus chromogenes*, *Staphylococcus simulans*, *Staphylococcus epidermidis*, *Staphylococcus xylosus* and *Staphylococcus hyicus* (Thorberg *et al* 2009, Park *et al* 2011). As, antimicrobials therapy has been an effective strategy for controlling CoNS IMI, it is important to monitor antimicrobial susceptibility of CoNS causing mastitis. In this regards, β-lactams are important antimicrobial

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agents used for the prevention and treatment of mastitis in dairy cows (Sawant *et al* 2009) and penicillin is recommended as the first choice for bacteria that inherently sensitive to it (Grave *et al* 1999). However, efficacy of this treatment could be compromised by staphylococci through the production of *blaZ*-encoded β-lactamases and through the production of *mecA*-encoded alternative penicillin binding protein 2A (PBP2A), which shows a reduced binding to all β-lactam antibiotics (Fuda *et al* 2005). Penicillin-resistance encoding gene *blaZ* can be located on mobile elements, such as transposons, insertion sequences (IS) and plasmids (Dyke 1997), but chromosomal location seems the most frequent in all bovine staphylococci (Olsen *et al* 2006). The *mecA* is located on a 21- to 67-kb mobile genetic element called staphylococcal cassette chromosome *mec* (SCC*mec*) that can be transferred between different species of staphylococci (Hanssen & Ericson Sollid 2006). Therefore, carriage of antimicrobial resistance genes by CoNS species in cattle represents a significant public health risk, both through lateral transfer of resistance genes between staphylococcal species, including *S. aureus*, and through direct transmission of resistant pathogens such as methicillin resistant *S. epidermidis* between humans and animals (Walther & Perreten 2007). An increase in the incidence of infections cause by methicillin-resistant CoNS (MR-CoNS) in human has been reported by several studies (Bouza *et al* 2004, Cuevas *et al* 2004, Garza-Gonzalez *et al* 2010). In Iran, methicillin-resistant *S. aureus* (MRSA) has been described recently as a rapidly developing problem in human medicine where the study of Askari *et al* (2012) demonstrated the high relative frequency (RF) of MRSA in different regions of Iran. Methicillin resistance is also widespread among CoNS commonly associated with human infections (Rahbar *et al* 2001, Pourakbari *et al* 2012). Nevertheless, there are limited published reports on penicillin and methicillin resistant CoNS at species level from farm animals in Iran. So in a 'One Health' perspective, epidemiological studies are required to characterize bacteria from animals with respect to antibiotic resistance genes to ensure optimal results of antimicrobial use and minimize the risk for development and spread of antimicrobial resistance. The aim of this study was to investigate different CoNS species isolated from bovine mastitis for the carriage of *blaZ* and *mecA* genes in the northwest of Iran.

MATERIALS AND METHODS

Coagulase-negative staphylococci (CoNS) isolates: A total of 108 coagulase-negative staphylococci isolates (20 of them were isolated from clinical and 88 from subclinical bovine mastitis cases) were studied. Some specifications of these isolates have been described in 2014 (Hossein-zadeh & Saei, 2014). In brief, they were isolated from 158 milk samples obtained from cattle with mastitis from 7 dairy herds (H1-H7) located in two Iranian provinces of East and West Azerbaijan during 2012. These isolates were identified to the species level by *gap* PCR-RFLP as described previously (Yugueros *et al* 2000, Yugueros *et al* 2001) and consisted of nine staphylococcal species: *S. haemolyticus* (n=44), *S. chromogenes* (n=17), *S. epidermidis* (n=11), *S. warneri* (n=11), *S. cohnii* (n=11), *S. simulans* (n=6), *S. hominis* (n=4), *S. capitis* (n=3) and *S. xylosus* (n=1). In clinical mastitis, out of 20 CoNS isolates recovered, 9, 6, and 5 isolates were *S. haemolyticus*, *S. warneri* and *S. chromogenes*, respectively.

Detection of blaZ gene: From all isolates, DNA was prepared using genomic DNA purification kit (Thermo Science, Germany). Primers 5'-AAG AGA TTT GCC TAT GCT TC-3' and 5'-GCT TGA CCA CTT TTA TCA GC-3' were used to amplify the *blaZ* gene (Vesterholm-Nielsen *et al* 1999). PCR was performed in a 25 µl mixture containing 12.5 µl 2X master mix (SinaClon, Iran), 0.4 µM of each primer and 2 µl of template DNA. Amplification reactions were carried out in a CORBETT thermocycler (model CP2-003, Australia) under the following conditions: initial denaturation at 94 ºC for 5 min followed by 35 cycles of denaturation at 94 ºC for 30 s, annealing at 55 ºC for 30 s, extension at 72 ºC for 30 s, and ended with a final extension at 72 ºC for 10 min (Haveri *et al* 2005).

Staphylococcus aureus ATCC 29213 was used as a positive control. PCR using distilled water instead of nucleic acid was used as a negative control.

Detection of mecA gene: All CoNS isolates were tested by PCR for the presence of the *mec*A gene using the primers 5'-AAA ATC GAT GGT AAA GGT TGG C-3' (corresponded to nucleotides 1282 to 1303) and 5'-AGT TCT GCA GTA CCG GAT TTG C-3' (complementary to nucleotides1793 to 1814) described by Murakami *et al* (2007). The composition of the PCR mix was the same as that described above, but containing 0.4 µM *mecA* primers. After an initial denaturation step for 5 min at 94 ºC, 40 cycles of amplification were performed as follows: denaturation at 94 ºC for 30 s, annealing at 55 ºC for 30 s and DNA extension at 72 ºC for 1 min, completed with a final extension step at 72 ºC for 5 min. The strains *S. aureus* ATCC 25923 (*mecA* negative) and *S. aureus* ATCC 33591 (*mecA* positive) were used as controls.

Agarose gel electrophoresis: The representative samples of each PCR amplicons were electrophoresed in 1% agarose gel containing ethidium bromide (0.5 µg/ml) at 100 V for 1 h and 20 min, and visualized under UV light.

RESULTS

Using the PCR assay, detection of *blaZ* and *mecA* genes were performed through successful amplification of 518 bp and 533 bp specific products, respectively (Figure 1).

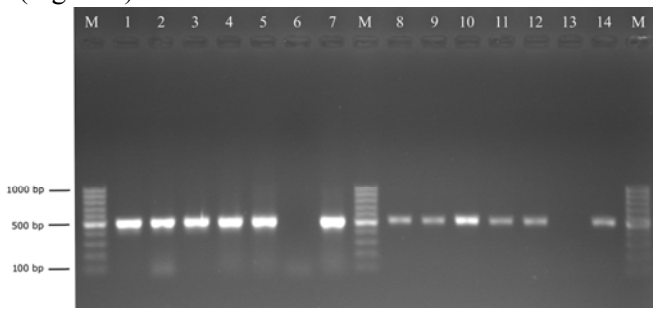

Figure 1. Detection of *blaZ* and *mecA* in isolates by PCR. Lanes M: 100 bp molecular weight marker (Thermo Scientific, Germany); Lanes 1 to 5 showing amplified product in representative *blaZ*-carrying CoNS; Lane 7: negative control for *blaZ* PCR; Lane 7: positive control for *blaZ* PCR; Lanes 8-12 showing amplified product in representative *mecA*-carrying CoNS; Lane 13: negative control for *mecA* PCR ; Lane 14: positive control for *mecA* PCR.

Overally, the 108 coagulase-negative staphylococci (CoNS) isolates can be classified into three groups according to the presence of *blaZ* and/or *mecA* genes as described in table 1: isolates having *blaZ* only (n=66; 61.1%), isolates having both *blaZ* and *mecA* (n=5; 4.6%) and isolates had neither *blaZ* nor *mecA* genes (n=37; 34.3%).

Table 1. Percentage of *blaZ* gene and *mecA* mediated β-lactam resistance among tested isolates of CoNS.

Species	Total	Genotypic antibiotic resistance		
		blaZ$^+$	*blaZ*$^+$ and *mecA*$^+$	*blaZ*$^-$ and *mecA*$^-$
S. haemolyticus	44	33	1	10
S. chromogenes	17	13	-	4
S. epidermidis	11	8	1	2
S. warneri	11	5	1	5
S. cohnii	11	2	-	9
S. simulans	6	3	-	3
S. hominis	4	-	2	2
S. capitis	3	1	-	2
S. xylosus	1	1	-	-
	108	66 (61.1%)	5 (4.6%)	37 (34.3%)

Considering the most common species, PCR analysis of 44 *S. haemolyticus* and 17 *S. chromogenes* detected *blaZ* gene in 34 (77.3%) and 13 (76.5%) isolates, respectively. The detection rate among the third most common CoNS species was 82% in *S. epidermidis* (9/11), 54.5% in *S. warneri* (6/11) and 18.2% in *S. cohnii* (2/11). As shown in table 1, five *blaZ*-carrying CoNS isolates were also found to be positive for *mecA* gene (4.6%) and comprised 2 *S. hominis*, 1 *S. haemolyticus*, 1 *S. epidermidis* and 1 *S. warneri* isolates. The distribution of the *blaZ*- and *mecA*-carrying CoNS species over the different herds is presented in Table 2. As shown, a high detection rate of the *blaZ* gene was found in herds H5 (15/18; 83.3%) and H7 (14/17; 82.3%), while the corresponding rates in herds H3, H1, H4, H2 and H6 were 66.7% (8/12), 55% (11/20),

54.5% (6/11), 52.9% (9/17) and 44.4% (8/18), respectively. As result, the *blaZ* harboring *S. haemolyticus* was detected in all of the studied herds, one of which was also positive for the *mecA* gene and detected in herd H3. The incidence of *mecA*-positive CoNS seems to be herd dependent, as they were detected in two herds. *mecA*-carrying *S. hominis* and *S. haemolyticus*, both identified in herd H3, while *mecA*-harboring *S. epidermidis* and *S. warneri*, both detected in herd H6.

Table 2. Percentage of *blaZ*-carrying CoNS species (*n* isolates) isolated from the studied herds (H1-7).

CoNS species	East Azerbaijan province					West Azerbaijan vince	
	H1 (20)[a]	H2 (17)	H3 (12)	H4 (11)	H5 (18)	H6 (18)	H7 (17)
S. haemolyticus (*n*=34)	8	5	4[b]	4	6	2	5
S. chromogenes (*n*=13)	1	2	1	1	7	-	1
S. epidermidis (*n*=9)	-	1	1	-	-	3[b]	4
S. warneri (*n*=6)	2	-	-	1	-	2[b]	1
S. cohnii (*n*=2)	-	1	-	-	1	-	-
S. simulans (*n*=3)	-	-	-	-	-	1	2
S. hominis (*n*=2)	-	-	2[c]	-	-	-	-
S. capitis (*n*=1)	-	-	-	-	1	-	-
S. xylosus (*n*=1)	-	-	-	-	-	-	1
Total	11 (55%)	9 (52.9%)	8 (66.7%)	6 (54.5%)	15(83.5%)	8 (44.4%)	14(82.3%)

[a] Total number of CoNS isolates recovered from each herds
[b] One of these isolates also harbored *mecA* gene
[c] These two isolates also harbored *mecA* gene

DISCUSSION

Mastitis-causing coagulase-negative staphylococci (CoNS) tend to be more resistant to antimicrobials than *S. aureus* and may be a source of β-lactam resistance genes (Taponen & Pyorala 2009). So, studying antimicrobial susceptibility of CoNS at the species level can provide valuable information about species-specific differences that can be vital data for effective mastitis therapy and control. The detection of the *blaZ* (code for β-lactamase) and *mecA* (code for alternative penicillin-binding proteins) genes is considered the gold standard for the determination of penicillin and methicillin resistance in staphylococci, respectively (Haveri *et al* 2005, John *et al* 2009). Thus, the purpose of the current study was to determine the presence of *blaZ* and *mecA* genes among 108 CoNS isolates belonging to nine species isolated from bovine mastitis by PCR. In the present study, a higher occurrence of *blaZ* was observed among CoNS of all species (n=71; 65.7%), indicating high resistance rate to penicillin and that β-lactamase production was the main mechanism associated with CoNS resistance to β-lactams. This finding is consistent with those of Gooraninejad *et al* (2007) and Moniri *et al* (2007) who reported high resistance rate to penicillin in staphylococci from clinical and subclinical bovine mastitis in other regions of Iran. A possible explanation for this might be the frequent use of β-lactams in intra-mammary infusions for mastitis treatment in Iranian dairy farms. However, the reported percentage of *blaZ* carrying for CoNS isolated from bovine mastitis was 23.8% in Switzerland (Frey *et al* 2013), 80.6% in Netherlands (Sampimon *et al* 2011), and 28.6% in Egypt (Asfour & Darvish 2011). These discrepancies can be explained in part by

the inter-country variation among the most common CoNS species causing bovine mastitis. Sampimon and colleagues (2011) concluded that CoNS species from bovine milk differ significantly in phenotypic and genotypic antimicrobial resistance profiles (Sampimon *et al* 2011). Other possible explanation for this might be different veterinary antibiotic policies and practices. During the recent years, methicillin-resistant CoNS (MR-CoNS) are of increasing importance to animal and human health and have mostly been studied as pathogens in ruminant mastitis (Rajala-Schultz *et al* 2009). It has been hypothesized that MR-CoNS of livestock animals may serve as important reservoirs of *mecA* that has potential to transfer to susceptible *Staphylococcus* strains, including *S. aureus* (Hiramatsu *et al* 2001, Hanssen *et al* 2004). According to PCR result, out of 108 CoNS isolated from bovine mastitis, 5 (4.6%) were carrying the *mecA* gene, including 2 *S. hominis*, 1 *S. warneri*, 1 *S. haemolyticus*, and 1 *S. epidermidis*. Reports on MR-CoNS associated with mastitis are limited but it has been demonstrated in *S. haemolyticus*, *S. xylosus*, *S. chromogenes*, *S. equorum*, *S. sciuri*, *S. warneri*, *S. succinus*, *S. epidermidis*, *S. saprophyticus*, *S. capitis*, and *S. fleurettii* from cattle (Sawant *et al* 2009, Fessler *et al* 2010, Sampimon *et al* 2011, Frey *et al* 2013, Gindonis *et al* 2013), *S. epidermidis* from sheep (Onni *et al* 2011), *S. capitis*, *S. sciuri* and *S. simulans* from goat (Rodrigues da Silva *et al* 2004). These imply that CoNS from mastitis could serve as a reservoir for the spread of *mecA* gene, and this represent a major concern for public health. Of most concern is the carriage of *mecA* by *S. haemolyticus* and *S. epidermidis*, as these two species are of importance for human infections (Huber *et al* 2011). Because of that, and of the potential for spread of MR-CoNS through the food chain, monitoring these micro-organisms in raw milk and different types of dairy products is important. In recent years, the isolation of MR-CoNS from bulk tank milk (BTM) and cheese was reported (Huber *et al* 2011, Fontes *et al* 2013). However, the role of MR-CoNS transmission through milkers' hands at milking should not be neglected in MR-CoNS positive herds (H3 and H7). It has been suggested that CoNS is more likely to spread from humans to dairy cattle than vice versa (Thorberg *et al* 2006). Further studies are needed to demonstrate the direction of interspecies transmission and possible herd management issues that have influenced their spread. Considering the number of each CoNS species tested, it seems that *S. hominis* is more likely to carry the *mecA* gene and *S. cohnii* was the only species exhibited low *blaZ* carrying. Species-based differences have been previously observed in the prevalence of β-lactam resistance determinants among CoNS isolated from bovine milk (Sawant *et al* 2009). In a study carried out in Sweden comparing antimicrobial resistance among CoNS species in clinical and subclinical bovine mastitis, Waller and colleagues (2011) also found some variations in β-lactamase production between CoNS species (Waller *et al* 2011). These suggest that the reservoir of different antimicrobial resistance genes is likely to be larger in certain CoNS species, and that the resistant CoNS may spread clonally within and among herds. Further genotypic analyses are necessary to confirm this. There is evidence of clonal transmission of a bovine methicillin-resistant *S. epidermidis* (MRSE) strain (Gindonis *et al* 2013). As discussed above, 71 and five isolates of CoNS from bovine mastitis were categorized as resistant to penicillin and methicillin, respectively. This phenomenon may pose difficulties to control and treatment of mastitis in cattle. Recovery rates for mastitis caused by penicillin-resistant CoNS have been reported to be about 20% lower than those for mastitis caused by penicillin-sensitive CoNS (Pyorala & Pyorala 1998). However, presence of resistance genes may not be always indicative of resistance phenotypes, and vice versa. Also, the relationship between presence of resistance genes and clinical response to treatment is largely unexplored terrain (Sampimon *et al* 2011). Further exploration of species-associated resistance profiles to develop new strategies for the prevention and treatment of CoNS infections would be of interest. In conclusion, it was found that *blaZ* widely distributed

among CoNS isolated from bovine studied cases. As it has been shown by other similar studies in other countries, this study indicates that dairy cattle can be possible reservoir for the *mecA* gene for humans.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Confict of Interest

Hereby, I declare "no conflict of interest exists" regarding submitted article.

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