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Short communication

ANTIMICROBIAL RESISTANCE PATTERNS OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM BOVINE SUBCLINICAL MASTITIS IN ALBORZ PROVINCE, IRAN

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Summary

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The aim of this study was to determine antimicrobial resistance patterns of *Staphylococcus aureus* isolated from bovine subclinical mastitis. California Mastitis Test was used for 2,160 quarters of 540 dairy cattle in 8 commercial dairy farms of Alborz province, Iran. Antimicrobial susceptibility test was performed by the disk diffusion method on Mueller Hinton agar. The results indicated that *Staphylococcus* genus was isolated from 84 out of 420 milk samples collected from suspected quarters. Out of 84 positive samples, 50 (59.5%) of them were reported as coagulase-positive *S. aureus;* 45 strains were further identified as *Staphylococcus aureus* by PCR amplification of the specific 23S rDNA gene. All *S. aureus* isolates showed resistance against penicillin and ceftiofur but no resistance to gentamicin, enrofloxacin and lincomycin. In addition, 38 (84.4%) of *S. aureus* isolates were resistant to at least 3 antimicrobial agents. According to the results ceftiofur, penicillin, ampicillin was the predominant pattern (22.2%) among seven different antimicrobial resistance patterns. Therefore, carrying out antimicrobial susceptibility tests before drug prescription seems necessary.

Key words: bovine, resistance pattern, *Staphylococcus aureus,*subclinical mastitis

Inflammation of the mammary gland (mastitis) is identified by an increase in the number of somatic cells in the milk as well as pathological changes in the mammary tissue (Sharma, 2007). Mastitis in both clinical and subclinical forms is one of the most significant causes of economic losses to the dairy industry in Iran and other countries around the world (Seleim *et al.,* 2002; Donovan *et al.,* 2005; Huijps *et al.,* 2008; Sahebekhtiari *et al.,* 2011; Hosseinzadeh & Dastmalchi Saei, 2014) due to reduced production and quality of milk and also high costs of treatment. The subclinical form of this disease is more important economically due to its higher prevalence (Rahim *et al.,* 2010; Balqees, 2012). The milk of dairy animals with

subclinical mastitis due to invisible changes can enter the bulk tank (Leitner *et al.,* 2008) and may represent potential health hazard to milk and dairy products consumers (Jorgensen *et al.,* 2005).

Among the wide spectrum of bacterial mastitis pathogens, *Staphylococcus aureus* is recognised as the most frequent isolate from clinical and subclinical bovine mastitis (Taponen *et al.,* 2006). The high prevalence of this pathogen in milk has been attributed to poor hygiene practices (Ateba *et al.,* 2010). The rapid detection of this pathogen in mastitis is significant to achieve treatment of the disease. Therefore, bacterial identification and susceptibility tests play important roles for selecting the appropriate antimicrobial agent when treating mastitis (Gentilini *et al.,* 2000). Antibiotic resistance of *S. aureus* has been attributed to extensive utilisation of antibacterial agents in bovine mastitis which has been reported by several researchers (De Oliveira *et al.,* 2000; Pitkala *et al.,* 2004). This antimicrobial resistance may be due to the occurrence and transmission of antimicrobial-resistant strains of *S. aureus* or their genes (Lowy, 2003) which can be the major reason of low treatment rate of mastitis (Barkema *et al.,* 2006; Gao *et al.,* 2012). Therefore, the aim of the study was to determine the antimicrobial resistance of *S. aureus* isolates from subclinical bovine mastitis in Iran.

California Mastitis Test (CMT) was used for 2,160 quarters of 540 dairy cattle in 8 commercial farms of Alborz province, Iran. After cleaning the teats and dipping them in a disinfectant and also shedding the initial milking, sampling was done from 420 quarters with positive CMT test. Thirthy mL for each sample were collected into sterile tubes and immediately transported to laboratory under temperature-controlled conditions.

Somatic cell count (SCC) of collected milk samples was estimated in the laboratory. Bacterial culturing of milk samples which had SCC greater than 200,000 cfu/mL were done. For bacteria identification, a loopful of each collected milk sample was streaked on blood agar, Mac Conkey agar and Baird Parker agar. After growth, staphylococci were identified on the basis of colony characteristics, Gram staining, pigment production, haemolysis and the following biochemical reactions: catalase activity and oxidase test. Tube coagulase test was done to detect the type of *Staphylococcus*, also PCR on 23S rDNA gene was used with a few modification. (Straub *et al.,* 1999). Briefly, DNA extraction was performed using a boiling method as a DNA template. Three overnight cultures in 2 mL nutrient broth were centrifuged for 5 min at 5,000 rpm. The bacterial pellet was re-suspended in 200 µL of distilled water and boiled for 10 min. Tubes were centrifuged again, and the supernatant was used as template DNA. Primer set (Forward 5'- ACG GAG TTA CAA AGG ACG AC-3' and Reverse 5'- AGC TCA GCC TTA ACG AGT AC-3') encoded a 1250 bp product. For PCR amplification, the reaction mixture (30 µL) contained 1 µL of primer F (10 pmol/µL), 1 µL of primer R (10 pmol/µL), 0.6 µL of deoxynucleoside triphosphate (10 mmol/L; Cinna-Gene), 3 µL of 10× PCR buffer (Cinna-Gene), 1.8 µL of $MgCl_2$ (25 mmol/L; Cinna-Gene), 0.1 µL of *Taq* DNA polymerase (5 U/µL, Cinna-Gene) and 20 µL of distilled water. Finally, 2.5 µl of DNA preparation was added to each 0.2 mL reaction tube.

Antimicrobial susceptibility test was performed by disk diffusion method on Mueller Hinton agar (CLSI, 2007). The following antibiotics were used: penicillin

(10 IU), ceftiofur (30 µg), ampicillin (10 µg), streptomycin (10 µg), oxytetracycline (30 µg), enrofloxacin (5 µg), sulfamethoxazole-trimethoprim (23.75 µg /1.25 µg), tetracycline (30 µg), gentamicin (10 µg), lincomycin (2 µg), neomycin (30 µg), tylosin (30 µg), florfenicol (30 µg). As a quality control a reference strain (*S. aureus,* ATCC 29213) was inoculated in each plate. After 24 h incubation at 37 °C plates were examined and the zone of inhibition was measured.

The results indicated that *Staphylococcus* genus was isolated from 84 among the 420 milk samples collected from suspected quarters. Out of 84 positive samples 50 (59.5%) were reported as coagulase-positive *S. aureus* from which 45 (90%) strains were further identified as *S. aureus* by PCR amplification of the specific 23S rDNA gene. The antimicrobial resistance profiles of the *S. aureus* isolates against 13 antimicrobial agents arepresented in Table 1. All *S. aureus* isolates showed resistance against penicillin and ceftiofur although no resistance has been observed to gentamicin, enrofloxacin and lincomycin. Moreover, resistance to ampicillin, tetracycline, neomycin, tylosin, streptomycin, oxytetracycline, sulfamethoxazole-trimethoprim and florfenicol was reported for 91.1%, 84.4%, 48.8%, 28.8%, 17.7%, 11.1%, 11.1% and 2.2% of isolates respectively. In addition 38 (84.4%) of *S. aureus* isolates were resistant to at least 3 antimicrobial agents. (Table 2). Furthermore according to the results CEF, PEN, AMP was the predominant pattern (22.2%) among the seven different antimicrobial resistance patterns.

Mastitis is the most common reason for using antimicrobial agents in dairy farms. Antimicrobial resistance is recognised as one of the most significant phe-

Table 1. Antimicrobial resistance of 45 *S. aureus* bovine subclinical mastitis isolates

Antimicrobial agents	Resistant isolates	
	Number	Percentage
Penicillin	45	100.0
Ceftiofur	45	100.0
Ampicillin	41	91.1
Tetracycline	38	84.4
Neomycin	22	48.8
Tylosin	13	28.8
Streptomycin	8	17.7
Oxytetracycline	5	11.1
Sulfamethoxazole-trimethoprim	5	11.1
Florfenicol	1	2.2
Gentamicin	0	0
Enrofloxacin	0	0
Lincomycin	0	0

nomena around the world. Increasing antimicrobial resistance of *S. aureus* as the most frequent cause of clinical and subclinical bovine mastitis has been reported in several previous investigations (Daka *et al.,* 2012; Mubarack *et al.,* 2012). In the present study *S. aureus* isolates showed the highest level of resistance to penicillin (100%) which was much higher than rates reported in South Africa (20%), South Ethiopia (67.9%), Turkey (63.3%) and Italy (69.1%) (Guler *et al.,* 2005'Ateba *et al.,* 2010; Daka *et al.,* 2012). Contrary to our findings, the results of previous studies in Poland and other countries showed lower resistance rates of *S. aureus* to ampicillin (7% to 68.9%) (Calvinho *et al.,* 2002; Erskine *et al.,* 2002; Malinowski *et al.,* 2002; Corti *et al.,* 2003; Daka *et al.,* 2012) but a resistance rate higher than ours was reported in Italy (98.5%) (Moroni *et al.,* 2006). The high resistance rate in Iran can be due to wide usage of β-lactam antibiotics such as penicillin and ampicillin for the treatment of bovine

Table 2. Antimicrobial resistance patterns of the 45 *S. aureus* isolates

Antibiotic resistance patterns[1]	Multidrug resistance	
	Number	Percentage
CEF, PEN, AMP	10	22.2
CEF, PEN, AMP, NEO	9	20.0
CEF, PEN, AMP, NEO, TYL	6	13.3
CEF, PEN, AMP, S	5	11.1
CEF, PEN, AMP, NEO, TYL, FFC, LIN	3	6.6
CEF, PEN, AMP, NEO, TYL, TET, OXT, SXT, S	3	6.6
CEF, AMP, NEO, TYL	2	4.4
Total	38	84.4

[1]PEN: Penicillin; SXT: Sulfamethoxazole-trimethoprim; AMP: Ampicillin; OXT: Oxytetracycline; TE: Tetracycline; S: Streptomycin; GM: Gentamicin; CEF: Ceftiofur; ENR: Enrofloxacin; FFC: Florfenicol; S: Streptomycin; TYL: Tylosin; NEO: Neomycin, LIN: Lincomycin

mastitis although the frequency of β-lactam antibiotics resistance varies among countries (Guler *et al.,* 2005). The third highest resistance rate occurred against tetracycline (84.4%). Lower resistance rate were reported by other researchers (Mubarack *et al.,* 2012; Oliveira *et al.,* 2012). Overuse of antimicrobials for several purposes including treatment and growth promotion in different fields can be the most significant reason for high resistance rates in Iran (Sodagari *et al.,* 2015). Moreover, our data demonstrated that *S. aureus* isolates were more resistant to oxytetracycline, sulfamethoxazole-trimethoprim and less resistant against streptomycin compared to those reported in previous studies (Shitandi & Sternesjö, 2004; Guler *et al.,* 2005; Mubarack *et al.,* 2012). None of *S. aureus* isolates was resistant against gentamicin, enrofloxacin and lincomycin in the present investigation which is in agreement with results reported in Turkey (Guler *et al.,* 2005), India (Mubarack *et al.,* 2012) and Italy (Moroni *et al.,* 2006) indicating that *S. aureus* isolates are still largely susceptible to these three antimicrobial agents and they can be used effectively in treatment of clinical and subclinical bovine mastitis.

The emergence of multiple drug resistance (MDR) in *S. aureus* strains has become a major challenge in the treatment of bovine mastitis and public health issue (Davies, 1994). Resistance to at least 3 antimicrobial agents was detected in 38 (84.4%) of *S. aureus* isolates. There are several reports related to MDR in staphylococcal isolates around the world (Gentilini *et al.,* 2002; Guler *et al.,* 2005; Ateba *et al.,* 2010; Daka *et al.,* 2012). In general the high level of MDR in this investigation can be due to overuse and misuse of antimicrobial agents for treatment of mastitis. Poor efficacy of antimicrobial therapy due to poor penetration of scar tissue barriers in animals with chronic *S. aureus* mastitis has been proved (De Oliveira *et al.,* 2000). Therefore refusal to insist on treatment of infected cows and culling them from the dairy herds is one of the most reasonable recommendations for decreasing the level of antimicrobial resistance in dairy farms (De Oliveira *et al.,* 2000). Furthermore, carrying out antimicrobial susceptibility test before drug prescription seems necessary.

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