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Increasing Resistant Coagulase Negative *Staphylococci* in Bovine Clinical Mastitis

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Abstract: The aim of this study was to determine Coagulase Negative *Staphylococci* (CNS) and other bacteria for their resistance to antimicrobial agents approved for the control of pathogens involved in clinical bovine mastitis. This descriptive study was done on 106 milk samples obtained from clinical mastitis in dairy cattle husbandry from April 2006 through August 2006 in Kashan, Iran. From the total of 106 milk samples collected from clinical mastitis, 96 (90.6%) lead to positive culture. Coagulase negative *Staphylococci* isolated in 51 out of 96 samples (53.1%), *Staphylococcus aureus* isolated in 21 out of 96 (21.9%), gram negative bacilli isolated in 14 out of 96 (14.6%) and *Enterococci* isolated in 4 (4.2%). The highest rate of resistant CNS observed to penicillin (56.6%) and the highest rate of sensitivity to enrofloxacin 100%, followed by kanamycin, streptomycin and neomycin, 92.2, 82.3 and 82.3%, respectively. The highest rate of resistance *S. aureus* exhibited to penicillin (66.6%); while the highest rate of sensitivity showed to trimethoprim-sulphamethoxasole (81%), followed by kanamycin and enrofloxacin both at 76.2%. The highest rate of resistance gram negative bacilli exhibited to ampicillin and erythromycin at 71.4%. Their highest rate of sensitivity observed to enrofloxacin (78.6%), followed by kanamycin, (71.4%). In recent years, CNS is emerging as important minor mastitis pathogens and can be the cause of substantial economic losses. The high resistance rate to penicillin and other antibiotics found in this study emphasize the importance of identification of CNS when a bovine clinical mastitis is present.

Key words: Bovine clinical mastitis, antibacterial resistant, coagulase negative *Staphylococci*

INTRODUCTION

Mastitis is one of the most economically important diseases affecting the worldwide dairy industry with huge financial losses due to treatment costs, loss of the affected animals and genetics through death or culling, lost milk production during and after disease and the withdrawal time need for removal of antibiotics in produced milk. Increasing antimicrobial resistance has become a serious concern worldwide and antimicrobial use in animal is currently under scrutiny (Tenhagen *et al*., 2006; Roesch *et al*., 2006). In many countries, the prevalence of the classic contagious bacteria such as *Streptococcus agalactiae* and *Staphylococcus aureus* has decreased. Coagulase-Negative *Staphylococci* (CNS) and *Corynebacterium bovis*, which are traditionally considered as minor mastitis pathogens, have become more common (Myllys *et al*., 1998; Huxley *et al*., 2002).

S. aureus and CNS are common causes of bovine intramammary infections. *S. aureus* infections, may present as clinical or subclinical forms, frequently persist for a long time and the infected mammary glands serve as reservoirs for the organisms that may spread to other cows within a herd and perhaps to other herds (Waage *et al*., 2002; Bergonier *et al*., 2003). The effectiveness of therapeutic methods depend on the organisms, their antibiotic susceptibility test, dosage and duration of treatment and how early treatment is initiated (Sordillo and Scott, 1995). Bacteriological cultures of milk specimens from clinical mastitis cases are useful in early detection of potential herd problems. Identification of the infectious agents provides a focal point for preventive actions. The outcome of treatment for various types of mastitis should also be monitored to assess the efficacy of the used antibiotic and antibiotic sensitivity patterns. The purpose of this study was to identify the bacterial agents involving in clinical mastitis and their antibacterial resistance in bovine clinical mastitis in Kashan, Iran.

MATERIALS AND METHODS

This study was done on 106 Holstein Frisian cattle with clinical mastitis in 10 industrial dairy farms from April 2006 through August 2006 in Kashan, Iran. After physical

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examination and confirmation of clinical mastitis, the clinical data from sick animals collected in questionnaires. Then 20 mL milk sample was taken aseptically from all quarters of animals suffering from clinical mastitis and immediately transferred cool to the bacteriology laboratory, Kashan University of medical Sciences. This study was approved by the Ethics Committee of the Kashan University of Medical Sciences.

Amount of 0.01 mL of each milk sample was cultured on blood agar and MacConkey agar plates which incubated at 37°C for 48 h. Isolated microorganisms were identified by standard methods. A quarter was identified as infected when a single pathogenic bacterium was isolated (Casadevall and Pirofski, 2000).

For susceptibility testing, isolates were suspended in trypticase soy broth and the suspension was adjusted with a turbidity equivalent to 0.5 McFarland standards. Drug susceptibility testing was performed by the agar disk diffusion method. The following antibacterial disks were used: penicillin 10 IU; ampicillin 10 μg; erythromycin 15 μg; oxytetracycline 30 μg; doxycycline 30 μg; streptomycin 10 μg; neomycin 30 μg; kanamycin 30 μg; spectinomycin 100 μg; enrofloxacin 5 μg and trimethoprim-sulfamethoxazole 1.25/23.75 μg; manufactured by HiMedia, India). Sensitivity testing on isolated bacteria was performed using disk diffusion methods in accordance with the standards, setting out by the National Committee for Clinical Laboratory Standards (NCCLS) (1999). The isolated bacteria were categorized as susceptible, intermediate and resistant based upon interpretive criteria. The results were analyzed statistically by the Chi square and Fischer's exact tests.

RESULTS AND DISCUSSION

The mean age of examined cattle were 4.9±2.1 years (2 to 14). From a total of 106 milk samples collected from clinical mastitis cases, 96 (90.6%) lead to positive culture. The hygienic condition of the studied farms was not suitable and the milking procedure was, too. The majority (84 out of 106; 79.2%) of herds had low hygiene quality. Absolutely, none of the herdsman (100%) applied any disinfectant to teats before milking; instead all of them washed the teats by water solely without drying with individual paper towel. The post-milking teat disinfection by Povidone Iodine derivatives were done in all farms (100%). The most (71 out of 106; 67%) of the milking machines and pipelines did not clean and disinfected properly. Previous history of clinical mastitis recorded in 43 cases (40.6%). Fifty-two (49.1%) of cases had history of recent antibiotic usage. Oxytetracycline was the most common used drug (46 out of 52; 84.4%). The isolated bacteria from 96 positive specimen cultures were as follow: CNS isolated in 51 (53.1%) (Fig. 1), *S. aureus* from 21 (21.9%), gram negative bacilli from 14 (14.6%) and *enterococci* from 4 (4.2%), *corynebacterium* sp. in 4 (4.2%), *S. pyogenes* in 1 (1.0%) and *micrococci* in 1 case (1.0%). The most frequently isolated udder pathogens among heifers were CNS 91.7% (11 out of 12) and *S. aureus* 8.3% (1 out of 12). In comparison, the main isolated pathogen in multi-parous cows with clinical mastitis at parturition were CNS 47.6% (40 out of 84), followed by *S. aureus* 23.8% (20 out of 84) and *E. coli* 10.7% (9 out of 84).

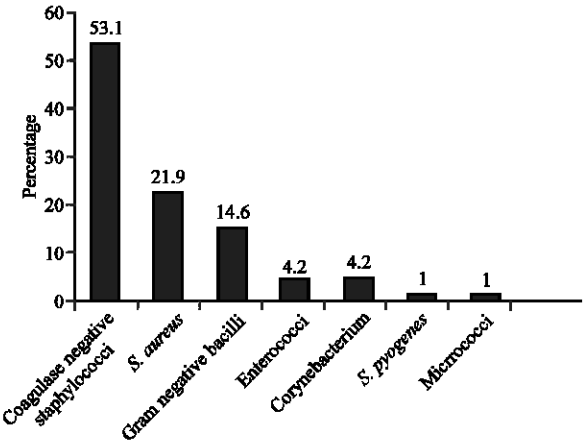

Fig. 1: The percentage of bacterial isolates from bovine clinical mastits in Kashan

Table 1 presented the resistance rate of gram negative bacilli to 10 tested antibacterials. The highest rate of sensitivity observed to enrofloxacin 78.6% (11 out of 14) followed by kanamycin and SXT, 71.4 and 64.3%, respectively. The sensitivity rate of 9 isolated *E. coli* to enrofloxacin and kanamicin were 100 and 88.9%, respectively. The lowest sensitivity rate exhibited to erythromycin 0%, followed by ampicillin and oxytetracyclin 7.1%. The highest resistance rate of *E. coli* observed against ampicillin and erythromycin, 77.8% and 66.7%, respectively. In Table 2 the resistance rate of isolated *S. aureus* to 11 tested antibacterial are presented. The highest resistance rate (66.6%) exhibited to penicillin (14 out of 21), followed by doxycycline and streptomycin both at 47.6% and oxytetracycline at 42.8%. The highest sensitivity rates showed to trimethoprim-sulphamethoxasole (81%), followed by kanamycin and enrofloxacin (76.2%). The *in vitro* activities of CNS against 11 selected antimicrobial agents are summarized in Table 3. The highest resistance rate of CNS observed to penicillin (56.6%), followed by streptomycin 51%, while the highest rate of sensitivity observed to enrofloxacin (100%), followed by kanamycin (92.2%), neomycin and spectinomycin (82.3%).

Table 1: *In vitro* susceptibility of 14 isolated gram negative bacilli from bovine clinical mastitis to 10 selected antimicrobial agents

Antibiotics	Resistance No. (%)	Intermediate No. (%)	Sensitive No. (%)
Ampicillin (10 μg)	10 (71.4)	3 (21.4)	1 (7.1)
Erythromycin (15 μg)	10 (71.4)	4 (28.6)	0
Oxytetracycline (30 μg)	5 (35.7)	8 (57.1)	1 (7.1)
Doxycycline (30 μg)	7 (50)	5 (35.7)	2 (14.3)
Streptomycin (10 μg)	5 (35.7)	4 (28.6)	5 (35.7)
Neomycin (30 μg)	2 (14.3)	8 (57.1)	4 (28.6)
Kanamycin (30 μg)	2 (14.3)	2 (14.3)	10 (71.4)
Spectinomycin (100 mcg)	5 (35.7)	5 (35.7)	4 (28.6)
Enrofloxacin (5 μg)	2 (14.3)	1 (7.1)	11 (78.6)
Trimethoprim-sulfamethoxazole (1.25/23.75 μg)	4 (28.6)	1 (7.1)	9 (64.3)

Table 2: *In vitro* susceptibility of 21 isolated *S. aureus* from bovine clinical mastitis to 11 selected antimicrobial agents

Antibiotics	Resistance No. (%)	Intermediate No. (%)	Sensitive No. (%)
Penicillin (10 IU)	14 (66.6)	1 (4.8)	6 (28.6)
Ampicillin (10 μg)	3 (14.3)	5 (23.8)	13 (61.9)
Erythromycin (15 μg)	7 (33.3)	3 (14.3)	11 (52.4)
Oxytetracycline (30 μg)	9 (42.8)	3 (14.3)	9 (42.9)
Doxycycline (30 μg)	10 (47.6)	0	11 (52.4)
Streptomycin (10 μg)	10 (47.6)	1 (4.8)	10 (47.6)
Neomycin (30 μg)	3 (14.3)	4 (19)	14 (66.6)
Kanamycin (30 μg)	4 (19)	1 (4.8)	16 (76.2)
Spectinomycin (100 mcg)	2 (9.5)	7 (33.3)	12 (57.2)
Enrofloxacin (5 μg)	1 (4.8)	4 (19)	16 (76.2)
Trimethoprim-sulfamethoxazole (1.25/23.75 μg)	2 (9.5)	2 (9.5)	17 (81)

Table 3: *In vitro* susceptibility of 51 isolated coagulase-negative *Staphylococci* from bovine clinical mastitis to 11 selected antibacterial agents

Antibiotics	Resistance No. (%)	Intermediate No. (%)	Sensitive No. (%)
Penicillin (10 IU)	29 (56.9)	0	22 (43.1)
Ampicillin (10 μg)	9 (17.7)	7 (13.7)	35 (68.6)
Erythromycin (15 μg)	9 (17.7)	11 (21.5)	31 (60.8)
Oxytetracycline (30 μg)	9 (17.7)	7 (13.7)	35 (68.6)
Doxycycline (30 μg)	13 (25.5)	4 (7.8)	34 (66.7)
Streptomycin (10 μg)	26 (51)	4 (7.8)	21 (41.2)
Neomycin (30 μg)	1 (1.9)	8 (15.7)	42 (82.3)
Kanamycin (30 μg)	1 (1.9)	3 (5.9)	47 (92.2)
Spectinomycin (100 mcg)	3 (5.9)	6 (11.8)	42 (82.3)
Enrofloxacin (5 μg)	0	0	51 (100)
Trimethoprim-sulfamethoxazole (1.25/23.75 μg)	6 (11.8)	0	45 (88.2)

Mastitis is the most frequent and costly disease in dairy cattle. The losses due to clinical mastitis easily observed and including transient reductions in milk yields, discarded milk and premature culling (Fetrow *et al*., 2000). The majority of bovine mastitis and those of significant economic importance are caused by species of *Staphylococci*, *Streptococci* and the gram-negative bacilli (coliform bacteria) (Smith and Hogan, 1993). *E. coli* is widely distributed in nature and is frequent cause of bovine mastitis (Jones *et al*., 1990). The microorganisms isolated in this study indicate that other pathogens have emerged to fill the niche vacated by *S. aureus* and *S. agalactia*. Organisms such as CNS and gram negative bacilli are increasingly implicated in mastitis of dairy cattle in Kashan. They are the most frequent organisms isolated from milk samples in herds that have controlled major pathogens (Harmon and Langlosis, 1995). The CNS bacteria lives on teat skin and can colonize into the teat canal. Anything that decreases the potency of the teat sphincter facilitates the occurrence of infections and may lead to both sub-clinical and clinical mastitis forms. In this study the most common isolated udder pathogens among heifers was CNS (91.7%) and in multi-parous cows were CNS (47.6%), followed by *S. aureus* (23.8%) which is differ from results that reported by Kalmus *et al.* (2006). In their study the most frequently isolated udder pathogens among heifers were *E. coli* (22.1%), followed by *Streptococcus uberis* (19.1%) and CNS (8.8%) and the main pathogen in multi-parous cows with clinical mastitis at parturition was *S. aureus* (11.2%).

In present study gram negative bacilli exhibited the highest rate of sensitivity to enrofloxacin and kanamycin, 78.6 and 71.4%, respectively and the lowest sensitivity rate (0%) to erythromycin, followed by ampicillin and oxytetracycline both at (7.1%) and neomycin (28.6%). Present findings are comparable with the results provided by Corti *et al.* (2003) from Switzerland that 90% of the *E. coli* strains were sensitive to kanamycin, but disagree with the results for ampicilin (80%) and neomycin (90%) sensitivity rates. In present study the highest resistance rate of *S. aureus* observed to penicillin 66.6% (14 out of 21). The highest rate of sensitivity showed to trimethoprim-sulphamethoxasole 81%, followed by kanamycin and enrofloxacin at 76.2%. Calvinho *et al.* (2002), showed the highest resistance rate of *S. aureus* against penicillin and ampicillin (47.6%) and the lowest rates against erythromycin (2%), pirlimycin (4%) and neomycin (2.9%). In another study on 336 strains of *S. aureus*, 160 (47.6%) were resistant to penicillin (Gianneechini *et al*., 2002). The other researchers showed that the prevalence of penicillin resistance among *S. aureus* infected cows was 12% (95% confidence interval: 6-19%). The proportion of isolates resistant to penicillin in Denmark was low compared to studies in other countries except Norway and Sweden (Bennedsgaard *et al*., 2006). Based on the high prevalence of penicillin resistance of *S. aureus* in our study, penicillin should be substituted by SXT as antimicrobial choice for treatment of bovine intra-mammary infection caused by *S. aureus* in Kahsan, Iran.

Rajala-Schultz *et al.* (2004) reported that CNS is increasing its importance as cause of bovine mastitis throughout the world in recent years. CNS has been isolated from cows with clinical and sub-clinical mastitis in several countries (Watts *et al.*, 1995; Owens *et al.*, 1997; Myllys *et al.*, 1998). Penicillin-resistance found in our study is higher than that previously reported for CNS (Myllys *et al.*, 1998; Gianneechini *et al.*, 2002), but in agreement with the results of other study by Owens *et al.* (1997), from the USA at 57%. The resistance rate of CNS against erythromycin (17.7%) that reported in present study was higher than those found in previous studies (Owens *et al.*, 1997; Myllys *et al.*, 1998). Kudinha and Simango (2002) showed that all of the CNS isolates were susceptible to erythromycin and more than 90% of the isolates were susceptible to neomycin, penicillin and streptomycin. Gentilini *et al.* (2002), showed that resistance rate of isolated CNS from bovine clinical and sub-clinical mastitis were 27.6 and 5.7% for penicillin and erythromycin, respectively. The CNS isolated in their study showed a low level of resistance to all tested antimicrobial agents (0-7.4%) except for ampicillin (18.1%) (Gentilini *et al.*, 2002). The resistance patterns of the CNS isolated during the study is concordant with antimicrobial usage in the studied herds. This is in agreement with the generally accepted notion that selection pressure from the use of antibiotics is a main factor in development of antibiotic resistance (Rajala-Schultz *et al.*, 2004). The resistance rate in their study was high, if the occurrence of resistant bacteria increases, the appropriate therapy may become a serious clinical problem. It is essential that CNS resistance in dairy herds in Iran monitored continuously in order to develop susceptibility patterns, to establish trends in CNS resistance and to apply the appropriate treatment program in dry cows. Bacterial identification and susceptibility tests are vital for selecting the appropriate antimicrobial agents in treatment of bovine mastitis. Tremendous progress has been made in the control of contagious mastitis. Mastitis due to pathogens such as CNS and gram negative bacilli has emerged to fill the niche vacated by control of the major pathogens. The high resistance to penicillin and other antibiotics found in this study emphasize the importance of identification of CNS when a bovine clinical mastitis is present. Antimicrobial susceptibility patterns should be identified for CNS, as current susceptibility data are necessary to select appropriate antibiotics for a successful treatment.

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