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Full Length Research Paper

Bacterial pathogens of intramammary infections in Azeri buffaloes of Iran and their antibiogram

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The aim of this study was to determine the bacterial causes, their antibiogram and prevalence of intramammary infection (IMI) in the buffaloes and quarters of Azeri ecotype in Tabriz, Iran and assess any relationships between IMI and somatic cell count (SCC). After conducting California Mastitis Test (CMT) in farms, quarter- based milk samples were collected from 300 buffaloes. Also 150 samples were collected for bacterial culture and count. Based on CMT, 13.87% of quarters and 23.66% of buffaloes had subclinical mastitis, the sensitivity and specificity of CMT for infections with all bacteria and infections with major pathogens were 55 and 67.39, 70 and 50% respectively. Coagulase-negative staphylococci (CNSs) were the most common pathogens. Isolated bacteria had no significant effects on mean SCC of infected quarters. The most effective antibiotics against all isolated agents were Cephtiofur and Trimetoprim+ Sulfametoxazol with a sensitivity rate of 97.3and 94.6% respectively. The SCC and total bacterial count (TBC) of infected quarters were significantly higher than healthy ones (p< 0.05). Based on the results of current study, CMT has acceptable sensitivity and specificity in diagnosis. Coliforms are not probably very important in buffaloes intramammary infections.

Key words: Buffalo, mastitis, intramammary infection, SCC, bacterial culture.

INTRODUCTION

Azeri ecotype buffaloes, originating from the Indo valley (Indian buffalo), are classified as a river type; are recognized to have economic significance among livestock animals in terms of milk and meat yields in the North West of Iran (IRAN's Country Report, 2004). Buffalo milk is more valuable and its price is nearly two fold more than cow milk in Iran.

Although mastitis is one of the most important diseases of dairy herds, its importance is not well recognized in buffaloes. Because of the limited distribution of buffaloes in the world, there is little information about buffalo mastitis. Although the buffalo has been traditionally considered less susceptible to mastitis than cattle (Wanasinghe, 1985), some researchers have shown similar mastitis frequencies for the 2 species (Badran, 1985; Bansal et al., 1995; Kalra and Dhanda, 1964). CNSs followed by *Corynebacterium* spp. and *Streptococcus* spp. are the most frequently isolated bacteria from milk samples of buffaloes affected with mastitis in previous studies (Chander and Baxi, 1975; Costa et al., 1997; Naiknaware et al., 1998; Paranjabe and Das, 1986; Saini et al., 1994).

SCC is widely used as an inflammatory indicator in diagnosing mastitis in the bovine mastitis. SCC has also been used in buffalo mastitis diagnosis. In fact, according to some studies (Dhakal et al., 1992; Singh and Ludri, 2001), it seems probable that an SCC > 200,000/ml is an indicative value of udder infection.

The European Union Directives set a limit of 400,000 cells/ml for SCC in buffalo milk but in Iran there is not any definite limitation. The goals of the present study were:

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Table 1. Frequency of quarters in different CMT scores.

Percentage	Frequency	CMT
38.4	461	Negative
47.1	566	T
12.7	152	1+
1.2	14	2+
0.08	1	3+
99.4	1193	Total
0.6	7	Missing
100	1200	Total

(1) To assess the prevalence of IMI in Azeri buffaloes of Tabriz region, Iran.
(2) To identify the bacterial causes of IMI.
(3) To study the phenotypic relationships among udder infection and SCC.
(4) To examine the sensitivity of isolated bacteria to antibiotics.

MATERIALS AND METHODS

Animals and milk sampling

The statistical population of this study was the adult lactating buffaloes of Tabriz (almost 3000 buffaloes). On the basis of the formula:

$$N = \frac{NZ^2P(1-P)}{(N-1)D^2 + Z^2P(1-P)}$$

P = 30%, d = 0.05, N = 3000, α = 0.05

300 buffaloes (1200 quarters) were selected randomly. The mean age, parity and weight were 6(2 to 15.5) years old, 3.31(1 to 13) and 435.5(250 to 800) kg, respectively. The average milk yield was 4.93 ± 1.9 kg/day. All of the buffaloes were clinically healthy with normally appearing milk during sampling. In most cases no premilking preparations were done. However in some of quarters washing without drying was performed by farmers. Milking was done twice daily in the morning and evening without any teat dipping. Sampling was done in the morning milking session and after teat cleaning (if contaminated by feces) in the winter (2008 to 2009).

CMT, SCC, bacteriological evaluation and antibiogram

CMT (CMT; Bovi-Vet, Kruuse, Denmark) was done for each quarter foremilk and results were classified as (-), trace, (1+), (2+) and (3+). Somatic cell count was carried out by an electronic fossomatic counter (Model 5000, Foss Fact., Denmark) from whole quarter milk samples. For statistical analyses, SCCs were converted to SCS using the standard $\log_2$ transformation of Ali and Shook (1980). In order to perform bacterial culture and TBC, 150 samples were collected aseptically on the basis of CMT scores (30 sample from each CMT score).

TBCs were determined according to the standard methods (Houghtby, 1992) in the microbiology laboratory. Serial dilutions of milk samples were made and 0.1 ml aliquot of each dilution was surface cultured on plate count agar and incubated in 37°C for 24 h, and finally colonies were counted.

For bacteriological evaluation 10 µl of each milk sample was spread on blood agar plates (5% defibrinated sheep blood). Plates were incubated aerobically at 37°C and examined after 24 h. Colonies were provisionally identified on the basis of Gram stain, morphology, and haemolysis patterns, and the numbers of each colony type were recorded. Representative colonies were then subcultured on blood agar plates and incubated aerobically at 37°C for 24 h to obtain pure cultures. Catalase and coagulase production was tested for Gram-positive cocci. Gram negative isolates were identified by using colony morphology, Gram-staining characteristics, oxidase, and biochemical reactions on MacConkey's agar. Specific identification of all isolates was performed using standard methods of National Mastitis Council (1999).

Contagious pathogens, *Staphylococcus aureus* and *Streptococcus agalactiae* were considered to cause IMI if at least one colony (≥ 100 cfu/ml) was isolated. For other microorganisms, IMI was defined by the isolation of ≥ 500 cfu/ml and 1 to 3 colony types. Milk samples which comprised > 3 colony types or < 500 cfu/ml of any isolated microorganisms were regarded as contaminated or uninfected, respectively (Moroni et al., 2006).

In-vitro susceptibility of the organisms to various commercial antibiotic agents was tested by the disc diffusion technique on Muller-Hinton agar. The plates were incubated at 37°C. Zones of growth inhibition were evaluated according to CLSI (2002).

Statistical analyses

One way analysis of variance (ANOVA) method was used for the variance analysis of SCC among CMT groups and isolated bacteria. Independent T test was carried out to compare mean levels of SCC, SCS and TBC between infected and healthy quarters, with a confidence level of α= 0.05, using SPSS software (ver.15). Discriminant analysis was used for the accuracy of SCC in diagnosis of subclinical mastitis severity.

RESULTS

Prevalence of IMI and somatic cell count

On the basis of CMT among total 1200 quarters from 300 examined buffaloes the prevalence of infection was 13.87% (167) of quarters and 23.66% (71) of buffaloes (Table 1). On the basis of bacteriological culture, 34

Table 2. Mean SCC (cells/ml) and SCS in quarters with different CMT scores.

CMT	No. (Quarters)	Mean (SCC× 10^3)	Mean(SCS)	Max.(× 10^3)	Min.(× 10^3)
Negative	376 450	131[a]	3.38	492	3
Trace		179[a]	3.83	678.5	10
1 +	120	375.78[b]	4.91	2365	58
2 +	12	1685.167[c]	7.07	13550	270

a,b.c: There are significant differences between different characters on the basis of ANOVA and Turkey post Hoc test (p< 0.001).

Table 3. Agreement between CMT and SCC in diagnosis of subclinical mastitis with divers severity in buffalo's quarters.

SCC agreement			
Percentage	**No.**	**Total**	**CMT**
65.2	245	376	Negative
28.7	450	129	T
30.8	120	37	1+
16.7	2	12	2+
100	1	1	3+

Table 4. The isolated bacteria from infected mammary quarters and their related mean SCC (cells/ml).

Isolated bacteria	No.	Percentage	Mean (SCC× 10^3)
Staphylococcus aureus	1	2.95	65
Staphylococcus aureus +Corynebacterium bovis	4	11.75	156
Staphylococcus aureus + Streptomyces	1	2.95	98
Streptococcus agalactiae	2	5.87	136
Streptococcus agalactiae+ Rhodococcus equi	1	2.95	110
Corynebacterium bovis	4	11.75	4531
Staphylococcus saprophyticus	6	17.64	108
Staphylococcus saprophyticus+ Proteus mirabilis	1	2.95	255
Staphylococcus simiulance	1	2.95	172
Staphylococcus hycus	2	5.87	199
Staphylococcus epidermidis	2	5.87	671
Staphylococcus epidermidis+ Micrococcus	1	2.95	606
Staphylococcus lentus	1	2.95	124
Staphylococcus intermedius	1	2.95	174
Bacillus Subtilis	4	11.75	367
Bacillus Subtilis+ Staphylococcus saprophyticus	1	2.95	23
Bacillus Subtilis+ Staphylococcus simiulance	1	2.95	671
Total	34	100	-

(22.66%) quarters from 150 selected samples showed the presence of IMI. By using bacterial culture as a golden test, the sensitivity and specificity of CMT were calculated as 75 and 67.39% respectively for all isolated bacteria but they were 70 and 50% for the major pathogens respectively. The mean SCC and SCS in all quarters were 592.73 × 10^3 and 4.79 respectively and they increased as the CMT scores raised (Table 2).

On the basis of Discriminant analysis, Wilks' lambda was 0.859 (p< 0.001), meaning that the SCC had a significant predictive value in diagnosis of the severity of subclinical mastitis in negative, trace, 1+, 2+ and 3+ scores like CMT. Agreement percentage between CMT and SCC in each mentioned scores were 65.2, 28.7, 30.8, 16.7 and 100%, respectively and overall agreement for all groups was 43.2% (Table 3).

Bacterial identification and antimicrobial susceptibility

From the 150 cultured milk samples, 34 were infected, 110 healthy and 6 contaminated (Table 4). The most

Table 5. The mean value of SCC, SCS, TBC in intact and infected mammary quarters.

	Quarter	No.	Mean	Standard dev.	St. err. of mean
SCC*(cells/ml)	Intact	95	221.821×10^3	185.523×10^3	19.034×10^3
	Infected	32	779.203×10^3	2417.096×10^3	427.286×10^3
SCS	Intact	95	3.68	1.27	0.13
	Infected	32	4.21	1.75	0.3
TBC*(cfu/ml)	Intact	95	97.7	128.53	13.18
	Infected	34	6.894×10^3	16.022×10^3	2.747×10^3

*There are significant differences between infected and intact quarters ($p<0.05$).

Table 6. Antibiotic Susceptibility pattern of different bacterial isolates from infected quarters.

	No.m of Isolates	Lincomycin	Amikacin	Ampicillin	Bacitracin	Cephazolin	Cephalotin	Cloxacillin	Enrofloxacin	Erythromycin	Gentamycin	Novobiocin	Penicillin	Tetracyclin	Trimetoprim+Sulfamet oxazol	Tylozin	Cephquinome	Cephtiofur
S. Aureus	4	3	4	1	0	4	3	0	4	3	4	2	0	3	4	3	4	4
Str. Agalactiae	3	1*	0	0	0	0	0	0	3	3*	0	0	0	3*	3*	1*	3	3
S. Lentus	1	1	1	1	0	1	1	0	1	1	1	1	0	1	1	1	1	1
S. Intermedius	1	1	1	1	0	1	1	0	1	1	1	1	1	1	0	0	1	1
S. Epidermidis	4	4*	4	4	0	4	4	0	4	4	4	4	2	4	4	4	4	4
S. Saprophyticus	8	7	8	6	1*	7	4	0	8	8*	8	1	2	8	7	7*	8	8
S. Simulance	2	2	2	2	0	2	2*	1*	1	2	2	2	1	2	2	2	2	2
S. Hycus	2	1*	2	1*	0	2	2	0	2	0	2	0	0	2	2	2	2	2
Proteus Mirabilis	1	0	1	0	1	0	0	0	0	0	1	0	0	1	1	0	1	1
Cory. Bovis	5	2	3	4	3*	3	2	0	4	3	5	1	1	3	5	2	5*	5*
Bacillus Subtilis	6	2*	6	2	0	6	6	1*	6	6*	6	1	2*	5	6	6	0	5

* There is an intermediate susceptibility.

predominant bacteria isolated from infected quarters were the CNSs (Coagulase Negative Staphylococci) rather than *S. aureus*. The highest mean SCC was associated with *Corynebacterium bovis* infection and the lowest SCC was related to simultaneous infection with *Bacillus subtilis* and *Staphylococcus saprophyticus.* The kind of isolated bacteria had no effect on SCC means. But there was a significant difference in the mean TBC and SCC between intact and infected quarters ($p< 0.05$) (Table 5).

All isolated bacteria from infected quarters were tested for antimicrobial susceptibility. *S. aureus* was susceptible to at least 14 common antimicrobials except for Penicillin, cloxacillin and bacitracin. *S. agalactiae* had the highest susceptibility to the cephquinome and cephtiofur. The susceptibility of CNSs were similar to *S. aureus.* However it was highest to cephquinome, cephtiofur, gentamycin and amikacin and moderate to novobiocine. The Gram negative bacterium *Proteus mirabilis* was susceptible to aminoglycosids, bacitracin, tetracycline, trimetoprim+ sulfametoxazol and cephalosporins. *C. bovis* was not susceptible to cloxacillin and had poor responses to Penicillin and novobiocin. *B. Subtilis* was resistant to bacitracin, cephquinome with a poor susceptibility to novobiocin, Penicillin, cloxacillin and lincomycine (Table 6).

DISCUSSION

Subclinical mastitis is important due to the fact that is 15 to 40 times more prevalent than clinical form, is of long duration, difficult to detect, adversely affects milk quality and production of dairy animals and constitutes a reservoir of microorganisms that can affect other animals within the herd due to its contagious nature (Schultz et al., 1978).

Buffaloes have some characteristics that may contribute to greater risk of mastitis. For example, the udder is more pendulous and teats are longer in

comparison with cattle. Conversely, buffaloes have a long narrow teat canal, which may be expected to prevent the invasion of microorganisms (Moroni et al., 2006). In a study in Turkey in 1637 milk samples of Anatolian buffaloes, Ozenc et al. (2008) showed that 12.6% of quarters had subclinical mastitis on the basis of CMT. However in a similar study in Pakistan 6.7% of quarters were affected (Ahmad, 2001). Alacam et al. (1989) reported the distribution of subclinical mastitis as 4.7 to 16.3% in buffaloes, according to CMT results. In the current study 13.87% of quarters and 23.66% of buffaloes were diagnosed as affected by IMI. However, prevalence of IMI in quarters by means of bacterial culture was higher (22.66%) that reflects higher sensitivity of microbial culture in comparison to CMT. Such rate of prevalence is similar to bovine infection rate. Bacterial culture in other countries has shown varying results; in an Italian study which was conducted on 46 buffaloes and 1936 quarters, prevalence of IMI was high; 63% of quarters were infected and no buffalo remained free from IMI according to bacterial culture (Moroni et al., 2006), in India 8% of quarters and 21.7% of buffaloes were affected (Dhakal, 2006). It has also been pointed out that SCC is always compared with bacteriology, and these tests can never agree completely. Schalm et al. (1971) have reported that although subclinical mastitis can be diagnosed by means of CMT, it may not always be possible to isolate all of the microorganisms causing infection. Furthermore, factors including age, trauma, lesions of the teats, metabolic diseases, stress, genetic factors, management and feeding of animals, and viral mastitis are considered to influence yield of trace or positive CMT results (Ucar, 1998).

The relative high sensitivity of CMT (70%) in diagnosis of infected quarters with major pathogens in this study suggests that it can be used for cases suspected of these pathogens. However, for the diagnosis of subclinical mastitis in quarters affected with other bacteria; it is better to use CMT together with bacterial culture due to intermediate sensitivity (55%) of CMT in diagnosing such cases, but with regard to relative high specificity (67.39%) of CMT, its use can be recommended in screening of clinically healthy quarters. In a recent study in Turkey the sensitivity and specificity of CMT for subclinical mastitis were 45.6 and 90.4%, respectively for all pathogens (Ozenc, 2008).

In bovine, microbial cultures of individual or mixed quarter milk samples are used in diagnosing of IMI in order to monitor herd mastitis. In dairy cow mastitis, the most isolated bacteria were *Staphylococcus, Streptococcus* spp. and *C. bovis* (Wilson et al., 1997; Mylrea et al., 1977; Wilson and Richards, 1980) and results showed that Gram negative bacteria were not common pathogens (Wilson et al., 1997). Sears et al. (1993) reported low prevalence (0.6%) of *E-coli* and *Klebsiellal* mastitis and stated that IMI due to Gram negative bacteria had short infection period and they always could not be isolated by culturing.

The type of bacteria most frequently isolated in milk samples of buffaloes, with mastitis in some studies, has been CNSs (Chander and Baxi, 1975; Costa et al., 1997). *S. aureus* was the most important microorganism responsible for mastitis in buffaloes in one study (Jaffery and Rizvi, 1975). Moroni et al. (2006) reported that in Italian buffaloes CNSs were the most common pathogens (66% of all bacteria) and *Streptococcus, Bacillus* and *Proteus* spp. with the prevalence of 15, 2.1 and 0.2% were the next. Also in this study, the most common isolated bacteria were *staphylococcus* spp. specially the CNSs and gram negative bacteria were not recognized responsible for IMI in most cases.

Milk somatic cell count is an indirect indicator of mastitis in cows and has been used in buffaloes. It has been reported that, an SCC > 200 × 10^3 can be used as the cutoff point in diagnosis (Dhakal, 1992; Singh and Ludri, 2001). In this study the mean SCC in infected quarters was predominantly higher than intact ones ($p< 0.05$). In comparison with cattle, this elevation in mean SCS of infected quarters (SCS= 4.21) is not too high. It has been shown that intramammary infections in cattle (Djabri et al., 2002) and goats (Moroni et al., 2005) were associated with high SCS and SCC. This may be due to differences in phagocytic activity of the neutrophils in various species and also diversity in concentrations of hydrolases enzymes (including lysozyme) between cattle and buffaloes. Disparate concentrations of these enzymes have been reported to affect SCC amounts required for phagocytosis in each species (Sahoo et al., 1998).

Although the highest SCC was associated with *C. bovis,* the kind of isolated bacteria from infected quarters did not have any effect on the mean SCC of quarters in this study. A meta-analysis of 21 articles in dairy cattle (Djabri et al., 2002) found that *S. uberis* was one of the bacteria associated with the greatest elevation in SCC and the least SCS was related to *Staphylococcus* spp (SCS= 2.8). Different studies in other species have demonstrated that *S. aureus* is associated with more greatly elevated SCS than other staphylococci (Djabri et al., 2002; Moroni et al., 2005). In Moroni et al. (2006) studies in buffaloes the greatest SCS was observed in quarters infected by streptococci. The mean SCS in all quarters in the current study was close to dairy cattle as reported by Schutz (1994).

The most effective antibiotics determined against IMI agents isolated from buffaloes infected quarters under *in-vitro* conditions were Cephtiofur and trimetoprim+ sulfametoxazol with a sensitivity rate of 97.3 and 94.6%, respectively. Penicillin, cloxacilin and bacitracin had the lowest sensitivity for all isolates. It seems that cephalosporins and enrofloxacin are the best antibiotic agents against *S. aureus*. Others have reported that in buffaloes, amoxicillin + clavulanic acid were most effective, with a sensitivity rate of 92% (Alacam et al.,

1989; Ozenc, 2008).

Conclusion

According to the results of this study the prevalence of IMI in Azeri buffaloes is similar to that of Anatolian and Murrah buffaloes. CMT may have acceptable sensitivity and specificity in the diagnosis of subclinical mastitis in the absence of somatic cell count and microbial culture in most buffalo breeding farms. Isolated bacterial pattern of infected quarters are similar to dairy cattle and most probably coliforms are not important in buffalo mastitis.

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