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Full Length Research Paper

Bovine mastitis: Prevalence of bacterial pathogens and evaluation of early screening test

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Mastitis is the most complex and costly disease of dairy cows occurring throughout the world. In Algeria, the disease is not well investigated. A cross-sectional study to elucidate its magnitude, distribution, bacterial causes, and to investigate the reliability of a test for early diagnosis of mastitis in cattle in Blida and Ain Defla governorates, central region of Algeria, was carried out from May to December 2011 in a total of 108 milking cows using California mastitis test, clinical inspection of udder and bacteriological analysis. After conducting california mastitis test (CMT) in farms, quarter-based milk samples were collected from 108 cows. Also, 50 positive samples with CMT were collected for bacterial culture. Based on CMT, 29.20% of quarters and 29.62% of cows had subclinical mastitis; the sensitivity of CMT to infections with all bacteria was 96%. *Staphylococcus aureus* were the most common pathogens (40%). Based on the results of the current study, CMT has very acceptable sensitivity in diagnosis.

Key words: Bacteriology, CMT, milk, sensitivity, subclinical mastitis.

INTRODUCTION

Bovine mastitis is the most costly disease to the dairy industry worldwide, with losses estimated at 1.7 billion dollars per year in the United States (Sahoo et al., 2012).

This disease, characterized by an increase in somatic cells, especially leukocytes, in the milk and by pathological changes in the mammary tissue (Ranjan et al., 2010), causes colossal economic losses, but also hold the risk for the transmission of zoonotic diseases like tuberculosis, brucellosis, leptospirosis and streptococcal sore throat to human beings (Bachaya et al., 2011). Mastitis thus has become major area of concern in the field of veterinary clinical practice. Various forms of clinical and subclinical mastitis occur in bovines. In the clinical mastitis, the five cardinal signs of udder inflammation (redness, heat, swelling, pain and loss of milk production) are present, while the subclinical form, 3-40 times more common than the clinical one, is bereft of any manifestation of inflammation (Nielsen, 2009). Subclinical mastitis is recognized by laboratory examination of milk (Hokmabad et al., 2011) or animal-side tests (Bachaya et al., 2011). So, early detection of mastitic cows is important for most dairy farmers to reduce production losses and to enhance prospects of recovery but the common farmers are not so much familiar with these techniques.

Considering this point in view, the present study was conducted to determine the quarter-wise and animal-wise prevalence of subclinical mastitis in cows and mastitic agents in and around center region of Algeria. A further objective was to determine the reliability of a test for early

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Table 1. Distribution of cows according to their stage of lactation.

Study area	Stage of lactation (%)		
	Early (1st month)	Mid (2nd-4th month)	Late (≥5 months)
Ain Beniane	5 (38)	2 (15)	6 (46)
Boumedfaa	18 (39)	14 (30)	14 (30)
Hoceinia	9 (38)	9 (38)	6 (25)
Chiffa	6 (24)	16 (64)	3 (12)
Total of 4 areas (%)	139 (34,75)	147 (36,75)	113 (28,25)
Total cows (%)	38 (35)	41 (38)	29 (27)

diagnosis of mastitis in cattle using an easy animal-side test, California Mastitis Test (CMT).

MATERIALS AND METHODS

In the study, farm selection was based on the easiness of access to farms, availability of farmers and their willingness to participate. The animals lived nearly under the same conditions of breeding from the habitat, hygiene and feeding systems. They were often provided with some supplementary diet in addition to the natural pasture and agricultural by-products. The stage of lactation of selected cows is detailed in Table 1.

Collection of milk samples

A total of 428 milk samples were collected from 108 clinical healthy cows from different governorates in centre region of Algeria, based on the absence of clinical signs of mastitis: no fever, no inappetence, normal appearance and no consistency changes in the udder. The milk samples were collected aseptically by the same operator and subjected to California Mastitis Test. Relevant history of individual animal was recorded.

California mastitis test

The first few jets of milk were discarded and small quantity was used to realize the CMT (Raidex, Milt-04-10112009, Cyclovet, 2003 uncolored, Germany). A qualitative measurement of the SCC in milk is a screening test for subclinical mastitis that can be used easily at the cow side (Leslie et al., 2002; Bafitan et al., 2008). Quarter milk samples and surf solution were then mixed in equal quantities in Petri-dishes separately for each quarter. The change in consistency of milk indicated mastitis, while no change in consistency of milk indicated healthy samples. The CMT reaction was graded from 0 to 4. The scores were ranked according to an increase in viscosity. Animals were considered positive for mastitis when CMT score was ≥1+ and SCC value was $\geq 2\times10^5$/ml of milk (threshold value).

Collection of milk samples

Prior to sampling, the examined udder was thoroughly washed, dried with a clean towel and the teats were sprayed with 70% ethanol. The initial milk stripped from each udder (one udder each cow) was discarded and the next 10 ml were collected in a sterile container.

Separate samples were chilled to 4°C and transported to the laboratory with a minimum of delay for routine culture techniques.

Culture and identification of microorganisms

Bacterial pre-culturing

Milk samples were subjected to a bacteriological study by inoculating approximately 10 µl of each milk sample 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.

Isolation and identification of E. coli

From each pre-cultured sample, a loopfull of bacteria suspension was streaked on Mackonkey agar plate and then incubated at 37°C for 24 h under aerobic conditions. Some red colored colonies were randomly selected and transferred to individual plate of nutrient agar (1000 ml nutrient broth, 20 g agar) to make pure culture of bacteria isolates. After 24 h incubation under the same condition, each presumptive bacteria isolate was characterized on the basis of colony morphology, Gram-staining characteristics, oxidase and conventional biochemical tests, including indole test, methyl red test, Voges -Prokauer test, citrate test and sugar (glucose, lactose, maltose, mannose and sucrose) fermentation test using API 20 E for typing to the species level (BioMérieux, Craponne, France).

Isolation and identification of Staphylococcus

From each pre-cultured sample, a loopfull of bacteria suspension was streaked on Mannitol Salt agar (MSA) (10 g tryptone, 1 g beef extract, 75 g NaCl, 10 g mannitol, 20 g agar , 6 ml 0.4% phenol red solution, H_2O to 1 L, pH 7.4) that was widely used to cultivate *Staphylococcus* from clinical specimens and then incubated at 37°C for 48 h under aerobic conditions (Boynukara et al., 2008). Some representative colonies were randomly selected and transferred to individual plate of nutrient agar obtain pure culture of bacteria isolates. Following incubation for 24 h under the same condition, a single colony of bacteria was streaked on MSA plate. Yellow colored colonies were mannitol positive and suspected as *S. aureus* or *S. saprophyticus*, while red colored colonies were mannitol negative and suspected as *S. epidermids* or *S. saprophyticus* (Boynukara et al., 2008). Subsequently , gram staining, pigment producing, maltose fermentation test, alkaline phosphatase test, catalase test and coagulase test using fresh rabbit plasma (tube method) were used for the presumptive identification of all isolates (Gundogan et al., 2006). Bacteriologic examinations

Table 2. Quarter-wise prevalence of subclinical mastitis in cows in 4 zones of center region of Algeria.

Area	Total quarters tested	Affected quarter		One positive quarter		Two positive quarters	
		Number	% Age	Number	% Age	Number	% Age
Ain Benian	52	8	15.4	4	7.7	4	7.7
Boumedfaa	184	36	19.6	16	8.7	16	8.7
Hoceinia	96	33	34.3	16	16.6	17	17.7
Chiffa	96	48	50	24	25	24	25

Over all affected quarters = 29.20%.

Table 3. Animal-wise prevalence of subclinical mastitis in cows in 4 zones of center region of Algeria.

Area	Animals tested	Affected animals	Affected % Age
Ain Benian	13	2	15.4
Boumedfaa	46	9	19.6
Hoceinia	24	9	37.5
Chiffa	25	12	48

Over all affected animals = 29.62%.

were subsequently identified using API-Staph (for genus *Staphylococcus*, BioMérieux, Craponne, France). The API system enables the classification of strains in species based on biochemical features.

Isolation and identification of Streptococcus

From each pre-cultured sample, a loopfull of bacteria suspension was streaked on blood agar plate and then incubated at 37°C for 48 h under aerobic conditions. Pinpoint and dewdrop like colonies were randomly selected and transferred to individual plate of nutrient agar with 10% blood to make pure culture of bacteria isolates. Following incubation for 24 h under the same condition, each presumptive bacteria isolate was identified as presumptively belonging to the genera *Streptococcus* by colony morphology, gram staining and conventional biochemical tests, including catalase assay (Ericsson et al., 2009),but were not further characterized for this paper.

A milk sample was classified as positive if at least one colony forming unit (CFU) of *S. aureus* or *Streptococcus* spp was isolated. For other agents, the presence of at least three CFUs was needed for positive classification.

Samples were classified as contaminated if three or more bacterial types were isolated from one milk sample and growth of a major udder pathogen was not identified. If growth of a major udder pathogen was found in combination with contaminating species and if the CMT was high, the sample would be diagnosed as positive for growth of the major udder pathogen.

Statistical analysis

Prevalence of mastitis was determined as the proportion of affected cows out of the total examined. The statistical analysis was performed with the Statistica software (V. 6), ANOVA. Thus, all obtained results we processed with the method of absolute and relative frequency and their testing is done with Z – test for comparison at the level of significance α = 0,05.

RESULTS AND DISCUSSION

CMT test results

Quarter-wise and animal-wise prevalence of subclinical mastitis in selected cows in center region of Algeria using CMT is shown in the Tables 2 and 3, respectively.

The quarter-wise prevalence was 29.20% while animal-wise prevalence was 29.62%. Contrary to our finding, Heleili et al. (2012) reported higher prevalence animal-wise prevalence of subclinical mastitis in cows (96.36%).

The value of incidence of subclinical mastitis reported in this study (29.2%) was similar to that obtained in France. Longo et al. (1994) reported a prevalence of 25% in cows on the basis of California Mastitis Test. In Spain, Ares et al. (1995) used cultural examination and reported a figure of 33.5% for cows. In Venezuela, Ferraro et al. (1999) reported a prevalence of 30.18% of subclinical mastitis in cows and cattle, on the basis of direct, indirect and cultural examination.

Hilali (2003) observed the prevalence of 50% in cows (quoted by Bouaziz, 2005). Bachaya et al. (2011) used Surf Field Mastitis test (SFMT) and found that 45% buffaloes suffered from subclinical mastitis.

Differences in management conditions, methods of detection, breeds of the animals, immune response of animals and climatic conditions, animal health, stress,

Table 4. Result of bacteriological analysis and frequency of bacterial strains isolation from subclinical Mastitis.

Organisms isolated	Number	Frequency (%)
Staphylococcus aureus	12	24.00
Streptococcus spp.	3	6.00
E. coli	1	2.00
Pseudomonas spp.	1	2.00
Staphylococcus Xylosus	3	6.00
Staphylococcus Warneri	1	2.00
Klebsiella oxytoca	1	2.00
Serratia odorifera	1	2.00
Enterobacter cloacae	1	2.00
Pneumotropica heamolytica	1	2.00
Providencia stuartii	1	2.00
Providencia alcalifacians	1	2.00
Mixed infection	21	42.00
Contaminated sample	1	2.00
Sterile collection	1	2.00
Total	50	100.00

Mixed infection= *Staphylococcus lentus* + *Klebsiella ornitholytica.*

hence immunity decreases and management conditions are probably risk factors of mastitis and may be explain the difference in prevalence of subclinical mastitis observed in the present and previous studies.

The studied factors were determined as risk factors affecting mastitis as breed (Bendixen et al., 1988), season, age (Hultgren, 2002), management conditions (Faull et al., 1985), environment (McDougall, 2003) and hygiene (Ward et al., 2002).

Mammary gland infection is the most important factor affecting somatic cell count in milk in the subclinical mastitis by increasing the number of somatic cells in milk (Bachaya et al., 2011). The presence of mastitis in dairy cows in our field conditions is due, in our opinion, to the absence of practicing regular screening tests for subclinical mastitis in the field by the farmers. Furthermore, cow therapy, pre and post-milking teat dip are not practiced at most of the milking sheds. Milking procedure in our conditions is accompanied with unhygienic conditions.

Bacteria isolated during subclinical mastitis

Many bacterial strains have been isolated representing a rate of infection of about 98% of CMT positive samples. This result was similar to those of Trinidad et al. (1990) who reported an infection rate of 96.9% and those of Heleili et al. (2012) who reported an infection rate of 87.25%. Among 25 dairy cows, harbor pathogens that are incriminated in subclinical mastitis correspond to 31 different germs while one cow (4%) presented a negative isolation but could harbor germs we could not isolate. Some of the microorganisms, like *Listeria* spp. and *Mycoplasma* spp., require specific culture media (Ranjan et al., 2010). Negative bacteriological results can be due to the presence of antibiotic residues (Longo et al., 1994).This could be the case because the withdrawal time is not respected in our herds.

A higher incidence of *Staphylococcal mastitis* was revealed following cultural examination of positive samples which is in agreement with Harini and Sumathi (2011) and Heleili et al. (2012).

Our results had revealed the predominance of *S. aureus* with 40%. This is in accordance with data from other countries (Busato et al., 2000; Ben Hassen et al., 2002; Tenhagen et al., 2006; Molalegne et al., 2010; Heleili et al., 2012). In the second plan was *Streptococcus* spp. with 12%. Other bacteria were isolated with variable and low frequency as shown in Table 4.

Most common pathogens isolated from mastitis were contagious. As the milking procedures were accompanied with unhygienic conditions, these pathogens were exposed to non-mastitic animals from milker's hands, because no preference of milking non-mastitic animals first is done. There is close contact between healthy and diseased animals in common grazing and wallowing places.

As weaning is not practiced, so un-weaned calves often cause injury to the udder because of biting, pulling and hitting and create a focus for infection. While open grazing in the field is large, pendulous and hanging udders are often exposed to injury and infection develops.

Table 5. Test results compared to CMT test and bacteriological culture.

Study area	Number of quarter			
	Tested at CMT	Positive at CMT (%)	Tested in Bact.	Bact. + (%)
Ain Benian	52	8 (15.38)	2	2 (100)
Boumedfaa	184	36 (19.56)	9	8 (88.88)
Hoceinia	96	33 (41.66)	27	27(100)
Chiffa	96	48 (48)	12	12 (100)
Total	428	125 (25)	50	49 (98)

Table 6. Distribution of bacteria species (nature and number) by CMT scores.

Total of samples per score	Score CMT	Results of bacteriology				
		Number of species				Nature and number
		0	1	2	3	
10	1 (±)	1	7	2	0	*S. aureus (1); Strep.* spp*. (1); Enterobacteriaceae (1); S. aureus + E. coli (2);*
14	2 (+)	0	6	8	0	*S. Xylosus (3); Providencia alcalifacians(1).* *S. aureus (1), Pseudomonas* spp. *(1), Streptococcus* spp. *(2); S. aureus + Streptococcus* spp. *(1), S. aureus+ Mycoplasma* spp. *(1); S. Lentus + Klebsiella ornitholytica (6); Serratia odorifera (1); Enterobacter cloacae (1).*
9	3 (++)	0	6	2	1	*S. aureus (4), S. aureus+ Streptococcus* spp. *(2), Streptococcus* spp. *+ S. aureus + E. coli (1) ; Pneumotropica heamolytica (1) ; Providencia stuartii (1)*
17	4 (+++)	0	8	9	0	*S. aureus (6), Streptococcus* spp. *+ E. coli (2); S. Lentus + Klebsiella ornitholytica (7); S. Warneri (1); Klebsiella oxytoca (1)*
50	Total of isolates	0	27	40	0	

Thus, this explains the high prevalence of mastitis pathogens contagious in our farms.

Correlation between results of CMT and bacteriological culture

On the basis of CMT, we reported a prevalence of 29.62% in cows. On the basis of cultural examination, we found a rate of 98% (49/50) of positive samples to CMT. These have been really subclinical mastitis in cows. Thus, the rate of positive isolates is 98%. All samples testing positive at CMT contain pathogenic bacteria except one (Tables 5 and 6).

The present results show a good correlation between the results of CMT and isolation for the identification of intra-mammary infections in cows. This suggests a high reliability of the test used CMT. Same results were obtained by Ruegg and Reiman (2002), Kivaria et al. (2004) and Rasmussen et al. (2005). These authors concluded that the CMT is still the superior screening diagnostic aid for subclinical mastitis, while bacteriological examination is still the most suitable for identifying mastitis but not feasible as a routine test to identify subclinical mastitis because some logistical and financial constraints limit its use especially in developing countries.

Conclusion

The prevalence of mastitis observed in this study was 29.62%. *S. aureus* and *Streptococcus* spp. were the main pathogens isolated that cause subclinical mastitis in dairy animals in center region of Algeria. In the absence of bacteriological analysis, the CMT is an interesting alternative to identify cows with mastitis before considering treatment.

To reduce the incidence of mastitis to an appreciable extent and the production increased, the following management measures should be adopted:

1) Keeping the animals on wet and dirty floors should be discouraged,
2) Pacca floor must be even and properly bedded,
3) Full hand milking should be practiced,
4) All cows should be treated at drying off with antibiotics,
5) Mastitic animals should be kept and milked separately. Otherwise, infected animals should be milked after non-infected ones,
6) Post-milking teat dipping with a germicidal dip is recommended,
7) After milking, the animal should not be allowed to sit immediately, because after milking the teat sphincter remain open for some time and if animal sits at that time there are maximum chances of infection due to contact of teat with unhygienic places,
8) Using a simple screening test like CMT, farmers should test the dairy animals before purchasing, if positive, avoid buying such animals and mastitic milk is unwholesome for human consumption, and
9) Culling of chronically infected animals.

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