



Original Research

Prevalence and Diagnosis of Subclinical Mastitis in Sahiwal Dairy Cows

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Abstract

The present study was conducted on 102 Sahiwal dairy cows to assess the prevalence of subclinical mastitis, type of bacteria present and ability of different parameters to diagnose subclinical mastitis. The prevalence of subclinical mastitis was found to be 43.12% animal wise and 18.14% quarter wise. The coagulase negative staphylococci (CNS) were the most common isolate both in specific mastitis (59%) and latent infections (47%). The *Corynebacterium* was found another important isolate in latent cases (33%). The evaluation of different markers in the diagnosis of subclinical mastitis revealed N-acetyl-beta-D-glucosaminidase (NAGase) as the most sensitive (84.29%) and the California Mastitis Test (CMT) most specific (89.09%) test. The milk SCC showed a good correlation with CMT (0.68) and NAGase (0.59). The CMT and NAGase possessed a correlation coefficient of 0.60. Overall, the CMT, NAGase and electrical conductivity (EC) were found as best markers for identification of subclinical mastitis quarters in Sahiwal cows.

Key words: Etiology, Diagnosis, Prevalence, Sahiwal Cows, Subclinical Mastitis

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Introduction

Sahiwal, a breed of Zebu cattle, produces the most milk of zebu breeds, and has advantages over the exotic/crossbred cattle that it possesses high level of tick and parasite resistance along with tolerance to high environmental temperature. However, despite its adaptability to native conditions, a very little research has been done on this breed. Mastitis, the inflammation of udder occurs mainly due to infection, and resulted in huge economic losses of Rs. 7165.51 crores per annum in India (Bansal and Gupta, 2009). The severity of the inflammation can be classified into subclinical and clinical mastitis. The subclinical mastitis is difficult to detect due to the absence of any visible indications. However, certain markers of inflammation



in the milk can be used for its detection. The golden standard to measure the inflammation of the mammary gland is the cytological investigation, the milk SCC (Hamann, 2002). The activity of the enzyme N-acetyl- β -d-glucosaminidase (NAGase) in milk has also been shown associated with the udder inflammation (Hovinen *et al.*, 2016). The major source of NAGase in milk appears to be the mammary gland secretory cells while other sources (white blood cells, blood serum) contribute only a minor proportion (Kitchen *et al.*, 1978). The others changes in mastitis include invasion of phagocytic cells and leaking of ions, proteins and enzymes from the blood into the milk, and a decrease in the synthetic capacity of the gland, resulting in decreased concentrations of certain milk constituents such as lactose. These changes in composition of milk have been exploited as mastitis diagnostic tests like electrical conductivity, pH, lactose and California mastitis test (Bansal *et al.*, 2005). Most of the research related to bovine mastitis has been concentrated on exotic and crossbred species. Also, a few studies showed some difference in test behaviour when applied in indigenous breeds (Jingar *et al.*, 2014, Kausar *et al.*, 2015). Due to this, present study was planned to evaluate the udder health in Sahiwal cows.

Material and Methods

The study involved 102 lactating Sahiwal cows kept on an organized dairy farm. The quarter foremilk (QFM) samples were collected aseptically at the routine evening milking and analyzed for various parameters as under-

Bacteriology

The isolation and identification of microbial organisms from QFM samples were done as per standard microbial procedures of the National Mastitis Council (Brown *et al.*, 1969). The blood agar media was used for primary isolation of bacteria. Each plate was divided into four equal parts marked for individual quarters, and 0.05 ml of quarter milk sample was streaked with sterilized platinum loop. The plates were incubated aerobically at 37°C and examined after 18-24 hours for the presence of any bacterial growth. The *Staphylococcus* species was suspected if round, shiny and golden yellow colonies with or without a zone of haemolysis was seen on blood agar. On staining with grams stain, the Gram positive cocci were seen in clusters for staphylococci, and in chains for streptococci. The individual bacterial colonies were picked up and subjected for identification. The catalase test (slide test) was conducted to differentiate streptococci and staphylococci. Here, a small amount of bacterial colony was transferred to a clean, dry glass slide using a wooden stick and a drop of 3% H₂O₂ was put on to the slide and mixed. The staphylococci which were catalase positive resulted in the rapid evolution of O₂ gas (within 5-10 seconds) as evidenced by bubbling, on reacting with 3% H₂O₂. The streptococci which were catalase negative showed no bubbles. After conducting the test, slide was disposed off in the biohazard glass disposal jar. To differentiate *S. aureus* from other CNS, Mannitol salt agar was used. The *S. aureus* produced yellow colonies with yellow zones,



whereas other staphylococci produce small pink or red colonies with no color change to medium. In addition, Baird Parker agar was used as a selective medium for the isolation and identification of *S. aureus* which produced dark grey to black colonies and an opaque zone of precipitation due to lipase activity. The corynebacteria were identified as Gram positive, non-spore forming, short, pleomorphic rods appearing in clusters joined at angles like Chinese letters. These organisms produced very small, circular, opaque, and white to creamy colonies on blood agar. The colonies were seldom visible before 48 hours of incubation.

- Somatic cell count using milk somatic cell counter from DELTA Instruments, The Netherland. Results were expressed in $\times 10^3$ cells/ ml.
- California mastitis test (CMT) as per standard method described by Pandit and Mehta (1969).
- Electrical conductivity (EC) with the help of digital conductivity meter (Eutech Instruments, CON 700). The results were expressed in milli Siemens per cm (mS/cm) at 25°C.
- pH using Systronics, μ pH system 361.
- Biochemical composition of milk using milk analyzer Lactoscan LA from Milkotronic Ltd., Bulgaria. The results were expressed in %.
- NAGase using the spectrophotometric method of Kitchen *et al.* (1978).

The quarter health status of the cows was defined considering SCC and culture results of quarter foremilk using International Dairy Federation (IDF) criteria as detailed below-

Milk SCC (cells/ml)	Microbial pathogen	
	Not detected	Detected
$\leq 500\ 000$	Healthy	Latent infection
$> 500\ 000$	Nonspecific mastitis	Specific mastitis

The data so obtained was subjected to discriminant function analysis for evaluation of different markers as indicators of subclinical mastitis. The threshold values for each parameter was set to achieve the highest discrimination ratio in differentiating healthy and mastitis quarters. Here, the mastitis group represented both the non-specific and specific subclinical mastitis quarters. The latent infections were not considered as they usually initiated no inflammatory reaction in the udder. At udder level, the cows with all at least one quarter with specific or nonspecific mastitis were categorized into mastitis udder but otherwise considered healthy i.e. cow with all the four healthy quarters. The interrelationship among different markers was studied by statistical correlation expressed as Pearson correlation coefficient.

Result and Discussion

Prevalence of Subclinical Mastitis

The prevalence of subclinical mastitis in the Sahiwal cows was found to be 43.13% animal wise and 18.14% quarter wise with 9.56% and 8.58% quarters representing specific and nonspecific mastitis, respectively (Table 1). The different workers have reported variable occurrence of mastitis in different breeds of cattle, and even the least squares analysis carried by Khate and Yadav (2010) revealed that breed, season of calving



and milk yield had a significant effect on the incidence of mastitis. These workers found the overall occurrence of mastitis in Sahiwal cows as 26.43%. Similarly, Sudhan *et al.* (2005) reported 43.33% prevalence of subclinical mastitis in crossbred cows.

Table 1: Prevalence of subclinical mastitis in Sahiwal dairy cows

Quarter Level			Animal Level		
Health Status	N	%	Health Status	n	%
Healthy	281	68.87	Healthy	58	56.87
Latent infections	49	12.01			
Nonspecific mastitis	35	8.58	Mastitis*	44	43.13
Specific mastitis	39	9.56			
Blind	4	0.98		-	
Overall	408			102	

*Udders representing at least one non-specific or specific mastitis quarter

In other recent studies on crossbred cows in India, Kumar (2012), Meir *et al.* (2014) and Abebe *et al.* (2016) found the prevalence of subclinical mastitis at 57.80%, 50.81% and 62.6%, respectively. Bangar *et al.* (2015) conducted a systematic review of the prevalence of subclinical mastitis in dairy cows for the period 1995-2014 in India and found the quarter wise subclinical mastitis to be 23.25%. Patel and Trivedi (2015) also in their study on crossbred cows found 23% infected quarters. Hence, probably it may be concluded that Sahiwal cows as compared to crossbred cows suffered less from mastitis. The variation in prevalence of disease in different studies may be attributed to various risk factors responsible for development of mastitis. These factors are present at both cow and quarter level.

Etiology of Mastitis

The staphylococci with predominance of CNS (59%) were found responsible for almost 85% cases of specific subclinical mastitis (Table 2).

Table 2: Pathogens of specific mastitis and latent infections in Sahiwal cows

Type of Infection/ Organism	Specific Mastitis		Latent Infections	
	%	%	Number	%
Coagulase Negative Staphylococci	23	58.97	23	46.94
<i>S. aureus</i>	10	25.64	6	12.24
<i>Corynebacterium</i>	4	10.26	16	32.65
Streptococci	2	5.13	4	8.16
Total	39		49	

Although, again CNS was the chief organism in latent infections, the corynebacteria represented another important isolate. The proportion of *S. aureus* was 25.64% in specific mastitis and 12.24% in latent cases. Worldwide, several studies have investigated the importance of the CNS in udder health. Kumar (2012)



and Mir *et al.* (2014) reported similar findings of high CNS prevalence in crossbred cows. In Finland, CNS was isolated from 50% of the quarters positive for bacterial growth in a nationwide survey (Pitkala *et al.*, 2004). Again in Canada, the CNS was found to be the most common bacteria (51%) causing intramammary infection at dry off (Lim *et al.*, 2007). Similarly, Haenni *et al.* (2010) and Jakeen *et al.* (2013) reported 13.7% and 16.6% cases of subclinical mastitis due to CNS, respectively. The CNS has become the most common bacterial pathogens isolated from milk samples in many countries, causing bovine intramammary infections (Radostits *et al.*, 2006) and could be described as emerging mastitis pathogens (Pyorala and Taponen, 2009). They are opportunists and adhere to metal devices to produce a protective biofilm. The ability to produce biofilm enables the CNS to persist on milking equipment as well as on the milker's hands, which serves a major source of staphylococcal spread (Pilipčincová *et al.*, 2010). Also the CNS has traditionally been considered to be normal skin microbiota, which as opportunistic bacteria can result in intramammary infections in the absence of proper teat disinfection.

Corynebacteria have been isolated from 33% cases of latent infections and 10% cases in specific mastitis. The increased frequency of coryneform infections has been noted elsewhere (Pitkala *et al.*, 2004, Kumar, 2012 and Mir *et al.*, 2014). This could probably be due to changes in herd management and consequent bacteriological ecology in the herd environment. Proper milking procedures, including the use of effective post-milking teat disinfectants, will help to reduce the number of new infections. In the farm under our study, teat dipping was not regularly practiced. The suboptimal post milking teat disinfection may result in a high herd prevalence of minor pathogens (Lam *et al.*, 1997). *C. bovis* persists with routine application of teat disinfection but is eliminated with dry cow antibiotic therapy. The *C. bovis* may cause mastitis but are generally considered as opportunistic pathogens and inhabitants of teat canals (Rainard, 1986).

The prevalence of *S. aureus* in our study was found to be less as compared to previous studies in other breeds of cattle (Lingathurai and Vellathurai, 2010, Kumar, 2012 and Kaur *et al.*, 2016). The high proportion of *S. aureus* in specific mastitis may be attributed to many factors, including the indiscriminate use of antibiotics that may have led to the emergence of more resistant strains (L-form). Some strains of organisms are present deep in alveolar tissue and capable of forming microabscess around them, so that they may escape the drug action and hence elimination from the udder. At the same time, the organism can survive for longer periods in environment and are widely distributed over the body surface of lactating animals and teat cups of the milking machine, and so can easily gain entry into the teat canal.

Discrimination Ability of Mastitis Markers

The ability of various markers to differentiate between healthy and mastitis quarters is presented in Table 3. The discrimination ratio showed the highest level for CMT (86.74%). The 89.09% of the quarters with SCC $\leq 500,000$ cells/ml showed < 1 CMT score (Table 3), with only 10.91% giving false-positive CMT

reactions. Similarly, false-negative predictions for CMT were calculated to be 22.22%. This is comparable to the findings of Kumar (1998) who, while using CMT at an SCC threshold of 100, 000 cells/ml of milk in German Black Pied cows could correctly identify 88.31% of quarters. The positive predictive value/sensitivity of CMT reported in the present study is also comparable with several previous reports (Saluja *et al.*, 2004, Muhammad *et al.*, 2009, Dubal *et al.*, 2010, Sharma *et al.*, 2010 and Langer *et al.*, 2014). However, the efficacy of CMT in identifying mastitis quarters was found much higher than that reported by Chahar (2007).

Table 3: Discrimination ability of different markers in diagnosing subclinical mastitis in Sahiwal cows

Parameter	Thres-hold	Sensitivity	Specificity	Discrimination ability	Positive Predictive value	Negative Predictive value	Diagnostic odds ratio	95% CI	Z statistic	Significance
CMT	1	77.78	89.09	86.74	65.12	93.87	28.58	14.59- 56.01	9.77	P<0.001
EC	5.55	82.54	63.01	66.99	36.36	93.37	8.05	3.99-16.21	5.84	p<0.001
Lac-tose	5.3	78.12	60	63.4	35.21	90.79	5.36	2.80-10.25	5.07	p<0.001
NA-Gase	77	84.29	73.41	75.67	45.38	94.78	14.81	7.36-29.77	7.56	P<0.001
pH	6.59	66.13	53.46	56.27	28.87	84.67	2.24	1.24-4.04	2.68	P = 0.007

The EC with a positive predictive value of 36.36% resulted in 33% misclassification in differentiating healthy and mastitis quarters. This is comparable to the 38.40% reported by Bansal *et al.* (2005). Langer *et al.* (2014) reported a higher positive predictive value of 61.7% and was supported by findings of Chahar (2007). Similarly, Holdaway *et al.* (1996) reported an accuracy of 58% for the EC of bucket milk samples. The NAGase with an overall discrimination ratio of 75.67% possessed highest sensitivity (84.29%) among all the markers tested. This is in close agreement with the findings of Holdaway *et al.* (1996) and Bansal *et al.* (2005) who also reported more than 70% efficacy for NAGase activity of foremilk in differentiating the healthy and mastitis quarters. Pyorala (2003) also concluded the NAGase concentration in milk is the best candidate for detection of subclinical mastitis. Reasonable accuracies were observed for lactose in discriminating the healthy and mastitis quarters; the discrimination ability being 63.40% (Table 3). Holdaway *et al.* (1996) with an accuracy of 60% of the bucket milk also found the lactose a relatively less sensitive indicator of mastitis. However, this is away from the findings of Bansal *et al.* (2005) where lactose content permitted correct identification of 80.85% of quarters and 75.66% of mastitis cows.

In overall, pH was not found to be a good inflammatory indicator of udder, the probability of misclassification being more than 43% (Table 4). Holdaway *et al.* (1996) and Bansal *et al.* (2005) also reported milk pH as less efficacious in detecting subclinical mastitis in cows. Bansal *et al.* (2005) observed that only 30% samples from mastitis quarters had a pH > 6.60; a threshold value selected for obtaining maximum accuracies in differentiating healthy and mastitis quarters. However, higher sensitivity was



reported for this parameter (Langer *et al.*, 2014) where the positive predictive value of 58% was seen unlike 28.87% in the present study.

Table 4: The correlation between different markers used in the diagnosis of subclinical mastitis in Sahiwal cows

	CMT	pH	EC	SNF %	Lactose	NAGase
SCC	0.68	0	0.35	-0.26	-0.24	0.59
CMT		-0.02	0.44	-0.3	-0.29	0.6
pH			0.01	-0.04	-0.03	-0.02
EC				-0.56	-0.43	0.35
SNF %					0.76	-0.13
Lactose						-0.05
NAGase						

Correlation between Markers

Table 4 depicts correlation coefficients between the various mastitis indicators. The highest correlation was found between SCC and CMT (0.68) and the SNF and Lactose (0.76). The present findings are in agreement with Vihan and Sahni (1987) and Bansal *et al.* (2005) who also reported that SCC and CMT had a high mutual agreement. The NAGase also showed a good correlation with the SCC and CMT; 0.59 and 0.60, respectively. This is in accordance with the findings of Andrei *et al.* (2009), who reported direct correlation between enzyme activity and SCC in milk samples. NAGase activity has got a good correlation with SCC (Kitchen, 1978, Leitner *et al.*, 2003 and Pyorala, 2003). Hence, it could be concluded that NAGase can be employed as a diagnostic test for subclinical mastitis. The EC, even though had an average correlation coefficient as compared to relationship between other variables, but this is fairly significant.

Conclusion

There seems to be a less prevalence of mastitis in Sahiwal cows in the present study when compared with available data on crossbred cow populations. However, due to limited data related to a single herd only, more studies are required for this breed under different climatic and management conditions to arrive at a definitive conclusion. The etiological agents were found similar to that reported in crossbred cow, but comparatively the lower prevalence of *Staphylococcus aureus* was seen in Sahiwal cows. Like crossbred cows, the CMT, NAGase and EC were found the reliable methods for diagnosis of subclinical mastitis in Sahiwal cows also.

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