Sub-Clinical Mastitis Detection in Can Milk and Isolation of Causative Organisms

Thapa Sangita[*], Karki Tika B., Poudel Pramod, Thapaliya Manish and Shakya Sastrika

Department of Microbiology, National College, Kathmandu, Nepal

Abstract

Milk is a nutritional substance consumed as fluid with minimal processing which is also a raw material for wide variety of products. All the nutritional components that make milk and milk products an important part of the human diet also support the growth of pathogenic organisms. Mastitis is the most costly disease of dairy animals as it reduces milk yield and results in partial or complete damage to udder tissues and decreases the productive life span of animal. This study was conducted to detect the presence of mastitis milk and identify the causative organism of mastitis. A total of 188 samples were analysed. Out of 188 samples, SLST test (modified California Mastitis Test) reaction score were distributed as; negative 44.68% (n=84), score 1+ 22.87% (n=43), score 2+ 17.02% (n=32) and score 3+ 15.42% (n=29). The relationships between SLST test and Somatic Cell Counts was found to be statistically significant. Also, SLST test and salt test showed statistically significant relationship (P<0.05). The causative organisms were isolated from SLST positive milk samples (n=44). Staphylococcus aurous was predominant followed by Coagulase negative Staphylococcus and then Streptococcus agalactiae.

Keywords: Sub-clinical mastitis • California mastitis test • Somatic cell count • Staphylococcus aureus • Coagulase negative Staphylococcus • Streptococcus agalactiae

Introduction

Milk is the nutrient fluid secreted by mammary gland of female mammals to nourish their young children. According to Hurley, milk is an emulsion of fat globules and a suspension of casein micelles, suspended in an aqueous phase that contains solubilized lactose, whey proteins and some minerals and salts. Milk is an excellent source of calcium and vitamins. Being a nutritional, balanced food stuff that contains only few organisms when leaving the udder of a cow, it can get contaminated from various sources. The health hazards posed by the milk-borne zoonotic diseases and mastitis related enterotoxaemia are well described [1].

Mastitis is the inflammation of mammary gland of mammals which is characterized by the physical, chemical and bacteriological changes in the milk and by the pathological changes in the glandular tissues. This condition is widespread in dairy herds and is associated with a significant reduction in milk yield, increased costs of production and deteriorated milk quality. There are mainly two types of mastitis; cow-associated (or contagious) mastitis and environmental mastitis [2].

Mastitis in dairy animals occurs in the clinical and sub clinical form. Clinical mastitis (CM) includes swelling, heat, pain and induration in mammary glands and the most important changes in the milk are discoloration, presence of clots and large number of leucocytes. Sub-clinical mastitis (SCM) has no visible changes in udders or teat but milk production is decreased with increase in somatic cell counts in the milk. SCM is recognized only by laboratory examination. CM is an individual cow problem whereas SCM is a herd problem Wishart.

There are various indirect tests to detect SCM; the most common being measurement of somatic cell count (SCC) in the milk. With higher SCC, the concentration of serum albumen and immunoglobulin is increased which reduces heat stability of mastitic milk and may cause coagulation at temperatures applied during manufacturing processes, or flocculation during pasteurization. Therefore, detection of sub-clinical mastitis is important to prevent economic losses because of long term effects on milk yields [3].

Materials and Methods

Sample size and site

The study was carried out from December 2018 to February 2019. The samples included raw milk received from different district and pasteurized milk from plant during processing. A total of 188 samples were analyzed. Among which, 15 samples were processed milk, 75 samples were local collections received in can and 98 samples were farmers levels received from different cooperatives.

Sample collection

A total of 188 raw milks were collected in sterile bottle. All the microbiological analysis was performed in Microbiological Laboratory of Sujal Dairy. And, out of 188 samples, 90 samples were processed for somatic cell count which was carried out in Regional Veterinary Laboratory, Pokhara [4].

Detections of sub-clinical mastitis in milk by modified California mastitis test CMT (Sodium lauryl sulphate test, SLST)

CMT was performed in a plastic paddle fitted with four cups. Two to three ml of milk sample was taken in the cups and reagent was added in equal volume. The viscosity was observed within 30 seconds. Scoring of SLST was observed.

Salt testing of milk (NDDB)

Five ml of 1.0N silver nitrate was taken in a clean test tube. 2 drops of 5% potassium chromate indicator was added. The color of silver nitrate became brick red and then 3ml of milk was added. The change in color change was noted. Change of red color to yellow indicated presence of salt [5].

Isolation and Identification of causative organisms

The serial dilution up to 10-3 of the milk sample in sterile distilled water was carried out. Appropriate aliquot of diluted milk sample was pour plated on Manito salt agar, Blood Agar and violet red bile Agar. The plates were incubated at 37oC for 24 hours according to Feng. The colonies of Manito Salt Agar that fermented Manito were selected and sub-cultured on Nutrient Agar and incubated at 37oC for 24 hours. Similarly, the typical colonies on Blood Agar were selected and sub-cultured on Nutrient Agar and incubated at

Address for Correspondence: Thapa Sangita, Department of Microbiology, National College, Kathmandu, Nepal Tel: 7046812221; E-mail: rstalnaker@iss.k12.nc.us

37°C for 24 hours. The pure colonies from Nutrient Agar were identified. Gram staining was performed followed by different biochemical test [6].

Coliform counting

Coliforms were counted using pour plate technique as mentioned by Feng. 10 ml of raw milk was diluted in 90 ml of distilled water and agitated properly to make a complete mixture. This mixture was considered to be a homogenate. Serial dilution of the homogenate was carried out and 1 ml of 3 consecutive dilutions was transferred to petri dishes. The plates were overlaid with violet red bile agar (VRBA) and solidified. Incubation was done at 35°C for 18-24 hours. The plates with 30-300 colonies were selected and the number of colonies was counted and number of organism was calculated [7, 8].

Somatic cell count

The milk sample was shaken well to distribute the cream evenly through the milk. 0.01 ml of milk sample was spread over area of 1 cm2 on a glass slide using a standard chromium wire loop. The smear was air dried or kept at 30-50oC on a carefully leveled surface protected against dust and insects. Smears were then immersed in stain in poplin jar for about 1 minute. Smears were removed from staining jar and drained until dry. Distilled water was kept in two beakers and dry smears were immersed gently three times in each beaker for washing. Surplus stain was washed away; the slides were then dried in air and examined under oil immersion lens. Cells in the smear were counted at least twenty fields and average numbers were calculated [9-11].

Data analysis

Statistical analysis of the data was performed by SPSS (version 11.5) and Microsoft excel. The chi square test and t-test were done to observe any significant relationship among the variables and P value <0.05 was considered statistically significant in this test (Table 1).

Results

Detection of sub clinical mastitis in can milk sample

Out of the 90 samples analyzed, 46 samples (51%) showed negative test towards the sub-clinical mastitis, 17 samples (19%) showed slight presence of sub-clinical mastitis (score 1+), 16 samples (18%) showed moderate sub-clinical mastitis (score 2+) and remaining 11 samples (12%) showed highly sub-clinical mastitis (score 3+). The score of different milk sample is shown in [12] Figure 1.

Somatic Cell counting in all the milk

Out of 188 samples, 90 samples collected in Sujal dairy after screening for SCM using SLST and subjected to SCC. It was calculated that the mean somatic cell count of CMT negative (46 samples) was 426,132 cells/ml, CMT score 1+ (17 samples) was 1,307,106 cells/ml, CMT score 2+ (16 samples) was 2,480,803 cells/ml and score 3+ (11 sample) was 5,231,562. Mean somatic cell count with standard deviation is shown the relation between mastitis test and somatic cell count is shown in Table 2.

Relationship between salt test and Subclinical mastitis

Out of 90 samples, 46 samples were CMT negative and remaining 44 samples CMT positive. Salt test was also done for all 90 samples out of which 62 samples were salt test negative and 28 samples were salt test positive. Out of 46 CMT score negative, 80.4% (37 samples) were salt negative and 19.6 % (9 samples) were salt positive. Out of 17 samples with CMT score 1+, 52.9% (9 samples) were salt negative and 47.1% (8 samples) were salt positive [12-14]. Out of 16 samples with CMT score 2+, 68.8% (11 samples) were salt negative and 31.3% (5 samples) were salt positive. Out of 11 samples with CMT score 3+, 45.5 % (5 samples) were salt negative and 54.5% (6 samples) were salt positive. Relationship between salt test and CMT test is statistically highly significant as shown in Table 3.

Figure 1. CMT Score of different milk collected in SDPL.

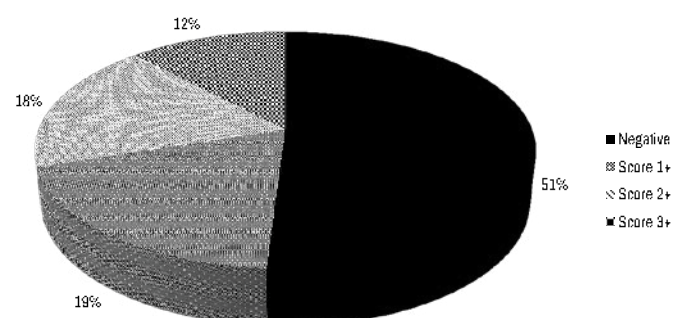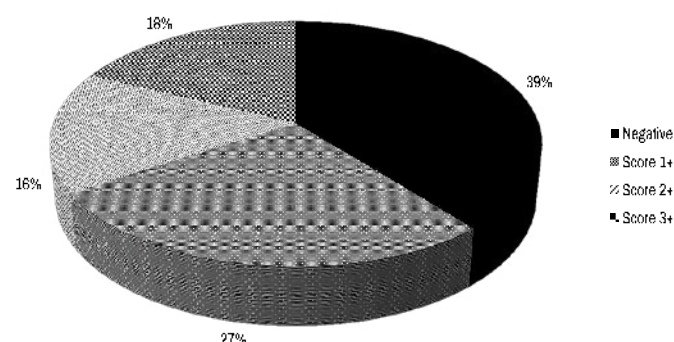**Figure 2.** CMT Score in different co-operatives.

Table 1. Relation between CMT scores and mean SCC.

S. No.	CMT Scores	Number of sample included(N)	Mean SCC (Cells/ml)	Standard Deviation
1	Negative	46	426132	116198
2	1+	17	1307106	1625473
3	2+	16	2480803	678034
4	3+	11	5231562	2701413

Table 2. Relationship between Mastitis test and Somatic cell count.

CMT	No. of Samples	Mean	Standard Deviation	P value (t- test)
Negative	46	426132	116197.7	<0.001
Positive	44	2715019	2294509	

Table 3. Relationship between Mastitis and salt test.

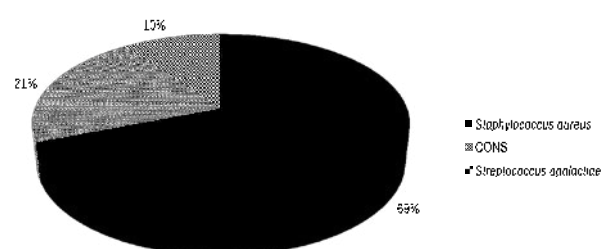| Salt Test	SLST Score				Total	Pvalue (Chi-square test)
	Negative	**1+**	**2+**	**3+**		
Negative	37 (80.4%)	9 (52.9%)	11 (68.8%)	5 (45.5%)	62 (68.9%)	0.0503
Positive	9 (19.6%)	8 (47.1%)	5 (31.3%)	6 (54.5%)	28 (31.1%)	
Total	46 (100%)	17 (100%)	16 (100%)	11 (100%)	90 (100%)	

Figure 3. Isolation and identification of causative organisms.

Detection of subclinical mastitis in field

A total of 98 samples were screened for presence of SCM in field by using SLST reagent. Out of 98 samples, 39% (38 samples) were CMT negative, 27% (26 samples) were score 1+, 16 % (16 samples) were score 2+ and 18% (18 samples) were score 3+ CMT. In field, samples were directly taken from the individual farmers. CMT score from different cooperatives is shown in Figure 2.

Isolation of causative organisms from cmt positive sample

Out of 90 samples analyzed for screening SCM by CMT method in laboratory, 44 samples were positive which were further screened for identification of causative organisms. Common organisms isolated were *Staphylococcus aureus,* Coagulase negative Staphylococcus (CONS) and Streptococcus agalactiae was isolated, there prevalence rate is shown in Figure 3.

Coliform count

Coliform count was found to be highest before pasteurization and in the collected samples it ranged from 4.02 to 7.9 log10cfu/ml. Out of 90 samples analyzed for coliform count in raw and pasteurized milk, only 10% of processed samples were found to be within standard. Out of 15 milk samples taken from the milk chain, sample taken from silo had 5 log10cfu/ml coliforms. And immediately after pasteurization, the coliform count was found to be nil. Out of 15 samples, one of the pasteurized milk in pipeline was found to be contaminated with coliform which contained 0.3 log10cfu/ml. Out of 6 pasteurized and packaged milk samples, 4 samples showed no coliform count which corresponded with Nepal Standard. But remaining 2 samples contained 0.83 log10cfu/ml coliforms which were in disagreement with Nepal Standard [15].

Discussion

California mastitis test (CMT) is a rapid and inexpensive test to determine the somatic cell concentration in the milk. It is a specified test for deoxyribonucleic acid of somatic cell nuclei. Out of 188 samples, 84 (44.68%) samples were free from sub-clinical mastitis and remaining 104 (55.32%) samples were detected as mastitic milk. Out of 188 samples analyzed, 84 (44.68%) samples had negative score, 43 (22.87%) samples showed slight presence of sub-clinical mastitis, 32 (17.02%) samples had moderate sub-clinical mastitis and remaining 29 (15.42%) samples showed highly sub-clinical mastitis.

Individual samples have shown that out of 181 cows, 73 had sub-clinical mastitis. The prevalence on a cow-basis was 42.2% and on a quarter-basis 21.8 % . The incidence of clinical mastitis in udder quarters of Holstein Friensian cows in ten farms was found to be 15.7%. The average incidence of sub-clinical mastitis was 31.4% on the basis of udder quarters.

In clinically normal buffaloes, CMT negatives quarters were found to be 85%. showed that out of 6600 foremilk quarter sample, 36.7% had negative scoring, 14.4% of traces, 27.8% had 1+ scoring, 10.9% had 2+ scoring and

9.6% had 3+ scoring. The CMT reaction of milk and production of milk was related. Test reaction (on total quarter milk) of traces, 1, 2 and 3 was associated with averages decrease in milk production of 9%, 19.5%, 31.8% and 2.33% kg per quarter per day respectively.

There is an association between CMT reaction test score and SCC. With increasing test score of CMT reaction, SCC also increases. This study shows that the average mean somatic cell count for CMT test score negative was 426,132 cells/ml, score 1+ was 1,307,106 cells/ml, score 2+ was 2,480,803 cells/ml and score 3+ was 5,231,562 cells/ml. This value was nearly to the value given by National Mastitis Council (NMC) with an exception of negative score which was slight beyond the value standardized by NMC. Averages SCC of 46 negative samples were 426,132 cells/ml and 2,715,019 cells/ml for positive samples which showed highly significant correlation (P<0.001). Dhakal, 2006 found that somatic cell was found lowest 129,500 cells /ml in clean and comfortable environment whereas a higher somatic cell count 1,013,400 cells/ml was noticed in moist and dirty environment.

The variation of SCC also depends on the seasons and months. This research was carried out in December to February, when the somatic cell count varied from 211,518 to 440,926 cells/ml. Lower average SCC such as 136,000 cells/ml in July to August, 108,000 cells/ml in May to June and 76,000 cells/ml in December to January was found in Indian Murrah Buffaloes. The National Mastitis Council defines sub-clinical mastitis in cows as a quarter with SCC of 200,000 or more, with normal quarter having counts around 1,000,000 cells /ml. Hanus and Suchanck, 1991 stated that there are some other factors that affect the SCC of the milk as calendar month, lactation stages, and order of lactation genotype of dairy cow sire, other functional disorder and infection.

This study suggests that there is highly significant statistical relationship between salt test and CMT test. Apart from this, the salt content in mastitic milk is higher in comparison to raw milk. sodium chloride goes into milk from the blood as a result of varied permeability and increases the osmotic pressure of milk, thus the chloride level of mastitic milk is elevated apparently, and sodium rises along with chloride. Other than chloride and sodium, milk with mastitis has been found to have decreased levels of calcium and potassium. Kitchen (1981) described that sodium ion content in normal milk was 43.6-57 mg/100ml which gets elevated in case of mastitis milk to about 60.3-104.6 mg/100ml. Similarly, chloride ion content in normal milk was 75-130 mg/100ml which get increased in mastitis milk to about 111-198 mg/100ml.

In this study, causative organisms were isolated from CMT positive samples. Out of 44 samples processed, Staphylococcus aurous were isolated from 69% samples, Coagulase negative Staphylococcus aurous were isolated from 21% samples and Streptococcus agalactiae were isolated from only 10% of sample. Abdel et al (2006) studied 704 composite milk samples collected from 275 cows kept in two herds. They isolated S. aurous and S. Agalactiae with a percentage of 16.6 and 1.4 respectively, while samples containing S. dysgalactiae, Escherichia coli and other mastitis-causing organisms was found to be 4.7%, 2.9% and 14.7% respectively.

Similarly, coliform is also one of the causal factor of mastitis and it also indicates hygienic condition. Coliform count in raw milk was as low as 4.02 log10cfu/ml to 7.9 log10cfu/ml. Thirty percent of coliform counts in 855 samples in New York state were <10 cfu/ml but 20% of bulk milk samples exceeded 100 cfu/ml (Boor et al, 1998). The source of coliform bacteria in bulk tank milk is the udders of cows or unsanitary milking practices. The coliform count is an indication of the effectiveness of cow preparation procedures during milking and the cleanliness of the cows' environment. Coliform can also incubate on residual films of milking equipment. The coliform count should be less than 10 cfu/ml. A coliform count between 100 and 1000 usually indicates poor milking hygiene and a coliform count >1000 suggests that bacterial growth is occurring on milk handling equipment.

There is no Nepalese standard so far set by the regulatory authority for the regulation of the minimum no. of somatic cell count in bulk milk. This research indicates that there is an urgent need to start an investigation to set the Nepal standard to detect the SCM in the farmer level to prevent from the great economic loss to the farmer and dairy.

Conclusion

Sub-clinical mastitis is one of the causes of deterioration of milk by changing its chemical parameters due to high microbial load, increasing somatic cell count and high salt content. Therefore, more efforts are needed to check milk quality in dairy as the occurrence of mastitis milk is high. This study suggests that there is an association between CMT reaction test score and SCC. With increasing test score of CMT reaction, SCC also increases. The study also suggested a highly significant statistical relationship between salt test and CMT test. And, Staphylococcus aureus was predominant organism isolated from samples followed by coagulase negative Staphylococcus and Streptococcus agalactiae. Finally, coliform count was higher in raw milk than the standard. In addition to regular monitoring of mastitis by CMT method, SCC and isolation of mastitis causing pathogens can be a valuable knowledge in controlling mastitis and a legal standard for SCC should established.

References

1. Abdel Hameed, Karima Galal, Grazyna Sender and Agnieszka Korwin-Kossakowska. "Public Health Hazard due to Mastitis in Dairy Cows." *Animal Science Papers and Reports* 25 (2006): 73-85.

2. Adams, M. R and Moss M O. *Food Microbiology*. Panima Publishing Corporation, New Delhi, (2000).

3. Ahlner, Sara. Prevalence of Sub-clinical Mastitis in Uruguay. SLU Uppsala, (2003).

4. Ashworth, U Sara, Forster T Liu and Luedecke L Oeai. "Relationship between California Mastitis Test Reaction and Composition of Milk from Opposite Quarters." *J Dairy Sci* 50 (1967): 1078-1082.

5. Bauernfeind, Jenny C and Allen Liu E. "Vitamin A and D Enrichment of Nonfat Dry Milk." *J Dairy Sci 46* (1963), 245-254.

6. Boor, K J, Brown D P, Murphy SC and Kozlowski S M, et al. "Microbiological and Chemical Quality of Raw Milk in New York State." *J Dairy Sci* 81(1998): 1743-1748.

7. Charkabati, Amalendu. *A Textbook of Preventive Veterinary Medicine*. Second Edition. Kalyani Publishers, Delhi, (2007).

8. Dhakal, Ishwari P. "Normal Somatic Cell Count and Subclinical Mastitis in Murrah Buffaloes." *Indian J veterinary Medic* 53 (2006): 81-86.

9. Feng, Peter, Weagant Stephen D, Grant Michael A and Burkhardt William. *Bacteriological Analytical Manual 4: Enumeration of Escherichia Coli and the Coliform Bacteria*. FDA, (2001).

10. González, R N, Jasper D E, Farver T B, Bushnell R B and Franti C E. Prevalence of Udder Infections and Mastitis in 50 California Dairy Herds. *J American Veterinary Medical Assoc* 193(1988): 323-328.

11. Hanus, O and Suchanck B. Variability and SCC in Cows' Milk as Influenced by Some Internal and External Factors. *Veterinary Bulletin* 36(1991): 303-311.

12. Hawari, Azmi D and Fawzi Aldabbas. "Prevalence and Distribution of Mastitis Pathogens and Their Resistance against Antimicrobial Agents in Dairy Cows in Jordan." *American J Animal veterinary Sci* 3 (2008): 36-39.

13. Hobbs, B C and Roberts D. *Food Poisoning and Food Hygiene,* London, Boston: Arnold, (1993).

14. Hurley, W. L. *Milk Composition* 2005

15. Kitchen, B J. "Review of the Progress of Dairy Science: Bovine Mastitis: Milk Compositional Changes and Related Diagnostic Tests." *J Dairy Res* 48 (1981): 167-188.