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Comparative Analysis of Hand v/s Machine Milking on Bovine Intramammary Infection

Mamta Singh[1]*, Bhagirathi[1], Reena Mukherjee[1] and Mukesh Shakya[2]

[1]Department of Medicine, ICAR-Institute, Izatnagar, Bareilly (U.P.)-243122, India
[2]Division of Parasitology, ICAR-IVRI, Izatnagar, Bareilly (U.P.) - 243122 India

**Corresponding author*

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A B S T R A C T

Mechanization has significantly altered the working conditions of humans and livestock in dairy industries over the past hundred years. Machine milking is a common practice from past decades in many organised dairy farms in most of milk producing country. The production of good quality and hygienic milk are essential to assess the impact of manual and machine milking method on udder health. California mastitis test (CMT) and Somatic cell count (SCC) widely used to predict the mammary health status of quarters (cows) and for the suitability of milk for human consumption. The objective of this study was to investigate the relationship of milk somatic cell counts, and mastitis causing *Staphylococcus aureus* with regard to the milking practices followed in organized farms.

Introduction

According to the present circumstances mastitis has symbolized itself as a most challenging disease in high yielding dairy animals in India next solely to FMD (Foot and Mouth Disease) (Varshney and Mukherjee, 2002). However as per many reports of its occurrence in dairy animals, it places itself at first position with its prevalence reported in more than 90% of high yielding cows (Reshi, 2015). Annual misfortunes in the dairy business due to mastitis have been around 2 billion dollars in the USA and 7156.53 crores in India (NAAS, 2013). In present scenario clean milk production is very challenging task in most of recognised milk producing countries. It is well known that bacterial, environmental or management, and cow factors may change the susceptibility to mastitis. Many microbial species such as *Escherichia coli*, *Klebsiella pneumoniae*, *Streptococcus agalactiae* and *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus dysgalactiae subsp. dysgalactiae* or *Staphylococcus chromogenes*, are common bacterial causes of bovine mastitis (Zadoks *et al.*, 2011) among which *Staphylococcus*

aureus is the most widely recognized causative organism of bovine mastitis (Li *et al.,* 2017). The management and environment likely favour the factors involves in causing mastitis; housing (Osteras and Lund, 1988), nutrition (Smith *et al.,* 1984; Barkema *et al.,* 1999), milk production, milking procedures (Schukken, 1990), and dry cow treatment (Berry and Hillerton, 2002) have been found to be associated with Intramammary infections.

Normal milk does contain cells, and the concentration of these cells is almost always less than 100,000 cells/ml in milk from uninfected/uninflamed mammary quarters (Barbano, 1999; Dohoo and Meek, 1982; Hamann, 1996; Harmon, 1994; Hillerton, 1999). This is based on twice-daily milking at regular intervals. A cell count of 200,000 cells/ml or greater is a clear indication that an inflammatory response has been elicited (subclinical mastitis), the quarter is likely to be infected, and the milk has reduced manufacturing properties such as reduced shelf life of fluid milk, and reduced yield and quality of cheese (Barbano, 1999; Dohoo, and Meek, 1982). Based on the likelihood of infection and altered manufacturing properties, milk from a mammary quarter with a SCC equal to or greater than 200,000 cells/ml, with or without clinical signs, is abnormal milk (National mastitis council, 2011).

Monitoring udder health performance is not feasible without reliable and affordable diagnostic methods (Zadoks and Schukken, 2006). The most often used diagnostic methods are CMT, SCC and bacteriological culturing of milk. Currently, methods such as measurement of N-acetyl-β-D-glucosaminidase (NAGase), lactate dehydrogenase activity (LDH), electric conductivity (EC) on milk, are used less frequently.

Milking is one of the main and final operations that determine profitability of a dairy farm. However, farmers are faced with several challenges that include low productivity, poor hygiene and routines for manual milking. The type of milking, whether by machine or by hand, can affect the incidence of intramammary infections. Hand milking exposes dairy animals to injury, disease transmission hazards and incomplete emptying udder that complicate the cow's health as well as subsequent milk yield (Dzidic, 2004; Christine, 2018). Hand milking is also slow, very tiresome and unhygienic. These challenges can be mitigated by investing in machine milking (Shem *et al.,* 2001). Therefore, many organized dairy farms have embraced machine milking to overcome these difficulties. The aim of this research is to determine the effect of two distinct milking methods (hand vs. machine milking) on somatic-cell-count and microorganisms in milk.

Materials and Methods

Place of study

Present Study was conducted in dairy cows specifically the Vrindavani crossbred cattle in an organized dairy farm in Bareilly (U.P.). A total 395 useful udder quarters of 100 lactating Vrindavani cows were screened randomly. Out of 100 cows, 50 are from the group in which hand milking is practiced and rest 50 are from the group in which machine milking is practiced.

California mastitis test

California mastitis test California mastitis test (CMT) was done on the spot of collection for milk samples. Milk samples were examined for noticeable changes and screened by the CMT according to Quinn *et al.,* (1999) prior to sample collection for bacteriological

examination. A squirt of milk sample was placed on the CMT paddle in each of the cups from every quarter of the udder, and an equal amount of 3% CMT reagent was added to each cup and mixed well. Reactions were graded as 0 and Trace for negative, +1, +2 and +3 for positive.

Collection of milk sample

Milk samples were collected according to the procedures recommended by National Mastitis Council (NMC, 1990). The milk sample from affected quarters from each cow was collected after proper disinfection of hand and teat surface with 70% ethyl alcohol. The first 3-4 streams of milk were discarded. The collecting vial was held as near horizontal as possible and by turning the teat to a near horizontal position, approximately 10 ml of milk was collected aseptically in a sterilized glass test tube. After collection, samples required for the further study were placed in icebox and processed in the same day.

Somatic cell count (SCC)

The SCC in milk was performed according to Schalm *et al.,* (1971) method with appropriate modification. The milk samples were thoroughly mixed by shaking the vials and 10µl of milk was taken over a grease-free clean glass micro slide on the predawn area of one sq cm, which was smeared uniformly with a fine sterile rod. The smear was dried and examined after staining them with modified Newman's Lampert stain. Cell counting in 10 different fields was carried out under oil immersion lens (100X) and counting was repeated thrice per smear to assess average number of somatic cell in 30 fields. The total number of cell in the milk was estimated by multiplying total number of cells in 10 fields to the working factor of microscope and expressed per ml of milk sample.

Bacteriological examination of milk sample

Microbiological analysis was performed according to adapted National Mastitis Council methodology (Oliver *et al.,* 2004), with the following ' Bacterial Identification Protocol' provided by Kloos and Schleifer (1975) for the identification of Pathogenic *Staphylococcus aureus*. The identification of causative organism in collected milk samples were carried out by inoculating 10 µl of milk, which spread over 5% bovine blood agar plates. The isolated organism from milk samples were identified initially on the basis of colony morphology, zone of hemolysis and smell on 5% blood agar as per Cruickshank (1962).

Culturing methods

Culture grown in 5% bovine blood agar was further grown on Mannitol Salt Agar, Bairds' Parker agar and MeReSa agar plates. The suspected colonies from 24 to 48 hrs old culture grown in 5% bovine blood agar were further grown on Mannitol Salt Agar .Yellowish coloration of the media due to lactose fermentation with bacterial colonies indicating coagulase positive Staphylococci which can be further confirmed by coagulase test. Coagulase positive *S. aureus* was isolated using technique given by Baird Parker, (1962). Enriched samples were streaked on Baird Parker Agar (BP agar) and the plates were incubated at 37ºC for 24-48 hours. The appearance of jet black colonies surrounded by a halo was presumably considered to be *S. aureus*.

Molecular characterization of S. aureus

Isolation of genomic DNA from bacterial cultures

Single colony of bacteria from nutrient agar was inoculated in 2ml Luria Bertini broth

aseptically and kept in shaker incubator at 37°C overnight. 1ml of bacterial culture suspension was placed into a 1.5 ml micro centrifuge tube, and centrifuge for 5 min at 5000 x g (7500 rpm. Supernatant was discarded, bacterial pellet was suspended in 180µl of the 20mg/ml Lysozyme solution and incubated for 30 min at 37°C. Calculate the volume of the pellet or concentrate and add Buffer ATL (supplied in the QIAamp DNA Mini Kit) to a total volume of 180µl). Add 20µl proteinase K, mix by vortexing, and incubate at 56°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a shaking water bath or on a rocking platform. Add 200µl Buffer AL to the sample, mix for 15 s with pulse-vortexing, and incubate at 70°C for 10 min. Add 200µl ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15s. Suspension from the micro centrifuge tube was carefully transferred to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim and centrifuge at 6000 x g (8000 rpm) for 1 min. Then the QIAamp Mini spin column was placed in a clean 2 ml collection tube and discard the tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 500µl Buffer AW1 without wetting the rim. Then close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate. Then carefully open the QIAamp Mini spin column and add 500µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min. Place the QIAamp Mini spin column in a new 2 ml collection tube and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube, and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200µl Buffer AE or distilled water. Incubate at room temperature for 1 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min. The filtrate containing DNA was collected, labelled, sealed and stored at 20°C for future use.

Amplification of *staphylococcal* 16 S ribosomal gene (16 S rRNA) and mecA gene

The following Published primers were used for the amplification of 16S rRNA gene (Lovseth *et al.*, 2004) and mecA gene (Kamal *et al.*, 2013). PCR reaction was carried out in thin wall PCR tubes in 25µl reaction volume. Genomic DNA (70ng) was used as template for amplification of 16S rRNA gene and mecA gene. The PCR mixture consisted of 2µl of forward and reverse primers, 0.5µl of each dNTPs and 0.3µl of *Taq* DNA polymerase with 10x *Taq* DNA polymerase buffer. The volume of the reaction was made upto 25µl with nuclear free water.

The cycling conditions used for amplification of the genes were as follows:

16S rRNA gene	**mecA gene**
Initial denaturation 95°C for 5 min.	Initial denaturation 95°C for 5 min.
Denaturation 95°C for 1 min.	Denaturation 95°C for 30 sec.
Primer annealing 64°C for1 min 35 cycles.	Primer annealing 58°C for30 sec. 35 cycles.
Primer elongation 72°C for 1 min.	Primer elongation 72°C for 30 sec.
Step 5: Final extension 72°C for 10 min.	Step 5: Final extension 72°C for 5 min.

The PCR amplified products were resolved on 2% agarose gel in 1X Tris Borate EDTA (TBE) buffer. The agarose gel stained with ethedium bromide was documented under UV light in a gel documentation system (Molecular Imager® Gel Doc ™ XR+System, BIO Rad, USA).

Statistical analysis

Descriptive statistics were used for all the variables. Chi-square (x2) was used for assessing the statistical associations of various factors with mastitis.

Results and Discussion

A total 395 useful udder quarters of 100 lactating cows from organised herd were screened for intramammary infection on the basis of CMT. A total of 7.59% quarter samples were detected CMT positive, of which 3.03% samples were from machine milked cows and 4.55% from hand milked cows. No significant difference was observed between hand and machine milking methods in chi squire test with respect to CMT (Table 1).

The difference, in SCC between the two groups was not significant, most probably due to the great variance of the values. During the study period, 3 % and 1.5 % of hand and machine milking samples, respectively, contained more than 200,000 somatic cells ml−1. The milk samples which had between 1, 00000 to 200,001 somatic cells ml^{-1} were 3.75% and 2.5%, respectively (Table 2). SCC in the group of machine milked cows was not found significant as compared to that of the other group. However, Kalyan *et al.,* (2011) reported that the introduction of machine milking, there is an increase in milk SCC which may increase the chance of mammary infection. Some of researchers observed difference in SCC was not significant (P> 0.05), regardless of the different milking methods (Zeng and Escobar, 1996). Shel-drake and co-workers (198 1) reported the lowest average 4.4 X 10^5 SCC ml^{-1}in a herd milked by hand and highest average 1.7 X $l0^6$ SCC ml^{-1} in another herd milked by machine. But Dang and Anand (2007) found that average values of SCC were higher (P<0.01) in hand milked animals than machine milked cows. There was a tendency of higher SCC in the milk of cows that were milked by hand.

Our finding revealed that, there was no significant impact of hand and machine milking method to cause *Staphylococcus* mastitis in bovine and the findings were similar as observed by Zeng and Escobar (1996). The results were suggested that if milking practice done by trained milkers with proper hygiene than risk factor to spread mastitis causing pathogen by different method could be avoided. Some early reports (Burkey and Sanders, 1949) indicated a higher incidence of mastitis in machine-milked animals than in animals milked by hand. Spencer (1998) noted that the milking machine could influence new intra mammary infection (IMI) by serving as a fomite, allowing cross-infections within cows, damaging teat sphincters or creating teat impacts, he was one of the first to point out that the milking machine is rarely a direct cause of new IMI. The mastitis situation caused by S. *aureus, C. bovis*, *S. agalactiae* and coagulase negative *staphylococci* could be improved by improving milking procedures and hygiene (Haltia *et al.,* 2006). Another hand according to some reports, Therefore the risk of contamination is usually considered higher during manual milking than in mechanic milking (De Luca, 2004; Salimei, 2016). The milkers' hands can be a major factor in the spread of udder infections, tend to reverse this situation, providing machine milking is done properly.

Table.1 CMT score wise milk samples

S.No.	CMT Grade	Hand milked sample (n=196)	Machine milked sample (n=199)	Chi square value
1	Trace	2.04% (4)	2.01%(4)	3.822(a)*
2	week positive	2.55%(5)	3.01%(6)	
3	Distinct positive	3.57%(7)	1.00%(2)	
4	Strong positive	1.02%(0)	0	

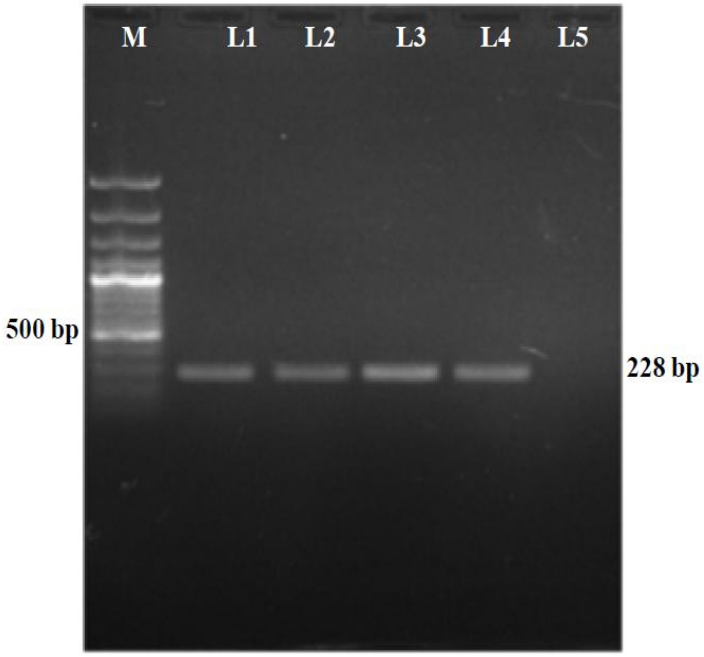
*non significance (p>0.05)

Table.2 Somatic cell count (SCC) of milk samples

SN	Somatic cells count/ml milk	Hand milked sample (n=18)	Machine milked sample (n=12)	Chi square value
1	<1, 00,000	27.77%(5)	33.33%(4)	0.255(a)*
2	1, 00,000-200,000	38.88%(7)	41.66%(5)	
3	>200,000	33.33%(6)	25% (3)	

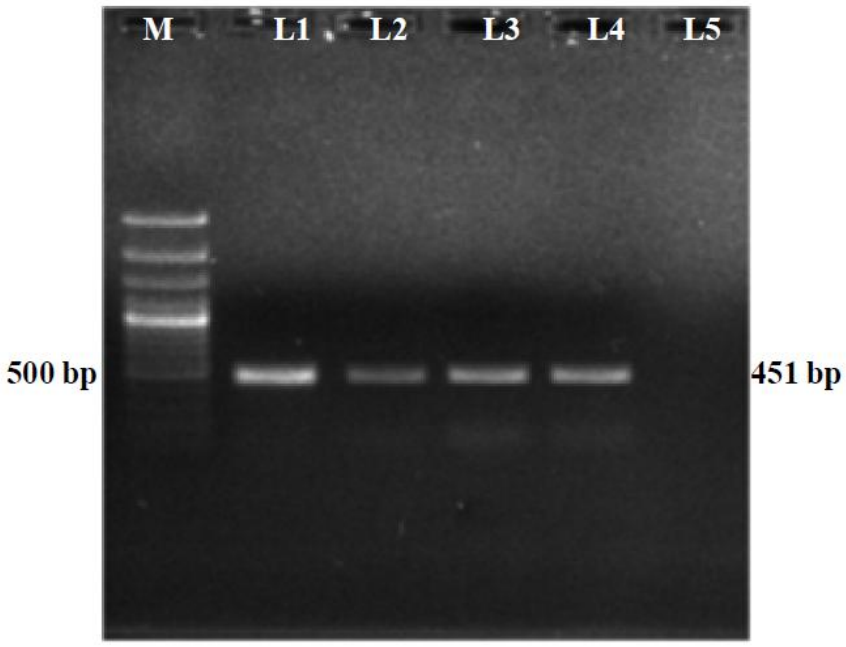
*non significance (p>0.05)

Fig.1 Agarose gel showing amplified 16S rRNA gene from mastitis milk samples



Lane M: 100 bp DNA ladder
Lane 1-4: PCR amplification of 16S rRNA gene in mastitis milk samples affected with *Staphylococcus* infection.
Lane 5: No template control (NTC).

Fig.2 Agarose gel showing amplified mecA gene from mastitis milk samples



Lane M: 100 bp DNA ladder
Lane 1-4: PCR amplification of mecA gene in mastitis milk samples affected with *Staphylococcus aureus* infection.
Lane 5: No template control (NTC)

Based on PCR amplification of 16S rRNA (228bp) and mecA (451bp) gene in mastitis milk samples, 3.06% samples were found positive for *Staphylococcus* infection out of which 1.53% samples were also detected positive for Methicillin- resistance *Staphylococcus aureus* in hand milked animals. However, in machine milked animals 1.50% samples were found positive for *Staphylococcus* infection and all samples were found negative for Methicillin resistance *Staphylococcus aureus* (Fig. 1 and 2). Amplification of 16S *rRNA* gene sequences is the most commonly used method for identifying and classifying bacteria, including staphylococci (Petti *et al.,* 2005; Mohammad *et al.,* 2007). Bacterial 16S *rRNA* genes generally contain nine "hypervariable regions" that demonstrate considerable sequence diversity among different bacterial species and can be used for species identification (Van de Peer *et al.*1996).

PCR based molecular methods are considered to be the gold standard for MRSA detection (Brown *et al.,* 2005).MRSA isolates have intrinsic resistance to penicillinase-resistant beta-lactam antibiotics like cloxacillin, oxacillin. This resistance is based on "*mec*A" gene encoding penicillin-binding protein 2a (PBP2a), an altered form of PBP that has low affinity for binding β-lactam antibiotics (Kaszanyitzky *et al.,* 2001).

In conclusion, the milking methods direct or indirect offer multiple opportunities for bacteria to be cause intramammary infection in cows. From last decade it is a controversy which method is better in respect to minimize the infection in quarters. Introducing of machine milking instead of hand milking can improve the hygienic quality of milk and increased the work efficiency on farms, but no difference in causing to bovine mastitis.

The PCR based methods for detection of *Staphylococcus aureus* mastitis is gold standard if possible.

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Conflict of interest

The authors declare that they have no conflict of interest.

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