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Research Article

Epidemiological Investigation of Some Indirect and Reference Tests for Screening of Bovine Subclinical Mastitis

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Abstract

The present research work was conducted to investigate the epidemiological comparison of some indirect screening tests for diagnosis of sub-clinical mastitis in dairy cows. A total of 2976 quarter milk samples from 744 milking cows in and around Parbhani district from various organized and unorganized farms, gaushalas and local dairy farms were screened for sub-clinical mastitis using modified California mastitis test (MCMT), pH and electrical conductivity (EC) in comparison to somatic cell count (SCC) as a reference standard test. The threshold value of milk SCC, EC and pH were found to be 196.078×103 cells/ml, 4.50 mS/cm and 6.6, respectively in indigenous cows and 239.651×103 cells/ml, 5.25 mS/cm and 6.7 in crossbred cows, for identification of subclinical mastitis quarters. The prevalence of sub-clinical mastitis was found 27.15%, 24.73%, 20.43 and 18.01% detected by using SCC, MCMT, pH and EC, respectively. Out of 2976 quarter milk samples subjected to MCMT, pH and EC, the per cent accuracies were found to be 92.00, 90.15 and 67.23 respectively. The highest sensitivity was found with MCMT (73.26%) while the highest specificity was found with EC (97.22%). Positive predictive value was found highest in EC (80.95%) whereas highest negative predictive value was found in MCMT (94.30%) as compared to other screening test for detection of sub-clinical mastitis in cattle. Apparent prevalence was found 17.84%, 16.30%, 13.51% and 12.01% detected by SCC, MCMT, pH and EC, respectively. The κ values of all the three screening tests were above 0.4 with highest MCMT (0.7183) indicating significant agreement with the gold standard test control. The results showed that MCMT was more reliable and can be used efficiently with SCC test for pinpoint diagnosis of sub-clinical mastitis in dairy bovines under field condition.

Keywords: Epidemiology; Investigation; Screening Test; Diagnosis; Mastitis; Cow

Abbreviations

EC: Electrical Conductivity; HF: Holstein Friesen; MCMT: Modified California Mastitis Test; SCC: Somatic Cell Count; WF: Working Factor; TP: True Positive; TN: True Negative; FP: False Positive; FN: False Negative; N: Total No. of Test Samples; OP: Observed Proportional Agreement; EP: Expected Proportional Agreement

Introduction

India is a premier milk producing country of the world and fabulously rich in dairy cows. Mastitis is the top most disease of dairy animals. Mastitis causes physical, chemical and bacteriological changes in milk and pathological changes in glandular tissue of udder [1]. A primary stage of mastitis, subclinical mastitis, is an inflammation of the mammary gland without noticeable signs. Sub-

clinical mastitis is a herd problem because it remains a reservoir of infection which could be transmitted to other animals of herd. Also prevalence of subclinical mastitis was observed four times more than clinical mastitis in dairy cows under different managemental conditions [2].

Diagnosis of mastitis at sub clinical stage is vital because abnormal changes already occur unnoticed at this level. Early detection of subclinical mastitis is an important tool for sustainable dairy and implementation of efficient strategies for control and prevention of mastitis. The invisible changes in subclinical mastitis can be detected indirectly by several diagnostic tests such as Somatic cell count (SCC), Modified California mastitis test (MCMT), pH and EC. These tests are preferred for screening of subclinical mastitis as they can be used easily and rapidly with satisfied results under filed conditions [2-4].

Inflammation of mammary gland is directly accompanied by an increase of somatic cell count (SCC) in milk. Therefore SCC is a significant marker for detection of subclinical mastitis. Modified California Mastitis test (MCMT) has been accepted as a quick and simple test to predict SCC from quarter milk sample and increase in MCMT score corresponds to the increase in SCC values [5]. Electrical conductivity (EC) of mastitic milk increases due to an increased concentration of Na^+ and Cl^-. However other factors like breed, lactation stage, milking interval and milk composition may affect EC of milk [6-8].

Therefore, the present research work was conducted to investigate the epidemiological comparison of some indirect screening tests like modified California mastitis test (MCMT), pH and electrical conductivity (EC) in comparison to somatic cell count (SCC) as a reference standard test for diagnosis of sub-clinical mastitis in dairy cattle.

Materials and Methods

Sample collection

Before collection of milk samples, udder and teats were washed, cleaned and dried. First few strips of the foremilk were discarded and about 10-20 ml of milk sample was collected from individual quarter of each cow in a sterilized bottle during routine milking hours. The indirect screening tests viz. MCMT, pH and EC were performed on the spot while SCC test was conducted in the laboratory within 1-2 hours after collection.

A total of 2976 quarter milk samples from 744 milking cows in and around Parbhani district from various organized and unorganized farms, gaushalas, local dairy farms and dairy unit of College of Veterinary and Animal Sciences, MAFSU, Parbhani, Maharashtra, India were screened for mastitis. Only healthy cows were selected for screening of mastitis. Cows which were having apparent symptoms of disease or abnormality were excluded from study.

Screening tests

The milk samples were processed for indirect and reference screening tests with following procedures.

Modified california mastitis test (MCMT)

The MCMT was performed as per the method described by Pandit and Mehta (1969). The MCMT reagent was prepared by adding 2 ml stock solution – B (Bromocresol purple reagent) to make volume 100 ml by adding remaining volume of stock solution - A (Sodium lauryl sulphate reagent) and stored at room temperature.

Preparation of stock solution - A (Sodium lauryl sulphate reagent)

3% aqueous solution of sodium lauryl sulphate was prepared by mixing 3 gm of sodium lauryl sulphate powder in 100 ml of distilled water. The suspension was heated to 50 °C to make a clear solution and stored in screw capped bottle. The pH of the solution was adjusted to 8.0 by using HCl or NaOH as per the need [1].

Preparation of stock solution - B (Bromocresol purple reagent)

0.5% aqueous bromocresol purple reagent was prepared by adding 0.5 gm of bromocresol purple reagent powder to dissolve in 100 ml distilled water and stored in amber colored bottle.

Procedure

Plastic paddles with four chambers or shallow cups were used to perform the test. The test was carried out with approximately 3 ml of milk from each quarter into the respective four cups of CMT paddle viz. LF, LH, RF and RH. To ensure equal quantity of milk in each cup, the paddle was tilted slightly at an angle of 45° to allow overflow of excess of the milk samples, if any in any cup. Then, approximately an equal amount of the MCMT reagent was added in each cup containing milk sample. The mixture of sample and reagent was gently mixed by circular movement of the paddle in horizontal plane with minimum agitation. Immediately after mixing, the reaction was recorded in 10 - 15 seconds. The degrees of precipitation or gel formation reflect the amount of total cell count present in the milk sample which was graded as.

MCMT Score	Reaction Observed	Interpretation
N	No change. The mixture remains fluid without thickening or gel formation.	Negative
T	A slight slime formation is observed which disappeared with continuous movement of paddle.	Trace
1+	Distinct slime formation occurs immediately after mixing solutions but no gel formation. This slime may disperse over time.	Weak positive
2+	Distinct slime formation occurs immediately after mixing solutions. When the paddle is swirled the fluid forms a viscous peripheral mass and the bottom of the cup is exposed.	Moderate positive
3+	Distinct slime formation occurs immediately after mixing solutions. Gel formation with convex or domed projection which do not disperse even after swirling movements of the paddle.	Strong positive

Table 1: Modified California mastitis test reactions for bovine milk.

Milk pH

The pH of milk samples were measured by an electronically operated pen type digital pH meter (Eco Tester pH-1) and procedure was followed as per the instructions manual given by manufacturer.

Procedure

The instrument was calibrated against standard buffer solutions of known pH (pH 4, 7 and 9.2) before actual testing of samples. The pH was indicated on the instrument display bar and reading was recorded directly. Thereafter, the pH reading of milk samples were recorded. For better accuracy instrument was put in ample amount of milk sample with given sufficient time to stabilize the reading (5 minutes) before taking the reading. The pH meter was recalibrated timely as needed with recommended by the supplier company.

Electrical Conductivity (EC)

The electrical conductivity of milk samples was measured by an EZ-1 pen type electrical conductivity meter. The procedure was followed as per the instructions manual given by manufacturer and the results were expressed in mS/cm.

Calibration

The instrument was calibrated against standard 0.1 N KCl solutions prior to actual sample testing. For calibration, potassium chloride 0.1491 gm was dissolved in 100 ml distilled water which gives conductivity value 1.413 mS/cm of the solution. The electrode was washed with distilled water and immersed in enough amount of milk sample to cover the electrode properly and electrical conductivity was measures directly. The electric conductivity and temperature were indicated on the instrument display and reading was recorded.

Procedure

After calibration of the instrument, testing of milk samples was conducted. The milk sample was taken in a flask and instrument was dipped in it. For better accuracy, ample amount of sample was taken and the instrument was put in sample for sufficient time to stabilize the reading (5-10 minutes) before taking actual reading. Direct reading was recorded on digital screen in mS/cm. The electric conductivity meter was recalibrated timely as recommended by the supplier company.

Somatic Cell Count (SCC)

The SCC was determined by microscopic method with procedure described by Schalm., *et al*. (1971).

Preparation of milk smear

The test milk sample was thoroughly mixed by gentle shaking the vials. The milk smears were prepared from the milk samples on the pre drawn one square cm (1 cm^2) marked area over a clean, grease free glass slide. Exactly 10 µl (0.01 ml) of milk was taken with a calibrated micropipette and spread over the area of 1 cm^2 on a microscopic glass slide which was uniformly smeared with a standard sterilized bacteriological platinum loop. The smear was allowed to air dry at room temperature while protecting from dust.

Staining of milk smear

These dry milk smear slides were stained by Newman-Lampart single dip stain. Slides were placed in a covered coplin jar containing the stain for 1-2 minutes. Excess of stain was drain off. The slides were gently washed in tap water and dried rapidly in air. The dried stained smears were examined under the oil immersion lens of the light microscope.

Counting of cells

The counting of cells in 30 different fields was done under oil immersion objective lens (100x). Total number of cells/ml of milk was estimated by multiplying total number of cells in 30 fields with working factor of the microscope (WF = 21786.50) used.

Microscopic factor determination

The common microscopic factor was determined by Prescott and Breed (1910) method as follow:

Diameter of an oil immersion microscopic field of microscope = 0.014 cm

Radius of oil immersion microscopic field = 0.007 cm

Area of oil immersion microscopic field = πr^2

= $22/7 \times (0.007)^2$ cm^2

= 0.000153 cm^2

$$\text{No. of field in 1 cm}^2 \text{ area} = \frac{1 \text{ cm}}{\text{Area of an oil immersion microscopic field}}$$

$$= \frac{1}{0.000153}$$

= 6535.95

The number represents total number of fields for the volume of 0.01 ml. Hence, 1 ml of volume will give 6, 53,595 fields.

Microscopic Factor (MF) = No. of field in 1 cm^2 area x 100

= 6535.95 x 100

= 6, 53,595

Thus, each cell in the field taken at random would be equal to 6, 53,595 cells per ml of milk.

$$\text{Working Factor (WF} = \frac{\text{MF}}{\text{No. of Fields Counted}}$$

$$= \frac{6, 53, 595}{30}$$

= 21786.50

SCC per ml of milk = Total no. of cells in 30 fields x WF

The same calibrated microscope was used throughout the course of research study.

Interpretation of screening tests

The interpretation of indirect screening tests viz., MCMT, pH and EC with SCC as reference test was calculated on the following basis: 1) each reading of an indirect test was compared with reference test. 2) If positive readings of any indirect tests correspond with reference test, it was considered as true positive (TP). 3) When sample showed negative results for both indirect and reference test, it was considered as true negative (TN). 4) Samples which were positive with indirect test and negative on reference test were considered as false positive (FP) and 5) samples which were negative on indirect test, but positive on reference test were taken as false negative (FN).

The following epidemiological characteristics of screening tests were determined using SCC test as a gold standard control

Sr. No.	Formula		
1.	Prevalence =	No. of positive animals / No. of tested animals	X 100
2.	Accuracy =	TP + TN / TP + FP + TN + FN	X 100
3.	Sensitivity =	TP / TP + FN	X 100
4.	Specificity =	TN / TN + FP	X 100
5.	Positive Predictive Value =	TP / TP + FP	X 100
6.	Negative Predictive Value =	TN / TN + FN	X 100
7.	Apparent Prevalence =	TP + FP / n	X 100
8.	OP =	TP + TN / n	
9.	EP =	[{(TP+FP)/n} x {(TP+FN)/n}] + [{(FN+TN)/n} x {(FP+TN)/n}]	
10.	Kappa (k) =	OP - EP / 1 - EP	
11.	Positive Likelihood Ratio =	Sensitivity / 1 - Specificity	X 100
12.	Negative Likelihood Ratio =	1 – Sensitivity / Specificity	X 100
13.	Odds =	Probability of event / 1 - Probability of event	
14.	Probability =	Odds / 1 + Odds	X 100

Table 2: Diagnostic Test Assessment Formulae [10].

*TP: True Positive; TN: True Negative; FP: False Positive; FN: False Negative; n: Total no. of test samples; OP: Observed proportional agreement; EP: Expected proportional agreement

Statistical analysis

The data were statistically analyzed using mean and standard error. Correlations between the tests were calculated using Cohen's kappa and Pearson's correlation.

Results and Discussion

A total of 2976 quarter milk samples from 744 milking cows were examined with different screening tests and the results were compared. The values of diagnostic tests for healthy and mastitis animal presented in Table 3.

Breed	Status	SCC/ml (x10^3)	pH	EC (mS/cm)
Deoni	Healthy	160.081 ± 2.693	6.595 ± 0.006	4.599 ± 0.020
	Mastitis	217.864 ± 8.672	6.708 ± 0.018	5.029 ± 0.072
Gir	Healthy	157.999 ± 2.562	6.587 ± 0.006	4.581 ± 0.019
	Mastitis	210.984 ± 8.021	6.698 ± 0.017	4.994 ± 0.067
Red kandhari	Healthy	154.251 ± 3.058	6.582 ± 0.007	4.554 ± 0.024
	Mastitis	215.211 ± 9.466	6.704 ± 0.019	5.031 ± 0.080
Non-descript	Healthy	157.105 ± 2.689	6.590 ± 0.006	4.594 ± 0.022
	Mastitis	221.346 ± 7.529	6.717 ± 0.015	5.021 ± 0.062
Hostein Friesen Cross	Healthy	180.455 ± 3.241	6.552 ± 0.007	4.553 ± 0.029
	Mastitis	246.022 ± 8.603	6.694 ± 0.019	5.032 ± 0.0674

Table 3: Statistical analytical results of diagnostic tests in examined cow's milk samples.

The threshold value of milk SCC, EC and pH were found to be 196.078 × 10^3 cells/ml, 4.50 mS/cm and 6.6, respectively in indigenous cows and 239.651 × 10^3 cells/ml, 5.25 mS/cm and 6.7 in crossbred cows, for identification of subclinical mastitis quarters. Moderate prevalence was recorded as 27.15%, 24.73%, 20.43% and 18.01% using SCC, MCMT, pH and EC respectively in tested cows (Table 4).

Screening Test	Tested cow	Positive	Prevalence (%)
SCC	744	202	27.15
MCMT	744	184	24.73
pH	744	152	20.43
EC	744	134	18.01

Table 4: Detection of sub-clinical mastitis using indirect & reference screening tests in cows.

Percent accuracy of indirect tests viz. MCMT, pH and EC were recorded 92.00%, 90.15% and 67.23% respectively with SCC (100%) as reference test control (Table 5).

Apparent prevalence was found 17.84%, 16.30%, 13.51% and 12.01% by applying SCC, MCMT, pH and EC respectively (Table 6).

Measurement of agreement of SCC, MCMT, pH and EC tests were 0.7183, 0.6278 and 0.5937 respectively (Table 6). Percent of post-test probability was found 78.42, 73.66% and 72.68% in MCMT, pH and EC tests respectively (Table 7).

Modified California mastitis test (MCMT) is a simple, inexpensive and rapid screening test for detection of subclinical mastitis. The principle of the test is the reactive reagent which reacts with

Screening Test	Sensitivity	Specificity	PPV	NPV	Apparent Prevalence	OPA	EPA	k	r
SCC	100	100	100	100	17.84	1.000	0.707	1	100.00
MCMT	73.26	96.07	80.21	94.30	16.30	0.920	0.716	0.7183	71.83
pH	60.26	96.64	79.60	91.80	13.51	0.901	0.734	0.6278	62.78
EC	54.42	97.22	80.95	90.76	12.01	0.896	0.744	0.5937	59.37

Table 6: Interpretation, agreement and correlation of indirect diagnostic tests for subclinical mastitis in cow with SCC as reference test.

*PPV: Positive Predictive Value; NPV: Negative Predictive Value; OPA: Observed proportional agreement; EPA: Expected proportional agreement; k: Kappa value (measure of agreement); r: Pearson's correlation (regression value).

Screening Test	Positive Likelihood Ratio	Negative Likelihood Ratio	Odds		Post–test Probability (%)
			Pre-test	Post-test	
MCMT	18.64	0.28	0.195	3.635	78.42
pH	17.93	0.41	0.156	2.797	73.66
EC	19.57	0.47	0.136	2.661	72.68

Table 7: Likelihood ratio, pre-test odd and post-test probability of indirect diagnostic tests for subclinical mastitis in cow with SCC as reference test.

the DNA of somatic cell nuclei after the dissolution of their outer wall and the nucleus cell wall with the formation of filamentous mass which is proportional with the somatic cells count [24]. A higher concentration in somatic cells leads to a higher MCMT score. This test has specificity for leucocytes in the milk. California mastitis test (CMT) was developed by Schalm and Noorlander (1957) using Schalm reagent (triethanolamine sulphonate and bromocresol purple) which was later modified by Pandit and Mehta (1969) by using Sodium lauryl sulphate reagent because of unavailability of Schalm reagent.

MCMT was found more accurate (92%) which was in agreement with Aarsharaj., *et al*., (2017); Sharma., *et al*. (2008); Patel., *et al*. (2000); Badiuzzaman., *et al*., (2015); Bhat., *et al*., (2017); Galfi., *et al*. (2017); Guha and Gera, 2011; Hoque., *et al*., 2015; Reddy., *et al*., (2014). The sensitivity was found higher in MCMT (73.26%) as reported by Aarsharaj., *et al*., (2017); Sharma., *et al*. (2008); Tanwar., *et al*. (2001); Badiuzzaman., *et al*., (2015); Bhat., *et al*., (2017); Galfi., *et al*. (2017); Guha and Gera, 2011; Hoque *et al*., 2015; Reddy *et al*., 2014. MCMT (96.07%) also showed slightly lower specificity and similar findings were reported by Aarsharaj *et al*., 2017; Sharma *et al*. (2008); Badiuzzaman., *et al*., 2015; Bhat., *et al*., 2017; Galfi., *et al*. 2017; Guha and Gera, 2011; Hoque., *et al*., 2015; Reddy., *et al*., 2014, Shelke., *et al*. (2019), Shaikh., *et al*., 2019, Chaunde., *et al*. 2023.

The pH may serve as the best indicator to assess the udder health status of the animal and food value of the milk. Mastitis increases the alkalinity of milk. As the severity of mastitis increased, pH value also increased. During inflammation of the mammary gland, permeability of the blood capillaries increases which allows alkaline blood constituents (sodium and bicarbonate ions) to enter the milk and consequently to increase milk pH [1]. The pH showed moderate (90.15%) accuracy, moderate sensitivity (60.26%) and slightly lower specificity (96.64%) which was correspond to findings of Tiwari., *et al*., (2018).

Electrical conductivity (EC) of milk is determined by sodium, potassium, calcium, magnesium, chlorine and other ions. Concentration of ions in secretion changes not only because of increased throughput of blood capillaries but also due to damaged active ion transport system. Secretory cells of mammary gland are distinguished by an active transport system when transporting Na+ to the extracellular fluid and K+ back into the cell. Na+ and K+ are transported from the secretory cells to milk in a passive manner. After disintegration of cells, ions contained in the extracellular fluid enter the alveolar chamber. Changes in ion concentration cause an increase in electrical conductivity. The change in electrical conductivity is one of the earliest manifestations associated with new infections making the early detection and recording of possible mastitis cases routine. Mastitic milk has a higher electrical conductivity than normal milk [1]. EC of cow milk have high correlation with mastitis and can give useful information about udder health status [6,22,23]. Also EC can be used as the decision criteria to treat or to cull the animals in herds with high prevalence of subclinical mastitis [3,8,19].

EC showed lowest (67.23%) accuracy [3,11,24]. Lowest sensitivity was found in EC (54.42%) which was corresponds by findings of Aarsharaj., *et al*., (2017); Reddy., *et al*., 2014. The specificity was found higher in EC (97.22%) which was in accordance with Aarsharaj., *et al*., (2017); Langer., *et al*. (2014) and Reddy., *et al*., (2014), Chaunde., *et al*. (2023).

Somatic cell count (SCC) is a useful predictor of intramammary infection (IMI) that includes leucocytes i.e., neutrophils, macrophages, lymphocytes, erythrocytes and epithelial cells [12]. The measurement of somatic cells in milk is known as somatic cell count. Leucocytes increase in response to bacterial infection, tissue injury and stress. Somatic cells are indicators of both resistance and susceptibility of cows to mastitis and can be used to monitor the level or occurrence of subclinical mastitis in herds or individual

cows. Das., *et al*. (2018) concluded that SCC can be used as a biomarker for prompt diagnosis of subclinical mastitis.

Moderate prevalence was detected by indirect and reference screening tests in given cow population which is may be due to differences in the management and rearing system. Higher prevalence was recorded by using reference test than indirect tests which may be due to difference in specificity and sensitivity of tests.

It was accepted that k (Kappa) values i.e., value of agreement <0.4 indicate poor agreement, values between 0.4 and 0.75: fair to good agreement and values >0.75: excellent agreement. The κ values of all the three tests were above 0.4 indicating significant agreement with the gold standard test control. This findings were in agreement with findings of Badiuzzaman., *et al*., (2015); Bhat., *et al*., (2017); Hoque., *et al*., 2015. While some researchers were found poor agreement with MCMT [11], pH [21] and EC [11,14].

The results of indirect screening tests were influenced by many causative agents and factors which were not taken in present study. The reasons behind the low percentage of false results could be the variation in the concentration of milk constituents during the different stages of lactation, secondary pathogens causing only mild inflammatory reactions with almost no or very little biochemical changes in milk composition and stage of infection. Also acidic pH of milk in early stages of lactation may gives false negative results to some extent.

Conclusion

In conclusion, there is low apparent prevalence of subclinical mastitis in tested cow population in study area. Although, there are different screening tests available to diagnose subclinical mastitis in cows under field conditions but their diagnostic accuracy, sensitivity and specificity vary greatly. The threshold value of milk SCC can be used as 196.078×10^3 cell/ml and 239.651×10^3 cells/ml in indigenous cow and crossbred cow for identification of subclinical mastitis quarter. MCMT was the test with highest accuracy and sensitivity while the kappa statistical analysis showed significant agreement with SCC test for all the three tests indicating more reliability of diagnostic testing in cows. Hence, MCMT was more reliable and can be used effectively with SCC test for pinpoint diagnosis of sub-clinical mastitis in dairy bovines.

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Conflict of Interest

There is no any conflict of interest during research and publication of this article.

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