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Evaluation of milk samples for detection of mastitis

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Abstract
Bovine mastitis is inflammation of the mammary gland caused by multiple pathogens with huge economic loss to the dairy industry. Indiscriminate use of antibiotics to treat such diseases in animals has led to the emergence of drug-resistant bacteria like *E.coli*, *Staphylococcus aureus* etc. Transmission of these AMR pathogens in the food chain continues to be a matter of great concern globally. In current study, The NDRI-developed Enzyme strip-based assay was used for evaluating 120 milk samples for their mastitis profile. Out of 120 samples, 54 samples were found to be normal as indicated by no change in colour, 45 samples were found to be subclinical and 21 were Clinical mastitis milk samples as indicated by intensity of colour development on strip. Further, the assay results were validated through conventional methods including CMT score and Somatic cell counter which confirmed the accuracy of strip based technology with neither false positive nor false negative results.

Keywords: Bovine mastitis, enzyme strip-based assay, CMT score, somatic cell counter

1. Introduction
India is the world's largest milk producer with 24% of the total milk produced worldwide in 2021–2022. Despite the fact that India produces the most milk globally, the availability of milk per capita remains low. One of the main reasons behind this is the unhygienic environment and lack of safety during handling due to which there exist some challenges in the dairy sector including diseases in cattle. Among all the diseases, mastitis is the prevalent condition that causes both significant economic loss to the dairy industry and severe pain to cattle.
Mastitis, which refers to the inflammation of the mammary gland, poses significant economic losses worldwide (Kumar *et al.*, 2017) [1]. It has always been a challenge for veterinarians due to the involvement of multiple causative agents (Varshney *et al.*, 2012) [2]. The adverse effects of mastitis, including decreased animal productivity and compromised milk quality and quantity, present formidable obstacles for dairy and livestock owners as well as the industry as a whole. Furthermore, the presence of different forms of mastitis, such as subclinical, clinical, and acute, further complicates the situation for microbiologists (Varshney *et al.*, 2012) [2].
Several bacterial agents commonly found in the dairy environment are associated with bovine mastitis (Bradley *et al.*, 2012) [3]. Potential bacteria that can cause both clinical and subclinical mastitis include *Streptococcus, Staphylococcus,* and *Corynebacterium* species. Additionally, environmental bacteria such as *Escherichia, Acinetobacter, Pasteurella, Pseudomonas, Klebsiella, Arthrobacter, Bacillus, Enterobacter, Enterococcus,* and *Serratia* contribute to more severe mastitis cases. *E. coli* is the predominant coliform bacterium, it is the second most prevalent causative agent of mastitis in cows, following *Staphylococcus aureus* (Jingar *et al.*, 2017) [4]. The excessive and inappropriate use of antibiotics for treating bovine mastitis has resulted in various issues for the dairy industry. Additionally, the presence of antibiotic residues in milk is a matter of concern. These organisms often carry antimicrobial resistance (AMR) genes and are developing resistance to multiple drugs commonly used to treat mastitis (Botrel *et al.*, 2010) [5].
Besides disadvantages of using the conventional methods including CMT scoring, Somatic cell counting, electrical conductivity test, a rapid enzyme strip based technology has been developed at ICAR-NDRI, which is an innovative approach over conventional techniques. By analysing the specific enzyme activity, this technique offers a simple and cost-effective alternative to traditional detection methods, providing comparable sensitivity and selectivity. Such advancements hold promise for applications in various fields, including food safety and environmental monitoring.

2. Materials and Methods

2.1 Enzyme Strip -based assay for detection of Clinical and subclinical mastitis in milk

Initially, a total of 120 milk samples were collected from the Cattle yard, Animal health complex of ICAR-NDRI, Karnal. The samples were labelled serially, immediately kept in a cool ice pack, and taken to the laboratory for further microbiological analysis. Milk samples were analysed for sub-clinical and clinical mastitis conditions using rapid technology i.e., strip test developed at NDRI. Results were evaluated further using the reference method like CMT and Somatic Cell Counter. Paper strip-based test for detection of mastitis in milk has been developed employing marker (s) present in somatic cells which is released in milk during mastitis infection of dairy animals. The protocol for the detection of sub-clinical and clinical status from milk was performed as mentioned in (Fig.1).

Firstly, 250µL of raw milk sample along with positive and negative control was taken in each respective Eppendorf tube. 20µL of activator was suspended into each tube containing the sample and vortexed for 10 seconds. Substrate functionalized strips were inserted into the tubes and incubated in a block heater at 50 °C for 6 min. Change in colour of the strip was observed and results were interpreted.

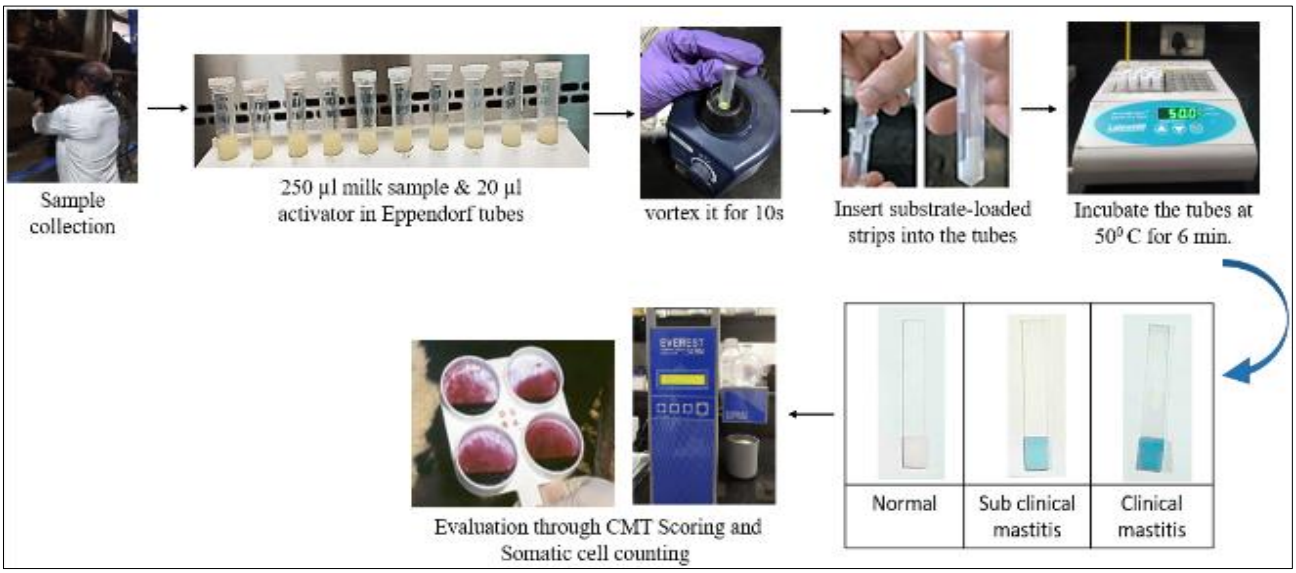

Fig 1: Enzyme strip protocol for rapid detection of sub-clinical and clinical mastitis

2.2 Analysis by CMT score and Somatic Cell Counter:

CMT is one of the most effective methods used for the detection of bovine mastitis at the farm level. It performs by rupturing the cell membrane of cells present in the milk sample, which enables the DNA in those cells to interact with the test reagent resulting in a gel formation. Based on gel formation scores were given and results were interpreted. A four-well plastic cup paddle was used where each well was dispensed with one milk sample along with testing reagent. The milk sample of 3mL was dispensed in a test paddle along with an equal amount of CMT reagent. The paddle was rotated immediately in a clockwise and anti-clockwise direction for 15-20 seconds and observed for gel formation. Based on the severity of the infection precipitation or gel formation was observed and results were interpreted.

The somatic cell count per mL of milk were estimated by using a Somatic Cell Analyzer which is designed for fast and reliable test for early detection of mastitis infections. The main principle is time taken for the flow of milk through the sample mixer capillary which in turn determines the number of somatic cells with respect to flowing time. After switching on the instrument, a setting button was pressed which shows "prepare sample" on the display, and the sample mixer with the flask was leaned forward. 5mL of milk Ekoprim solution was dispensed into the flask of somatic cell counter along with 10mL of milk sample and the OK button was pressed. The flask was shaken by the analyser and before stopping the liquid was allowed to flow through the capillary. When the measurement was finished, the somatic cell counts and time taken for analysing the sample was displayed on the screen which were recorded.

3. Results and Discussion

3.1 Evaluation of samples for detection of mastitis by Enzyme-strip based test

The NDRI-developed enzyme strip-based technology was used for evaluating 120 milk samples for their mastitis profile as mentioned in above section. Out of 120 samples, 54 samples were found to be normal milk samples as indicated by no change in colour, 45 samples were found to be subclinical as indicated by the change in colour of the strip into light blue whereas 21 samples were found to be clinical mastitis samples which showed a change in colour of the strip into intense blue colour.

3.2 Evaluation of samples by CMT score for mastitis

All 120 milk samples were evaluated by the CMT method for the detection of Clinical and subclinical mastitis**.** Based on the results obtained, it was interpreted that 54 samples were categorised as normal samples having a CMT score of 0, 12 samples were recorded with a CMT score of 1, 18 samples recorded a CMT score ranging from 1-2, 15 samples recorded a CMT score of 2, and 21 samples recorded a CMT score of 3. The majority of the Samples had CMT scores of 1 and 2 which were categorised as Subclinical mastitis, the remaining samples with CMT scores of 3 were classified as clinical mastitis (Fig. 2).

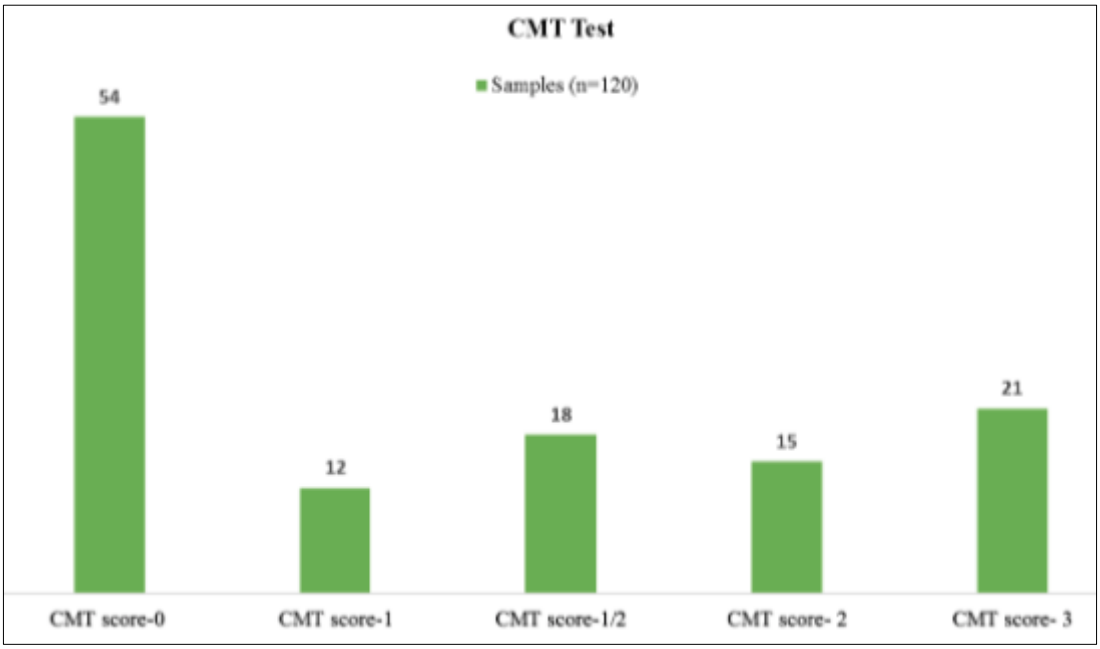

Fig 2: Evaluation of samples by CMT score for mastitis status

3.3 Evaluation of samples by Somatic cell counter

The milk samples were further evaluated for mastitis detection using commercial equipment called Somatic Cell Counter. The number of somatic cells present in mastitis milk is comparatively greater as compared to normal milk. When these mastitis milk samples were treated in the somatic cell counter along with the Ekoprim, an activator, gel precipitation was observed. The time taken for milk samples to flow through the capillary was recorded and the number of somatic cells present in the injected sample was measured and displayed. Results showed that 54 milk samples had SCC of 0.5-2 lakhs cells/mL i.e., normal milk samples, 45 samples recorded SCC of 2-25 lakhs cells/mL having subclinical mastitis, and 21 samples had SSC of 25 lakhs cells /mL and above showing the status of clinical mastitis.

By the overall evaluation of all the tests for the detection of mastitis in raw milk, it was found that 45% of normal milk, 37.5 % of Subclinical mastitis samples and 17.5% of samples had clinical mastitis status (Fig. 3).

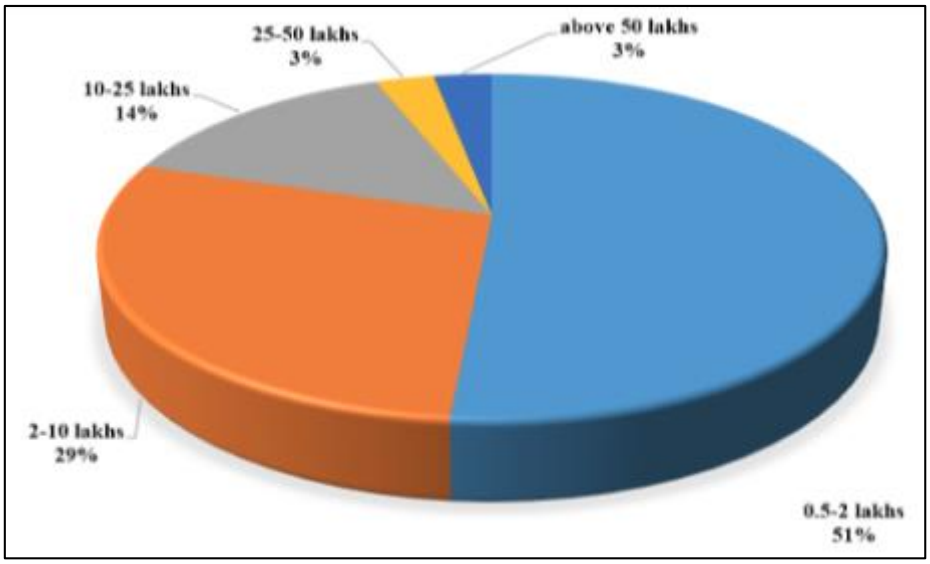

Fig 3: Evaluation of samples for mastitis condition by Somatic cell counter

Table 1: Analysis and comparison of the results obtained through developed strip test, CMT score, and Somatic cell counter.

No. of Samples	CMT Score	Somatic cell count/ml	Color development on mastitis strip	Result Interpretation
54	N/T	0.5-2 lakh	No color	Normal
12	1	2- 3 lakhs	Slight blue color	Sub-clinical mastitis
18	1-2	3-10 lakhs	Blue color	Sub-clinical mastitis
15	2	10-25 lakhs	Blue color	Sub-clinical mastitis
03	3	25-50 lakhs	Dark blue color	Clinical mastitis
18	3	Above 50 lakhs	Deep blue color	Clinical mastitis

4. Conclusion

The current study was focused on evaluation and validation of developed strips conducted under field conditions using raw milk samples collected from cattle yard of NDRI. The results of research work showed that 45% of normal milk, 37.5 % of subclinical mastitis samples and 17.5% of samples had clinical mastitis status. The results of an assay were validated through conventional methods whose outcomes were in correlated. In summary, the development of rapid, sensitive, on-site detection methods for mastitis milk, without the requirement for sophisticated equipment or highly skilled personnel, is of utmost importance at the field level. This method enables timely interventions, enhance disease management practices, and contribute to the overall health and productivity of dairy cattle. By addressing the challenges of accessibility and usability, this solution has the potential to revolutionize the field of bacterial detection in veterinary medicine, benefiting both farmers and veterinary professionals alike.

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