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Comparative Study of Different Tests for Diagnosis of Sub Clinical Mastitis in Buffaloes

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A B S T R A C T

In the present investigation, 235 quarter milk samples from 59 apparently healthy lactating buffaloes were collected to compare different diagnostic tests for detection of Sub-Clinical Mastitis (SCM) in Buffaloes. Out of 235 quarter milk samples, 17.02 per cent, 16.59 per cent, 16.17 per cent, 14.49 per cent, 14.46 per cent and 17.02 per cent quarters were tested positive for SCM by bromothymol blue (BTB) strip test, electrical conductivity (EC), California mastitis test (CMT), N-acetyl-β-D-glucosaminidase (NAG-ase), Somatic cell count (SCC) and Cultural Examination, respectively. While calculating percent sensitivity, specificity and predictive value of these tests taking cultural examination as standard, the NAG-ase test was found to have highest sensitivity (60%), Specificity (94.87%) and predictive value (70.58%) followed by SCC, CMT, EC and BTB strip test, respectively. NAG-ase test can be used as a preferential laboratory tool over SCC and cultural examination, however CMT can be used as a first screening tool for detecting suspected samples for further investigation for SCM.

Introduction

Mastitis is a disease complex with multifacetedetio pathogenesis, different degrees of intensity and variation in duration with diverse clinical syndrome. It is one of the most economically important disease of dairy animals, which not only causes heavy economic losses in terms of milk quality and quantity but also poses public health hazard due to persistence of antibiotic residues in the milk. Mastitis occurs in both clinical and subclinical forms. Clinical form of the disease is an individual animal problem and can be easily diagnosed during clinical examination whereas, subclinical mastitis (SCM) is a herd problem which may go unnoticed since no gross signs of inflammation and changes in the milk composition are evident and the milk and udder appear normal.

Subclinical mastitis is 15-40 times more prevalent than clinical form accounting for greater losses in terms of milk production. Some of these SCM cases later progress into clinical form causing more economic losses owing to cost of treatment, veterinarian's fee, discarded milk and culling of animals etc.

Several studies have been conducted on various diagnostic tests for early detection of SCM, viz. bromothymol blue (BTB) strip test, electrical conductivity (EC) of milk, California mastitis test (CMT), SCC, N-acetyl-b-D-glucosaminidase (NAG-ase) activity of milk and cultural examination. Of these, Somatic cell count (SCC) is one of the most commonly used test, though it is time consuming and cumbersome process. Estimation of N-acetyl-b-D-glucosaminidase (NAG-ase) activity of milk has been found to be a sensitive indicator of mammary gland damage (Kitchen *et al.,* 1980; Emanuelson *et al.,* 1987; Ball and Greer, 1991; Rana *et al.,* 2003). Various workers have studied either one of these tests alone or in combination. Therefore, all these tests need to be compared to assess their sensitivity and specificity.

Materials and Methods

A total of 235 quarter milk samples collected from 59 apparently healthy lactating Murrah buffaloes from Buffalo Research Centre, CCS Haryana Agricultural University, Hisar were the source of material for the study.

The animals which has calved recently (< 2 weeks) and those in late lactation (> 9 months) were not included in this study.

Bromothymol blue strip test

Bromothymol blue strips are cellulose based strips stabilized with ion sensitive indicator for detection of SCM. These strips are manufactured by Dabur Ayurvet Ltd, 22, Site IV, Sahibabad, Ghaziabad-201020, U.P; under trade name "Mastrip".

A drop of milk sample was put over the strip and change in colour, if any, was observed within 30 seconds and compared with the standard colour chart provided with the packing and interpreted as:

Yellow- Normal
Greenish yellow- Mild to moderate SCM
Green- Advanced (severe) SCM
Blue- Clinical mastitis

Electrical conductivity

Electrical conductivity of the quarter milk samples from lactating buffaloes was determined with the help of a portable mastitis detector manufactured by AHI Plastic Moulding Co; New Zealand which shows green, green-red, and red light output indicating three sensitivities.

As the battery of detector was switched "ON", green light appeared. The detector was held under the udder and a strip of milk from one quarter was directed into the teat cup.

Lights were observed and recorded for respective quarters. Interpretation was done using mastitis detection chart as follows:

Colour of light - Range

Green light - I
Green-red - II
Red - III

A red light (range III) shows a high conductivity level and indicates SCM. Normal infected quarters have a conductivity level in the range I. If the conductivity of all four quarters is in the range II, it is normal but if any one of the quarter is in range I and the rest in range II, it indicates SCM.

California mastitis test (CMT)

California mastitis test was performed using modified CMT reagent as per the method described by Pandit and Mehta (1969). Testing individual quarter sample requires use of a plastic paddle having four shallow cups marked A, B, C and D.

Approximately 2 ml of milk was taken in the respective cup of paddle. An equal amount of CMT reagent was added to milk. The paddle was then rotated in circular motion to mix the contents. Scoring was done within ten seconds keeping the paddle rotating. Interpretation and scoring was done as follows:

Negative - Mixture remains liquid, with no evidence of thickening

Trace - Slight thickening

I - Distinct thickening of liquid but no tendency towards a gel formation

II - Mixture thickens immediately with gel formation

III - A distinct gel was formed

Estimation of NAG-ase activity of milk

NAG-ase activity of milk samples was measured as per the method described by Kitchen *et al.,* (1978) using p-nitrophenyl-N-acetyl-b-D-glucosaminidase as substrate (Hi media Laboratories ltd, Mumbai-400086).

In brief 0.2 ml of whole milk was mixed with 0.3 ml of substrate solution (3.3 mM p-nitrophenyl- N-acetyl-b-D-glucosaminidase in 0.33 M citrate buffer, pH 4.6) in a 6 inch glass test tube. After incubation at 50ºC for 15 minutes, the reaction was stopped using 5.5 ml of 0.1 M carbonate buffer pH 10.

After termination of reaction, one ml chloroform was added and shake the tube vigorously for five seconds and centrifuge at 2000 rpm for 10 minutes. The top aqueous layer was removed and optical density was determined at 410 nm in a Spekol-1100 (Carl Zeiss Technology). The quarter milk samples reading optical density of 0.5 or more than 0.5 were taken as positive for NAG-ase activity.

By taking cultural examination as standard, per cent sensitivity, per cent specificity and predictive value of positive test were calculated as follows:

$$\text{Per cent Sensitivity} = \frac{\text{True positive}}{\text{True positive + False negative}} \times 100$$

$$\text{Per cent Specificity} = \frac{\text{True negative}}{\text{True negative + False positive}} \times 100$$

$$\text{Per cent predictive Value of positive test} = \frac{\text{True positive}}{\text{True positive + False positive}} \times 100$$

Somatic cell count

The SCC of milk was performed by the method as described by Schalm *et al.,* (1971). For staining of milk films Newman's lampert stain (Methylene blue 1.2 g, Ethanol (95%) 54 ml, Tetrachloroethane 40 ml and Glacial acetic acid 6 ml) was used.

Preparation of milk films

The samples were mixed thoroughly so as to obtain uniform distribution of the cells. The sample was allowed to stand for two to five minutes to permit air bubbles and foam to settle down. A clean grease free slide was placed on a level area over template to outline 1.0 cm^2 area. With help of a 4.00 mm diameter platinum loop, 0.01 ml of milk was spread evenly over the first template on the left side of slide. This procedure was repeated with sample from each quarter. Slides were air dried and subjected to staining.

Staining

Slides were immersed for 30 seconds in Newman's lampert stain. Excess stain was

drained off and the slides were air dried. Then slides were rinsed thrice under tap water, drained and rapidly air dried after gently blotting with filter paper. Somatic cells stained clearly with deep blue background.

Calculation of working factor (WF) of the microscope

A binocular microscope was used with 10X ocular and 1.8 mm oil immersion objective. The diameter of field was measured with the help of a stage micrometer.

Diameter of microscopic field= 0.18 mm = 0.018 cm.

Area of field= πr^2= 3.14X $(0.009)^2$ = 0.00025 sq. cm

Since 0.01 ml of milk was spread in 1.0 sq. cm area, the possible number of fields which could be counted in 1.0 sq. cm is 4000.

Milk volume represented by each field=1/4000 X 1/100= 1/4000 ml

Hence, microscopic factor = 4,00,000.

Working factor = Microscopic factor/ No of fields counted

4,00,000/ 25 = 16,000.

Counting of cells

The stained cells were examined under oil immersion objective.

The cells in the required 25 fields were counted. Total numbers of cells counted were multiplied by working factor of the microscope to obtain the number of cells per ml of milk. Milk samples containing more than 5,00,000 cells per ml was considered positive.

Cultural examination

After shaking the milk samples, 0.01 ml of milk from each quarter was streaked on five per cent sheep blood agar plates and MacConkey's lactose agar (MLA) plates separately with the help of a 4.00 mm diameter platinum loop. The plates were incubated at 37ºC for 24-48 hrs. The resulting growth from the respective plates of media was purified and identified on the basis of colony characteristics, Gram's reaction and morphology. On the basis of oxidase test, Gram-positive and catalase positive cocci were differentiated into staphylococci and micrococci. Staphylococci were further differentiated into coagulase positive staphylococci and coagulase negative staphylococci on the basis of coagulase test. The cultures were stocked in semisolid agar for further characterisation. The organisms, which were on preliminary examination found to be streptococci, were further identified on the basis of CAMP test (Christie *et al.*, 1944).

Results and Discussion

A total of 235 quarter milk samples from 59 buffaloes were subjected to different diagnostic tests i.e. Bromothymol blue (BTB) strip test, electrical conductivity (EC), California mastitis test (CMT) and N-acetyl-β-D-glucosaminidase (NAG-ase) test along with SCC and cultural examination for detection of SCM. Results are shown in table 1.

Out of 235 quarter milk samples, 17.02 per cent (40), 16.59 per cent (39), 16.17 per cent (38), 14.46 per cent (34), 14.46 per cent (34) and 17.02 per cent (40) quarters were tested positive for SCM by bromothymol blue (BTB) strip test, electrical conductivity (EC), CMT, NAG-ase, SCC and cultural examination, respectively, whereas 82.97 per cent (195), 83.40 per cent (196), 83.82 per cent (197), 85.53 per cent (201), 85.53 per cent (201) and

82.97 per cent (195) of the quarters were found negative for SCM, with the respective tests.

Table 2 and Figure 1 presents results of sensitivity, specificity and predictive value of all tests for detection of SCM taking cultural examination as standard. Out of 235 quarters examined, true positive quarters of SCM by BTB strip test, EC, CMT, NAG-ase and SCC were 21, 21, 22, 24 and 22, respectively, whereas true positive quarters were 40 according to cultural examination. Out of 195 culturally negative quarter milk samples 175, 177, 179, 185 and 183 quarter milk samples were found true negative by BTB strip test, EC, CMT, NAG-ase and SCC, respectively.

As many as 19, 18, 16, 10 and 12 quarter milk samples revealed false positive results by BTB strip test, EC, CMT, NAG-ase and SCC, respectively, whereas 20, 19, 18, 16 and 16 quarter milk samples were false negative with the respective tests.

The NAG-ase test was found to have higher percent specificity (94.87 per cent) and predictive value (70.58 per cent) followed by SCC (93.84 per cent and 64.70 per cent), CMT (91.79 per cent and 57.89 per cent), EC (90.76 per cent and 53.84 per cent) and BTB strip test (90.25 per cent and 52.50 per cent), respectively. Percent sensitivity for detection of SCM by BTB strip test, EC, CMT, NAG-ase and SCC was found to be 52.20, 52.20, 55.50, 60.00 and 55.50 per cent, respectively.

In the present investigation, five tests viz. bromothymol blue (BTB) strip test, electrical conductivity (EC), California mastitis test (CMT), N-acetyl-β-D-glucosaminidase (NAG-ase) and somatic cell count (SCC), were compared for detection of SCM taking cultural examination as standard. Out of 235 quarter milk samples from 59 lactating buffaloes examined, 40 (17.02%), 39 (16.59%), 38 (16.17%), 34 (14.46%) and 34 (14.46%) quarters were found positive for SCM with BTB strip test, EC, CMT, NAG-ase and SCC, respectively. The percent positivity of quarters for SCM by these tests varied negligibly and was in fairly agreement among themselves.

While calculating per cent sensitivity of different tests, NAG-ase test was found to have maximum sensitivity (60%) as compared to other tests. Nauriyal and Pachauri (1999) and Bulla (2002) have also reported NAG-ase test as a sensitive indicator of udder infections. Sensitivities of CMT and SCC (55.50 per cent each) were more than those of BTB strip test and EC (52.20 per cent each). Buragohain and Dutta (1998), Reddy *et al.*, (1998) and Sahay *et al.*, (2002) also reported a higher sensitivity of CMT as compared to other tests. Bulla (2002) reported somewhat lower sensitivity of SCC (47.91%) as compared to that recorded in the present study, however Reddy *et al.*, (1998) reported higher sensitivity of SCC (65.21%). In our study sensitivity of EC was 52.20 per cent, which corresponds with the findings of Reddy *et al.*, (1998). Sensitivity of BTB strip test for detection of SCM, was found to be 52.20 per cent, however other workers Buragohain and Dutta (1998), Tiwari and Sisodia (2000) and Sahay *et al.*, (2002) reported a higher sensitivity of BTB strip test ranging between 69.38 to 83.15 per cent.

In our study NAG-ase test was found to have highest specificity (94.87%) and predictive value of positive test (70.58%) as compared to SCC (93.84 and 64.97 per cent), CMT (91.79 and 57.89 per cent), EC (90.76 and 53.84 per cent) and BTB strip test (90.25 and 52.50 per cent). Ball and Greer (1991) and Bulla (2002) also reported higher specificity and predictive value of NAG-ase test for detection of SCM as compared to SCC and the values of per cent specificity and predictive values were also almost similar to that in the present study.

Fig.1 Graphical representation of percent sensitivity, specificity and predictive value of different tests

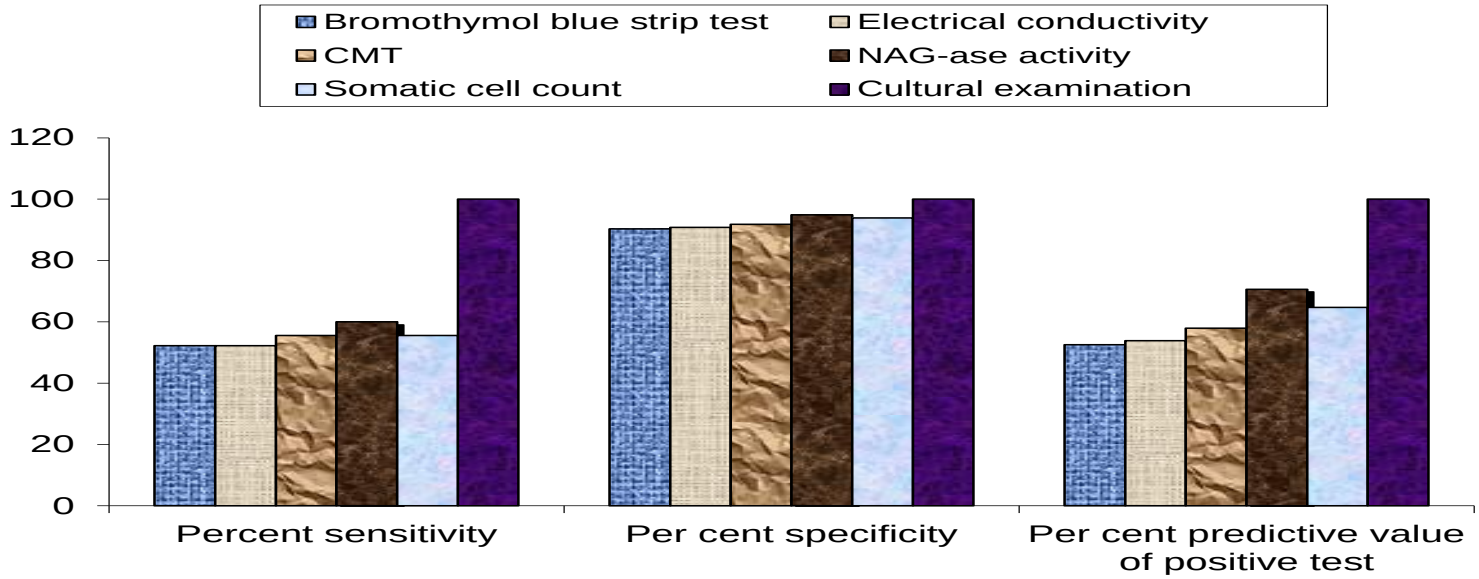

Table.2 Sensitivity, specificity and predictive value of different diagnostic tests taking cultural examination test as standard

Name of the Test	Number of samples				Per cent	Per cent	Per cent sensitivity of positive test
	True positive	False positive	False negative	True negative			
Bromothymol blue strip test	21	19	20	175	52.20	90.25	52.50
Electrical conductivity	21	18	19	177	52.20	90.76	53.84
CMT	22	16	18	179	55.50	91.79	57.89
NAG-ase activity	24	10	16	185	60.00	94.87	70.58
Somatic cell count	22	12	18	183	55.50	93.84	64.70
Cultural examination	40	0	0	195	100	100	100

Table.1 Detection of subclinical mastitis in 235 quarter milk samples from 59 buffaloes by different diagnostic tests

Name of the Test	Number of samples	
	Positive	**Negative**
Bromothymol blue strip test	40 (17.02)	195 (82.97)
Electrical conductivity	39 (16.59)	196 (83.40)
CMT	38 (16.17)	197 (83.82)
NAG-ase activity	34 (14.46)	201 (85.53)
Somatic cell count	34 (14.46)	201 (85.53)
Cultural examination	40 (17.02)	195 (82.97)

Values in parentheses indicate percentage

Sahay *et al.,* (2002) also reported higher specificity of CMT for detection of SCM as compared to BTB strip test. However, Reddy *et al.,* (1998) recorded higher specificity of EC as compared to SCC and CMT for detection of SCM. Results of specificity of CMT was almost similar with the findings of Sahay *et al.,* (2002), however specificity measured by Reddy *et al.,* (1998) was lower (75.75%) as compared to that in our study. Results for SCC were in accordance with the findings of Ball and Greer (1991) and Bulla (2002). Reddy *et al.,* (1998) also reported specificity and predictive value of EC lower than the values recorded in this study. Sahay *et al.,* (2002) reported higher specificity of BTB strip test as compared to that in our study. This variation in the specificity of BTB strip test may be because of slight change in pH, which was not appreciable by the strips.

NAG-ase test revealed least false positive (10) and false negative (16) quarters as compared to SCC (12 and 18), CMT (16 and 18), EC (18 and 19) and BTB strip test (19 and 20) taking cultural examination as standard, indicating that NAG-ase test is more reliable than the other indirect tests. BTB strip test was proved to be least advantageous as it showed more false positive and false negative results.

On the basis of these findings, it may be inferred that NAG-ase test is better than other routine tests for the detection of SCM. SCC is a very cumbersome and time-consuming test and it is limited to fresh milk samples only.

Besides this, several workers have suggested the variation in the SCC level due to many factors such as stage of lactation (Cullen, 1966), number of lactation (Natzke *et al.,* 1972; Blackburn, 1968) and stress caused by poor farm management. When compared with bacteriological examination, NAG-ase test is more convenient and rapid because techniques

of milk collection and handling are not so critical. No strict asepsis is required and samples can be frozen without harm for a long time for NAG-ase test. NAG-ase test can be used as a preference laboratory test in comparison to other laboratory tests to detect SCM in buffaloes.

It can also be used as a screening method for selecting samples for further analysis of SCC and bacteriological examination. This finding is in accordance with suggestions of Ball and Greer (1991) and Bulla (2002) who recommended NAG-ase test as a screening test.

In comparison to direct tests, CMT, EC and BTB strip test have shown their validity and specificity and predictive values of these test were almost similar in the present study. Buragohain and Dutta (1998) also reported close agreement between BTB strip test and CMT. However sensitivity, specificity and predictive value of CMT was slightly more than BTB strip test and EC. There are certain other conditions such as illness, estrous and advanced lactation which can cause rise in EC of milk (Linzell and Peakar, 1975; Sheldrake *et al.*, 1983). Therefore, when interpretation is based on this single test, the other factors viz. history or any signs of systemic disease, estrous, stage of lactation and recent antibiotic treatment etc. should be kept in mind.

It is well established that increase in SCC is an early indicator of SCM as compared to change in pH. Kapur and Singh (1977) have shown CMT to be more reliable in detecting SCC-positive samples. Moreover, BTB strip also has some disadvantage that it can give false positive results in cases of bovine ketosis; and since it is a visual test, slight change in the colour of strip may not be appreciable. Though the sensitivity of these three tests are low, yet because of high predictive value and specificity, these tests can be used as the decision criteria to cull the animals for control of SCM in high prevalence herd. Similar recordings were made by Reddy *et al.*, (1998). On the basis of these observations, it may be concluded that CMT may be practical and useful screening test to identify buffaloes that should undergo further testing by cultural examination and NAG-ase test.

All the 235 quarter milk samples were subjected to different diagnostic tests viz. Bromothymol blue (BTB) strip test, electrical conductivity (EC), California mastitis test (CMT), N-acetyl-β-D-glucosaminidase (NAG-ase) test, SCC and cultural examination for detection of SCM. As many as 17.02 per cent, 16.59 per cent, 16.17 per cent, 14.46 per cent, 14.46 per cent and 17.02 per cent quarters were tested positive for SCM by bromothymol blue (BTB) strip test, electrical conductivity (EC), CMT, NAG-ase, SCC and cultural examination, respectively, whereas 82.97 per cent, 83.40 per cent, 83.82 per cent, 85.53 per cent, 85.53 per cent and 82.97 per cent of the quarters were found negative for SCM, with the respective tests.

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