Comparison of newly standardized ‘Latex milk agglutination test’, with ‘Indigenous milk ELISA’ for ‘on spot’ screening of domestic livestock against Mycobacterium avium subsp. paratuberculosis infection

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Mycobacterium avium subspecies paratuberculosis (MAP), the cause of Johne’s disease in animals, has also been associated with Crohn’s disease (CD), Inflammatory bowel disease (IBD) and auto-immune disorders of humans. Increased consumption of milk and milk products made from pasteurized milk led to the sharp rise in the cases of IBD/CD in India. Milk and milk products are the main source of transmission of MAP from animals to humans, since MAP is not inactivated during pasteurization. Lack of rapid and sensitive ‘field test’ is the major stumbling block in estimating bio-incidence of MAP and threat it poses to human population. In the present study, newly standardized ‘Latex milk agglutination test’ (LMAT) was compared with ‘Indigenous milk ELISA’ test. Of the 900 raw milk samples of domestic livestock screened, 36.4 and 51.4% were positive in milk ELISA and LMAT, respectively. In milk ELISA, 38.7, 71.4, 35.1 and 33.5% milk samples of goats, sheep, cattle and buffaloes were positive for MAP, respectively. Whereas in LMAT, 60.6, 90.4, 45.9 and 44.7% milk samples of goats, sheep, cattle and buffaloes were positive for MAP, respectively. LMAT had 70.5% overall rate of agreement with milk ELISA. LMAT had sensitivity and specificity of 80.1 and 65.0%, respectively on comparison with ‘Indigenous milk ELISA’ and Kappa value of 0.416. Strength of agreement between two tests was ‘fair’. Study showed that LMAT has potential to be developed as field based ‘spot test’ for the rapid screening of milk samples of lactating domestic livestock for the detection of MAP.

Keywords: Indigenous ELISA test, lactating animals, Latex Agglutination test, Mycobacterium avium subsp. paratuberculosis, raw milk, spot test

Introduction

Johne’s disease (JD) caused by Mycobacterium avium subspecies paratuberculosis (MAP) is characterized by chronic granulomatous enteritis, highly prevalent and endemic in the domestic livestock population worldwide. It is potentially fatal and highly infectious disease causing huge losses to the livestock industry globally. Recent categorization of MAP as potential human pathogen provided additional reason for the initiation of country-wide programmes for the control of JD in domestic livestock. Infected animals shed bacilli via milk, faeces, saliva, semen, vaginal discharges etc., contaminating the environment. MAP has been associated with Crohn’s disease (CD) in human beings, a systemic intestinal disorder characterized by chronic enteritis. MAP has been isolated from the intestinal biopsies of CD patients and from the breast milk of women infected with MAP. MAP has been recovered from raw and pasteurized milk and milk products (commercial supplies), pooled and individual milk samples of lactating animals and India. MAP bacilli have been recovered from commercial pasteurized milk and milk products globally and India. MAP biotype recovered from the milk samples was novel bio-type (‘Indian Bison Type’),
Milk samples were screened using indigenous milk ELISA Test (milk ELISA) as per Singh et al. with modification (instead of milk whey, whole milk was used). Briefly, each well of flat bottom 96 well ELISA plate was coated with 0.1 µg of protoplasmic antigen in 100 µL of carbonate-bicarbonate buffer (pH 9.6) per well and incubated at 4°C overnight. Plates were washed thrice with PBST (PBS with 0.05% Tween 20), followed by blocking in 100 µL of 3.0% skimmed milk in PBS, incubated for 1 h at 37°C. Plates were washed three times with PBST and then 100 µL of whole milk, diluted in PBST with 1.0% BSA in ratio of 1:1, was added as sample in duplicate wells and incubated for 2 h at 37°C. Plates were washed thrice, followed by addition of 100 µL of optimally diluted rabbit anti-bovine (1:6000 in 1× PBS)/caprine (1:5000 in 1× PBS) conjugate and again incubated for 1 h at 37°C. Finally, after five times washing, 100 µL of freshly prepared OPD substrate was added and incubated till the colour developed (3-5 min) at 37°C. Absorbance was read at 450 nm in ELISA reader (i-Mark microplate reader, Biorad). Milk whey from weak and culture positive, and healthy and culture negative buffaloes were used as positive and negative controls, respectively. Optical densities (ODs) were transformed and expressed as sample-to-positive (S/P) ratios. Samples in low positive, positive and strong positive categories of S/P ratio were taken as positive for MAP infection.

**Materials and Methods**

**Collection of Milk Samples**

Milk samples (900) were collected between February 2015 and March 2016 from lactating domestic livestock (goats, 305; sheep, 21; cows, 174; & buffaloes, 400) belong to farms/dairies owned by the individual households and farmers herds/flocks located in Mathura and Agra districts of Uttar Pradesh. Udder of each animal was cleaned, dried and 15 mL of milk was collected from each animal. Milk samples were stored at −20°C till further processing. 'Latex milk Agglutination Test' (LMAT) was standardized on milk samples as first time LAT was standardized on milk samples as 'Latex milk Agglutination Test' (LMAT) for the screening of JD in lactating animals. In the present study, efficiency of in-house LMAT was compared with 'indigenous milk ELISA kit'.

**Latex Milk Agglutination Test (LMAT)**

**Preparation of MAP Antigen Coated Latex Beads**

Polystyrene latex beads (3.0 µm mean size, 10 µL; Sigma Aldrich) were washed four times in distilled water and re-suspended in 20 µL of 0.5 M glycine saline buffer (1.4 g glycine, 0.07 g sodium hydroxide, 1.7 g sodium chloride, 0.1 g sodium azide in 100 mL of triple distilled water) (pH 8.6). To which 20 µL of antigen (4 mg/mL) was added and incubated for 3 h at 37°C in incubator. The mixture was centrifuged at 5000 rpm for 10 min and after aspirating the supernatant, mixture was re-suspended in blocking buffer (1% BSA in 1× PBS) and mixed in incubator for 45 min at 37°C. Finally, beads were washed twice in 1× PBS.

**Test Proper**

LMAT was performed by mixing 4 µL of milk sample with 2 µL of coated latex beads using tip of a pipette on a glass slide. The slide was shaken gently for 2 min. Samples were considered positive, if agglutination was observed within 2 min and negative if no agglutination was observed within 2 min (Fig. 1).
Statistical Analysis
To measure statistical significance between two tests, McNemar's test and kappa agreement statistical analysis methods were applied by Graph Pad software, USA. Sensitivity and specificity of the tests were measured by Med-Calc software, Belgium.

Results
Of the 900 milk samples of domestic livestock screened, 328 (36.4%) and 463 (51.4%) were positive in milk ELISA and LMAT, respectively. Of the 574 milk samples from large ruminants, 195 (33.9%) and 259 (45.1%) were positive in milk ELISA and LMAT, respectively. Of the 400 milk samples of individual buffaloes screened, 134 (33.5%) and 179 (44.7%) were positive in milk ELISA and LMAT, respectively. Further, of the 174 milk samples of cows screened, 61 (35.1%) and 80 (45.9%) were positive in milk ELISA and LMAT, respectively. Of the 326 milk samples of small ruminants consisting of goats and sheep, 133 (40.7%) and 204 (62.5%) were positive in milk ELISA and LMAT, respectively. Species-wise, of the 21 milk samples of sheep screened, 15 (71.4%) and 19 (90.4) were positive in milk ELISA and LMAT, respectively. Whereas, of the 305 milk samples of goats screened by milk ELISA and LMAT, 118 (38.7%) and 185 (60.6%) were positive, respectively. Livestock species-wise, bio-incidence of MAP was highest in sheep (71.4%), followed by goats (38.7%), cows (35.1%) and buffaloes (33.5 using milk ELISA test (Table 1). Similar, results were obtained using LMAT but bio-incidence reported here was higher in comparison to milk ELISA with respect to LMAT with a kappa value of 0.416.

Discussion
Johne's disease is endemic in the domestic livestock population of the country. High to very high bio-incidence of MAP has been reported in domestic livestock in past 30 years in India\(^2\) and outside India\(^1\). Milk is very attractive sample for the detection of MAP in lactating animals, due to ease of collection and sampling from large ruminants in our conditions. In the absence of proper restraining devices (cattle trevis) for large ruminants, collection of blood has been difficult and was major impediment for the survey of animal diseases in the country. Therefore, milk and faeces are two convenient clinical samples for screening in large ruminants. In case of non-lactating animals, collection of feces per rectum without trevis is challenging in large ruminants. In India, bio-load of MAP is very high in the milk of cattle, buffaloes, goats and sheep\(^2\). Our domestic livestock continually get repeated infection of MAP, since we are yet to initiate National Programme for the control of JD. Milk has been reported to be a potential source of transmission of MAP to human population\(^2\), since MAP is not killed during pasteurization. Live MAP bacilli has been frequently

### Table 1—Screening of milk samples for the presence of \textit{M. avium} ssp. \textit{paratuberculosis} using LMAT and indigenous milk ELISA

<table>
<thead>
<tr>
<th>Livestock species screened</th>
<th>Milk samples ((n))</th>
<th>Milk ELISA</th>
<th>LMAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Small ruminants ((1+2))</td>
<td>326</td>
<td>133 (40.7)*</td>
<td>204 (62.5)</td>
</tr>
<tr>
<td>1 Goats</td>
<td>305</td>
<td>118 (38.7)*</td>
<td>185 (60.6)</td>
</tr>
<tr>
<td>2 Sheep</td>
<td>21</td>
<td>15 (71.4)</td>
<td>19 (90.4)</td>
</tr>
<tr>
<td>B. Large ruminants ((3+4))</td>
<td>574</td>
<td>195 (33.9)</td>
<td>259 (45.1)</td>
</tr>
<tr>
<td>3 Cattle</td>
<td>174</td>
<td>61 (35.1)</td>
<td>80 (45.9)</td>
</tr>
<tr>
<td>4 Buffaloes</td>
<td>400</td>
<td>134 (33.5)</td>
<td>179 (44.7)</td>
</tr>
<tr>
<td>Grand total ((A + B))</td>
<td>900</td>
<td>328 (36.4)</td>
<td>463 (51.4)</td>
</tr>
</tbody>
</table>

*All figures in parenthesis are per cent

strong positive [4 (0.4%)]. In milk ELISA, a total of 328 (36.4%) milk samples were positive for MAP infection (Table 2). A total of 70.5% milk samples showed perfect agreement, while 29.4% showed mismatch between the two tests. True positives (positives in both the tests) and true negatives (negatives in both the tests) were 29.2 and 41.3% milk samples, respectively (Tables 2 & 3). Statistical comparisons were made and p-value was calculated to be <0.0001 (extremely significantly different) and the strength of agreement was ‘moderate’ for milk ELISA with respect to LMAT.

![Fig. 1—Visual definition of agglutination reactions for the presence/absence of \textit{M. avium} ssp. \textit{paratuberculosis}: (a) Present; & (b) Absent.](image-url)
recovered from commercial pasteurized milk and milk products not only outside India \(^\text{28,29}\) but also in India \(^\text{13,30}\). Diagnostic tests for the detection of MAP in milk samples are either based on antibody (indigenous ELISA & LMAT) or antigen (microscopy, culture, indirect fluorescent antibody & PCR). Culture, though ‘Gold’ standard, is time consuming, costly and takes several months for the appearance of minute colonies. ELISA, PCR, iFAT and microscopy are laboratory based tests, requiring sophisticated instrument and techniques. Therefore, before employing control measures for JD in domestic livestock, the key issue to address is to estimate national bio-incidence (status) of JD in the domestic livestock population (>500.0 million). To meet this challenge, a sensitive, specific and cost effective ‘field based test’ is needed.

LAT has been employed for the screening of several diseases \(^\text{31,32}\) of animals \(^\text{15-18}\) and human beings \(^\text{19-22}\). LAT is simple to perform and quick (requiring less than 2 min for detection). The advantage of LAT is the rapid antigen antibody reaction, which gives desired results without the use of species-specific secondary antibodies under field conditions, thereby providing results quickly to initiate action either repeat testing by other tests or segregation or culling of infected animals. Present study evaluated potential of latex milk agglutination (LMAT) to be used as rapid field based ‘spot test’ for the detection of MAP in milk samples (raw or pasteurized; individual animals or pooled).

In the present study, LMAT was used for the detection of MAP in the milk. In majority of the MAP studies, LAT was employed for the detection of bovine tuberculosis. In 2004, Koo and coworkers \(^\text{33}\) evaluated the potential of latex bead agglutination assay for the diagnosis of bovine tuberculosis (bTB), wherein latex beads were coated with ESAT-6p (early secretory antigenic target), a portion of the ESAT6 protein that is secreted during the early or active phase of mycobacterial infection. This assay had the sensitivity and specificity of 95.7 and 100.0%, respectively, and kappa value of 0.85. Authors concluded that the assay could be used along with ‘skin test’ for the accurate detection of bTB by reducing the frequency of misdiagnosis. In the following year, Koo extended his research with the use of rMPB70 a recombinant form of PMB70, a major secreted immunogenic protein component of tuberculin, and found that assay had the sensitivity and specificity of 86.7, and 97.8%, respectively.

Other studies of mycobacterial detection were carried out by Ganju and co-workers \(^\text{34}\) wherein latex beads were coated with *Mycobacterium*, a saprophytic organism with antigenic epitopes that are cross-reactive with *M. leprae* and *M. tuberculosis*. The study revealed that LAT had the sensitivity of 90.4 and 91.6% for the diagnosis of Hansen’s disease (HD)

### Table 2—Comparison of LMAT *vis a vis* indigenous milk ELISA for the screening of milk samples (*n*=900) of domestic livestock against *M. avium* ssp. *paratuberculosis*

<table>
<thead>
<tr>
<th>Indigenous milk ELISA</th>
<th>Status of MAP infection in milk ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>n (%)</em></td>
<td>Negative (N)</td>
</tr>
<tr>
<td></td>
<td>450 (50.0)*</td>
</tr>
<tr>
<td>LMAT <em>n (%)</em></td>
<td>Negative (N)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>Positives (P)</td>
<td>132 (14.7)</td>
</tr>
<tr>
<td>Total</td>
<td>P=200 (22.2) or FP</td>
</tr>
</tbody>
</table>

FP= False positive; TP= True positive

*All figures in parenthesis are per cent

### Table 3—Comparison of LMAT and indigenous milk ELISA for the detection of *M. avium* ssp. *paratuberculosis* in milk samples of domestic livestock

<table>
<thead>
<tr>
<th>Tests</th>
<th>Combinations, <em>n (%)</em></th>
<th>Total positives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk ELISA</td>
<td>+ - - +</td>
<td>328 (36.4)</td>
</tr>
<tr>
<td>LMAT</td>
<td>+ - + -</td>
<td>463 (51.4)</td>
</tr>
<tr>
<td>Total (<em>n=900</em>)</td>
<td>263 (29.2) 372 (41.3) 200 (22.2) 65 (7.2) 528 (58.6)</td>
<td></td>
</tr>
</tbody>
</table>

Kappa value, 0.416; SE, 0.029 (95.0% confidence interval 0.359 to 0.472).

Type of agreement between tests was fair.
and tuberculosis (TB), respectively, when immune complexes from patients' sera were used.

In view of the above findings, where LAT was reported as the most reliable diagnostic assay, present study evaluated the efficacy of LMAT vis a vis a standard 'Indigenous milk ELISA' for the screening of milk samples against MAP infection. Results of new LMAT, as the field based 'Spot test', were compared statistically with indigenous milk ELISA by calculating sensitivity, specificity, kappa value and strength of agreement. The kappa value was found to be 0.416 and LMAT had moderate agreement with indigenous milk ELISA, when samples in low positives, positives and strong positives categories were considered positives in milk-ELISA. About 70.5% similarity was observed between LMAT and milk ELISA as compared to 29.4% mismatch. The 65 (7.2%) milk samples missed by LMAT and detected in milk ELISA might be due to the differences between the two tests with respect to the reading of results. In indigenous milk ELISA test, results were read with the help of ELISA reader (quantified) as compared to naked eye reading of the results (qualitative) in LMAT. However, an interesting finding was that the two tests behaved differently in small and large ruminants. In large ruminants, difference between LMAT and milk ELISA were small and LMAT was nearly 10.0% superior in detecting MAP. Whereas this difference was more pronounced (20.0%) in small ruminants, with respect to the sensitivity of LMAT vis a vis milk ELISA.

In milk samples, with respect to milk ELISA, the sensitivity and specificity of LMAT was 80.1 and 65.0%, respectively. Large number of milk samples were positive (22.2%) in LMAT exclusively and not detected by milk ELISA (Table 3). This may be due to the better efficiency of LAT, wherein suspended latex beads provide larger surface area for attachment of antigen and antigen anti-body reaction as compared to solid matrix provided on the surface of wells in the ELISA plates. The other reason may be that LMAT is a qualitative test (positive or negative), while milk ELISA is a quantitative test, wherein milk samples in 'suspected' categories are considered as negatives. Of 22.2% milk samples exclusively positive in LMAT, 14.7 and 7.6% were, respectively, in negative and suspected categories in milk ELISA. These results can be further evaluated using other diagnostic tests (microscopy, iFAT, PCR, dot-ELISA etc.). This problem can also be addressed by repeating the tests at short or definite intervals. Since both these tests are screening tests, inclusion of confirmatory tests (culture, PCR, iFAT) may help to further elucidate the test results. Assays giving 'different' results in comparison to other tests were also noted during the standardization of dot-ELISA with indigenous milk ELISA, since other tests used were not the 'Gold standard'. In our other studies, two new tests (milk dot-ELISA & indirect milk FAT) were standardized as alternative tests for the screening of milk samples for the detection of MAP, where both the tests had better sensitivity than LMAT. LMAT has the advantage over iFAT, since microscope is essential with FAT facility. Though both dot-ELISA and LMAT are ‘field tests’, LMAT is much quicker test as compared to dot-ELISA and results can be obtained within 2 min. Sensitivity of LMAT was slightly lower than iFAT (sensitivity 84.7% with respect to microscopy) and lower than dot ELISA (sensitivity 86.2% with respect to indigenous milk ELISA). Specificity of LMAT was lower than dot ELISA (specificity 73.8% with respect to indigenous milk ELISA) and iFAT (specificity 90.4%) with respect to microscopy. In the above study, majority of samples had similar results in milk ELISA, dot ELISA and iFAT. Minor variations in three tests may be due to endemic and spectral nature of JD.

In our recent study, of 1310 milk samples screened using iFAT, PCR, Microscopy, dot-ELISA, milk-ELISA and LMAT, only 4.2% samples were detected by single test; 1.0% LMAT, 2.1% dot-ELISA, 0.3% microscopy and 0.07% milk ELISA (personal communication). Hence, only 1.0% samples detected positive in LMAT may be false positive, which was significantly lower than other four (BPAT, SAT, RBPT & LAT) tests used in Brucella infection. In the Leptospirosis infection of human beings, mean overall sensitivity of serum LAT was found at par with that of IgM ELISA and MAT (80.1%) unlike present study, Bhalaria et al reported lower sensitivity (66.6%) of LAT in detecting HBsAG in voluntary blood donors. However, Ramadass et al reported slightly higher (87.9%) sensitivity of LAT in the diagnosis of Leptospirosis.

Using milk ELISA, bio-incidence of MAP in livestock, species-wise, was the highest in sheep (71.4%), followed by goats (38.7%), cows (35.1%) and buffaloes (33.5%) (Table 1). Though similar results were obtained using LMAT but at higher value.
of incidence (sheep, 90.4; goats, 60.6; cows, 45.9; & buffaloes, 44.7%). These results showed that both milk ELISA and LMAT were good screening tests to estimate bio-incidence of MAP in lactating animals. Of 900 milk samples screened for the presence of MAP, 59.7% were positive in two tests (milk ELISA, 36.4 & LMAT, 51.4%). This may be due to the higher sensitivity of the LMAT and milk ELISA tests. Lack of ‘field test’ has been major impediment in estimating bio-incidence of MAP in domestic livestock at National level.

Conclusion
As compared to indigenous milk ELISA, newly standardized LMAT (using semi-purified protoplasmic antigen from native MAP strain S 5) for screening the milk samples of domestic livestock against MAP was superior, sensitive, simple, cost effective and rapid (takes 5 min) field based ‘spot test’.

Conflict of Interest
No conflict of interest to declare.

References