Evaluation of new ‘indigenous milk dot-ELISA’ as ‘Field Test’ vis-à-vis milk plate-ELISA for the detection of Mycobacterium avium sub species paratuberculosis infection in lactating domestic livestock

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Mycobacterium avium subspecies paratuberculosis (MAP), the cause of incurable Johne’s disease (JD), is endemic in both domestic livestock and human population of the country. It is the major cause of low per animal productivity and reduced productive life in domestic livestock, wherein buffaloes, goats and sheep go for early slaughter for harvesting meat and cows are left to roam on the roads of cities and towns. In the present study, milk dot-ELISA test was standardized in efforts to develop a ‘field test’ for the detection of MAP infection in lactating animals using milk samples. Results of newly developed ‘milk dot-ELISA’ were compared with well standardized ‘indigenous milk plate-ELISA kit’, with known sensitivity and specificity. Of the 276 milk samples screened, 43 (32.0%) and 51 (35.9%) were positive in plate-ELISA and dot-ELISA (True Positives), for bovine and caprine paratuberculosis, respectively. Sensitivity and specificity of dot-ELISA vis-à-vis plate-ELISA was 86.2 and 73.8%, respectively. Newly standardized dot-ELISA was found to be highly sensitive, cost effective, quick, repeatable and efficient ‘field test’ for the screening of milk samples of lactating cattle, buffaloes, sheep and goats against MAP infection.

Keywords: Paratuberculosis, dot-ELISA, plate-ELISA, milk, lactating goats and cattle,

Introduction

Johne’s disease (JD), caused by the Gram-positive Mycobacterium avium subspecies paratuberculosis (MAP), is endemic both in the domestic livestock and human population of the country¹. MAP has also been linked with a number of human infections, especially Crohn’s disease². In developed countries, Johne’s disease has been one of the most researched disease of animals, since disease has huge impact on health (animal & human) and productivity. Isolation of MAP from retail milk supplies³ has raised alarm bells, suggesting consumption of milk and milk product from infected livestock may possibly be the primary source of MAP infection to human beings⁴. Despite low per animal productivity due to MAP infection, other countries besides USA, Canada, Europe, Japan, New Zealand and Australia had paid very little attention to investigate the bio-prevalence of disease or estimate production losses. This is mainly because of the lack of indigenous, cost-effective and field based kits for the diagnosis of Johne’s disease/MAP infection in animals using milk.

Allergic reaction using Johnin is the only field test available but has serious limitation in terms of sensitivity and specificity⁵. Of the range of available tests (faecal microscopy, faecal culture, blood PCR, plate-ELISA & IFN-y), none has potential to be used or qualify as field test. In the present study, newly developed serum based ‘dot-ELISA test’, for the diagnosis of Johne’s disease in buffaloes, has been standardized and evaluated for the detection of MAP infection in milk samples of lactating domestic livestock⁶-⁸. Further, indigenous milk plate-ELISA (p-ELISA) test (quantitative) developed, standardized

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and evaluated extensively for the diagnosis of Johne’s disease in lactating domestic livestock was used as the parallel (quantitative) test to validate newly developed ‘indigenous milk dot ELISA (qualitative) test’.  

### Materials and Methods

#### Sampling

Milk samples were collected from domestic livestock around the Central Institute for Research on Goats (CIRG) campus located in Mathura district of South Uttar Pradesh (North India). Animals (goats & bovines, viz., cows & buffaloes) were owned by individual households and some milk samples were also collected from nearby dairy farms. To collect the samples, the udder of each animal was first washed, cleaned and dried with towel and then 15 mL of milk was collected from each animal and subjected to centrifugation at 4500 rpm for 45 min. Intermediate whey layer was separated and stored at −20°C. A total of 274 animals (134 bovines & 142 goats) were screened between February and October 2015.

### Diagnostic Tests

Newly developed indigenous ‘dot-ELISA’ test was standardized using same semi-purified protoplasmic antigen (sPPA) and was compared with indigenous p-ELISA kit for the screening of lactating livestock using milk. For the twin tests, native strain (S 5) of MAP, bio-typed as novel ‘Indian Bison Type’ and a major ‘biotype’ infecting domestic livestock population, was used as the antigen source for the preparation of sPPA.

#### Indigenous p-ELISA

Milk based indigenous ‘p-ELISA’ kit was used as a standard test for screening of animals for MAP infection and was parallel test for comparative studies. The 0.1 µg of antigen in 100 µL of carbonate-bicarbonate buffer, (pH 9.6) was added to each well of flat bottom 96 well ELISA plate 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% skimmed milk in PBS, incubated for 1 h at 37°C. Plate was further washed 3 times with PBST and then test samples (100 µL of 1:4 diluted whey) were added in duplicate wells and incubated for 2 h at 37°C. Plates were washed thrice with PBST, followed by the addition of 100 µL of optimally diluted rabbit anti-bovine/caprine conjugate and again incubated for 1 h at 37°C. Finally, after 5 times washing with PBST, 100 µL of freshly prepared OPD substrate was added and incubated until the colour developed at 37°C. Absorbance was read at 450 nm in ELISA reader (iMark micro-plate reader, Biorad). Whey samples from culture positive and culture negative buffaloes and goats were used as positive and negative controls, respectively. Optical densities (OD) were transformed and expressed as sample-to-positive (S/P) ratios as per the method of Collins et al.

#### Indigenous Dot-ELISA (d-ELISA)

d-ELISA was performed as per Singh et al with minor modifications. Briefly, tips of 12 legged immune-diffusion combs (Advanced Micro Devices Pvt. Ltd., Ambala (Haryana), India) fixed with nitrocellulose membrane were coated with 1 µL (1 µg of sPPA in 1 µL of carbonate-bicarbonate buffer) and incubated at 4°C overnight. Plates were washed thrice with PBST, followed by the addition of 100 µL of optimally diluted rabbit anti-bovine/caprine conjugate and again incubated for 1 h at 37°C. Finally, after 5 times washing with PBST, 100 µL of freshly prepared OPD substrate was added and incubated until the colour developed at 37°C. Absorbance was read at 450 nm in ELISA reader (iMark micro-plate reader, Biorad). Whey samples from culture positive and culture negative buffaloes and goats were used as positive and negative controls, respectively. Optical densities (OD) were transformed and expressed as sample-to-positive (S/P) ratios as per the method of Collins et al.

#### Analysis of OD (Absorbance) Values

S/P ratio value = 

\[
\frac{\text{[Sample OD–Negative OD]}}{\text{[Positive OD–Negative OD]}}
\]

Value of samples to positive ratios and corresponding status of Johne’s disease in animals was determined as per Singh et al. Samples in low positive (LP), positive (P) and strong positive (SP) categories of S/P ratio were considered as positive for MAP infection in milk p-ELISA.

### Table 1—Evaluation of d-ELISA vis-à-vis indigenous p-ELISA for the screening of bovine milk (n=134) samples against M. avium ssp. paratuberculosis infection

<table>
<thead>
<tr>
<th>Screening tests</th>
<th>Indigenous p-ELISA n (%)</th>
<th>Low positive (LP)</th>
<th>Positive (P)</th>
<th>Strong positive (SP)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=83 (61.9)</td>
<td>13 (9.7)</td>
<td>38 (28.3)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>d-ELISA n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negatives</td>
<td>63 (47.0)</td>
<td>4 (2.9)</td>
<td>5 (3.7)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Total</td>
<td>63 (47.0) or TN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positives</td>
<td>19 (14.1)</td>
<td>9 (6.7)</td>
<td>33 (24.6)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Total</td>
<td>20 (14.9) or FP</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figures in parenthesis are percent; Total samples (n)=134; N=Negative, P=Positive, TP=True positive, TN=True negative, FN=False negative, FP=False positive
buffer, pH 9.6) of sPPA spot in middle of nitrocellulose paper and incubated for 2 h at 37°C. Combs were dipped in blocking solution (3.0% skimmed milk powder in PBS) for 1 h at 37°C. After further washing in PBST the combs were dipped in test samples (200 µL clear whey in 1:4 dilution in 1% BSA in PBS) for 1 h, followed by again washing in PBST and then the combs were incubated with 200 µL of rabbit anti-bovine and/or caprine HRP conjugate solution at 37°C for 30 min. Finally, the combs were dipped in 200 µL of 3,3'-diaminobenzidine (6 mg/10 mL of 1× PBS) at room temperature till the development of colour (1-3 min). Once the spot was visible, the combs were dipped in water to stop the reaction. Positive and negative controls used in the study were confirmed through IS900 PCR and microscopy were used on two legs of each comb to assist in reading of the test samples.

Statistical Analysis

To measure the statistical significance between both the test, Mc Nemar’s test and kappa agreement statistical analysis methods were applied by Graph Pad software, USA and sensitivity and specificity of the tests was measured by Med-Calc Software, Belgium.

Results

Of the 134 bovine milk samples screened by milk p-ELISA, 51 (38.0%) and 83 (61.9%) were positive and negative, respectively for MAP infection (Table 1). p-ELISA being quantitative test, therefore on the basis of S/P ratio, 82 (61.1%), 1 (0.7%), 13 (9.7%) 38 (28.3%) and none of the milk samples were in negative, suspected, low positive, positive and strong positive categories, respectively (Table 1). d-ELISA being qualitative test showed 62 (46.2%) and 72 (53.7%) milk samples positive and negative, respectively for MAP infection. Thus, the data for true positives (positives in both the tests) and true negatives (negative in both the tests) were 31.3 and 49.2%, respectively. A total of 21.6% (6.7% false negative & 14.9% false positive) samples showed mismatch, while 78.4% (31.3% true positive & 49.20% true negative) of milk samples showed perfect agreement within two tests. Statistical comparisons were made with p-value and found to be ‘not significantly different’ (Table 3). The strength of agreement was ‘moderate’ for p-ELISA with respect to d-ELISA, having a kappa value of 0.559.

Of 142 milk samples screened in case of goats by indigenous p-ELISA, 54 (38.0%) and 88 (61.9%) were positive and negative, respectively for MAP infection (Table 2). Based on the S/P ratio, 81 (57.0%), 7 (4.9%), 18 (12.6%), 36 (25.3%) and none of the milk samples were in negative, suspected, low positive, positive and strong positive categories, respectively. Further, in d-ELISA, 64 (45.0%) and 78 (54.9%) milk samples were negative and positive, respectively for MAP infection. A total of 21.1% milk samples exhibited mismatch in results (2.1% false negatives & 19.0% as false positives), while 112 (78.8%) milk samples had matching results (true positives and true negatives were 35.9 and 42.9%, respectively) in both the tests. Statistical comparisons were evaluated with p-Value and found to be ‘significantly different’ (Table 3). The strength of agreement was estimated to be ‘moderate’

<table>
<thead>
<tr>
<th>Screening tests</th>
<th>Status</th>
<th>N</th>
<th>Suspected (S)</th>
<th>Low positive (LP)</th>
<th>Positive (P)</th>
<th>Strong positive (SP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-ELISA n (%)</td>
<td>Total</td>
<td>58 (40.8)</td>
<td>3 (2.1)</td>
<td>2 (1.4)</td>
<td>1 (0.7)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>N=88 (61.9)</td>
<td>Positive</td>
<td>23 (16.1)</td>
<td>4 (2.8)</td>
<td>16 (11.2)</td>
<td>N=3 (2.1) or FN</td>
<td>35 (24.6)</td>
</tr>
<tr>
<td>P=27 (19.0) or FP</td>
<td></td>
<td>P=51 (35.9) or TP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Milk samples</th>
<th>Tests</th>
<th>P-value</th>
<th>Kappa</th>
<th>Strength of agreement</th>
<th>95% Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine</td>
<td>p-ELISA vs d-ELISA</td>
<td>1.0000</td>
<td>0.559</td>
<td>Moderate</td>
<td>0.418 to 0.700</td>
</tr>
<tr>
<td>Goats</td>
<td>d-ELISA</td>
<td>&gt;0.0001</td>
<td>0.587</td>
<td>Moderate</td>
<td>0.463 to 0.711</td>
</tr>
</tbody>
</table>
for p-ELISA with respect to d-ELISA, having a kappa value of 0.587.

Discussion
India is the largest milk producer in the world, which accounts for 16.0\% of the global milk production\textsuperscript{12}. However, the per animal productivity is very low mainly due to endemicity of MAP in domestic livestock\textsuperscript{3}. Shedding of MAP in milk of the infected livestock is a matter of great concern since recent studies have established that zoonotic potential and consumption of raw and pasteurized milk was the main source of transmission of MAP in humans\textsuperscript{13}. A comprehensive study by Singh et al\textsuperscript{1} revealed that the average bio-load of MAP infection in livestock population of the country was 23.3\% in the last 28 years\textsuperscript{1}. Further, 5 yearly average of infection has shown increasing trend and, in the absence of control measures, in last one year bio-load has reached alarmingly high levels in 4 domestic livestock species. To prevent transmission and spread of infection need giving priority to disease control and use of good ‘herd management practices’. However, in chronic infections like MAP, efficient rapid diagnostic test is the basic to any efforts to either prevent the spread or manage the disease at herd/flock or national level. Since quick and accurate identification of infected animals could help to isolate infected animals and stop the spread of infection, the present study evaluated the diagnostic potential of d-ELISA vis-à-vis p-ELISA for the diagnosis of MAP infection in milk samples of lactating domestic livestock. d-ELISA, a highly versatile solid phase immunoassay for antibody detection, is not new in the diagnostic field and has proved to be excellent alternative field based test for decades in other diseases. It has been used for a wide range of human and animal diseases like amoebiasis, malaria, toxid tick infestation etc\textsuperscript{14}. Barbuddhe et al\textsuperscript{15} used d-ELISA for the detection of Brucella antibodies in human serum samples and it was found more sensitive (88.8\%) and specific (76.9\%) compared to other standard tests, like Rose Bengal plate test (RBPT), serum agglutination test (SAT) or complement fixation test (CFT)\textsuperscript{14}. Singh et al\textsuperscript{16} developed a d-ELISA kit for the detection of brucella infection in goats\textsuperscript{15}. This kit was validated by comparing with other standard test like SAT, micro-CFT and p-ELISA. A good correlation was observed when compared with standard tests and thus d-ELISA was found to be suitable field based test.

So far, no parallel studies have been reported for the detection of MAP in milk using d-ELISA. In the present study, indigenous p-ELISA kit was used for the comparison as it has already undergone continued validations over the years\textsuperscript{9}. In bovines, 134 milk samples were screened where 29 (21.6\%) samples showed mismatch between p-ELISA and d-ELISA. Of the mismatch, 20 (14.9\%) were categorised as false positive, wherein samples negative in p-ELISA were positive in d-ELISA. In case of goats, 30 (21.1\%) milk samples showed mismatch, of which 27 (19.0\%) were false positive. This discrepancy has been reported in most studies pertaining to d-ELISA and may be due to lack of pipetting errors and better sensitivity of d-ELISA\textsuperscript{6,16,17}. A study in 1994 evaluated diagnostic potential of d-ELISA for detection of JD in M. phlei absorbed bovine sera samples\textsuperscript{18}. Findings revealed that though the specificity of d-ELISA was highly compatible with other techniques, the sensitivity of such a test remained low at 34.1\% as compared to 65.9\% for non absorbed sera. Due to very low sensitivity, absorption with M. phlei was not considered in this study as an alternative to lower the number of false positive results. Moreover, corresponding author has long experience of culturing MAP and has seen sharp decline in the presence and population of the fast growing Mycobacteria (non-pathogenic) in the gut of the domestic livestock in general and in case of goat gastro-intestinal tract/fecal samples in particular. These mycobacteria must be providing some kind of protection against pathogenic MAP bacilli, due to sharing of nearly 60.0\% antigens. Therefore pre-absorbance of test sera with saprophytic (non-pathogenic) Mycobacteria like M. phlei was not considered in the present study as an option/step. Of the total 276 milk samples (134 bovines & 142 goats) screened, none was in the strong positive category. This is possible due to the fact that MAP infection is initially localized in the intestine and intestinal immune system, since MAP does not primarily infects the mammary glands and is not closely linked with respect to lymphocytes circulation in ruminants. Therefore, milk antibodies produced by plasma cells local to the mammary glands are not easily influenced by MAP infection\textsuperscript{18}.

Kappa values were 0.559 and 0.587 for bovine and goat milks, respectively. In both cases, d-ELISA gave a moderate level of agreement with milk p-ELISA, when low positives, positives and strong positives
were considered as positives in milk p-ELISA. Sensitivity and specificity of d-ELISA for bovine milk samples was 82.3 and 75.9%, respectively and that for goat samples was 94.4 and 69.3%, respectively. It is evident that milk d-ELISA was highly sensitive with a combine sensitivity of 88.3% and with moderate specificity of 72.6%, with respect to milk p-ELISA. In milk d-ELISA, false negative milk samples were quite low both in bovine (6.7%) and caprine (2.1%) milk samples but the concern was higher number of false positives (14.9% in bovine & 19.0% in caprine samples). These problems can be overcome by repeated standardization of d-ELISA, repeating p-ELISA on same milk samples, since it is highly sensitive test and comparison with other tests like milk culture, microscopy, LAT, iFAT, PCR etc. ‘Herd screening’ for possible infection requires that the diagnostic test should be highly specific to limit the time and efforts spent in confirming the diagnosis. However a highly sensitive test is evidently important for screening of herds in vaccination control programmes as such tests can accurately identify all animals that are infected with MAP. In view of the inherent disadvantages of the Johnin test or allergic tests, such as, low specificity and lack of agreement in tests results with respect to criteria of interpretations, we lack a ‘spot test or field based test’ at present for the diagnosis of JD. However, present milk d-ELISA using native protoplasmic antigen for native MAP (S 5) strain (Indian Bison Type) has potential to be developed as first line ‘screening test’ at field level along with microscopy and p-ELISA. For countries as diverse as India with huge population of low to very low yielding (milk & meat) domestic livestock and with little emphasis on health care, may be due to low resources and lack of knowledge, d-ELISA has the potential to be developed as quick, cost-effective, sensitive spot test for the identification of MAP infection.

**Conclusion**

Present study showed that d-ELISA using semi-purified protoplasmic antigen from native strain (‘S 5’) of MAP (Indian Bison Type) of goat origin was quick, cost-effective and sensitive field test. Therefore, it can be recommended as ‘screening test’ for the detection of Johne’s disease in lactating bovines and caprine population of the country.

**Conflict of Interest**

There is no conflict of interest to declare.

**References**


