

## Comparative potential of modified indigenous, indigenous and commercial ELISA kits for diagnosis of *Mycobacterium avium* subspecies *paratuberculosis* in goat and sheep

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In the present study, modified indigenous ELISA kit (kit 1) was compared with indigenous ELISA kit (kit 2) and commercial ELISA kit (kit 3) for its sensitivity and specificity with respect to faecal culture for diagnosis of Johne's disease in goats and sheep under natural conditions. Of the 64 positive animals, serum of 42.1, 48.4 and 18.7% animals yielded positive infection in kit 1, 2 and 3, respectively. Specificity of kit 1 (95.1%) was maximum followed by kit 3 (93.7%) and kit 2 (83.4%). Kit 1 showed superior diagnostic potential than the other two kits. Kit 1 may be used as single screening test regimen for diagnosis of MAP infection in the population of goats and sheep in India.

**Keywords:** Absorbed ELISA, Commercial ELISA, Johne's disease, *Mycobacterium avium*, Paratuberculosis

Johne's disease (JD), a chronic infection of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is a global animal health problem. Few studies report high prevalence of disease in India<sup>1-3</sup>. Information on prevalence of JD in small ruminants in India is limited due to high cost of imported diagnostic kits. JD is most effectively identified by culture of MAP from faeces<sup>4,5</sup> but being expensive, low in sensitivity and time consuming<sup>6,7</sup>, its use is limited. PCR cannot distinguish between pass-through bacilli or those arising from colonization of intestinal tract. Thus, serology provides rapid and cost-effective alternative diagnostic tool. Sensitivity of ELISA is highest in comparison to other serological tests. To replace commercial ELISA kits and a cattle based kit with variable sensitivity<sup>7</sup>, a new indigenous ELISA kit has been developed for goats

and sheep using protoplasmic antigen from local 'Indian Bison type' MAP strain of goat origin<sup>8</sup>. Specificity of the indigenous ELISA kit was moderate<sup>9</sup>, therefore it was modified ('modified indigenous ELISA kit') to improve its specificity. In the present study, 'modified indigenous ELISA kit' has been compared with 'indigenous ELISA kit' and a 'commercial ELISA kit' for its sensitivity and specificity with respect to faecal culture in order to identify a potential 'single screening test' for the screening of Johne's disease (MAP infection) in goats and sheep population in Indian conditions.

Farm animals (209; 134 goats and 75 sheep) were screened for MAP infection by three ELISA kits and faecal culture.

### Samples collection

*Goat samples (Jamunapari breed)* - Goats samples (134) belonged to 2 farms of elite Jamunapari breed [40 from Central Institute for Research on Goats (CIRG), Mathura and 94 samples from State Animal Husbandry Department, Etawah, Uttar Pradesh] were considered for the study. Both the farms were source of improved breeding bucks for farmer's herds.

*Sheep samples (Muzaffarnagri and Bharat Merino breeds)* - Of the 75 farm sheep screened, 43 were from elite farm of Muzaffarnagri breed located at CIRG, Mathura, India and rest 32 were from Bharat Merino sheep maintained at Southern Regional Center of Central Sheep and Wool Research Institute, Mananvanur, India. Faecal and serum samples were aseptically collected from goats and sheep of the selected farms.

### Screening of samples

*Faecal samples*—Faecal samples (209) were cultured for isolation of MAP as per the modified method of Singh *et al.*<sup>10</sup>.

*Serum samples*—Each of the 209 serum samples were screened by three ELISA kits.

*Modified indigenous ELISA kit (Kit 1)*—Highly sensitive Indigenous ELISA kit (Kit 2) used for the screening of MAP infection in animals and human beings<sup>1,11</sup> was modified to improve the specificity by adding absorption step in the protocol<sup>12</sup>. Briefly,

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10 µg per plate of MAP 'Bison type' protoplasmic antigen<sup>1,9</sup> was taken in 10 ml of carbonate-bicarbonate buffer (pH 9.6) and coated in duplicate wells of flat bottom 96 well ELISA plates. Plates were incubated at 4°C for overnight, washed thrice with washing buffer (PBS + 0.05% Tween-20, pH 7.4), blocked (3% bovine serum albumin fraction V / skimmed milk powder in PBS) and incubated at 37°C for 1 hr. Plates were washed thrice (PBST) and stored at 4°C till further use. *M. phlei* (Allied Monitor Inc. USA) was mixed in the serum dilution buffer before dilution of each test serum sample. 'Dilution plate' was incubated for 60 min with frequent manual agitation at 37°C. After incubation, 100 µl of adsorbed serum was transferred to duplicate wells of ELISA plate and was incubated for 2 hr at 37°C. The plates were washed three times with PBST for 5 min each, 100 µl of optimally diluted (1:8,000) rabbit anti-goat horse radish peroxidase conjugate (Genei, Bangalore) added to all wells and incubated at 37°C for 1 hr. Plates were washed three times with PBST for 5 min each and 200 µl of freshly prepared substrate (OPD, 5 mg / plate in substrate buffer, pH - 5.0) was added to each well and was incubated in dark for 30 min at room temperature. Absorbance was read at 450 nm in ELISA reader (Multiskan, Finland).

*Indigenous ELISA kit (Kit 2) and commercial ELISA (Kit 3)*—Serum samples were also screened by Indigenous ELISA kit (kit 2)<sup>9</sup> and commercial ELISA kit (kit 3) gifted by ID Vet (France) as per recommended protocol.

Positive and negative serum controls in kit 1 and 2 were selected from goats positive and negative in culture in earlier study<sup>13-15</sup>. Samples were run in duplicate and mean OD was taken as final OD. OD values of test samples were transformed to S/P ratio<sup>16</sup> and animals in strong positive category were considered as positive in kit 1 and 2. In kit 3 manufacturer's instructions were followed to decide positive animals.

*Calculation of sensitivity and specificity of ELISA tests*—Sensitivity and specificity of ELISA kits were calculated using method of Arizmendi and Grimes<sup>17</sup>.

*Kappa scores and ROC analysis of ELISA test*—Performance of 3 ELISA kits with respect to 'Gold Standard' faecal culture were compared by calculating Kappa Scores (Proportional Agreement) as per method described earlier<sup>18</sup> (0 <, poor; 0.0-0.20, slight; 0.21-0.40, fair; 0.41-0.60, moderate;

0.61-0.80, substantial and 0.81-1.00, almost perfect) and ROC analysis were performed.

*MAP infection in goats*—Of the 134 goats screened, 27.6, 16.4, 28.3 and 8.9% were positive in faecal culture using kits 1, 2 and 3, respectively. Using kit 1, 2 and 3, there was agreement in 81.3, 72.3 and 72.3% and mismatch in 18.6, 27.6 and 27.6%, respectively.

*MAP infection in sheep*—Of the 75 sheep screened, 36.0, 16.0, 22.6 and 12.0% were positive in faecal culture using kits 1, 2 and 3, respectively. For kit 1, 2 and 3, there was agreement in 74.6, 73.3 and 68.0% and mismatch in 25.3, 26.6 and 32.0%, respectively.

*Sensitivity and specificity of ELISA kits*—With respect to faecal culture, sensitivity and specificity of ELISA kit 1, 2 and 3 in goats and sheep was 42.1, 48.4 and 18.7% and 95.1, 83.4 and 93.7%, respectively. In goats, the sensitivity and specificity of kit 1, 2 and 3 was 45.9, 51.3 and 16.2% and 94.8, 80.4 and 93.8%, respectively. In sheep, the sensitivity and specificity of kit 1, 2 and 3 was 37.0, 44.4 and 22.2% and 95.8, 89.5 and 93.7%, respectively.

*Kappa and ROC analysis*—Kappa and ROC values for kit 1, 2 and 3 in goats and sheep were 0.78, 0.72 and 0.70 and 0.686, 0.659 and 0.562, respectively. In goats, kappa values were, 0.81, 0.72 and 0.72, using kit 1, 2 and 3, respectively. In sheep, kappa values were 0.74, 0.73 and 0.68 using kit 1, 2 and 3, respectively.

Effective control and eradication of Johne's disease has been hampered due to lack of rapid, sensitive and specific diagnostic tests. No single test can detect all JD positive animals in a farm, therefore use of multiple tests is suggested<sup>19</sup>. In absence of indigenous diagnostic kits, National JD control program could not be implemented. Lower sensitivity and cost are major constraints in cattle based commercial kits<sup>9</sup>. Faecal culture is 'Gold Standard' for diagnosis of MAP<sup>5</sup>. Serological screening offered potential for rapid and effective screening at individual level in herds. Strict browsing nature, dwindling grazing area and high lactation stress are primary causes of higher prevalence of MAP in Jamunapari breed despite regular screening and culling. Lower prevalence of MAP in Etawah, India, might be due to replacement of old stock with new goats from farmer's herds<sup>2,20-22</sup>. Prevalence of MAP in Muzaffarnagri sheep was significantly higher as compared to Bharat Merino in this study. Singh<sup>20</sup> has also reported higher prevalence of JD in Muzaffarnagri sheep.

Indigenous ELISA kit (kit 2) was improved ('modified indigenous ELISA kit'; kit 1) by pre-adsorption of test serum with *M. phlei*<sup>12</sup> and lower prevalence with kit 1 as compared to kit 2 was due to removal of cross-reacting antibodies against environmental mycobacteria. Use of native antigens in kit 1 and 2 might have detected significantly higher number of culture positive animals than kit 3 (commercial) using purified protoplasmic antigen (PPA) of MAP 316 strain of cattle origin as suggested by earlier observations<sup>3,9,23</sup>.

Kit 1 and 2 had comparable sensitivity, but two kits were significantly more sensitive than kit 3. Adsorption of test serum improved the specificity of kit 1 to the level of commercial kits, even better. With respect to sensitivity, kit 2 was more sensitive than kit 1 and 3, since least number of positive animals in faecal culture were missed (false negative) by kit 2 followed by kit 1 and 3. Similar effect of adsorption has been reported earlier<sup>12,24,25</sup>. Kit 1 is therefore recommended for screening of farm goats and sheep in India as 'single test regime'. Kit 1 showed comparable specificity and sensitivity with commercial ELISA kits.

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