‘Indigenous’ and ‘Ethanol Vortex’ ELISA kits for diagnosis of Mycobacterium avium subsp. paratuberculosis infection in cattle: 
Is there a ‘globally relevant kit’ in the ‘Reverse Ice-burg’ environment?

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Highly versatile and robust 'Indigenous ELISA kit' for the diagnosis of Mycobacterium avium subsp. paratuberculosis infection in cattle herds was compared with 'Ethanol Vortex (EV)' ELISA kit of USA. Of 160 (118 vaccinated and 42 non-vaccinated) cattle screened, 129 and 35 were positive in 'Indigenous' and 'Ethanol Vortex' ELISA kits, respectively. 'I-ELISA', using 'semi-purified protoplasmic antigen' from native highly prevalent biotype ('Indian Bison type') of MAP of goat origin, was highly sensitive (91.4%) as compared to the 'EV-ELISA. 'I-ELISA kit using whole cell sonicate from native 'S 5' ('Indian Bison type') strain of MAP as 'antigen source' was significantly superior than EV-ELISA kit using surface antigens from 'Linda' strain ('cattle type') of cattle origin in USA. Therefore, 'I-ELISA kit' may be recommended for the screening of domestic cattle herds against MAP infection in India. The present study has demonstrated that in the diagnosis of chronic infections and diseases, such as Johne's disease, 'Indigenous kits' are significantly superior to kits made in other countries, EV-ELISA, in the present case, particularly screening of native cattle herds endemically infected with MAP.

Keywords: Cattle, i-ELISA kit, EV-ELISA kit, Indian Bison type strain, Johne's disease, 'Linda' strain, MAP infection

Mycobacterium avium subsp. paratuberculosis (MAP), the cause of Johne’s disease (JD) is endemic in the cattle herds of the country. MAP has recently been associated with Crohn’s disease (CD) in human beings. JD is a debilitating, incurable and terminal disease characterized by chronic enteritis, diarrhea, weakness, loss in body weights, reduction in productivity (milk and meat) and fertility, emaciation and eventual death of infected animals. In view of the insidious nature of the disease, laboratory confirmation of sub-clinically infected animals and those in latent stages of infection is always challenging. In case of JD, an ideal diagnostic kit, which is sensitive, specific and globally relevant has not been developed so far. This is primarily due to the spectral nature of the disease as well as immune response in the infected animals. Diagnostic tests are either based on detection of bacilli (direct) or immune response (indirect). Direct methods (culture, microscopy, PCR) heavily depend on shedding of bacilli in faecal samples, which is highly variable with respect to number of bacilli and time interval. Microscopy is not considered sensitive and specific test. Culture, besides time consuming, needs mycobactin J for the growth of MAP. PCR based tests being complex and costly cannot be used for routine screening of animals in herds and flocks. Serological tests like ELISA are good for the large scale screening of herds and flocks. 'Indigenous ELISA' (i-ELISA) kit developed by our group has reasonably good sensitivity and specificity for diagnosis of MAP infection. Milk being convenient sample to collect from large number of lactating cow herds in quick time, therefore has potential to be used as 'mass screening' test for dairy herds. 'Indigenous ELISA' (i-ELISA) kit has also reported both serum and milk based 'i-ELISA kit' as good screening tests for the diagnosis of JD in lactating dairy herds.

Initially, MAP bacilli could not be cultured by our team. Therefore, we first focussed on developing 'i-ELISA test kit' in 1989 (personal communication), using 'wild type antigen' by harvesting MAP bacilli from the intestines of a goat suffering from highly advance form of disease. The test kit was later improved using 'native isolate' of MAP recovered from a goat as 'antigen source' and was compared with antigens driven from ‘TEPS strain’ of UK, obtained from Biological Products Division, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh.

In an effort to develop sensitive and specific ‘i-ELISA kit’ at ICAR-CIRG, we replaced 'wild type' antigens' harvested from MAP recovered from the
intestines of goats naturally infected with novel native strain (‘S 5’) of MAP from a goat, as the ‘antigen source’. This goat strain (‘S 5’) has since been characterized as ‘Indian Bison type’, a new and novel bio-type, reported first time was the dominant bio-type colonizing domestic livestock population and wild ruminants, and was also recovered from raw milk supplies, pasteurized milk and milk products and also from human population. This goat based ‘i-ELISA kit’ developed initially for the screening of goat herds, has since been standardized and validated successfully for use in cattle, buffaloes, sheep and human beings. ‘i-ELISA kit’ has been under continuous validation and upgradation, ever since it was developed first time.

In this study, ‘i-ELISA kit’ using semi purified protoplasmic antigen (sPPA) from the native strain (‘S 5’), a new bio-type (‘Indian Bison type’) of MAP, was compared with highly sensitive (100%) and specific (97.4%) Ethanol Vortex (EV) ELISA kit using purified surface antigens dislodged from ‘Linda strain’ of MAP of USA, as part of our efforts to find a globally relevant ‘ELISA kit’ for the diagnosis of Johne’s disease and screening of native cattle herds of country endemically infected with MAP.

Materials and Methods

Samples

Serum samples were collected before (zero day) and after JD vaccination (variable time interval) from Holstein Friesian, Hariana, Sahiwal and non-descript breeds of cattle located at different farm herds in the northern parts of the country during the field trials of the ‘Indigenous vaccine’ using same ‘S 5’ strain against Johne’s disease in cattle herds. Serum samples were collected from 160 (118 vaccinated and 42 non-vaccinated) cows. Serum samples were stored at −20°C until screening.

Indigenous ELISA kit (i-ELISA):

Kit was initially developed for goats. has been standardized for the screening of dairy cattle. Whole cell sonicate (semi-purified protoplasmic antigen-sPPA) was prepared from the novel native strain (‘S 5’) of MAP bio-typed as ‘Indian Bison type’ recovered from a terminal case of JD in a goat. The antigen (sPPA) was standardized at 0.1 microgram per well. Serum samples were used in the 1:50 dilution and anti-cattle horseradish peroxidase conjugate (Sigma Aldrich, USA) in 1:3000 dilution.

Serum samples from culture positive and negative cattle were used as positive and negative controls, respectively. Optical densities (OD) were expressed as sample-to-positive (S/P) ratios as per Collins. Sensitivity and specificity of the ‘i-ELISA kit was 83.3 and 90.0%.

Analysis of OD (absorbance) values:

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S/P\text{ ratio value } = \frac{OD\ at\ 450\ nm\ of\ test\ serum - OD\ at\ 450\ nm\ of\ negative\ control}{OD\ at\ 450\ nm\ of\ positive\ control - OD\ at\ 450\ nm\ of\ negative\ control}
\]

As per the S/P ratios corresponding status of Johne’s disease in cattle was determined:

Condition A: Cattle in positive and strong positive categories of S/P ratio were considered as ‘Positives’ for Johne’s disease.

Condition B: Cattle in low positive, positive and strong positive categories of S/P ratio were considered as ‘Positive’ for JD or MAP infection.

Ethanol Vortex (EV) ELISA kit, USA

The EV ELISA kit was supplied by Dr. Shigetoshi Eda, NIMBioS, USA, and brief protocol of the kit is as under.

Preparation of antigens

MAP culture [Linda strain (‘Cattle type’)] harvested from liquid medium at stationary phase was centrifuged at 2600 × g for 10 min. Pellet was resuspended in 80.0% ethanol, by agitating in vortex at room temperature for 2 min, and centrifuged at 10621 × g for 10 min. The 50 µL of supernatant was inoculated into each well of a 96-well micro-titre plates. Plates were incubated overnight with the covers removed in a fume hood at the room temperature to allow coating of the antigens (sPPA) to the surface of wells.

Each well of a micro-titre plate coated with antigens was incubated with 200 µL of buffer B at the room temperature for 1 h, washed twice with 250 µL of PBST (10 mM phosphate-buffered saline, pH 7.0, containing 0.5% Tween 80), and inoculated with 50 µL of MAP-positive serum, MAP-negative, was
serum prepared by diluting each serum sample 100 times with buffer B and incubating it at the room temperature for 1 h. After wells were washed four times with 250 µL of PBST, each well was inoculated with 50 µL of horseradish peroxidase (HRP)-labeled rabbit anti-bovine immunoglobulin G (IgG) polyclonal antibody (1:500 dilution) and incubated at the room temperature for 1 h. Wells were washed five times with 250 µL of PBST, ABTS tablets (2, 2-azinobis [3-ethylbenzthiazoline-6-sulfonic acid] and di-ammonium salt) were used to develop colour reactions as per manufacturer’s instructions, reaction was stopped by 1% SDS solution and optical densities were taken using a microplate reader at 415 nm.

Samples from culture positive and negative cattle were used as positive (PC) and negative controls (NC), respectively. These serum samples were used to determine SOD/POD values, which were calculated by using following formula: [(SOD-NOD)/(POD-NOD)] X 10, where SOD is the optical density of a sample, NOD is the optical density obtained using the NC serum, and POD is the optical density obtained using the PC serum. Calculated values of S/P ratio and corresponding status of JD in animals was determined as under (Table 2). Animals with S/P ratio of more than 0.24 were considered as positive for MAP infection and sensitivity and specificity of EV-ELISA was 100 and 97.4%.

Statistical analysis

McNemar’s test was applied to measure the significant difference between results of both tests.

Results and Discussion

Of the 160 serum samples from Holstein Friesian, Hariana, Sahiwal and non-descript cattle screened for MAP infection, 35 (21.8%) and 129 (80.6%) were positive by EV-ELISA and i-ELISA kit, respectively (Table 3). Of the 129 serum samples positive in i-ELISA, 19 (11.8%) and 110 (68.7%) were in strong positive (super shedders) and positive categories, respectively (Table 4). Two tests in combination detected 132 (82.5%) animals positive (Table 5). Of the 35 (21.8%) EV-ELISA positive cows, 2 (1.2%), 0 (0.0%), 1 (0.6%), 25 (15.6%) and 7 (4.3%) were in negative, suspected, low positive, positive and strong positive categories, respectively (Table 5).

Comparison of EV-ELISA kit, with respect to our ‘i-ELISA kit’, where for all practical purposes and routinely we consider serum samples (animals) in positives and strong positives range in ‘i-ELISA kit’, as positives (Condition A). Of the 35 (21.8%) EV-ELISA positive cows, 32 (20.0%) and 3 (1.8%) were positive (strong positives 4.3% and positives 15.6%) and negatives (negative 1.2% and low positives 0.6%) in i-ELISA and were regarded as true positives (TP 20.0%) and false positives (FP 1.8%), respectively. Similarly, of the 125 (78.1%) cows negative in EV-ELISA, 97 (60.0%) and 28 (17.5%) were positives (positives 53.1% and strong positives 7.5%) and negatives (Negatives 7.5%, suspected 3.1%, low positives 6.8%), respectively, in i-ELISA and were considered false negative (FN) and as true negatives (TN), respectively. Therefore, by screening of 160 cows using two tests and two different MAP antigens, 129 (80.6%) and 31 (19.3%) cows were positives and negatives.
As per EV-ELISA kit instructions, if S/P ratio was >0.24 then serum sample/animal was considered positive for MAP infection. Contrary to above, condition A (where 0.25 to 0.39 were in low positive category and considered negative), if we consider Eda’s cut off to differentiate between positive and negative results (condition B), then LP category in S/P ratios has to be considered positive in ‘i-ELISA kit’). Of 35 (21.8%) cows positive in EV-ELISA, 33 (20.6%) and 2 (1.2%) were positive and negative in i-ELISA and were considered true positives (TP) and false positives (FP), respectively (Table 5). Whereas, of 125 cows negative in EV ELISA, 17 (10.6%) and 108 (67.5%), were negative and positive in i-ELISA, were considered true negative (TN) and false negative (FN), respectively. Therefore, by screening 160 cows using two tests (EV-ELISA and i-ELISA) and two different sets of MAP antigens, 19 (11.8%) and 141 (88.1%) cows were negatives and positive, respectively (Table 5).

Considering ‘EV-ELISA kit’ as standard, sensitivity and specificity of i-ELISA was 91.4 and 15.4% and 94.2 and 13.6% in condition A and condition B, respectively (Table 6). Considering ‘i-ELISA kit’ as standard, sensitivity and specificity of EV-ELISA was 17.6 and 90.3% and 23.4 and 89.4% in condition A and condition B, respectively (Table 6). The P value for McNemar Chi-square test showed that proportions of positive results for serum samples using two different ELISA tests (i-ELISA and EV-ELISA) were significantly different ($P<0.0001$) and level of strength of agreement between results of i-ELISA and EV-ELISA was consider to be poor (Kappa = 0.070) and the 95% confidence interval was from 0.006 to 0.134 (Table 7).

JD is endemic in domestic livestock worldwide and also in India. Tiwari et al. reported 12.7% of 61 cows (Canadian dairy herd) sero-positive for MAP, mean loss was $2992$ (95% C.I., $143 to $9741) annually, or $49 per cow per year. In India, Rawat et al. reported loss of Rs. $54442.5$ (US$ 837.5) per cow per lactation in dairy farm consisting of high yielding Holstein Friesian cows. Losses in milk production were 74% of the total production losses. In USA and other developed countries, disease exhibits typical ‘ice-burg’ phenomenon, wherein clinical cases are at tip. Wherein for one animal in the advanced stage, it is assumed that there are 1-2 in the clinical stage, 4-8 in the sub-clinical stage, and 10-14 fourteen in the silent stage. These ratios are arbitrary based on little evidence and disease is progressive in nature following four distinct stages: silent, sub-clinical, clinical and advanced clinical stage (anergy). In the absence of control measures JD is not only endemic (37.3% in last 28 years) in bovine population of country but has shown sharp rise (68.4%) in recent times. In India and other developing countries, there is ‘Reverse ice-burg’

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### Table 5 — Status of EV-ELISA negative and positive samples of cattle in i-ELISA kit

| Tests          | EV ELISA | I-ELISA | Positive – 35 (21.8) | N | S | LP | P | SP | N | S | LP | P | SP |
|----------------|----------|---------|----------------------|---|---|----|---|----|---|---|----|---|----|---|
|                |          |         |                      | 12| 5 | 11 | 85| 12 | 2 | 0 | 1 | 25| 7 |
| Condition A    |          |         |                      | (7.5)| (3.1)| (6.8) | (53.1) | (7.5) | (1.2) | (0.0) | (15.6) | (4.5) |
| Condition B    |          |         |                      | 17| 108| 2 | 2 | 18 | 2 | 3 | 108| 3 |
|                |          |         |                      | (10.6)| (67.5) | (1.2) | (0.0) | (2.0) | (97) |

| Tests          |          |         |                      | 17| 108| 2 | 2 | 18 | 2 | 3 | 108| 3 |
| Condition A    |          |         |                      | (10.6)| (67.5) | (1.2) | (0.0) | (2.0) | (97) |
| Condition B    |          |         |                      | 33| 108| 2 | 2 | 18 | 2 | 3 | 108| 3 |
|                |          |         |                      | (1.2) | (67.5) | (13.6) | (0.0) | (1.2) | (9.4) | (13.6) |

[Total samples (n)=160, TN - True negative, FN - False negative, FP - False Positive, TP - True Positive, N- Negative, S- Suspected, LP- Low Positive, P- Positive, SP- Strong Positive. Figures in parenthesis are percent. Evaluation of EV-ELISA with respect to ‘Indigenous ELISA kit’. Condition A: Positive and strong positives taken as positive for MAP infection: Negatives – 31 (19.3%). Positives – 129 (80.6%). 160 - 32 (20.0)- TP and 28 (17.5) – TN, 160 – 97 (60.0)- FN and 3 (1.8) – FP. Condition B: Low positives, positive and strong positives taken as positive for MAP infection: Negatives – 19 (11.8%). Positives 141 (88.1%). 160 - 33 (20.6)- TP and 17 (10.6) - TN, 160 - 108 (67.5)- FN and 2 (1.2)- FP]
phenomenon. Wherein majority of animals are in clinical to advance clinical stage at the top and is biggest slot, followed by sub-clinically infected animals. Animals, which can be considered negative for JD or clean for MAP infection are just ‘Tip’ of this ‘Reverse Ice-burg’. Since reporting of losses in dairy industry in USA, the estimated reported prevalence of JD infected dairy herds in US has tripled. It is likely that in India also the economic burden of JD has increased over the years. Singh et al. (2014) reported significantly high bio-load of MAP in domestic livestock of country in general and in cattle population in particular, using four diagnostic tests. This high bio-load of MAP in animals is being regularly passed to the human population through consumption of milk and milk products made from pasteurized milk, wherein MAP bacilli does not get killed.

Global efforts for ‘Internationally proven diagnostic kit’ for the diagnosis of JD; has been subject of intense research. Researchers attempt to improve performance of sero-diagnostic tests by identifying useful species specific proteins and non-protein molecules (e.g., Cord factor and lipo-arabinomannan) as diagnostic antigens. Eda and co-workers first time developed EV-ELISA a near perfect test for the screening of animals in US (cattle herds, red deer, goats and sheep) using serum and milk for diagnosis of tuberculosis and paratuberculosis. Therefore, in the present study, we compared EV-ELISA with respect to i-ELISA kit for the diagnosis of JD in Indian dairy cattle.

Performance of EV-ELISA with respect to i-ELISA as standard test, exhibited sensitivity of 24.8 and 23.4% and specificity of 90.3 and 89.4%, in condition A and condition B, respectively considered positive in i-ELISA. But specificity of ‘i-ELISA was significantly poor (22.4 and 13.6% in condition A and B) with respect to EV-ELISA as standard. However, sensitivity was 91.4 and 94.2% in condition A and B, respectively (Table 6). These findings (low sensitivity) of EV-ELISA are contrary to the findings of Eda et al. in US dairy cattle and may be due to difference in the ‘Bio-type profile of MAP’ bacilli (Indian Bison Type) infecting cattle population in India.

In this study, of 160 serum samples screened, 129 were positive by i-ELISA whereas, only 35 serums were positive by EV-ELISA. Which showed that epitopes of MAP strain ‘S 5’ ‘Indian Bison type’ and ‘Linda strain of Cattle type’ are distinct that is why i-ELISA kit was superior to the EV-ELISA kit for the screening of Indian dairy cattle population against JD.

EV-ELISA, which contains only surface antigens of MAP as compared to i-ELISA, which uses ‘antigen mix’ consisting of both surface and PPA. The antigen (sPPA) had both protein and non-protein antigens, in the ‘whole cell lysate’ and were capable of detecting all the stages of JD; early (sub-clinical), middle (clinical) and late (advance clinical). Present study established that high species specificity as reported by Eda et al. cannot be a ‘global phenomenon’ but is purely a ‘local phenomenon’ in chronic infections like JD. This was due to the differences in molecular epidemiology with respect to distribution of ‘MAP bio-types’. Therefore, it is better that each country develop their own ‘i-ELISA kit’ using locally prevalent ‘MAP bio-type’ (local strain) as antigen source. Another important variable in the two studies was the significant differences in bio-load of MAP or endemicity of JD in the cattle population screened by two tests. JD is endemic in the country and rate of infection is also high, which influenced the performances of two tests developed in two separate conditions. Similarly, Eda and co-workers reported poor sensitivity of Biocur-ELISA (a commercial ELISA kit) as compared to EV-ELISA, which detected 13.7, 25.0 and 96.2% cattle in low, middle and high level sheds. The ‘i-ELISA kit has been extensively evaluated for sensitivity and specificity in different models (serum ELISA to fecal culture, PCR and microscopy; milk ELISA to milk culture, PCR and microscopy; serum ELISA to tissue culture, PCR and microscopy in different species of livestock (goats, cattle, sheep and buffaloes)’. ‘Indian Bison type’ is the predominant biotype infecting domestic livestock and wild ruminants and other animals in India, hence ‘i-ELISA’ kit performed exceedingly well in screening of bovine, caprine, ovine, buffaloes population in Indian conditions. For estimating sensitivity and specificity of i-ELISA in serum samples was best when compared with fecal culture.

Recent developments of diagnostic have led to the propagation of new diagnostic tests that grab assurance for the improvement in control and

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Table 7 — Statistical analysis of EV ELISA and i-ELISA by Mc Nemar test

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management of MAP infection. MAP has emerged as a major threat for both human and animals not only in US\textsuperscript{35} but also in India\textsuperscript{3}. EV-ELISA was developed using ethanol extract of Linda strain of 'cattle type' of MAP for JD\textsuperscript{36,37} and was found significantly better than Biocor-ELISA, in screening of low and middle level MAP shedders\textsuperscript{37}. Many workers recommended the use of species specific antigens in ELISA for better results\textsuperscript{36}. Wadhwa \textit{et al.}\textsuperscript{30} recommended the use of multiple antigens to improve the accuracy of sero-diagnosis of the mycobacterial diseases. Similar findings were reported when performance of EV-ELISA was evaluated for screening of serum samples collected from herds of domestic and wild animals (red deer and cattle) including other experimentally infected animals with MAP\textsuperscript{36}.

Various studies compared sensitivity and specificity of ELISA tests developed using bovine strains of MAP to screen wild and domestic animals world-wide\textsuperscript{41}. The 'i-ELISA' exhibited enhanced sensitivity and specificity when compared with commercially available ELISA kits\textsuperscript{17}. The i-ELISA was sensitive, cost effective and reliable for screening of domestic animals against JD\textsuperscript{9}. Results of i-ELISA were correlated with culture and were optimally good both as screening and as confirmatory tests, for estimating JD\textsuperscript{17}. Similar other comparative study was carried out, where i-ELISA kit showed superior diagnostic potential had higher specificity as compared to commercial ELISA kit\textsuperscript{42}. Other studies also reported that i-ELISA kit improved detection rate of MAP in test samples\textsuperscript{2}. Studies reported higher correlation between fecal culture and ELISA tests, where animals with high ELISA readings (thus high level of antibodies), then sensitivity of fecal culture would be in range of 60 - 70\%\textsuperscript{43}. Similarly, in Indian conditions where JD was highly endemic in domestic livestock population, while using i-ELISA as diagnostic test, we have recommended S/P ratios of positive and strong positives (Condition A) as positive for MAP infection, which correlated best with fecal culture in our studies\textsuperscript{23,24}. However, in some of the animals positive in culture were missed (negative and suspected in S/P ratio) or not taken as positive being in 'Low positive' category in S/P ratio in i-ELISA\textsuperscript{26}. The i-ELISA test was effectively used as 'mass screening' test in animals wherein considerable increase in the detection rate of bio-load of MAP \{28.9\% (2001–2010) to 47.0\% (2011–2013)\}\textsuperscript{2} was seen. Since i-ELISA was developed using native source of MAP it was found to be superior than AGPT in the diagnosis of MAP infection in cattle and other commercially available ELISA kits\textsuperscript{17,42}. Findings of present study also confirm that the i-ELISA was highly sensitive in detecting early, middle and late stages of infections of MAP.

Similarly, Yadav \textit{et al.}\textsuperscript{24} used i-ELISA for the screening of slaughtered buffaloes, none and 46.7\% buffaloes were positive using purified protoplasmic antigen (PPA) of bovine origin (Allied Monitor Inc., USA) and sPPA of native strain ('S 5') bio-typed as 'Indian Bison type' of MAP, respectively. This study showed change in species from cattle to buffaloes exhibited very low sensitivity. Kumar \textit{et al.}\textsuperscript{22} indicated greater sensitivity of species specific antigens. Chaubey \textit{et al.}\textsuperscript{44} have reported i-ELISA (Indigenous g-ELISA) with respect to commercial antigen based b-ELISA and commercial sr-ELISA had 100\% sensitivity (both) and 44.4 and 11.4\% specificity, respectively. Pahangchopi \textit{et al.}\textsuperscript{45} using antigens from native MAP strain ('S 5'), proved superiority over AGPT using commercial antigen (ATCC 19698, UK used for making Johnin) in India. All these studies showed major problem of lowered sensitivity or cross reactivity of antigens used in commercial ELISA kits, was mainly due to difference in bio-type used as antigen source. This small change in antigen source and purification of antigens led to major changes in sensitivity and specificity of ELISA kits.

This study clearly established that in case of chronic infection like Johne's disease there cannot be standard 'International or Globally relevant kits'. Therefore, ELISA based diagnostic kits should use antigens from locally prevalent strains of MAP, which is critical for the sensitivity of the test. This is why 'i-ELISA' kit using sPPA from native MAP strain ('S 5') biotype 'Indian Bison Type' was significantly superior in the screening of MAP infection in cattle herds of India as compared to EV-ELISA kit of USA. 'i-ELISA' was a good diagnostic test to screen domestic livestock population against MAP infection in Indian conditions.

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