EVALUATION OF “INDIRECT FLUORESCENT ANTIBODY TEST” AS POTENTIAL SCREENING TEST FOR *Mycobacterium avium* SUBSPECIES *paratuberculosis* USING MILK OF LACTATING DOMESTIC LIVESTOCK

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ABSTRACT

In the present study, Indirect Fluorescent antibody test (iFAT) has been used as the “screening test” for the detection of MAP bacilli in the milk samples of lactating domestic livestock. A total of 372 milk samples from lactating animals were screened by iFAT and results were compared with microscopy of milk samples and milk ELISA test. Comparative analysis of the results of three tests showed that iFAT had fairly good sensitivity (84.7%) and specificity (90.4%), with respect to ZN staining (microscopy) with kappa value of 0.735 and ‘good’ strength of agreement. Similar comparison with milk ELISA test revealed, sensitivity (73.3%) and specificity of (72.6%) with kappa value of 0.443 and strength of agreement was moderate. Lower or higher difference in sensitivity and specificity of iFAT with respect to ZN staining and milk ELISA may be due to the difference in detection ‘target’ of the test i.e., antigen or antibody. It was concluded that iFAT was a reliable and sensitive diagnostic test for the detection of MAP in milk and can also be used for the ‘mass screening’ of the milk samples.

KEYWORDS

*Mycobacterium avium* subsp. *paratuberculosis*

Indirect Fluorescent antibody test

ZN staining

Milk
1 Introduction

*Mycobacterium avium* subspecies *paratuberculosis* (MAP), cause the incurable Paratuberculosis or Johne’s disease, it is an infectious disease of the intestinal tract, primarily infects domestic livestock (Singh et al., 2011; Singh et al., 2014a). It is globally known that milk and milk products made from pasteurized milk of endemically infected domestic livestock contain live MAP bacilli. Therefore, consumption of contaminated pasteurized milk and milk products by human population is no longer safe. MAP is close to be considered as ‘zoonotic pathogen’ and has high public significance due to large scale presence in the milk of domestic livestock (Singh et al. 2014b). Ability of MAP to survive at pasteurization temperature has made milk the primary source of human infection, especially infants, who are increasingly dependent on milk powders (Singh & Vihan, 2004; He & Buck, 2010; Galiyo et al., 2015). Crohn’s Disease (CD), a systemic disorder in humans where principal clinico-pathological manifestation is the chronic inflammation of the intestines. Increasing evidences suggest that Crohn's disease has an infectious etiology and MAP being the most plausible candidate since it has been frequently reported from commercial milk and milk products globally (Grant et al., 2002; Ikonomopoulos et al., 2005; Ellingson et al., 2005; Ayele et al., 2005; Paolicchi et al., 2012) and has also been isolated from patients suffering from CD (Singh et al., 2008; Naser et al., 2014).

Vaccination is the best available therapy for the treatment and recovery of animals suffering from Johne’s disease or MAP infection (Singh et al., 2010; Bannantine & Talat, 2015). It is important that disease must be identified at an early stage so that infected animals can be timely vaccinated for treatment of disease in order to prevent the spread of infection (Chaubey et al., 2016). Critical issues in the diagnosis of MAP infection are; endemicity of Johne’s disease, poor specificity of cellular tests, lowered sensitivity of serological tests, infrequent shedding of MAP, fastidious nature of bacilli and high cost of imported kits (Singh et al., 2013). Johnin based allergic field test, lacks sensitivity and specificity and is also well known for false positive reactions (Kalisi et al., 2003; Singh et al., 2014a). To address these issues, there is need for continued efforts to develop new diagnostic assays with high sensitivity and specificity. Tests that can be used both in the field and laboratory with minimum facilities and cost will have more acceptability as compared to only ‘laboratory based’ or imported costly kits. Ideal test assays are those that can be formatted for use in multiple species. In our previous studies, we evaluated “dot-ELISA”, as an alternative test to be used in the field as “herd screening test” for the detection of MAP in the milk of lactating animals. Results of ‘dot-ELISA’ with respect to ‘indigenous plate-ELISA’ revealed good specificity and sensitivity (unpublished data).

In the present study, ‘indirect Fluorescent Antibody Test’ (iFAT), an antigen based detection test was evaluated to determine the sensitivity and specificity of the test for the detection of MAP in the milk of lactating domestic livestock. Results of iFAT were also compared with other two screening tests namely microscopy (antigen detection) and ‘indigenous ELISA kit’ (antibody detection).

2 Materials and Methods

2.1 Sampling

Udder of each lactating animal was washed and cleaned before milk sample (15 ml) was collected and stored at -20°C, till further processing. A total of 372 animals (142 goats and134 bovines (Cattle and bovine) were screened between February and October 2015. Milk samples were collected from lactating livestock belonging to individual households / dairy farms in villages around Central Institute of Research on Goats (CIRG) campus. Milk samples were screened for the presence of MAP by iFAT, microscopy using ZN staining and indigenous ELISA kit.

2.2 Ziehl–Neelsen (ZN) staining

Smears were prepared from the 20 µl of whole milk, stained by Ziehl Neelsen (ZN) stain as per Singh et al. (2008) and examined under oil under immersion (×100) for pink coloured acid-fast bacilli (AFB) indistinguishable to MAP (Singh et al., 2008).

2.3 Indirect Fluorescent antibody test (iFAT)

The Fluorescent antibody test was adopted for use in milk from the protocol developed by D’Haese et al. (2005) and Paliwal et al. (1983) with some modifications. Clean slides were taken and smears were prepared from 20µl of whole milk per slide and smear was allowed to air dry at room temperature followed by gentle heat fixing. Slides were then dipped in a solution of 30.0% H2O2 in 90.0% methanol (3:7 ratio) and then incubated for 10 minutes at 37°C, followed by dipping of slides in phosphate citrate buffer (2.1% citric acid and 3.56% disodium hydrogen phosphate in 100 ml triple distilled water, pH- 5) and were placed in microwave for heating for 30 seconds (15 cycles) with rest for 20 seconds after each cycle (total time 10 minutes). Slides were taken out from solution and air dried at room temperature. Then primary antibody (whey) in ratio of 1:4 in serum dilution buffer (1% BSA in PBST) was added on the slides. The slides were incubated for 1 hour at 37°C in BOD incubator, followed by washing of slides in 1X PBS (3 times). Anti species secondary antibody (FITC conjugate) was added in the ratio 1:750 in 1X PBS (pH-7.6). Slides were then incubated in dark for 1 hour at 37°C followed by washing of slides 5 times in 1X PBS. Slides were then air dried at room temperature. Finally slides were mounted with glycerine and covered with cover slip and then observed immediately under fluorescent microscope. Slides positive for MAP infection exhibited green fluorescence.
2.4 Indigenous milk ELISA test

The test was performed as per the method of Singh et al. (2007). Briefly, Milk based indigenous ‘p-ELISA’ kit was used as standard test for screening of animals for MAP infection and parallel test was used as for the 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 one hour at 37°C. Plates were washed thrice with PBST and then test samples (100µl of whole milk in 1:2 dilution with 1% BSA in 1X PBST) and incubated for two hrs 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 one hour at 37°C. Finally, after five 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 (i Mark micro-plate reader, Biorad). Whey samples from weak culture positive and healthy 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 (2002). OD (absorbance) values were analysed as per following equation:

\[
S/P \text{ ratio value} = \left[\frac{(\text{Sample OD} - \text{Negative OD})}{(\text{Positive OD} - \text{Negative OD})}\right]
\]

Values of sample to positive ratios and corresponding status of Johne’s disease in animals was determined as per Sing et al. (2016) The samples in low positive (LP), positive (P) and strong positive (SP) categories of S/P ratio were considered as positive for MAP infection in p-ELISA (Singh et al. 2016).

3 Results

Of 372 milk samples (207 goats and 165 bovines) screened by microscopy, 184 (49.4%) animals {122 (58.9%) goats and 62 (37.5%) bovines} were positive for MAP infection (Table 1). However, in iFAT, 174 (46.7%) {126 (60.8%) goats and 48 (29.0%) bovines} were reported positive by iFAT for MAP infection (Table 1). Comparison of iFAT with microscopy showed significant correlation between the two tests and there was perfect agreement in 87.6 % and a mismatch of 12.3%. True positives (positives in both the tests) and true negatives (negative in both the tests) were 41.9% and 45.6% respectively (Table 2). Similar comparison with milk ELISA test revealed, sensitivity (73.3%) and specificity (72.6%) with kappa value of 0.443 and strength of agreement was moderate (Table 3 and 4).

Statistical comparisons showed that the two-tailed P value equals 0.1845. By conventional criteria, this difference is considered to be not statistically significant and the strength of agreement was ‘good’ for microscopy with respect to iFAT with a kappa value of 0.753 (Table 4).

| Animal species | Milk samples, n | Positives, n (%) | Positives, n (%) | Microscopy | Indirect FAT | Indigenous plate ELISA |
|----------------|----------------|-----------------|-----------------|------------|--------------|-----------------------|
| Goats          | 207            | 122 (58.9)      | 126 (60.8)      | 102 (61.4) |              |                       |
| Bovine         | 165            | 062 (37.5)      | 048 (29.0)      | 64 (38.5)  |              |                       |
| Total          | 372            | 184 (49.4)      | 174 (46.7)      | 166 (44.6) |              |                       |

*Figures in parenthesis are percent, Total samples (n) =372, N: Negative, P: Positive, TP: True positive, TN: True negative, FN: False negative, FP: False positive,
Table 3 Comparison of iFAT vis a vis indigenous p-ELISA for the screening of milk samples (n= 372) against *M. avium* sub-species *paratuberculosis* infection.

| Tests | Indigenous p-ELISA status n (%) | Total n (%) |
|-------|--------------------------------|------------|
|       | Negative (N) | Suspected (S) | Low Positive (LP) | Positive (P) | Strong Positive (SP) |       |
|       | 202 (54.5) | 32 (0.8) | 43 (11.6) | 94 (25.4) | 1 (0.2) | 372 (100.0) |
| Reference N and P | | | | | | |
|       | N - 232 (62.7) | P - 138 (37.2) | | | | |
| iFAT Status n (%) | | | | | | |
| Negative | 153 (41.1) | 17 (4.5) | 20 (5.3) | 16 (4.3) | 0 (0.0) | 206 (55.3) |
| Total | 170 (45.6) N- or TN | 37 (9.9) N- or FN | | | | |
| Positive | 49 (13.1) | 15 (4.0) | 23 (6.1) | 78 (20.9) | 1 (0.2) | 166 (44.6) |
| Total | 64 (17.2) P- FP | 102 (27.4) P or TP | | | | |

Table 4 Comparison of iFAT with microscopy and indigenous p-ELISA for the detection of *M. avium* sub-species *paratuberculosis* by Mc-Nemar test and Kappa agreement.

| Tests | iFAT | Comparative Test | indigenous p-ELISA |
|-------|------|------------------|--------------------|
| Sensitivity | 84.7% | 73.3% |
| Specificity | 90.4% | 72.6% |
| Disease Prevalence | 49.4% | 37.2% |
| p-value | Not significantly different | very statistically significant |
| Strength of agreement | Good | Moderate |
| Kappa | 0.753 | 0.443 |

Figure 1 Milk indirect fluorescent antibody stained milk smear showing the presence of *Mycobacterium avium* subspecies *paratuberculosis* bacilli in milk samples.
4 Discussions

Ability of MAP to survive at pasteurization temperature is by making clumps on heating, therefore within the clumps bacilli remains live and heating has no effects. Zoonotic potential of MAP has been generated renewed concerns, since bacilli has been frequently reported from the foods of animal origin, specially milk and milk products made from pasteurized milk (Singh et al., 2007). Human exposure to MAP by consumption of pasteurized milk and milk products has been regarded as a major risk factor for the development of number of chronic, incurable and auto-immune type disorders (Crohn's disease, type-1 diabetes, Sarcoidosis, Multiple sclerosis and Hashimoto's thyroiditis among others) (Sechi & Dow, 2015). Over the past few years sufficient information has been generated on the culture of live MAP bacilli from milk of clinically and sub-clinically infected cows and other domestic livestock (Ellingson et al., 2005; Ayele et al., 2005). Recent surveys have reported presence of MAP in commercial supplies of pasteurized retail milk globally (Grant et al., 2002; Ellingson et al., 2005; Gill et al., 2011). In order to find the efficient, specific, sensitive and cost effective test for the detection of MAP in milk, in present study effectiveness of iFAT was analyzed, which has potential to be developed as screening test for testing of large number of milk samples.

In clinical laboratories, FAT has been used over the years for the detection of various diseases like Lyme disease, Rocky Mountain spotted fever and Pemphigus vulgaris among others (Judd & Mescon, 1979; Magnarelli et al., 1984; Kaplan & Schonberger, 1986). Indirect FAT test is more commonly used for the antibody detection while direct FAT technique is used for antigen detection through fluorescence (Figure 1). Earlier fewer researchers have evaluated fluorescent antibody test as alternative test for the detection of Johne’s disease (Abbas et al., 1983). The major part of the work on FAT was done in the 1970’s. In 1976, Gilmore did an extensive study on the ability of FAT to be used as an alternative to the standard recognized Complement Fixation Test (Gilmore, 1976). Initially, 5 calves were infected with MAP and evaluation of the serum found that the serological response of FAT was specific while the first diagnosis of the disease was done at 4 months and lasted up to 5 months. In the same year large scale screening of serum samples of cattle and rabbits from different farms were screened and authors concluded that FAT was as sensitive as CFT (Gilmore, 1976; Goudswarda et al., 1976).

Fluorescent Antibody Technique had also been developed and evaluated for the diagnosis of paratuberculosis infection in goats and cattle by demonstrating MAP antibodies (Paliwal et al., 1983). Abbas and co-workers in 1983 showed that, FAT gave similar results to complement fixation test, but had lower specificity and sensitivity for the detection of sub-clinical cases of paratuberculosis (Abbas et al., 1983). The serum samples of the animals from farms with history of Johne’s disease were examined by fluorescent antibody tests (FAT) using macrophage uptake (MU) and defined antigen substrate spheres (DASS) system. A good correlation was found between the results of the DASS/FAT and those of the MUFAT, which is known to be a sensitive and specific test in the diagnosis of Johne’s disease in cattle. Author suggested the possibility of the FAT/DASS system being used in routine diagnostic procedures. To the best of our knowledge, no new researches have been done with respect to fluorescent antibody based detection test with respect to paratuberculosis disease.

Milk has been regarded as the main source of transmission of MAP from infected animals to human beings. In this study ability of iFAT was evaluated to detect MAP bacilli in whole milk samples of lactating animals. In the present study, diagnostic potential of iFAT was also compared with the vis a vis microscopy using ZN staining for the detection of MAP in milk samples. FAT is regarded as a highly sensitive and specific procedure for the detection of antigens in samples; therefore we evaluated its ability in the detection of MAP bacilli in milk samples specifically (Dean et al., 1996; Foral et al., 2007). In the present study, microscopy using ZN staining was regarded as the reference antigen detection test and of the 372 milk samples screened 326 (87.6 %) samples were in agreement with ZN staining and a mismatch of 46 (12.3%) was recorded. The strength of agreement between the two tests was good with high specificity and sensitivity of 84.7% and 90.4% respectively. The results of present study showed that iFAT was as sensitive and specific alternative to available antigen based detection tests for the screening of milk samples of lactating animals endemically infected with MAP. False negative (4.8%) in iFAT with respect to microscopy may be actually positive, since FAT uses better detection system as compared to microscopy, which is colour based and fewer individual bacilli may be missed due to over sight. But 18(4.8%) false positive may be either due to processing error in staining with FAT or during visualization. However, since only 20 µl of milk was taken to make smear, there are chances of sampling error. Therefore, it may be advised to use minimum of 2 or 3 smears during comparison studies. Similarily in cases of mismatch, the two tests can be repeated.

Mis-match between microscopy with iFAT and ELISA test was of 107 samples (28.7%). Of these, 6.7% cases were detected new (which were negative in both ZN staining and iFAT) (Table 5). This may be due to low shedding of MAP by these animals in milk samples or due to onetime sampling. If these animals are sampled repeatedly then some of the animals will definitely come positive in ZN staining and iFAT.

About 21.2% cases positive in both microscopy and iFAT were missed by ELISA. This may be due low infection therefore poor titer against MAP; therefore these samples did not reached above cutoff levels in ELISA. Again it may be possible because of poor health of some animals. Table 2, showed that 11.6% samples were in low positive range in ELISA, (which were considered negative), and of these 6.1% were positive in iFAT.
Similarly of 0.8% and 54.5% samples in suspected and negative range, 4.0 and 13.1% were positive in iFAT, respectively. In chronic infection like Johne’s disease, shedding of bacilli in feces / milk or development of immune response in serum / milk are two independent phenomenon and therefore at particular point of time in spontaneous cases of chronic and insidious infection like Johne’s disease, it is difficult to get optimum detectable level of immune response and optimum shedding levels, which are detectable. These processes are also governed by other factors like state of health and nutrition, physiological state of animal, production status etc., therefore it is always prudent to use both immunological and bacilli detection tests at same time. Of the immunological tests ELISA is best. However sensitivity of ELISA also depends on the type of antigen used. Our indigenous-p-ELISA test developed using antigen mix from our native and local strain of MAP (‘S5’) has proved to be highly sensitive and specific at very low levels (0.1 to 0.5 microgram per well of indigenous-p-ELISA).

Comparison of ‘Indigenous –p-ELISA test kit’ with other commercially available international kits showed that our indigenous-p-ELISA kit was much superior in sensitivity. Similarly for detection of bacilli we can use microscopy (ZN staining), culture, PCR etc. Since culture of MAP takes very long time and PCR depends on number of bacilli per sample for detection of DNA, we preferred ZN staining, due to the ease of performing.

Long time centrifugation of samples help in improving the sensitivity of ZN staining test. Despite standardization of the microscopy test and our competency, we know this test will not gain the acceptability due to scientific reasons. This is why we developed iFAT, which is more technically sound, simple to perform and has acceptability as compared to microscopy. Besides we can select or develop other tests (dot ELISA etc.), and compare with iFAT, since it is simpler than either iFAT or microscopy. However, repeat testing of mis-match samples was more prudent way to resolve or involve few more tests on these samples.

Conclusion

Unlike microscopy which may detects all kinds of acid fast mycobacterium and milk ELISA detects antibody response, iFAT was highly sensitive and specific for the detection of the presence of MAP bacilli in the milk samples of lactating animals. Based on the above findings, we recommend that iFAT may be used in the large scale screening of milk samples.

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Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

References

Abbas B, Riemann H, Behymer DE (1983) Evaluation of the fluorescent antibody test for diagnosis of paratuberculosis. American Journal of Veterinary Research 44:720-721.

Ayele WY, Svastova P, Roubal P, Bartos M, Pavlik I (2005) Mycobacterium avium Subspecies paratuberculosis Cultured from Locally and Commercially Pasteurized Cow's Milk in the Czech Republic. Applied and Environmental Microbiology 71: 1210–1214. doi: 10.1128/AEM.71.3.1210-1214.2005.

Bannantine JP, Talaat AM (2015) Controlling Johne's disease: vaccination is the way forward. Frontiers in Cellular and Infection Microbiology 5: 2. doi: 10.3389/fcimb.2015.00002.

Chaubey KK, Gupta RD, Gupta S, Singh SV, Bhatia AK, Jayaraman S, Kumar N, Goel A, Rathore AS, Shazad, Sohal JS, Stephen BJ, Singh M, Goyal M, Dhamma K, Derakhshanadeh A (2016) Trends and advances in the diagnosis and control of paratuberculosis in domestic livestock. Veterinary Quarterly 36: 1-25. DOI: 10.1080/01652176.2016.
Collins MT (2002) Interpretation of a commercial bovine paratuberculosis enzyme-linked immunosorbent assay by using likelihood ratios. Clinical and Vaccine Immunology 9:1367-1371. doi: 10.1128/CDLI.9.6.1367-1371.2002.

Dean DJ, Ableseth MK, Atanasiu P (1996) The fluorescent antibody test. In: Meslin F-X, Kaplan MM, Koprowsky H (Eds.) Laboratory Techniques in Rabies. 4th edition. Geneva, Switzerland: WHO, Pp. 88-96.

D’Haese E, Dumon I, Werbrouck H, De Jonghe V, Herman L (2005) Improved detection of Mycobacterium paratuberculosis in milk. Journal of Dairy Research 72: 125-128. DOI: http://dx.doi.org/10.1017/S0022029905001226.

Ellingson JL, Anderson JJ, Koziczkowski RP, Radcliff SJ, Sloan SE, Allen SE, Sullivan NM (2005) Detection of viable Mycobacterium avium subsp. paratuberculosis in retail pasteurized whole milk by two culture methods and PCR. Journal of Food Protection 68: 966-972.

Foral TM, French RA, Van Kruiningen HJ, Garmendia AE (2007) Fluorescent antibody test for rapid detection of West Nile virus antigen in avian tissues. Avian Diseases 51: 601-605. DOI: 10.1637/0005-2086(2007)51[601:FAFTHD]2.0.CO;2.

Galiiero A, Fratini F, Turchi B, Colombani G, Nuvoloni R, Cerri D (2015) Detection of Mycobacterium avium subsp. paratuberculosis in a sheep flock in Tuscany. Tropical Animal Health and Production 47: 1567-71. doi: 10.1007/s11250-015-0899-z.

Gill CO, Saucier L, Meadus WJ (2011) Mycobacterium avium subsp. paratuberculosis in dairy products, meat and drinking water. Journal of Food Protection 74: 480–499.

Gilmour NJ (1976) The specificity of the fluorescent antibody test using the sera of rabbits inoculated with strains of mycobacteria. Research in Veterinary Sciences 20: 1

Goudsward J, Gilmour NJ, Dijkstra RG, Van Beek JJ (1976) Diagnosis of Johne's disease in cattle: a comparison of five serological tests under field conditions. Veterinary Record 98: 461-462.

Grant IR, Hitchings EI, McCartney A, Ferguson F, Rowe MT (2002) Effect of commercial-scale high-temperature, short-time pasteurization on the viability of Mycobacterium paratuberculosis in naturally infected cows' milk. Applied and Environmental Microbiology 68:602-607. doi: 10.1128/AEM.68.2.602-607.2002.

He Z, Buck JD (2010) Localization of proteins in the cell wall of Mycobacterium avium subsp. paratuberculosis K10 by proteomic analysis. Proteome Science 8: 21. doi: 10.1186/1477-5956-8-21.

Ikonomopoulos J, Pavlik I, Bartos M, Svastova P, Ayele WY, Roubal P, Lukas J, Cook N, Gazouli M (2005) Detection of Mycobacterium avium subsp. paratuberculosis in Retail Cheeses from Greece and the Czech Republic. Applied and Environmental Microbiology 71: 8934–8936. doi: 10.1128/AEM.71.12.8934-8936.2005.

Judd KP, Mescon H (1979) Comparison of different epithelial substrates useful for indirect immunofluorescence testing of sera from patients with active pemphigus. Journal of Investigative Dermatology 6: 314-316. DOI: http://dx.doi.org/10.1111/1523-1747.ep12531752.

Kalis CHJ, Collins MT, Hesselink JW, Barkema HW (2003) Specificity of two tests for the early diagnosis of bovine paratuberculosis based on cell mediated immunity: the Johnin skin test and the gamma interferon assay. Veterinary Microbiology 97: 73-86. http://dx.doi.org/10.1016/j.vetmic.2003.07.003.

Kaplan JE, Schonberger LB (1986) The sensitivity of various serologic tests in the diagnosis of Rocky Mountain spotted fever. The American Journal of Tropical Medicine and Hygiene 4: 840-844.

Magnarelli LA, Meegan JM, Anderson JF, Chappell WA (1984) Comparison of an indirect fluorescent-antibody test with an enzyme-linked immunosorbent assay for serological studies of Lyme disease. Journal of Clinical Microbiology 20: 181-184.

Naser SA, Sagramsingh SR, Naser AS, Thanigachalam S (2014) Mycobacterium avium subspecies paratuberculosis causes Crohn’s disease in some inflammatory bowel disease patients. World Journal of Gastroenterology 20: 7403–7415. doi: 10.3748/wjg.v20.i23.7403.

Paliwal OP, Rajya BS and Krishna SG (1983) Fluorescent antibody technique (FAT) in the diagnosis of Mycobacterium johnei infection in goats. Indian Journal of Veterinary Pathology 7: 6-14.

Paolicchi F, Cirone K, Morrella C, Gioffré (2012) First isolation of Mycobacterium avium subsp Paratuberculosis from commercial pasteurized milk in Argentina. Brazilian Journal of Microbiology 43 : 1034–1037. doi: 10.1590/S1517-838220120003000028.

Sechi, LA, Dow CT (2015) Mycobacterium avium ss. paratuberculosis Zoonosis - The Hundred Year War - Beyond Crohn’s Disease. Frontiers in Immunology 6:96. doi: 10.3389/fimmu.2015.00096.

Singh AV, Singh SV, Makharia GK, Singh PK, Sohal JS (2008) Presence and characterization of Mycobacterium avium subspecies paratuberculosis from clinical and suspected cases of Crohn's disease and in the healthy human population in India. International Journal of Infectious Diseases 12: 190-197.
Singh SV, Audarya SD, Singh M, Stephen BJ, Chhabra D, Chaubey KK, Gupta S, Sahzad, Pachoori A, Jayaraman S, Aseri GK, Sohal JS, Bhatia AK, Dhama K (2016) Development of New ‘Indigenous Dot-ELISA Kit’ as Sensitive Field Based Herd Screening Test for the Diagnosis of Johne’s Disease in the Domestic Buffalo Population. Asian Journal of Animal and Veterinary Advances 11: 44-52. DOI: 10.3923/ajava.2016.44.52.

Singh SV, Singh AV, Singh PK, Singh B, Ranjendran AS, Swain N (2011) Recovery of Indian Bison Type Genotype of Mycobacterium avium subsp. paratuberculosis from Wild Bison (Bos gourus) in India. Veterinary Research 4: 61-65. DOI: 10.3923/vr.2011.61.65.

Singh SV, Singh AV, Singh R, Sandhu KS, Singh PK, Sohal JS, Gupta VK, Vihan VS. (2007) Evaluation of highly sensitive indigenous milk ELISA kit with fecal culture, milk culture and fecal-PCR for the diagnosis of bovine Johne's disease (BJD) in India. Comparative Immunology, Microbiology and Infectious Diseases 30: 175-86.

Singh SV, Singh PK, Gupta S, Chaubey KK, Singh B, Kumar A, Singh AV, Kumar N. (2013) Comparison of microscopy and blood-PCR for the diagnosis of clinical Johne’s disease in domestic ruminants. Iranian Journal of Veterinary Research 14: 345-349.

Singh SV, Singh PK, Singh AV, Sohal JS, Kumar N, Chaubey KK, Gupta S, Kumar A, Bhatia AK, Srivastav AK Dhama K (2014b) ‘Bio-Load’ and Bio-Type Profiles of Mycobacterium avium subspecies paratuberculosis Infection in the Domestic Livestock Population Endemic for Johne's Disease: A Survey of 28 years (1985–2013) in India. Transboundary and Emerging Diseases 61: 43–55. DOI: 10.1111/tbed.12216.

Singh SV, Singh PK, Singh AV, Sohal JS, Sharma MC (2010) Therapeutic Effects of a New “Indigenous Vaccine” Developed Using Novel Native “Indian Bison Type” Genotype of Mycobacterium avium subspecies paratuberculosis for the Control of Clinical Johne's Disease in Naturally Infected Goatherds in India. Veterinary Medicine International DOI:10.4061/2010/351846.

Singh SV, Sohal JS, Kumar N, Gupta S, Chaubey KK, Rawat KD, Chakraborty S, Tiwari R, Dhama K (2014a) Recent approaches in diagnosis and control of mycobacterial infections with special reference to mycobacterium avium subspecies paratuberculosis. Advances in Animal and Veterinary Sciences 2: 1-12.

Singh SV, Vihan VS (2004) Detection of Mycobacterium avium subspecies paratuberculosis in goat milk. Small Ruminant Research 54: 231–235. http://dx.doi.org/10.1016/j.smallrumres.2003.12.002.