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Molecular and Serological Detection of *Mycobacterium avium* Subspecies *Paratuberculosis* in Ruminants of Jabalpur Region

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ABSTRACT

Background: Paratuberculosis is a chronic infectious disease of wild and domestic ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). The present study aimed to conduct the molecular and serological detection of MAP in ruminants of Jabalpur region.

Methods: A total of 261 faecal, 124 blood and 51 tissue samples were collected from goats, buffaloes and cattle of the region irrespective of their age, sex and breed. ZN staining of faecal and tissue samples was performed as a preliminary screening test followed by anti-MAP antibodies detection in serum samples using indirect ELISA and *IS900* PCR analysis of ZN positive faecal and tissue samples.

Result: A pattern of acid-fast bacilli shedding in faecal samples of ruminants was obtained based on ZN staining with a majority of +1 shedders. In ELISA, 33.87% of serum samples were found positive for anti-MAP antibodies whereas, 22.50% ZN positive faecal samples and 100% ZN positive tissue samples were found positive in *IS900* PCR. Therefore, a conclusion of moderately prevalent MAP infection in ruminants of the Jabalpur region can be drawn.

Key words: Bovines, ELISA, Goats, MAP, Paratuberculosis, PCR.

INTRODUCTION

Paratuberculosis or Johne's disease (JD) is one of the most important chronic infectious diseases of domestic, wild and zoo animals. It is considered to be endemic within the dairy sector worldwide. Paratuberculosis results in heavy economic losses due to its impact on livestock production including milk, meat, fiber and leather, increased mortality as well as the cost of treatment (Haque *et al*., 2022). The disease is caused by *Mycobacterium avium subspecies paratuberculosis* (MAP). It is slow growing, acid-fast intracellular bacilli belonging to the family Mycobacteriaceae. It is quite resistant to heat and cold due to the presence of mycolic acid in cell wall. A number of studies have proven clinical and pathological similarities between Johne's disease and Crohn's disease, a zoonotic infection caused by *Mycobacterium avium subspecies paratuberculosis* in humans (Eslami *et al*., 2019).

Following faeco-oral infection, MAP invades the specialized M cells of follicle associated epithelium (FAE) of intestinal lymphoid tissues. Subsequently, they get engulfed by intestinal macrophages and accumulate in the lamina propria of intestine where they remain for several months to years before replicating. After latency of several months, extensive multiplication of the bacteria results into thickening and corrugation of intestinal mucosa. Hence, affected animals may suffer from malabsorption, diarrhoea, progressive emaciation and loss of body condition (Tripathi, 2008). Apart from the intestine, MAP may also be disseminated to udder, uterus, supramammary lymph nodes, vaginal and other tissues subsequently getting secreted in colostrum, milk, foetus, semen *etc*. (Singh *et al*., 2018).

Effective and early detection of MAP infection is an integral part of disease management in ruminants. Prevalence of paratuberculosis is variable world-wide with animal level prevalence ranging from 02.31-29.80% being reported in Asia (Elmagzoub *et al*., 2020). In India, bio-load of MAP has been reported to be 28.3-48.0% in buffaloes, 06.0-39.3% in cattle and 09.4-20.1% in goats (Gupta *et al*., 2019). Prevalence of antemortem and postmortem sub-clinical paratuberculosis was found to be 01.25% and 04.28%, respectively by using PCR in goats of Mahakoshal region of Madhya Pradesh (Jatav *et al*., 2018). However, upon perusal of available published literature no studies regarding paratuberculosis had been conducted in clinically

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affected large ruminants of Jabalpur region of Madhya Pradesh.

MATERIALS AND METHODS

Study material

In the present study, a total of 162 antemortem (116 faecal and 46 blood) and total 51 ruminant carcasses (21 goats, 20 buffaloes and 10 cattle) were examined irrespective of age, sex and breed were included. The samples were collected from different areas of Jabalpur region in period of seven months (March 2022 to September 2022). The blood samples were aseptically collected from external jugular vein in clot activating vacutainers. The serum was separated and stored at -20°C for ELISA. Faecal samples were collected directly from the rectum and placed in sterile zipped polythene bags which were sealed properly. Representative tissue samples from intestine, mesenteric and ileo-caecal lymph nodes were collected and transported to laboratory under refrigerated condition (4°C). Individual tissue was divided into two parts, for smear preparation and molecular diagnosis (-20°C). All the samples were properly labelled and documented.

Direct microscopy of cytological smears for detection of AFB by Ziehl-Neelsen staining

For preliminary screening, the faecal and tissue samples collected in the study were stained with Ziehl-Neelsen staining (K005-1KT, Hi-Media) as per the method described by Chauhan (2003). Briefly, approximately 2 gms of faecal sample was properly crushed in distilled water to make 15 ml homogenized suspension. This was allowed to settle for 2-3 hours at room temperature, followed by centrifugation of supernatant at 4000-5000 rpm for 5 minutes. The sediment was smeared on clean glass slides followed by air drying and heat fixation. Similarly, tissue smears were prepared from intestinal scrapings and impressions of mesenteric and ileo-caecal lymph nodes. Smears exhibiting presence of short acid-fast bacilli (AFB) in clumps was considered positive for MAP, in dispersed form as suspected for MAP and negative if neither of the two forms could be observed.

Serodetection by indirect ELISA

The indirect ELISA was carried out using 46 serum samples with standard commercially PARACHEK® 2 KIT, an *in vitro* Enzyme Linked Immuno Sorbent Assay kit (Prionics).

Detection of MAP specific gene by PCR

The DNA was extracted from the faecal and tissue samples which were found positive or suspected in ZN staining using the method described by (Jatav *et al*., 2018). Extracted DNA samples were amplified using specific IS 900 (F90 and R91) primers F 90; 5′- GAA GGG TGT TCG GGG CCG TCG CTT AGG -3 and R 91; 5′- GGC GTT GAG GTC GAT CGC CCA CGT GAC -3′.

Briefly, in a volume of 12.5 µl of PCR master mix, 0.5 µl forward primer and 0.5 µl reverse primer, 9.5 µl nuclease free water and 2.0 µl of genomic DNA (total volume 25 µl). Total of 37 cycles were performed in a thermal cycler for complete amplification reaction. Thermal cycling conditions were initial denaturation at 94°C for 5 minutes (1 cycle), followed by 37 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, synthesis at 72°C for 30 seconds and final extension at 72°C for 7 minutes. For every reaction, positive control from genomic DNA extracted from a culture of the MAP and a negative control were used to cross check for any contamination of foreign DNA in reaction component. The amplified product was visualized as a single compact fluorescent band of expected size 413 bp under U-V light and documented by a gel documentation system.

RESULTS AND DISCUSSION

Presence of AFB in Ziehl-Neelsen stained cytological smears

On examination of total 261 faecal smears, 15.32% faecal smears were found positive for the presence of acid-fast bacilli (AFB), graphically presented in Fig 1. Amongst these, 21.55%, 12.72% and 8.90% faecal smears of goats, buffaloes and cattle were found acid-fast positive, respectively. On examination of ZN-stained tissue smears, overall 29.41% smears revealed the presence of AFB. Out of these, 38.09%, 25.00% and 20.00% tissue smears from goats, buffaloes and cattle, respectively showed typical acid-fast bacilli either individually or in clumps.

AFB shedding pattern in faecal samples of ruminants

The shedding pattern of AFB in faeces was estimated. Among 40 faecal samples, 18 were graded as +1 shedders, 11 as +2 shedders, 06 as +3 shedders and only 01 buffalo as +4 shedders. 25 goat samples were found positive for the presence of AFB out of which, 09 goats were identified as +1 AFB shedders, 07 goats as +2 AFB shedders and 05 goats as +3 AFB shedders. Out of 07 AFB positive faecal samples of buffaloes, 03 were categorized as +1 shedders, 02 as +2 shedders, 01 each as +3 and +4 shedders. Similarly, of 08 and 07 AFB positive faecal samples of cattle, 06 samples were identified as +1 shedders and 02 samples were categorized as +2 shedders (Table 1 and Fig 2).

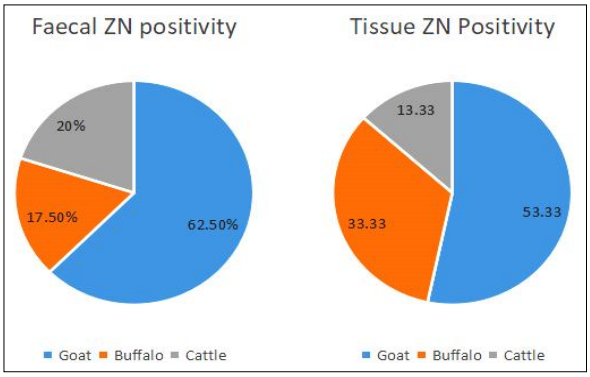


Fig 1: Graphical representation of percent positivity in ZN staining of faecal and tissue samples.

Faeces is a major source of infection for disease transmission and hence considered as first choice for clinical specimen of paratuberculosis (Eamens *et al*., 2000). Faecal shedding of the bacteria is reported to occur even before appearance of noticeable clinical signs and during subclinical infection. ZN staining provides information regarding shedding load and shedding pattern of AFB by the animals which is beneficial in terms of diagnosing the animal either in clinical or subclinical infectious stages. Additionally, in impression smears or sections of tissues (intestine and mesenteric lymph nodes with gross lesions), visualization of groups of brightly pink coloured bacilli within the resident macrophages in the lesions is highly suggestive of PTB (Chaubey *et al*., 2016).

Presence MAP specific gene in ZN positive samples

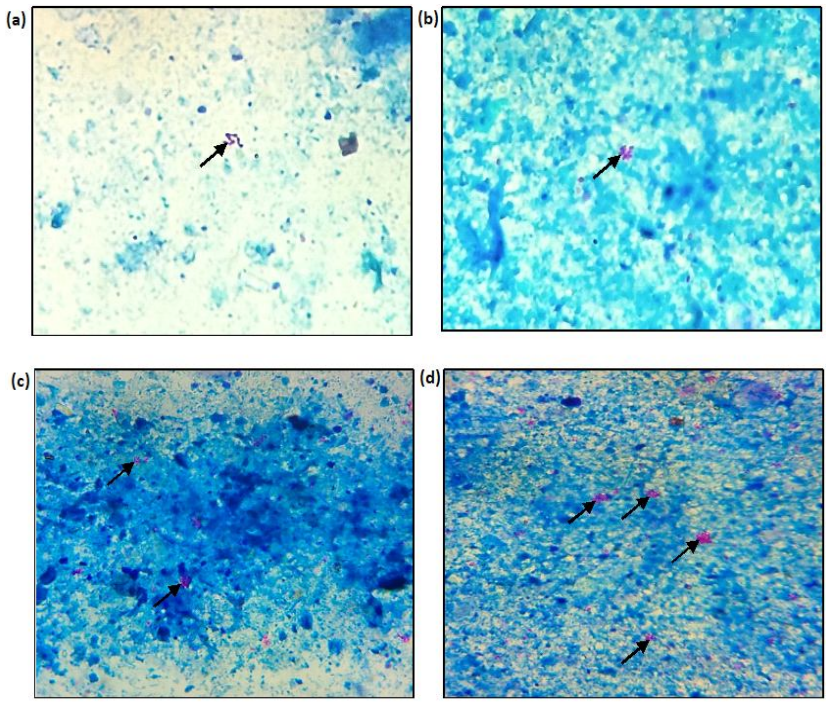
A total of 40 ZN positive faecal samples were subjected to *IS900* PCR, in which 22.50% faecal smears were found positive for MAP specific gene. Amongst these, 28.00% and 28.57% faecal samples of goats and buffaloes, respectively revealed *IS900* specific amplicon of 413 bp. From a total of 15 ZN positive tissue samples including intestine and lymph nodes, all (100%) samples comprising of 53.33% goats, 33.33% buffaloes and 13.33% cattle were positive for *IS900* PCR (Fig 3).

Table 1: Shedding pattern of AFB in faecal microscopy.

Rate of shedding	Positive (n)			
	Goats	Buffaloes	Cattle	Total
+1	09	03	06	18
+2	07	02	02	11
+3	05	01	00	06
+4	00	01	00	01
Total	25	07	08	40

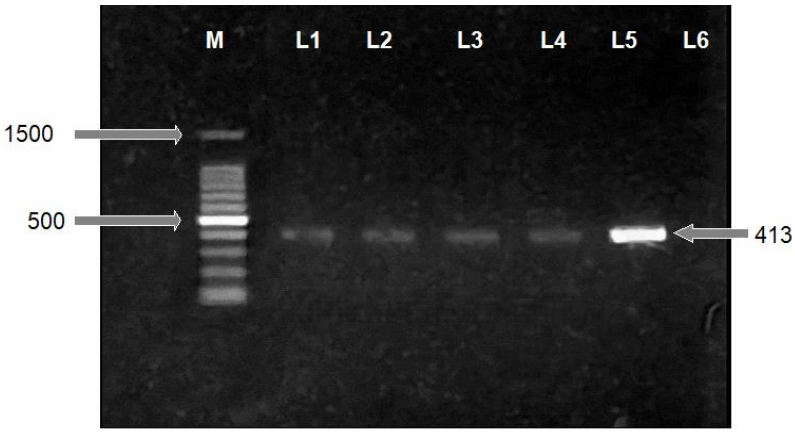
Polymerase chain reaction (PCR) is most specific and highly sensitive test for detection of MAP in faeces and in tissue samples. It is a powerful alternative diagnostic method for bacterial culture by which specific amplification of MAP DNA can be done (Biswal *et al*., 2020 and Singh *et al*., 2020). The *IS900* gene is an ideal target for direct faecal and tissue PCR with high sensitivity as greater number of copies of this gene are present in Map genome than other genes like ISMav2, ISMap02 or F57 (Kaur *et al*., 2011; Singh *et al*., 2013 and Hassan *et al*., 2019). Therefore, in the present study, suspected faecal and tissue samples were screened using *IS900* PCR on extracted DNA to obtain the frequency of MAP distribution in goats, buffaloes and cattle.

Around 28% of the AFB positive faecal samples of goats and buffaloes, along with 100% of the AFB positive tissue samples were found positive for MAP genome by PCR in the present study. Unfortunately, ZN positive faecal samples of cattle did not yield the amplified product on PCR in our study. This may be due to the presence of low amounts of DNA in the sample, the presence of PCR inhibitors and irrelevant DNA or by loss of MAP during the faecal DNA extraction steps. However, such barriers are not present in



(a) Low shedders +1. (b) Moderate shedders +2. (c) Moderate shedders +3. (d) Super shedders +4. ZN×1000.

Fig 2: Grading pattern of AFB shedding in faeces of ruminants.



M: 100 bp DNA ladder; L5: Positive control DNA; L1, L2, L3 and L4: Positive DNA samples; L6: Negative control.

Fig 3: PCR amplification of 413 bp of IS900 gene for identification of MAP in tissue samples.

detection of MAP genome by tissue PCR protocol. Therefore, refinement of the method could lead to widespread application of faecal PCR in detection of both clinical and subclinical cases.

Serodetection of MAP antibodies

A total of 124 ruminant serum samples comprising of 46 goats, 25 buffaloes and 53 cattle were screened for the presence of specific antibodies against MAP using indirect ELISA. Out of a total 124 serum samples of ruminants, 33.87% of serum samples were found positive for anti-MAP antibodies. Among these, 56.52%, 20.00% and 20.75% serum samples of goats, buffaloes and cattle, respectively, revealed the presence of anti-MAP antibodies.

Similar findings were also observed by Tripathi (2005); Sulficar *et al.* (2009); Singh *et al*. (2010); Barad *et al*. (2013); Singh *et al*. (2013); Abraham *et al*. (2014); Derakhshandeh *et al*. (2018); Hamid *et al.* (2018); Biswal *et al*. (2020); Bhat *et al*. (2021); Elsohaby *et al*. (2021) and Haque *et al*. (2022) in goats, Bhide *et al.* (2006); Shankar *et al*. (2008); Singh *et al.* (2014) and Elsohaby *et al*. (2021) in cattle and Sivakumar *et al.* (2006); Yadav *et al.* (2008); Chaturvedi *et al.* (2017), Pereira *et al*. (2020) and Audarya *et al.* (2021) in buffaloes. In the current study, results obtained indicate that the ZN staining detects higher positivity in faecal samples when compared with the MAP detection by PCR. This considerable gap of positivity might be due to the presence of other environmental mycobacteria in faecal samples and hence showing positivity by ZN staining.

Enzyme linked immunosorbent assay (ELISA) was conducted in the present study for the detection of humoral immune response to clinical and subclinical MAP infection in ruminants. The correlation of extend of shedding in faecal microscopy and seropositivity in ELISA, helps in confirmatory diagnosis of paratuberculosis along with determination of severity of infection (Kumar *et al*., 2020).

CONCLUSION

The present study confirms presence of paratuberculosis infection in goats, buffaloes and cattle of Jabalpur region of Madhya Pradesh, India. Faecal shedding pattern of acid-fast bacilli of ruminants revealed that the majority of the shedders were of +1 category. Molecular and serological examination of biological samples revealed 43.64% prevalence of MAP in ruminants (goats, buffaloes and cattle) in the area, emphasizing the need to design and implement better prevention and management programs for controlling further spread of the disease in this area.

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Conflict of interest

No conflict of interest observed by the authors.

REFERENCES

Abraham, A., Thomos, N., Joseph, S. and Raghavan, K.C. (2014). Comparitive study on the efficacy of ELISA and IS*900* PCR for the diagnosis of paratuberculosis in goats. Biotechnology in Animal Husbandry. 30(4): 661-667.

Audarya, S.D., Singh, M., Chaubey, K.K., Gupta, S. and Sikrodia, R. (2021). Antibodies against *Mycobacterium avium* subspecies paratuberculosis in cattle of Indore District in Madhya Pradesh. Indian Journal of Animal Research. 1: 1-6. DOI:10.18805/IJAR.B-4773.

Barad, D.S., Chandel, B.S., Dadawala, A., Chauhan, H., Kher, H.S., Shroff, S., Bhagat, A.G., Singh, A.V., Sohal, S.J., Gupta, S. and Chaubey, K.K. (2013). Comparative potential of traditional versus modern diagnostic tests in estimating status of caprine johne's disease. Advances in Animal and Veterinary Sciences. 1: 35-40.

Bhat, A.M., Malik, H.U., Chaubey, K.K., Hussain, T., Mir, A.Q., Nabi, S.U., Gupta, S. and Singh, S.V. (2021). Bio-typing of *Mycobacterium avium* subspecies paratuberculosis isolates recovered from the Himalayan sheep and goats. Tropical Animal Health and Production. 53(2): 1-9.

Bhide, M., Chakurkar, E., Tkacikova, L., Barbuddhe, S., Novak, M. and Mikula, I. (2006). IS900-PCR-based detection and characterization of *Mycobacterium avium* subspecies *paratuberculosis* from buffy coat of cattle and sheep. Veterinary Microbiology. 112(1): 33-41.

Biswal, S., Rath, A.P., Singh, S.V., Sahoo, N., Gupta, S., Singh, M. and Chaubey, K.K. (2020). Detection of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) from subclinical caprine paratuberculosis cases of Odisha. Indian Journal of Animal Research. 54(6): 709-715. DOI: 10.18805/ijar.B-3691.

Chaturvedi, S., Singh, S.V., Srivastava, A.K., Gangwar, N.K., Kumar, N., Rawat, K.D., Gupta, S., Chaubey, K.K., Singh, R., Singh, R. and Dhama, K. (2017). Comparative evaluation of FAT, IS900 PCR and microscopy vis a vis histo-pathology for the detection of *Mycobacterium avium subsp paratuberculosis* infection in tissues of goats naturally died in herds endemic for Johne's disease. Indian Journal of Animal Science. 87(6): 658-693.

Chaubey, K.K., Gupta, R.D., Gupta, S., Singh, S.V., Bhatia, A.K., Jayaraman, S., Kumar, N., Goel, A., Singh Rathore, A., Sahzad Sohal, J.S., Stephen B., Singh, J.M., Goyal, M. and Derakhshandeh, A. (2016). Trends and advances in the diagnosis and control of paratuberculosis in domestic livestock. Veterinary Quarterly. 36(4): 203-227.

Chauhan, R.S. (2003). Veterinary laboratory diagnosis, 1st Edn., International book distributing Co., Lucknow, pp: 285-286.

Derakhshandeh, A., Namazi, F., Khatamsaz, E., Eraghi, V. and Hemati, Z. (2018). Goat paratuberculosis in Shiraz: Histopathological and molecular approaches. Veterinary Research Forum. 9(3): 253-257

Eamens, G.J., Whittington, R.J., Marsh, I.B., Turner, J.J., Saunders, V., Kemsley, P.D. Rayward, D. (2000). Comparative sensitivity of various faecal culture methods and ELISA in dairy cattle herds with endemic Johne's disease. Veterinary Microbiology. 77: 357-368.

Elmagzoub, W.A., Adam, N.M., Idris, S.M., Mukhtar, M.E., Abdelaziz, S.A., Okuni, J.B., Ojok, L., El Wahed, A.A., Elteyab, E., Gameel, A.A. and Eltom, K.H. (2020). Seroprevalence of *Mycobacterium avium* subspecies *paratuberculosis* in dairy cattle in Khartoum state, Sudan. Veterinary Sciences. 7(4): 209. doi: 10.3390/vetsci7040209.

Elsohaby, I., Fayez, M., Alkafafy, M., Refaat, M., Al-Marri, T., Alaql, F.A., Al Amer, A.S., Abdallah, A. and Elmoslemany, A. (2021). Serological and molecular characterization of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) from sheep, goats, cattle and camels in the Eastern province, Saudi Arabia. Animals. 11(2): 323. doi: 10.3390/ani11020323.

Eslami, M., Shafiei, M., Ghasemian, A., Valizadeh, S., Al Marzoqi, A.H., Shokouhi Mostafavi, S.K., Nojoomi, F. and Mirforughi, S.A. (2019). *Mycobacterium avium paratuberculosis* and *Mycobacterium avium* complex and related subspecies as causative agents of zoonotic and occupational diseases. Journal of Cellular Physiology. 234(8): 12415-12421.

Gupta, S., Singh, S.V., Singh, M., Chaubey, K.K., Karthik, K., Bhatia, A.K., Kumar, N. and Dhama, K. (2019). Vaccine approaches for the therapeutic management of *Mycobacterium avium* subspecies *paratuberculosis* infection in domestic livestock. Veterinary Quarterly. 39(1): 143-152.

Hamid, M.A., Mohammed, G.E.E., Bakheit, A.O. and Saeed, E.M. (2018). Histopathological and RT-PCR detection of mycobacterium paratuberculosis in tissues of clinically suspected small ruminants. International Journal of Life Sciences. 4(5): 2012-2018.

Haque, M.I., Khan, F.A., Sadique, U., Khan, H., Rehman, Z., Khan, S., Khan, H., Ahmad, F., Rahman, M., Rehman, F.U., Saeed, M., Ali, M. and Nawaz, S. (2022). Molecular, serological and pathological detection of *Mycobacterium avium* subspecies *paratuberculosis* infection in small ruminants in Peshawar, Pakistan. Pakistan Journal of Zoology. 1: 1-8.

Hassan, N., Randhawa, C.S., Narang, D., Singh, S.V., Sharma, S. and Singh, S. (2019). Bio-incidence and bio-type of *Mycobacterium avium* subspecies paratuberculosis in diarrheic dairy cattle and buffaloes of Punjab area in India. Indian Journal of Animal Research. 53(7): 926-931. doi: 10.18805/ijar.B-3694

Jatav, G.P., Jayraw, A.K., Shrivastava, N., Sikrodia, R., Agrawal, V. and Karmore, S.K. (2018). Detection of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in faeces of buffaloes (*Bubalus bubalis*) from Malwa region of Madhya Pradesh. International Journal of Livestock Research. 8(1): 73-78.

Jatav, M., Verma, Y., Dubey, A., Swamy, M. and Singh, A.P. (2018). Pathology of subclinical paratuberculosis in goats of Mahakausal region. Indian Journal of Veterinary Pathology. 42(3): 165-170.

Kaur, P., Filia, G., Singh, S.V., Patil, P.K., Kumar, G.V.P.P.S.R. and Sandhu, K.S. (2011). Molecular epidemiology of *Mycobacterium avium* subspecies *paratuberculosis* IS900 PCR identification and IS 1311 polymorphism analysis from ruminants in the Punjab region of India. Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases. 34: 163-169.

Kumar, S., Kumar, S., Singh, R.V., Chauhan, A., Kumar, A., Bharati, J. and Singh, S.V. (2020). Association of genetic variability in CD209 gene with bovine paratuberculosis disease: A case-control study in the Indian cattle population. Animal Biotechnology. 1-8.

Pereira, H.D.M., Santos, H.P., de Oliveira, E.AA., Rocha, T.B., Araujo, I.M.S., Soares, D.M., Junior, F.G., de Albuquerque, P.P.F. and Mota, R.A. (2020). High prevalence of subclinical paratuberculosis in buffaloes (*Bubalus bubalis*) in Maranhao, Brazil. Brazilian Journal of Microbiology. 51(3): 1383-1390.

Shankar, H., Singh, S.V., Singh, P.K., Singh, A.V. and Sohal, J.S. (2008). Recovery of live *Mycobacterium avium* subspecies *paratuberculosis* from milk and milk products from north India. Indian Journal of Comparative Microbiology Immunology and Infectious Diseases. 29(1 and 2): 31-35.

Singh, M., Gupta, S., Singh, S.V., Chaubey, K.K., Sohal, J.S. and Dhama, K. (2018). Bio-incidence of *Mycobacterium avium* subspecies *paratuberculosis* in the pasteurized liquid milk, flavoured milk and milk powder commercially sold by leading market brands in India. Journal of Experimental Biology and Agricultural Sciences. 6(1): 188-203.

Singh, M., Gupta, S., Singh, S.V., Kumaresan, G., Sharma, D., Aseri, G.K., Yadav, P., Polavarapu, R. and Sohal, J.S. (2020). Development and standardization of visual loop mediated isothermal amplification (LAMP) assay for specific diagnosis of johne's disease. Indian Journal of Animal Research. 54(3): 293-299. DOI: 10.18805/ijar.B-3775.

Singh, P.K., Singh, S.V., Kumar, H., Sohal, J.S. and Singh, A.V. (2010). Diagnostic application of IS900 PCR using blood as a source sample for the detection of *Mycobacterium avium* subspecies *paratuberculosis* in early and subclinical cases of caprine paratuberculosis. Veterinary Medicine International. 10: 1-8.

Singh, S.V., Singh, P.K., Singh, A.V., Sohal, J.S., Kumar, N., Chaubey, K.K. and Dhama, K. (2014). 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.

Singh, S.V., Singh, P.K., Gupta, S., Chaubey, K.K., Singh, B., Kumar, A., Singh, A.V. and 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(4): 354-349.

Sivakumar, P., Tripathi, B.N., Singh, N. and Sharma, A.K. (2006). Pathology of naturally occurring paratuberculosis in water buffaloes (*Bubalus bubalis*). Veterinary Pathology. 43(4): 455-462.

Sulficar, S., Saseendranath, M.R., Krishnan, N.G., Tresamol, P.V. and Pillai, U.N. (2009). Comparative efficacy of various diagnostic tests for caprine paratuberculosis: A field study. Journal of Veterinary Animal Sciences. 40: 35-36.

Tripathi, B.N. (2005). Paratuberculosis (Johne's disease) in cattle. International Journal of Cow Science. 1(1): 16-26.

Tripathi, B.N. (2008). Paratuberculosis (Johne's disease) in animals: Pathogenesis, diagnosis and zoonotic significance. Indian Journal of Veterinary Pathology. 32(2): 187-199.

Yadav, D., Singh, S.V., Singh, A.V., Sevilla, I., Juste, R.A., Singh, P.K. and Sohal, J.S. (2008). Pathogenic 'Bison-type' *Mycobacterium avium* subspecies *paratuberculosis* genotype characterized from riverine buffalo (*Bubalus bubalis*) in North India. Comparative Immunology, Microbiology and Infectious Diseases. 31(4): 373-387.