



First Genotyping of *Mycobacterium avium* Subspecies *Paratuberculosis* from Large Ruminant Population at Satna District in Madhya Pradesh in India to Bison Type

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ABSTRACT

Background: Paratuberculosis or Johne's disease in large ruminants is caused due to *Mycobacterium avium* subspecies *paratuberculosis*. The bacterial disease in large ruminants is characterized by chronic diarrhea, weakness and emaciation. There are no reports on Paratuberculosis in this area's large ruminant population and its genotyping.

Methods: A total of 76 samples (26 fecal and 50 milk) of large ruminants from Satna district in Madhya Pradesh were investigated (microscopical; for the presence of acid-fast organisms and molecular methods; for gene-specific amplification) for the presence of *Mycobacterium avium* subspecies *paratuberculosis* infection.

Result: Microscopical examinations of fecal samples revealed 11.54% positivity and milk samples 14% positivity of *Mycobacterium avium* subspecies *paratuberculosis* infection. A single fecal and 2 milk samples were found positive in the IS900 polymerase chain reaction test (413 bp product). Specific amplification of 608 bp IS1311 product followed by restriction endonuclease analysis proved the genotype of *Mycobacterium avium* subspecies *paratuberculosis* as Bison type.

Key words: Bovines, Genotyping, IS1311, IS900, Paratuberculosis, Prevalence.

INTRODUCTION

Paratuberculosis or Johne's disease in domestic livestock population is caused by the bacteria, *Mycobacterium avium* subspecies *paratuberculosis* (MAP). A long-lasting enteritis of large ruminants characterizes the disease. So, regular testing of the livestock population to monitor their health status is essential to prevent and control animal infectious diseases and thereby contain losses to the dairy sector. Paratuberculosis is diagnosed by traditional agent isolation and serological methods as well as modern molecular techniques (Sahzad *et al.*, 2017). Recently marker assay to differentiate infected and vaccinated cows has been developed based on recombinant proteins (Chaubey *et al.*, 2019). In the present investigation, fecal and milk samples from large ruminants of Satna district in Madhya Pradesh, India were tested by different screening tests for detecting the presence of MAP.

MATERIALS AND METHODS

About the district satna

Satna district is surrounded by five districts of the state of Madhya Pradesh namely, Rewa, Shahdol, Umaria, Katni and Panna and one district of Uttar Pradesh state called as Banda (<https://satna.nic.in/en/map-of-district/> Accessed on 12th June 2022. It has an area of 7,502 square kilometers with 2,125 villages. Its population is over 2 million (2,228,619) (<https://satna.nic.in/en/> Accessed on 21st May 2023) that have literacy rates of 65.12% slightly below the country's 65.38% and above the state's 64.11%. The district

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Satna has seven tehsils namely; Raghuraj Nagar, Rampur Baghelan, Nagod, Unchehra, Amarpatan, Ram Nagar, and Maihar. It has a population density of 249 per square kilometer above the state (196) but below the country (324). Most of the agricultural laborers are women (https://cdn.s3waas.gov.in/s38e6b42f1644_ecb1327dc03ab345e618b/uploads/2016/04/2018041238.pdf Accessed on 12th June 2022). The location of Satna in the Madhya Pradesh state and India is depicted in Fig 1 using assistance of Google Maps 2022 (<https://satna.nic.in/en/map-of-district/> and https://en.wikipedia.org/wiki/Satna_district#/media/File:MP_Satna_district_map.svg Accessed on 12th June 2022).

Livestock, collection and transportation of samples

In the present study, a representative randomly selected 26 fecal samples from two different dairy farms Rajouri dairy (adult Murrah buffalo) and Kushwaha dairy (adult cattle Sahiwal and Jersey breeds) and 50 milk samples from a Private farm located at Satna district in Madhya Pradesh were collected for investigating on Paratuberculosis or Johne's disease infection. The animals were healthy but weak in bodily conditions. These samples were collected in February in the year 2019. After collection, these samples were then transported and stored in the deep freezer (-40°C) to the Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Nanaji Deshmukh Veterinary Science University, Dr. Ambedkar Nagar-Mhow, Indore, Madhya Pradesh, India. These samples were further transported at cold chain conditions to the Microbiology Laboratory, Animal Health Division, Central Institute for Research on Goats (CIRG), Makhdoom-Farah, Mathura, Uttar Pradesh, India. Fecal and milk samples were tested for the presence of MAP infection by different tests during the year 2019-2021. Processed fecal and milk samples were examined by microscopical and molecular methods for the presence of *Mycobacterium avium* subspecies *paratuberculosis* and gene-specific amplification from extracted genome from the organisms, respectively.

Processing of fecal samples and microbial staining of fecal and milk smears

Individual fecal sample, 2 g was taken into sterile mortar and pestle and crushed well after adding 10 ml Phosphate Buffer Saline (PBS) solution. This mixture was poured in a 15 ml centrifuge tube and it was kept for centrifugation for 45 minutes at 4,500 revolutions per minute (rpm). The resulting supernatant was discarded and from the middle layer, a fecal smear was prepared on a glass slide. The smear was further examined after acid-fast staining for the presence of acid-fast bacilli. Milk smears were also prepared on glass slides and examined microscopically as above.

Isolation of deoxyribonucleic acid (DNA) from feces and milk samples

2 g of fecal material was taken in a sterile mortar containing 4 ml of sterile PBS solution. A pestle homogenized the fecal material. The resulting homogenate was transferred to a 15 ml centrifuge tube containing 8 ml of sterile PBS. The tube was centrifuged at 3,500 revolutions per minute for 45 minutes to concentrate bacilli. The tube was kept undisturbed for 1 hour at room temperature. The supernatant was discarded and the semisolid middle layer was collected with a sterilized swab and transferred to 300 µl of PBS in an Eppendorf® tube. The mixture was suspended to heat at 95°C for 10 minutes. A volume of 40 µl of Lysozyme (20 mg/ml) was added and the tube was incubated at 37°C for 2 hours with continuous shaking. Then, 20 µl of Proteinase K (10 mg/ml) and 50 µl of 10% Sodium Dodecyl Sulfate (SDS) were added to the mixture and heated at 56°C for 2 hours with continuous shaking. After the addition of 64 µl Cetyltrimethylammonium bromide-Sodium Chloride (CTAB-NaCl) solution in the mixture, it was heated at 65°C for 30 minutes. Equal volume of Chloroform-Isoamyl alcohol (24:1) was added to the tube and on mixing properly it was centrifuged at 10,000 rpm for 20 minutes at 4°C. The aqueous layer (upper layer) was transferred to the fresh 1.5 ml Eppendorf® tube. On addition 0.6 volume of isopropanol to the aqueous layer, it was

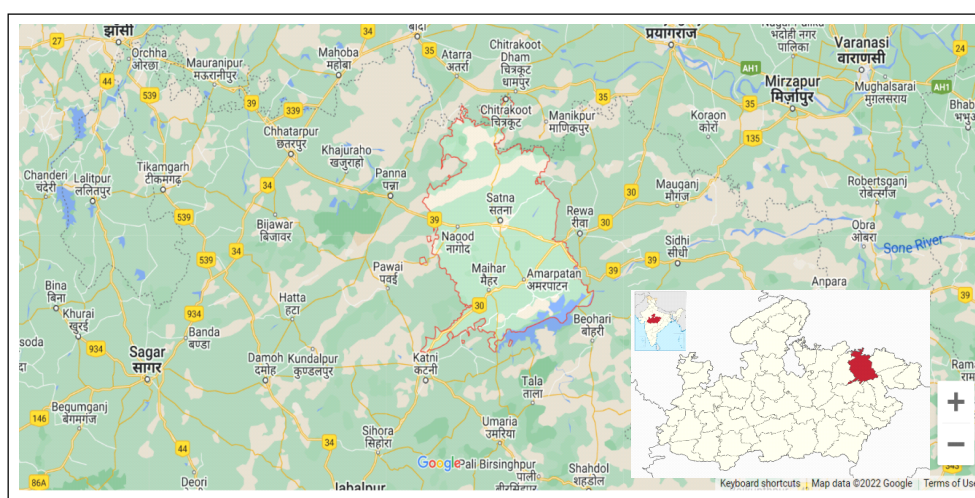


Fig 1: Location of Satna district in Madhya Pradesh and India.

mixed by gentle inversion and DNA precipitation was allowed at -20°C for overnight. Then, the tube was centrifuged at 10,000 rpm for 20 minutes at 4°C and discarded the supernatant. The resulting pellet was washed with 1 ml of 70% ethanol by centrifugation at 10,000 rpm for 10 minutes at 4°C. The supernatant was discarded and air dried the pellet. The pellet was resuspended in 30 µl of Tris Ethylenediaminetetraacetic acid (TE) buffer and stored at 4°C overnight to dissolve the DNA. It was further stored at -20°C for long-term usage. Nucleic acid extraction from milk samples was performed as per Matoli *et al.* (2018).

IS900 polymerase chain reaction (PCR)

Primers sequences used were specific to MAP (IS900 P90/91) (Forward primer: 5'-GAAGGG TGT TCG GGGCCGTCG CTT AGG-3' and Reverse primer: 5'-GGC GTT GAG GTC GATCGC CCA CGT GAC-3') (Millar *et al.*, 1995; Millar *et al.*, 1996; Singh *et al.*, 2010). A reaction master mix (with deoxyribonucleotide triphosphates, dNTPs; Taq DNA polymerase; assay buffer, Magnesium Chloride, MgCl₂ and loading dye) was used to perform the test. A total reaction volume of 25 µl containing 2.5 µl of a test DNA sample and 0.5 µl of each primer. Positive (DNA from native Bison type S 5 strain of MAP) and negative controls were also kept. Thermocycler chain reaction conditions were initial denaturation at 94°C for 3 minutes and subsequently for the next 30 cycles (denaturation at 94°C for 30 seconds, annealing at 63°C for 15 seconds and extension at 72°C for 1 minute) and final extension at 72°C for 10 minutes and at the end of the reaction it was kept at 4°C. The PCR product (413 bp) was analyzed on a 1% agarose gel in 1X Tris Borate Ethylenediaminetetraacetic acid (TBE) buffer containing

0.5 µg/ml of Ethidium Bromide at 80 V for 1 hour. IS900 PCR product from MAP S 5 Indian Bison type DNA as positive control and 1.5 kb DNA ladder or Marker (Fermentas) were also run.

IS1311 PCR amplification for genotyping of MAP

A 25 µl reaction volume consisted of 12.5 µl master mix (Genei, Bengaluru), 5 µl template, 0.5 µl of each of primers (Forward primer (M56): 5'-GCG TGA GGC TCT GTG GTG AA-3' and Reverse primer (M119): 5'-ATG ACG ACC GCT TGG GAG AC-3'; Sevilla *et al.*, 2005) and remaining amount of nuclease-free water. Thermocycler chain reaction conditions were initial denaturation at 94°C for 5 minutes and subsequently for the next 37 cycles denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds and extension at 72°C for 1 minute and final extension at 72°C for 10 minutes and at the end of the reaction it was kept at 4°C. No template control and a positive control from reference strain, were included in each PCR amplification. The DNA band of 608 bp was considered PCR positive after separation in 1% agarose gel stained with Ethidium Bromide. The PCR products (608 bp) were digested using HinfI/MseI enzymes and patterns were compared as Cattle type/Sheep type/India Bison type (Whittington *et al.*, 2001).

RESULTS AND DISCUSSION

Mycobacterium avium subspecies *paratuberculosis* (MAP) bacteria in large ruminants cause chronic progressive diarrhea in large ruminants. This bacterial disease, characterized by enteritis in animals, is named Johne's disease or Paratuberculosis. MAP infection from the state of Madhya Pradesh in India was reported in the livestock population (Audarya *et al.*, 2013; Audarya *et al.*, 2016; Singh

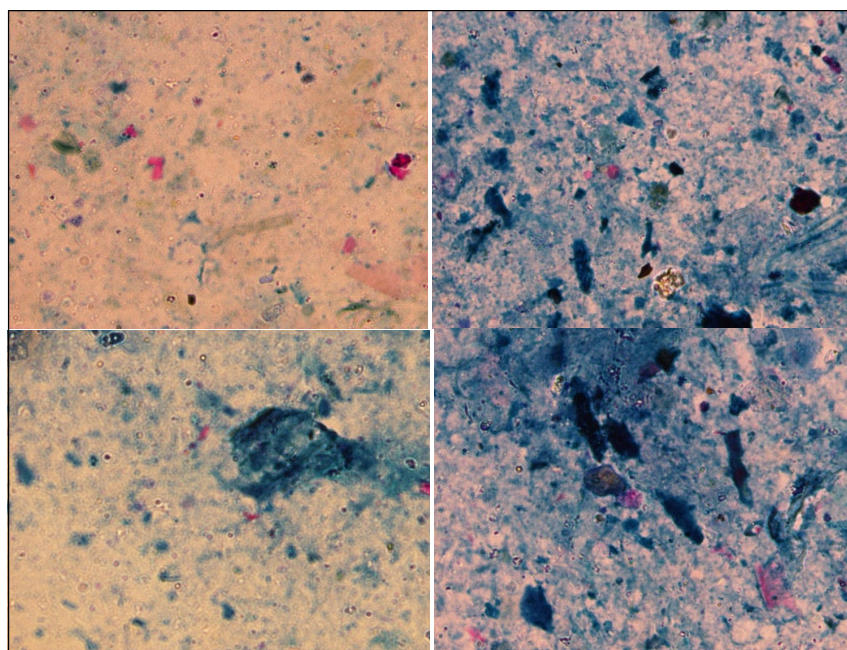


Fig 2: *Mycobacterium avium* subspecies *paratuberculosis* bacilli visualized by using the microscope.

et al., 2016; Jatav *et al.*, 2017; Audarya *et al.*, 2018; Matoli *et al.*, 2018; Audarya *et al.*, 2022; Verma *et al.*, 2024). The disease also has zoonotic concerns with Crohn's disease (Jain *et al.*, 2020). MAP is also associated with an increasing number of inflammatory and autoimmune diseases such as sarcoidosis, Blau syndrome, autoimmune diabetes, autoimmune thyroiditis, multiple sclerosis and rheumatoid arthritis and Parkinson's disease. MAP can impair autophagy and promote insulin resistance. In India, if considering human bio-load of MAP; 30% of more than 28,000 tested individuals were found to harbor, or to have harbored, MAP (Singh *et al.*, 2014; Dow, 2021). Prevalence of Paratuberculosis appears in decreasing order, in Africa (53%), India (23.3%), Europe (20%), South America (18.3%), North America (16.9%) and Australia (6.8%) (Agrawal *et al.*, 2019).

In the present study, pink-colored acid-fast bacilli closely resembling MAP were observed microscopically on acid-fast staining (Fig 2). Genomic DNA was extracted from fecal and milk samples and used in various molecular methods (Fig 3). Molecular identification of MAP was confirmed in IS900 PCR from 1 sample by the presence of 413 bp product which was visualized under the gel documentation system (Fig 4). Rasool *et al.*, 2017 used IS900 PCR for the detection of MAP in bovine fecal samples. These IS900 PCR-positive samples were further confirmed in IS1311 PCR by detecting a product size of 608 bp (Fig 5). Digested IS1311 PCR product from fecal samples and milk samples showed positive results for Indian Bison type (67 bp, 218 bp and 323 bp digested products) (Fig 6). Vaccination is considered the method of choice for the control of Paratuberculosis in livestock. The efficacy of vaccines depends on the genotype of the candidate strain used. Therefore, knowledge of the genotypes infecting domestic livestock species is critical for designing disease control strategies. MAP isolates are grouped into three

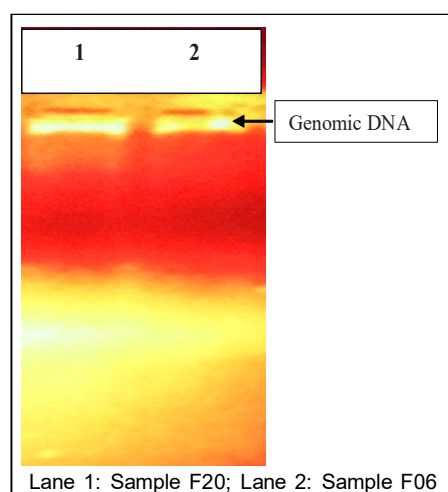


Fig 3: Genomic deoxyribonucleic acid bands visualized by using gel document system.

genetically distinct genotypes (Cattle type, Sheep type and Bison type) based on the IS1311 PCR-restriction endonuclease analysis (REA) method. Bison type genotype was first reported from wild bison of Montana, USA and later similar genotypes have been reported as major genotypes infecting domestic livestock, wild ruminants and the human population in India (Singh *et al.*, 2015). A single

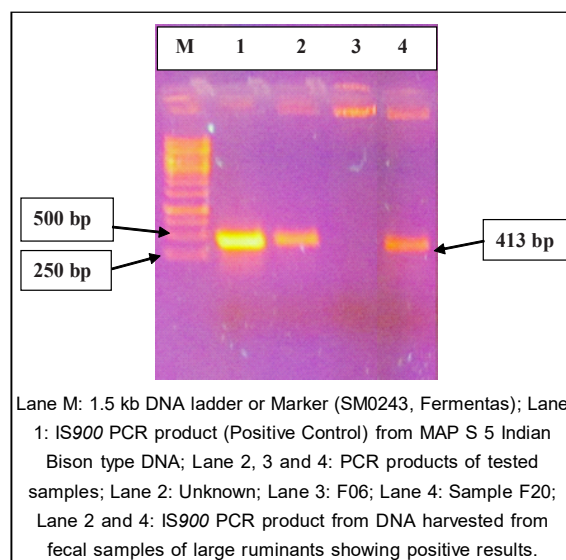


Fig 4: Visualization of 413 bp product of IS900 polymerase chain reaction by using gel documentation system.

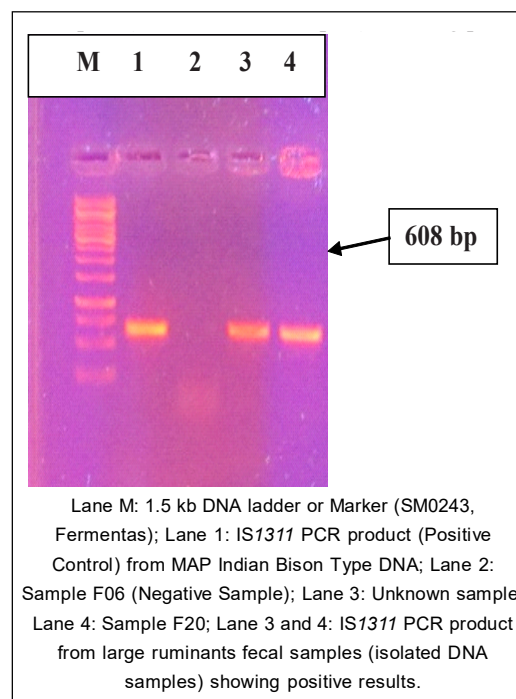


Fig 5: Visualization of 608 bp product of IS1311 polymerase chain reaction by using gel documentation system.

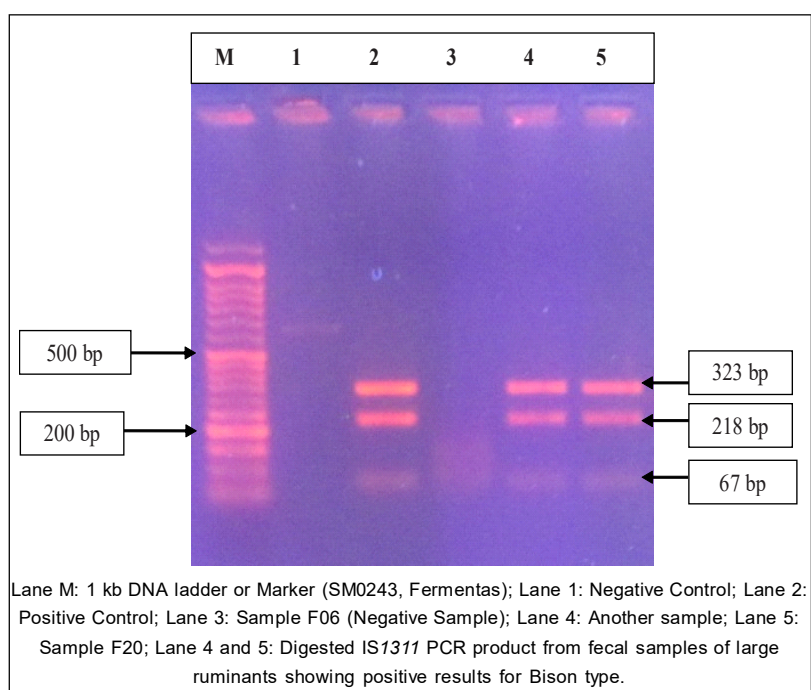


Fig 6: Genotyping of *Mycobacterium avium* subspecies *paratuberculosis* from large ruminants at Satna of Madhya Pradesh state in India.

Table 1: *Mycobacterium avium* subspecies *paratuberculosis* presence in the fecal samples of large ruminants detected by different screening tests.

Code	Subfamily	Microscopy	IS900PCR	IS1311PCR	IS1311 genotyping
F01	Bovinae	N	N	N	-
F02	Bovinae	N	N	N	-
F03	Bovinae	N	N	N	-
F04	Bovinae	+1	N	N	-
F05	Bovinae	N	N	N	-
F06	Bovinae	+1	N	N	-
F07	Bovinae	N	N	N	-
F08	Bovinae	N	N	N	-
F09	Bovinae	N	N	N	-
F10	Bovinae	N	N	N	-
F11	Bovinae	N	N	N	-
F12	Bovinae	N	N	N	-
F13	Bovinae	N	N	N	-
F14	Bovinae	N	N	N	-
F15	Bovinae	N	N	N	-
F16	Bovinae	N	N	N	-
F17	Bovinae	N	N	N	-
F18	Bovinae	N	N	N	-
F19	Bovinae	N	N	N	-
F20	Bovinae	+1	P	P	Bison type
F21	Bovinae	N	N	N	-
F22	Bovinae	N	N	N	-
F23	Bovinae	N	N	N	-
F24	Bovinae	N	N	N	-
F25	Bovinae	N	N	N	-
F26	Bovinae	N	N	N	-

P- Positive; N- Negative; PCR- Polymerase chain reaction.

Table 2: *Mycobacterium avium* subspecies *paratuberculosis* presence in the milk samples of large ruminants detected by different screening tests.

Code	Subfamily	Microscopy	IS900PCR	IS1311 PCR	IS1311 genotyping
M1	Bovinae	N	N	N	-
M2	Bovinae	1+	P	P	Bison Type
M3	Bovinae	1+	N	N	-
M4	Bovinae	N	N	N	-
M5	Bovinae	N	N	N	-
M6	Bovinae	N	N	N	-
M7	Bovinae	N	N	N	-
M8	Bovinae	1+	N	N	-
M9	Bovinae	N	N	N	-
M10	Bovinae	N	N	N	-
M11	Bovinae	N	N	N	-
M12	Bovinae	1+	N	N	-
M13	Bovinae	N	N	N	-
M14	Bovinae	N	N	N	-
M15	Bovinae	N	N	N	-
M16	Bovinae	N	N	N	-
M17	Bovinae	N	N	N	-
M18	Bovinae	N	N	N	-
M19	Bovinae	N	N	N	-
M20	Bovinae	1+	N	N	-
M21	Bovinae	1+	N	N	-
M22	Bovinae	N	N	N	-
M23	Bovinae	N	N	N	-
M24	Bovinae	N	N	N	-
M25	Bovinae	N	N	N	-
M26	Bovinae	1+	P	P	Bison Type
M27	Bovinae	N	N	N	-
M28	Bovinae	N	N	N	-
M29	Bovinae	N	N	N	-
M30	Bovinae	N	N	N	-
M31	Bovinae	N	N	N	-
M32	Bovinae	N	N	N	-
M33	Bovinae	N	N	N	-
M34	Bovinae	N	N	N	-
M35	Bovinae	N	N	N	-
M36	Bovinae	N	N	N	-
M37	Bovinae	N	N	N	-
M38	Bovinae	N	N	N	-
M39	Bovinae	N	N	N	-
M40	Bovinae	N	N	N	-
M41	Bovinae	N	N	N	-
M42	Bovinae	N	N	N	-
M43	Bovinae	N	N	N	-
M44	Bovinae	N	N	N	-
M45	Bovinae	N	N	N	-
M46	Bovinae	N	N	N	-
M47	Bovinae	N	N	N	-
M48	Bovinae	N	N	N	-
M49	Bovinae	N	N	N	-
M50	Bovinae	N	N	N	-

P- Positive; N- Negative; PCR- Polymerase chain reaction.

Table 3: Final status of *Mycobacterium avium* subspecies *paratuberculosis* infection in the fecal and milk samples from large ruminants as well as overall positivity when tested by different screening tests.

Samples	Health Status	Age	Animals positive in			
			Microscopy	IS900 PCR	IS1311 PCR	IS1311 genotyping
26 fecal	Healthy (Weak)	Adult	3 (11.54%)	1 (3.84%)	1 (3.84%)	Bison type in cattle /large ruminant
50 milk	Healthy (Weak)	Adult, lactating	7 (14%)	2 (4%)	2 (4%)	Bison type in cattle/ buffalo/ large ruminant
76 overall	Healthy (Weak)	Adult, lactating	10 (13.16%)	3 (3.95%)	3 (3.95%)	Bison type in cattle/ buffalo/ large ruminant

fecal sample and 2 milk samples were found positive in the IS900 PCR (Table 1, Table 2). In the present study, microscopically 11.54% MAP positivity in fecal and 14% MAP positivity in milk samples were detected (Table 3). Specific amplification of 608 bp IS1311 product followed by restriction endonuclease analysis proved the genotype of MAP as Bison type which is the major biotype infecting domestic animals in the country (Hassan *et al.*, 2019).

In India, the presence of MAP was reported in various species of animals and their products, human beings and environmental resources from 1960 to till date (Singh *et al.*, 2016a). Madhya Pradesh state in India has a shared border with many states. There was a report regarding the illegal transportation of buffaloes from Chhattisgarh state to Uttar Pradesh state via Madhya Pradesh state in India (<https://www.bhaskarhindi.com/state/news/police-chased-and-caught-a-truck-loaded-with-cattle-376998> Accessed on 21st May 2023). So, animal certification and transportation must be regulated with strict measures regarding the movement of animals for trade, fair and other purposes.

CONCLUSION

The results of the study indicate the presence of Johne's disease in a large ruminant population in the Satna district of Madhya Pradesh. The MAP detected in the present study from Satna district of Madhya Pradesh was further confirmed as Bison type in the genotyping study. An indigenously developed vaccine against the disease is available. This vaccine is meant for both prophylactic and therapeutic purposes. A thorough investigation of the livestock population of the adjacent region (cattle, buffalo, goats) of Satna district is recommended for ascertaining the presence of this disease and its magnitude. Vaccination against paratuberculosis is recommended for the susceptible livestock population of the area in the state.

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Conflict of interest

All author declare that they have no conflict of interest.

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