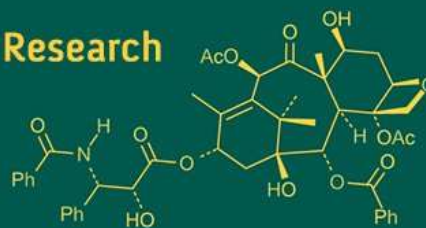
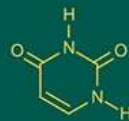
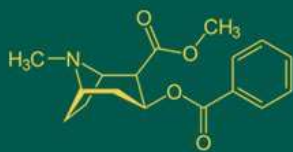


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Pathology and molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis* infection in goats: Age variations, clinical features and diagnostic confirmation in Malwa region

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Abstract

This study investigated the prevalence, pathological features and molecular detection of *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the causative agent of paratuberculosis (Johne's disease) in goats. A total of 250 goats slaughtered at the Cantonment Board Slaughterhouse in Mhow, Indore, were investigated using multiple sample types: faecal, blood, intestinal tissue, and mesenteric lymph nodes. The overall prevalence of paratuberculosis was 38.8%, with 97 goats (38.8%) testing positive via Ziehl-Neelsen-stained faecal smear examination. Clinical signs observed antemortem included chronic diarrhoea, pasty or unformed faeces, progressive emaciation, and a dull, rough hair coat, classic indicators of advanced infection. Age-specific prevalence varied significantly. The highest rate was recorded in adult goats older than 2 years (44.93%), followed by those aged 6-12 months (35.71%), while the lowest occurred in the 1-2-year age group (29.76%). This pattern suggests that older animals are more likely to exhibit detectable infection, possibly due to longer exposure or progression to the clinical stage. No notable sex-based difference was observed, with prevalence nearly identical in females (38.89%) and males (38.64%). For confirmation, MAP was successfully cultured on Herrold's Egg Yolk (HEY) medium from 26.67% of faecal samples and 25% of tissue samples. Subsequent Polymerase Chain Reaction (PCR) targeting the IS900 gene (a widely used marker for MAP) confirmed the bacterium in 20% of faecal isolates and 16.67% of tissue isolates. These findings highlight a substantial burden of subclinical and clinical paratuberculosis in this goat population, emphasizing the need for improved diagnostic strategies, herd management practices, and control measures to mitigate economic losses and potential zoonotic risks associated with MAP.

Keywords: *Mycobacterium avium* subspecies *paratuberculosis* (MAP), Prevalence, Herrold's Egg Yolk medium, IS900 gene

Introduction

Small ruminants such as sheep and goats are crucial to the socioeconomic fabric of rural India and other developing countries. They serve as a primary source of livelihood for impoverished communities, generating income through milk, meat, fibre and manure, while bolstering food security and resilience for smallholder farmers in low-resource environments. Johne's disease (paratuberculosis), triggered by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), manifests as a persistent, incurable granulomatous enteritis in ruminants. In affected goats, it results in gradual wasting, intermittent or resistant diarrhoea, diminished output (e.g., lower milk or growth rates) and death (Gupta *et al.*, 2012 and Richards *et al.*, 2021) [7, 17]. Clinical signs usually manifest late, after years of subclinical infection. Unlike cattle, where profuse watery diarrhoea is common, small ruminants typically exhibit milder faecal changes (softened stools) alongside prominent emaciation and cachexia. Goats tend to be more vulnerable than sheep, with accelerated progression, abbreviated latent phase, and earlier faecal shedding—frequently preceding immune detection. Pathologically in goats, key gross findings encompass intestinal wall thickening and corrugation, granular or pleated mucosa, serous fat atrophy in the mesentery, and lymphatic vessel hypertrophy. Mesenteric lymph nodes commonly appear swollen, oedematous, corded, and sometimes calcified, indicative of longstanding granulomatous

response. Fueled by international livestock movement, Johne's disease occurs worldwide, inflicting major economic damage via decreased productivity, early culling, carcass devaluation, and management expenses. Documented in the United States for more than 75 years, it impacts diverse hosts including cattle, sheep, goats, deer, bison, and beyond. In India, MAP remains widespread and endemic among small ruminants, with studies documenting collective prevalence around 22.5% in sheep and goats, and bioload estimates up to 23% in goats across decades (Kumar *et al.*, 2007; Singh *et al.*, 2008; Narnaware and Tripathi, 2017; Chaubey *et al.*, 2017; Ganesan and Sravani, 2024) [13, 22, 16, 3, 5]. Earlier nationwide surveys revealed an average bioload of 23.3% in screened livestock, with species variations (e.g., up to 29% in organized cattle/buffalo herds). Regional data show elevated seroprevalence in sheep (often >30-40% in areas like Rajasthan or Kashmir) and persistent molecular evidence of transmission. Goats generally display greater susceptibility and shedding intensity relative to sheep. The insidious, protracted course and testing limitations (prolonged culture, inconsistent test sensitivity) hinder effective containment. In India, where small ruminants sustain countless rural families, uncontrolled Johne's disease intensifies hardship via lost production, herd depletion, and economic strain. Robust strategies, enhanced diagnostics, strict biosecurity, removal of shedders, and stakeholder education are essential to curb losses and address potential zoonotic implications. Diagnosis of Johne's disease (paratuberculosis) in ruminants primarily relies on detecting *Mycobacterium avium* subsp. *paratuberculosis* (MAP) shedding in faeces and the host's immune response. A common field-applicable method involves Ziehl-Neelsen (ZN) staining of faecal smears to visualize acid-fast bacilli, indicating MAP excretion. Molecular approaches enhance sensitivity: MAP DNA is extracted from faeces, blood, or tissue samples, followed by amplification via IS900 PCR, a widely used target sequence specific to MAP for confirmation (Singh *et al.*, 2013a) [20]. This technique enables rapid detection without waiting for bacterial growth. Although MAP is fastidious and challenging to culture *in vitro* due to its slow growth and specific requirements (e.g., mycobactin J supplementation), bacterial culture remains the gold standard for definitive MAP identification (Ristow *et al.*, 2006) [18]. Culture confirms viability and allows strain characterization, but it is time-consuming (often 8-16 weeks or longer) and resource-intensive (Stabel, 1998) [24]. Consequently, other methods like ZN staining and PCR are frequently validated against culture results to ensure reliability. Various cultivation protocols for MAP from faecal specimens exist across laboratories, incorporating decontamination steps and specialized media to overcome contamination and growth hurdles. Combining direct detection (ZN, PCR) with culture provides a comprehensive diagnostic strategy, balancing speed, sensitivity, and specificity for accurate Johne's disease diagnosis in affected animals.

Materials and Methods

This study was carried out in the Department of Veterinary Pathology, College of Veterinary Sciences and Animal Husbandry, Mhow (Madhya Pradesh), a constituent college of Nanaji Deshmukh Veterinary Science University, Jabalpur.

A total of 250 goats representing various breeds were examined at the Mhow Cantonment Board Slaughterhouse. Comprehensive ante-mortem and post-mortem evaluations were performed on each animal. The goats originated from multiple areas within the Malwa tract of Madhya Pradesh, including Mhow, Manpur, Dhar, Indore, and Maheshwar. Prior to slaughter, each goat underwent a thorough physical examination to determine age, sex, and body condition. Body condition was assessed and scored on a scale from 0 to + 4 (physical profile), classifying the animals as: healthy (+ 4), normal (+ 3), weak (+ 2), or emaciated (+ 1). Faecal samples were collected rectally from each goat before slaughter using sterile plastic gloves. Approximately 3-5 g of faeces was placed in labeled polythene bags, transported promptly to the Department of Veterinary Pathology and stored at -20 °C for further analysis of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). During detailed post-mortem inspection, 3-5 pieces (each 1-2 cm) of affected small intestine (near the ileo-caecal junction) and involved mesenteric lymph nodes were harvested. Tissue samples were placed in sterile plastic sachets and transported to the laboratory as quickly as possible at 4 °C. To minimize contamination, intestinal tissues were rinsed with sterile water to remove adherent faeces prior to transport. No preservatives were added to the tissue samples intended for culture. These sampling protocols ensured hygienic collection, proper preservation, and suitability for subsequent Ziehl-Neelsen staining, culture on Herrold's Egg Yolk medium and IS900 PCR-based molecular confirmation of MAP.

Processing of Faecal sample

Faecal samples (~2 g) were crushed within polythene bags and then ground in a sterile pestle and mortar with 3-5 mL distilled water to form a fine paste. This paste was transferred to 15 mL centrifuge tubes using an additional 7-8 mL distilled water. Tubes were centrifuged at 3500 rpm for 45 minutes at room temperature to concentrate bacilli. Post-centrifugation, tubes were undisturbed for 1-2 hours at 4 °C, revealing three layers: top clear supernatant, middle semi-solid, and bottom solid sediment. The supernatant was discarded, and the middle layer was decontaminated in 25 mL 0.9% Hexadecyl Pyridinium Chloride (HPC) for 18-24 hours at room temperature. After sedimentation, excess supernatant was removed, leaving ~1 mL sediment. Smears were prepared from this sediment using a wooden stick on clean, grease-free slides and air-dried.

Ziehl-Neelsen Staining of Faecal Smears

Air-dried, heat-fixed smears were flooded with 1.0% carbol fuchsin and gently heated to steaming for 10 minutes to enhance dye penetration. After cooling, slides were rinsed with distilled water, then decolorized with 3% acid-alcohol for 30 seconds, followed by another water rinse. Slides were counterstained with 0.1% methylene blue for 1 minute, rinsed, and blot-dried. Under oil-immersion microscopy, pink-colored short rods (0.5-1.5 µm) in bunches, clumps, or singly, morphologically consistent with MAP were recorded as positive for Johne's disease/MAP infection.

Microscopic Examination of Tissue Samples Tissue Processing

Thawed intestinal (near ileo-caecal junction) and mesenteric lymph node tissues were rinsed with sterile 1X PBS to

remove residual faeces. Connective tissue and fat were trimmed using sterile instruments. Approximately 2 cm³ of tissue was finely minced, ground with sterile sand and 8-10 mL sterile 1X PBS in a pestle and mortar. The suspension was collected in test tubes and allowed to sediment for 5-6 hours at room temperature. The top clear fluid (5-7 mL) was used for further processing; intestinal and lymph node samples were handled separately.

Staining of Tissue Smears and Sections

Smears from processed tissues were prepared on clean slides, air-dried, heat-fixed, and stained by the same Ziehl-Neelsen protocol as faecal smears. Pink short rods in characteristic arrangements indicated MAP positivity. For tissue sections, a slightly modified Ziehl-Neelsen acid-fast staining was applied: sections were deparaffinized on a hot plate, cleared in xylene (10 min), rehydrated through descending alcohol grades (100%, 90%, 70%, 50%; 2 min each), and rinsed in distilled water. Sections were flooded with carbol fuchsin, intermittently heated for 30 minutes, gently washed (8 min), decolorized with 1% acid-alcohol (1-2 min), rinsed, counterstained with methylene blue (1-2 min), washed, dehydrated in absolute alcohol (two changes, 1 min each), cleared in xylene (two changes, 2 min each), and mounted in DPX. Oil-immersion examination revealed pink rod-shaped acid-fast bacilli in positive sections.

Isolation and Confirmation of *Mycobacterium avium* subsp. *paratuberculosis* (MAP)

Faecal samples underwent decontamination with 0.9% hexadecylpyridinium chloride (HPC) to suppress contaminating bacteria. After centrifugation, the middle semi-solid layer was collected and swirled into 40 mL 0.9% HPC, then left undisturbed for 18-24 hours at room temperature. Decontaminated sediment (0.2 mL) was inoculated onto Herrold's Egg Yolk Medium (HEYM) supplemented with mycobactin J using sterile swabs, spreading evenly across slants. Slants were incubated at 37 °C for up to 26 weeks, initially slanted for 4-5 days with loose caps, then upright after moisture evaporation. Cultures were monitored every 15 days for 180 days (Denaives *et al.*, 2007). HEYM preparation involved a mineral salt solution (beef extract, peptone, NaCl, sodium pyruvate, glycerol, agar; pH 7.6-7.8), autoclaved, then mixed at 45-50 °C with 2% malachite green, homogenized egg yolk, and mycobactin J (dissolved in ethanol). Medium (15-20 mL) was dispensed into McCartney bottles, slanted at 45°, solidified, sterility-checked overnight at 37 °C, and stored at 4 °C. Suspected MAP colonies were harvested (5-6 loopfuls) in saline, heat-inactivated (72 °C, 2 h), washed, and processed for DNA extraction using lysozyme, SDS, proteinase K, CTAB/NaCl, chloroform:isoamyl alcohol extraction, and isopropyl alcohol precipitation. Purified DNA was screened by IS900 PCR with P90/P91 primers (Millar *et al.*, 1995), yielding a 413 bp product visualized on

2% agarose-ethidium bromide gel electrophoresis for molecular confirmation.

Results

In the present study, 250 goats (162 females, 88 males) slaughtered at the Cantonment Board Slaughterhouse in Mhow (Indore) were screened for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection, the causative agent of paratuberculosis (Johne's disease). The animals originated from the Malwa region (Indore, Dewas, Mhow, Manpur, Maheshwar) and ranged in age from 6 months to 4 years. Overall prevalence was 38.80% (97/250), based on gross pathological lesions and Ziehl-Neelsen-stained faecal smear examination, which revealed characteristic pink short rods (0.5-1.5 µm) indicative of MAP. Tissue smear analysis showed 24.40% (61/250) positivity in intestinal samples and 21.60% (54/250) in mesenteric lymph nodes.

Faecal microscopy confirmed MAP in 97 goats (38.80%). Results are summarized in Table 1, with visual documentation in Fig 1-2. Faecal smear examination using Ziehl-Neelsen staining is a highly effective, traditional, and widely used method for detecting *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in both clinical and subclinical cases, offering superior sensitivity for Johne's disease diagnosis (Manning and Collins, 2001) [14].

Prevalence varied significantly with body condition score (BCS): emaciated goats (+ 1) showed the highest group prevalence (61.11%, 11/18 positive), followed by weak (+ 2) at 56.86% (29/51), normal (+ 3) at 35.29% (36/102), and healthy (+ 4) at the lowest (26.58%, 21/79). Overall contributions: emaciated 4.40%, weak higher impact due to numbers (Table 2). Age-wise, among 250 goats: highest MAP prevalence in >2 years (44.93%, 138 goats), followed by 6-12 months (35.71%, 28 goats) and lowest in 1-2 years (29.76%, 84 goats) (Table 3). Out of the 250 goats screened (162 females, 88 males), MAP prevalence was higher in females (25.20%) than males (13.60%) (Table 4). After 0.75% HPC decontamination, 15 faecal and 12 tissue samples were cultured on Herrold's Egg Yolk Medium (HEYM) at 37 °C for 180 days, observed biweekly. Growth appeared after 8-16 weeks: no colonies at 0-4 weeks; yellowish discoloration at 4-8 weeks; pinpoint to 1 mm translucent colonies at 4-8 weeks; 1-2 mm opaque, rough, granular colonies at 8-12 weeks; and 4-5 mm rough colonies with nipple-like centers at 12-16 weeks. Positive growth occurred in 3/15 (20%) faecal and 4/12 (33.33%) tissue samples. (Fig 3, 4, 5 and 6) The MAP growth (5-6 loopful) was harvested and the DNA was screened by IS900 PCR using P90 and P91 primers (Millar *et al.*, 1995), then presence of a specific PCR product (413 bp) was visualized on 2% agarose ethidium bromide gel electrophoresis. (Fig 7) Out of the 15 faecal sample culture isolates, 3 (20%) were tested positive for MAP infection, while 2 (16.67%) of the 12 tissue sample culture isolates were positive for the MAP infection.

Table 1: Prevalence of paratuberculosis in goats based on faecal smear examination

No.	Particulars	No. of goats (n = 250)	Percentage (%)
1.	Positive for MAP	97	38.80
2.	Negative for MAP	153	61.20

Table 2: Prevalence of paratuberculosis based on physical profile and body condition score (BCS) in goats

S. No.	Physical profile	Body condition score	No. of goats (n = 250)	Positive for MAP	Group prevalence (%)	Overall prevalence (%)
1.	Emaciated	+ 1	18	11	61.11	4.40
2.	Weak	+ 2	51	29	56.86	11.60
3.	Normal	+ 3	102	36	35.29	14.40
4.	Healthy	+ 4	79	21	26.58	8.40

Table 3: Age-wise prevalence of paratuberculosis in goats

Age group	Number of Goats (n = 250)	Positive for MAP	Group prevalence (%)	Overall prevalence (%)
6-12 months	28	10	35.71	4.00
1-2 years	84	25	29.76	10.00
Above 2 years	138	62	44.93	24.80

Table 4: Sex-wise prevalence of paratuberculosis in goats

Sex	Number of goats (n = 250)	Positive for MAP	Group prevalence (%)	Overall prevalence (%)
Female	162	63	38.89	25.20
Male	88	34	38.64	13.60

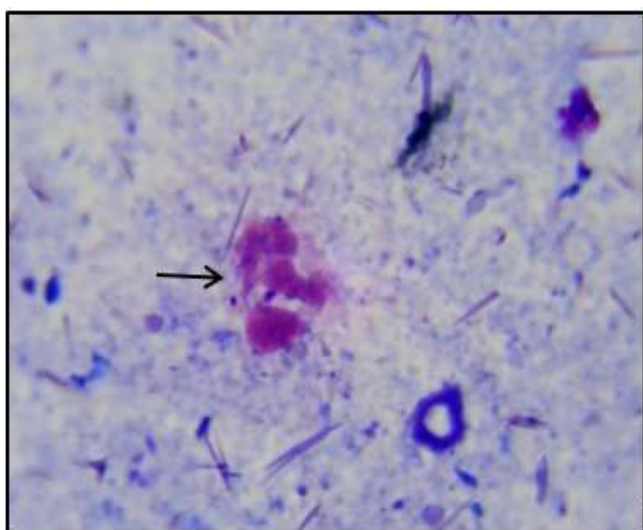
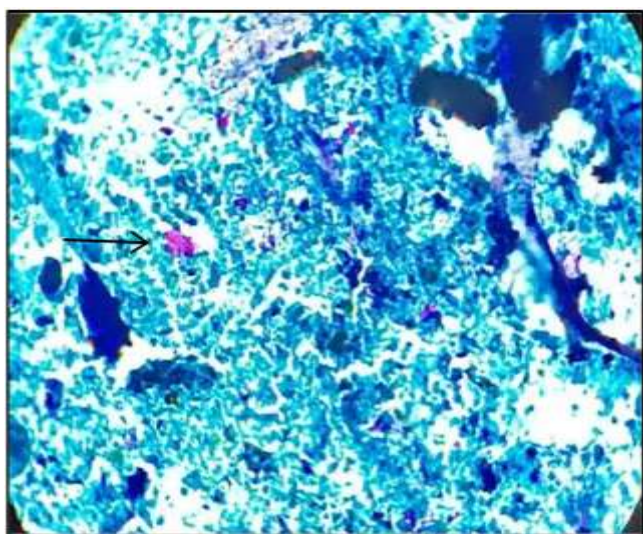
**Fig 1:** Photomicrograph showing clump of pink coloured MAP organisms in the faecal smear (Ziehl-Neelsen stain, ×1000) (arrow)**Fig 2:** Photomicrograph showing clump of pink coloured MAP organisms in the faecal smear (Ziehl-Neelsen stain, ×1000) (arrow)**Fig 3:** Primary colonies very small 1mm diameter (4 weeks)**Fig 4:** Colourless, translucent and hemispherical colonies (8 weeks)



Fig 5: Margins round and even, smooth and glistening surface (12 weeks)



Fig 6: Roughness increased with age (16 weeks)

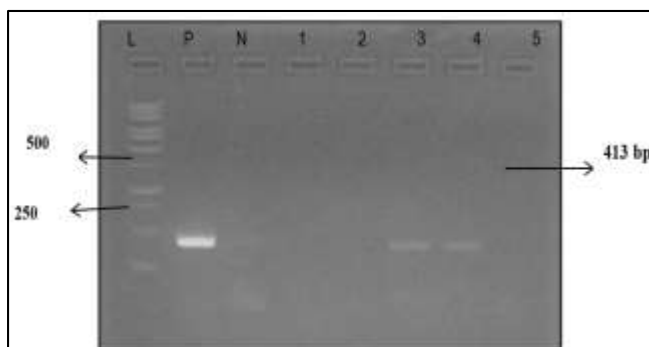


Fig 7: Agarose gel electrophoresis (2%) showing MAP specific amplicon (413 bp) using IS900 PCR in harvested HEYM culture growth
Lane L: 250 bp ladder
Lane P: Positive control (MAP DNA)
Lane N: Negative control (NFW)
Lane 3 and 4: DNA samples positive for IS900
Lane 1, 2 and 5: DNA samples negative for IS900

Discussion

The study should be elaborately discussed with the significance of the results with the help of earlier work and reports. The 38.80% prevalence of paratuberculosis observed through faecal smear examination in this study aligns closely with findings reported by Singh *et al.* (2007) [23], Shah *et al.* (2012) [23], Singh *et al.* (2013a) [20], Hajra *et al.*

(2014) [8], Bhat *et al.* (2018) [2] and Hamid *et al.* (2018) [9]. However, lower prevalence rates were noted in studies by Sulficar *et al.* (2009) [26], Barad *et al.* (2014) [1] and Haque *et al.* (2022) [10]. The body condition scoring method in the present study was adopted from the body condition narrated by the Ghosh *et al.* (2019) [6] in goats. Windsor (2014) [30] reported that the progression of MAP infection to clinical paratuberculosis can result in profound weight loss. Similarly, Singh *et al.* (2013a) [20], Tafti and Rashidi (2000) [27], Idris *et al.* (2021) [12] and Hernandez *et al.* (2022) [11] stated the same in their findings. In the present study, the results were in contrast to the previous findings and most positive for MAP was recorded in apparently healthy animals which supports the investigation of Tharwat *et al.* (2025) [28] that older goats were at higher risk than younger goats due to the incubation period ranging up to 1 year and apparent clinical signs. In the present study paratuberculosis infection among goat population was more common in goats aged above 2 years followed by 1-2 years and least among the goat population of 6-12 months. In accordance with the present study similar results were published by Singh *et al.* (1998) [21], Tripathi *et al.* (2006) [29], Hajra *et al.* (2014) [8] and Hamid *et al.* (2018) [9]. In contrast to the above findings supported by researchers Haque *et al.* (2022) [10] and Stefanova *et al.* (2023) [25] reported that inoculated animals showed MAP after 1 year became faecal shedder post infection while showed cell mediated immune response from 9 weeks post inoculation Bhat *et al.* (2018) [2] screened at 26 weeks confirmed MAP infection in 4 (26.67%) faecal isolates and 3 (25%) tissue isolates, validated by IS900 PCR testing. The growth pattern of MAP on different culture media reveals pin point size colonies appearing during initial stages of growth. Singh *et al.* (1998) [21] revealed 22.7% faecal culture positive while Corpa *et al.* (2000) confirmed 76% faecal culture positive.

Conclusion

This study investigated paratuberculosis in 250 goats analyzing faecal, blood, intestine, and lymph node samples. The prevalence of MAP was recorded as 38.80%, with 97 goats positive via faecal smear, confirmed by Ziehl-Neelsen staining. Prevalence varied by body condition: emaciated (61.11%), weak (56.86%), normal (35.29%), healthy (26.58%). Adult goats (>2 years) had the highest prevalence (44.93%), followed by 6-12 months (35.71%) and 1-2 years (29.76%). Sex-wise, the non-significantly different prevalence was noted in females (38.89%) and males (38.64%). Antemortem findings like diarrhoea, emaciation, and rough hair coats were evident during the study. Culture on HEY medium showed growth in 4/15 faecal (26.67%) and 3/12 tissue (25%) samples, with IS900 PCR confirming MAP in 3 faecal (20%) and 2 tissue (16.67%) samples.

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