Molecular and Pathological Characterization of *Mycobacterium Avium* Subspecies *Paratuberculosis* Infection in Buffaloes of Jabalpur Region

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Abstract: ***Background**: Mycobacterium avium subspecies paratuberculosis (MAP) causes chronic and debilitating disease in ruminants called paratuberculosis or Johne's disease. It not only affects production of animals but also downturns the economy of the country. Thus, the aim of the present study was detection and documentation of MAP infection in buffaloes of Jabalpur region of Madhya Pradesh, India. **Methods**: A total of 148 buffaloes were screened in the study and 55 faecal, 25 blood and 20 tissue samples were collected from suspected animals. Preliminary test was performed with Ziehl-Neelsen (ZN) staining of faecal and tissue samples followed by indirect ELISA, histopathology and IS900 PCR analysis of ZN positive samples. **Result**: The results of present study revealed 20% seropositivity in buffaloes of the region. Further, 28.57% ZN positive faecal samples and 100% ZN positive tissue samples were found positive in IS900 PCR. The most significant pathological findings included gross thickening, congestion and severe corrugation on mucosal surface of ileo-caecal region of intestine. Microscopically, granulomatous reactions with cellular infiltration were observed in various regions of the intestine. Therefore, a conclusion of moderately prevalent MAP infection with moderate to severe pathological changes in buffaloes of the Jabalpur region of Madhya Pradesh can be drawn.***

Keywords: Paratuberculosis, Johne's disease, buffalo health, MAP infection, PCR detection, pathological changes.

1. Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) causes one of the most important chronic infectious diseases of domestic, wild and zoo animals called paratuberculosis or Johne's disease (JD). It results in huge economic losses to the livestock farmers due to decline in livestock production, increased morbidity and mortality as well as the finances of treatment (Eslami *et al*., 2019). MAP is a slow growing bacillus with acid-fast property attributing to presence of mycolic acid in its cell wall. These bacilli enter into the body commonly through faeco-oral route and become latent in macrophages of intestinal epithelium and lymphoid tissues. After several months, they multiply extensively in intestine causing malabsorption, diarrhoea, progressive emaciation and loss of body condition (Tripathi *et al*., 2007). Subsequently, MAP gets disseminated into udder, supra mammary lymph nodes, vaginal and other organs (Singh *et al.*, 2018).

Paratuberculosis is endemic in various regions of the world with animal level prevalence ranging from 02.31% to 29.80% 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 ante-mortem and post-mortem sub-clinical paratuberculosis was documented 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 review of available published literature no study regarding paratuberculosis had been conducted in clinically affected buffaloes of Jabalpur region of Madhya Pradesh, India. Therefore, the present work was carried out to conduct the molecular and serological detection of *Mycobacterium avium* subspecies *paratuberculosis* in biological samples and to document the MAP associated pathological changes in buffaloes of the region.

2. Materials and Methods

Study material

In this study, around 148 buffaloes belonging to various organized and unorganized dairy farms in and around Jabalpur were screened for the present study irrespective of age, sex and breed. Samples were collected from 95 animals showing clinical features suggestive of paratuberculosis including 55 faecal, 25 blood and 20 tissue samples over the period of seven months. Faecal samples were directly collected from the rectum and placed in sterile containers which were properly sealed and labelled. The blood samples were aseptically collected from external jugular vein in clot activating vacutainers. The serum was separated and stored at -20ºC for ELISA. Representative tissue samples from intestine and mesenteric lymph nodes (MLN) were collected, labelled and transported to laboratory under refrigerated condition (4ºC). Individual tissue was divided into three parts, for cytological smear preparation, histological tissue processing and molecular diagnosis (-20ºC).

Direct microscopy of cytological smears

The cytological smears were prepared from the collected faecal and tissue samples followed by staining with Ziehl-Neelsen (ZN) stain as per the method described by Chauhan (2003). Briefly, the heat fixed faecal and tissue smears (prepared from intestinal scrapings and impressions of mesenteric and ileo-caecal lymph nodes) were flooded with carbol fuschin and heated with low flame. After thorough washing, the smears were decolourized with acid-fast decolourizer and counterstained with methylene blue. Smears exhibiting presence of short acid-fast bacilli (AFB) in clumps or in dispersed form were considered positive, and if neither of the two forms could be observed then the sample was considered negative.

Serodetection by indirect ELISA

The indirect ELISA was conducted for detection of anti-MAP antibodies in serum samples using standard commercially available in vitro Enzyme Linked Immuno-Sorbent Assay kit (PARACHEK® 2, M/s. Thermo fisher scientific). Briefly, 100 µl of diluted serum samples and controls were added to the designated wells of the antigen coated plate. After proper incubation and washing, conjugate and enzyme substrates were added to the plate. Optical density was measured at 450nm absorbance.

Detection of MAP specific gene by PCR

For molecular detection, the DNA was extracted from the faecal and tissue samples which were found positive in ZN staining using the method described by Verma *et al*. (2025). Extracted DNA samples were amplified using *IS 900* specific primers as mentioned below.

a) F'90: 5'- GAA GGG TGT TCG GGG CCG TCG CTT AGG -3'

b) R'91: 5'- GGC GTT GAG GTC GAT CGC CCA CGT GAC -3'

Briefly, initial denaturation of extracted DNA sample was conducted 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. Positive and negative controls were used for every reaction and amplified product of expected size 413bp was visualized under UV light.

Gross and histopathology

During the present study, detailed postmortem examinations of 27 buffaloes was conducted and history, body condition of carcasses, and gross lesions were recorded. Representative tissue samples from intestine and mesenteric lymph nodes (MLN) of all the carcasses were collected in buffered formalin, labelled, processed and stained with Hematoxylin and Eosin (H&E) stain and ZN stain as per the standard procedure (Gridley, 1960).

3. Results and Discussion

Clinical observations

Out of 75 buffaloes included in ante-mortem examination of the study, three buffaloes were clinically weak, emaciated and non-diarrheic with shedding AFB in faeces and four buffaloes were found in lethargic condition with rough body coat, anemia, emaciation and diarrhea which was evident by soiled hind quarter, enteritis and fecal shedding of AFB. Similar results have been reported by Khan *et al*. (2010) and Jatav *et al*. (2020).

Detection of AFB in faecal smears

On examination of faecal smears from 55 buffaloes**,** 07 (12.72%) faecal smears were found positive for the presence of acid-fast bacilli (AFB). Pink coloured short bacilli were observed in positive smears as shown in Figure 1. This positivity was correlated with the age of the animals and it was recorded that 01/15 (06.66%) animals of 2-4 years, 03/24 (12.50%) animals of 4-6 years and 03/10 (30.00%) animals of above 6 years of age were shedding AFB in faeces. However, animals belonging to age group of 0-2 years were negative for presence of AFB in faecal microscopy. This observation indicated that buffaloes of above 6 years age had a higher predilection for AFB in faecal samples.

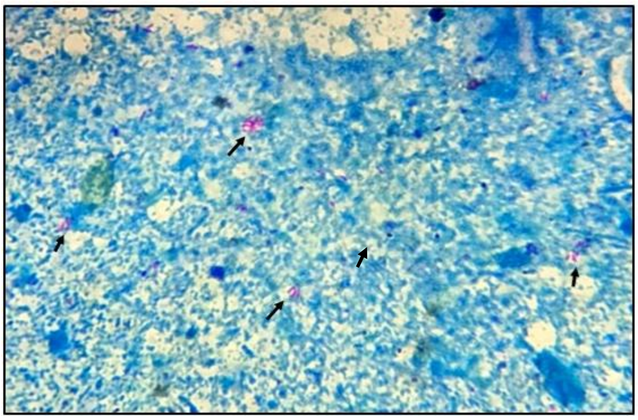

Figure 1: Microphotograph of acid fast bacilli (arrows) in faecal smear; ZN X1000.

Similarly, a correlation of AFB shedding in faecal samples and gender of the animals was observed. Out of 11, one (09.90%) male buffalo was found positive for AFB in faecal samples whereas, 06 (13.63%) out of 44 female animals were positive for AFB in faecal samples. This indicates that a higher proportion of females have the presence of AFB in faecal samples as compared to males.

The present findings are in accordance with the findings of Kaur *et al.* (2011), Sikander *et al*. (2012), Singh *et al.* (2018), Sharma *et al*. (2020) and Agrawal *et al*. (2021) in large ruminants. Shedding of MAP bacteria in faeces starts even before onset of clinical infection making it potent source of infection (Eamens *et al*., 2000). Thus, faecal microscopy is considered as an excellent screening test for detection of clinical and subclinical MAP infection in a herd (Agrawal *et al*., 2021). In this study, increased faecal AFB shedding was recorded with age which is indicative of prolonged incubation periods (Singh *et al*., 2016).

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Detection of AFB in tissue smears

During the study period total 20 buffaloes' carcasses were examined and tissue smears were prepared from mucosal surface of intestine and lymph nodes. On examination of ZN stained tissue smears, 05 (25.00%) buffaloes were positive with their tissue smears revealing typical acid-fast bacilli either individually or in clumps (Figure 2). Observation of AFB in cytological tissue smears is highly suggestive of paratuberculosis infection.

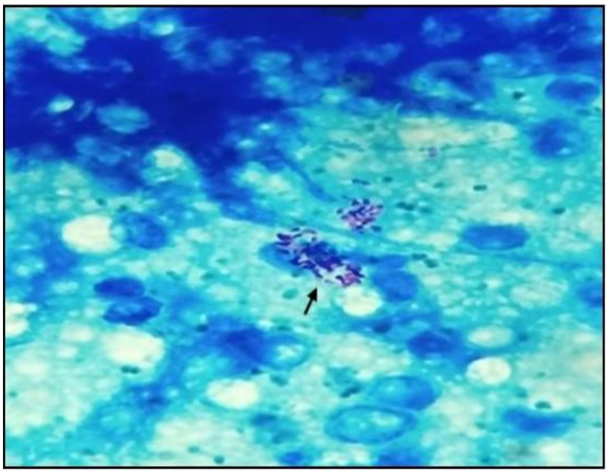

Figure 2: Microphotograph of AFB in tissue smear of buffaloes; ZN X1000.

The presence of AFB in tissue smears was correlated with age of the animals and it was observed that 01 (06.66%) out of 04, 01 (08.33%) out of 06, and 03 (30.00%) out of 06 tissue samples were belonging to animals above 2 years, 4 years and 6 years of age, respectively. This correlation revealed that buffaloes above 6 years of age had a higher predilection for AFB in tissue samples. Similarly, higher proportion of female buffaloes (16.66%) showed the presence of AFB in tissue samples as compared to male buffaloes (21.05%). This higher AFB positivity in females may be attributed to decreased immunity allowing proliferation of MAP in susceptible animals and the development of clinical symptoms such as diarrhoea, decreased milk production, and weight loss (Cheng *et al*., 2020).

Serodetection of anti-MAP antibodies

Presence of anti-MAP antibodies was detected in serum samples of 25 buffaloes and 05 (20.00%) samples were found highly positive. One (11.11%) out of 09 and 04 (30.76%) out of 13 serum samples belonging to animals of 4-6 years and above 6 years of age, respectively were found seropositive indicating a higher predilection for anti-MAP antibodies in buffaloes of above 6 years of age. Similarly, a higher proportion of female ruminants (21.05%) showed the presence of anti-MAP antibodies as compared to male ruminants (16.66%).

Results observed in the present study are in harmony with previous reports (Desio *et al*., 2013; Gupta *et al*., 2019; Sharma *et al*., 2020; Zhao *et al*., 2021). Enzyme linked immunosorbent assay (ELISA) is conducted in the present study to detect the humoral immune response to clinical and subclinical MAP infection in buffaloes. Serological survey is a highly recommended method for assessing the prevalence of disease in endemic areas. It is used by most of the PTB control programmes to detect potential infection of MAP at herd and animal levels as it is cost-effective and less time-consuming (Gupta *et al*., 2019; Sharma *et al*., 2020).

Higher seropositivity for anti-MAP antibodies observed in female buffaloes suggested higher susceptible in females due to stress of calving and higher milk production. Cruz-Estupinan *et al.* (2022) reported higher seroprevalence of anti-MAP antibodies in older bovines as observed in this study and reported older age as a risk factor associated with MAP infection. Further, the incubation period of paratuberculosis is much longer in large ruminants supporting higher seropositivity in older large ruminants.

Molecular detection of MAP specific gene

In order to confirm the presence of MAP specific gene, Ziehl-Neelsen (ZN) positive faecal and tissue samples were processed for DNA extraction and the extracted DNA was analyzed by IS*900* PCR. A total of 12 ZN positive samples were subjected to IS*900* PCR comprising of 07 faecal samples and 05 tissue samples. Of these, total 07 samples including 02 (28.57%) faecal samples and all 05 (100%) tissue samples, revealed IS*900* specific amplicon of 413bp (Figure 3) and were considered positive for MAP specific gene. These positive buffaloes were considered infected with paratuberculosis.

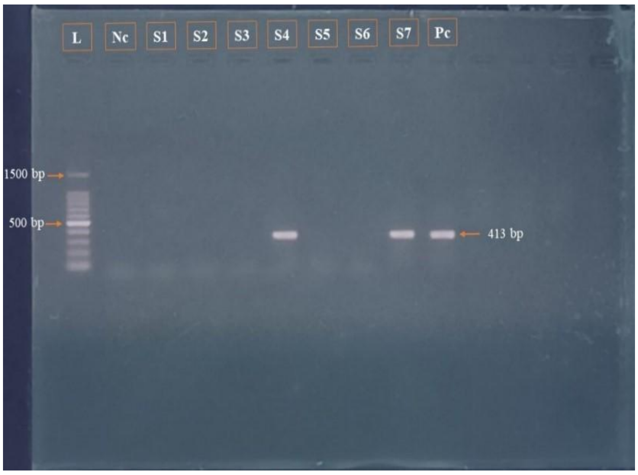

Figure 3: PCR amplification of MAP specific *IS900* gene (413bp) in buffalo faecal samples. L – Ladder; Nc - Negative control; S1-8 - Samples; Pc - Positive control.

The results of the present study corroborate with the observations of Sivakumar *et al.* (2006); Tripathi *et al.* (2007); Khan *et al.* (2010); Kaur *et al*. (2011); Pereira *et al.* (2020); Verma *et al.* (2025) in buffaloes. PCR is highly sensitive and specific diagnostic method for identification of MAP infection in faecal and tissue samples which leads to amplification of specific gene of MAP genome (Jatav *et al.,* 2018). Since *IS900* gene is more prevalent in the MAP genome it makes an excellent target for direct faecal and tissue PCR and thus, used for molecular detection in this study (Kaur *et al*., 2011 and Singh *et al.,* 2016).

In contrast to 100% positivity observed in ZN positive tissue samples, only 28% of ZN positive faecal samples were found positive in PCR. This gap in positivity may be due to presence of environmental acid fast bacteria (e.g.

Mycobacterium bovis & *Nocardia*) in faecal samples, presence of inhibitors or less quantity of MAP organism present per gram faeces (Tripathi *et al*., 200; Singh *et al.,* 2016; Sharma *et al*., 2020).

Gross pathology

In the present study, intestine and corresponding lymph nodes of MAP specific gene positive carcasses were included for detailed pathological examination. The MAP positive buffalo carcasses included in pathological studies were above 4 years of age and revealed rough body coat, emaciated, pale to anaemic mucus membrane and soiled hind quarter as shown in Figure 4. The subcutaneous and visceral fat of all the carcasses was gelatinized with straw discolouration.

Figure 4: Emaciated carcass of MAP positive buffalo.

Severe thickening of intestinal wall was observed in most of the animals. This thickening was found predominantly near ileo-caecal junction of the intestine. Mucosal surface of thickened parts of intestine showed congestion along with severe corrugations which did not disappears on stretching in almost all the infected animals. However, one case was observed with moderate corrugation in the intestine. The corrugations were observed mostly in colo-rectal (Figure 5) and ileum regions of the intestine.

The mesenteric lymph nodes (MLN) were enlarged and congested with thick capsule in all the animals (Figure 6). Caseation in lymph nodes is commonly reported in MAP affected goats, however, it was not observed in any of the cases included in the present study. These pathological finding are in corroboration with those reported by Sivakumar *et al*. (2006); Khan *et al.* (2010); Gautam *et al.* (2018) and Jatav *et al.* (2018). The thickening of intestinal wall and corrugations were observed due to oedema and excessive accumulation of mononuclear inflammatory cells and epithelioid macrophages in the mucosa and submucosa of the intestine (Kordshouli *et al.,* 2016). Further, thickening of MLN may have occurred due to fibrous connective tissue proliferation (Jatav *et al*., 2018).

Figure 5: Severe corrugations and haemorrhages in mucosa surface of rectum in MAP positive buffalo

Figure 6: Enlarged, corded and congested mesenteric lymph nodes (arrow) of MAP positive buffalo

Histopathology

Microscopically, intestinal sections revealed sloughing of epithelial lining and thickening of the intestinal villi with flat and wide tips (Figure 7). The granulomatous reaction with infiltration of lymphocytes, macrophages, epithelioid and multinucleated giant cells was observed in mucosa of the various parts of the intestine in most of the cases (Figure 8 and 9).

The microscopic sections of MLN, revealed fibrous thickening of capsule with infiltration of inflammatory cells and congestion of blood vessels. Pink coloured acid-fast bacilli were observed individually or in clusters in the epithelioid macrophages of ZN stained tissue sections from intestine and lymph nodes suggestive for MAP (Figure 10).

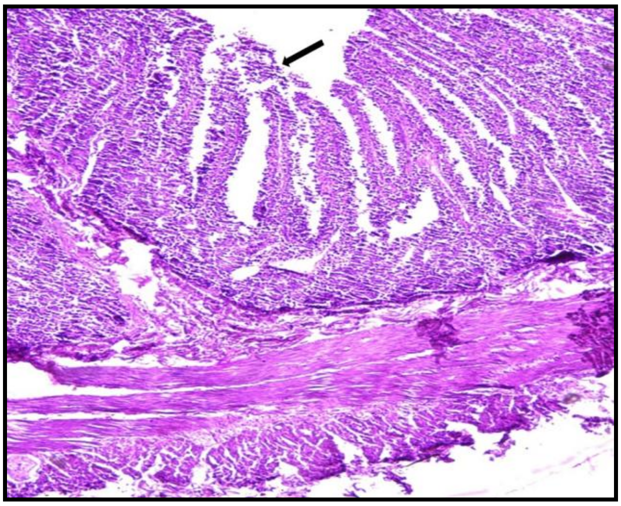

Figure 7: Microscopic section of jejunum of buffalo showing eroded epithelium and severe cellular infiltration with disruption of muscularis mucosae. H&E X40.

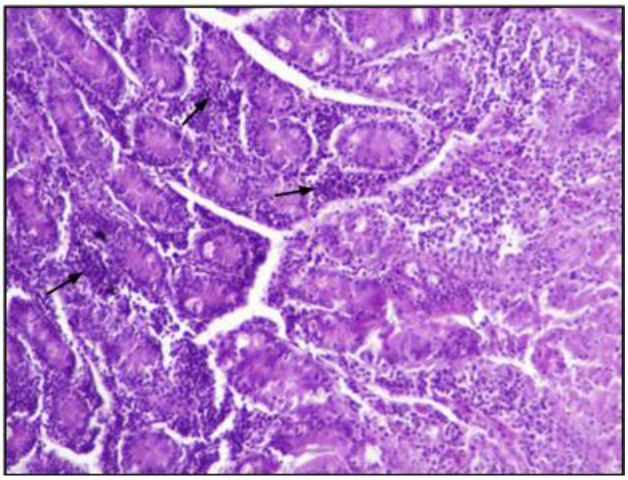

Figure 8: Microphotograph of intestinal section from MAP positive buffalo showing infiltration of inflammatory cells (arrows) in between crypts; H&E X100.

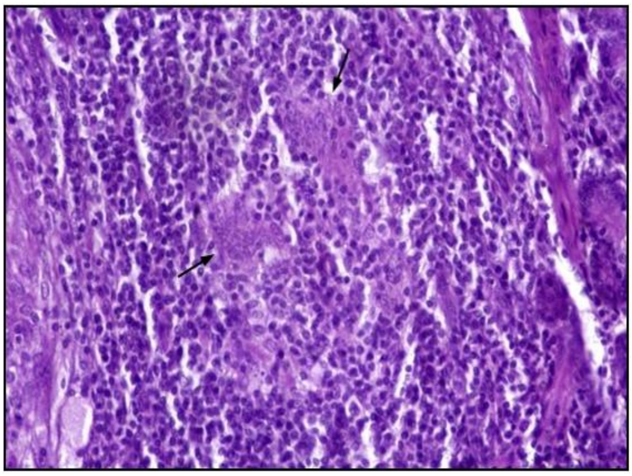

Figure 9: Microphotograph of ileal section from MAP positive buffalo showing early granuloma formation (arrows) in the centre of peyer's patches along with cellular infiltration. H&E X200.

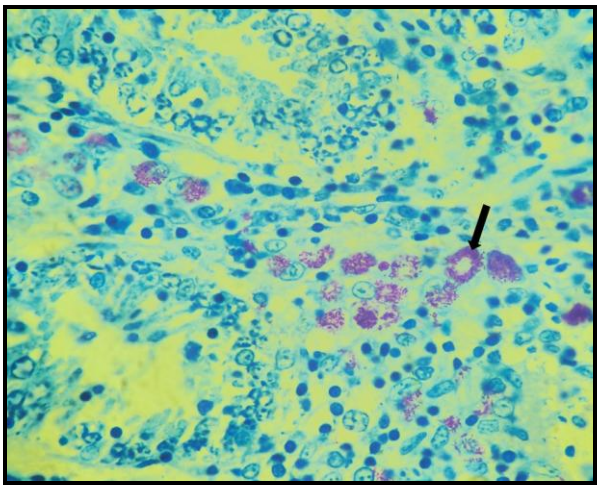

Figure 10: Microphotograph of intestine section from MAP positive buffalo showing Pink coloured acid-fast bacilli in clusters in the epithelioid macrophages. ZN 400X.

Increased thickness of mucosa and submucosa and villous atrophy in affected intestine can be due to infiltration of inflammatory cells and associated oedema as also suggested by Kordshouli *et al.* (2016) and Pereira *et al*. (2020). This infiltration of inflammatory cells suggests a strong cell mediated immune response that might have restricted the replication of bacteria by phagocytosis. The lesion around the blood and lymphatic vessels suggested the possibility of haematogenous and lymphatic route of transmission of MAP throughout the body of the affected animals.

4. Conclusion

Serological, molecular and pathological examinations were conducted in the present study for detection of MAP in buffaloes of Jabalpur region in Madhya Pradesh, India. The results obtained in the study indicate active presence of paratuberculosis infection in the buffaloes of the region. Since most of the infected animals shed the bacteria in faeces, they can be considered as potent source of infection for other susceptible animals and humans of the region. This emphasizes on the need for regular surveillance and implementation of measures to control and eradicate the disease.

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