



ASSESSMENT OF *Mycobacterium paratuberculosis* BIO-LOAD IN PATIENTS SUFFERING WITH THYROID AND ARTHRITIS DISORDERS USING MULTIPLE TESTS

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(Received 12 August, 2024; accepted 10 December, 2024)

ABSTRACT

Johne's disease in ruminants is caused by *Mycobacterium avium paratuberculosis* (MAP), linked to inflammatory bowel and Crohn's disease in humans, with milk from infected ruminants being the primary transmission source. This study was aimed to estimate the bio-presence of MAP from the confirmed cases of thyroid and arthritis clinical samples (blood and serum) which were collected from the Community Healthcare Center, Bhangel campus, Noida NCR region, India. One hundred and eleven blood samples (76 thyroid and 35 arthritis samples) were collected and screened by IS900 blood PCR, indigenous ELISA, and Taqman probe qPCR IS1311 PCR_REA. Of the serum samples (111) screened by indigenous ELISA, 19 (17.1%) were positive for MAP antibodies (thyroid, 18.4% and arthritis, 14.2%). Of the blood samples evaluated, 10.8 and 11.7% were confirmed positive for MAP by IS900 PCR and Taqman probe qPCR, respectively. The bio-incidence of MAP in thyroid and arthritis patients was 9.2-14.2 and 10.5- 14.2%, respectively, as assessed by IS900 PCR and Taqman probe qPCR. Substantial agreement was found between ELISA and PCR, and IS1311 PCR_REA molecular strain typing showed that 'Indian Bison type' was prevalent biotype. The study indicated moderate exposure of human population to MAP in Noida NCR region and presence 'Indian Bison type' biotype suggests a possible zoonotic transmission pathway for MAP from cattle products to humans. In order to validate these findings, in broader cohorts, larger sample size of thyroid and arthritis patients is required.

Keywords: Crohn's disease, Indian bison type, IS900 PCR, indigenous ELISA, *Mycobacterium avium* subspecies *paratuberculosis*

INTRODUCTION

Paratuberculosis (ParaTB) is an infectious disease of animals, caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). ParaTB has widest host range as it affects both domestic and wild ruminants and human (Chaubey *et al.*, 2016; Gupta *et al.*, 2019). MAP peptides and human homologs show high level of homology which support a possible mycobacterial role in triggering the autoimmune illnesses (Sechi *et al.*, 2008; Moghadam *et al.*, 2022; Ozana *et al.*, 2022). MAP is a common environmental trigger in inflammatory and autoimmune diseases. It is the known cause of Johne's disease, Crohn's disease, sarcoidosis, juvenile sarcoidosis, autoimmune diabetes, autoimmune thyroiditis, multiple sclerosis, rheumatoid arthritis, and Parkinson's disease (Chaubey *et al.*, 2020; Moghadam *et al.*, 2022). While MAP may trigger autoimmune antibodies through its heat-shock proteins, it is more difficult to assign a role in diseases where auto-antibodies are a primary feature, as it may participate in the granuloma (Dow, 2024). Mycobacterial heat shock protein-65 shares conformational and sequential elements with a number of human proteins. It is predicted that MAP

can trigger auto-antibodies, leading to auto-immune disorders such as multiple sclerosis, CD, type 1 diabetes, and thyroiditis, among others, through molecular mimicry mechanisms (D'Amore *et al.*, 2010; Dow, 2011). The pathophysiology of autoimmune hepatitis, biliary cirrhosis, and rheumatoid arthritis disease has also been linked to mycobacterial HSP65 (Moudgil *et al.*, 1997; Dow, 2011). Additionally, anti-glutamic acid decarboxylase antibodies are predicted to be triggered by MAP HSP60/65, leading to the destruction of the pancreas (Jones *et al.*, 1993; Naser *et al.*, 2013).

The MAP transmission occurs through various routes; for instance in human population it passes through milk as it escapes pasteurization process and is a one health emergency (Gupta *et al.*, 2021; Dow and Alvarez, 2022). MAP is found in the tissues and blood of inflammatory bowel disease (IBD) patients (Gupta *et al.*, 2021), human breast milk, and blood samples of CD patients (Naser *et al.*, 2004; Aitken *et al.*, 2024). The development of CD is associated with the NOD2 gene; NOD2 mutations leads to compromised innate response to mycobacterial infection and may lead to inefficient clearance of intracellular MAP (Ogura *et al.*, 2001; Goyette *et al.*, 2007; Azzam *et al.*, 2012). In India, MAP sero-prevalence was estimated to be 23.4% in suspected human populations (Singh *et al.*, 2011; Singh *et al.*, 2014b). However, the information on its association with various health problems is not acknowledged and considered seriously by Indian scientists and physicians. Few studies confirmed that patients with MAP infection successfully resolved IBD and Crohn's disease using anti-MAP antibiotics, demonstrating the link between treatment and disease in humans (Singh *et al.*, 2016; Gupta *et al.*, 2021). MAP genetics and genomics data suggest molecular diagnostic assays could overcome the limitations of conventional microbiology tests for this fastidious organism (Singh *et al.*, 2014a). The study aimed to assess the prevalence of MAP in NCR region's clinically affected population using microscopy, IS900 blood and fecal PCR test and indigenous ELISA test. Single test is not sufficient for the absolute detection of MAP however culture-based detection is the gold standard method (Chaubey *et al.*, 2016). Therefore, this study was aimed to estimate the bio-presence of MAP from the confirmed cases of thyroid and arthritis clinical samples (blood and serum) collected from the Community Healthcare Center, Bhangel campus, Noida NCR region, India.

MATERIALS AND METHODS

The present study was approved by the Institutional Biosafety Committee (IBSC) vide No. GLAU/IBSC/07/2023 and Registration No. GLAU231120211995). The patients were fully informed about the sampling process and subsequent works on their samples.

Sample collection

A total of 111 blood samples were collected from the Community Healthcare Center Bhangel campus, Noida NCR region (India). Using indigenous ELISA, IS900 PCR, and Taqman probe qPCR, 111 blood samples were screened for MAP in 76 thyroid and 35 arthritis samples.

Indigenous ELISA

The study used 'Indigenous ELISA kit' developed by our group to screen serum samples for MAP infection (Singh *et al.*, 2007). Serum samples were screened using soluble protoplasmic antigen from a 'Indian Bison type' biotype of MAP strain 'S 5'. Serum samples from MAP positive patients confirmed by IS900 PCR were used as positive and negative controls were healthy MAP-negative humans. Optical densities (OD) were measured at 450 nm using iMark ELISA reader (BioRad) and converted as a S/P (sample to positive) ratio in compliance with Collins (2002) in order to determine the extent of MAP infection. Serum samples were considered positive for MAP infection if they were within the cut-off range (>0.40) for the S/P ratio.

IS900 PCR

DNA was isolated and screened for MAP using specific IS900 PCR primers as per Millar *et al.* (1996) and Singh *et al.* (2011). Template DNA, Taq polymerase, 10X PCR buffer, MgCl₂, dNTPs, and primers were used to set up the PCR in a 20 µL volume. Conditions for thermal cycling were established, with an initial 5 min denaturation at 94°C, 37 cycles of denaturation, 30 sec annealing at 64°C, and 30 sec extension at 72°C. Amplicon of 413 bp was considered positive for MAP after separation on agarose-ETBR gel electrophoresis.

Taqman probe qPCR targeting IS900 gene

The study used MAP IS900 primers and probe sequences to MAP conserved regions of IS900 gene as per Gupta *et al.* (2017). The PCR thermal cycling conditions were as follows: initial denaturation, 37 cycles of denaturation, annealing, and extension, followed by 5 min final extension at 72°C.

IS1311 PCR_REA

IS1311 PCR was performed on IS900 PCR positives, with some modifications, using M56 and M119 primers, as described by Sevilla *et al.* (2005). Briefly, PCR was performed in a 30 µL volume with template DNA, 10X PCR buffer, MgCl, dNTPs, and Taq polymerase. Thermal cycling was carried out, with product size of 608 bp considered positive. IS1311 PCR-restriction endonuclease analysis (REA) was carried out using HinfI and MseI (Fermentas, USA) and incubated at 37°C for 2 h. Following electrophoresis on 3.0% agarose gel and ethidium bromide staining, band patterns were observed. The genotype profiles were interpreted as per Whittington *et al.* (2001).

Statistical analysis

The data was analysed statistically using kappa agreement and Fisher's exact test with Graph Pad Instat software to determine the significance of the tests.

RESULTS AND DISCUSSION

The study estimated the presence and level of MAP infection in a pilot scale screening of a community healthcare center at Noida NCR region, using the indigenous ELISA test, a method previously established for other livestock species (Singh *et al.*, 2014a). Nineteen (17.1%) out of 111 serum samples examined by indigenous ELISA test were positive for MAP antibodies. Nevertheless, using an indigenous ELISA kit, 5 patients (14.2%) and 14 patients (18.4%) out of 35 arthritis patients who were screened had thyroid disorders confirmed by third-generation chemiluminescent assays tested positive for MAP antibodies (Table 1). The existence of MAP in human population suffering from diabetes, IBD, and CD have been documented (Singh *et al.*, 2014b; Gupta *et al.*, 2021). Additionally, a population-based study found 35% sero-positivity rates; however, rates were the same for CD and UC patients as well as healthy controls (Collins *et al.*, 2000). No significant difference was found in the rates of MAP infection among the patients suspected of having arthritic and thyroid. Sevilla *et al.* (2005) discovered the presence of high sero-positivity levels in 100% CD patients, 75% animal attendants, and 38% apparently healthy humans. The local ELISA test yielded positive results for 1.9 and 4.2% animal keepers suspected of having CD and those not, respectively (Singh *et al.*, 2011).

Table 1: Percentage bio-incidence of MAP in thyroid and arthritis patients using multiple tests

Disorders/tests	Indigenous ELISA <i>positives (%)</i>	IS900 blood PCR <i>positives (%)</i>	Taqman probe qPCR <i>positives (%)</i>
Thyroid (n - 76)	14 (18.4)	7 (9.2)	8 (10.5)
Arthritis (n - 35)	5 (14.2)	5 (14.2)	5 (14.2)
Total (n - 111)	19 (17.1)	12 (10.8)	13 (11.7)

Further, during screening process, animal attendants who worked with endemic goatherds were more likely to have MAP infection than those who had never handled an animal.

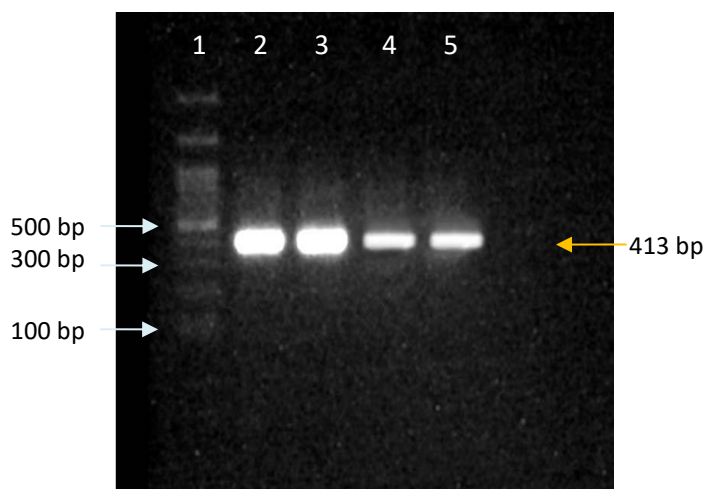


Fig. 1: IS900-specific primers are used to create MAP-specific amplicons (413 bp). Lanes 3-5: DNA samples, Lane 2: Positive control, and Lane 1: 100 bp ladder

The 76 thyroid and 35 arthritis patients were screened by IS900 PCR and Taqman probe qPCR, and 10.8 and 11.7% were found positive for MAP infection, respectively (Table 1; Fig. 1). The analysis of a standard curve plotting using serially diluted (10-fold dilutions) MAP DNA showed 98.5 efficiency and R² value of 0.997 for MAP infection detection. According to a previous study by Singh *et al.* (2011), using indigenous adsorbed ELISA test, CD patients had a higher seroprevalence of MAP (80.0%) than UC patients (4.5%) or presumably normal individuals (15.3%). A higher MAP bio-load was found in domestic and wild ruminants and

animal products, with 34.0, 33.3, 32.8, 25.0, 17.7, and 12.5% of serum samples tested positive in Punjab, Uttarakhand, New Delhi, Himachal Pradesh, Haryana, and Uttar Pradesh, respectively. The presence of higher MAP bio-load in animal milk and milk products suggests that animals are a direct and indirect source of MAP infection in human population (Kumar *et al.*, 2010; Shankar *et al.*, 2010; Singh *et al.*, 2010). Animal keepers and attendants are more vulnerable to MAP exposure due to the moderate to heavy MAP bacterial load in animals and the environment (Dow and Alvarez, 2022).

Table 2: Comparison of diagnostic efficacy of multiple tests in MAP detection

Tests	Combinations (percentage)								
Indigenous ELISA kit	+	-	+	+	-	-	+	-	-
IS900 blood PCR	+	-	+	-	+	+	-	+	-
Taqman probe qPCR	+	-	-	+	-	-	-	-	+
Total (n = 111)	12	92	3	0	1	0	2	0	1
	(10.8%)	(82.8%)	(2.7%)	(0.0%)	(0.9%)	(0.0%)	(1.8%)	(0.0%)	(0.9%)

Comparative diagnostic sensitivity and specificity of indigenous ELISA vs traditional PCR was 93.8 and 97.9%, indigenous ELISA vs Taqman probe qPCR was 100 and 95.0%, and Taqman probe qPCR vs IS900 PCR was 92.9 and 97.0%, respectively (Table 2 and 3). The 50% CD patients and 22% UC patients had MAP bacteria in their blood, indicating that they can spread to various organs and have harmful effects at infection sites (Naser *et al.*, 2004). They have also been connected to an etiological role in Crohn's disease (Naser *et al.*, 2009).

Table 3: Statistical analysis by Quantify agreement with Kappa and Fisher's exact test

Test combinations	Kappa value ± SE	Sensitivity (%)	Specificity (%)
IS900 blood PCR vs Taqman probe qPCR	0.846 ± 0.075	92.9	97.0
Indigenous ELISA kit vs Taqman probe qPCR	0.769 ± 0.090	100.0	95.0
Indigenous ELISA kit vs IS900 blood PCR	0.893 ± 0.061	93.8	97.9

*p-value between the tests was < 0.0001; Kappa agreement: 0.21-0.40 = Fair, 0.41-0.60 = Moderate, 0.61-0.8 = Substantial, and 0.81-0.99 = Perfect.

Recent research indicates that *Mycobacterium paratuberculosis* (MAP) infection is associated with various disorders such as type 1 diabetes, multiple sclerosis, autism, sarcoidosis, autoimmune thyroiditis, and autoimmune arthritis (Cossu *et al.*, 2013; Gupta *et al.*, 2021; Moghadam *et al.*, 2022). Mycobacterial heat shock protein (hsp65), which shares sequence and conformational elements with human proteins, is predicted to trigger auto-antibodies through molecular mimicry mechanisms, leading to autoimmune disorders like multiple sclerosis, type 1 diabetes, thyroiditis, and CD. Mycobacterial hsp65 pathogenesis has been linked to various diseases, including rheumatoid arthritis, scleroderma, Kawasaki disease, autoimmune hepatitis, and primary biliary cirrhosis (Dow, 2024). NOD2 is a crucial susceptibility gene for Crohn's disease, linked to structuring, ileal involvement, and surgery risk. Therefore, NOD2 genetic variants are promising for precision medicine development (Kayali *et al.*, 2024). Mce-family proteins, recognized by the host's immune system, play a role in the virulence of Mycobacteria, with their expression potentially influenced by stress and nutritional status (Hemati *et al.*, 2019).

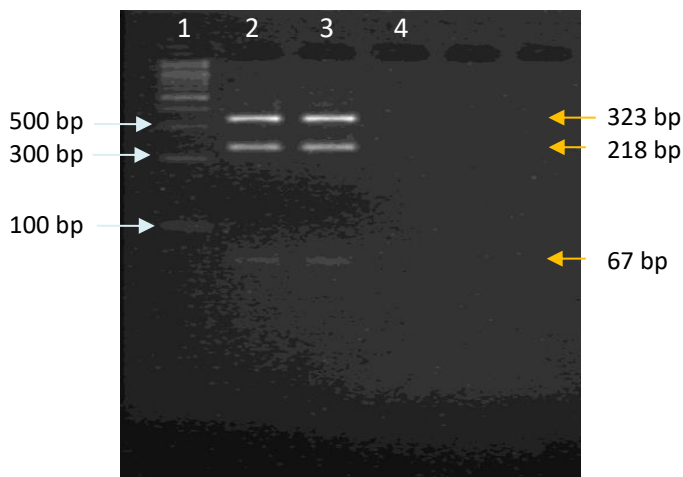


Fig. 2: IS1311 PCR-REA analysis, Lane 1: 100 bp DNA Marker, Lane 2: MAP positive digested DNA as control, Lane 3: Digested product ('Indian Bison type'), Lane 4: MAP negative control

Taqman probe qPCR was used to increase the rate of MAP infection detection in patients with rheumatoid arthritis and thyroid conditions. Comparison between ELISA and PCR assays showed substantial agreement by kappa values and statistical significance with p-value <0.0001 by Fisher's exact test (Table 3). Moreover, IS1311 PCR-REA molecular strain typing showed 'Indian Bison type' was the most prevalent biotype (Fig. 2). Compared to the healthy, MAP IS900 was found in a significantly higher percentage of the cases of Hashimoto's thyroiditis (46.6%). However, the sera tested from above cases displayed higher frequency (38.3%) of reactivity

against MAP3865c (Moghadam *et al.*, 2022). As per previous study, 29.5% patients suspected of having thyroid tested positive for MAP infection in indigenous ELISA (Singh *et al.*, 2014b). Whittington *et al.* (2001) developed PCR-REA method for the characterization of native strains of MAP that infect human and animals. This assay showed that this particular MAP biotype acquired genetic variations as compared to MAP strain K10 and other MAP isolates (Fiocchi, 1998). The presence of MAP in human populations in a variety of suspected conditions indicates that moderate exposure to MAP was experienced by the residents of India's Noida NCR region. Molecular and serological testing helped to establish the presence of MAP in clinical samples and its possible association with various physiological and pathological conditions.

Conclusion: MAP is more pernicious to human health due to its higher survivability. Even with genome sequencing not much is known about how common it is in India. This study employed molecular and serological techniques to identify MAP in clinical samples and explore its correlation with a range of physiological and pathological conditions. Mathura district in UP India had high bioload of MAP. More studies are needed to address the high bio-load of MAP in various disease conditions in India. This will make it easier for diagnosticians and researchers to

comprehend the epidemiological state of illnesses like CD. MAP's impact on animal and human health and environment is significant, prompting one health and other global regulatory agencies to acknowledge its slow-motion zoonosis threat and implement public health mitigation measures.

Acknowledgment: Authors are thankful to Prof. Shoor Vir Singh, Head, Department of Biotechnology and Director, IAH, GLA University, Mathura (India) for providing financial and laboratory facilities. The help extended by Ms. Samiksha Agrawal, Mr. Naresh Pathak and Mr. Deen Dayal is thankfully acknowledged.

Conflict of interest: Authors report no conflicts of interest in this work.

Ethical statement: The Institutional Biosafety Committee (IBSC) has approved the work (under No. GLAU/IBSC/07/2023 and Registration No. GLAU231120211995) and the patients were fully informed about the sampling process and subsequent works on their samples.

Authors contribution: Sheetal Rajput contributed in data collection, analysis, results interpretation, and manuscript preparation; while Saurabh Gupta contributed in conception and critical review.

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