Comparison of IS900 PCR with ‘Taqman probe PCR’ and ‘SYBR green Real time PCR’ assays in patients suffering with thyroid disorder and sero-positive for *Mycobacterium avium* subspecies *paratuberculosis*

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*Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the cause of chronic incurable granulomatous enteritis in domestic livestock and has been associated with number of human autoimmune disorders like thyroiditis. Indigenous ELISA kit was used to monitor the sero-status of MAP infection in the patients of thyroiditis confirmed by pathology laboratories using 3rd generation chemi-luminescent assays. Sero-positive patients for MAP infection were further investigated using traditional PCR and newer (Taqman probe PCR & SYBR green Real time PCR) assays targeting IS900 gene. Screening of 76 patients suffering with thyroid disorders, 36.8% (n=28) were sero-positive for MAP infection. Further screening of blood samples of 28 sero-positive patients by IS900 PCR, Taqman probe and SYBR green Real time based IS900 PCR, 25.0, 32.1 and 35.7% were positive for MAP infection, respectively. Molecular assays targeting IS900 gene revealed ‘good agreement’ in the tests. Taqman probe and SYBR green Real time based IS900 PCR assays were 100.0 and 85.7% sensitive, respectively and highly specific as compared to IS900 PCR for the detecting MAP infection. Study indicated the need for investigating the role of MAP in initiation and progression of thyroid disorders and on the genetic susceptibility of thyroid patients to MAP infection. In the absence of control programmes in the domestic livestock population, there is large scale exposure of human population to MAP infection.

**Keywords:** *Mycobacterium avium* subspecies *paratuberculosis* (MAP), thyroid disorders, indigenous ELISA kit, Taqman probe PCR, Real time IS900 PCR

**Introduction**

*Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the cause of chronic and incurable Johne's disease (JD) in domestic ruminants leading to granulomatous inflammation of intestines. Infected lactating cows, buffaloes and goats1 excrete MAP in their milk. Thus, bacilli are continuously entering human population through milk and milk products2,3. Pasteurized milk and dairy products like cheese may not be always free of MAP infection4. Epidemiological evidences correlated exposure of MAP with incidence of a number of human autoimmune disorders like Crohn’s disease, Rheumatoid arthritis, Type 1 diabetes mellitus (T1DM), Hashimoto's thyroiditis (Hypo thyroidism), Autoimmune arthritis, Multiple sclerosis etc5-10. Clinical symptoms of Crohn’s disease closely mimic those found in animals suffering with Johne’s disease11. Similarly contaminated baby milk powder exposes children and immuno-compromised people (at high risk) to MAP infection12. Consumption of raw milk has been recognized as major risk factor in the development of autoimmune diseases like Hashimoto's thyroiditis. Environmental microorganisms including MAP have been thought to trigger autoimmune responses in genetically susceptible individuals13. In the absence of cost effective indigenous diagnostic kits and high cost of imported kits, there is lack of information on the bioload and biotype profiles of MAP infecting both animals and human population in the country. Despite

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report (Singh et al) of high bioload of MAP in human patients suffering from different kinds of noninfectious (diabetes, liver disorders, anemia, thyroid disorder, abdominal disorders, inflammatory illness & ion imbalance) and infectious disease conditions (tuberculosis & typhoid) from North India, control of MAP infection in the domestic livestock population has not received the highest priority.

It is estimated that 42 million of the total 1.25 billion human population in India suffer from thyroid disorders. Hypothyroidism is the most common ailment affecting one out of ten adults and prevalence of hypothyroidism in India is 11.0% as compared to 2.0% in UK and 4.6% in USA. Sisto et al reported the use of species-specific IS900 probe based PCR to detect MAP infection in the family members suffering from Hashimoto’s thyroiditis. MAP has also been identified in an Italian patient with Hashimoto’s thyroiditis using RT-PCR. Recent study by Masala et al have reported the identification of zinc transporter 8 and MAP3865c homologous epitopes from Sardinia population with Hashimoto’s thyroiditis, a common target with type 1 diabetes. Reports on the bioload of MAP infection in human patients suffering with thyroid disorders using PCR based diagnostic tests are scanty. In the present study, traditional IS900 PCR has been compared with newer assays (‘Taqman probe’ and ‘SYBR green Real time’ based PCR targeting IS900 gene) for the screening of the human patients suffering with thyroid disorders and found positive for MAP infection using indigenous ELISA kit.

Materials and Methods

Clinical Samples

A total of 76 patients were diagnosed and confirmed suffering with ‘Thyroid disorders’ using 3rd generation chemi-luminescent assays at the Lahiri Pathology Laboratory, Agra (Uttar Pradesh), India. Informed consent and other necessary clearances were taken before drawing blood samples from these 76 patients for screening against MAP infection. Normal TSH range from 0.4 to 4.0 mU/L was the reference range and people with normally functioning thyroid gland fall in this range. If TSH measures >4.0 mU/L, a second test (T4) was performed to verify the results. TSH >4.0 mU/L with a low T4 level indicated hypo-thyroidism. If TSH is >4.0 mU/L and T4 level was normal, then serum was tested for anti-thyroid peroxidase (anti-TPO) antibodies. Presence of these antibodies indicates autoimmune thyroid disorder, which is an important risk factor in developing hypothyroidism.

Indigenous ELISA Kit (i_ELISA)

Human serum samples were screened by ‘Indigenous ELISA kit’ for the presence of MAP infection. ‘Kit’ uses semi-purified protoplasmic antigen (sPPA) from highly pathogenic native strain (‘S 5’) of MAP, a novel biotype (‘Indian Bison type’), recovered from a terminally sick goat with JD. Absorbance was read at 450 nm in micro ELISA reader (BioRad Laboratories). Positive and negative control serum samples were simultaneously run with test samples. Optical density (OD) values were converted to sample to positive (S/P) ratios as per Collins to determine status of MAP infection. Samples in positive and strong positive categories were considered positive for MAP infection. Ratio between mean OD of positive and negative control (≥ 4 times) was critical for calculating S/P ratio and categories were (0.00-0.09, negative; 0.10-0.24, suspected; 0.25-0.39, low positive; 0.40-0.99, positive; and 0.61-0.80, strong positive).

Traditional IS900 Blood PCR

DNA was isolated from blood samples as per van Embden method with some modifications and resulting DNA samples were subjected to specific IS900 PCR using P90 and P91 primers. Presence of specific PCR product (413 bp) was considered positive for MAP infection (Fig. 1).

Taqman Probe Real Time PCR Targeting IS900 Gene

Primers and probe were designed for MAP specific contiguous conserved region of IS900 gene. About 10 NCBI IS900 sequences from several loci have been aligned using Clustal-W and the conserved regions were mapped. MAP IS900 primers and probe sequences (100 nano molar, HPLC purified) were used in the study (Table 1). 0.25 µL of probe (10 pico mol/µL), 7.25 µL of nuclease free water and
2 μL template DNA were added (20 μL total volume). Thermal cycling conditions (2 step PCR) were: initial denaturation (94°C for 5 min), followed by 37 cycles of denaturation (94°C for 20 sec), annealing and extension (61°C for 30 sec), and a final extension (72°C for 5 min).

**SYBR Green Real Time PCR Targeting IS900 Gene**

Extracted DNA of MAP from blood samples (n=28) was subjected to screening by IS900 SYBR green Real time PCR (SYBR RT_PCR) following the protocol as follows. Briefly, in a volume of 10.0 μL of 2× SYBR green master mix with ROX Ref. dye (Thermo Scientific), 0.5 μL each of forward (10 pmol/μL) and reverse primer (10 pmol/μL), 7.0 μL of nuclease free water and 2 μL template DNA were added (20 μL total volume). Thermal cycling conditions were: initial denaturation (94°C for 5 min), followed by 37 cycles of denaturation (94°C for 20 sec), annealing (61°C for 20 sec), extension (72°C for 20 sec), and a final extension (72°C for 7 min). MAP specific IS900 primer sequences used as per Vary et al21 were:

150C: 5’-CCGCTAATTGAGAGTGCAGTTGG-3’ (Forward primer) and 921: 5’-AATCAACTCCAGCAGCGGCGCCTG-3’ (Reverse primer). Standard curve was plotted for the positive control MAP strain-S5 DNA by serially diluting with the starting concentration at 100 ng (10² ng) with 10-fold dilutions descending up to 10⁻² ng. The reaction conditions were set up according to the standard conditions as described above. The standard curve generated by the machine software (CFX96, Biorad) was further analyzed for regression coefficient and efficiency.

**Statistical Analysis**

To measure significance between the tests, data were analysed statistically by Fisher’s exact test and kappa agreement using Graph Pad software, USA.

**Results**

Of the 76 thyroid patients (suffering with thyroid disorders and positive by 3rd generation chemiluminescent assays) screened, 28 patients were positive (36.8%) for the bioload of MAP using indigenous ELISA kit (Table 2). Sero-positive thyroid patients (28 or 36.8%) were further screened by traditional IS900 PCR, newly developed Taqman probe and SYBR green Real time IS900 PCR assays and 7 (25.0%), 9 (32.1%) and 10 (35.7%) were found positive for MAP infection, respectively (Table 2; Figs 1-3). Sero-assay using i-ELISA revealed that

<p>| Table 1 — Primers and probes used for IS900 based Taqman RT-PCR for detection of M. avium subspecies paratuberculosis (MAP) |</p>
<table>
<thead>
<tr>
<th>No.</th>
<th>Primer &amp; probe</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>P1F (forward)</td>
<td>5’-ATCTGGACACTGAGTTACGGAG-3’</td>
</tr>
<tr>
<td>2</td>
<td>P1R (reverse)</td>
<td>5’-ATCGCTGCGCGTCGTCGTT-3’</td>
</tr>
<tr>
<td>3</td>
<td>Probe IS900*</td>
<td>5’6FAM-AAGGCCCAGCATTACTGATGG-BHQ1 3’</td>
</tr>
</tbody>
</table>

*Modification: 6FAM reporter dye in 5’ end and BHQ1 Quencher in 3’ end

<p>| Table 2 — Serostatus of MAP in human patients suffering from thyroid disorder using ‘Indigenous ELISA kit’ |</p>
<table>
<thead>
<tr>
<th>Categories</th>
<th>Serostatus in ‘indigenous ELISA’</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Strong positive (SP)</td>
</tr>
<tr>
<td>Sub-total</td>
<td>6 (7.9)</td>
</tr>
<tr>
<td>Total (n=76)</td>
<td>P= 28 (36.8)</td>
</tr>
</tbody>
</table>

Fig. 2 (A & B) — Real time PCR based assay for the quantitative diagnosis of MAP infection: A: Standard curve generated by graphing the log of the DNA concentration (serial dilutions from 0 to 10⁵ folds) of MAP DNA (10 ng/μL) vs the CT value showing 98.5 efficiency (R²=0.997) for detection of MAP; & B: Melting peak of IS900 amplification product for tested MAP DNA samples.
bioburden of MAP infection in the human population suffering with thyroid disorder was high (36.8%). This fact was further confirmed using PCR based molecular assays targeting specific IS900 gene. Analysis of standard curve plotted between the 10-fold dilutions of serially diluted MAP DNA based on CT values (Cq) showed 98.5 (E) efficiency and R² value=0.997, with the slope -3.3, for the detection of MAP infection. The Y-intercept generated in the standard curve, the machine theoretically indicates the cycle threshold taken by one copy of DNA. Meanwhile, the slope of the regression curve reflects the efficiency of the PCR reaction based on the formula E=[10^{1/slope–1}]. Comparison between traditional PCR, Taqman probe and SYBR green Real time PCR assays revealed good agreement by kappa values and was considered statistically significant (Table 3). Comparative diagnostic sensitivity of Taqman probe PCR vs Traditional PCR was 100.0% and by SYBR green PCR vs Traditional PCR assay was 85.7% (Table 4). Comparative diagnostic specificity of Taqman probe PCR vs traditional PCR was 90.5% and SYBR green PCR vs Taqman probe PCR was 89.5% and SYBR green PCR vs traditional PCR was 80.9% (Table 4). Use of Taqman probe and Real Time PCR assays improved the detection rate of MAP infection in human patients suffering with Thyroid disorders.

**Discussion**

MAP the cause of Johne’s disease (JD) is responsible for chronic granulomatous enteritis in domestic and wild ruminants1, and has also been associated with number of human autoimmune disorders22,23. Milk and water supplies have been suggested the vehicles for transmission of MAP from animals to human beings2,24. Recently, MAP infection attracted considerable interest owing to the rapidly growing body of scientific evidences suggesting similarities between Johne’s disease of domestic livestock and Crohn’s disease in human beings. This has led to the speculation that MAP might be one of the causative agents of Crohn’s disease25 and other similar human autoimmune disorders (Hashimoto Thyroiditis)15. A recent study in India has reported sharp rise in the bioload of MAP in domestic livestock species1. Clinically and sub-clinically infected animals excrete huge quantities of MAP bacilli in their feces, milk, semen etc. MAP is also known to survive pasteurization temperature2,26 and

<table>
<thead>
<tr>
<th>Tests</th>
<th>Combinations</th>
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<tbody>
<tr>
<td>Taqman Probe RT-PCR</td>
<td>+</td>
</tr>
<tr>
<td>SYBR green RT-PCR</td>
<td>-</td>
</tr>
<tr>
<td>Traditional PCR</td>
<td>+</td>
</tr>
<tr>
<td>Total (n-28)</td>
<td>6 (21.4)</td>
</tr>
</tbody>
</table>

*Figures in parentheses are percentage

<table>
<thead>
<tr>
<th>Blood</th>
<th>Tests</th>
<th>Combination</th>
<th>Two tailed P value*</th>
<th>Kappa±SE (agreement)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>SYBR green PCR vs Traditional PCR</td>
<td>&lt;0.0001</td>
<td>0.826±0.117 (Very good)</td>
<td>85.7</td>
<td>80.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Taqman PCR vs Traditional PCR</td>
<td>0.0033</td>
<td>0.583±0.162 (Moderate)</td>
<td>100.0</td>
<td>90.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SYBR green PCR vs Taqman PCR</td>
<td>0.0001</td>
<td>0.761±0.129 (Good)</td>
<td>88.8</td>
<td>89.5</td>
<td></td>
</tr>
</tbody>
</table>

*The two-sided P value is considered extremely significant
similar high bioload of live MAP has been reported in raw milk and milk products (ice cream, paneer, condensed milk, baby milk powder etc) made from pasteurized milk in many countries (UK, USA, Canada, Europe, Japan, New Zealand, Australia) including India. Therefore, it is a serious public health concern worldwide. In India, raw milk serves as base for many traditional and Ayurvedic medicines, which pose huge risk of human infection through milk. This threat has recently been increased many folds in the absence of control programmes for MAP infection in domestic livestock species.

Several studies have shown association between MAP and Crohn’s disease. Recently, MAP has been proposed as the risk factor in genetically susceptible individuals for the development of autoimmune diseases like Hashimoto’s thyroiditis. Momotani et al. have shown that MAP antigen has potency to induce colitis and recommended that people who may have genetic predisposition to Crohn’s disease should avoid dairy products specially those contaminated with MAP antigen. However, some researchers believed there is no evidence that MAP as individual pathogen can be involved in the pathogenesis of inflammatory bowel diseases. While others have reported that MAP may asymptotically colonize apparently healthy individuals. Association between MAP and chronic inflammatory bowel disease has been reported and found to persist in cell wall-deficient form escaping clearance by the host immune system. Masala et al. have reported the evidence accounting for a cross-recognition of MAP3865c/ZnT8 homologs sequences in Type 1 diabetes and proposes MAP as an Hashimoto’s thyroiditis environmental trigger (acting through molecular mimicry mechanism). It is thus natural to consider MAP for a causal role in Hashimoto’s thyroiditis. Moreover, viable MAP was also identified in an Italian patient with Hashimoto’s thyroiditis and Melkersson-Rosenthal syndrome using RT-PCR. Sisto et al. proposed a relationship between MAP infection and Hashimoto’s thyroiditis in family members by using species-specific IS900 probe PCR.

Diagnosis of MAP infection is difficult due to prolonged incubation, intermittent shedding of bacilli, variability in host immune response, fastidious nature and high cost of imported kits. For the diagnosis of MAP in different samples (feces, milk & intestinal tissues), several diagnostic tests (culture, ELISA & PCR) are used routinely but long incubation (12-16 wk) and low sensitivity (>50.0%) limit the use of culture test. Utility of serological tests is also limited due to low specificity and sensitivity, as immune response may not be detectable either due to anergy or late appearance of antibodies in the pathogenesis. Reports on the bioload of MAP infection in human patients suffering with thyroid disorder using Real time PCR based assays are scanty. In the present study, apart from estimating bio-load of MAP in human patients suffering with thyroid disorder in Northern parts of country, we also evaluated comparative sensitivity and specificity of PCR based diagnostic tests. To formulate the best strategy for the screening of human subjects, various PCR tests combinations were evaluated on the same set of samples (Table 2). Sensitivity of ‘Taqman probe’ and ‘SYBR green’ Real time PCR were compared with traditional PCR targeting MAP specific IS900 gene in human patients. It was found that for the detection of MAP infection sensitivity of both Taqman probe and Real time PCR based assays was superior besides being highly specific as compared to regular IS900 PCR. Combination of IS900 PCR in Ziehl-Neelsen stained smears and IS900 PCR in tissues would increase sensitivity of diagnosis. However, IS900 PCR has proved to be highly specific and sensitive in detecting MAP. IS900 PCR has sensitivity and specificity equal or greater than culture and takes only hours to complete the test as compared to 6-12 weeks by culture. Sensitivity of Taqman probe PCR versus traditional PCR was 100.0%, followed by SYBR green PCR versus traditional PCR (85.7%). There was little difference in relative specificity of Taqman probe PCR versus traditional PCR, and it was 90.5% compared with SYBR green PCR versus Taqman probe PCR (89.5%) and SYBR green PCR versus traditional PCR (80.9%). IS900 sequence is the most commonly targeted sequence but 100 percent uniqueness of this sequence is doubtful. Potential cross-reactions can result in false-positive results because of the homologous nature of IS900-like elements. Our earlier study on mass screening of human population, 3093 blood and 23,196 serum samples were randomly collected from different pathology laboratories in Mathura and Agra region of South Uttar Pradesh. Screening of the human serum samples using ‘indigenous ELISA kit’ revealed 34.0% positivity for MAP infection in random screening of patients suffering with different kinds of clinical disorders (infectious or non-infectious) and results
were comparable with findings of present study. However in the random study, disease profile-wise, 29.5% patients with thyroid disorders were found positive for MAP infection, which was lower than the human patients suffering with thyroid disorders screened in the present study and reported 35.8% as positives for MAP infection using indigenous ELISA kit. However, positivity for MAP infection was 8.4% in blood samples using IS900 PCR\(^2\), which was again significantly lower than the positivity found in the present study (25.0%), using IS900 PCR. This may be due to screening of small number of blood samples and screening of sero-positive patients for MAP infection in indigenous ELISA using traditional IS900 PCR and improved PCR assays (Taq man probe and SYBR green Real time) in the present study. Whereas in the earlier study, Singh et al\(^3\) performed general screening of human population and not specifically those patients suffering with thyroid disorders. Similar higher (43.7%) bio-incidence of MAP infection was reported by Singh et al\(^7\), when they screened human patients confirmed for suffering diabetes using IS900 PCR as compared to suspected healthy patients (39.4%).

**Conclusion**

The present study shows that bioburden of MAP infection in the human population suffering with thyroid disorder was high in India. This was possible only when samples were screened with highly specific and sensitive tests like i-ELISA, Taqman probe and SYBR green Real Time based PCR assays. Use of Taqman probe and SYBR green Real time PCR assays increased detection rates of MAP infection (upto 10 fg of DNA) with improved specificity of 100.0%. The study indicates the urgent need for larger study to investigate the role of MAP in precipitation and progression of thyroid disorder in human population and genetic susceptibility of individuals to MAP infection on exposure through consumption of milk and milk products.

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**Conflict of Interest**

No potential conflict of interest to declare.

**References**


