Nested PCR and its Comparison with other Diagnostic Test in the Diagnosis of Paratuberculosis (Johne’s Disease) in Goats

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Abstract: Mycobacterium avium subsp. paratuberculosis (MAP) causes Johne’s disease (JD) in ruminants. There are many methods for diagnosis of Johne’s disease in goats. Among these, bacterial isolation was still considered as reference standard for diagnosis of JD in spite of its long turn over time. Molecular targets like IS900 were routinely used for diagnosis, since it is present only in MAP. But lately presence of IS900 like sequence in closely related members of Mycobacterium avium complex (MAC) make IS900 based PCR a less sensitive method. Therefore, IS900 PCR positive sample should be confirmed by PCR assay targeting another gene within the genome of the organism. This led to the discovery of f57 gene, which is unique to this organism and not present in other members of MAC. The present study was carried out to evaluate the nested PCR method (targeting IS900 & f57 gene) to diagnose Johne’s disease in goats. The efficacy of this nested PCR was compared with other serological tests like agar gel immunodiffusion (AGID) and absorbed enzyme-linked immunosorbent assay (ELISA). Out of the 265 goat faecal and sera samples, positive results were; AGID 36 (13.59%); absorbed ELISA 51 (19.25%) and nested PCR 58 (21.88%). This nested PCR was also compared with intra-dermal Johnin test in 65 animals, of which, positive results recorded were; Johnin test 21 (32%) and nested PCR 28 (43%). The nested PCR showed higher sensitivity compared to other diagnostic tests. Hence, this method can be used for diagnosis of clinical and sub clinical JD in goats.

Keywords: Agar Gel Immunodiffusion, ELISA, Nested PCR, Paratuberculosis

Introduction

Mycobacterium avium subsp. paratuberculosis (MAP) causes paratuberculosis or Johne’s disease (JD) in cattle, sheep and goats. Johne’s disease is prevalent worldwide and cause substantial economic losses to farming industry. The rate of prevalence may be higher than that of reported due to difficulty in the diagnosis of this disease, particularly during the preclinical stages. Generally, diagnosis of paratuberculosis is based on the detection of the organism or the immune response. Cultivation of bacteria from the faecal samples is considered as reference standard in diagnosis of paratuberculosis, but this method is more time consuming in sheep and goats as compared to cattle (Carrigan and Seaman, 1990; Collins et al., 1993). Furthermore, the sensitivity of faecal culture is also too low (Chioldini et al., 1984).

The host immune response to infection is initially cell mediated (CMI). As the infection progresses from subclinical to clinical disease, CMI responses are replaced by strong humoral responses characterized by the presence of antibodies. So the early identification of MAP infected animals can be detected by delayed type hypersensitivity (DTH) skin test or release of interferon (IFN)-γ in the blood sample (Gwozdz et al., 2000) that can be measured by ELISA (Billman-Jacobe et al., 1992; Stable, 1996). The specificity of these tests is low resulting in many false-positive results (Jungerson et al., 2002; Huda et al., 2003; Manning et al., 2003; Reddclifff and Whittington, 2003). On the other hand, the humoral immune response occurs relatively late in infection and can be detected by various serological test like agar-gel immunodiffusion (AGID), enzyme linked immunosorbent assay (ELISA) and complement fixation test (CFT). The sensitivity and specificity of these assays is relatively high in clinically affected animals, but low in sub clinically infected animals, as antibody generally develop late in this infection (Milner et al., 1987, 1990). In general the absorbed ELISA is considered to have the highest specificity and sensitivity among various serological tests (Hilbink et al., 1994; Rajukumar et al., 2001). In spite of its lower sensitivity and specificity, intradermal Johnin and AGID tests are the diagnostic test available at the field level.

The application of molecular method for the diagnosis of JD is under constant development and modification. A number of genes and sequence unique to MAP have been identified over the years. The insertion element IS900 has been routinely used to detect the presence of MAP in clinical samples. However, sequence related to IS900 like IS902 (Wood pigeon mycobacterium), IS901 (Mycobacterium avium subsp. avium), IS1626 (Mycobacterium avium subsp. avium and M. intracellularum) were reported (Cousins et al., 1999; Puyang et al., 1999; Englund et al., 2002) and hence reduces its specificity. Therefore, a positive IS900 PCR should be confirmed by subsequent nested PCR or by
a PCR assay targeting another gene. Another sequence named f57 has been identified and this sequence does not have any homology with any known sequences (Poupart et al., 1993; Coetsier et al., 2000). In the present experiment, the samples from the goats were screened for Johne’s disease by nested PCR method of both IS900 and f57 gene. Furthermore, the efficacy of this nested PCR is compared with other diagnostic tests like AGID, absorbed ELISA and Johnin skin test.

Materials and Methods
Serum and faecal samples from 200 adult goats (>1 year of age) of both sex (male and female) were collected randomly from slaughter house of different regions in India. Additional faecal and serum samples from 65 adult goats of both sexes (male and female) were collected from Indian Veterinary Research Institution farm and these 65 animals were also used for Johnin skin testing.

Bacterial Strain and Preparation of Antigens
Standard culture of Mycobacterium avium subsp. paratuberculosis ATCC 19698 and Mycobacterium phlei was procured from Biological Product Division, Indian Veterinary Research Institute, India. It was maintained in Lowenstein Jenson (LJ) medium containing mycobactin J. For large scale production of antigen, the culture was grown as a surface pellicle on Watson and Reid synthetic broth containing mycobactin J. and incubated for eight weeks at 37°C. Bacterial growth was killed by heating at 72°C for 2 h and separated by filtration. The cells were washed thrice with PBS and centrifuged to get cell mass. It was re-suspended in PBS containing 0.2mM phenyl methyl sulfonyl fluoride and sonicated at 16µ amplitude for 45 min in ice with intermittent intervals. Sonicated preparation was centrifuged to get cell mass. It wa

**Table 1. Details of the primers, its target and the PCR product size**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Nucleotide sequence</th>
<th>Target gene</th>
<th>Product length</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS900 F</td>
<td>5'-GGGTGAGTCTGGGACAATGACGGTTA-3'</td>
<td>IS900</td>
<td>572</td>
</tr>
<tr>
<td>IS900 R</td>
<td>5'-AGCCGGCGACGGCTTGTGTT-3'</td>
<td>IS900</td>
<td></td>
</tr>
<tr>
<td>IS900FN</td>
<td>5'-GGAGGTGGTGTTGGCCAACACCTGT-3'</td>
<td>IS900</td>
<td>452</td>
</tr>
<tr>
<td>IS900RN</td>
<td>5'-CGATCGACCCAGCACTCGGAA-3'</td>
<td>IS900</td>
<td></td>
</tr>
<tr>
<td>F57 F</td>
<td>5'-CCCTCTGCTAATTCGTACGCGCTAGA-3'</td>
<td>f57</td>
<td>432</td>
</tr>
<tr>
<td>F57 R</td>
<td>5'-TCAGCTATTTGCTGACGAGGATG-3'</td>
<td>f57</td>
<td></td>
</tr>
<tr>
<td>F57 RN</td>
<td>5'-TGGTGATCCACATTGTTGTTGTTCAC-3'</td>
<td>f57</td>
<td>424</td>
</tr>
</tbody>
</table>

In 2nd amplification (nested), IS900 FN and IS900 RN were used to anneal the amplified product targeting the region within IS900 from the first PCR run. Similarly, F57 F and F57 RN were used to anneal the amplified product of targeting the region within f57 from first PCR run.

PCR reaction mixtures for 1st and 2nd amplification of IS900 and f57 gene were made in total volume of 25 µl. Final concentrations of different constituents in reaction mixture were made, respectively as tris-Cl (pH 9); 10mM, KCl; 50 mM. MgCl2; 1.6 mM, dNTP mix; 800 µM total, primers; 0.8 µM each, Red Taq polymerase; 0.5 U, triton-X100; 0.01% and DNA

Raising Hyper-Immune Sera
Two healthy goats free from MAP infection by PCR and ELISA techniques were chosen and used for hyper-immune sera production. Hyper-immune serum was raised as per methods described previously with suitable modifications (Castelnuovo et al., 1969; Johle, 2008). The antigenic mixture consisting of 200 mg of whole cells, 10 mg of sonicated antigen and 4 ml of sonicated sediment of MAP was mixed with equal amounts of Freund’s incomplete adjuvant (Difeo, USA). Each goat was inoculated with 1 ml of the antigen mixture subcutaneously at weekly intervals for 6 weeks. Antibody titre was monitored by agar gel immunodiffusion test after fifth injection. Serum were collected from goats one week after the last injection and stored at 4°C. The serum collected before immunization was used as negative controls.

Extraction of Genomic DNA from Faecal Samples
Approximately, 200 mg faecal samples were used to isolate genomic DNA by using QIAmp stool DNA kit (Qiaagen, Germany) according to manufacturers’ instruction. Extracted DNA was stored at -20°C until further use.

Nested Polymerase Chain Reaction
The primers which were used to amplify the target gene were taken from published literature (Vansnick et al., 2004). The details of the primers, its target and product length are given in Table 1. Amplification of both sequences was based on the nested PCR approach; PCR product of the first PCR run used as the template for the 2nd amplification (nested). Briefly, for the first PCR run, primers IS900 F and IS900 R were used for amplification of IS900 gene, whereas primers F57 F and F57 R for amplification of f57 gene.
templates 10-20 ng. For positive control, DNA isolated from standard MAP strain ATCC 19698 was used. Whereas nuclease free water was used as nontemplate control.

Amplification of both sequence were performed in thermocycler (Biometra, India) with amplification condition respectively in sequence, as, initial denaturation (94°C) for 4 min; followed by 40 cycles (25 cycles for 2nd PCR run) of denaturation (94°C), annealing (68°C) (the annealing is same for both) and extension (72°C), each for 45 sec and at last, final extension (72°C) for 10 min was given.

PCR products were analyzed by electrophoresis in 1X tris-acetate EDTA (TAE) buffer for 2 h at 50 V. PCR products along with DNA marker were loaded in 2% (w/v) agarose gel, made in 1X TAE containing 0.5 µg/ml (w/v) ethidium bromide. Separation of DNA was visualized by UV at 260 nm and documented.

Absorbed Indirect ELISA
The absorbed ELISA was developed by the method previously described (Cox et al., 1991). Briefly all the wells in the microtitre plate (NUNC, Maxisorp) were coated with 100 µl of 0.02 µg/µl (2 µg/well) capture antigens (sonicated antigens) in coating buffer (sodium bicarbonate buffer) and incubated at 37°C for 1 h. Unbound antigen in the plate was washed thrice with washing buffer PBS with 0.5% Tween-20 (PBST). Blocking of unbound site was done by adding 100 µl blocking buffer (2% BSA in PBST) and incubated at 37°C for 1 h. Plate was washed thrice with wash buffer. The 100 µl of sera sample were incubated at 37°C with 0.40 µg/µl absorbed antigen (sonicated M. phlei antigen) for 30 min. 100 µl of each serum was added in duplicate wells and incubated for 1 h at 37°C. Plate was washed thrice with wash buffer. 100 µl of rabbit anti-goat HRPO conjugate (Genei, Bangalore) was added per well (1:5000) and incubated for 1 h at 37°C. Plate was washed thrice with wash buffer. 100 µl 1x TMB solution (Genei, Bangalore) was added per well and observed for colour development. After 10-20 min the reaction was stopped by adding 100 µl of 1 M sulphuric acid per well and reading was taken at 450 nm in an ELISA reader. All ELISA results were determined by the procedure described previously (Rajukumar et al., 2001).

Agar-Gel Immunodiffusion Assay
One percent agarose gel was prepared in PBS (pH 7.4) containing sodium azide (0.02% w/v). Gel was cast into sterile plastic petriplates and allowed to solidify at 4°C for 1 h in the humid chamber. Wells of 3 mm diameter were punched out in a hexagonal pattern with six peripheral wells for sera and one centre well for the antigen at equidistance of 5 mm between them. The central well was filled with sonicated antigen with optimum concentration (1 mg/ml). The test sera was charged in duplicate in six peripheral wells and incubated at 4°C overnight in the humid chamber along with positive control and negative control. Gels were examined after 24 and 48 h and in suspected cases after 72 h. White precipitation line between antigen and sera wells were taken as positive whereas absence of precipitation line was recorded as negative. Hyperimmune sera were used as positive control whereas distilled water was used as negative control.

Johnin Intradermal Skin Test
This test was carried out by the single intradermal inoculation of 0.1 ml of Johnin purified protein derivatives (1 mg/ml). An increase in skin fold thickness more than 4 mm or more and/or with clinical signs of inflammation and clinical edema was considered as positive reactors.

Data Processing and Analysis
Statistical analysis of the data was accomplished using the Kappa statistic which gives a chance-related measure of agreement (Fleiss, 1981). This statistic can vary from -1 (no agreement) to +1 (perfect agreement). The diagnostic test sensitivity determination for the four tests was also compared by using McNemar’s test (Remington and Schork, 1970). The test results were evaluated by binomial distribution (Remington and Schork, 1970). All statistical calculation and interpretation were done using Graphpad QuickCals (San Diego, CA, USA).

Results
Nested PCR of Faecal DNA Sample
Primary PCR with primers targeting IS900 and f57 of MAP resulted in specific amplification of products of 572 bp and 432 bp, respectively (Figure 1). Secondary PCR (nested PCR) with primers targeting the region within IS900 and f57 also resulted in specific amplification of products 452 and 424 bp, respectively (Figure 2). Among the DNA samples isolated from 265 fecal samples from different flocks and from institutional animal house, 56 animals were positive for both nested targeted genes (IS900 and f57) (Table 1).

Figure 1. Nested PCR of IS900 gene for detection of MAP in faecal sample. Lane M: 100 bp DNA ladder; Lane 1-3: Primary PCR product (572 bp); Lane 4-6: Nested PCR product (452 bp).
Absorbed Indirect ELISA
The absorbed ELISA (Figure 3) detected a total of 51 animals out of total of 265 serum samples collected from different flock and institutional animal house (Table 2).

Agar Gel Immunodiffusion Assay
A precipitation line (Figure 4) indicating a positive reaction in AGID was observed in 36 animals out of 265 serum samples collected (Table 3).

Table 2. Results of serological tests in 265 sera sample collected from different flock

<table>
<thead>
<tr>
<th>Flock</th>
<th>Status of flock</th>
<th>No. of samples</th>
<th>No. of animals positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Unknown</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Negative</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Unknown</td>
<td>37</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Unknown</td>
<td>33</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>Positive</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>Positive</td>
<td>33</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>Unknown</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>Negative</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>Positive</td>
<td>65</td>
<td>24</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>265</td>
<td>36</td>
</tr>
</tbody>
</table>

Table 3. Result of 65 animals from which both serum and faecal samples were collected and Johnin test was performed

<table>
<thead>
<tr>
<th>Johnin</th>
<th>Faecal PCR</th>
<th>ELISA</th>
<th>AGID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Johnin +</td>
<td>15</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Johnin -</td>
<td>5</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

P= PCR; E= ELISA; A= AGID; + = Positive; - = Negative.

Johnin Intradermal Skin Test
An increase in skin fold thickness more than 4 mm or more and/or with clinical signs of inflammation and edema indicating positive reaction was observed in 21 animals out of a total of 65 animals from institutional farm house (Table 4).

Table 4. Statistical analysis of the results of different diagnostics tests for JD using Kappa statistics

<table>
<thead>
<tr>
<th>Tests</th>
<th>Kappa value *</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Faecal PCR Vs. ELISA</td>
<td>0.961</td>
<td>Perfect agreement</td>
</tr>
<tr>
<td>ELISA Vs. AGID</td>
<td>0.872</td>
<td>Perfect agreement</td>
</tr>
<tr>
<td>AGID Vs. Faecal PCR</td>
<td>0.834</td>
<td>Perfect agreement</td>
</tr>
<tr>
<td>AGID Vs. Johnin</td>
<td>0.774</td>
<td>Substantial agreement</td>
</tr>
<tr>
<td>ELISA/PCR Vs. Johnin</td>
<td>0.667</td>
<td>Substantial agreement</td>
</tr>
</tbody>
</table>

**Discussion**

The evaluation of the nested PCR in diagnosis of JD was determined by comparing it with other diagnostic tests that commonly used for JD diagnosis. The evaluation of nested PCR with a golden standard is always advisable. Due to more time consumption and the low sensitivity of the faecal culture method (Chiodini et al., 1984; Carrigan and Seaman, 1990; Collins et al., 1993) absorbed ELISA was used for evaluation. Flocks with negative, unknown and positive status of infection were included in order to determine a proper diagnostic accuracy of the test. When the results of the 65 animals for which all tests (Nested PCR, ELISA, AGID and Johnin test) were performed are compared (Table 3), one animal with negative PCR and positive ELISA result may be due to shedding of bacteria below the detection limit at the time of sampling. Two animals with positive PCR and negative ELISA results indicate that these animals may not have yet sero-converted or that it may be in early stage of infection. AGID test showed lower sensitivity and false positive reaction when compared with nested PCR and ELISA.

The higher sensitivity of nested PCR was due to the ability of this test to detect even low fecal shedders and as the humoral response come to play in later part of the infection. ELISA or AGID was not able to detect these low faecal shedders which are seen during earlier part of infection (Hope et al., 2000; Sergeant et al., 2003). On the other hand, the three animals which were negative by PCR and other serological test were found positive by Johnin test. This indicates the less specificity and cross reactivity of Johnin test with another environmental mycobacterial organism (Huda et al., 2003).

When the different test results were compared statistically using Kappa statistic with 95% confidence interval, the kappa value for these tests varied from 0.667 to 0.961 (Table 4). There was perfect agreement between PCR, AGID and ELISA (K= 0.961). Substantial agreement (K= 0.774) of Johnin test with another test like PCR and ELISA was observed. Furthermore, when the test were compared for sensitivity using Mc Nemar’s test, significant difference ($p > 0.01$) was not noticed between the sensitivity of ELISA, AGID and PCR. However, the sensitivity of PCR and ELISA was significantly high ($p < 0.001$) when compared with that of Johnin test. These relationships suggested that absorbed ELISA was better option for screening animal for JD. However, ELISA fails to detect low shedder, which was detected by nested PCR test. When the PCR was compared with the reference test like ELISA, there was no statistically significant difference ($p > 0.01$) in their sensitivity, indicating that ELISA and PCR have similar results in diagnosis of Johnne’s disease.

**Conclusion**

The nested PCR was used for diagnosis of Johnne’s disease in goat and its relative sensitivity and specificity was determined by comparing with other diagnostic tests. Though the test possesses several advantages like rapidity and specificity, it may not be used for screening purpose due to high cost involvement. However, this technique detected earlier stage of infection and low shedder animal. Hence, this method can be used for early diagnosis of Johnne’s for effective control of the disease.

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**Conflict of Interests**

The authors declare that they have no competing interests.

**References**


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