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Detection of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in alcohol-fixed tissues of sheep by IS*Mav2* gene PCR and its comparison with histopathology, bacterial culture and IS*900* PCR

B. Debroy[a], B.N. Tripathi[a,b,*], G.G. Sonawane[b], R.B. Bind[a]

[a] *Division of Veterinary Pathology, Indian Veterinary Research Institute, Izatnagar 243 122, Bareilly, UP, India*
[b] *Animal Health Division, Central Sheep & Wool Research Institute, Avikanagar 304 501, Rajasthan, India*

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ABSTRACT

The objective of the present investigation was to study the efficiency of an IS*Mav2* polymerase chain reaction (PCR) in detecting *Mycobacterium avium* subspecies *paratuberculosis* (MAP) DNA in archived alcohol-fixed sheep tissues and compare with Ziehl Neelesen (ZN) staining, bacterial culture on Herrold's egg yolk medium and IS*900* PCR on fresh tissues. Tissue samples preserved in 70% alcohol for 6–8 months from 23 naturally infected paratuberculosis sheep and 7 healthy sheep were used for DNA extraction. In PCR amplification targeting IS*Mav2* gene of MAP, 19 (82.6%) were found to be positive. Bacterial culture, ZN and fresh tissue IS*900* PCR detected 65%, 100%, and 95% cases, respectively. It was concluded that alcohol could be an alternative fixative for transportation of tissues for molecular detection of MAP genome in tissues by IS*Mav2* PCR, which compared well with fresh tissue IS*900* PCR for the diagnosis of paratuberculosis in sheep. This may be useful in tropical countries, where shipment of fresh tissues for molecular diagnosis may be expensive proposition and most of the times facilities for maintaining cold chain are not available.

1. Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) causes chronic granulomatous enteritis of domestic and wild ruminants popularly called as paratuberculosis or Johne's disease. The disease is prevalent world-wide and causes huge economic losses due to decrease in the milk, meat and wool production, and direct losses due to death of the infected animals (Harris and Barletta, 2001). *M. a. paratuberculosis* has been also implicated in Crohn's disease (CD) of humans (Behr and Kapur, 2008).

Johne's disease can be diagnosed by histopathology, immunohistochemistry, bacterial isolation, skin testing and serological assays (Clarke, 1997; Tripathi et al., 2002). Bacterial culture techniques are generally recognized as the most specific tests for MAP infection, but the widely used conventional methods require a long incubation period of 8–16 weeks on solid media and up to 8 weeks in liquid medium (Stabel and Bannantine, 2005; Florou et al., 2009). Nucleic acid detection methods by PCR have led to rapid and specific detection of MAP genome in a variety of biological samples reducing the time of detection to 2–3 days. Majority of the previous studies have targeted IS*900* gene of MAP as it is present in approximately 17 copies in the genome, thereby providing a higher level of sensitivity (Stabel and Bannantine, 2005). However, false-positive results because of presence of IS*900*-like sequences in the environmental mycobacteria may limit its use (Englund et al., 2002).

* Corresponding author at: Animal Health Division, Central Sheep & Wool Research Institute, Avikanagar 304 501, Rajasthan, India.
Tel.: +91 1437 220142; fax: +91 1437 220163.
E-mail address: bntripathi1@yahoo.co.in (B.N. Tripathi).

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The other specific multicopy genes of potential diagnostic value identified in MAP are IS*Mav2*, IS*Map02* and IS*Map04* but have been less frequently assessed on a variety of clinical samples (Paustian et al., 2004; Li et al., 2005). The IS*Mav2* gene, present in at least 3 copies, has been used in the conventional as well as real-time (RT) PCR as a sensitive and specific method for detection of MAP in faeces and milk in some previous studies (Strommenger et al., 2001; Stratmann et al., 2002, 2005, 2006; Shin et al., 2004; Wells et al., 2006). In the past two decades, reports have shown that PCR can be used to detect MAP genome in formalin-fixed paraffin embedded tissue sections (Whittington et al., 1999; Tripathi and Stevenson, 2011). However, as formalin has adverse effects on the quality of mycobacterial DNA (Srinivasan et al., 2002), variable sensitivity of the procedure has been reported depending on the duration of fixation and the size of the DNA fragment to be amplified (Fiallo et al., 1992; Williams et al., 1995). Tissues fixed in alcohol and other fixatives such as methacarn, acetone, and OmniFix used for DNA extraction and subsequent PCR amplification have shown superior performance over formalin-fixed tissues (Fiallo et al., 1992; Greer et al., 1991; Uneyama et al., 2002). Except for a report of detection of *Mycobacterium leprae* DNA from alcohol-fixed human tissues (Fiallo et al., 1992) and *M. tuberculosis* genome in sputum sediments (Williams et al., 1995), the efficacy of amplification of MAP DNA in alcohol-fixed tissues of animals has not been evaluated. Therefore, the purpose of this study was to determine the efficacy of an IS*Mav2* PCR, a less frequently used target sequence for diagnosis of MAP infection, on DNA extracted from alcohol-fixed tissues of naturally infected paratuberculosis sheep and its comparison with pathology, bacterial culture and fresh tissue IS*900* PCR.

2. Material and methods

2.1. Animals

Twenty-three adult sheep of either sex, originating from known paratuberculosis infected flocks in a semiarid district of Rajasthan were used in the study. These sheep were diagnosed for paratuberculosis on the basis of regular clinical and faecal examinations and were culled from the flock or brought dead to the post-mortem room of the Animal Health Division for necropsy. Clinical signs in these sheep were soft pasty faeces with occasional diarrhoea and progressive loss of body weights. Some animals were found to be severely emaciated at the time of necropsy. Sheep belonged to the Avikalin, Chokla and Garole breeds. All experimental procedures and care of animals were carried out as per the recommendations of the Institute Animal Ethics Committee (IAEC).

2.2. Necropsy and sample collection

Detailed necropsy examination was conducted on the dead and slaughtered animals, and gross lesions were recorded. The representative tissue sections from the small intestines (duodenum, jejunum, ileum and ileo-caecal valve) and associated mesenteric lymph nodes (MLN) were collected in 10% buffered neutral formalin for histopathology. Three adjacent portions of the ileum were collected in sterile vials: one for culture and another for IS*900* PCR were brought on ice to the laboratory and stored at −20 °C until used. The third portion was collected in 70% alcohol and kept at room temperature for 6–8 months before subjected to DNA extraction and PCR analysis. Comparable tissue samples from seven healthy sheep slaughtered at small animal slaughterhouse, Bareilly, were also collected for histopathology, culture, and fresh tissue and alcohol-fixed tissue PCRs.

2.3. Histopathology

Tissues after fixation were cut into pieces of 2–3 mm thickness and processed conventionally, embedded in paraffin, sectioned at 4 μm thickness and stained with haematoxylin and eosin. Adjacent sections of intestines and mesenteric lymph nodes (MLN) were subjected to Ziehl Neelsen's (ZN) staining technique (Culling, 1974).

2.4. Bacterial culture

About 2 g of tissue sections from ileum were cut and chopped finely and homogenized in 15 ml sterile distilled water (DW) in a homogenizer (Ultra Turrex, Willington, USA). The homogenate was allowed to settle down for 30 min in a test tube. Three ml of tissue supernatant from each animal was transferred to a 15 ml centrifuge tube containing 12 ml of 0.9% hexadecyl pyridinium chloride (HPC). These tubes were kept on shaker at room temperature for 16–18 h (overnight). The tubes were then centrifuged at 6000 × *g* for 20 min. The supernatant was discarded and 0.1 ml of the sediment was inoculated onto each of 3 slants of Herrold's egg yolk medium (HEYM), two with mycobactin J (Allied monitor Inc., Fayette, USA) and one without it. The inoculated tubes were incubated at 37 °C in the BOD incubator for 12–16 weeks. The tubes were regularly examined at weekly intervals for bacterial growth. Acid-fast colonies appearing on mycobactin J tubes were further confirmed by IS*900* PCR as described previously (Sivakumar et al., 2005).

2.5. Extraction of DNA from fresh and alcohol-fixed tissue samples

Extraction of DNA from fresh tissue sample was performed as per the method described previously (Sivakumar et al., 2005). One gram of alcohol-fixed tissue (ileum) from each animal was weighed and kept under running tap water for 30 min and finally washed with three changes of sterile distilled water (DW). Tissues were then homogenized in 4 ml sterile distilled water (DW) and allowed to settle. A quantity of 200 μl of homogenate was taken into a sterile microcentrifuge tube and added with 500 μl of TE buffer and kept undisturbed for 15–20 min. The mixture was then centrifuged at 13,000 × *g* for 10 min and supernatant was discarded. The DNA was extracted using a commercial kit with slight modification (Himedia Laboratories, Mumbai, India). The collected sediments after addition of 180 μl of lysis solution and 20 μl of proteinase K (20 mg/ml) was incubated at 56 °C overnight (16–18 h). The subsequent procedures were followed as per manufacturer's instructions.

2.6. IS900 gene polymerase chain reaction on fresh tissue samples

The genomic DNAs of MAP isolated from fresh tissues were amplified in 50 μl reaction mixture containing 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM each of dATP, dGTP, dCTP and dTTP, 1 U of Taq polymerase (MBI Fermantas, MO, USA), 0.5 pM of primers (BA5: 5′-CTG GCTACC AAA CTC CCG A-3′, BA6: 5′-GAA CTC AGC GCC CAG GAT-3′) (Bauerfeind et al., 1996) and 5 μl of purified genomic DNA solution. Amplification was carried out in a programmable thermal cycler (Eppendorf Master Cycler, Hamburg, Germany) with initial denaturation at 94 °C for 5 min, and 30 cycles each of denaturation (94 °C, 45 s), primer annealing (58 °C, 1 min), and synthesis (72 °C, 1 min), and a final cycle of extension (72 °C, 5 min). The amplified PCR products were analysed by agarose gel electrophoresis using 1% agarose containing ethidium bromide.

2.7. ISMav2 gene polymerase chain reaction

Oligonucleotide forward IS*Mav1*-5′GTA TCA GGC CGT GAT GGC GG3′ and reverse IS*Mav2*-5′CCG CAC CAG CGC TCG ATA CA3′ primers flanking 313 bp nucleotides of IS*Mav2* gene of *M. a. paratuberculosis* were synthesized commercially (Imperial Life Science, USA) as described previously (Stratmann et al., 2002). The bacterial genomic DNA isolated from alcohol-fixed tissues was used as template for amplification of IS*Mav2* gene specific sequences. The PCR mixture of 50 μl contained 1× PCR buffers, 200 μM dNTPs, 1.0 pM of each primer, 1 U Taq polymerase, 1.5 mM $MgCl_2$, 10 μl of 50% glycerol, and 5 μl DNA template. Amplification was carried out in a thermal cycler (Eppendorf Master Cycler) with initial denaturation at 94 °C for 5 min, 30 cycles each of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, and synthesis at 72 °C for 30 s, final elongation at

72 °C for 5 min and holding at 4 °C. The amplified PCR products were analysed by agarose gel electrophoresis using 1% agarose containing ethidium bromide.

2.8. Sequencing of PCR product

The PCR products of test samples (CSWRI-23/CS 2836) and a positive control (IVRI/C-132) used in the study were sequenced in an automated nucleotide sequencer at Delhi University, South Campus, and New Delhi and submitted in the NCBI database (GU045496.1, GU045498.1). The sequences were analysed and compared with published sequences of IS*Mav2* gene available in the database (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

2.9. Statistical analysis

The sensitivities of all tests were analysed statistically using Chi-square test (Snedecor and Cochran, 1989).

3. Results

3.1. Clinical signs, pathology and ZN staining

More than 75% of sheep were emaciated with moderate to severe loss of body weights. These sheep had soft and pasty faeces but diarrhoea was observed only in a few sheep. Wool shedding and submandibular oedema was observed in five sheep. Grossly, small intestinal lesions such as thickening and corrugation of mucosa were prominently observed from ileocaecal valve (ICV) to mid jejunum in almost all the animals. Thickening in the duodenum, caecum, colon and rectum were usually less severe. Enlarged and oedematous mesenteric lymph nodes (MLN) and ileocaecal lymph nodes (ICLN) were observed in all the sheep. Microscopically, lesions were characterized by the presence of multifocal to diffuse infiltration of epithelioid macrophages many a times in sheet form, in the flat, fused and club shaped villi of the jejunum and ileum (Fig. 1a). The epithelioid cells were intermixed with mild to moderate infiltration of lymphocytes and polygonal macrophages and occasional giant cells. Focal to multiple granulomas were observed in the Peyer's patches. Submucosae were thickened due to infiltration with macrophages and lymphocytes, and many a times with sheet of epithelioid macrophages. The serosa was thickened due to oedema and mixed population of lymphocytes and macrophages around blood vessels. Lesions in the duodenum, caecum, colon and rectum were less extensive, and consisted of focal to multiple granulomas, mainly in the caecum. Multiple granulomas consisting of large macrophages or epithelioid cells were observed in the paracortical and interfollicular areas of MLN/ICLN of all sheep. These granulomas sometimes were observed in the diffuse pattern and extended towards medullary regions. In ZN staining, acid-fast bacilli (AFB) were demonstrated in all of the animals in variable numbers. AFB in the ileum and jejunum mucosae were numerous, in small clumps or in clusters in epithelioid macrophages (Fig. 1b). Other regions of the intestines had usually lesser number of bacilli. AFB were also demonstrated in MLN and ICLN of all cases but were generally lesser in numbers in comparison to those demonstrated in the jejunum and ileum. Histological examination of tissues from healthy control sheep did not show any changes compatible with paratuberculosis.

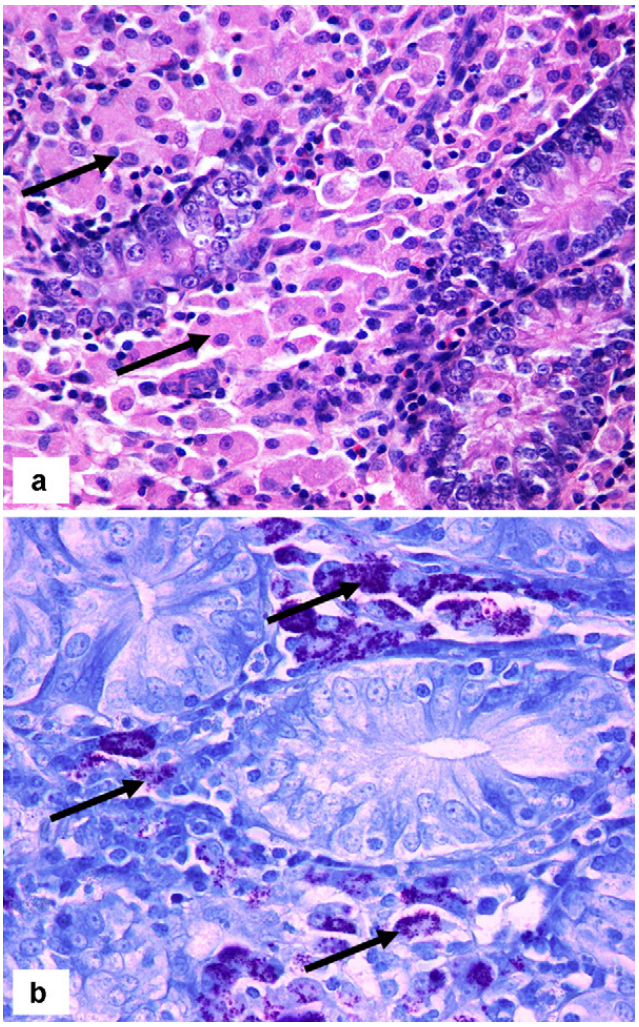

Fig. 1. (a) Ileum of sheep: mucosa showing granulomatous lesions consisting of epithelioid cells and (b) epithelioid cells showing clusters of acid-fast bacilli.

3.2. Bacterial culture and PCR

The results of histopathology, acid-fast bacilli demonstration by ZN method, fresh tissue IS*900* PCR and IS*Mav2* PCR on alcohol-fixed tissues on naturally infected and healthy control sheep are presented in Table 1. Comparative sensitivities of all diagnostic tests are given in Table 2. Out of 23 cases, 19 (82.6%) were found to be positive in IS*Mav2* PCR (Fig. 2). All the animals in control group were

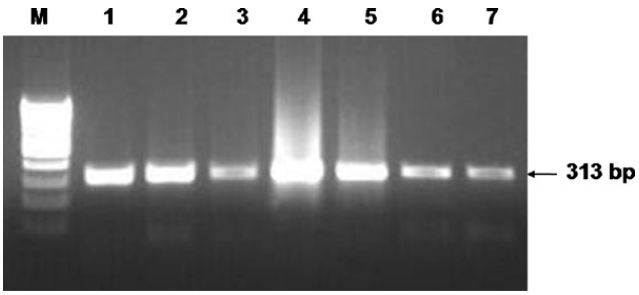

Fig. 2. IS*Mav2* PCR on alcohol-fixed tissues: Lane M: 100 bp DNA ladder, Lane 1: Positive control, Lane 2: S-17, Lane 3: S-18, Lane 4: S-19, Lane 5: S-20, Lane 6: S-21, and Lane 7: S-22.

Table 1
Results of various test conducted on tissues of infected and healthy sheep.

Case No.	Bacterial culture	Characteristic clinical signs, H/P lesions	Acid-fast bacilli	IS*900* PCR (fresh tissue)	IS*Mav2* PCR (alcohol-fixed tissue)
S1	+	+	+	+	+
S2	–	+	+	+	+
S3	–	+	+	+	+
S4	–	+	+	+	+
S5	+	+	+	–	–
S6	NT	+	+	NT	–
S7	+	+	+	+	–
S8	+	+	+	+	+
S9	+	+	+	+	+
S10	NT	+	+	NT	–
S11	NT	+	+	NT	+
S12	–	+	+	+	+
S13	–	+	+	+	+
S14	+	+	+	+	+
S15	+	+	+	+	+
S16	–	+	+	+	+
S17	+	+	+	+	+
S18	+	+	+	+	+
S19	+	+	+	+	+
S20	+	+	+	+	+
S21	+	+	+	+	+
S22	–	+	+	+	+
S23	+	+	+	+	+
Control					
C-1	–	–	–	–	–
C-2	–	–	–	–	–
C-3	–	–	–	–	–
C-4	–	–	–	–	–
C-5	–	–	–	–	–
C-6	–	–	–	–	–
C-7	–	–	–	–	–

NT, not tested; +, positive; –, negative; H/P, histopathology.

Table 2
Comparison of results of various tests.

	Bacterial culture	Histopathology & ZN staining	IS*900* fresh tissue PCR	IS*Mav2* alcohol fixed tissue PCR
Number tested	20	23	20	23
Number positive	13	23	19	19
Percentage	65^{b}	100^{a}	95^{a}	82.6^{ab}

Values bearing different superscripts across the row differ significantly ($P < 0.05$).

found to be PCR negative. The sequence of the PCR products from a test sample (CSWRI-23/CS 2836) and the positive control showed 98.1% and 98% homology, respectively, with published sequences available in the NCBI database (www.ncbi.nlm.nih.gov/blast/Blast.cgi, Genbank accession no. GU045498). The IS*900* PCR carried out on fresh tissue samples on these sheep was positive in 95% cases (Fig. 3). The bacterial culture on HEY medium detected 65% of all cases tested. None of the tissues from healthy control sheep was positive in any of the tests.

4. Discussion

Histopathology is the oldest and time-tested method for diagnosis of paratuberculosis in animals (Clarke, 1997; Perez et al., 1996). But due to possible involvement of other mycobacteria in causation of granulomatous enteritis and lymphadenitis, laboratory confirmation by specific detection of MAP or its genome is required. In most of the developing countries especially tropical ones, shipment of fresh biological samples is practically not feasible, and therefore, alternative methods are required to be developed. During necropsy of sheep dying at organized farms tissue samples are routinely collected in 10% formalin for

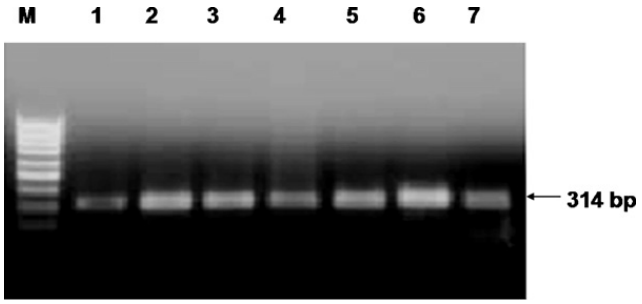

Fig. 3. IS*900* PCR on fresh infected tissues: Lane M: 100 bp DNA ladder, Lane 1: Positive control, Lane 2: S-17, Lane 3: S-18, Lane 4: S-19, Lane 5: S-20, Lane 6: S-21, and Lane 7: S-22.

histopathology. Most of the time, these tissues are not available for histological processing to laboratory within a week or so, rather it reaches laboratory after several weeks or even months for retrospective diagnosis. Whilst these tissues are good enough for histopathology, they are not suitable for nucleic acid detection as formalin has deteriorating effect on the quality of DNA and, therefore, on the sensitivity of PCR detection methods (Lewis et al., 2001; Srinivasan et al., 2002; Williams et al., 1995). A number of studies have indicated that non cross-linking alcoholic reagents generally yielded superior results as nucleic acid fixatives than aldehydes (Giannella et al., 1997). In the present study, we collected tissues from infected and healthy sheep in 70% alcohol and stored for over 6 months before extraction of DNA and evaluation of IS*Mav2* gene PCR.

The infected animals included in this study were from endemically infected organized farms and had shown clinical signs and gross and histological changes with large numbers of AF bacilli compatible with paratuberculosis (Perez et al., 1996; Clarke, 1997). Our procedure of DNA extraction and IS*Mav2* PCR on alcohol-fixed tissues could detect 82.6% of all cases tested which had demonstrable AF bacilli in tissue sections. The results were comparable to IS*900* PCR that was carried out on the freshly collected tissue samples. Despite the wide difference between the copy number of these two genes: IS*900* (approximately 17 copies) and IS*Mav2* (3 copies), non-significant differences in the detection rate of two PCRs suggest the utility of alcohol-fixed tissue PCR. In one of the previous studies in our laboratory, sensitivity of IS*900* PCR and *251* gene PCR did not differ significantly, despite the fact that *251* gene locus is found in single copy (Sonawane, 2009). Twenty cases for which data were available for both PCR, IS*Mav2* on alcohol-fixed tissues detected 90% (18/20), whereas IS*900* PCR on fresh tissues detected 95% (19/20) indicating a negligible difference in the sensitivity. In a previous study, fixation of human skin biopsy specimens in 50% or 70% alcohol had minimal effect on the PCR detection of *Mycobacterium leprae* (Fiallo et al., 1992).

Amplification of a 123-bp fragment of the *M. tuberculosis*-specific IS*6110* was not affected from *M. tuberculosis* spiked sputum samples fixed in 50, 70, or 95% ethanol for up to 24 h (Williams et al., 1995). It was further observed that sputum samples fixed in 70% ethanol and stored for 6 months at room temperature showed no detrimental effects on the PCR detection of *M. tuberculosis*. In another study evaluating the effect of short- and long-term storage (2 h to 30 days) and size of DNA amplification products (268–1327 bp), Greer et al. (1991) reported decreasing trend in DNA amplification products with increasing period of storage in 10% formalin, whilst there was no effect on DNA amplification of 1372 bp from tissues fixed in 95% ethanol. In the present study, even after fixation of tissues in 70% alcohol for over 6 months, we could able to amplify a DNA fragment of 313 bp, which was in agreement with the results on other mycobacteria or human DNAs (Srinivasan et al., 2002; Williams et al., 1995). However, more studies involving amplification of larger fragments of MAP genome from alcohol-fixed tissues are required.

The most ideal way of assessing the effect of alcohol fixation on detection of MAP genome by PCR could have been conducting the IS*Mav2* PCR on fresh tissue as well as on fixed tissues in alcohol. However, we intended to compare the results of IS*Mav2* PCR on alcohol fixed tissue with those of IS*900* PCR results on fresh tissue because of comparable size of the amplified products and also this gene has been most extensively used for the diagnosis of MAP infection in animals. The specificity of IS*Mav2* gene to MAP has been reported on clinical samples, MAP isolates and non-MAP isolates (Strommenger et al., 2001; Shin et al., 2004; Schonenbrucher et al., 2008; Wells et al., 2006). In a diagnostic set up, it is generally required to confirm the correctness of PCR products by DNA hybridization, restriction enzyme analysis or sequencing (Shin et al., 2004). In the present study, we sequenced PCR products and compared with IS*Mav2* gene sequence of MAP available in the database. There was more than 98% homology with published sequences of IS*Mav2* genes of cattle and sheep isolates (Accession numbers: GU045497.1, AE016958.1, AF286339.1). Thus our results showed the correctness of the PCR products as well as specificity of IS*Mav2* gene of MAP. A recent study (Mobius et al., 2008), comparing specificity of primer pairs for various specific genes of MAP applied as diagnostic targets, reported false positivity and formation of by-products with the primers of IS*Mav2* gene (Stratmann et al., 2002), which, however, were not confirmed subsequently by sequencing or by other methods.

In the present study, only 65% sensitivity of bacterial culture could be obtained on Herrold's egg yolk medium during an observation period of 16 weeks. In previous studies, it has been demonstrated that sensitivity increases, if culture from sheep tissue is made on the LJ medium as some strains prefer to grow on this medium (Aduriz et al., 1995), which was not carried out in this study. Also variation in the severity of lesions and number of bacilli and segmental nature of lesion in ovine paratuberculosis could be reasons for variation in mycobacterial load between tissue samples from the same animal and thereby affecting the sensitivity of the bacterial culture (Clarke, 1997; Perez et al., 1996).

Thus, based on the previous reports and our results, alcohol appears to be a superior fixative for PCR analysis, but it is generally not recommended for histologic analysis because of excessive shrinkage and reproduction of tissue architecture. Therefore, if PCR analysis is anticipated for laboratory confirmation of MAP infection, tissue samples would be split into two halves; one half to be fixed in 10% formalin and other half in 70% alcohol. Though this aspect was not investigated in the study and could be a subject for further research, it is expected that tissue specimen fixed in alcohol could provide opportunity for detailed analysis of DNA for molecular typing or other purposes especially for the organism like MAP which is fastidious and slow grower.

5. Conclusion

It is concluded that IS*Mav2* PCR on alcohol-fixed tissues is a sensitive method in comparison to culture, and comparable to IS*900* PCR on fresh tissue samples. The test offers an alternative or an additional method for the rapid detection of MAP in the biological samples from suspect animals.

Taken together, PCR testing of fresh or alcohol-fixed tissues in combination with bacterial culture increases effectiveness of laboratory diagnostic efforts to detect and identify paratuberculosis, the most common mycobacterial disease of ruminants.

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