

## Evaluation of four methods of DNA recovery from *Mycobacterium avium* subspecies *paratuberculosis* present in intestine tissue of goats and comparative sensitivity of IS900 PCR with respect to culture for diagnosis of Johne's disease

P K Singh, S V Singh\*, A V Singh & J S Sohal

Microbiology Laboratory, Animal Health Division, Central Institute for Research on Goats, Makhdoom,  
PO - Farah, Mathura, 281 122, India

Received 10 July 2007; revised 2 June 2008

Low sensitivity of PCR reaction for detection of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in tissues and fecal samples is mainly attributed to false negative results. Present study was undertaken to compare four methods of DNA isolation from tissues of infected animals and to determine most sensitive protocol for the recovery of DNA, suitable for IS900 PCR based detection of Johne's disease infection. Method I, the traditional van Soolingen<sup>2</sup> method of DNA isolation was adopted for the isolation of DNA from tissues. Method II was modification (hexadecyl pyridinium chloride-HPC treatment) of van Soolingen<sup>2</sup> method. Method III was traditional tissue DNA isolation method based on tissue lysis buffer. Method IV was modification of method III (HPC treatment). Using four methods of DNA isolation from 25 intestinal tissues of clinically infected goats, DNA was isolated from 15 (60.0%), 18 (72.0%), 13 (52.0%) and 13 (52.0%) tissues using method I, II, III and IV, respectively. All isolated DNA preparations were positive for MAP in IS900 PCR. HPC treatment enhanced the recovery of DNA from tissues of infected animals using method II. Therefore, method II can improve the diagnosis MAP infection using IS900 PCR.

**Keywords:** DNA, Goat, IS900 PCR, Intestine tissues, Johne's disease, *Mycobacterium avium paratuberculosis*,

Johne's disease (JD) control programs in India hampered due to lack of suitable diagnostic method. Screening of target tissues is more confirmatory as compared to faeces in the diagnosis of JD. Culture usually takes up to 4 months and ovine strains of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) are difficult to cultivate. Culture is inadequate to distinguish MAP and *M. avium* and other closely related mycobacterial species. IS900 PCR is rapid method for identification of MAP, though sensitivity of test is highly variable. False negative reactions result in low sensitivity, which may be due to inappropriate DNA isolation and presence of PCR inhibitors. Cell wall of MAP is difficult to break causing problems in DNA isolation. Thus, selection of suitable method for isolation of DNA is crucial for the detection of MAP by IS900 PCR test. Present study was undertaken to compare the sensitivity of four methods of DNA isolation from intestine tissue of goat.

### Materials and Methods

**Animal**—Entire study including collection of tissue samples from naturally infected goats suffering from clinical Johne's disease were sacrificed for experimental purpose was approved by the Institute Ethics Committee (IEC) and Institute (CIRG, Makhdoom) has been registered with Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA), Government of India, after approval of the ongoing projects, of which this study was a part.

**Collection of samples**—Thirty intestine tissues (near ileocaecal junction) were collected from goats (*Capra hircus*) of farm herds of Central Institute for Research on Goats, Makhdoom. All goats were adults (> 4 years) and suffering from clinical JD (multi-bacillary status in fecal culture). Tissues were swollen, corrugated and also positive (25 samples) on test under culture conditions.

**Culture and DNA isolation**—About 4 cm<sup>2</sup> tissue samples were homogenized in 15 ml sterilized distilled water and allowed to settle for 3-4 hr at room temperature. The supernatant (1.5 ml) of each sample

\*Correspondent author

Ph: + 91 565 2763260 – 269 Ext (O), Fax: + 91 565 2763246

E-mail: shoovir.singh@gmail.com

was taken in each of five tubes (4 tubes for DNA isolation by four different methods and 1 tube for culture) of 2 ml capacity. Hexadecylpyridinium chloride (HPC), an anionic detergent recently recommended for decontamination of tissue samples for improved detection of MAP<sup>1</sup>, has been used in this study for MAP bacilli and lysis of other bacteria/fungi/mycobacteria. Therefore, harvested supernatant was decontaminated with 0.9% of hexadecylpyridinium chloride (HPC) for overnight and about 1 ml of sediment containing MAP and HPC was starting material for isolation of MAP DNA (method II and VI), as for culture. Two bottles of Herrold's egg yolk medium (HEYM) supplemented with mycobactin J and one without mycobactin J were inoculated from each sediment<sup>2</sup>. MAP colonies were identified and confirmed on the basis of culture characteristics, mycobactin J dependency, acid fastness and slow growth. Genomic DNA was isolated using 4 methods as follows from 25 tissues, which showed positive test under culture conditions.

*Method I*—Supernatant was pelleted, washed twice with PBS (pH 6.5) and DNA was isolated as per method of van Soolingen *et al*<sup>2</sup> employed for MAP cultures.

*Method II*—Supernatant was decontaminated by keeping it for HPC (0.9%) over night and sediment (1 ml) was pelleted and washed twice with PBS (pH 6.5). Pellet was subjected to DNA isolation by the method of van Soolingen *et al*<sup>3</sup>.

*Method III*—Supernatant was pelleted and washed twice with PBS (pH 6.5). The DNA extraction from this pellet was done as per Fekete *et al*<sup>4</sup>. Pellet was digested with 400 µl of tissue lysis solution [ 2%, Triton-X; 1%, SDS; 100mM, NaCl; 10mM, Tris HCl; pH 8.0] and 5 µl of proteinase K (20 mg/ml) and incubated for 30 min at 50<sup>0</sup>C. Saturated phenol (400 µl) was mixed thoroughly and centrifuged for 5 min at 10,000 rpm. Aqueous layer was transferred to fresh tube and equal volume of chloroform-iso-amyl alcohol (24:1) was added and mixed thoroughly and centrifuged for 5 min at 10,000 rpm. This step was repeated once more and aqueous layer was transferred to a fresh tube. Double volume of chilled absolute alcohol was mixed and stored overnight at -20<sup>0</sup>C. DNA was recovered by centrifuging samples for 10 min at 12,000 rpm. Pellet washed with 70% ethanol, air dried and suspended in 30 µl TE buffer [pH 8.0].

*Method IV*—Supernatants was first decontaminated as per method II and DNA was isolated using Method III.

Genomic DNA was purified by Qiagen kit and checked for quality and quantity on agarose gel and by spectrophotometry, respectively.

*IS900 PCR*—DNA obtained from aforementioned 4 methods was screened by *IS900PCR*<sup>5</sup>. PCR reaction mix (50 µl) contained; 0.2 mM each of 4 dNTPs, 1µM each of 2 primers, 1.5 mM of MgCl<sub>2</sub>, 1 U of Taq polymerase, and template DNA (50-100 ng). PCR reactions were conducted (M J Research, PTC-200) under the conditions- 94°C for 4 min; 30 cycles at 94°C for 10 sec, 61°C for 10 sec, 72°C for 10 sec and finally 72°C for 10 min. PCR products were separated in agarose gel (2%) at 5 V/cm for 30 min, stained with ethidium bromide, and expected PCR products (229 bp) were visualized by gel document system. DNA from known local MAP culture of Indian 'Bison type' strain (S-5) was positive control and sterilized distilled water was negative control. Expected size of amplicons (229 bp) obtained in all 4 methods were further confirmed by sequencing of PCR products and by subjecting assembled sequence data to BLAST (NCBI).

*Statistical analysis*—Relative sensitivity, specificity, and kappa values were calculated comparing each PCR test (using different DNA isolation methods) with tissue culture.

## Results

*Bacterial culture*—Multibacillary colonies were recovered in all 25 samples from intestine tissues of goat. All colonies were typical MAP colonies having mycobactin J dependency, acid fastness and slow growth.

*IS900 PCR*—The 10, 7, 12 and 12 DNA samples extracted by method I, II, III and IV, respectively, were not intact and not suitable for PCR amplification. However, all 25 samples were subjected to *IS900 PCR* and specific 229 bp amplicons (Fig. 1) were obtained from 15, 18, 13, and 13 DNA extracted by methods I, II, III and IV, respectively (Fig. 2). As compared to other three methods, method II was most efficient for isolation of DNA of MAP from tissues. Relative sensitivity of PCR using DNA from 4 different methods with 'tissues culture' was 60.0, 72.0, 52.0 and 52.0% for methods I, II, III and IV, respectively. Results of PCR using DNA from methods I, II, III and IV on

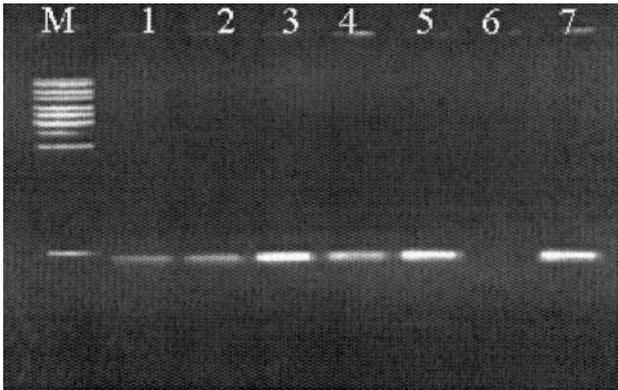


Fig. 1—MAP specific amplicons using IS900 PCR for MAP in tissues. [Lane M: 100bp DNA marker, Lane 1-5: test samples, Lane 6: negative control, Lane 7: positive control].

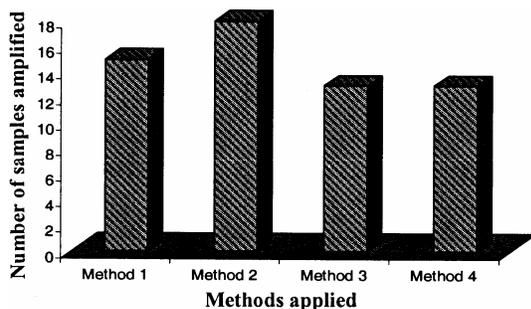


Fig. 2—Results of IS900 PCR to detect MAP infection in tissues samples using four different methods of MAP DNA isolation methods

comparison with culture gave Kappa scores of 0.60, 0.72, 0.52 and 0.52, respectively.

**DNA sequencing of IS900 PCR products—**Assembled sequence data was subjected to BLAST (NCBI) and sequences were confirmed as IS900 specific for MAP.

## Discussion

Rapid and early detection of MAP infection is important for the control of JD in herds. It reduces risk of transmission of MAP to healthy animals and human beings. MAP is difficult to control in herds if detected late. Isolation of MAP by culture is most definitive, but it takes up to 16 weeks<sup>2</sup>. Direct amplification of MAP DNA from clinical samples has potential to reduce time required for culture, but it suffers from false negative results due to poor recovery of DNA, thick lysis resistant cell wall of MAP and presence of inhibitory substances in clinical specimens. To address some of the limitations, different methods of cell wall lysis and removal of

inhibitors have been developed. Culture is not sensitive enough, especially in sheep and goats<sup>6</sup>. Dimareli-Malli *et al.*<sup>7</sup> have reported sensitivity of culture in intestinal samples of goats and sheep as 74.0 and 46.0%, respectively. Mesenteric lymph nodes of goats and sheep have sensitivity of 60.0 and 36.0%, respectively. On comparing the confirmed histo-pathological changes with culture, 54.0% sensitivity has been reported by them. Poor sensitivity of culture may be due to presence of small number of bacilli in sub-clinical stage of disease or presence of spheroplastic form in tissues<sup>8,9</sup>. In the present study, culture of tissues of goats with clinical JD and prior positive status in fecal culture showed 100 % sensitivity to detect MAP from necropsy tissues. All tissues were positive in culture, therefore used as reference test to check efficiency of 4 methods of DNA isolation.

In this study, efficacy in terms of extraction of MAP DNA by 2 classical methods, Method I and III (using tissues lysis buffer) with modified Method II and IV (treatment with HPC) were compared. Electrophoresis of DNA isolated by Methods I and II showed that DNA was of good quality than isolated by other 2 methods. Accordingly, results of PCR from Methods I and II were better than other 2 methods. Kumar *et al.*<sup>10</sup> have also used method II for detection of MAP in goat kids and of 9 intestinal tissues 3 (33.3%) were positive by IS900 PCR that was in agreement with results of culture and pathomorphology. Sivakumar *et al.*<sup>11</sup> have compared efficacy of culture and IS900 PCR for the detection of MAP. Of 20 (4.9%) animals showed histological lesions suggestive of paratuberculosis, 14 (70%) and 6 (30%) were positive in PCR and culture, respectively. PCR is more sensitive than culture in detection of sub-clinical paratuberculosis in water buffaloes.

Treatment of samples with HPC prior to DNA isolation proved to be more efficient as described earlier<sup>1</sup> compared to classical DNA isolation methods (except method IV by which same number of samples were amplified as by method III). Treatment of tissues with HPC has been used for decontamination. HPC, an anionic detergent, has influence on cell wall that probably enables better DNA isolation<sup>1</sup>. Decontamination also helped in concentration of MAP. Though culture is as sensitive as PCR, differences may still occur because live undamaged bacilli are necessary for culture, but only qualitatively

preserved DNA are required for a PCR<sup>12</sup>. Sensitivity of method II with tissues culture was 72.0%, which is in close agreement with 79.0% sensitivity reported by Dimareli-Malli *et al.*<sup>7</sup> against histo-pathological study of tissues. In this study, assessing the agreement of PCR tests to tissues culture, only method II showed substantial agreement, whereas K values for method I, III and IV suggested good agreement with culture results. Although, method I lie in the same categories of 'good agreement' with method III and IV, the proportional agreement is nearly on the borderline (0.60) of substantial agreement. This, further indicated the crucial role of HPC in isolation of DNA from tissues for detection of MAP. It can be concluded that for rapid diagnosis of MAP infection in goats, IS900 PCR using HPC assisted DNA isolation (Method II) was best, however samples from clinically diagnosed cases should also be processed by culture method.

## References

- 1 Pislak M, Ocepek M, Zabavnik Piano J & PogaNik M, Comparison of four methods for isolation of *Mycobacterium avium* subsp. *paratuberculosis* DNA from tissue samples. *Slov Vet Res*, 40 (2003) 91.
- 2 Singh N, Singh S V, Gupta V K, Sharma V D, Sharma R K & Katoch V M, Isolation and identification of *Mycobacterium paratuberculosis* from naturally infected goatherds in India. *Indian J Vet Path*, 20 (1996) 104.
- 3 van Soolingen D, Hermans P W, de Haas P E, Soll D R & van Embden J D, Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. *J Clin Microbiol*, 29 (1991) 2578.
- 4 Fekete A, Bantle J A & Halling S M, Detection of *Brucella* by polymerase chain reaction in bovine fecal and maternal tissues. *J Vet Diag Invest*, 4 (1992) 79.
- 5 Vary P H, Andersen P R, Green E, Hermon-Taylor J & McFadden J J, Use of highly specific DNA probes and the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in Johne's disease. *J Clin Microbiol*, 28 (1990) 933.
- 6 Choy E, Whittington R J, Marsh I, Marshall J & Campbell M T, A method for purification and characterization of *Mycobacterium avium* subsp. *paratuberculosis* from the intestinal mucosa of sheep with Johne's disease. *Vet Microbiol*, 64 (1998) 51.
- 7 Dimareli-Malli Z, Sarris K, Papadopoulos O, Iliadis N, Xenos G & Papadopoulos G, Caprine and ovine isolates of *Mycobacterium paratuberculosis*. *Paratubercul News*, 9 (1997)18.
- 8 Chiodini R J, Van Kruiningen, H J, Thayer W R & Coutu J A, Spheroplastic phase of mycobacteria isolated from patients with Crohn's disease. *J Clin Microbiol*, 24 (1986) 357.
- 9 Miller J M, Aljeny J B & Payer R, PCR detection of *Mycobacterium tuberculosis* complex and *Mycobacterium avium* organisms in formalin fixed tissue from culture negative ruminants. *Vet Microbiol*, 87 (2002) 15.
- 10 Kumar P, Singh S V, Bhatiya A K, Sevilla I, Singh A V, Whittington, R J, Juste R A, Gupta V K, Singh P K, Sohal J S & Vihan V S, Juvenile Capri-Paratuberculosis (JCP) in India; Incidence and characterization of by six diagnostic tests. *Small Rumin Res*, 73 (2007) 45.
- 11 Sivakumar P, Tripathi B N & Singh N, Detection of *Mycobacterium avium* subsp. *paratuberculosis* in intestinal and lymph node tissues of water buffaloes (*Bubalus bubalis*) by PCR and bacterial culture. *Vet Microbiol*, 108 (2005) 263.
- 12 Altwegg M, General problems associated with diagnostic applications of amplification methods. *J Microbiol Methods*, 23 (1995) 21.