

Rapid detection of *Mycobacterium avium subsp. paratuberculosis* from cattle and zoo animals by Nested PCR

¹Joseph Erume, ²Joachim Spargser and ²Renate Rosengarten

¹Faculty of Veterinary Medicine, Makerere University, P.O.Box 7062, Kampala, Uganda. ²Institute of Bacteriology, Mycology and Hygiene, University of Veterinary Medicine Vienna, Veterinärplatz 1, A-1210 Vienna, Austria.

ABSTRACT

Paratuberculosis, caused by *Mycobacterium avium subsp. paratuberculosis*, a suspect causative agent of Crohn's disease in man, is an emerging disease of international proportions affecting all ruminants. Early stage detection of *Mycobacterium avium subsp. paratuberculosis* infection would accelerate progress in control programmes. Despite new molecular approaches the standard diagnostic test for this disease is at present still the time consuming classic isolation procedure. Therefore, alternative diagnostic tests such as PCR, are needed for quick detection of infected animals. In this study, the conventional enrichment and isolation procedure and two IS900-based PCR methods for detection of *Mycobacterium avium subsp. paratuberculosis* in clinical samples from zoo animals and cattle were compared. A total number of 48 different clinical specimens obtained from animals suspected of having paratuberculosis were examined. The samples included faeces (n = 15) and organ tissues (n = 33). Of the faecal specimens two were identified as positive by nested PCR, whereas none was positive by single PCR or by culture. 28 organ specimens were found positive by culture. *Mycobacterium avium subsp. paratuberculosis* DNA was detected by nested PCR in 82% of the organ specimens identified positive by culture (23 samples) as opposed to 57% by single PCR (16 samples). Nested PCR also identified two positive samples that were not detected by either culture or single PCR. These findings show the great potential of nested PCR as a useful tool for the rapid diagnosis of paratuberculosis in animals.

African Health Sciences 2001; 1(2):83-89

INTRODUCTION

Paratuberculosis or Johne's disease is a chronic progressive infectious disease that affects all categories of domestic and wild ruminants including cattle, goats, camels, buffaloes and farmed deer ^{1,2}. It is caused by *Mycobacterium avium subsp. paratuberculosis*, a small, fastidious acid-fast bacterium. This organism is also suspected to be a potential causative agent of Crohn's disease in humans ^{3,4}. The disease occurs throughout the world and is responsible for considerable economic losses. There is no therapy and it invariably leads to the death of the affected animal ⁵. Control of the disease in ruminants is dependent on the early detection and culling of infected animals. Attempts to the control are, however, severely hampered by inadequate diagnostic procedures. At present, the standard diagnostic procedure involves culture of the organisms from faeces ^{6,7}. The problem with the culture procedure is that it is time consuming and requires 8-16 weeks of incubation and use of specialised culture medium ⁸. Despite decon-

tamination steps, cultures are often lost because of contamination. In addition, clinically infected animals may shed organisms sporadically during disease and therefore false negative results may occur ⁹. Furthermore, though the slow growth rate and the dependence on exogenous mycobactin for *in vitro* growth are characteristic for *Mycobacterium avium subsp. paratuberculosis*, these are not enough to differentiate it from other mycobacteria in particular *Mycobacterium avium subsp. avium* and *Mycobacterium avium subsp. silvaticum* ¹⁰. Due to the limitations associated with the culture method, a number of other tests have been developed to aid in the diagnosis of paratuberculosis ^{6,8,11}. The technique right now that is the most focus of attention is the polymerase chain reaction (PCR). The potential value of the PCR in diagnosing *Mycobacterium avium subsp. paratuberculosis* infections has been recognised for some time and this technique has been applied in a variety of clinical samples ¹². A break through in the use of the PCR for diagnosis of paratuberculosis was the detection of IS900, a repetitive DNA insertion element unique to *Mycobacterium avium subsp. paratuberculosis*. IS900 can be specifically detected by PCR ^{9,13,14}.

The PCR technique offers specific and rapid detection of *Mycobacterium avium subsp. paratuberculosis*, however, reduced levels of sensitivity have been encountered when it has been applied on clinical samples, and these reductions have been attributed to the presence of inhibitors ^{9,12}. Although inhibitors undoubtedly are involved, the poor sensitivity is also likely a result of inefficient amplification of DNA. This follows a result of findings that

Correspondence to:

Dr. Joseph Erume

Faculty of Veterinary Medicine

Makerere University

P. O. Box 7062 Kampala, Uganda

Tel: 256(41)533002; Fax: 256(41)534336

Email: erujoseph@yahoo.com

sensitivity of amplification may be enhanced by nested polymerase chain reaction, a two-step amplification procedure¹⁵. The purpose of this study was to assess the potential of nested PCR for the rapid detection of *Mycobacterium avium subsp. paratuberculosis* in clinical samples from animals and compare its performance with single PCR and bacteriological culture.

METHODS

Samples

We have tested 48 clinical specimens from animals suspected of having paratuberculosis. Of these samples 33 were tissues (ileum, mesenteric lymph nodes and liver) and 15 were faeces. The specimens were obtained by clinicians from zoo animals including ibex, markhor, elk, tapir, waterbuck, wild goat, wild swine, bison and from sheep and cattle. Faeces and tissue samples were collected ante-mortem and at necropsy respectively. After collection the samples were immediately put in sterile plastic bags and placed in cooling boxes. They were transported to the Institute of Bacteriology, Mycology and Hygiene, University of Veterinary Medicine, Vienna, where they were kept at -20°C.

Ziehl-Neelsen Staining

The faecal and tissue clinical samples from cattle and zoo animals submitted to the Institute were thawed within one week and were screened for acid-fast bacilli compatible with Johne's bacillus using the standard Ziehl-Neelsen (ZN) procedure¹⁶.

Culturing of bacteria from clinical samples

Culturing from faecal samples was performed according to the suggested standardised procedure⁸. After decontamination of the samples with 0.9% hexadecyl pyridinium chloride (HPC) (final concentration, 0.75% HPC), each was inoculated into 3 tubes of Herrold egg yolk medium (HEYM) (self prepared according to the previous procedure¹⁷) with mycobactin J and 1 tube without mycobactin J. Tubes were incubated at 37°C in a horizontal position with loosened caps for about 1 week. Caps were tightened and incubation continued with tubes in a vertical position for 3-4 months.

Culturing from tissues was carried out on HEYM after decontamination with HPC as for faecal samples. First 1 g of tissue was weighed and homogenised in 0.5 ml sterile distilled water using a mortar and pestle. The homogenate was then transferred to a 50 ml centrifuge tube. 25 ml of 0.9% HPC was added to the homogenised material, shaken, and the material allowed to stand for 30 minutes. After settling, the cellular fraction (minus tissue fragments) was transferred to a second 50 ml centrifuge

tube. Decontamination of the sample was carried out overnight at laboratory temperature. This was followed by centrifugation at 1700 x g for 20 minutes. The supernatant was discarded and 0.1 ml of sediment per tube was inoculated on HEYM after resuspending the pellet in 0.5 ml sterile water. Inoculation and incubation of samples was done as for faecal samples.

Extraction of DNA from faecal samples

An aliquot of faecal samples was suspended in 500 µl of lysis buffer (20 mM Tris-HCl, 1 mM EDTA, 30 mM DTT, 0.5% SDS) supplemented with 0.4 mg/ml proteinase K (Boehringer, Mannheim, Germany). The samples were kept overnight in a heating block set at 55°C. After the lysis, samples were heated at 95°C for 10 minutes. To each sample, 80 µl 10% Cetyl trimethyl ammonium bromide (CTAB) (No. 85, 582-0 Aldrich) and 100 µl 5M NaCl were added, mixed and incubated at 65°C for 10 minutes. The DNA was then extracted with an equal volume of chloroform isoamylalcohol (24:1 v/v) and similarly with phenol chloroform isoamylalcohol (25:24:1 v/v/v) and precipitated with 0.6X of ice cold isopropanol. The DNA was pelleted by centrifugation at 12000 x g for 20 minutes and the pellet washed with 70% ethanol and air dried. The DNA was finally resuspended in 50 µl of sterile distilled water.

Isolation of DNA from tissue samples

The extraction technique was a modification of that described previously¹². Portions of 0.1 g tissue were mixed in microcentrifuge tubes with 0.5 ml water and ground. 0.5 ml NALC (3% NaOH; 1.45% sodium citrate; 0.5% N-acetyl-L-Cystein) was added and the samples were vortexed and left at ambient temperature for 30 minutes. The samples were vortexed after addition of 0.2 ml of xylene and left at ambient temperature for 30 minutes. The upper creamy layer was transferred to new tubes and the mycobacteria were recovered by centrifugation at 12000 x g for 10 minutes. The supernatant was removed and the pellet washed once with 0.5 ml 100 % isopropanol (pellet was dissolved in the 0.5ml isopropanol and centrifuged at 12000 x g for 10 minutes). The pellet was then dissolved in 0.5 ml PBST (67 mM PBS; (pH 6.8), 0.5% Tween 80) followed by centrifugation at 12000 x g for 10 minutes. The final pellet was resuspended in 100 µl TE digestion buffer (100 mM Tris-HCl buffer (pH 8.5), 0.5 mM EDTA; 1% Tween 20), supplemented with 300 µg of proteinase K per ml and incubated at 55°C overnight¹⁸. After digestion, the specimens were centrifuged briefly at 12000 x g and incubated at 95°C for 10 minutes to inactivate the proteinase K. Before PCR, the samples were briefly centrifuged 12000 x g and 5 µl of the super-

nants were used in the PCR.

DNA extraction and subculture of colonies

Isolation of DNA from suspect colonies and from those of the reference strain (ATCC 19698) (DNA L. Engstrand, Akademiska sjukhuset, Uppsala, Sweden) was done using TE digestion buffer supplemented with proteinase K as for tissue samples above. Colonies were picked and suspended in 500 µl NaCl (0.85%). For colonies from samples, a drop of suspension was examined by ZN and a portion subcultured on HEYM to determine mycobactin dependency. The remaining suspension was centrifuged at 16000 x g for 10 mins. The pellet was then washed once with 500 µl PBS at 16000 x g for 10 mins. Final pellet was suspended in 100 µl digestion buffer and incubated at 55°C for 4-5 hours. Samples were then incubated at 95°C for 10 mins before being frozen at -20°C until analysed by PCR.

Analysis of clinical samples with Single PCR

Enzymatic amplification of DNA was performed by a standard PCR technique described previously¹⁹. Primers, Mp3 (5'-CTGGCTACCAAACCTCCCGA-3') and Mp4 (5'-GAACTCAGCGCCAGGAT-3') derived from the published DNA sequence of IS900² of *Mycobacterium avium subsp. paratuberculosis* resulting in a 314 base pair product were used. The PCR was performed as described earlier but with the bead beating step omitted²⁰. Amplification was carried out in a 50 µl final volume. This consisted of a 5 µl DNA sample mixed with a 45 µl master mix containing 67 mM Tris-HCl pH 8.8, 2 mM MgCl₂, the four deoxyribonucleotide triphosphates, dATP dCTP dGTP dTTP (100 µM each), 1 µM of each of the oligonucleotide primers, 2.5 U *Tag* polymerase (Promega, Madison WI, USA) in a 0.5 ml Eppendorf tube. Samples were subjected to an initial denaturation step of 94°C for 3 min and then to 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min in a Perkin Elmer 480 thermal cycler (Perkin Elmer Cetus, USA). A negative control (sterile water), and a positive control DNA from *Mycobacterium avium subsp. paratuberculosis* strain ATCC 19698, were included in each amplification run.

Analysis of clinical samples with Nested PCR

For nested PCR, amplification was first performed with the primers, para 1 (5' TGA TCT GGA CAA TGA CGG TTA CGG A 3') and para 4 (5' CGC GGC ACG GCT CTT GTT 3') based on IS900 of *Mycobacterium avium subsp. paratuberculosis* targeting a 563 base pair product¹⁵. The final reaction mixture and amplification conditions were similar to those used for Single PCR above.

To reamplify the DNA, 1 µl of each of the first

amplification products was transferred to tubes containing the same reaction mixture described above except that oligonucleotide primers para 1 and para 4 were replaced by oligonucleotides para 2 (5' GCC GCG CTG CTG GAG TTG A 3') and para 3 (5' AGC GTC TTT GGC GTC GGT CTT G 3'), which recognise sequences contained within the sequence amplified by para 1 and para 4 and an amplicon target of 210 base pairs¹⁵. The samples were subsequently amplified for an additional 35 cycles as described above.

Agarose gel electrophoresis

The PCR products were subjected to agarose gel electrophoresis for 40-45 minutes. The gel contained 1% agarose in 1 x TBE buffer. The gels were stained for 20 min in Ethidium bromide, rinsed in distilled water and visualised by UV-light transillumination.

RESULTS

We have tested 48 clinical specimens from animals suspected of having paratuberculosis. Of these samples 33 were tissues (ileum, mesenteric lymph nodes and liver) and 15 were faeces. These specimens were obtained from zoo animals including ibex, markhor, elk, tapir, waterbuck, wild goat, wild swine, bison and from sheep and cattle.

Detection of infection from tissue samples

28 of the 33 tissue samples were found to be culture positive. Colonies compatible with *M. avium subsp. paratuberculosis* were first noted at 8 weeks of incubation. But the majority of growths were seen from 10-16 weeks of incubation. 11 samples grew colonies other than *M. avium subsp. paratuberculosis*. Of these samples, one showed colonies within two weeks while in the remaining 10 samples contaminant colonies were observed at 4 weeks of incubation. In all these samples, contaminants were seen in all the 4 tubes inoculated per sample. Later colonies compatible with *M. avium subsp. paratuberculosis* were also seen in the 11 samples.

Suspect colonies were characterised by ZN staining and confirmed by PCR as of *M. avium subsp. paratuberculosis*. Furthermore, isolates were found to be mycobactin dependent when subcultured. The identity of contaminants was not determined. The 33 different clinical tissue samples were tested by single PCR and 16 of the 33 tissue specimens were found positive.

When nested PCR was applied on the DNA from the clinical samples, 23 were found positive. None of the 5 tissue samples that were found negative by culture were positive by nested PCR. The details of results of culture, ZN staining and PCR on tissue samples are presented in Table 1.

Table 1 Paratuberculosis: Detection of infection from tissue samples by PCR, bacteriological culture and ZN staining

Animal species	Number of Tissues taken Samples	Direct ZN+	Culture results				Single PCR +	Nested PCR +
			Positive		Negative			
			ZN +	ZN -	ZN +	ZN -		
Ibex	18	7	14	1	0	3	9	12
Cattle	5	4	4	0	0	1	4	4
Markhor	4	2	3	1	0	0	1	2
Tapir	1	0	0	1	0	0	0	1
Wild swine	1	1	1	0	0	0	0	1
Sheep	1	0	1	0	0	0	0	1
Wild goat	2	2	2	0	0	2	2	2
Elk	1	0	0	0	1	0	0	0
Total		33	16	25	3	5	16	23

Detection of infection from faecal samples

M. avium subsp. paratuberculosis was also cultured from all the 15 faeces samples. No isolates were obtained from any of these samples even in the bovine sample from which acid-fast bacilli were earlier seen by direct microscopy. When single PCR was performed on the 15 faecal samples, no *M. avium subsp. paratuberculosis* DNA was amplified in any of the samples. Furthermore, of all the 15 faecal samples found negative by culture and single PCR, two samples were found positive by nested PCR. These included one sample from wild swine and the other from a cow. Table 2 shows a summary of culture, ZN, and PCR results on the faeces samples.

Table 2 Paratuberculosis: Detection of infection from faecal samples by PCR, bacteriological culture and ZN staining

Animal species	No. of faeces samples	ZN Positive	Culture Positive	Single PCR Positive	Nested PCR Positive
Cattle	11	1	0	0	1
Elk	1	0	0	0	0
Waterbuck	1	0	0	0	0
Wild Swine	1	0	0	0	1
Bison	1	0	0	0	0
Total	15	1	0	0	2

Comparison of PCR , ZN and bacteriological culture

Culture is taken as the „gold standard“ for the diagnosis of *M. avium subsp. paratuberculosis* infection in animals. Of the 28 organ samples found positive by culture 16 were identified as positive by single PCR representing a detection rate of 57% (16/28). Nested PCR on the other hand detected 23 of the 28 samples, a detection rate of 82% (23/28) as compared to culture. Direct microscopy on the clinical organ samples identified acid-fast bacilli compatible with Johne's disease organisms in 17 samples.

One of these samples was found to be negative by culture and by PCR and was therefore classified as false positive. In this study, direct microscopy showed a detection rate of 57% (16/28) similar to that of single PCR. 10 clinical organ samples tested by both methods of PCR are shown in figure 1 (single PCR) and figure 2 (nested PCR).

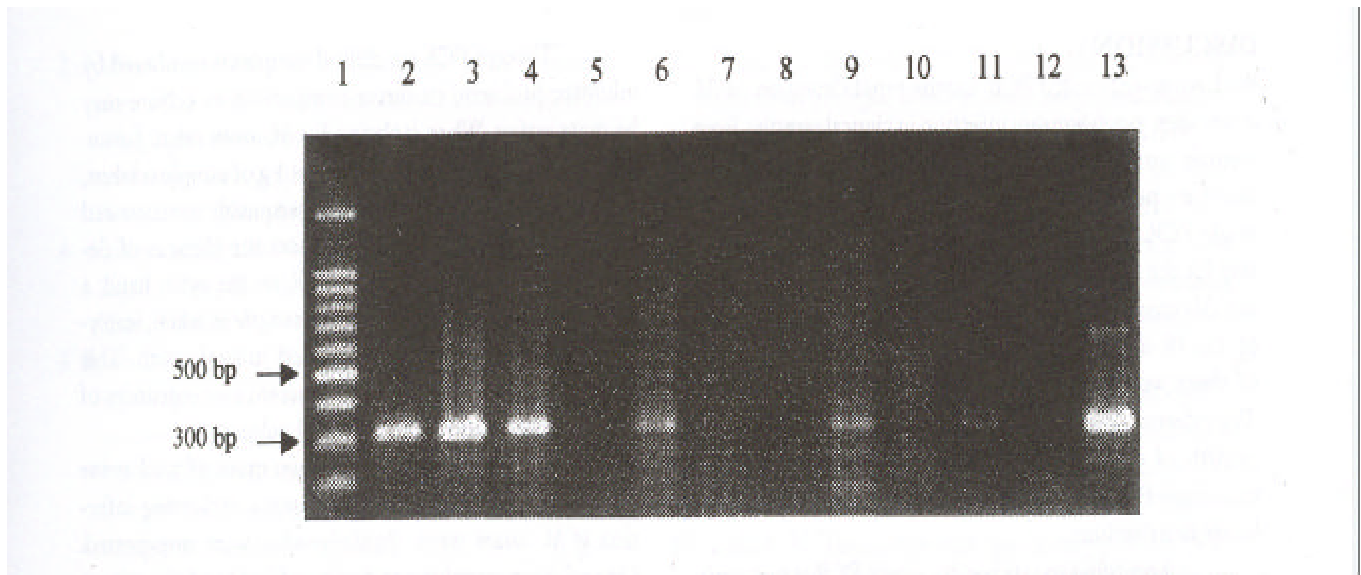


Figure 1 Ethidium bromide stained gel of PCR product after amplification in the single PCR for *M. avium subsp. paratuberculosis*. Lane 1 molecular weight standard (100 base pair ladder), lane 2 to 11 ten clinical organ samples tested [lanes 2,3,4 were intestines, lymphnodes and lymphnodes tissues from cattle respectively, lanes 5 and 6 were intestinal tissues from ibex, lane 7 lymphnode from ibex, lanes 8,9 and 10 were intestines from ibex and lane 11 lymphnode from markhor], lanes 12 and 13 negative and positive controls respectively. The lower arrow indicates the position of migration expected for the amplified fragment produced by the amplification protocol (314 bp).

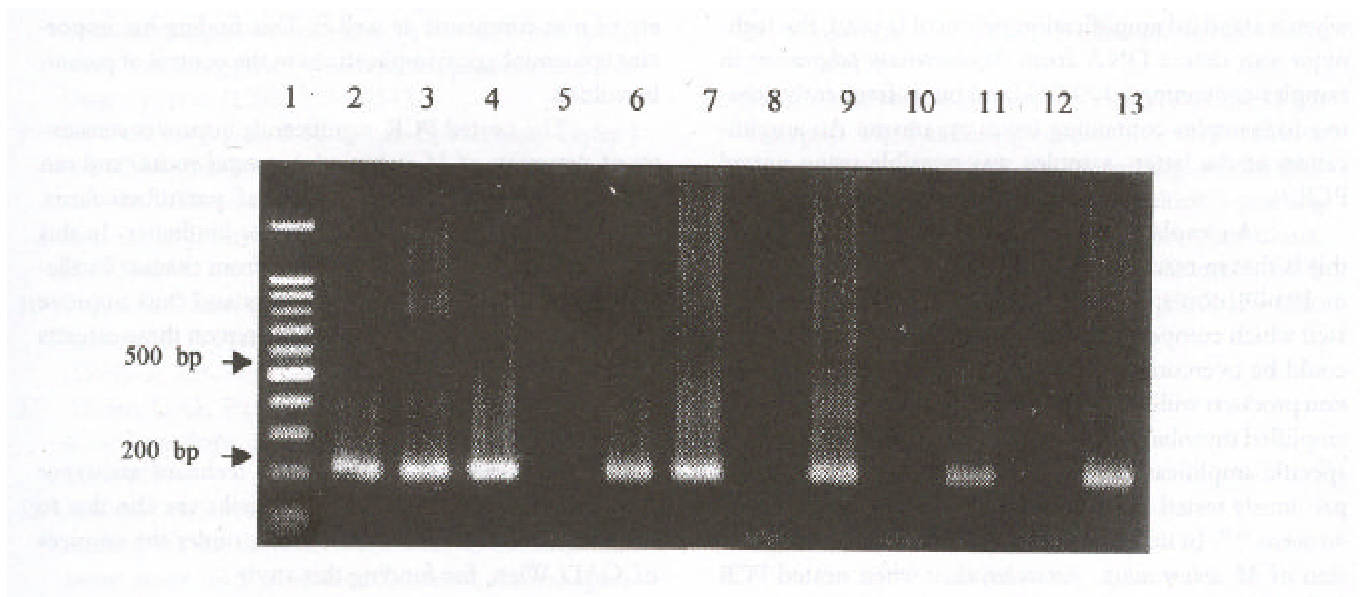


Figure 2 Analysis of the 10 clinical samples shown in figure 1 above by nested PCR. Lane 1 molecular weight standard (100 base pair ladder), lane 2 to 11 samples tested for *M. avium subsp. paratuberculosis*, lane 12 negative control, lane 13 positive control. The lower arrow indicates the position of migration expected for the amplified fragment produced by the reamplification protocol (210 bp).

Overall, 30 clinical samples were identified as positive, with culture showing a detection rate of 93% (28 samples), nested PCR 83% (25 samples) and both single PCR and ZN a rate of 53% (16 samples). Nested PCR increased sensitivity for the detection of *M. avium subsp. paratuberculosis* approaching that of culture. Moreover it detected 2 faecal samples that were not identified by culture or single PCR.

DISCUSSION

We have tested nested PCR for the rapid detection of *M. avium subsp. paratuberculosis* infection in clinical samples from animals suspected of having paratuberculosis and compared its performance with bacteriological culture and single PCR. Bacteriological culture is currently the main stay for the diagnosis of this disease but its time consuming. *M. avium subsp. paratuberculosis* was isolated from 28 of the 48 clinical samples tested (Table 1 and 2). Sixteen of these were identified by single PCR test as infected. The infected samples detected by single PCR test showed growth of colonies at 8 weeks of incubation implying that single PCR is able to detect clinical samples with high levels of infection.

According to our results, single PCR is not sensitive enough as a diagnostic test for clinical samples. This low sensitivity of single PCR has been reported previously and was attributed to the presence of inhibitors of the amplification^{9,12}. Studies on *Mycobacterium tuberculosis* have, however, found that this lower sensitivity of the standard amplification protocol when applied on clinical samples is neither solely due to presence of inhibitors nor to extraction of mycobacterial DNA-. It was found that when a standard amplification protocol is used, the technique can detect DNA from *Myobacterium tuberculosis* in samples containing >100 CFU/ml but is frequently negative for samples containing fewer organisms. An amplification of the latter samples was possible using nested PCR²¹.

An explanation that has been put forward for this is that in reactions containing low numbers of target molecules, non-specific amplification products are generated which compete with the mycobacterial DNA¹⁹. This could be overcome by reamplifying the initial amplification products with primers which recognise the inefficiently amplified mycobacterial DNA but not recognise the non-specific amplification products. This approach has been previously tested for detection of *M. avium subsp. paratuberculosis*^{15,18}. In this present study, the sensitivity of detection of *M. avium subsp. paratuberculosis* when nested PCR was used was evidently superior to that obtained when single PCR was applied. Interestingly, Nested PCR detected 2 positive faeces samples which even culture had not identified. The nested PCR detection rate of 82% from the clinical organ samples is somewhat disappointing because it showed a lower sensitivity compared to bacteriological culture. The main reason for this lower sensitivity of nested PCR in this present study was its failure to amplify 5 tissue samples that were found positive by culture.

The failure to amplify these samples could be attributed to the presence of inhibitors of *Tag* polymerase in the original samples.

Though PCR on clinical samples is cumbered by inhibitor problem, its direct comparison to culture may be quite unfair. When isolating for *M. avium subsp. paratuberculosis* from clinical samples about 1 g of sample is taken, decontaminated and organisms subsequently concentrated before culture. This greatly enhances the chances of detecting the positive cases. For PCR on the other hand, a comparatively smaller amount of sample is taken, implying that few organisms are targeted to begin with. This may be another reason for the apparent low sensitivity of PCR as compared to conventional culture.

On the other hand, the two cases of wild swine that responded to IS900 PCR primers, indicating infection of *M. avium subsp. paratuberculosis* were unexpected. One of these samples was a piece of liver and the other a fecal sample. On culture, the tissue sample showed organisms other than *M. avium subsp. paratuberculosis* within 4 weeks of incubation. Colonies compatible with Johne's disease bacilli were observed later after 8 weeks of incubation and on tubes with mycobactin J only. This was probably a case of a mixed infection. Previously *M. avium subsp. paratuberculosis* was thought to infect only ruminants, however, increasingly infection is being detected in a variety of non-ruminants as well²². This finding has important epidemiological implications in the control of paratuberculosis.

The nested PCR significantly improves sensitivity of detection of *M. avium subsp. paratuberculosis* and can be useful for the quick diagnosis of paratuberculosis. However, PCR inhibitors are a major hindrance. In this study we used crude DNA extracts from tissues. To alleviate the problem of PCR inhibitors and thus improve the sensitivity, a phenol chloroform step on these extracts could be of help.

ACKNOWLEDGEMENT

We thank Martina Wukovits for the technical assistance she rendered towards this work. Thanks are also due to the Federal Government of Austria, under the auspices of ÖAD, Wien, for funding this study.

REFERENCES

- 1 Chiodini R J; Van Kruinigen H J; Merkal R S. Ruminant paratuberculosis (Johne's disease): The current status and future prospects. *Cornell Vet.* 1984; 74: 218-262.
- 2 Bauerfeind R; Benazzi S; Weiss R; Schliesser T; Willems H; Baljer G. Molecular characterisation of *Mycobacterium paratuberculosis* isolates from sheep, goats and cattle by hybridisation with a DNA probe to insertion element IS900. *J. Clin. Microbiol.* 1996; 34: 1617-1621.

- 3 McFadden J J; Butcher P D; Chiodini R and Hermon Taylor R. Crohn's disease- associated bacteria are identical to *M. paratuberculosis*, as determined by DNA probes that distinguish between mycobacterial species. *J. Clin. Microbiol.* 1987; 25: 796-801.
- 4 Sanderson J D; Moss M T; Tizard M L V; Hermon-Taylor J. *Mycobacterium paratuberculosis* DNA in Crohn's disease tissue. *Gut* 1992; 33: 890-896.
- 5 Pavlik I; Bejckova L; Pavlas M; Rozsypalova Z; Koskora S. Characterisation by restriction endonuclease analysis and DNA hybridisation using IS900 of bovine, ovine, caprine and human dependent strains of *Mycobacterium paratuberculosis* isolated in various localities. *Vet. Microbiol.* 1995; 45: 311-318.
- 6 Colgrove G S; Thoen C O; Blackburn B O; Murphy C D. Paratuberculosis in cattle: a comparison of three serologic tests with results of faecal culture. *Vet. Microbiol.* 1989; 19: 183-187.
- 7 de Lisle G W; Samagh B S; Duncan J R. Bovine paratuberculosis. II: A comparison of faecal culture and antibody response. *Can. J. Comp. Med.* 1980; 44: 183-191.
- 8 Whipple D L; Callihan D R; Jarnigan J. Cultivation of *Mycobacterium paratuberculosis* from bovine faecal specimens and a suggested standardised procedure. *J. Vet. Diagn. Invest.* (1991) 3: 368-373.
- 9 Vary P H; Andersen P R; Green E; Hermon-Taylor J and McFadden J. Use of highly specific DNA probes and the PCR reaction to detect *Mycobacterium paratuberculosis* in Johne's disease. *J. Clin. Microbiol.* 1990; 28: 933-937.
- 10 Cocito C; Gilot P; Coene M; de Kesel M; Poupart P; Van-nuffel P. Paratuberculosis. *Clin. Microbiol. Rev.* (1994). 7: 328-345.
- 11 Thoen C O; Baum K H. Current knowledge of paratuberculosis. *J. Am. Vet. Med. Assoc.* 1988; 192: 1609-1611.
- 12 Challans J A; Stevenson K; Reid H W; Sharp J. A rapid method for the extraction and detection of *Mycobacterium avium subsp paratuberculosis* from clinical specimens. *Vet. Rec.* 1994; 95-96.
- 13 Chollins D M; Gabric D M; De Lisle G W. Identification of repetitive DNA sequence specific to *Mycobacterium paratuberculosis*. *FEMS Microbiol. Lett.* 1989 60: 175-178.
- 14 Green E P; Tizard M L V; Moss M T et al. Sequence and characteristics of IS900, an insertion element identified in human Crohn's disease isolate of *Mycobacterium paratuberculosis*. *Nucleic Acids Res.* 1989; 17: 9063-9073.
- 15 Englund S; Ballargi-Pordany A; Bolske G; Karl-Erik J. Single PCR and nested PCR with mimic molecule for detection of *Mycobacterium avium subsp. paratuberculosis*. *Diagn. Microbiol. Infect. Dis.* 1999; 33: 163-171.
- 16 Bisping W; Amtsberg G A. colour atlas for the diagnosis of bacterial pathogens in animals. Paul Parey Scientific Publishers. Berlin and Hamberg. 1988; 337.
- 17 Merkal, R.S., McCullough, W.G.; A new mycobactin, mycobactin J, from mycobacterium paratuberculosis. *Current Microbiol.* 1982; 7, 6, 333-335.
- 18 Ros Bascunana C; Belak K. Detection and identification of mycobacteria in formalin-fixed, paraffin-embedded tissues by nested PCR and restriction enzyme analysis. *J. Clin. Microbiol.* 1996; 34: 2351-2355.
- 19 Saiki R K. The design and optimisation of the PCR. In H. E. Erlich (Ed), *PCR Technology*. Stockholm Press, New York. 1989; 7-16.
- 20 Ahrens P; Giese S B; Klausen J; Inglis N F. Two markers, IS901-IS902 and p40, identified by PCR and by using monoclonal antibodies in *Mycobacterium avium* strains. *J. Clin. Microbiol.* 1995; 33: 1049-1053.
- 21 Pierre C; Lecossier D; Bousougant Y et al. Use of a reamplification protocol improves sensitivity of detection of *Mycobacterium tuberculosis* in clinical samples by amplification of DNA. *J. Clin. Microbiol.* 1991; 29: 712-717.
22. Greig A; Stevenson K; Perez V; Pirie A A; Grant J M; Sharp J M; Paratuberculosis in wild rabbits (*Oryctolagus cuniculus*). In: Proceedings of the fifth international colloquium on paratuberculosis: a meeting of the international association for paratuberculosis, Madison; Wisconsin; USA. Ed R J Chiodini, M E Hines, M T Collins. Evergreen press. 1997; 190-195.