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Comparative evaluation of PCR in Ziehl-Neelsen stained smears and PCR in tissues for diagnosis of *Mycobacterium avium* subsp. *paratuberculosis*

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Thirty six tissues from sheep, previously diagnosed with paratuberculosis, were tested by PCR in positive Ziehl-Neelsen staining smears of tissues, and PCR in tissues targeting *IS900* specific for *Mycobacterium avium* subsp. *paratuberculosis*. DNA amplification was achieved in 33.3% Ziehl-Neelsen smears, and in 61.1% tissue samples. Combination of both techniques found 66.7% samples as positive. Combination of techniques would, therefore, increase the sensitivity of diagnosis.

Keywords:*Mycobacterium avium* subsp. *paratuberculosis*, PCR, tissues, Ziehl-Neelsen

Ovine paratuberculosis, a chronic wasting disease of the intestinal tract caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*), is responsible for large economic losses worldwide[1,2]. Rapid laboratory diagnosis of infection relies on recognition of acid fast bacilli indistinguishable by Ziehl-Neelsen staining (Z-N). Z-N staining and histopathology are the two most commonly used methods for diagnosis for *Map* in tissues[2-4]. Z-N staining is cheap and easy to carry, however it evidences cell wall of the bacillus, and does not differentiate *Map* from other acid fast bacilli[5]. Z-N identifies only whole forms of bacilli, which affects *Map* identification when bacteria are degraded or spheroplasts predominate in the sample[2,6]. Other techniques in tissues include *in situ* methods in formalin-fixed samples[5], histological, molecular and culture[1,7]. Diagnosis of infected animals is time-consuming using traditional techniques[4]. Molecular methods have potential to both detect and characterize *Map* directly from clinical samples[1]. Several PCR protocols and DNA extraction methods have been published for the detection of *Map* in infected animals[1,8]. Application of PCR to detect genome directly from tissues is valuable for laboratory confirmation of infectious diseases[7].

This work aimed to compare PCR from Z-N stained smears to detect *Map* infection with PCR from tissues of sheep with paratuberculosis.

Thirty six specimens from ileum, ileocecal valve or mesenteric lymph nodes were used. Specimens belonged to 17 adult sheep previously diagnosed with paratuberculosis[9]. Specimens were stored at -20ºC for one year. Slide smears prepared from tissues impressions were stained by the Z-N technique and microscopically examined for the presence of typical clumps of acid-fast bacilli. The smears were classified as positive for *Map*. After microscopy mineral oil was removed with xylene and the material on slide was gently scraped by addition of 50 µl of sterilized distilled water[10]. Scraped material was mixed with 500 µl of TE-Triton X100. Specific *Map* DNA was detected following the extraction of genomic DNA from segments of ileum, ileocecal valve or lymph node, each one corresponding to a stained slide and from scraped slide material.

Tissue preparation to PCR analysis was done as per by Garrido *et al*.[8]. In brief, 1 g of tissue was homogenized in a Stomacher lab blender (Seward Stomacher 400) for 30 sec. in 20 ml of a solution containing 5% sodium dodecyl sulfate (SDS). After allowing to sediment for 15 min, the upper aqueous phase was transferred into 15 ml tubes and centrifuged. The pellet was washed three times in PBS. The pellet was resuspended in 2 ml of PBS and transferred into a 2 ml screw-cap tube. The suspension was centrifuged at 9600 *g* and the supernatant discarded. The pellet was transferred to an eppendorf tube and either processed immediately for DNA extraction or stored at -20°C. Prior to DNA extraction the tissue pellet were resuspended in 500 µl

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of TE-Triton X100. The DNA extraction of tissue samples and stained smears method was performed as per the protocol (freeze-boiling) of Garrido *et al*.[8].

Each specimen was used in duplicate in a PCR assay. The primers RJ1 (GTT CGG GGC CGT CGC TTA GG) and PT91 (CCC ACG TGA CCT CGC CTC CA) flanking a region of 389 bp were used for amplification of the *IS900* sequence of *Map*[11]. A 5µl DNA extract aliquot was added to a PCR mix containing 0.1 (M of each primer, 1.5mM of $MgCl_2$ and 2U of *Taq* polymerase in a total volume of 25 µl l in 200 µl tubes, covered by mineral oil. The cycling conditions with the thermal cycler (Perkin Elmer Cetus® 480) were one cycle at 96°C for 2 min, 40 cycles at 95°C for 30s, 55°C for 30s and 72°C for 1 min and a final extension time of 72°C for 10 min.

Aliquots of each amplification mixture were electrophoresed in a 2% agarose gel containing ethidium bromide. The results were analyzed blind, and a sample was scored as positive only if a 389-bp DNA fragment of the expected size was identified on the gel, and no signal was obtained with the negative control[8]. To monitor occurrence of false-positive PCR results, 20 negative controls (smears of sterilized water) were included during slide preparation. DNA obtained from *Mycobacterium avium* subsp. *paratuberculosis* ATCC19698 were used as positive control.

PCR in tissues found 22 (61.1%) specimens positive and 14 (38.9%) negative. The DNA extraction control in the PCR negative probe was negative. Among DNA samples extracted from Z-N stained slides, in 12 (33.3%) amplification was achieved from *IS900* and 24 (66.7%) were found negative. Sensitivity of PCR in tissues was 61.1% (22/36) and in stained smears was 33.3% (12/36), and specificity was 100% for both tests. Both tests were positive in 10 samples (27.8%). Two of the PCR in smears-positive samples were PCR in tissues-negative (5.6%). Twelve of the PCR in tissues-positive samples were negative (33.3%) in smear PCR. Combination of both techniques found 24 (66.7%) positive samples. The kappa (κ) value observed between the PCR in smears and PCR in tissues was 0.276.

Studies comparing PCR amplification methods for detection of ovine paratuberculosis are scarce[1,4,9]. This study report the recovery and amplification of acid fast bacilli from Ziehl-Neelsen (Z-N) stained smears from different samples that had been used for microscopy, as a potential sampling method for PCR. Whereas, tissue PCR could detect 61.1% of diagnosed paratuberculosis samples in this study. Sivakumar *et al*.[3] have reported higher sensitivity (70%) for tissue PCR in buffaloes with sub-clinical paratuberculosis.

The present study demonstrated that sufficient *Map* DNA could be extracted from stained slides using a rapid, and inexpensive procedure. This diagnostic approach had advantage of using the same specimen for both microscopic and molecular studies, and possibility of conducting retrospective studies from archival or stored samples. Amplification of DNA by PCR in stained smears has been used to diagnose several infectious diseases caused by *Mycobacterium* in human beings[10,12]. This is the first application of molecular epidemiological techniques in acid fast bacilli stained smears in paratuberculosis.

PCR from tissues was more sensitive than PCR from smears. Number of bacilli per smear differs as bacilli are not equally distributed in tissues and the number of bacilli fixed on the slide can also differ. Result depends on the material and choosing the right material may improve the diagnostic[13].

Low sensitivity in PCR from smears may be due to preserve of inhibitory substances in extracted slide material in PCR amplification[10]. Negative PCR results in both techniques could possibly be false negative[14] due the low number of mycobacteria present in tissues, i.e. below the detection limit of the procedure used in the study.

Although further work with a larger number of specimens is required, laboratory diagnosis sensitivity could be maximized by combining PCR in tissues and PCR in Z-N smears.

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