Short communication

Detection of *Mycobacterium avium* subsp. *paratuberculosis* in the mesenteric lymph nodes of goats by PCR and culture

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Abstract  The efficacy of bacterial cultures and IS900-specific polymerase chain reaction (PCR) was compared for the detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) from the mesenteric lymph nodes of goats. Samples were collected from 75 goats slaughtered in Ilam, in southwest of Iran. Tissue homogenates were inoculated onto four media. The genomic DNA was extracted directly from mesenteric lymph nodes and also from grown bacteria. The purified DNA was utilized as template DNA in the PCR targeting IS900 marker of MAP. IS900 PCR was compared with conventional culture methods. PCR allowed amplification of IS900 element in 27 (36%) of the mesenteric lymph nodes. In comparison, 13 (17.3%) MAP isolates were cultured on Löwenstein–Jensen + mycobactin J. Moreover, the DNA of all 13 MAP isolates was amplified by PCR, confirming the results of cultures. The number of recovered MAP on HEY+ mycobactin J was six isolates (8%). The study found that LJ + mycobactin J was a more appropriate medium for primary isolation of Map bacilli in mesenteric lymph nodes as well as the first documentation of molecular detection of Map directly from naturally infected goat tissues in southwest of Iran.

**Keywords:** Detection, *Mycobacterium avium* subsp. *paratuberculosis*, goat, PCR, culture, IS900, Mycobactin J

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Introduction

*Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the infectious agent of Johne’s disease or paratuberculosis in domestic and wild ruminants and is responsible for considerable economic losses to the livestock industry worldwide (Garrido et al., 2000; Vazquez et al., 2014). *Mycobacterium avium* subsp. *paratuberculosis* has also been implicated in the pathogenesis of Crohn’s disease in humans (Feller et al., 2007; Behr et al., 2008; Juste et al., 2008).

*Mycobacterium avium* subsp. *paratuberculosis* is a slowly growing fastidious acid-fast bacillus that requires ferric mycobactin for in vitro growth in the culture. Cultivation of Map from faecal and tissue specimens still remains the most definitive method for detecting animals with paratuberculosis (Whittington, 2010) and is also an essential step for the application of most molecular typing assays. Despite widespread use, cultivation techniques are not standardized and the ability of different laboratories to cultivate varies considerably. It is also difficult to isolate bacteria in culture from subclinical cases due to intermittent shedding and low number of bacilli in feces and tissues (Vazquez et al., 2014). Conversely, MAP detection by PCR represents a rapid, alternative diagnostic tool. This technique has been routinely used both to directly determine the presence of Map from different sources (faeces, milk, intestinal tissues and mesenteric lymph nodes), and to confirm the identification of isolates obtained by cultural methods. It has certain insertion sequences (IS900 and ISMav2) that help in differentiating this bacterium from genetically related mycobacteria of *M. avium* group (Strommenger et al., 2001; Arrazuria et al., 2015).

However, a variety of studies used both culture and PCR to measure MAP prevalence (Corti and Stephan, 2002; Stephan et al., 2002; O’Reilly et al., 2004; Haghkhah et al., 2008; Wells et al., 2009). Usually, faeces or milk were investigated as sample matrices. However, shedding of Map in faeces or milk often also occurs intermittently. Therefore, diagnostic tests may lead to false negative results and infected animals could represent a potential source for later infections. Direct pathogen detection by PCR or culture at the primary site of bacterial multiplication seems to be the most suitable method. Intestinal tissues and mesenteric lymph nodes
are generally accepted to be the main locus of MAP colonization (Wu et al., 2007; Stabel et al., 2009). The aim of the present study was to carry out a small experiment on the frequency of mycobacterial microorganisms detected by four different culture media and by a PCR targeting a specific genetic marker (IS900) for MAP in mesenteric lymph nodes of apparently healthy goats in southwest of Iran. Moreover, a comparison of culture and PCR in terms of sensitivity was also made based on the results obtained.

Material and methods

Sample collection and bacterial culture

Mesenteric lymph node samples were collected from 75 goats slaughtered in a slaughterhouse in Ilam, in southwest of Iran. Tissue specimens were collected from suspected clinical cases with emaciation and those elderly cases with more than two years old. The tissue samples were homogenized, and a routine Ziehl–Neelsen (ZN) staining was performed on the smears prepared from homogenates. Then, after decontamination by hexadecylpyridinium chloride (HPC) (Sigma) for five hours, centrifugation at 3,000 g for 30 min was carried out. The pellets were resuspended in 0.5 mL sterile distilled water. 100 µL were inoculated onto one slope of four media as follows: Herrold’s egg yolk medium (HEY) (Herrold, 1931), HEY supplemented with mycobactin J (HEY+ mJ), Löwenstein– Jensen (LJ) (Kalis et al., 2000) and LJ supplemented with mycobactin J (LJ+ mJ). The inoculated slopes were incubated at 37 °C up to 16 weeks and examined every week for bacterial growth.

DNA extraction and PCR

Briefly, a loopful of bacterial growth was washed twice in PBS and resuspended in 500 µL extraction buffer (EB) (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). In screw-capped microcentrifuge tubes, the suspension was subjected to 3 consecutive cycles of 5 min freezing at –20 °C and 12 min heating in a boiling water bath. In the case of mesenteric lymph tissues, approximately 500 µL of the homogenate was spun briefly and the tissue pellet then resuspended in 500 µL EB as described above. Additional purification of the genomic DNA was then carried out by phenol/chloroform/isoamyl alcohol extraction, followed by ethanol precipitation as described by Ausubel et al. (Ausubel et al., 1992). The genomic DNA content was quantified using Eppendorf spectrophotometry. Approximately 500-1000 ng of purified DNA preparation was utilized as template DNA in the PCR reaction.

Amplification of IS900 described by Shin et al. (2010) was used for identification of both DNA isolated from mesenteric lymph nodes and bacteria recovered from tissue homogenates (Fig. 1). Three to five µLs of each respective DNA sample was used for amplification, in 25 µL PCR reactions using Taq DNA polymerase (Fermentas, Ukraine). Each reaction contained 1.5 units of enzyme, 200 mM of deoxynucleoside triphosphate, 2 mM MgCl2, and 10 pmol of each primer (Table 1) plus appropriate amount of PCR water. Cycling was performed in a gradient thermal cycler (Eppendorf, Germany) as follows: 95 °C for 5 min; 35 cycles for 95 °C, 60 °C and 72 °C each for 1 min followed by a final extension stage at 72 °C for 7 min. 5 µL of each reaction mixture was analyzed on 1.2% agarose gels stained with ethidium bromide for visualization of PCR products. A no- DNA template reaction was included along with each PCR run as negative control.

Results

Out of the 75 intestinal lymph samples 13 were cultured positive on LJ+ mJ (accounted for approximately 17.3% of the goat samples). The number of recovered MAP on HEY+ mJ was eight isolates (6%). Acid fast bacilli were constantly found, characterized by morphology and clumps density within ZN staining of bacterial colonies. No MAP was grown on either plain HEY or LJ media. Additionally, eight rapidly growing mycobacterial species were detected on the plain LJ media, which their identity was checked by ZN staining and mycobacteria genus-specific PCR, but these were not found to be MAP.

![Figure 1](image.png)

**Figure 1.** The PCR products obtained from amplification of IS900 for a representative selection of Map.

M: molecular size marker 100bp ladder, Lane 1: –ve control, Lanes 2 to 6: Map isolates recovered in this study.

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<th>Table 1. Primers used in this study</th>
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<td>Gene target</td>
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PCR allowed amplification of the 398-bp, MAP-specific DNA fragment of the IS900 element in 27 (36%) of the mesenteric lymph nodes that were directly extracted from tissues (prior to cultivation) (data not shown). Moreover, the DNA of all 13 MAP isolates was amplified by PCR, confirming the results of mycobactin-dependant cultures (Table 2).

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**Discussion**

Conventionally, MAP has been isolated from milk, feces, and other farm wastes. However, paratuberculosis is an extremely slow progression disease; therefore, infected animals normally appear healthy, without shedding MAP in milk or feces, while the intestinal lymphatic tissue is known to be the predominant locus of MAP proliferation. Hence, the mesenteric lymph node is regarded to be the optimal sample matrix for direct pathogen detection in subclinically infected goats. In the present study, MAP was successfully detected with the aid of the IS900-PCR technique either directly from lymph tissues or from MAP isolates recovered from these tissues.

Various genes, particularly ribosomal RNA, i.e., 5S, 16S and 23S rRNA have been targeted to differentiate between the members of *M. avium* complex. However, these genes are highly conserved within the member of *M. avium* complex. Thus, PCR amplification targeting IS900 sequence of MAP was carried out in this investigation. IS900 is present in 15–18 copies in MAP genome (Bull et al., 2000). Abattoir-based MAP prevalence studies using tissues have already been published (Wells et al., 2009; Arrazuría et al., 2015). The PCR assay, used in this study, produced the expected result for 100% of samples, indicating that MAP present among the population of AFB, which had been recovered from the mesenteric lymph nodes. The detection of MAP from such tissue samples in this study is evidence of true infection, not simple transient of recently ingested contaminated materials.

Of the 75 samples processed in the present investigation, MAP bacilli were detected in 27 and 13 mesenteric lymph nodes by direct PCR and bacterial culture methods, respectively. The poor sensitivity of the bacterial culture may be due to the presence of small number of bacteria in the tissues (Miller et al., 2002). Isolation of MAP by culture relies on efficient decontamination of the sample. Therefore, it is possible that harsh decontamination step in the bacterial culture reasons the poor sensitivity.

The influence of the type of culture medium on growth of MAP strains has been reported (Cernicchiaro et al., 2008). Of the two culture media that were used in this study, LJ+ mJ was found to be effective for the purpose of isolating MAP from mesenteric lymph nodes. Some investigators have suggested that bacterial culture using supplemented material has greater analytical sensitivity than that using only plain media (Corpa et al., 2000; Ellingson et al., 2004; Cernicchiaro et al., 2010). Giese and Ahrens (2000) reported better results using alternative medium and avoiding decontamination step instead of routinely used method for cultivation of MAP from feces and tissues using HEY+ mJ and HPC decontamination. However, Grant et al. (2001) could not recover viable MAP, though they simultaneously tried to cultivate bacilli, despite using two culture media (HEY and BACTEC).

The reports on identification of MAP directly from infected tissue without previous culturing are scarce (Slana et al., 2010; Miranda et al., 2011). This study is the first report of the presence of cultivable MAP bacilli in the goat mesenteric lymph nodes, as well as the first documentation of molecular detection of MAP directly from naturally infected goat tissues in southwest of Iran. More work is needed to determine other likely routes of disease transmission such as the milk and feces.
References


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چکیده
در این مطالعه نقش کشت‌های باکتریایی و واکنش زنجیره‌ای پلیمراز (PCR) اختصاصی عنصر الحاق‌شونده (IS900) در شناسایی مایکوباکتریوم اویوم زیر‌گونه پاراتوبرکلوزیس از عقده‌های لنفاوی بز‌های برزان بوشیله (PCR) مورد بررسی قرار گرفت. در مجموع، تعداد 75 نمونه از بزهای کشت‌دار در کشت‌گاه صنعتی ایجم مویوری گردید. نمونه‌های منتفی‌شده به آزمایش‌های صورت‌گیری و واکنش‌های PCR مثبت نشان‌دهنده بر روی چرخه مهیج کشت‌شونده بودند. برای استخراج DNA از نمونه‌های هم‌وهم‌زدایش‌شده و یا گونه‌های مختلف از بالکنی های رشد بافت‌های بر روی چرخه محیط نمونه مثبت کشت‌شونده شدند. با بره‌گیری عنصر الحاق‌شونده IS900 از نمونه‌های مثبت کشت‌شونده، 75 نمونه از نمونه‌های مثبت کشت‌شونده DNA به کشت‌های باکتریایی مایکوباکتریوم اویوم در نمونه‌های عقده‌های لنفاوی بز‌های برزان بوشیله ارائه شدند. نتایج این مطالعه نشان دهنده حسایش‌های تعداد بزرگی از نمونه‌های مثبت کشت‌شونده DNA از نمونه‌های مثبت کشت‌شونده را که در سطح مولکولی مایکوباکتریوم اویوم زیر‌گونه پاراتوبرکلوزیس داشتند و دارای کوپتری‌های مثبت کشت از مایکوباکتریوم اویوم بودند. این مطالعه نشان داد که تشخیص دادن، در حالی که تعداد نمونه‌های رشد بافت‌های مایکوباکتریوم اویوم بر روی محیط کشت‌شونده- جنین حاوی ماکروکبتهای (Δ) به (13/17发展潜力) مورد بررسی قرار گرفت. از نمونه‌های کشت‌شونده این میکروب در کشت‌شونده- جنین حاوی ماکروکبتهای (Δ) به (13/17发展潜力) مورد بررسی قرار گرفت. از نمونه‌های کشت‌شونده این میکروب در کشت‌شونده- جنین حاوی ماکروکبتهای (Δ) به (13/17发展潜力) مورد بررسی قرار گرفت.