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 IS*900*-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 IS*900* marker of *MAP*. IS*900* PCR was compared with conventional culture methods. PCR allowed amplification of IS*900* 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* from goat tissues. This is the first report of presence of cultivable *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, IS*900*, Mycobactin J

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 (IS*900* and ISMav*2*) 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 (IS*900*) 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

spectophotometry. Approximately 500-1000 ng of purified DNA preparation was utilized as template DNA in the PCR reaction.

Amplification of IS*900* 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 $MgCl_2$, 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 approximately17.3% 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.
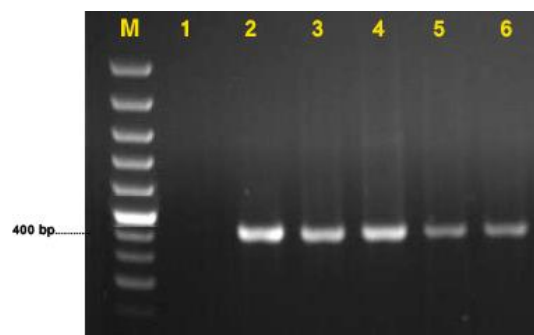

Figure 1. The PCR products obtained from amplification of IS*900* for a representative selection of *Map*.
M: molecular size marker 100bp ladder, Lane 1: –ve control, Lanes 2 to 6: *Map* isolates recovered in this study.

Table 1. Primers used in this study

Gene target	Primer sequence (5´ → 3´)	Amplicon size (bp)	Reference
IS*900*	TGGACAATGACGGTTACGGAGGTGG CGCAGAGGCTGCAAGTCGTGG	398	Shin et al., 2010

Table 2. Diagnostic test results

samples (Mesenteric lymph node[*])	LJ	LJ+mJ	HEY	HEY+mJ	Acid fast bacilli (ZN)	PCR IS*900*
Numbers (%) 75	0	13 (17.3%)	0	6 (8%)	15 (20%)	27 (36%)

ZN: Ziehl–Neelsen (ZN) staining
LJ: Löwenstein–Jensen
mJ: mycobactin J
HEY: Herrold's egg yolk medium
[*]Collected from goats with emaciation and those elderly cases with more than two years old

PCR allowed amplification of the 398-bp, *MAP*-specific DNA fragment of the IS*900* 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 IS*900*-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 IS*900* sequence of *MAP* was carried out in this investigation. IS*900* 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; Arrazuria 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 faeces 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.

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جداسازی مایکوباکتریوم اویوم زیرگونه پاراتوبرکلوزیس از عقده های لنفاوی مزانتر بزان بوسیله کشت و واکنش زنجیره ای پلیمراز (PCR)

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چکیده   در این مطالعه نقش کشت های باکتریایی و واکنش زنجیره ای پلیمراز (PCR) اختصاصی عنصر الحاق شونده (IS900) در شناسایی مایکوباکتریوم اویوم زیر گونه پاراتوبرکلوزیس در داخل عقده های لنفاوی مزانتر بزها مورد مقایسه قرار گرفت. در مجموع تعداد ۷۵ نمونه از بزهای کشـتار شـده در کشـتارگاه صنعتی ایلام جمع آوری گردید. نمونه های منتقل شـده به آزمایشـگاه به صـورت هموژنیزه در آمده و بر روی چهار نوع محیط کشت منتقل شـدند. برای استخراج DNA از نمونه های هموژنیزه شده و یا کلونی های رشد یافته بر روی چهار نوع محیط کشت استفاده شد. با بهره گیری از PCR، عنصـر الحاق شونده IS900 مایکوباکتریوم اویوم در ۲۷ (۳۶٪) مورد از نمونه های عقده لنفاوی مزانتری بزها تشخیص داده شد، در حالی که تعداد نمونه های رشد یافته مایکوباکتریوم اویوم بر روی محیط کشت لوانشتاین— جنسن حاوی مایکوباکتین (J) به ۱۳ (۱۷/۳٪) مورد از نمونه های مورد بررسـی رسـید. آزمایش PCR هویت این باکتری های رشـد یافته را که در مورفولوژی مایکوباکتریوم اویوم زیرگونه پاراتوبرکلوزیس داشتند، را تایید کرد. تنها شش مورد (۸٪) از نمونه های رشد داده شده بر روی محیط هرولداگ یاک منتج به رشد مایکوباکتریوم اویوم گردید. این مطالعه ارجحیت محیط کشـت لوانشتاین– جنسن حاوی مایکوباکتین (J) را در جداسازی مایکوباکتریوم اویوم را در نمونه های عقده های لنفاوی بز نشـان داد. حضور باسیل های قابل کشت مایکوباکتریوم اویوم در عقده های لنفاوی مزانتر و قابلیت تست های مولکولی در شناسایی بزهای عامل این باکتری در ایلام برای اولین بار در این مطالعه گزارش می شود.