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# Goat paratuberculosis in Shiraz: Histopathological and molecular approaches

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## Abstract

In the present study, *Mycobacterium avium* subsp. *paratuberculosis* (MAP) was investigated in goats slaughtered in Shiraz abattoir using histopathological examinations and polymerase chain reaction (PCR). Ilium and mesenteric lymph node samples from 66 suspected goat carcasses to Johne's disease were collected. Among 66 examined slaughtered goats, nine (13.63%) goats were positive for MAP in both histopathological and PCR examinations. Eight goats were positive in PCR method while no lesion related to Johne's disease was observed in their histopathological sections. All positive goats in histopathological examination were also positive in PCR. Based on the results of PCR, the detection rate of MAP in Shiraz abattoir was 25.80% (17 goats). According to the present findings, although both histopathological and PCR methods are appropriate for detecting Johne's disease, PCR is more sensitive than histopathological examination.



## پاراتوبرکولوز بزی در شیراز: رهیافت های هیستوپاتولوژی و مولکولی

### چکیده

در مطالعه حاضر، حضور *مایکوباکتریوم اویوم تحت گونه پاراتوبرکولوزیس* در کشتارگاه شیراز با استفاده از آزمایش‌های هیستوپاتولوژی و واکنش زنجیره‌ای پلی‌مراز (PCR) مورد تحقیق قرار گرفت. نمونه‌های ایلئوم و عقده‌های لنفاوی مزانتریک از لاشه های تعداد ۶۶ بز مشکوک به بیماری یون جمع آوری گردید. از میان ۶۶ لاشه بز مورد مطالعه، تعداد نه بز (۱۳/۶۳ درصد) در هر دو روش هیستوپاتولوژی و PCR مثبت بودند. نتایج واکنش PCR در هشت بز مثبت بود در حالی که هیچگونه ضایعه هیستوپاتولوژیک مربوط به بیماری یون در آنها مشاهده نشد. همه بزهای با نتایج هیستوپاتولوژیک مثبت دارای نتیجه PCR مثبت بودند. براساس نتایج PCR، میزان حضور *مایکوباکتریوم اویوم تحت گونه پاراتوبرکولوزیس* در کشتارگاه شیراز ۲۵/۸ درصد (۱۷ بز) بود. براساس نتایج حاضر، می‌توان گفت که اگرچه روش های هیستوپاتولوژیک و PCR روش های مناسبی برای تشخیص بیماری یون هستند، اما واکنش PCR حساسیت بیشتری دارد.

**واژه های کلیدی:** بز، بیماری یون، *مایکوباکتریوم اویوم تحت گونه پاراتوبرکولوزیس*، واکنش زنجیره ای پلیمراز ، هیستوپاتولوژی

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## Introduction

*Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the causative agent of paratuberculosis (PTB), also known as John's disease. The disease has worldwide distribution and in Iran its prevalence has been investigated in a number of research works most of them focused on the detection of MAP in cattle's milk bulk.[1] The MAP is an acid-fast staining small rod-shaped and slow-growing bacterium. Its unique cell wall structure which is rich in complex lipids is responsible for the persistence of this bacteria, both in the environment and inside the host.[2]

Because of MAP detection in environmental samples, surviving up to one year in contaminated pastures and possibility of remaining viable after milk pasteurization, its real threat is clearly underestimated for both ruminants and human. Paratuberculosis is one of the most economically important disease affecting cattle, sheep and goats by increasing the mortality and premature culling risks, lower reproductive efficiency, compromised growth rates and decreased milk yield.[3,4] There is no therapy for John's disease and the control of this disease in ruminants depends on the early detection and culling of infected animals.[5]

The major route of MAP infection in ruminants is via ingestion, so the first step in infection is uptake of MAP through mucosal surfaces primarily in the ileum, via M cells (specialized absorptive mucosal cells) residing in the Peyer's patches and then phagocytosed by subepithelial macrophages. Eventually, the infected macrophages migrate into local lymphatics and the infection spread to regional lymph nodes.[6]

Several techniques such as Ziehl Nielsen staining (ZN), histopathology, immunohistochemistry (IHC), *in situ* hybridization (ISH) and polymerase chain reaction (PCR) were tested for detection of MAP, but their performances are different. Traditionally, the gold standard for diagnosis of John's disease is fecal culture for MAP.[7] However, fecal culture of MAP is time-consuming and detects only 38.00 to 50.00% of infected cows.[8] The most convenient tests for detecting MAP are ZN and IHC tests. Although these tests have high sensitivity for MAP detection, false negative can arise when infection is recent or bacilli are scanty.[9] Histopathology for detection of MAP in tissue samples is an important tool and will be necessary when experimental infections are induced. *In situ* hybridization has high specificity, but it is expensive, difficult to perform, and its interpretation may be difficult because of the low obtained signals.[10] Molecular methods based on the PCR are quick with high sensitivity and specificity for detection of MAP.[11] The most common target locus for the direct detection of MAP by PCR is the multiple copy insertion sequence IS900[12] that is present in genome in 12 to 18 copies.[13] The objective of this study was to investigate the presence of MAP in goats slaughtered in Shiraz abattoir using histopathology and IS900 PCR methods for examining 66 suspected goats to John's disease.

## Materials and Methods

**Sample collection.** During six months (fall and winter 2015-2016), a number of 66 suspected goats to John's disease slaughtered in Shiraz abattoir were sampled. Tissue samples including ileum and mesenteric lymph nodes were collected.[9] Necropsy findings of suspected carcasses were emaciation, intestinal mucosal thickness, enlarged lymph nodes and occasionally serous atrophy of fat. The samples were immediately put in sterile plastic bags, placed in cooling boxes and were transported to the microbiology laboratory. Samples of suspected carcasses were considered for both tissue staining procedure and DNA extraction.

**Histopathological examinations.** The tissue samples from ileum and mesenteric lymph nodes were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm in thickness and stained with hematoxylin and eosin (H & E) and ZN staining.[14,15]

**DNA extraction.** One gram of each small intestine and lymph node was separately homogenized in 1.00 mL distilled water and centrifuged (13000 *g*, 30 sec). Supernatant was removed and 1.50 mL distilled water was added to the tissue pellet, resuspended and centrifuged (13,000 *g*, 30 sec). This step was repeated twice for washing the tissues. An amount of 500 μL of lysis buffer (Tris-HCL, EDTA, 0.2% Tween; Sinaclon, Tehran, Iran) and 50 μL proteinase K (Sinaclon) was added and incubated overnight at 37 ˚C. The enzyme digested samples were mixed with 500 μL of phenol:chloroform:isoamylalcohol (25:24:1) for 15 min. Then, the mixture was centrifuged (10000 *g*, 10 min). The DNA was purified from the supernatant by adding equal volume of phenol: chloroform:isoamylalcohol (25:24:1) and was centrifuged at 10000 *g* for 1 min. The DNA was precipitated from the aqueous phase by addition of 2.50 volume of absolute ethanol and incubated at -20 ˚C for 1 hr. The resultant DNA pellet was washed with 70.00% ethanol twice, dried and resuspended in 50 μL distilled water.[16]

**PCR detection of MAP.** All 132 extracted DNA from goat's tissues were subjected to PCR for detection of MAP. The primers P90 (5' -GAA GGG TGT TCG GGG CCG TCG CTT AGG-3') and P91 (5' -GGC GTT GAG GTC GAT CGC CCA CGT GAC-3') which described previously were used.[17] The PCR contained 0.75 μL dNTPs (each at 0.20 mM; Sinaclon), 1.00 μL of each primer (20 pmol), 0.75 μL 50 mM $MgCl_2$ (Sinaclon), 2.50 μL 10X PCR buffer (Sinaclon), 0.20 *Taq* DNA polymerase (5.00 U $\mu L^{-1}$) (Sinaclon) and 2 μL of template DNA. Sterile distilled water was added to make the final volume of PCR to 25.00 μL. Polymerase chain reaction condition were as follows: 5 min at 94 ˚C, 30 cycles of 1 min at 94 ˚C, 1 min at 59 ˚C, 2 min at 72 ˚C, and a

final extension of 7 min at 72 ˚C. Negative and positive bacterial DNA (MAP ATCC43105, kindly provided by Prof. Leonardo Sechi, Department of Biomedical Sciences University of Sassari, Sassari, Italy) controls were included for each PCR. The PCR products were subjected to electrophoresis in 1.50% agarose gel containing Gel Red (2.00 µL per 50 gel) and the gel visualized under UV light.

## Results

**Macroscopic findings.** Thickened intestinal wall, mucosal folds, corrugated and granular mucosa, serous atrophy of fat, thickened mesenteric lymphatic vessels, enlarged and edematous mesenteric lymph nodes and occasionally calcium deposits were macroscopic lesions seen in the collected samples (Fig. 1A).

**Histopathological results.** In histopathological evaluation of the tissue samples using H&E staining, nine samples (13.63%) were positive for Johne's disease. Four positive samples had diffuse multibacillary lesions (44.40%), which were characterized by a diffuse granulomatous enteritis (Fig. 1B) and lymphadenitis with infiltration of large numbers of epithelioid macrophages in the intestinal lamina propria and cortex of lymph node. In some cases, there were submucosa lymphangitis and lymphangiectasia. Caseous necrosis and calcification were observed in lymph nodes of two cases. The rest of samples had diffused lymphocytic lesions (33.30%) and diffused mixed lesions (12.30%). Diffused lymphocytic lesions consisted of a diffused granulomatous enteritis (Fig. 1C) and lymphadenitis with infiltration of large numbers of lymphocytes and a few epithelioid macrophages in the intestinal lamina propria and cortex of lymph node. Caseous necrosis and calcification was seen in lymph node sections of one case. In diffused mixed lesions, a mixture of large numbers of lymphocytes and epithelioid macrophages were observed in the intestinal lamina propria (Fig. 1D) and cortex of lymph nodes. Caseous necrosis and calcification of lymph node was similar to diffused lymphocytic form.

The negative samples were screened and parasitic infection, particularly coccidiosis was the most common cause of intestinal lesions (Table 1). In ZN staining, eight intestinal sections (12.10%), (Fig. 1E) and eight mesenteric lymph nodes (12.10%), (Fig. 1F) were positive for Johne's disease (Table 1).

**PCR results.** The IS900 PCR amplified products of 413-bp in size were considered as positive. Seventeen ilium samples (25.80%) and 12 mesenteric lymph node samples (18.20%) were positive for Johne's disease (Fig. 2).

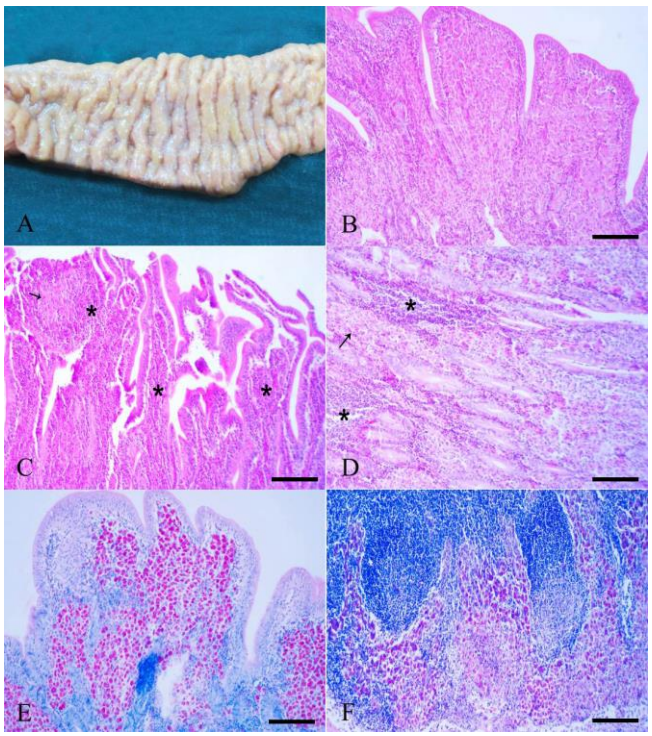


**Fig. 1. A)** Thickening and corrugation of the intestinal mucosa; **B)** Diffused multibacillary lesion with infiltration of numerous epithelioid macrophages (H&E); **C)** Diffused lymphocytic lesion with infiltration of numerous lymphocytes (*) and a few epithelioid macrophages (arrow) (H & E); **D)** Diffused mixed lesion with infiltration of mixture of lymphocytes (*) and epithelioid macrophages (arrow) (H&E); **E and F)** Acid fast bacilli in the cytoplasm of epithelioid macrophages in intestinal mucosa and mesenteric lymph node sections, respectively (ZN staining, Bars = 55 µm).

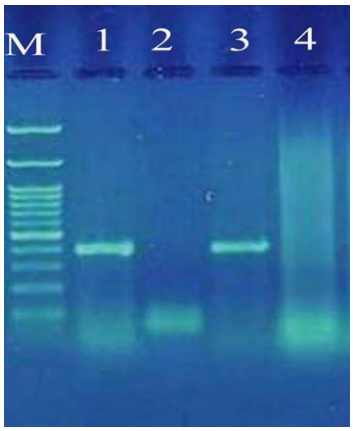


**Fig. 2.** Electrophoretic analysis (1.50% agarose gel) of DNA amplified fragments. M: DNA marker 100 bp (Sinaclon). Lane 1: positive control (MAP ATCC43105); Lane 2: negative control; Lane 3: positive sample; Lane 4: negative sample.

**Table 1.** Results of hematoxylin and eosin, Ziehl Nielsen staining and IS900 PCR in intestinal and mesenteric lymph node tissues.

| Sample | Number of samples | Direct H&E positive | Direct ZN positive | PCR positive |
|---|---|---|---|---|
| **Ilium tissue** | 66 | 9 (13.60%) | 8 (12.10%) | 17 (25.80%) |
| **Mesenteric lymph node** | 66 | 9 (13.60%) | 8 (12.10%) | 12 (18.90%) |

Generally, among 66 goats, nine samples of both lymph nodes and ilium tissues were positive in both PCR and histopathology approaches (13.63%). Eight samples were positive in PCR method while no lesion related to Johne'sdisease was observed in their histopathological sections. There was no sample with positive histopathological and negative PCR result.

## Discussion

Paratuberculosis is one of the most economically important diseases, affecting ruminants worldwide. In the present study, we investigated MAP in goats slaughtered in Shiraz slaughterhouse using IS900 PCR assay. PCR method and histopathology (H&E and ZN staining) were compared for their performance in detecting MAP using 132 tissue specimens of the ileum and the mesenteric lymph nodes suspected to paratuberculosis.

In the present study, MAP was detected in 17 goats (25.70%) using PCR assay. In a previous study on the bulk-tank milk of cattle in southern Shiraz, the herd prevalence of John's disease was 8.60 to 23.00% using PCR.[1] Also, the prevalence of John's disease was reported 2.00% using Ziehl-Neelsen staining on ileocecal valve samples of cattle slaughtered in Ahvaz abattoir.[18] In other countries, there are a few reports on the prevalence of paratuberculosis in goat populations. In Norway, Djonnea *et al.* reported paratuberculosis from 7.10% of milk samples of goats.[19] Based on Kruze *et al.*, 14.60% of the fecal cultures of dairy goats were positive for MAP, all of them were confirmed by PCR IS900.[20] The varied results reported by the different investigators can be related to the sample collection procedures, specimen type, diversity of geographic locations and sensitivity or specificity of the detection assays for identification of MAP.

In this study, the detection of MAP using PCR in the ileum samples was higher than mesenteric lymph nodes. Histopathologically, our results revealed that the most common lesion of this disease was diffused multibacillary (44.40%) that are similar to the previous studies.[21,22] The results can be explained by the fact that the major entrance route of infection in ruminants is via ingestion of MAP and the uptake of this agent through mucosal surfaces primarily in the ileum. Accordingly, it seems that the important site for the detection of MAP will be intestinal tissue especially the ileum.

In conclusion, the results of the present study demonstrated that H&E and ZN staining had the same sensitivity for detection of MAP and PCR assay was more sensitive than histopathological examination. Furthermore, it is critical to conduct in-depth epidemiological studies for identifying the prevalence of John's disease in small ruminants and its economic consequences throughout the country.

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## Conflicts of interest

The authors declare no conflict of interests.

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