

## Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* in Dairy Cattle Bred in Northern Iran by Nested-PCR

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**Abstract:** Johne's disease is a chronic intestinal infection in ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Infected livestock diffuses bacterium to the environment in different ways, of which milk is the most common way. Since a relationship is proposed between the causes of the Johne's disease in livestock and Crohn's disease in human being, a study on the presence of the bacterium in the milk can provide an important index to the rate of development of this infection in the livestock and, probably in human societies. The current study aimed to identify MAP infection in dairy cattle bred in the northern Iran. A total of 90 dairy cattle were tested by comparative tuberculin test and results of the positive, negative and suspicious cases were obtained in the forms of 3, 64 and 23, respectively. A nested PCR was also conducted using two pairs of primers to amplify a 194 bp fragment located on IS900 element to confirm the results obtained by the former method. According to the results, the level of infection in different regions of the studied area ranged from 4.2% to 7.7%.

**Key words:** *Mycobacterium avium* subsp. *paratuberculosis* • MAP • Johne's disease • Crohn's disease, • Dairy cattle • Iran

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### INTRODUCTION

Johne's disease is chronic granulomatous enteritis in domestic and wild ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). It slowly obliterates the intestinal tissue of the infected livestock, which leads to excessive weight loss, severe and permanent diarrhea, milk reduction, infertility and increased risk of stroke for other diseases [1].

Johne's is a fatal epidemic disease which brings about great economic loss to the countries [2-5]. The infection takes place in the intestine and lymph nodes. However, this intracellular bacterium can also be transferred to the other parts of the body by macrophages. MAP diffuses to the environment through the feces, milk, colostrums and sperm, of which milk is considered as one of the most common resources for its diffusion [6-8]. The importance of milk is highlighted as a possible relation proposed between the Johne's disease in livestock and Crohn's disease in human being [7, 9].

Crohn's disease is a chronic granulomatous infection of the intestine in the human being with a complex etiology. Detection of the MAP in the intestine of individuals with no precedent of the enteritis disease has revealed that the consumption of polluted milk is a major way of transmitting this acid fast bacillus from livestock to the human being [6, 10, 11]. According to the conducted researches, the number of MAP cells diffused into the milk of the infected cattle at the clinically manifested stage is about 100 CFU/ml, while, it is around 2 to 8 CFU/ml in those with no clinical signs [8, 12]. The infected livestock with no clinical signs are considered as the main reservoir of the Johne's disease with regard to the prolonged latency period of the disease which generally lasts 2 to 10 years [6, 7]. As a result, because of the irreparable damages caused by the Johne's disease to the raising cattle industry and also its issue regarding the human being's health, the accurate and efficient tests seem necessary to be used in order to identify the infected livestock. Microbial culture is one of the most common

methods used to detect MAP. However, due to the complex nutritional need of the bacterium and the long incubation time needed for the growth of MAP (12 and 18 weeks for feces and milk samples, respectively), this method may last up to 6 months [6, 13, 14].

In Iran, no screening test is used in order to identify the MAP infected cattle. However, the policy of Iranian Veterinary Organization is to identify and eliminate the sick livestock infected by both bovine brucellosis and bovine tuberculosis. This research aimed to study the outbreak of the MAP infection in dairy cattle bred in Tonekabon County in Northern Iran. The comparative tuberculin test was used as the initial screening method in order to identify the MAP infected cattle. However, due to the lack of sufficient sensitivity, a nested PCR method was also used to confirm the results.

## MATERIALS AND METHODS

**Animal Screening:** The study population included 20 dairy cattle herds selected from 3 different regions in Tonekabon County (Northern Iran) including Tonekabon, Solaiman abad and Nashtaroud. In this Study, the comparative tuberculin test was used as the preliminary test. An avian tuberculin (containing the extract of *M. paratuberculosis*) and a bovine tuberculin (containing the extract of *M. bovis*) were injected to the cattle and the dermal diameter of the injection areas were measured by the caliper 48 h after injection. The dermal diameter smaller than 2 mm was considered as negative, between 2 to 4 mm as suspicious and larger than 4 mm as positive cases [15].

**Milk Sampling:** The milk samples of 90 studied dairy cattle were collected during a 4 months period of time from May until September 2010. Twenty to 30 ml of milk was collected in the 50 ml sterile centrifuge tubes and kept at 4°C.

**DNA Extraction:** DNA was extracted from milk samples based on method described by Corti and Stephan [6] with minimal modifications. Briefly, 10 ml of each milk sample

was transferred into a centrifuge tube and centrifuged at 4000 rpm for 20 min at room temperature. Three hundreds µl of pellet was transferred into a 1.5 eppendorf tube after vortexing and incubated at 95°C for 20 min. The solution was incubated at 65°C for 20 min after adding 20 µl proteinase K (1mg/ml) (Fermentes, Russia) and vortexing. The procedure was followed by adding 200 µl mycobacterial lysis buffer (0.1 M NaCl; 10 mM Tris-HCl; 1 mM EDTA) and incubation of solution at 65°C for 15 min. Four hundreds µl of the phenol/choloroform/isoamyl alcohol (25:24:1) was added to the tube. The aqueous layer was transferred to the new tube after centrifuging at 10,000 rpm. Eight hundreds µl of the 96% ethanol was added to the tube and it was left at -20°C freezer for at least 1 h. After centrifugation at 14,000 rpm, the tube was washed with 500 µl of the cold 70% ethanol. Pellet of DNA was air-dried for 5 min and re-suspended in 40 µl of sterile water. The DNA was either used immediately or frozen at -20°C for later analysis.

**Nested-PCR:** A nested PCR (BIO-RAD, Germany) was conducted to amplify IS900 element based on the protocol described by Stabel *et al.* [14] with some minor modifications. The primers used are given in Table 1. A total of 3 µl of purified DNA was used as template for IS900 nested PCR. The PCR reaction volume was 25µl containing 10 pmol of each primer, 1 mM MgCl<sub>2</sub>, 200 µM dNTPs and 2.5 U Smart Taq polymerase (Cinagen, Tehran, Iran) in 1x reaction buffer. PCR cycling condition included 95°C for 3 min (1 cycle), 94°C for 30s, 62°C for 30s, 72°C for 45s (35 cycle) and 72°C for 7 min (1 cycle). Five µl of the PCR product was used as the template for the nested PCR with the previous mentioned compounds. The nested PCR cycling condition included 94°C for 2 min (1 cycle), 94°C for 30s, 58°C for 30s, 72°C for 40s (25 cycle) and 72°C for 7 min (1 cycle). After amplification a volume of 10 µl of the PCR product was run on 2.5% agarose gel by electrophoresis and bands were visualized by using a UV transilluminator (Uvitec, United Kingdom). A positive control (*M. paratuberculosis* DNA) and negative control (sterile water) were used in each of the reactions [14, 16, 17].

Table 1: Oligonucleotide primers to generate PCR amplification products.

Target gene	Type	Sequence 5'→3'	Length of product (bp)
Single PCR	Forward	G TTCGGGGCCGTCGCTTAGG	400
	Reverse	GAGGTCGATCGCCACGTGA	
Nested PCR	Forward	GCTTAGGCTTCGAATTGCC	194
	Reverse	CTCCGTAACCGTCATTGTCC	



Fig. 1: Agarose gel electrophoresis of PCR products obtained from milk samples following IS900 nested PCR. Lanes 1-2-3-4 & 7: negative samples. Lanes 5-6-8 & 9: positive samples with a specific amplification product of 194 bp. PC: positive control. NC: negative control. M: 100 bp molecular weight ladder.

Table 2: Relative frequency of MAP in the three studied regions of the Tonekabon town ship by PCR method

Region	Positive PCR		Negative PCR	
	Number	%	Number	%
Tonekabon	2	4.2	45	95.7
Solaiman abad	2	6.6	28	93.3
Nashtaroud	1	7.7	12	92.3
Total	5	5.6	85	94.4

Table 3: Comparison of the results of the PCR and tuberculin test

Milk PCR	Tuberculin status of dairy cows			Total
	Positive	Suspicious	Negative	
Positive	3	2	0	5
Negative	0	23	62	85
Total	3	25	62	90

## RESULTS

A 194 bp fragment was amplified by PCR in positive samples of the milk (Fig. 1). The results obtained by comparative tuberculin test showed that out of 90 cattle examined in the studied areas, 3 were positive, 23 suspicious and 64 negative.

The results obtained by nested PCR also showed that 5 out of 90 samples (5.6%) were positive for MAP. However, as shown in table 2, the frequency of infection was diverse in different regions, ranged from 4.2% to 7.7%.

The comparison between results of two methods used in this study revealed that all positive cases detected by tuberculin test were confirmed to be positive by nested PCR too, while only 2 suspicious cases were positively confirmed by PCR. The comparison of the results obtained by the tuberculin test and nested PCR is given in table 3.

## DISCUSSION

Regarding the negative effects of Johne's disease on the livestock industries as well as the hygiene of the human societies, paying attention to this disease is of the great importance. Excretion of the MAP happens through the milk and feces of infected cattle with or without the clinical signs, which leads to the infection of the forage, drinkable water and, finally, the diffusion of the infection to the whole herd [8, 14]. Johne's disease has been spread to many countries through exportation of the infected purebred race cattle. Researches in the Wisconsin region of the USA have shown that about 34% of the dairy cattle of this region are infected with MAP [14]. The difficulty with the diagnosis of the Johne's disease is related to the lack of prominent clinical signs as infected cattle are mostly suffering only from weakness. On the other hand, there are cattle suffering from weakness with thicken and crimped intestine (in the autopsy) which are not infected

by MAP [6]. Therefore, the methods relying on the clinical signs are not reliable for detection of infected livestock, as the tuberculin test used in this study was not able to distinguish between the infected and uninfected livestock in suspicious cases. Based on this method, the cattle are classified into negative, suspicious and positive groups through measuring of the dermal thickness 48 h after injection of the bovine and avian PPD. Test is construed as negative when the difference between thickness of the injection location and bovine tuberculin is less than 2 mm and the clinical signs including edema, necrosis and pain are not observed in the location. Test is declared as suspicious when the thickness difference increases about 2 to 4 mm and it is considered as positive when the difference is more than 4 mm, even if there is no clinical sign. The tuberculin test is not a reliable test because of the false positive and negative reactions and the minimum dermal sensitivity of subclinical cases. As a result, a supplementary test seems to be necessary to affirm the detection. Culturing is of the most common standard methods for identification of the MAP. However, this method may last a few months because of the complex nutrient needs of the bacterium and its slow growth, while it is able to identify only 50% of the infected cattle [9, 14, 18]. Therefore, a PCR based molecular method could be considered as a preferred supplementary test. The most common target used to identify the MAP by PCR is IS900 [9,12,17,19]. Many studies have been carried out to identify MAP in milk tanks by amplifying IS900. The study was done by Grant *et al.* [20] on 224 samples collected from milk tanks and showed that the prevalence of MAP infection was up to 7.8% in the United Kingdom. A similar study carried out by Corti and Stephan [6] on milk samples collected from different parts of Switzerland revealed that 19.7% of samples were MAP positive. Moreover, the researches done by Shaymaa and Mohamed [21, 22] on milk and feces samples of cattle in Greece using IS900 PCR method revealed that 69.2% of cattle with clinical symptoms were infected by MAP. These results and also the results obtained from present study show that dairy cattle are of the most important sources for spread of bacterium, which threaten human health through Crohn's disease. This has been proven through study on infant formulas infected by MAP [23].

Despite some multiple and dispersed studies done in Iran, the accurate prevalence of Johne's disease is not yet available in different regions of the country. Haghkhah *et al.* [9] reported that the prevalence of the infection was

23% in the Shiraz region by examination of 110 dairy cattle. In the herds in which Johne's disease has been detected by clinical, histopathology, or culture methods, the number of the subclinical cases is far more than the clinically detectable infected cattle. Johne's disease, in addition to the health complexities, leads to major economical loss including reduction in milk and meat production, decrease of fertility, stillbirth and death of adults. However, tuberculin test is the only routine method to identify MAP infection in Iran. Since this test does not benefit from the enough sensitivity in detection of positive livestock, especially in suspicious cases, the nested PCR method used in this study is suggested to be defined as a standard supplementary test by the Iranian Veterinary Organization to increase the accuracy of identification and eradication of the disease.

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