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RESEARCH ARTICLE

# Survey for the Presence of *Mycobacterium avium* subsp. *paratuberculosis* in the Bull Frozen Semen Samples and Blood Samples of Cattle, Sheep and Camel by Nested-PCR

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## Summary

*Mycobacterium avium* subsp. *paratuberculosis* (MAP) is an obligate, Gram positive, acid-fast bacterium in the genus *Mycobacterium*. It is the causative agent of Johne's disease in some animals and perhaps the human disease Crohn's disease. The aim of this study was to use nested- PCR as an exact and fast technique to trace MAP in cattle, camel and sheep from each samples. Blood samples were collected from 144 cattle, 110 sheep and 95 camels and 83 frozen semen samples were obtained from bulls were obtained and DNA was extracted. At that time, nested-PCR was performed by specific primers for IS900 gene of MAP. The PCR products with 230 bp length were estimated as a positive. The occurrence of MAP detected in 8 of the 83 (9.638%) frozen semen samples of bull's that used for artificial insemination, and was detected in 11 of the 144 (7.638%), 7 of the 95 (7.368%) and 16 of the 110 (14.545%) blood samples of cattle, camel and sheep in Iran, respectively. The results of this study were displayed nested-PCR is a good technique with high efficiency for detection of intracellular bacteria such as MAP in cattle, camel and sheep samples. Consequently, more attention to Johne's disease in cattle, camel and sheep to find MAP quickly is essential.

***Keywords:*** *Blood Johne's disease, Mycobacterium avium subsp. paratuberculosis, Polymerase chain reaction (PCR), Ruminant, Semen*

# *Mycobacterium avium* subsp. *paratuberculosis* Varlığının Dondurulmuş Boğa Semen Örneklerinde ve Sığır, Koyun ve Deve Kan Örneklerinde Nested-PCR İle Taranması

## Özet

*Mycobacterium avium* subsp. *paratuberculosis* (MAP) *Mycobacterium* cinsi içinde zorunlu, Gram pozitif, aside dirençli bir bakteridir. Bu bazı hayvanlarda "Johne's" hastalığı ve belki de insanlardaki "Crohn's" hastalığı etkenidir. Bu çalışmada nested-PCR ile sığır, deve ve koyunların herbir örneğinde MAP'ın belirlenmesi amaçlanmıştır. Kan numuneleri 144 sığır, 110 koyun ve 95 deveden toplandı ve 83 dondurulmuş semen örneği boğalardan elde edildi, takiben DNA izolasyonu gerçekleştirildi. Bu sırada, nested-PCR MAP'ın IS900 geni için özel primerler ile gerçekleştirildi. İkiyüz otuz bp uzunluğuna sahip PCR ürünleri, pozitif olarak tahmin edildi. İran'da suni tohumlamada kullanılan boğalardan elde edilen 83 semen örneğinin 8'inde (%9.638) ve sığır, deve ve koyunlardan alınan sırasıyla 144'te 11 (%7.638), 95'te 7 (%7.368) ve 110'da 16 (%14.545) kan örneğinde MAP belirlendi. Bu çalışmanın sonuçları, nested-PCR'ın sığır, deve ve koyunlarda MAP gibi hücre içi bakterilerin belirlenmesinde yüksek verimlilikli iyi bir teknik olduğunu göstermektedir. Bu sonuçlar sığır, deve ve koyun "Johne's" hastalığında MAP'in hızlı teşhisi için daha fazla dikkatin gerekli olduğunu ortaya koymaktadır.

***Anahtar sözcükler:*** *Kan, Johne's hastalığı, Mycobacterium avium subsp. paratuberculosis, Polimeraz zincir reaksiyonu (PCR), Ruminant, Semen*

## INTRODUCTION

*Mycobacterium avium* subspecies *paratuberculosis* (*MAP*) is most common and economically significant disease of ruminants. It is the causative agent of paratuberculosis or Johne's disease, a gastro intestinal inflammatory disorder

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and a chronic granulomatous enteritis in ruminants such as cattle, goats, sheep, deer and camelids and otherwise animals, and too possibly the human disease Crohn's disease (CD) [1-5]. *MAP* is recognized as a multi-host mycobacterial microorganism by a longtime special skill to start and keep general and chronic infection of the intestine of a variety of histopathological types in many animals, with primates [6].

Though animals by clinical infection are commonly picked from the herd, animals via sub clinical paratuberculosis may make happen cost-effective losses since of poor reproductive and lessened milk production show [7]. Johne's disease causes foremost cost-effective losses to the dairy farm industries and farmers. The range of infection in a herd rises overly time and if the disease is left unmanaged, the economic result of bovine Johne's disease becomes more and more necessary [8]. The infection is dispersed global and Iran is one among those nations that are bare to the disease [9,10]. The results of *MAP* on dairy farm operations were estimated at nearby $200 to $250 million a year in the USA [11].

Rapid weight loss, a protein enteropathy and diarrhea are the three chief signs of Johne's disease. Diarrhea may be fewer common in certain species of animals like goats and sheep [4,12,13]. Generally, the larger the herd the more probably it is to have animals infected by Johne's disease. Later the long incubation period, the chief clinical sign seen in infected animals are plentiful, long-standing watery diarrhea, occasionally, noticeable weight loss and sporadic fever. Johne's infected cows go on to eat even by serious diarrhea. Typically has no mucous or blood in the diarrhea. Therefore the clinical signs of Johne's disease are generally vague and should be make happen via various extra agents. Repeatedly, even in severe clinical cases, Johne's disease is not known and the animals are primarily showed to slaughter without worrying for the primary reason for the disease. Inside any infected herd, single a few infected cows will have diarrhea at any one time [4,13]. The seroprevalence of Johne's disease in Georgia beef and dairy farm cull cattle in United States in 2000 was calculable and in dairy cattle was 9.58%, in beef cattle it was 3.95% and in cattle of unknown breed it was 4.72% [8].

Notwithstanding the development of contemporary techniques and methods for isolation and identification of Johne's disease, its management remains a serious veterinary problematic [14]. One of the difficulties associated by the disease is that signs seem recently and after a long time [15]. Furthermore, identification and isolation procedure is awkward, time consuming and may take several months [16]. Serologic tests, e.g. agar gel immunodiffusion (AGID) test, ELISA and fecal culture are suggested to approve the diagnosis of paratuberculosis in a clinically affected animal or in an infected herd. Indirect diagnostic techniques based on immunological methods for example complement fixation test, skin testing by john in, interferon test, and enzyme linked immunosorbent assay have shown low specificity or sensitivity specificity [17]. Serological methods could too be used to find an earlier exposure to the disease however not an active infection. Nucleic acid hybridization methods were useful effectively for the detection of the disease. Polymerase chain reaction (PCR) provides speedy, sensitive and specific detection for an early identification of the disease [18].

The infection is especially limited to the small intestinal tract and its draining lymph nodes. Infection may spread to more intestinal sites as shown via effective cultural isolation of the organism from milk, lung, semen and fetus. Other the intestinal tract, these other organs does not elicit a characteristic inflammatory reply to the presence of the organism [17].

In the past research describe the isolation of *MAP* from semen in rams and semen and male accessory genital organs in bulls. The usage of molecular techniques for the detection of *MAP* in milk and different matrices were made probably via the detection of specific DNA sequences, particularly IS900 [19,20], however whether semen can send the disease via uterus is not fully investigated [21]. The detection of the IS900 insertion sequence within the *MAP* genome has offered a unique for the fast detection of the bacterium DNA in clinical samples [22].

The aims of this study were to differentiate the possible of PCR for the rapid, to develop and to evaluate for detection of *M. avium* subsp. paratuberculosis in semen and blood samples from Iranian cattle, camel and sheep and also to determine the frequency of this infection in cattle, camel and sheep.

## MATERIAL and METHODS

### *Sampling and DNA Isolation*

In this study, a total number of 432 samples of cattle, camel and sheep, were collected randomly from 83 bulls frozen semen and 144 cattle, 95 camel blood and 110 sheep blood between January 2013 and March 2013. Samples are removed aseptically with sterile instruments. Semen and blood samples were sent to the Biotechnology Research Center of Islamic Azad University of Shahrekord Branch in a cooler with ice packs. Each of the specimens was stored -20°C for further use. Genomic DNA was extracted from specimens using DNA extraction kit (Qiagen, Germany), according to the manufacturer's protocol. The overall DNA was measured at 260 nm optical density according to the technique described by Sambrook and Russell [23]. The extracted DNA of each sample were kept frozen (−20°C) until analysis.

### *Gene Amplification*

The PCR amplification was done with two sets of

primers: the outer oligonucleotide primers were ISo-1 F: 5´- GTTCGGGGCCGTCGCTTAGG-3´ and ISo-1R: 5´GAGGTCG ATCGATCGCCCACGTGA-3´ and the inner oligonucleotide primers were ISi-2F: 5´- CCGCTAATTGAGAGATGCGAT TGG-3´andISi-2R:5´-AATCAACTCCAGCAGCGCGGCCTCG-3´. The target sequence was amplified in a 50 µl reaction volume containing 100 ng of genomic DNA, 0.2 mM dNTPs, 1X Taq buffer, 2 mM $MgCl_2$, 100 ng of each primer and 1 unit of Taq DNA polymerase (Fermentas, Germany).

The first round of PCR was performed in a DNA thermal cycler at a denaturation temperature of: first denaturation for 5 min at 94°C, followed by 30 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min, with a last extension for 5 min at 72°C. Two to five µl from the first round amplicon was used as a template for the second round PCR with the same PCR program by inner oligonucleotide primers.

### *Analysis of PCR Products*

Amplified samples were studied by electrophoresis (120 V/208 mA) in 1.5% agarose gel. Negative and positive PCR controls were run with each series of amplifications. The gel was stained by 0.1% ethidium bromide (0.4 g/mL) and were images obtained in UVIdoc gel documentation systems (Uvitec, UK).

In the current study, 83 frozen semen specimens of bulls and 349 blood samples of cattle, camel and sheep were tested for *MAP* by a nested PCR assay. The nested PCR assay used in current study enabled the detection of IS900 gene of *MAP*. Nested PCR amplification of the *MAP* in specific insertion sequence IS900 and later agarose gel analysis of the amplified products showed a single band of 230 bp for the positive samples *(Fig. 1)*.

### *Statistical Analysis*

Data were analyzed by using SPSS (version 15) software.

## RESULTS

The presence of *MAP* DNA was detected by nested PCR in each samples were from 42 out of 432 samplings (9.722%). *MAP* infection was detected 11 of the 144 (7.638%) blood samples of cattle, 16 out of the 110 (14.545%) sheep blood samples and 7 out of the 95 (7.368%) camel blood samples were positive for the *MAP* DNA and 8 out of 83 (9.638%) bulls frozen semen samples are infected. The Chaharmahal va Bakhtiary province had the highest prevalence of *MAP* in cattle (8.695%) while The Isfahan province had the lowest prevalence of *MAP* in cattle (5.769%). The Chaharmahal va Bakhtiary province had the lowest prevalence of *MAP* in sheep (13.235%) while The Isfahan province had the highest prevalence of *MAP* in sheep (16.666%). Gender wise differences out of the 25 number positive were females and 17 numbers positive were males. Sheep is a highest occurrence of *MAP* while, camel is a lowest occurrence of *MAP*. These finding suggested that control and eradication programs for *MAP* infection it seems to be necessary in Iranian cattle, camel and sheep. The results of the prevalence of *MAP* infection in cattle, camel and sheep are shown in *Table 1*.

## DISCUSSION

*MAP* is a microorganism that is the cause of Johne's disease, was isolated from the feces of a giver bull in an artificial insemination stud [19]. *MAP* can alive in animals for years lacking inevitably inflicting clinical disease [24]. *MAP* may have a role within the development of Crohn's disease in humans via the consumption of contaminated milk and milk products [9,25]. Milk and milk products resulting from cows via clinical or different suspected para-tuberculosis are not usable even later pasteurization [24]. The signs of this infection in human are bowel disease and a chronic inflammatory that may be severe, extended and

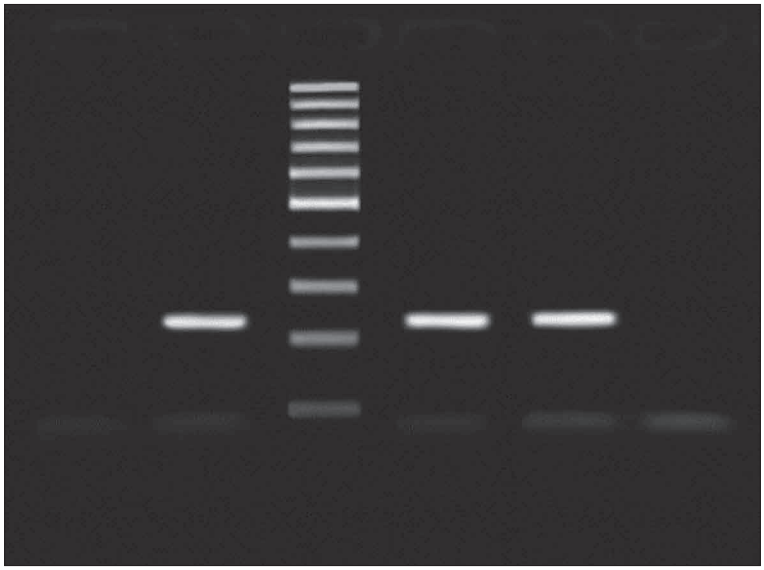

**Fig 1.** Agarose gel electrophoresis of PCR products (230 bp) for detection of *MAP* DNA in samples after PCR amplification. Lane 3: 100 bp DNA ladder (Fermentas, Germany); lanes 2, 4 and 5: positive samples (230 bp); lanes 1, 6: negative samples

**Şekil 1.** Örneklerde *MAP* DNA tespiti için PCR ürünlerinin (230 bp) agaroz jel elektroforezi. Şerit 3: 100 bp DNA merdiveni (Fermentas, Almanya); Şeritler 2, 4 ve 5: pozitif örnekler (230 bp); Şerit 1, 6: negatif örnekler

***Table 1.*** *The results of the prevalence of MAP in each specimens*
***Tablo 1.*** *Her bir örnekte MAP yaygınlığının sonuçları*

| Variables | Species | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Blood of Cattle | | Blood of Camel | | Blood of Sheep | | Frozen Semen of Bull | |
| | N | Prevalence: Positive Samples (n) (%) | n | Prevalence: Positive Samples (n) (%) | n | Prevalence: Positive Samples (n) (%) | n | Prevalence: Positive Samples (n) (%) |
| **Region** | | | | | | | | |
| Chaharmahal va Bakhtiary | 92 | 8 (8.695%) | 0 | 0 (0%) | 68 | 9 (13.235%) | Unknown | Unknown |
| Isfahan | 52 | 3 (5.769%) | 95 | 7 (7.368%) | 42 | 7 (16.666%) | Unknown | Unknown |
| **Sex** | | | | | | | | |
| Female | 108 | 9 (8.333%) | 28 | 4 (14.285%) | 75 | 12 (16%) | 0 | 0 (0%) |
| Male | 36 | 2 (5.555%) | 67 | 3 (4.447%) | 35 | 4 (11.428%) | 83 | 8 (9.638%) |
| Total | 144 | 11 (7.638%) | 95 | 7 ( 7.368%) | 110 | 16 (14.545%) | 83 | 8 (9.638%) |

incapacitating [3,7]. The identification of Johne's disease is very difficult, especially within the primary phases. This is payable to the long incubation period, the variable log phase associated via bacterial proliferation, and the multifocal distribution of bit via bit developing lesions [26].

There are 2 techniques for transmission of *MAP* is considered and contain direct faecal-oral cycle and indirect transmission, for instance through manure contamination of machinery used for feed sending and water bowls [9]. The diagnostic techniques contain bacteriological cultures and serological. Highest in number of the serological reactions quickly detect antibodies to *MAP* nevertheless not active infections [14].

Improvement diagnostic techniques are presently used in several research laboratories all over the globe. These techniques are based on molecular characterization of the specific microorganism isolates. These diagnostic exams use DNA probes, Restriction Fragment Length Polymorphisms (RFLPS), cloning and PCR [14,18].

PCR based on IS900 has been used for direct detection of *MAP*, lacking primary culture, from faecal specimens, milk, semen and human intestinal tissue and workers have been able to detect the presence of paratuberculosis DNA in intestinal tissue from patients by Crohn's disease and semen samples from bulls. For the goal that the clinical symptoms of Crohn's disease carefully imitator those found in animals via Johne's disease [3,9,27]. A sensitive and quick PCR-exam would help to check *MAP* in semen prior to artificial insemination [17,28], to induce high responsiveness of detection of *MAP* or different pathogens via IMS-PCR on faeces and milk [29-31].

Numerous researches have been focused on the association of Crohn's disease by *MAP*. Publications dealing by the culture detection of *MAP* in milk and milk products have too been increasing in number over the last ten years [13,32]. Larsen et al.[19] detached these microorganisms from the semen and genital organs of bulls, and showed eight of thirty one semen samples are infected. There have been data of detection of *Map* in the tissues [33-36], blood [37] of patients with CD or irritable bowel syndrome extra regularly than in control patients. The occurrence of *MAP* in bulk-milk samples using tracing of IS900 gene in Switzerland was 19.7% and indicated *MAP* can so often be transferred to humans via raw milk consumption [38]. These results largely identical to the outcomes of current study. Claus et al.[17] showed that *M. avium* subsp. paratuberculosis may give hematogenously to the semen and male reproductive tract as an extra intestinal place and agent reservoir.

*Map* was detected via culture in the intestinal lymph nodes or faeces of 34% of healthy dairy cows and 3% of healthy beef cows obtainable for slaughter [39]. *Map* was too cultured from the liver of 11.1% of the dairy cows and 0.7% of the beef cows tested [40].

Nebbia et al.[4] done molecular identification route for detection of mycobacterium in sheep and goat milk. *MAP* was especially found in 4 out of 14 seronegative and 9 out of 15 seropositive animals. Moreover, *MAP* DNA was sporadically recovered in milk samples from 13 out of 29 animals. A study of 200 retail ground beef samples were check in the USA via Jaravata et al.[41] and *Map* was not detected by PCR (IS900) and conventional culture techniques. In an examination of 133 minced beef samples found from a meat processing plant in the Republic of Ireland, no practical *Map* was detected [42].

*Map*-contaminated water and contact via infected animals or people have been recommended as vehicles of transmission to humans [43]. *Map* DNA was detected in 15% of the Canadian samples [44] and 9.8% of Irish pasteurised milk samples [45]. *Map* was detected in 11.6% of the pasteurized milk samples in a UK study [46]. Even though most studies have attentive on cattle milk, *Map* DNA has been detected in raw goats and sheep milk [47]. Researches

in India displayed high prevalence of *MAP* in many animals; for example: domestic (cattle, goats, buffaloes and) and wild (blue-bulls, hog deer, bison, etc) ruminants, other animals (camels, rabbits, etc), primates and human beings [48].

The results of present study show that the each samples in cattle, camel and sheep served as a reservoir of disease in Iran. Consequently it could be stated that the animal reservoirs increase the risk of the potential spread of disease to other animals and specially humans, and this deserves special attention.

In conclusion, the results presented high occurrence of *MAP* infection in cattle, camel and sheep samples and suggested that control and eradication programs for prevent and cut of economic loses of *MAP* infection it seems to be necessary. Study in order to knowledgeable about the physiological ecology of mycobacteria in the environment and wildlife is necessary to fully find out the effects that mycobacteria have on human health and to agree to new methods for controlling of their environmental and wildlife reservoirs. It is important to buy bulls, their semen and replacement heifers only from farms/regions which are declared paratuberculosis free. Evading several source of infection from manure contaminated feed, water, soil and a full comprehending of potential reservoirs in wild is significant in developing an effective control programme. Though it is not known whether *M. paratuberculosis* causes disease in populate, livestock diseases that are transmissible to human beings are presently affecting the confidence of consumers principally. So paratuberculosis infection in food animals should be controlled as a safety measures. Furthermore, the results of the current study suggest that PCR was highly sensitive and specific for identification and differentiation of *MAP* and that it could be a suitable tool for diagnosis of *MAP*. Thus, it is essential to screen in all area regularly to prevent the spread of the disease and laboratory support is a significant tool in the identification of the disease. Seemingly, PCR is one of the best ways to detect and characterize *MAP* as fast, less hazardous and sensitive method.

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