

Detection of *Mycobacterium avium* subspecies *paratuberculosis* infection in two different camel species by conventional and molecular techniques

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Article Info	Abstract
<p>Article history:</p> <p>Received: 13 October 2014 Accepted: 08 June 2015 Available online: 15 December 2015</p> <p>Key words:</p> <p>Acid fast staining Iranian Camel <i>Mycobacterium avium</i> Paratuberculosis PCR</p>	<p>Paratuberculosis (John's disease) is infectious and chronically progressive granulomatous disease which affects domestic and wild ruminants. The causative agent is <i>Mycobacterium avium paratuberculosis</i> (MAP), a slow growing mycobactin dependent acid-fast bacillus. We investigated the detection and frequency of MAP in apparently healthy dromedary and Bactrian camels by insertion sequence 900 (IS900) polymerase chain reaction (PCR) and acid fast staining of fecal samples in Iran. Acid fast staining results showed that 6/50 (12.0%) samples of dromedary camels and 4/26 (15.3%) samples of Bactrian camels were suspected to MAP. Although the percentage of positivity for PCR assay of fecal dromedary camel was 8.0%, no bands corresponding to MAP detect in all samples of Bactrian camels. In conclusion, Although the incidence of MAP infection was low, further studies should be conducted to get more information on MAP infection in camel population, especially in areas where camels are close to other ruminants such as dairy cow, sheep and goat.</p> <p>© 2015 Urmia University. All rights reserved.</p>

تشخیص عفونت ناشی از مایکوباکتریوم اوئیوم تحت گونه پاراتوبرکولوزیس در دو گونه مختلف شتر با روش های متعارف و مولکولی

چکیده

پاراتوبرکولوزیس (بیماری یون) یک بیماری گرانولوماتوز پیش رونده ی عفونی و مزمن است که نشخوارکنندگان اهلی و غیر اهلی را مبتلا می کند. عامل ایجاد بیماری مایکوباکتریوم اوئیوم پاراتوبرکولوزیس (باسیل اسید فاست وابسته به مایکوباکتین دارای رشد آهسته) می باشد. این مطالعه به منظور تشخیص و تعیین فراوانی مایکوباکتریوم اوئیوم پاراتوبرکولوزیس در شتران دو کوهانه و تک کوهانه ی به ظاهر سالم با روش واکنش زنجیره ای پلیمرز IS900 و رنگ آمیزی اسید فاست بر روی نمونه های مدفوع آنها صورت پذیرفت. نتایج رنگ آمیزی اسید فاست نشان داد که شش مورد از ۵۰ (۱۲/۰ درصد) نمونه مربوط به شتر تک کوهانه و چهار مورد از ۲۶ نمونه (۱۵/۳ درصد) مربوط به شترهای دو کوهانه آلوده به مایکوباکتریوم اوئیوم پاراتوبرکولوزیس بودند. اگر چه درصد مثبت بودن نمونه مدفوع شتر تک کوهانه با روش واکنش زنجیره ای پلیمرز ۸/۰ درصد بود اما هیچ باندی در مورد نمونه های شتر دو کوهانه مشاهده نگردید. از این مطالعه استنباط می گردد که اگر چه وقوع عفونت مایکوباکتریوم اوئیوم پاراتوبرکولوزیس پایین بود اما مطالعات بیشتری بایستی برای بدست آوردن شناخت بیشتر مایکوباکتریوم اوئیوم پاراتوبرکولوزیس در جمعیت شتر به ویژه در مناطقی که شتران در تماس نزدیک با دیگر نشخوارکنندگان از قبیل گاو شیری، گوسفند و بز هستند، انجام پذیرد.

واژه های کلیدی: پاراتوبرکولوزیس، رنگ آمیزی اسید فاست، شتر ایرانی، مایکوباکتریوم اوئیوم، واکنش زنجیره ای پلیمرز

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Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) causes Johne's disease (JD), also called paratuberculosis, in domestic and wild ruminants throughout the world which estimated economic losses are over \$250 per cow annually in highly infected dairy cattle herds,^{1,2} because of severe loss of slaughter weight and reduced milk production.^{3,4} Apart from its outbreak in cattle, sheep, goats and deer, paratuberculosis has also been diagnosed in a wide range of other free-ranging and domesticated ruminants including camels, llamas and alpacas.⁵⁻⁷

Dromedary camel (*Camelus dromedarius*) and Bactrian camel (*Camelus bactrianus*) are valuable domestic animals in tropical and subtropical areas used for their meat, milk and wool. Camel milk and meat are considered as an important source of protein for wide range of population.⁸

Paratuberculosis affects camels worldwide causing characteristic clinical manifestation of severe diarrhea resulting in death.⁹ The disease in camels may have a more rapid course than in cattle, with death occurrence after 4 to 6 weeks of onset of illness.¹⁰ In Bactrian camels, the disease was most severe in 3 to 5 year old animals.⁷

Paratuberculosis has been reported widely in many populations of camels in Asia, Middle East, Africa and the former Soviet Union.^{7,11,12} Paratuberculosis in Iranian camels is poorly documented in the literature even though the disease has been detected in some dairy cattle herds.¹³⁻¹⁷ To the best of our knowledge there has not been any published report of JD in Iranian camels using molecular approaches.

To reduce the infection rate in a herd, the test and culling strategy of JD control programs require sensitive and specific diagnostic techniques. Fecal culture is considered as the gold standard for the diagnosis of MAP infected animals but requires 12 to 16 weeks;¹⁸ therefore the development of a rapid, sensitive and specific diagnostic method for the detection of MAP is essential in the control of Johne's disease in economically important animals.¹⁹ The insertion sequence 900 (IS900) element is an insertion sequence considered to be a MAP-specific gene with 15 to 20 copies per genome and is a target for rapid detection of MAP by PCR.^{20,21} The aim of this study was to detect MAP in apparently healthy dromedary and Bactrian camels by IS900 PCR and acid fast staining of fecal samples in Iran.

Materials and Methods

Collection of samples. A total of 76 fecal (50 dromedary camel and 26 Bactrian camel) samples from apparently healthy camels with different ages were obtained during six months from Semnan and Ardebil provinces (Iran). Approximately 5 g of feces were collected from each animal with a gloved hand directly from the rectum and placed in

a sterile, leak proof container. The fecal samples were stored at 4 °C until they were transported to the laboratory. The unprocessed fecal samples were preserved at -20 °C for further studies.

Ziehl-Neelsen acid fast staining. For Ziehl-Neelsen staining, fecal smears were stained with carbol fuchsin Ziehl-Neelsen acid-fast stain. The smears were washed for 2 min in tap water, decolorized in two brief washes of acid alcohol (1% hydrochloric acid in 70% ethanol), washed for 2 min in tap water, and briefly counterstained with methylene blue.²² All smears were examined by three specialists, with multiple fields being evaluated.

DNA extraction. According to Stabel *et al.*, fecal samples (1 g) were diluted in 9 mL of 1× Tris-ethylene-diamine tetra-acetic acid (EDTA) buffer (10 mM Tris-HCl, 1 mM EDTA; pH 7.6) in 15-mL polypropylene conical tubes. Samples were vortexed for 5 sec and allowed to settle for 2 min and then vortexed again. Samples were centrifuged at 200 *g* for 30 sec, and the supernatants from each sample were transferred to new 15-mL conical tubes. The supernatants were then diluted 1:10, 1:100, or 1:1,000 in 1× TE (Tris EDTA) buffer. One milliliter of each dilution was placed into 1.5 mL sterile, DNase, RNase-free Eppendorf tubes (Eppendorf, Hamburg, Germany) and centrifuged at 13,000 *g* for 2 min. Supernatants were discarded and pellets washed 2 times with 1 mL of 1 × TE buffer. Pellets were resuspended in 500 µL of 1 × TE buffer and placed in a heating block at 100 °C for 10 min. A negative control containing 1 × TE buffer was included in the heating block as a sentinel for DNA cross-contamination during sample processing. After cooling to room temperature, 4 µL of RNAase (500 µg mL⁻¹) was added to each sample. Samples were stored at -20 °C until PCR analyses were performed.²³

Polymerase chain reaction. IS900 PCR was performed as described by Corti and Stephan with minor modification.²⁴ Resuspended DNA (5 µL) was used as the template for IS900 PCR. 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' were used for amplification cycles that generated a 413 bp product. The PCR mix consisted of 25 µL reaction volume containing 20 pmol of each primer, 5 mM MgCl₂ (CinnaGen Co., Tehran, Iran), 40 µM 2 deoxyribonucleoside-5-triphosphate (dNTP; CinnaGen), 0.5 U Taq polymerase (CinnaGen) in 1× reaction buffer (CinnaGen). PCR program was as follows: 94°C for 5 min (1 cycle); 94°C for 1 min, 59°C for 1 min, 72 °C for 2 min (30 cycles); 72 °C for 7 min (1 cycle). PCR products were visualized by gel electrophoresis on 1% agarose gel. *Mycobacterium avium* subspecies *paratuberculosis* ATCC 43105 was used as positive control for each PCR round.

Statistical analysis. Prevalence of positive and negative results for Ziehl-Neelsen acid fast staining and IS900 PCR were primarily analyzed by contingency tables. All proportions were compared using the Fisher's exact

test and a p -value less than 0.05 was considered significant by SPSS (version 16.0, SPSS Inc., Chicago, USA).

Results

Ziehl-Neelsen Staining analysis of the fecal smears.

Acid fast staining results showed that 6/50 (12.0%) samples of dromedary camels and 4/26 (15.3%) samples of Bactrian camels were suspected to MAP. But there was no significant difference ($p = 0.7$) between the prevalence of positive acid fast staining results in two groups of dromedary and Bactrian camels.

Polymerase chain reaction analysis. A total of four dromedary faecal samples out of 50 were positive by PCR assay for Map yielding an expected PCR product of size 413 bp (Fig. 1). The percentage of positivity for PCR assay of fecal dromedary camel was 8.0%. Although expected PCR bands size of MAP revealed in fecal samples of dromedary camels, there were not bands corresponding to MAP detection in all samples of Bactrian camels. No significant difference ($p = 0.2$) was found between the percentage of positivity for IS900 PCR assay in two groups of dromedary and Bactrian camels.



Fig. 1. Gel electrophoresis of IS900 PCR products from fecal samples of two different camel species. Lane M: 100 bp DNA ladder. Lane 1: Positive control MAP 43105, Lane 2: Negative control, Lanes 3, 5, 8 and 11: PCR product of 413 bp from fecal samples. Lanes 4, 6, 7, 9 and 10: No product from fecal sample.

Discussion

Johne's disease is an infectious disease of cattle and other ruminants, caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP).^{6,7} Diagnosis of paratuberculosis is difficult because of the fastidious growth pattern of the microorganism and the different host immune responses invoked during subclinical and clinical stages of infection. Traditionally, fecal culture for MAP is considered as the gold standard for diagnosis.

However, fecal culture is time-consuming and detects only 38.0 to 50.0% of infected animals.²³ Serological tests such as enzyme-linked immunosorbent assay (ELISA) are even less sensitive than fecal culture, particularly in

apparently healthy or sub-clinically infected animals.⁸ Use of nucleic acid probes combined with the PCR for detection of MAP in fecal samples have vastly improved in recent years, leading to an increased sensitivity in detecting of low shedders, including a detection level of one colony-forming unit(s) per gram of feces. This is done by amplifying the IS900 gene sequence which is the most reliable way of detecting cows shedding low levels of MAP in their feces.^{23,25,26}

Use of PCR in contrast to culture and serological tests, allowed to detect nonviable as well as viable microorganisms and would be a more sensitive detection method. Therefore, in comparison with serological or culture methods, detection of MAP directly from bulk-tank milk or feces by IS900 PCR could be considered a valuable test for the estimation of herd-level prevalence.²⁷

The MAP has been detected in dairy herds throughout Iran.¹³⁻¹⁵ A recent work in dairy cow herds in Fars province (southern Iran) showed a herd-level prevalence of 11.0% based on IS900 nested PCR on bulk-tank milk samples.²⁸ Another study done by nested PCR also showed that, 5 out of 90 samples (5.6%) were positive for MAP. However, the frequency of infection was diverse in different regions ranging from 4.2% to 7.7%.²⁶ Khaled *et al.*, confirmed infection with MAP by PCR by targeting the IS900 gene. They extracted DNA from lymph node and liver samples of the five suspected camels resulted in amplification of a 229-bp PCR product which is the specific product of *M. paratuberculosis*-IS900.²⁵

Alhebab and Alluwaimi reconfirmed the spread of MAP infection in the Saudi camel herds and concluded the ruminant ELISA was proved useful for the screening of the MAP infection in camel and could rule out the need for the species specific ELISA test. They have shown their feasibility as robust diagnostic tool for screening of John's disease in camel. They also proved that PCR was more practical than ELISA in detecting MAP.⁸ In this study, according to previous study,²³ we used a rapid and simple DNA extraction method for the detection of MAP in fecal samples which were taken from two different camel species. Acid fast staining of suspected tissues is rapid and requires little optimization. However, Ziehl-Neelsen staining has been reported to falsely identify *Nocardia* and *Corynebacteria* and cannot differentiate among the various mycobacterial species. Previous reports have determined the sensitivity of Ziehl-Neelsen to be 36.4%.²⁹

A total of 4 out of 50 dromedary camel samples were positive in faecal samples by PCR. The percentage of positivity for PCR detection in this study was 8.0%. This result is in agreement with that of Alhebab and Alluwaimi who reported only 97 positive samples out of 310 tested samples by PCR in dromedary camels.⁸ Although the outbreak of MAP infection in Bactrian camels (*Camelus bactrianus*) was 15.3% based on acid fast staining, acid fast staining could not completely predicative. The results

showed no bands on fecal samples by IS900 PCR, so it could be concluded that the genus *Camelus bactrianus* from our region are may be free of infection but further research is required. The study has confirmed the spread of MAP infection in the dromedary camel in Iran. But there is no significant association ($p > 0.05$) between the camel species and the prevalence of MAP infection by either acid fast staining or IS900 PCR methods.

In the present study, IS900 PCR assay proved to be a more sensitive and reliable than acid fast staining for the detection of MAP in faecal samples of camels since the PCR assay was able to detect significantly more positive cases than acid fast staining. This study suggests that IS900-PCR-based detection of MAP could be used as a potential diagnostic tool for rapid and effective Johne's disease surveillance in camels.

To the best of our knowledge, this study is the first description of a work of this kind performed in Iran. Although the incidence of MAP infection was low, further studies should be conducted to get more information on MAP infection in camel population, especially in areas where camels are close to other ruminants such as dairy cows, sheep and goats.

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References

1. Stabel JR. John's disease: A hidden threat. *J Dairy Sci* 1998; 81: 283-288.
2. Ott SL, Wells SJ, Wagner BA. Herd-level economic losses associated with Johne's disease on US dairy operations. *Prev Vet Med* 1999; 40:179-192.
3. Beaudeau F, Belliard M, Joly A, et al. Reduction in milk yield associated with *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection in dairy cows. *Vet Res* 2007; 38: 625-634.
4. Kudahl AB, Nielsen SS. Effect of paratuberculosis on slaughter weight and slaughter value of dairy cows. *J Dairy Sci* 2009; 92: 4340-4346.
5. Muskensa J, Bakkerb D, de Boera J, et al. Paratuberculosis in sheep: its possible role in the epidemiology of paratuberculosis in cattle. *Vet Microbiol* 2001; 78: 101-109.
6. Crawford CG, Ziccardi HM, Gonzales JB, et al. *Mycobacterium avium* subspecies *paratuberculosis* and *Mycobacterium avium* subsp. *avium* infections in a tule elk (*Cervus elaphus nannodes*) herd. *J Wildlife Dis* 2006; 42: 715-723.
7. Behr AM, Collins MD. Paratuberculosis, organism, disease, control. Oxfordshire, UK: CABI 2010; 179-183.
8. Alhebab MA, Alluwaimi MA. Paratuberculosis in camel (*Camelus dromedarius*): The diagnostic efficiency of ELISA and PCR. *Open Vet Sci J* 2010; 4: 41-44.
9. Manefield GW, Tinson AH. Camels. A compendium Sydney post graduate foundation vade mecum series C 1997; No. 22.
10. Wernery U, Kaaden OR. Infectious diseases of Camelids. Berlin, Germany: Blackwell Science 2002; 23-33.
11. Kaura P, Folia G, Singh SV, et al. Molecular epidemiology of *Mycobacterium avium* subspecies *paratuberculosis*: IS900 PCR identification and IS1311 polymorphism analysis from ruminants in the Punjab region of India. *Comp Immun Microbiol Infect Dis* 2001; 34: 163-169.
12. Tharwat M, Al-Sobayil F, Ali A, et al. Clinical, ultrasonographic, and pathologic findings in 70 camels (*Camelus dromedarius*) with Johne's disease. *Can Vet J* 2012; 53:543-548.
13. Khodakaram Tafti A, Rashidi K. The pathology of goat paratuberculosis: Gross and histopathological lesions in the intestines and mesenteric lymph nodes. *J Vet Med B* 2000; 47: 487-495.
14. Kasravi R, Nowrouzian I. Clinical paratuberculosis as a cause for higher culling rate in cows with left displaced abomasum and diarrhea in a Holstein dairy herd. *J Vet Med B* 2004; 51: 302-303.
15. Anzaby Y, Tabatabayi AH, Asgharzade M. A survey of the infection status of *Mycobacterium avium* paratuberculosis in dairy cattle using PCR and culture of Tabriz region [Persian]. *Iran J Vet Sci* 2006; 2: 297-310.
16. Shahmoradi AH, Arefpajohi R, Tadayon K, et al. Paratuberculosis in Holstein-Friesian cattle farms in central Iran. *Trop Anim Health Prod* 2008; 40:169-173.
17. Raoofi A, Hemmatzadeh F, Ghanaei AM. Serological survey in camels (*Camelus dromedaries*) to detect antibodies against bovine herpesvirus type-1 and *Mycobacterium avium paratuberculosis* in Iran. *J Camel Prac Res* 2012; 19:1-4.
18. Stabel JR, Whitlock RH. An evaluation of a modified interferon- γ assay for the detection of paratuberculosis in dairy herds. *Vet Immunol Immunopath* 2001; 79: 69-81.
19. Vansnick E, de Rijk P, Vercammen F, et al. A DNA sequence capture extraction method for detection of *Mycobacterium avium* subspecies *paratuberculosis* in feces and tissue samples. *Vet Microbiol* 2007; 122: 166-171.
20. Collins DM, Gabric DM, De Lisle GW. Identification of a repetitive DNA sequence specific to *Mycobacterium paratuberculosis*. *FEMS Microbiol Lett* 1989; 51: 175-178.
21. Ikonomopoulos J, Gazouli M, Pavlik I, et al. Comparative evaluation of PCR assays for the robust molecular detection of *Mycobacterium avium* subsp. *Paratuberculosis*. *J Microbiol Meth* 2004; 56: 315-321.

22. Quinn PJ, Carter ME, Markey BK, et al. *Clinical veterinary microbiology*. London, UK: Wolf Publishing 1994; PP: 21-66.
23. Stabel JR, Bosworth TL, Kirkbride TA, et al. A simple, rapid, and effective method for the extraction of *Mycobacterium paratuberculosis* DNA from fecal samples for polymerase chain reaction. *J Vet Diag Invest* 2004; 16: 22-30.
24. Corti S, Stephan R. Detection of *Mycobacterium avium* subspecies *paratuberculosis* specific IS900 insertion sequences in bulk-tank milk samples obtained from different regions throughout Switzerland. *BMC Microbiol* 2002; 2(1): 15. doi: 10.1186/1471-2180-2-15.
25. Khaled B, Alharbi KB, Al-Swailem A, et al. Pathology and molecular diagnosis of paratuberculosis of camels. *Trop Anim Health Prod* 2012; 44:173-177.
26. Sadati R, Jafarpour M, Mirinargesi M, et al. Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* in dairy cattle breed in northern Iran by nested-PCR *Glob Vet* 2012; 8: 259-263.
27. Soumya PM, Pillai MR, Antony PX, et al. Comparison of faecal culture and IS900 PCR assay for the detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine fecal samples. *Vet Res Commun* 2009; 33:781-791.
28. Haghkhah M, Ansari-Lari M, Novin-Baheran AM, et al. Herd-level prevalence of *Mycobacterium avium* subspecies *paratuberculosis* by bulk-tank milk PCR in Fars province (southern Iran) dairy herds. *Prev Vet Med* 2008; 86: 8-13.
29. Zimmer K, Drager KG, Klawonn W, et al. Contribution to the diagnosis of Johne's disease in cattle. Comparative studies on the validity of Ziehl-Neelsen staining, fecal culture and a commercially available DNA-Probe test in detecting *Mycobacterium paratuberculosis* in feces from cattle. *J Vet Med* 1999; 46:137-140.