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ORIGINAL ARTICLE

Subtype Genotyping Characterization of *Mycobacterium avium* subspecies *paratuberculosis* Isolated from Dairy Cattle of Tehran province

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ABSTRACT
Mycobacterium avium subspecies of paratuberculosis, called Mycobacterium paratuberculosis causes Johnes disease. This bacterium is a common infection factor between human and animals which is widespread throughout the world and requires a reliable method for prevention and control. The purpose of this study is to recognize genotypes of Mycobacterium avium subspecies paratuberculosis isolated from dairy flocks in Tehran province. In these research 100 cattle stools that suspicious to be infected by johns disease extracted. Then, Nested PCR reaction was arranged and carried out by using specific primers of IS900 gene and PCR products gained were electrophoresis on Agarose gel. Then the approved samples were PCR processed by using specific gene primers of GyrA and GyrB. From among positive cases isolated, 20 samples randomly selected were sequence. Then the resulted sequences were recorded in a gene bank and a phylogenic tree diagram was drawn for it using Bioedit and DNA Star software. Based on results of this study and by presenting two genes of GyrA and GyrB, we observed that resulted from our research are similar to that recorded in gene bank. According to the results obtained from observed that separation of MAP-GyrA-spain and MAP-GyrA- Iran was very similar and MAP-uk has had the biggest differences with separations of in GyrA gene. GyrB gene is most similar to the strains of MAP-GyrB-USA and the biggest difference is related to MAP- k10-USA.
Keywords*: Mycobacterium avium subspecies paratuberculosis, Johnes disease, Genotype*

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INTRODUCTION

Johnes Disease or *Paratuberculosis* is infection and chronic infection of ruminants, which epidemiologically perches in Class B (World Organization for Animal Health) (2.1). In terms of prevalence, this disease from an economic point widespread and of view, is one the most important diseases in animal husbandry [3]. The infectious agent is a bacillus (*Mycobacterium avium*subsp. *Paratuberculosis* or MAP) in Actinomycetal Class, Mycobacteriaceae Family, Mycobacterium Genus [4, 5, 6]. Decreased milk production, increased sensitivity to diseases, loss of genetic value, premature removal, and increased calving interval all economic results of this disease. Due to impacts on milk components, it also results in reduction of fats and proteins and increase of somatic cells. According to relevant studies, reduction of milk production was 1.58 to 7.2 kg/day. Since the incubation period of Jone's disease is very long, the infected animals excrete a lot number of the bacterium and pollute the environment. Thus, control of Jone's disease is impossible without a test to identify symptomless (clinical) vectors [7, 8, 9, 10]. A noteworthy feature of MAP is its relation with Crohns Disease (a chronic inflammatory bowel disease similar to Jone's in humans). Isolation of MAP from intestinal tissues of patients with Crohns disease suggests that *Mycobacterium aviumparatuberculosis* is also an factor of Crohns disease [4, 11, 12, 13].Human getting exposed to *M. paratuberculosis* raw and pasteurized milk as well as meat as a secondary source [14].

Since MAP was first identified, a wide range of methodologies including traditional diagnosis based on clinical signs, enzyme-linked immunosorbent assay (ELISA)[15], PCR-based methods (simple PCR [16], Nested PCR [17], Real – Time PCR [18, 19] and quantitative Real – Time PCR [20]) were applied to identify infected animals.

Polymerase Chain Reaction-Restriction Enzyme Analysis (PCR-REA) was also proposed long time ago [21]. Even poll solid phase cells were used and optimized [22]. It should be noted that cell culture is still used in laboratories as the main method to find MAP [23]. Among already mentioned methods, PCR methods are particularly important owing to high speed, low costs, and high sensitivity. Today, most PCR methods use MAP diagnosis based on identification of DNA sections embedded in IS900 [20]. The purpose of research is identification of genotypes of *Mycobacterium avium* subsp *paratuberculosis* isolated from dairy farms of Tehran.

MATERIALS AND METHODS

This research is a descriptive Cross-Sectional study conducted on 100 fecal samples of cows suspicious of John's disease (showing clinical symptoms) in the city of Tehran. DNA extraction kits (DNA Purification DNP ™kit) were used to extract DNA. The extracted DNA samples until PCR analysis were kept frozen in -20 °C. Of course, considering high concentration of PCR inhibitors in the feces, the DNA samples were diluted at a 1:50 ratio.

In Nested-PCR assay, primer pairs designed for identifying IS900 gene (DeMeneghi, 2005) as well as positive/negative controls were used. The standard strain of MAP was used as the positive control, and negative controls contain all PCR reagents except template DNA, which is replaced by the same volume of deionized water.

First PCR phase: To conduct the first PCR phase the following primer was used.

S1: 5'-GGGTTGATCTGGACAATGACGGTTA-3'
R3: 5'-AGCGCGGCACGGCTCTTGTT-3'

First phase PCR reagents were mixed at 25µl final volume. This mixture is consisted of 2.5µl template DNA, 0.2 µmol of each primer, 200 µmoldNTPMix, 1.5µmol $MgCl_2$, 2.5 µmol PCR buffer, and 2.5µmol DNA Polymerase. Then, 20µl mineral oil sterile was added to the PCR mixture to prevent from contamination and evaporation. PCR reagents were mixed on ice and the samples were immediately put in thermocycler (Mastercycler Gradient, Eppendorf, Germany), where the thermal schedule was: 94°C for 5min, then cycle (94°C for 30s, 55°C for 30s, 72°C for 30s, respectively) and finally 72°C for 7min.

PCR Phase2: in this phase, this PCR primer was used.

S2: 5'-GGAGGTGGTTGTGGCACAACCTGT-3'
R1: 5'-CGATCAGCCACCAGATCGGAA-3'

Every condition including mixing of PCR reagents, time and thermal cycle were similar to Phase1, but the used template DNA including 2.5µl PCR produce Phase1 is added to the mixture at ratio of 1:100. Then, PCR products were electrophoresed by agarose gel, colored by ethidium bromide and evaluated by UV rays. Then, using *GyrA* and *GyrB* gene primers,

F:TGTTCTTCACCACCCAGGGCCGGG
R:TTGAGCGACAGCAGGTAGTCGTCGGCG

Positive samples witch isolated by GyrA and GyrB gene, out of which 20 samples were randomly selected to determine their nucleotide sequence to be recorded in a genbank (http:/www.ncbi. nlm.nih.gov /BLAST) . Using Bioedit & DNA star software's, we identified and compared sub genotypes of this bacterium to other researchers' findings.

RESULTS

The quality of extracted DNA after electrophoresis on agarose gel were observed and confirmed and it was found suitable for Nested- PCR. Out of 100 samples tested for PCR, 18 samples were positive, of which 20 samples were sent for sequencing.

Since positive/negative controls were used in all phases, the samples reported positive for presence of MAP have adequate valuable diagnostic and applied accuracy.

Nucleotide sequencing and phylogenetic analysis:

After PCR assay was conducted using *GyrA* and *GyrB* primers, PCR product was sent Macrogen Company to be sequenced. The result of sequencing BLAST in NCBI databank was identical to the gene recorded for *avium*. To compare sequence of this genes with recorded gene in Genbank and evolutionary and mutational study, the obtained sequences with other sequences the recored in NCBI that were similarity

overwere *aligned* with Bioedit& DNA star. After target sequences were aligned with other sequences recorded in NCBI, Similarity Tree was drawn (Figure1-2).

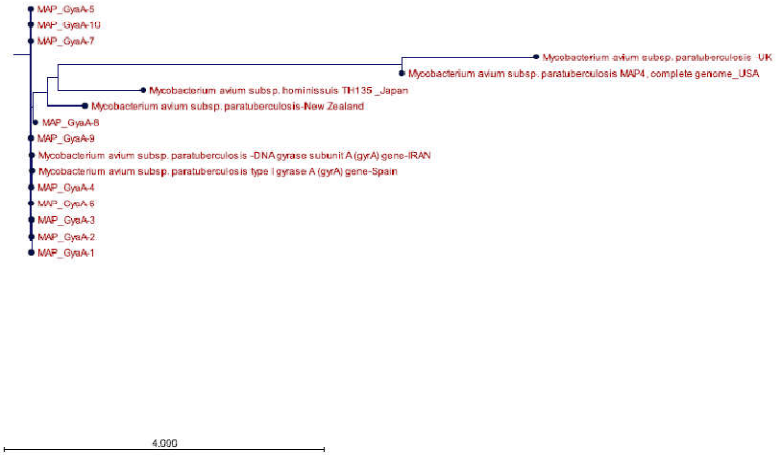

Figure 1: Phylogenetic tree isolate the gene *GyrA*, studied with isolates from around the world

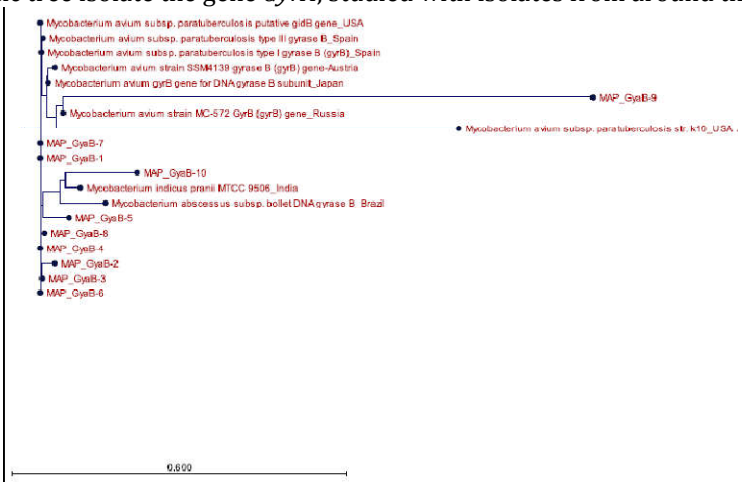

Figure 2: Phylogenetic tree isolate the gene *GyrB*, studied with isolates from around the world

DISCUSSION AND CONCLUSIONS

To be successful, John's disease requires a quick and accurate diagnosis method to identify and control its vector animals. Despite introduction of a variety of diagnosis methods to identify animal vectors of MAP, application of these methods involves enormous time and cost. Polymer Chain Reaction (PCR) researchers were encouraged to detect infectious agents, especially for but Non culturable or late growing microorganisms.

John's disease is an intestinal chronic disease, which may occur in many ruminants. Its transmission is often oral or fecal via infected milk or placenta [24] and it is of high importance due to zoonosis and losses from reduced milk production.

James et al [25] in an article entitled "study of genomic comparison of *Mycobacterium aviumparatuberculosis* between humans and animals" suggested that there are similarities between John's disease in humans and animals. Also, by PCR analysis on 30 MAP samples, they confirmed that disease isolates share VGI-17 and VGI-18 [25].

in "study of variations and differences of interspecies and interspecies genotyping of strains of *Mycobacterium aviumparatuberculosis*" usingIS900-RFLP and PFGE techniques Frank et al (2012) revealed that MAP strains are grouped to two major categories: S-type (Sheeps) and C-type (Cows), with some genetic similarities and differences between the isolates [26].

John et al (2010) in "analysis of genomic sequence of *Mycobacterium avium* subspecies *paratuberculosis* in sheeps and its relation to the host "showed that MAP strains in the sheep and cows are related, and this analysis can help diagnose various phenotypes differently [27].

In Milk-ELISA and IS900 PCR sensitivity analysis of feces, stool culture, and milk culture of the sheep and goats Singh et el [28] showed these sensitivity values: stool culture (84.6%), milk culture (96.1%), Milk-ELISA (88.4%), fecal PCR (23%).

In another study to identify MAP by fecal PCR and Milk-ELISA in 1808 samples of dairy cows, Wells and his colleagues showed these positive results: fecal PCR (23%) and Milk-ELISA (25.7%) [29].
By using IS900 specific primers and Nested PCR, Abbas Doosti and Saadat found some problems in an analysis of 120 fecal samples from cows suspicious of John's disease, i.e. out of 120 samples, 22 (18.33%) were infected with MAP, which had been tested positive in PCR [30].
In "Identification of *Mycobacterium aviumparatuberculosis* in fecal samples of Holstein-Friesian cows using culture and molecular methods" by taking 106 and 103 fecal samples from symptomatic and asymptomatic cattle, respectively using Nested PCR assay and Herrold's media with or without mycobactin J, Sideen et al found out that among symptomatic samples culture (81.3%) and molecular (87.15%) methods were positive, whereas asymptomatic samples showed that 11.7% and 9.7% of them were positive, respectively [31].
In an article entitled "identification and determination of *Mycobacterium aviumparatuberculosis* by PCR and REA based on IS900 and pieces*",* Jahandar and colleagues took 243 fecal and 56 raw milk samples of the suspicious cattle in dairy farms around Mashahd City and extracted DNA to detect samples infected to Insertion segments MAP-specific piece (IS900) and determine Insertion segments MAP-specific piece for sheep and cow isolates (IS1311). Then, the pieces were digested with the aid of HinfI coenzyme and revealed that 107 (out of 243 fecal samples) (44%) and 10 (out of 56 raw milk samples) (18%) were infected to MAP, which by enzyme digestion also showed that all MAP-infected samples were of cow isolates [32].
In "isolation of *Mycobacterium aviumparatuberculosis* of cows in Urumiyeh" Dilmaghani and colleagues found that out of 400 cows in Urumiyeh, fecal culture identified the isolated bacteria are MAP. The results were 12% positive (n=48) and the rest were negative [33].
In "Identification of *Mycobacterium aviumparatuberculosis* by Polymer Chain Reactions (PCR)in raw milk samples of cows in the Shahrekord City" Sherafati Chaeshtari and colleagues found 3% positive cases among 100 raw milk samples taken from industrialized and non-industrialized dairy farms of Shahrekord City by PCR analysis [12].
Haghkhah and colleagues, in a study about determination of prevalence of *Mycobacterium aviumparatuberculosis* in milk tanks of Fars Province by IS900 Nested PCR reported the infection rate between 8.6% and 23% [34].
Some research conducted in the recent years suggested that the simple and one-phase PCR method in some cases is not able to accurately detect *Mycobacterium aviumparatuberculosis* as infectious agent particularly in low levels of template DNA. Thus, a more reliable and more sensitive method (Nested-PCR) was used due to its ability to search and proliferate very low levels of DNA. On the other hand, detection of *Mycobacterium aviumparatuberculosis*in feces is especially important, because distribution of infected animal feces in the environment is the most important transportation route. One advantage of methods applied in this study is that the fecal samples can be processed directly and without any needs for culture or information on clinical conditions of the animal, and health or infection of the animal to MAP is reported with highest certainty. Since the infected animal does not appear clinical signs and due to high survival of MAP during pasteurization process, a quick and precise diagnosis method (Nested – PCR) is very important to identify and control this bacterium.
The results indicate ability and accuracy of the tests designed for proper detection and proliferation of IS900 gene in the reaction mixture, because MAP has 10 copies of IS900. Therefore, detection of John's disease in primary phases, which quantity of microorganisms is low, is possible by proliferation of this gene by PCR. In sequencing and study of subgenotypes with GyrA and GyrB it was observed that out strains are 98% comparable to those recorded in the Genbank.

REFERENCES

1. Collins M, (2003). Update on paratuberculosis: Epidemiology of Johne's disease and the biology of *Mycobacterium paratuberculosis*. *Veterinary Ireland Journal* 56: 565-574.
2. Vasnick E, Vercammen F, Bauwens L, D'Haese.E, Nelis h, Geysen D. (2005). A survey for Mycobacterium avium subspecies *partuberculosis* in the Royal zoological society of Abtwerp.*Vet . J.* 170:249-256.
3. Douarre P, et al. (2010). Isolation and detection of *Mycobacterium avium* subsp. paratuberculosis (MAP) from cattle in Ireland using both traditional culture and molecular based methods. *Gut Pathogens* 2: 11.
4. Jagdeep S, et al .(2009).Genomic analysis of local isolate of *Mycobacterium avium* subspecies paratuberculosis. *Veterinary Microbiology*. 134: 375–382.
5. Songer J. G. post k.w.(2005). Veterinary Microbilogy. *Elsevier, Saunders ltd , Philadelphia USA.* Pp. 7216-8717-20.
6. Alexander DC, Turenne CY, Behr Ma. (2009). Insertion and deletion events that define the pathogen mycobacterium avium subsp. Paratuberculosis.*J Bacteriol*. 19(3): 1018-1025.

7. Bhide M, et al. (2006). IS900-PCR-based detection and characterization of *Mycobacterium avium subsp. paratuberculosis*from buffy coat of cattle and sheep.*Vet. Microbiol*. 112: 33-41.
8. Radostits O, M, et al.2007.Veterinary Medicine. Saunders Ltd. London, UK. pp.1019 - 1040.
9. Beaudeau F, Belliard M, Joly A, SeegersH . (2007). Reduction in milk yield associated with mycobacterium avium subspecies paratuberculosis (MAP) infection in dairy Cows. Vet Res.38(4): 625-34.
10. Gonda MG, chang YM, shook GE , Collins MJ kirkpatrik BW. (2007). Effect of mycobacterium paratuberculosis infection on production, reproduction, and health traits in us Holsterins.*Prev vet med*. 80(2-3): 103-19.
11. Elena C, et al.(2012). Progress in molecular typing of *Mycobacterium avium* subspecies paratuberculosis; *Research in Veterinary Science* 92:169–179.
12. Sharafatichaleshtari F,et al. (2011).Detection of *Mycobacterium paratuberculosis* in raw milk University Polymer chain reaction. *Journal ofLaboratory Medicine*. 1(3):15-20.
13. Verlaine J.et al. (2011). How accurately can we detect *Mycobacterium avium* subsp. Paratuberculosis infection. *Journal of Microbiological Methods*.85 :1–8.
14. Donaghy JA, Rowe MT, Rademaker JL, Hammer P, Herman L, De Jomghe V, et al. (2008). An inter-Laboratory ring trial for the detection and isolation of mycobacterium avium Subsp. paratuberculosis from raw milk artificially contaminated with naturally infected faeces. *Food microbial*. 25(1):128-35.
15. Jorgensen JB, Jensen PT. (1978). Enzyme –linked immunosorbent assay (ELISA) for detection of antibody-ies to *Mycobacterium paratuberculosis* in cattle. *Acta Veterinaria Scandinavica*. 19:310-312.
16. Vary PH, Andersen PR, Green E, Hermon – Taylor J,Mc Fadden JJ. (1990). Use of highly specific DNA probe and the polymerase chain reaction to detect mycobacterium paratuberculosis in Jone's disease *Journal of clinical microbiology*. 28:933-937.
17. Stable J, Wells S, Wagner B. (2002). Relationships between fecal culture , ELISA, and bulktank milk test results for John's disease in us dairy herds . *Journal of Dairy science* .85:525-533.
18. O'Mahony J, Hill C. (2002). Rapid real-time PCR assay for detection and quantization of *Mycobacterium avium* subsp Paratuberculosis using SYBR Green and the light cycler .*Journal of microbiological.Methods*. 51: 283-293.
19. O'Mahony J, Hill C. (2004). Rapid real-time PCR assay for detection and quantization of mycobacterium avium subsp. Paratuberculosis DNA in artificially contaminated milk.*Applied and Environmental microbiology*.70:4561-4568.
20. Slana I, kralik p, karalovaA, pavlik I. (2008). on- farm spread of *mycobacterium avium* sp Paratuberculosis in raw milk studied by IS900 and F57 competitive real time quantitative PCR and culture examination *International Journal of Food Microbiology*.128: 250-257.
21. Eriks IS, Munck KT, Besser TE, Cator GH, Kapur V. (1996). Rapid differentiation of mycobacterium avium and M. Paratuberculosis by PCR and restriction enzyme analysis.*Journal of clinical microbiology*. 34:734-737.
22. D' Haese E, Dumon I, werbrouck H, J.V. D, Heman L. (2005). Improved detection of mycobacterium Paratuberculosis in milk.*The Journal of dary research*.72:125-128.
23. Douarre PE, Cashman W, Buckley J, coffey A, O'Mahony JM. (2010). Isolation and detection of mycobacterium avium subspParatuberculosis (MAP) from cattle in Ireland using both traditional culture and molecular based methods. *Gut pathogens*. 2:110.
24. Berghaus RD, Lombard JE, Gardner IA, Farver TB. (2005). Factor analysis of a Johne's disease risk assessment questionnaire with evaluation of factor scores and a subset of original questions as predictors of observed clinical paratuberculosis, *prev vet Med*. 12(3-4): 291-309.
25. James W,et al. (2011).Exploring the Zoonotic Potential of Mycobacterium avium Subspecies paratuberculosis through Comparative Genomics. *PLoS ONE,* www.plosone.org.6 (7)221-171.
26. Franck B,et al. (2012).Inter- and Intra-subtype genotypic differences that differentiate Mycobacterium avium subspecies paratuberculosis strains Biet et al. BMC Microbiology.12:1471-2180.
27. John P,et al. (2012). Genome sequencing of ovine isolates of Mycobacterium avium subspecies paratuberculosis offers insights into host association Bannantine et al. *BMC Genomics*.13:89.1471-2164.
28. Singh S,V,et al. (2007). Evaluation of highly sensitive indigenous milk ELISA kit with fecal culture, milk culture and fecal- PCR for the diagnosis of bovine Johne's disease (BJD) in India**.**Comp *ImmunolMicrobiol Infect Dis*.30(3):175-86.
29. Wells S,J, et al. (2006). Evaluation of a rapid fecal PCR test for detection of *Mycobacterium avium* subsp paratuberculosis in dairy cattle.*Clin Vaccine Immunol*.13(10):1125-3.
30. Doosti A, and Moshkelani S. (2009). Detection of *Mycobacterium paratuberculosis* by Nested PCR in dairy cattle's suspected to john's disease. *Journal of Microbial World*.2(1):19-23.
31. Seyyedin M. et al. (2010). Identification Of*mycobacterium Avium Subsp Paratuberculosis* In Fecal samples Of Holstein-Friesian Cattle Usingmolecular And Cultivation Methods; *J. Vet. Res.* 65, 2:135-140.
32. Jahandar M, H, et al. (2012).Identification and strain determination of *M. paratuberculosis*(MAP) by PCR and REA methods based on IS900 and IS1311 insertion segments methods based on IS900 and IS1311 insertion segments; J*ournal of Agricultural Biotechnology*.1(4)83-96.
33. Dilmaghani M, et al. (2012).Detection of *Mycobacterium avium*subsp. Paratuberculosis in cows from Urmia region. *Veterinary Journal (Pajouhesh & Sazandegi)*.93.: 13-17.
34. Haghkhah M, et al. (2008).Herd- level prevalence of Mycobacterium avium subspecies paratuberculosis by bulk-tank milk PCR in Fars province (Southern Iran) dairy herds. *Prev Vet Med*.;86(1-2):8-13.