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Molecular Identification of *Mycobacterium avium* subsp. *Paratuberculosis* isolated from ELISA-Positive Samples by Nested PCR

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ABSTRACT

Paratuberculosis (Johne's disease) is a chronic granulomatous small intestine disease caused by MAP. Diagnosing and isolating infected animals is the most important measure for controlling the disease. Therefore, this study aimed to molecularly identify mycobacterium isolated from ELISA-positive cows with Johne's disease by nested PCR from the samples from Markazi Province, Iran. For this purpose, 2938 samples were decontaminated and then cultured on the Herrold egg culture medium containing mycobactin and no mycobactin. After DNA extraction, PCR for *16S rRNA* was first performed, followed by nested PCR on positive samples. Of 2938 samples, 87 were positive, and 26 were suspected. All positive isolates were observed in Ziehl-Neelsen staining in microscopic expansion. A 543-bp band was observed in 26 tested samples and mycobacterium strains in PCR for *16S rRNA*, indicating the presence of mycobacterium in the above samples. Nested PCR was performed for all isolates and positive and negative control strains. A 398-bp band was obtained in the first stage, and a 298-bp fragment was obtained in the second stage, indicating the presence of MAP in the samples. Accordingly, nested PCR is suggested as a proper method for the quick and definitive diagnosis of disease cases.

Keywords

Mycobacterium avium, Johne's disease, 16S rRNA, Nested PCR

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Abbreviations

MAP: Mycobacterium avium subsp. paratuberculosis
RVSRI: Razi Vaccine and Serum Research Institute
bp: base pair
TE: Tris/EDTA

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Introduction

Johne's disease is a progressive chronic granulomatous enterocolitis in ruminants caused by MAP. MAP is a 0.5-2 µm, very slow-growing, acid-fast, mycobactin-dependent, and very stable pathogen that can survive in various media for a long time [1]. This bacterium enters the intestinal epithelial cells of cattle and other mammals, causing irreversible damage to infected animals, such as indigestion, diarrhea, vomiting, reduced reproduction, and death in various cases [2]. MAP is a silent, chronic, surprising infection considered a serious risk for the animal husbandry industry. Dealing with this disease is very costly and causes the early elimination of breeding livestock, reduction of livestock products, and heavy economic damage to stockbreeders.

Culturing is the technique used to precisely identify this bacterium, but this technique faces numerous challenges. The disease cannot be diagnosed in the early stages of culturing, and ELISA screening is recommended during this period. Furthermore, the incubation period of this disease is very long, with very slow growth. Consequently, molecular methods are used for the diagnosis and epidemiological investigations of this disease [3].

Johne's disease has existed for many years in Iran's ruminants. Considering similar clinical symptoms and molecular isolation and diagnosis of this bacterium in most patients with Crohn's disease, this bacterium is assumed to play a role in Chron's disease in humans. Therefore, scientists consider this bacterium a potentially serious threat to public health. Despite the high frequency of MAP in Iran's livestock [4-6], it is challenging to study the actual prevalence of the disease due to the high insensitivity of diagnostic methods and different testing and sampling methods in distinct countries. Notably, comprehensive research has not been conducted in Iran, but scattered studies have been performed using diverse methods.

A high-efficiency vaccine is required to prevent the disease caused by this bacterium. The disease situation in Iran must be first identified to run the disease control program, and control programs should be then performed accordingly.

This study aimed to isolate and determine the molecular identity of mycobacterium from ELISA-positive suspicious cows with Johne's disease from the samples of Markazi Province sent to RVSRI.

Results

ELISA. Of 2938 cow samples from dairy farms in Markazi and Alborz provinces, 87 positive and 26 suspicious samples were obtained using ELISA.

Culture. Initial isolation and MAP recultivation were performed using the Herrold egg culture medium (Figure 1; A). Ziehl–Neelsen staining confirmed the accumulation of acid-fast bacilli in 26 suspicious samples (Figure 1; B). Bright bacteria were also observed in the fluorochrome method (Figure 1; C).

PCR. The quantity (a concentration of 300-800 ng/mL and a wavelength of 260 nm) and quality (an obvious bond) of extracted DNA were suitable.

PCR for *16S rRNA*. At this stage, the 16S rRNA gene amplification confirmed isolates belonging to the mycobacterium genus. The PCR product length for this gene was 543 bp (Figure 2; A).

Nested PCR. The product length of 398 bp confirmed that isolates belonged to MAP (Figure 1; A). Finally, MAP was identified by producing a 298-bp fragment (Figure 1; C).

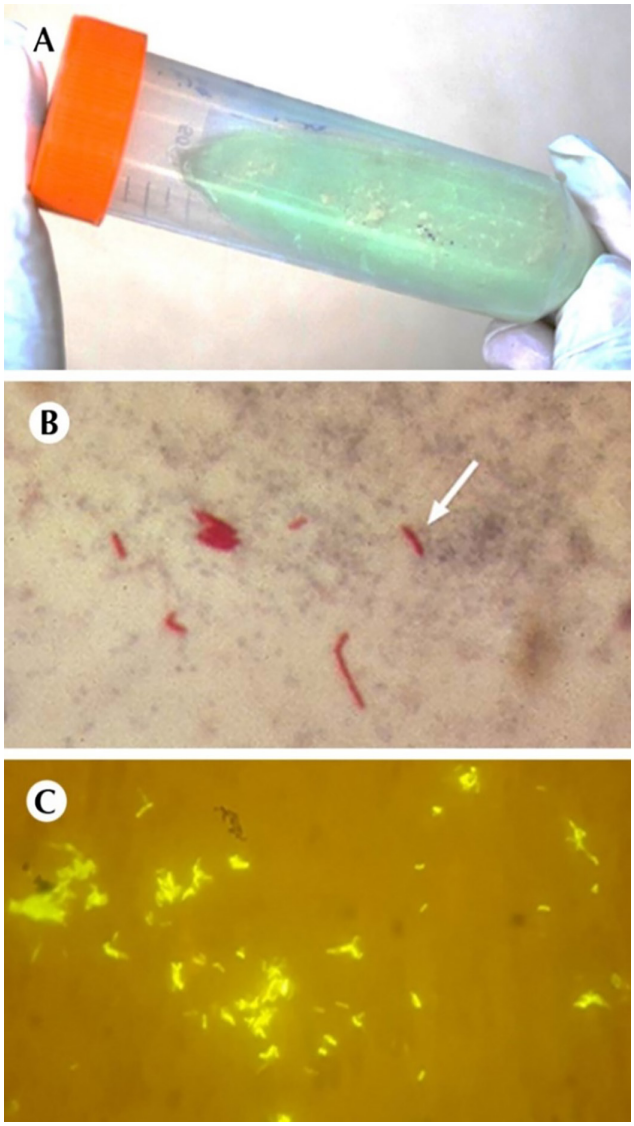

Figure 1.
MAP culture on Herald's egg yolk medium with mycobactin-J (A), Ziehl-Neelsen (B), and fluorochrome (C) staining of isolates

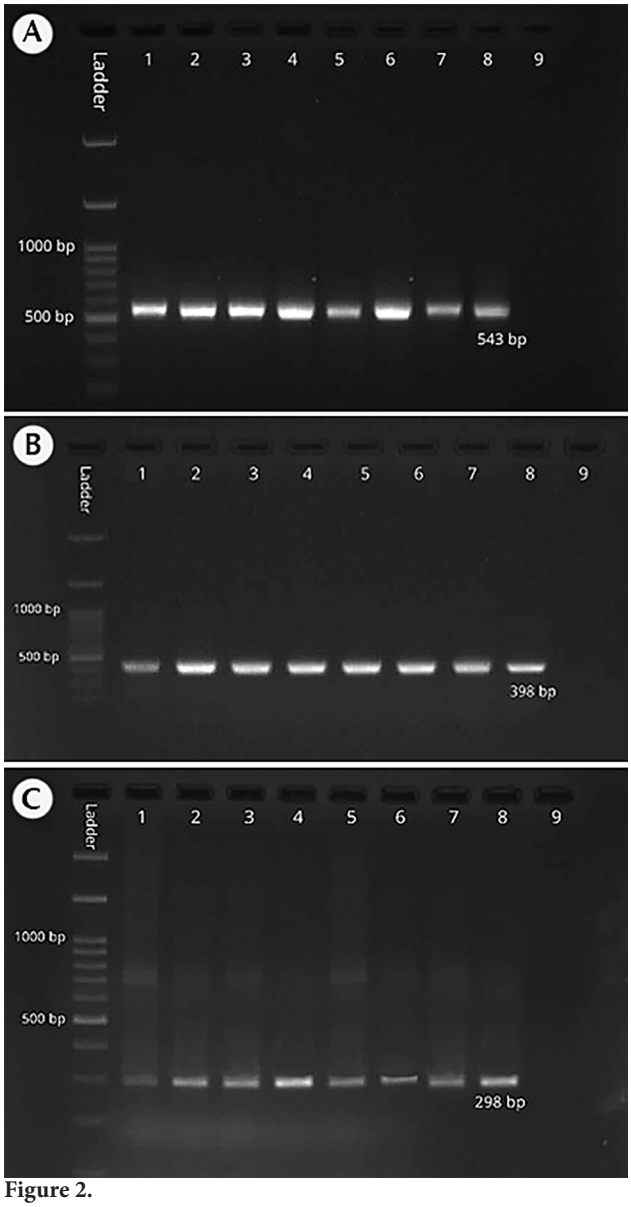

Figure 2.
PCR for identifying the isolates
PCR for 16S rRNA (A); Nested PCR for P90 & P91 (B); Nested PCR for AV1 & AV2 (C); [1-8: Isolates; 9: Negative control]

Discussion

Johne's disease is a serious problem due to the economic damage it causes to the livestock industry worldwide. Paratuberculosis also causes significant economic damage to the industry in Iran, but its extent is still unknown. Therefore, the diagnosis and control of diseases in ruminant herds is very important [8, 11]. The clinical symptoms of livestock are not solely reliable, as confirmed by the results of various studies on cows examined by the Johnin test, because this test fails to diagnose infected livestock among the suspected cases. Consequently, using molecular methods in Iran as a complementary test considerably helps dairy herds' health and, thereby, human society's health [10, 12].

Culturing on specific solid media is the most sensitive and specific method for diagnosing Paratuberculosis in livestock. However, culture-based methods are time-consuming and experimentally challenging for this bacterium [11]. Accordingly, new PCR-based molecular methods are used as proper alternatives for detecting the bacterium. Moreover, secondary contamination and drying of the culture medium may occur during long incubation periods, causing false negative results [10, 12].

The IS900 marker is specific to the MAP genome. However, unrealistic results may be observed depending on the type of selected primers due to the genetic similarity of the insertion sequence and its similar genetic units, known as pseudo-IS900 factors. There are reports on this case [13]. As a result, using equivalent genetic markers can improve the precision and correctness of identity tests. For this reason, in addition to the PCR-IS900 test in this study, nested PCRs using the P90 and P91 primers on the samples, and then the primers AV1 and AV2 were used.

Numerous PCR-based molecular techniques with high sensitivity and specificity have been proposed to quickly detect the bacterium [14]. However, nested PCR was used to confirm the isolates due to the lower sensitivity of usual PCR than nested PCR. Numerous studies have been recently conducted in this area, reporting the higher sensitivity of nested PCR than other methods in some cases. Abdolmohammadi et al. [8], Corti and Stephan [15], Haghkhah et al. [5], Jafari [16], and Doosti [17] identified MAP-infected cases using this technique.

Seyyedin et al. designed three primer pairs for the IS900 sequence, including Para1F/Para4R, Para2F/Para3R, and P90F/P91R, producing a 210-bp fragment. Their results showed that, except in one case, all positive cultured cases were consistent with nested PCR [18]. Soumya et al. reported a higher sensitivity of nested PCR than culture and ELISA [19].

Comparing the results of direct observation, ELISA, culturing, and nested PCR in examining the contamination of the milk tanks of dairy farms to MAP showed that 82 samples (82%) were positive in culture, 94 (94%) in nested PCR, 98 (98%) in ELISA, and only 33 (33%) in direct observation. Four samples were positive in ELISA but were negative in PCR. These 4 samples, along with the other 12 samples whose ELISA was positive (16 samples), did not have bacterial growth in the culture medium.[8]. Overall, the results of this study confirmed the superiority of nested PCR over other methods employed in this study. The contamination of the dairy cows of Kerman Province to MAP was examined and identified using microbial culture, PCR, and nested PCR [10]. Comparing these methods showed the superiority of nested PCR in detecting MAP, as confirmed by our results.

Abdolmohammadi et al. isolated MAP from

suspicious samples and examined them using nested PCR. Of 142 suspicious samples, 47 isolates were obtained. All positive isolates were acid-fast bacilli in Ziehl-Neelsen staining. In 83 samples of 142 samples and mycobacterium strains in PCR for 16S rRNA, a 543-bp band was observed, indicating the presence of mycobacterium in the samples. Nested PCR was performed on all isolates and positive and negative control strains, confirming the presence of MAP by generating a 398-bp fragment in the first stage and a 298-bp fragment in the second stage [8]. The consistency of our results with those reported by Abdolmohammadi et al. suggests that nested PCR can be used as a quick and definitive method for diagnosing the disease.

Some recent studies showed that the simple, one-step PCR cannot detect MAP in some cases, particularly at low DNA templates [20]. Single PCR is not sensitive enough as a diagnostic test for clinical samples. This low sensitivity of single PCR has been reported previously and was attributed to the presence of amplification inhibitors. Research on MAP has, however, found that this lower sensitivity of the standard amplification protocol when applied to clinical samples is neither solely due to the presence of inhibitors nor to the extraction of mycobacterial DNA [21]. It was found that when a standard amplification protocol is used, the technique can detect DNA from MAP in samples containing >100 CFU/mL but is frequently negative for samples containing fewer organisms [22]. An amplification of the latter samples was possible using nested PCR.

Consequently, the more reliable and sensitive nested PCR was used in this study to detect this infectious factor. This test was used due to its ability to search and amplify very low DNA contents. We have tested nested PCR for the rapid detection of paratuberculosis infection in suspicious clinical samples and compared its performance with bacteriological culture and single PCR. Nested PCR had an increased sensitivity for the detection of MAP, approaching that of culture.

Conclusion

It can be argued that ELISA can be very helpful in examining the status of samples and monitoring the progress of disease control programs. It is recommended as a screening method for Johne`s disease. However, this test may produce false positives and negative results. Therefore, culture or PCR should be used after detecting the infected cases by ELISA for the definitive diagnosis of the disease. Nested PCR significantly improves the sensitivity of detecting MAP and can be useful for the quick diagnosis of paratuberculosis. However, PCR inhibitors are a major hindrance. Accordingly, nested PCR is suggested as a proper method for the quick and definitive diagnosis of disease cases.

Materials & Methods

Collection of samples

Of 2938 cows from Markazi Province dairy farms (Arak: 497, Khomein: 101, Shazand: 66, Delijan: 328, Komijan: 26, Farahan: 17, Saveh: 498, and Zarandiye: 1405 samples), 6 mL blood was taken from the Jugular vein and sent to the RVSRI reference laboratory where the sera were separated. Microtubes were stored at -20°C for ELISA and PCR.

ELISA

ELISA was performed for all 2938 serum samples using an ELISA paratuberculosis kit (Cat No. RVJ99001, RVSRI) according to the standard instructions [7]. To this end, the wells were coated with MAP316F antigen. In the next step, 10 µL of the serum samples was mixed with 300 µL dilution buffer in a blank plate and then, the mixture was incubated at 25°C for 30 min, and 100 µL of this mixture was transferred to the main plate and the plate was incubated at 25°C for 30 min. After 5 times of washing, 100 µL of the conjugated bovine antibody was added to the mixture, and the mixture was incubated at 25°C for 30 min. After washing once again, 100 µL of substrate was added and the plate was incubated in a dark room at 25°C for 10 min. Finally, 100 µL of stop solution was added and the absorbance was read at 450 nm by an ELISA reader.

Culture

Initial isolation and MAP recultivation were performed using the Herrold egg culture medium containing mycobactin and without mycobactin, along with amphotericin B (5 mg), chloramphenicol (50 mg), and penicillin (100,000 units) [8]. The isolates were microscopically examined using the Ziehl–Neelsen (acid-fast) and fluorochrome methods.

Genomic DNA extraction

Bacterial DNA was extracted by Van Solingen's method using CTAB, isoamyl alcohol, and chloroform. Two complete loops of bacterial colonies were taken from the surface of the Herrold egg medium and slowly transferred into a microtube containing 400 µL of TE buffer. The resultant suspension was kept at 85°C for 30 min to deactivate bacteria [8, 9].

To extract DNA, 50 µL lysozyme (10 mg/mL) was added to each microtube containing mycobacterium suspension and stored overnight at 37°C after vortexing. Thereafter, 110 µL of protease K and SDS 10% was added to each microtube, vortexed, and incubated overnight at 50°C. Afterwards, 100 µL of 5 M NaCl and 100 µL of CTAB/NaCl solution were added and vortexed until a milky white solution appeared. The solution was stored at 65°C for 10 min. Then, 750 µL isoamyl alcohol-chloroform (1:24) was added to each microtube and vortexed for 10 min and then centrifuged at 11000 g for 8 min at 25°C. A volume of 450 µL of cold isopropanol was added to the aqueous phase (containing DNA) and kept at -20°C for 30 min. The microtubes were centrifuged at 11000 g for 15 min at 25°C, and the supernatant was discarded. One milliliter of cold ethanol 70% was added, and the microtubes were vortexed several times. The solution was then centrifuged at 11000 g for 5 min at 25°C, and the supernatant was discarded. Next, 20 µL of TE buffer was poured on the DNA precipitates, and the DNA quantity and quality were examined by electrophoresis and nanodrop.

PCR

PCR was first performed for the 16S rRNA gene (confirming that the isolates belonged to the mycobacterium genus) with primers 5'ACGGTGGGTACTAGGTGTGGGTTTC3' and 5'TCTGCGATTACTAGCGACTCCGACTTCA3'. After that, Nested PCR was performed using primers IS900 P90 (5'GTTCGGGGCCGTCGCTTAGG3') and IS900 P91 (5'GAGGTCGATCGCCCACGTGA3'), confirming that the isolates were MAP. The product was used as a pattern for the subsequent PCR using primers IS900 AV1 (5'ATGTGGTTGCTGTGTTGGATGG3') and IS900 AV2 (5'CCGCCGCAATCAACTCCAG3'). The final PCR volume was set at 12.5 µL (Table 1). In addition to the positive and negative mycobacterium DNA samples, a distilled water microtube containing all PCR components except DNA was used as a negative control. The microtubes were then placed in a thermocycler, and the temperature program of PCR was set according to Table 2 [8-10]. Electrophoresis was performed using Red Safe pre-stained 1% MP agarose with a genetic marker size of 100 bp for 90 min at 2 V/cm.

Table 1.
Thermal cycle conditions

PCR reaction	Initial heating	Denaturation	Annealing	Extension	Final extension	No. of cycles
16S rRNA	5 min, 94 °C	1 min, 94 °C	1 min, 60 °C	1min, 72 °C	10 min, 72 °C	25
MAP1	5 min, 95 °C	1 min, 95 °C	90 sec, 58 °C	90 sec, 72 °C	10 min, 72 °C	35
MAP2	5 min, 95 °C	1 min, 95 °C	90 sec, 58 °C	90 sec, 72 °C	10 min, 72 °C	35

Table 2.
PCR Materials

PCR reaction 16S rRNA	PCR buffer (µl)	dNTPs (µl)	MgCl2 (µl)	Primer forward (µl)	Primer reverse (µl)	DMSO (µl)	Taq polymerase (µl)	DNA template (µl)	PCR water (µl)
IS900 P	1.25	0.25	0.4	1	1	0.5	0.3	3	4.8
IS900 AV	1.25	0.25	1.25	0.4	0.4	0.5	0.5	3	4.95

Authors' Contributions

A.S. conceived and planned the experiments. M.S. carried out the experiments. M.S., and M.Sa. contributed to sample preparation. A.S., and M.Sa. contributed to the interpretation of the results. M.S. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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