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Original Article

Diagnostic advancements: Isolating *Mycobacterium avium* ssp. paratuberculosis and unveiling its molecular identity with nested-PCR

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Abstract

Mycobacterium avium subspecies paratuberculosis (MAP) is the causative agent of paratuberculosis, which is currently prevalent in many parts of Iran and produces severe economic loss. It is hence necessary to identify and isolate the animals infected with this bacterium, so this research aimed to isolate MAP from milk and fecal samples of ELISA-positive animals and determine the molecular identity of isolates. After performing ELISA on 3,700 bovine blood samples, 115 samples of milk and feces were taken from ELISA-positive cattle and were cultured on Herald's egg yolk medium with and without mycobactin-J and then the acid-fastness of positive samples was determined using Ziehl–Neelsen staining. The 16S rRNA-PCR test was performed after DNA extraction to determine the molecular identity of isolates. Primers IS6110 and IS901 were employed to ensure that the isolates were not related to members of *M. tuberculosis* complex and *M. avium*, respectively. Primer IS900 was also used to determine the molecular identity of MAP isolates. Also, expression levels of MAP-related genes (IS900, ISMAP02, F57, MAP2191, MAP4027) were evaluated via qPCR. Finally, positive samples were confirmed based on the Nested-PCR. Results showed that a total of 9 isolates were obtained from the culture of 90 ELISA-positive samples. The results revealed that all grown samples were positive for acid-fastness. The 16S rRNA-PCR test revealed the 543 bp band, which confirms the presence of Mycobacterium in the samples. The PCR test with Primer IS900 generated the 398 bp fragment in the first step and the 298 bp fragment in the second step, indicating the presence of MAP in samples. Also, relative expression analysis revealed that MAP-related genes were significantly higher in ELIZA-positive samples than in negative ones. Based on the study findings, it can be concluded that MAP-infected animals can be identified by ELISA. In addition, mycobacterium can be isolated by culturing the samples on appropriate media and then its molecular identity can be determined by using nested-PCR.

Keywords: *Mycobacterium avium*, Subspecies paratuberculosis, Paratuberculosis, Nested-PCR, Molecular Identity

1. Introduction

Mycobacterium avium subspecies paratuberculosis (MAP) is the causative agent of paratuberculosis, which is a chronic disease reported in almost all ruminants around the world [1]. In addition to infecting sheep and goats, MAP infects wild ruminants and probably plays a role in developing human Crohn's syndrome [2]. Therefore, it is considered a global challenge for veterinarians to control and fight the disease due to the possible transmission of its pathogen to humans.

Paratuberculosis was first identified in Iran in 1961, and its pathogen was isolated from the stool of Jersey cattle imported by Abadan Oil Refinery Company [3]. Due to the lack of a control program like in other countries in the region [4], paratuberculosis soon turned into an endemic disease of dairy cattle in almost all geographical areas of Iran. As a result, most Iranian animal husbandries are infected with this bacterium [5, 6].

A highly important part of paratuberculosis control programs is to isolate and identify MAP from infected animals without clinical manifestations in a herd that can transmit the disease through feces [7]. Although the bacterial culture of animal feces is time-consuming and expensive, it is still considered a reliable method that can detect MAP with higher sensitivity and specificity than ELISA [8].

Considering the intracellular nature of MAP, the human body's humoral immune system may fail to identify this pathogen in all cases. It is hence necessary to employ cellular diagnostic systems such as IFN-γ. Since performing such tests is highly expensive in Iran, ELISA is considered a good alternative option that produces a sensitivity of

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about 50% and a specificity of more than 90%. Although ELISA cannot identify all infected and subclinical cases, ELISA-positive cases can indicate infection with a specificity of 90%. Subclinical cases and primary infections are also tested using PCR and a negative culture. Therefore, ELISA can be used as a technique for rapid detection to prevent financial loss. Moreover, the pathogen can be identified by the gold standard investigation and its molecular identity can be determined and confirmed by PCR-IS900 and nested-PCR, respectively [9].

Among the wide range of MAP-specific genetic markers such as Locus 255, F57, and ISMav^2 [10], it is assumed that the IS900 sequence is the only MAP-specific marker. However, evidence suggests that other mycobacteria share the same gene sequence [11], but the F57 sequence is specific to MAP [12]. As a result, the IS900 locus is still one of the best conventional genetic markers for MAP identification [10]. In this study, the pathogen of paratuberculosis was isolated from suspected animals and then the isolates were confirmed by using nested PCR with emphasis on IS900.

2. Materials and methods

2.1. Bacterial sampling and isolation

The sample consisted of 3,700 heads of cattle selected from 10 animal husbandries in Markazi Province, Iran, from August to November 2020. Blood samples were taken from the tail of cattle using Venoject tubes and then they were centrifuged at 2,000 rpm for 10 minutes to separate their serum and keep them in 0.5-mL microtubes at -20°C for ELISA. From 90 ELISA-positive heads of cattle, 90 samples of feces and 61 samples of milk were collected in sterile enclosed containers (Table 1). The samples were put in ice and transferred to the Mycobacteriology Laboratory of Razi Vaccine and Serum Research Institute (RVSRI) to be stored at -70°C until culture. In addition, the vaccine strains of MAP, i.e. MAP III&V and MAP316F, were selected as the positive control and three strains close to MAP without IS900 sequence were selected as negative controls, which included *M. avium* subspecies avium (D4 strain) and *M. bovis* (AN5 strain) and *M. tuberculosis* (PN strain).

2.2. ELISA

ELISA was performed for all 3,700 serum samples using of ELISA Paratuberculosis kit (Cat No. RVJ99001, RVSRI) according to the standard instructions. To this end, the wells were coated with MAP316F antigen. In the next step, 10 µL of the serum sample 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 a substrate was added and then 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 [13].

2.3. Culture of samples

Feces. For decontamination, one gram of feces samples was transferred to a 50-mL Falcon tube containing sterile distilled water, and the content was stirred to become uniform. The resulting suspension was placed at room temperature for 30 minutes and then 5 mL of the supernatant was transferred to a tube containing 20 mL of 0.75% hexadecyl pyridinium chloride. The mixed content of the tube was placed at room temperature for 18 hours and then it was centrifuged at 4000-3000 rpm for 20 min. The resulting precipitates were rinsed with sterile saline three times. Finally, 0.5 mL of the remaining physiological serum along with the precipitates was inoculated in four Falcon tubes containing Herald's egg yolk medium with mycobactin-J, Herald's egg yolk medium without mycobactin-J, Herald's egg yolk medium with mycobactin-J, and the Herald's egg yolk medium produced by RVSRI [14].

Milk. For preparing the milk samples, 25 mL of raw milk sample was poured into a sterile porcelain jar containing 1 N solution of sodium hydroxide of the same volume. The resulting mixture was placed at room temperature for 15-20 minutes for decontamination. Then 5 to 6 mL of the mixture (depending on the milk fat, debris, etc.) was removed from the surface and margin of the porcelain jar using a sterile pipette and gradually poured into Falcon tubes (containing 5 mL of 1 N solution of hydrochloric acid and one drop of sterile bromothymol blue) until the acidic yellow color turned into olive green. After centrifuging the tubes at 4,500 rpm for 15 min, the liquid part of the milk was removed and 0.1 mL of cream and precipitates were inoculated on a culture medium. The precipitates ready for culture and grown colonies were evaluated by the microscopic Ziehl–Neelsen staining. The culture media were incubated at 37°C for 18 weeks and their growth and possible contamination were examined every week. After the growth of colonies was observed following the incubation, a loopful of bacteria was removed and transferred to a microtube containing 400 µl of 1% Tris-EDTA buffer

Table 1. Samples suspected of being infected with MAP from Markazi province.

City	Number of samples	Number of Host (Cow)	Number of feces/milk	Number of isolates
Arak	560	950	19/13	4
Khomain	101	100	5/2	1
Shazand	66	581	2/2	0
Delijan	328	800	24/21	1
Komijan	26	200	2/2	0
Farahan	17	100	4/3	0
Saveh	498	4000	7/4	1
Zarandiyeh	1405	750	19/14	2
	3738	7481	82/61	9

to make a uniform suspension. The microtube was then placed in a bain-marie at 85°C for 30 min to inactivate the bacteria [15].

2.4. qPCR

Using a cDNA synthesis kit and following the manufacturer's instructions, a traditional PCR was used to create cDNAs for mRNAs (Exiqon; Qiagen, Inc.). Transfected cells that produced scramble mimics were used as the adverse control. To measure the expression levels of the aforementioned genes, qPCR was carried out using a light cycler 96 (Roche Diagnostics) and SYBR Premix Ex Taq (BIO FACT Co., Ltd.). The primer sequences of genes are listed in Table 2.

2.5. Nested PCR

Bacterial DNA was extracted by the isoamyl alcohol-chloroform method [16]. The final PCR volume was adjusted to 12.5 µL (including 1.25 µL of PCR buffer, 0.2 µL of $MgCl_2$ (25 mM), 0.25 µL of dNTP (10 mM), 1 µL of each forward and reverse primer (5 pmol/µL; Table 3), 0.2 µL Taq DNA polymerase (5 U) and 4.5 µL sterile distilled water). Then 3 µL of extracted DNA sample (100-100 ng) was added to the mixture of each microtube. In addition to positive and negative mycobacterial DNA samples, a microtube of distilled water containing all PCR components, except the template DNA, was used as a negative control.

The following thermal cycle conditions were used: A initial denaturation step for 10 min (94°C); 35 cycles of denaturation for 60 s (95°C), annealing for 90 s (58°C), and elongation for 90 s (72°C); and, a final elongation cycle of 10 min (72°C). The amplified fragments were electrophoresed on a 1% agarose gel stained with ethidium bromide.

2.6. Statistical analysis

Statistical analysis was done using the statistical and graphics software Prism 6.0. The data are given as a mean of 3 to 5 experiments ± standard deviation (SD). The differences between the given groups were tested for statistical significance using Student's t-test (*$p<0.05$; **$p<0.01$; ***$p<0.001$). A p-value less than 0.05 was considered a significant difference.

3. Results

3.1. Growth of MAP colonies in culture tube

Of the 3,700 serum samples, 87 samples were strongly ELISA-positive, 3 samples were poorly ELISA-positive, and 26 samples were very poorly ELISA-positive (suspected). Only strongly and poorly ELISA-positive samples were cultured (Figure 1).

3.2. Evaluation of ELISA-positive samples

From the 90 ELISA-positive samples (strongly and poorly), 9 positive isolates were obtained, including 4 isolates from Arak (from 19 feces samples and 13 milk samples), one isolate from Khomein (from 5 feces samples and 2 milk samples), one isolate from Delijan (from 24 feces samples and 21 milk samples), one isolate from Saveh (from 7 feces samples and 4 milk samples), and 2 isolates from Zarandieh (from 19 feces samples and 14 milk samples).

Acid-fast bacillus (AFB) was observed in all samples that were cultured on Herald's egg yolk medium with my-

Table 2. Primer sequences of genes used in qPCR.

Gene	Sequences
IS900	F: 5'- GTT CGG GGC CGT CGC TTA GG -3' R: 5'- GAG GTC GAT CGC CCA CGT GA -3'
ISMAP02	F: 5'- GTG CGC GAT TCC TGT CGT AG -3' R: 5'- TCG CAC CAC GCT GTC TTG TT -3'
MAP2191	F: 5'- ACG GAA ACC GTC GTC TGT TC -3' R: 5'- CTC GGC GAC TGT CTT CTT TG -3'
F57	F: 5'- CGA GGA GCG CTA ACC GAT GA -3' R: 5'- CGC GTG CGG GTC TGA GTT TT -3'
MAP4027	F: 5'- CGA GGA GCG CTA ACC GAT GA -3' R: 5'- CGC GTG CGG GTC TGA GTT TT -3'

Table 3. Primers used in this research.

Primer	Sequence (5'-3')	Strains/Size	Product size (bp)	Ref
16S rRNA (f)	ACGGTGGGTACTAGGTGTGGGTTTC	Mycobacterium	543	[17]
16S rRNA (r)	TCTGCGATTACTAGCGACTCCGACTTCA			
IS*6110*INS1	CGTGAGGGCATCGAGGTGGC	*M. tuberculosis*	243	[15]
IS*6110*INS2	GCGTAGGCGTCGGTGACAAA			
IS900-P90	GTTCGGGGCCGTCGCTTAGG	*M. paratuberculosis*	398	[15]
IS900-P91	GAGGTCGATCGCCCACGTGA			
IS900AV1	ATGTGGTTGCTGTGTTGGATGG	*M. paratuberculosis*	298	[16]
IS900AV2	CCGCCGCAATCAACTCCAG			
IS901 (f)	GCAACGGTTGTTGCTTGAAA	MAP	1108	[18]
IS901 (r)	TGATACGGCCGGAATCGCGT			

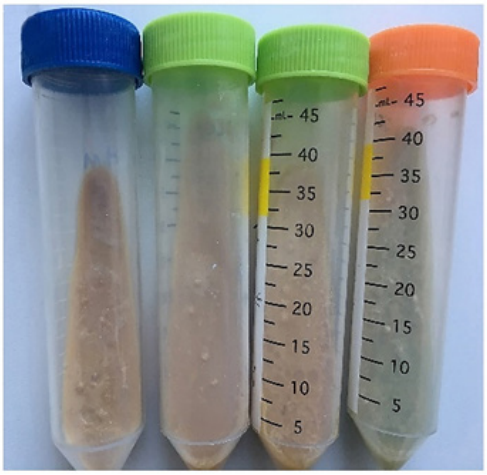

Fig. 1. Growth of MAP colonies in culture tube containing Herrold's egg yolk culture medium with Mycobactin-J.

cobactin-J.

3.3. Expression of MAP-related genes

Using the qPCR method, we analyzed the relative expression of MAP-related genes in ELISA-positive samples and ELISA-negative samples. Based on our results we showed that the expression of all IS900 (P value < 0.05*), ISMAP02 (P value < 0.05*), MAP2191 (P value < 0.01*), F57 (P value < 0.01*), and MAP4027 (P value < 0.01*) genes were significantly higher in ELISA-positive samples compared to ELISA-negative samples (Figure 2).

3.4. Validation of identity of MAP in isolates

PCR-16srRNA test produced a fragment of 543 bp in 9 isolates out of the 90 ELISA-positive samples as well as in positive and negative control strains, which indicated the presence of mycobacterium (Figure 3; A). The PCR-IS900 also produced fragments of 398 bp, which represented the existence of MAP (Figure 3; B). Finally, nested PCR confirmed the identity of MAP in all isolates by producing a fragment of 298 bp (Figure 3; C).

4. Discussion

Control programs for infectious livestock diseases, such as paratuberculosis, often begin with the diagnosis of an infection in the livestock population of an area through various methods. This aims to adopt the best diagnostic method in order to minimize the losses, especially economic loss, resulting from such diseases. In addition, the success of control programs for paratuberculosis requires a reliable and quick diagnostic method for detecting and isolating the infected animals.

Microbial culture is currently the most sensitive and specific method for diagnosing paratuberculosis in cattle; that is why it is referred to as the gold standard. Nevertheless, microbial culture cannot be applied in large-scale diagnostic programs because it needs time-consuming and extensive laboratory operations [6].

On the other hand, despite the high productivity of decontamination methods, some isolates may be lost due to the predominance of secondary contaminants or even the destruction of bacteria during the decontamination process [19]. Therefore, PCR-based direct bacterial detection methods will be a suitable alternative. In this study, the CTAB method was used to extract DNA from Mycobacteria. This method is suitable for the isolation of mycobacterial DNA, and the resulting DNA is both quantitatively and qualitatively suitable for performing molecular techniques. Researchers have proposed PCR-based molecular techniques with high sensitivity and specificity that detect bacteria in a short time. However, since simple one-step PCR methods may fail to accurately detect MAP, especially when the DNA template is low [20], a more reliable and sensitive method called nested PCR was employed in this study for detecting MAP because this method can explore and amplify very small amounts of DNA. Another advantage of this method is that it allows us to directly examine fecal samples taken from livestock and reliabi-

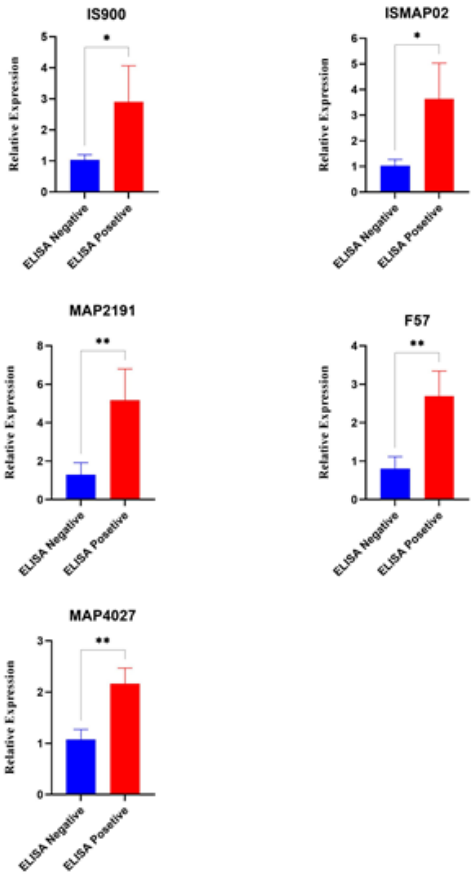

Fig. 2. As depicted, we showed that the expression of all IS900 (P value < 0.05*), ISMAP02 (P value < 0.05*), MAP2191 (P value < 0.01*), F57 (P value < 0.01*), and MAP4027 (P value < 0.01*) genes were significantly elevated in ELISA-positive samples compared to ELISA-negative samples. (P value < 0.05*, P value < 0.01**, P value < 0.001***, P value < 0.0001****).

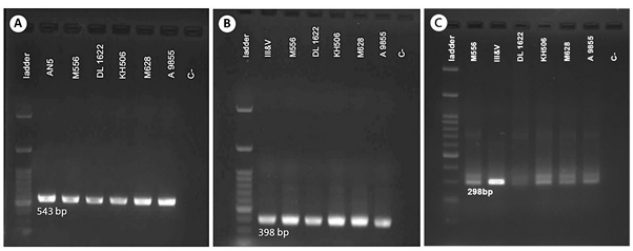

Fig. 3. PCR-16srRNA (production of a 543 bp fragment); **B:** PCR-IS900 P90/P91 (production of a 398 bp fragment); C: Nested-PCR AV1/AV2 (production of a 298 bp fragment. Wells from left to right: 1: Size marker, 2: Paratuberculosis vaccine (III & V), 3-7: MAP and 8: Negative control.

lity determine their health or contamination status without the need for bacterial culture or knowing about the clinical condition of livestock.

Although the IS900 sequence is widely used to identify bacteria due to its high specificity and sensitivity and the large number of its copies [10, 21], in some cases, the application of the IS900 marker alone can lead to incorrect results in some cases [22, 23]. As a result, P91/P90 [24] and AV1/AV2 [25], primers were employed in this study to confirm the isolates. The results of this study regarding the pathogenic strains of paratuberculosis and MAP (D4) and *M. bovis* strain AN5 are consistent with the findings of Bartos *et al*. and Thorne *et al*., respectively [25, 26].

Many studies conducted in recent years have sometimes reported the higher sensitivity of this method compared to other techniques. Corti and Stephan, and Haghkhah *et al*. employed this method for identifying MAP-infected cases in raw milk [27, 28]. This method has been also performed for identifying MAP in tissue samples of animals kept in zoos [29].

Jafari *et al*. and Doosti and Moshkelani used this technique for identifying MAP [30], and Seyyedin *et al*. designed three pairs of primers in the IS900 sequence (Para1F/Para4R, Para2F/Para3R and P90F/P91R) that created a fragment of 210 bp [21]. Their results indicated that all culture-positive cases, except one, were completely consistent with nested PCR [23]. Soumya *et al*. reported that nested PCR was more sensitive than bacterial culture and ELISA [20].

However, culture and PCR are only effective in the clinical form for diagnosis. PCR and culture cannot be used in the subclinical tests and the onset of infection. In such cases, tests such as ELISA can be useful.

The USDA protocols indicate that ELISA on serum or milk samples can be very useful in assessing their condition and monitoring disease control programs. Therefore, it can be used as a precise screening method for paratuberculosis infection. Considering the intracellular nature of MAP, the human body's humoral immune system may fail to identify this pathogen in all cases. It is hence necessary to employ cellular diagnostic systems such as IFN-γ. Since performing such tests is highly expensive in Iran, ELISA is considered a good alternative option for rapid detection to prevent financial loss. Moreover, the pathogen can be identified by the gold standard investigation and its molecular identity can be determined and confirmed by PCR-IS900 and nested PCR, respectively. Molecular epidemiological studies on isolates can be conducted by using genetic markers to determine how many strains there are in Iran and how they are geographically distributed.

5. Conclusion

According to the mentioned cases, in order to control paratuberculosis in the fields (herds), it is necessary to first perform screening test by ELISA and PCR and culture on cases 2 plus and more, and if the result is positive, the above animals shall be taken out from the herd. It is also better to prepare a colostrum and milk bank to feed the calves from cows on which symptoms of paratuberculosis disease are observed after the Mycobacterium ELISA test were performed.

Conflict of Interest

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

No human or animals were used in the present research.

Informed Consent

The authors declare not used any patients in this research.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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